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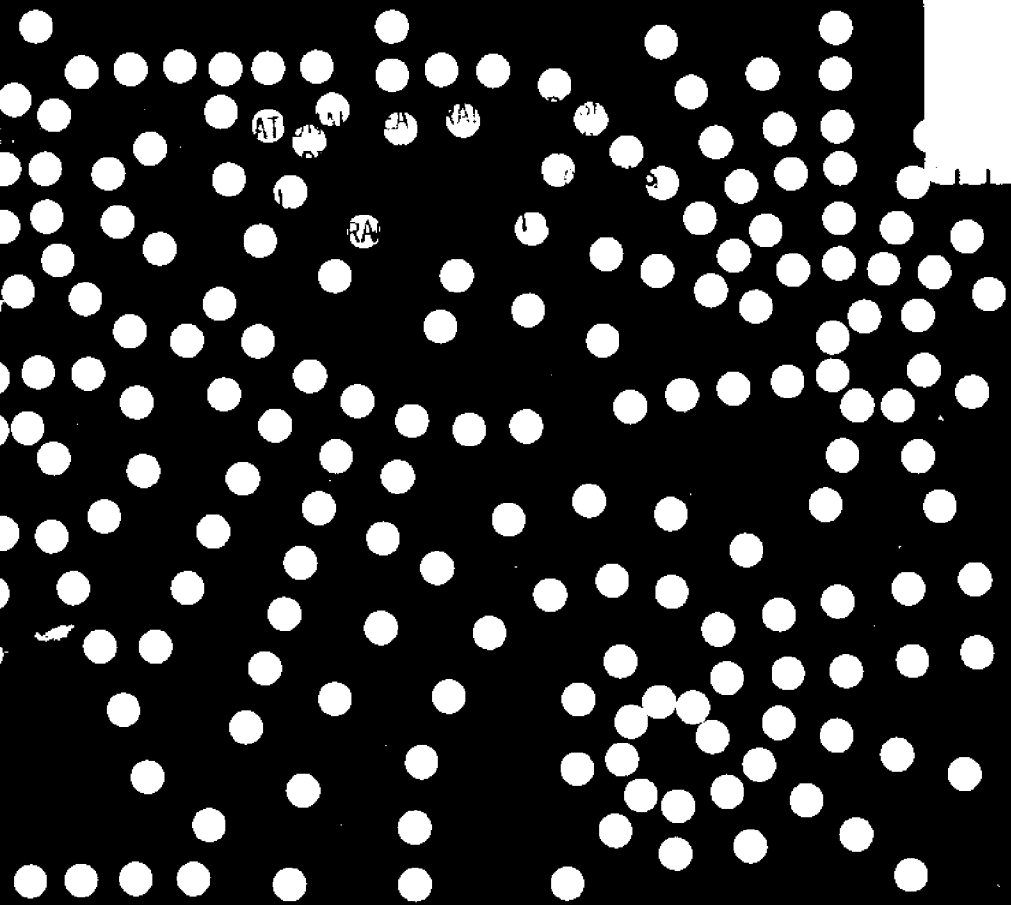
Proceedings of The First International Conference on Chitin/Chitosan



MIT Sea Grant Program

Massachusetts Institute of Technology
Cambridge, Massachusetts
02139

MITSG 78-7

PROCEEDINGS OF
THE FIRST INTERNATIONAL CONFERENCE ON
CHITIN/CHITOSAN

May 1978

Edited by

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The hosts for the conference, which was held in Boston, Massachusetts on April 11 through 13, 1977, were the MIT Sea Grant Program and the Massachusetts Science and Technology Foundation.

MIT Sea Grant Report MITSG 78-7
Index No. 78-307-Dmb

INTRODUCTION

The objective of the MIT Sea Grant Program and the Massachusetts Science and Technology Foundation in convening the First International Conference on Chitin/Chitosan was to bring together those experts and researchers from around the world who are working to expand the uses of this exciting, abundant, renewable natural resource. By providing a forum, we hoped to encourage the business and scientific communities to share insights and experiences, to stimulate new ideas, and to identify research that would advance the application of chitin and chitosan in pharmaceuticals, food processing, papermaking, agriculture, adhesive and textile manufacturing, and waste treatment.

We are publishing the proceedings of this conference because we believe that continued information sharing is essential if the advances in developing this valuable resource are to continue. We wish to express our appreciation to all those who participated in and contributed to the conference, and we sincerely hope that publication of the papers will serve in the future as a comprehensive reference source for them, as well as for those who could not attend.

Mr. Irving Sacks
Acting Director

Massachusetts Science and
Technology Foundation

Mr. Dean A. Horn
Director

MIT Sea Grant Program

15 May 1978

We dedicate these proceedings to the National Sea Grant Office, and especially to

Dr. Robert Abel and

Mr. Harold Goodwin

whose vision and wisdom are responsible for making the study of chitin and chitosan a matter of international concern.

R.A.A. Muzzarelli
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ACKNOWLEDGMENTS

We wish to acknowledge the cooperation and support received from the following organizations whose contributions made this conference possible.

University of Delaware Sea Grant Program
University of Washington Sea Grant Program
National Sea Grant Program, NOAA, DOC
Massachusetts Institute of Technology

The initiation, planning, organizing and managing of a meeting such as this conference always requires a taxing and protracted team effort involving many participants whose efforts it is impossible to acknowledge adequately. The names of at least two people must, however, be recorded here in appreciation of the great contributions they made: Mr. Vincent LoCicero, formerly of the Massachusetts Science and Technology Foundation who was largely in charge of pre-conference preparations and conference organization; the other is Ms. Marie Phaneuf, also of the Massachusetts Science and Technology Foundation, the organizing genius, who kept all major and minor details, correspondence and papers in mind and in order and who started the enormous and difficult job of retyping the scientific papers presented at the conference. Richard DeAmicis should also be mentioned here to acknowledge his important contributions in initiating the editing work on the papers. We owe a very special vote of thanks to Ms. Margaret M. Sevcenko who undertook the arduous and complicated task of editing, proofreading and organizing the text of these proceedings, which we feel she did superbly. Last, but by no means least, we must thank the whole MIT Sea Grant staff for its patient and efficient cooperation over the last year, but especially we would like to express our thanks to Ms. Joanne Sullivan for her meticulous, tireless and always cheerful efforts to ready the manuscript for final printing.

To co-host the conference and publish the proceedings of the First International Conference on Chitin/Chitosan, the MIT Sea Grant Program received support from the Office of Sea Grant in the National Oceanic and Atmospheric Administration, U.S. Department of Commerce, through grant number 04-6-158-44081, and from the Massachusetts Institute of Technology. The U.S. Government is authorized to produce and distribute reprints for governmental purposes notwithstanding any copyright notation that may appear hereon.

RELATED REPORTS

The following reports contain information related to the project described herein:

CHITIN AND CHITIN DERIVATIVES. MIT/Marine Industry Collegium Opportunity Brief. MITSG 76-5. Cambridge: Massachusetts Institute of Technology, August 1976. 21 pp. $2.50.

Ashford, Nicholas A., Dale Hattis, and Albert E. Murray. INDUSTRIAL PROSPECTS FOR CHITIN AND PROTEIN FROM SHELLFISH WASTES. MITSG 77-3. Cambridge: Massachusetts Institute of Technology, 1976. 99 pp. $12.50.

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PREFACE

CHITIN (kai'tin). *Zool.* and *Chem.* Also -ine.
[a. F. *chitine*, f. Gr. χιτών frock, tunic: see -IN.
(The etymological formation would be *chitonin*)]
The organic substance which forms the elytra and integuments of insects and the carapaces of crustacea.
The Oxford English Dictionary, 1961

One of the streets in the old quarter of Nancy, France, bears the name of Henri Braconnot, the discoverer of chitin. Braconnot, who lived from 1780 to 1856, studied chemistry and botany in Strasbourg and biology and geology in Paris. Still in his twenties, he became director of the Botanic Gardens of Nancy. The city honored him, however, apparently not for his scientific contributions, but for a generous sum of money he left to the town.

In the course of his work with mushrooms in 1811, Braconnot became intrigued with a cellulosic substance he had isolated which did not undergo change in an alkaline solution. He named the substance, "fungine."

Ten years later, in 1821, the name "chitine" was given to a material seemingly identical to fungine, but found by A. Odier while studying insects. Odier, who published the new name in the Mémoires de la Société d'Histoire Naturelle de Paris and in the Dictionnaire Classique d'Historie Naturelle, noted,"It is most remarkable to find the exoskeleton of insects to be identical with the cuticle of plants." Though Odier was incorrect in believing the two substances were identical, he is distinguished for not only naming chitin but for being the first person to observe the remarkable similarity between the function of cellulose in plants and that of "chitine" in insects.

Scientific interest in chitin continued for a time, but eventually much published work fell into disuse and was forgotten until almost 40 years ago when awareness that this natural polymer had an impressive number of potential applications stimulated new research. The editors of these proceedings believe that this curiosity and subsequent investigations into the uses of chitin contributed to the British war effort by providing a superior adhesive for the wooden fighter planes used by the British during the beginning of World War II.

Now more than a century and a half after Braconnot made his discovery, scientists throughout the world are fascinated by the potential large-scale, industrial applications for chitin. And they continue to be astonished by the number of living organisms in which they are finding chitin, its derivative chitosan, and enzymes capable of interacting with the two substances. Chitin has come of age in both basic and applied science and technology.

At a time when it is accepted, indeed imperative, that the wise utilization of natural resources is a conditio sine qua non for humanity's survival, it was logical, farsighted and in keeping with the best traditions of international scientific cooperation for the Sea Grant Program of NOAA, U.S. Department of Commerce, together with listed academic institutions and organizations, to co-sponsor and generously support the first international conference on chitin and chitosan, of which these are the proceedings.

Most of the papers in this volume were presented at the conference. Papers by E.R. HAYES, et al., page 103; K. SIMPSON, page 253; O.L. OKE, et al., page 327; M. TAKEDA, page 355; B.P. HETTICK, et al., page 464; M.L. BADE, page 472; S.C. SHRIVASTAVA, et al., page 492; C.J. BRINE, page 509; K. OKUTANI, page 582; and K.D. PARKER, page 606, however, were received after the conference and are included here because of the contributions they are making to the field.

TABLE OF CONTENTS

CHAIRMAN'S ADDRESS

CHITIN, AN IMPORTANT NATURAL POLYMER

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There are many natural polysaccharides commercially available today. They include cellulose, dextran, pectin, alginic acid, agar-agar, agarose, starch, carrageenans and heparin; all of them are either neutral or acidic. Chitin and chitosan, the more or less acetylated polymers of glucosamine, on the other hand, are the only natural polysaccharides that have sharply basic characteristics, and, because of their basicity, they have unique properties: for example, their solubility in various media, the viscosity of their solutions, their polyelectrolyte behavior, membrane-forming ability and polyoxysalt formation. Their optical and structural properties are due to the presence of regularly spaced amino groups on the polyanhydroglucose chain.

From the chemical point of view, chitosan is a primary aliphatic polyamine, and therefore it can undergo all those reactions typical of amines. Its reactions with carbonyl compounds include acylation with acid anhydrides to form novel derivatives whose applicability ranges from selective aggregation of cancer cells to special coatings. Aldehydes react very easily with chitosan at room temperature to form Schiff bases. Products have been obtained from these bases that have unpredictable characteristics; again, their applicability ranges over a wide field, from the cross-linking of a tobacco sheet with chitosan and glyoxal, to enzyme immobilization on chitosan in the presence of glutaraldehyde. The analytical determinations of chitosan are today performed with either p-aminobenzaldehyde or 3-methylbenzo-2-thiazolone hydrazone. These determinations are sensitive enough to permit the detection of early stages of biodegradation by fungi of hydrocarbon fuels and measurements of fungal infiltration of cellulosic materials.

Chelation of transition metals, mostly a result of the presence of free amino groups, is offering new perspectives in chelation chromatography and in ligand-exchange chromatography. The establishment of quality standards for chitosan to be used in chromatography would certainly contribute to its wider applications as a chromatographic support.

Many ways of derivatizing chitosan are available; most of them involve hydroxyl groups. Sodium chitin, which has been known for many years, deserves more attention than it has so far received as a versatile compound. Carboxyl group formation, sulfation, cyanoethylation, glycolation, xanthation are just a few examples of the many reactions that can be carried out with chitin and chitosan. New products have been obtained from such reactions; semipermeable membranes for example, have been made with glycolchitosan, desalting has been carried out with formaldehyde cross-linked chitosan membranes and with regenerated chitin membranes, and the delayed release of drugs coated with glycolchitin has been studied *in vivo*. There is hope that blood anticoagulants can be produced based on chitosan that may operate in a manner similar to heparin.

The magnitude of the natural-resource base from which chitin can be obtained has been established in the United States, and estimates of production costs have been calculated. Industrial manufacture of chitin and chitosan appears to be feasible; available resources should, of course, not only be exploited, but also protected.

Many potential applications of chitin chemistry are of great industrial importance. Paper and textile additives and finishes, adsorbents for harmful metal ions, cements for leather manufacture, drilling muds, photographic products and coagulants useful for flocculating suspensions are some of the topics that have already been examined in the literature. Since chitosan is biodegradable, vegetable-canning waste suspensions coagulated with chitosan can safely be fed to animals.

In recent years, biochemical research has shown increasing interest in the biosynthesis of chitin and in the relevant enzymology. Chitin and chitosan are two of the polymers that provide structural support to many organisms. Fungal and animal chitin are not the only forms that occur: the polysaccharide from bacterial cell walls, composed of alternate N-acetylmuramic acid and N-acetyl-glucosamine units, can be regarded as an ether of lactic acid and chitin. Chitin is hydrolyzed by lysozyme. The biochemistry of lysozyme has been elucidated with the help of oligochitosaccharides.

The pathway of chitin synthesis has recently received important elucidation from investigations carried out *in vivo*; many other experiments have been done to study the synthesis of chitin *in vitro*. The roles of chitin synthetase and ß-ecdysone are also under study. The purification of chitin synthetase and observations of the microfibrils that originate from chitosomes are still other recent accomplishments. Chitosomes have been described in terms of spheroidal organelles about 50 nm in diameter, which undergo a series of irreversible transformations when substrate and activator are combined to produce fibrils.

Chitin associations have received attention in recent years as well. For instance the concept of discrete layers of single components in fungi (α-glucan, protein, ß-glucan and chitin) has been rejected in favor of structural gradients. Chitin deacetylase has been found in certain microorganisms where chitosan is produced through enzymatic deacetylation of chitin. The chitin biosynthesis can be inhibited by various substances such as polyoxins and insecticides derived from substituted ureas. Here again chitin assumes importance in our life as it plays a role in the agricultural sciences and in entomology. The insecticidal action of microorganisms (including viruses) is enhanced by chitinase. Associations of glucanase and chitinase as antimicrobial agents have been proposed for use in agriculture. There is evidence that the growth of the hyphal tips of fungi depends on a delicate balance between wall synthesis and wall lysis; chitinase can alter this balance.

Chitosanase has only recently been discovered; the purified enzyme hydrolyzes chitosan, but not chitin. It prevents spore germination of *Mucor* strains and causes a decrease in the turbidity of germinated spores of this fungus, which is known to contain chitosan.

In the light of available scientific and technical information, chitin appears today to be a substance of much greater significance and relevance than it seemed to have only a few years ago. The progress made in our knowledge of chitin also

provides an excellent example of the value of interdisciplinary research. Its implications for ecology, resource conservation, pollution prevention and agricultural and food-industry uses are obvious. Chitin seems to fulfill a number of demands in our technological world and, and at the same time, to be a key polymer for the preservation of our environment.

This First International Conference on Chitin and Chitosan was convened to affirm the importance of chitin and to integrate our expertise and knowledge in a common endeavor.

I. SOURCES OF CHITIN

DISTRIBUTION AND QUANTITATIVE IMPORTANCE OF CHITIN IN ANIMALS

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ABSTRACT

Using a specific and quantitative enzymatic-identification procedure, the polysaccharide chitin has been found in a wide range of animal species. It is used by protozoans, mainly ciliates, to build up cyst walls. It also constitutes the bulk of the stalks or stems of most hydrozoan colonies, but it is rarely produced by Scyphozoa (jellyfishes) and Anthozoa, and is absent in sponges.

Chitin is the main structural polysaccharide of most invertebrates belonging to the Protostomia. Arthropods are the best known and most important chitin-producing animals; the dry organic matter of their cuticles can contain up to 80% chitin. Besides the arthropods, relatively large amounts of chitin may be found in the setae of annelids (from 20 to 38% of dry weight), in the skeleton of the colonies of Bryozoa and in the shells and other structures (jaws, radulae, gastric shield) of many mollusc species (up to 7% of the dry organic matter in gastropods and bivalve shells, and up to 26% in cephalopods). Chitin is only absent in free and parasitic flatworms (Platyhelminthes), nemerteans, sipunculids and leeches. In some other groups, such as nematodes and rotifers, chitin is present only in the egg envelopes.

Chitin synthesis has never been observed in echinoderms or vertebrates, but the tubes of some Pogonophora contain 33% chitin, while tunicates secrete a chitinous peritrophic membrane.

From an ecological point of view, besides crustaceans and molluscs, marine benthic animals are a rich source of chitin. Despite their small size, bryozoan and hydrozoan colonies yield a large biomass with relatively substantial amounts of chitin. Some bryozoans play a role in the epuration of fresh water and produce a considerable biomass of chitin-containing colonies as well.

The proportion of "free" chitin, i.e., not bound to other organic molecules, is generally low (less than 10%), although it can account for 80% of the total chitin in mollusc shells.

INTRODUCTION

The first comprehensive studies of chitin distribution in animals (9, 10,20,26,28) were based on histochemical methods, such as the chitosan test by Campbell (5). These methods lacked specificity, however, and were sometimes unreliable, especially with small animals or when the amount of chitin was low (23,25). The more recent x-ray diffraction method (27) gives accurate but only qualitative results.

In order to obtain both qualitative and quantitative data on chitin

occurrence and localization, an enzymatic method based on the use of purified chitinases was developed (13,14). Owing to the specificity of chitinases (Enzyme Nomenclature:3.2.1.14) for the β-1,4 glucosidic linkages in N-acetylglucosamine polymers, this enzymatic method is highly specific for chitin, provided the purified chitinase preparation is devoid of other hydrolase. Moreover, this method enables us to discriminate the "free" chitin from the chitin chemically bound to other substances (14).

This distribution of chitin biosynthesis in animals has already been discussed from an evolutionary point of view (13,14,15). The aim of the present paper is to try to summarize the numerous data so far obtained with regard to the main ecological features of chitin-containing animals.

METHODS

Chitin was identified and estimated by the enzymatic method of Jeuniaux (13,14). After desiccation under vacuum, the material was weighed, treated with 0.5 N HCl at room temperature, washed, weighed, then treated with 0.5 N NaOH for 6 hours at 100°C. After washing, the residual material was suspended in a buffer (citric acid 0.2 M - Na_2HPO_4 0.4M) at pH 5.2 and incubated for 4-8 hours at 37°C with 1 ml purified chitinase (0.9 mg/ml for 0.02-2 mg chitin), using thymol as an antiseptic. After centrifugation, an aliquot of the supernatant was incubated with chitobiase (lobster serum 10 times diluted with distilled water) at pH 5.2 at 37°C for 2 hours. The liberated N-acetylglucosamine was determined by the method of Reissig et al. (24). The results are expressed as mg of chitin per 100 mg dry organic matter (chitin %).

"Free" chitin is estimated by the same procedure, omitting any previous treatment with NaOH.

The enzymatic method was also used for the qualitative detection of chitin in small animals (3,13,15). After treatment with HCl and NaOH, the washed residues were stained with Congo Red, then incubated with chitinase and observed under the light microscope.

Chitinase was purified from submerged culture filtrates of Streptomyces antibioticus (11) following the procedure described by Jeuniaux (12,13,15).

RESULTS

Micro- and meiofauna

Among protozoa, chitin is used by most ciliates to build cyst walls (14 of 22 species so far studied) (4), or sheaths in the case of the sessile species (Folliculina) that can sometimes be abundant on marine substrates.

The mesopsammic meiofauna pluricellular species living in soft sediments are mainly Turbellaria, nemerteans, nematodes, rotifers and gastrotrichs, which are devoid of chitinous structures. The eggs of nematodes, rotifers and gastrotrichs are, however, often provided with chitinoproteic envelopes (14.6% chitin with respect to total dry weight of the amictic eggs of Brachionus leydigii) (6,17).

Kinorhynchs, small and scarce mesopsammic marine animals, are covered with a chitinous cuticle. Tardigrades, which are often more common, also

possess(contrary to the opinion of some authors) a relatively thick cuticle made of chitin, probably bound to proteins (3).

Endoparasites

Chitin was found neither in parasitic flatworms (Cestoda, Trematoda) nor in round worms (Nematoda), except in the egg envelopes of the latter (16.6% in ascarid eggs) (13).

Terrestrial invertebrates

Chitin is the structural polysaccharide of the cuticle of insects, arachnids and myriapods (20 to 80%). Terrestrial tardigrades (3) and onychophorans (27) also possess a chitinous cuticle. The setae of earthworms (Oligochaeta) are also typical chitinous structures (27). In the pulmonate gastropods, snail shells contain small amounts (3%) of chitin (13), mainly as "free" chitin.

Planktonic and pelagic animals

The marine zooplankton are a rich source of chitin, being mainly formed by small holoplanktonic crustaceans and, in certain periods, by the meroplanktonic larvae of pelagic or benthic crustaceans. Their cuticular organic matter contains about 60-80% chitin.

Planktonic and pelagic coelenterates (Hydrozoa, Scyphozoa, Ctenophora) are devoid of chitin, with the exception of the pelagic colonies of Chondrophoridae such as Velella, the floating apparatus of which is a chitinous perisarc, as previously stated (10,22), containing about 48% chitin, almost entirely as "free" chitin.

The most important chitin-producing pelagic animals are the cephalopods, mainly cuttlefishes, the shells of which sometimes accumulate on the beaches with the tide. Chitin accounts for 26% of the organic matter of Sepia officinalis shells, and for 17.9% of squid (Loligo vulgaris) pens, mainly as "free" chitin.

Benthic marine fauna

Most benthic marine invertebrates produce chitinous structures, with the exception of sponges, flatworms (Turbellaria), nemerteans, echinoderms, sipunculids, pterobranchs and enteropneusts (7,13,27). The thick mantle, or tunic, of the sessile tunicates (sea squirts) is devoid of chitin, but these animals secrete a chitinous peritrophic membrane (21).

In crustaceans, the proportion of chitin in the cuticle is about 65-85% of the dry organic matter.

In molluscs, chitin was found in variable amounts in a wide variety of morphological structures (8,13,23), mainly as "free" chitin. The proportion of chitin varied from 0.1 to 7.3% in the periostracum, traces to 0.2% in prisms, 0.1 to 1.2% in mother of pearl, and from 0.2 to 8.3% in the calcitostracum of bivalve shells, the higher values being found in burrowing species such as Glycymeris, Venus, Pholas, Zyrphaea and Mya. The gastric shield of bivalves is also made of chitin (27.7% in Zyrphaea crispata [1]). In marine gastropods, the proportion of chitin was 3.0 to 7.0% in

mother of pearl of 3 species of Prosobranchia, 19.7% in the radulae, and 36.8% in the gizzard teeth of an opisthobranch. In the shell plates of Acanthochites discrepans (Polyplacophora), chitin amounted to 12%.

In different kinds of setae of marine worms (Polychaeta), chitin accounts for 20 to 38% of the dry organic matter and is mainly bound to quinone-tanned proteins. The tubes secreted by tubicolous worms do not contain any chitin.

Besides this macrofauna, benthic communities of the continental shelf also include the encrusting or erected colonies of hydrozoans and bryozoans (Ectoprocta). In both cases, the cuticular organic matrix of these colonies is made of chitin more or less bound to proteins, calcified in many species. In hydrozoans, the amount of chitin varied from 3.2 to 30.3% (13). In whole colonies of bryozoans (Flustra, Scrupocellaria, Cellaria, Crisia), the amount of chitin was 1.6 to 6.4%. Despite their small size, hydrozoan and bryozoan colonies may comprise an important element in epifaunal communities and may thus represent a large biomass of chitinous organic matter.

Chitin was also found in the stalk cuticle of Endoprocta, in the tubes of Phoronidea (13.5%), and in those of Pogonophora (33%) (2,7). The shells of brachiopods seemed to be devoid of chitin, with the exception of those of some Inarticulata (Lingula, Discinisca) (9), in which chitin amounted to 29% of the dry organic matter (13). The cuticle of the pedicle was chitinous in all the brachiopods so far studied (9,13,27).

Benthic freshwater invertebrates

Besides insects and crustaceans, a few bryozoan species may develop important colonies in some semi-polluted waters and give rise to the production of a large biomass (19) mainly made up of a chitinoproteic exoskeleton (ectocyst) (9,13). The biomass of Plumatella fungosa colonies in a pond was estimated to be 15.6 tons/ha (fresh weight) and the annual production to be 112 Kg nitrogen/ha/year. These colonies play a prominent part in the process of water purification (18).

CONCLUSIONS

If chitin is mainly secreted by cells of epidermal origin, the endoderm layer is also able to synthesize this polysaccharide, not only in arthropods, annelids and even tunicates (peritrophic membranes), but also in molluscs (gastric shields and gizzard plates).

The biosynthesis of chitin is a very old property of the animal cell, already present in Protozoa. This property was retained by most invertebrate animals of those groups belonging to the protostomian evolutionary lineage. At the top of this lineage, arthropods have exploited to a maximum the ability to use chitin as a structural polysaccharide, chitin often constituting, indeed, more than 50% of the cuticular organic matter. However, chitin may also be found in appreciable amounts in annelids, molluscs and in hydrozoan and bryozoan colonies, which form an important part of the marine benthic biomass.

ACKNOWLEDGMENT

This work was supported by grant no. 2.4567.75 of the Fonds de la Recherche Fondamentale Collective.

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THE DISTRIBUTION AND QUANTITATIVE IMPORTANCE OF CHITIN IN FUNGI

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ABSTRACT

Chitin is the second most abundant organic compound on the earth, and fungi constitute its main source. Chitin is present in the vast majority of fungi as the principal fibrillar polymer of the cell wall. As such, it is responsible for the rigidity and the shape of the wall. Chitin is also present in the cytoplasm of some Oomycetes in the form of special granules (cellulin granules). The only major classes of fungi which lack chitin are the Schizomycetes, Myxomycetes and Trichomycetes. Those Oomycetes and Hyphochytridiomycetes which contain chitin also contain cellulose. Zygomycetes contain chitosan and chitin, whereas Ascomycetes, Basidiomycetes and Deuteromycetes, with the sole exception of yeasts, contain chitin as the only structural polymer. As a group, Euascomycetes are the fungi which contain the highest amounts of chitin, followed by Zygomycetes, Basidiomycetes and Deuteromycetes. Hemiascomycetes contain the lowest amounts of chitin. Highest values reported correspond to Allomyces macrogynus (58%) and Sclerotium rolfsii (61%). In general, conidia contain lower amounts of chitin than mycelium. In the case of dimorphic fungi, there is no correlation between cell shape and the relative amounts of chitin present in the cell wall.

INTRODUCTION

In 1811 Braconnot described a substance which he found in fungi and which he called "fungine" (31). It was not until the end of the century that the substance was rediscovered and identified as chitin, which had been known to be present in Arthropoda. X-ray diffraction studies have revealed that the degree of crystallinity of chitin from most fungi is similar to that present in invertebrates.

It was later demonstrated that chitin in fungi was located in the cell wall. Oomycetes belonging to the order Leptomitales contain, in the cytoplasm, special granules called cellulin granules. These granules were thought to be made of modified cellulose (28), but recent analyses of the genus Apodachlya have shown that they are made of β-1-3, β-1-6 glucans and chitin (20). These granules constitute the only exception where chitin is not present in the cell wall but in the form of a cytoplasmic inclusion.

Chitin is not the only compound present in the cell walls of fungi and, in fact, it may be a minor component. Other compounds present in the cell wall are cellulose, other polysaccharides, proteins and lipids.

Chemical composition of the cell walls of fungi

Two types of components can be recognized in the cell wall of fungi: structural components and amorphous components. Amorphous components comprise lipids, proteins and different types of polysaccharides. They are

important for the resistance of the wall and for the protection of the cell from different harmful compounds. Besides, some proteins of the cell wall have enzymatic activity. Structural components are responsible for the shape and rigidity of the cell wall. Using microchemical tests, van Wisselingh (40) recognized that fungi had either cellulosic or chitinous walls. Further refinement in the analytical methods sheds some doubts on the results obtained with microchemical reactions, but more recently it has become evident that fungi have chitin, cellulose and other β-glucans as their structural polymers.

Chitin is the sole structural component of fungal cell walls, where it is responsible for their shape and rigidity. As evidence for this statement, it can be shown after the removal of amorphous cell components from the cell that the cell wall maintains its original structure (33). Treatment of whole cells with chitinase seldom removes the cell wall or gives rise to protoplasts. This failure to hydrolyze the cell walls is due to the inaccessibility of the chitin to the enzyme. On the other hand, once the amorphous cell components are removed, chitin becomes accessible to chitinase, is hydrolyzed, and the shape of the cell wall is lost.

Particular fungi mutants, unable to synthesize chitin, form swollen hyphae and are osmotically sensitive (19).

Polyoxins, a group of antibiotics produced by Streptomyces cacoi, interfere with chitin biosynthesis (13). This antibiotic inhibits the growth of fungi (16,17), and at suboptimal concentrations induces morphological alterations of the cells (6,8).

Zygomycetes contain chitosan, besides chitin, in their cell wall. Apparently, in these fungi, chitosan plays an important role in the rigidity of the wall, since their cell walls are lysed by a chitosanase preparation obtained from Streptomyces sp.(29). This preparation does not lyse fungal chitin.

There is a strong correlation between the chemistry of the cell wall and the taxonomy of fungi. Bartnicki-Garcia (3) has distinguished 8 groups of fungi according to the chemistry of their cell walls (Table 1). These chemical groups coincide closely with accepted taxonomic and evolutionary concepts.

Chitin is present in most fungi and, according to this classification, it may be the only structural component (groups V and VII), or it may share this role with cellulose in group III and in some Oomycetes (22,23) not considered in Table 1, or with chitosan (group IV). In yeasts, chitin is only a minor component, and it is localized in the rim of the bud scars (10).

TABLE 1

CHEMOTYPES OF FUNGAL CELL WALLS*

	CHEMOTYPE	TAXONOMIC GROUP
I	Cellulose-glycogen	Acrasiales
II	Cellulose-glucan	Oomycetes**
III	Cellulose-chitin	Hyphochytridiomycetes
IV	Chitosan-chitin	Zygomycetes
V	Chitin-glucan	Chytridiomycetes
		Ascomycetes
		Basidiomycetes
		Deuteromycetes
VI	Mannan-glucan	Saccharomycetaceae
		Crytococcaceae
VII	Mannan-chitin	Sporobolomycetaceae
VIII	Polygalactosamine-galactan	Trichomycetes

*From Bartnicki-Garcia (3)
**Some members of the order Leptomytales have been shown to contain also chitin in the cell wall (22, 23).

METHODS

Detection of chitin in cell walls of fungi has been achieved by the classical microchemical test of van Wisselingh (40). This method may give erratic results, however, so its use has been mostly abandoned as a reliable test for chitin. Several authors have made use of the characteristic insolubility of chitin as a method of detection. These authors regard a compound which is insoluble in acid and alkali and releases glucosamine by hydrolysis with 6N HCl to be chitin. More reliable is the use of infrared spectroscopy to detect chitin. Michell and Scurfield (26) made a careful study of the infrared spectra of standard compounds and found that chitin was easily recognized from other cell wall components by its characteristic bands. They compared the spectra of isolated and extracted cell walls from several fungi with the standards and recognized the presence of chitin and cellulose in the different genera examined. Infrared spectroscopy has been used to detect the presence of chitin in Aspergillus (33), Morchella (35) and Choanephora cucurbitarum (21). The method par excellence to detect chitin is the use of x-ray diffraction. X-ray powder diagrams have been extensively used to detect chitin in whole cells, isolated cell walls and extracted cell walls.

Galun et al.(15) used an ingenious method to detect the presence of chitin in three fungi isolated from lichens. These fungi grow so meagerly that it was impossible to collect enough material to carry out chemical analysis. The authors regarded the incorporation of N-|^{3}H| acetyl glucosamine and the binding of fluorescein-conjugated wheat germ agglutinin as evidence for the presence of chitin in the cell walls of these lichen fungi.

Quantitative determination of chitin involves measurement of hexosamines in acid hydrolysates of the cell walls. Since during hydrolysis N-acetyl hexosamines are deacetylated, the method does not distinguish between polymers of hexosamines and N-acetyl hexosamines. Most authors first extract alkali-soluble glycoproteins and acid-soluble chitosan before measuring chitin. It is also important to determine whether all the hexosamine released is glucosamine or if there are also other hexosamines, of which galactosamine is the most abundant. A gentler and more specific method to determine chitin is the measurement of N-acetyl glucosamine released by enzymatic hydrolysis with chitinase and chitobiase. This method has been seldom used and then only for specific purposes (11).

Microfibrillar structure of the cell walls of fungi

Several fungi which contain chitin in their cell wall have been examined by electron microscopy. It is a general observation that the outer surface of the cell wall appears rather smooth, or at most granular, whereas the inner surface shows the presence of microfibrils. The microfibrillar appearance of the cell walls becomes more apparent when these are extracted with acid and alkali or treated with specific enzymes to remove amorphous compounds. Microfibrils have been observed by electron microscopy, mostly after shadowing, but also heavy metal replica have been prepared (21). Negative staining of cell walls from Histoplasma farciminosum (36) revealed the presence of microfibrils, both isolated and in bundles, measuring ca. 6 nm. Similar microfibrils 2-7 nm wide have been observed in oblique and tangential sections of Gilbertella persicaria (9).

In most fungi studied, microfibrils do not follow a particular orientation, but rather they are randomly oriented. Nevertheless, Scurfield (37) described that in the inner surface of Polyporus millitae microfibrils showed a strong tendency toward a transverse orientation. In cross walls of the same fungus, microfibrils were circularly arranged around a central pore. A similar arrangement of chitin microfibrils has been described in the septa of Chaetomium globosum (25).

Presence and content of chitin in fungi belonging to different taxonomical groups

As mentioned above, chitin is present in most fungi. Using microchemical tests, infrared spectrophotometry and x-ray diffraction, it has been shown to be present in the cell walls of selected species of most taxa: Chitridiomycetes, Hyphochytridiomycetes, Oomycetes, Zygomycetes, Deuteromycetes, Ascomycetes and Basidiomycetes. Chitin has not been detected in Schizomycetes, Myxomycetes and Trichomycetes.

It had been generally considered that Oomycetes lacked chitin in their cell walls, but x-ray diffraction studies by Lin and Aronson (22) revealed the presence of chitin and cellulose in the cell wall of Apodachlya sp. and more recently in the cell wall of the related species Leptomitus lacteus (23). These fungi contain in their cytoplasm unique granules, cellulin granules (28), which are composed of glucan and chitin (20). By x-ray diffraction, chitin and cellulose have been found also to co-exist in the cell wall of the hyphochytridiomycete Rhizidiomyces sp. (14) and in the ascomycete Ceratocystis ulmi (32). By use of infrared spectrophotometry it was found that the deuteromycete Epicoccum sp. possibly

contained both chitin and cellulose (26). This result has not been corroborated by use of x-ray diffraction.

Quantitative data of the content of glucosamine in the cell walls of fungi belonging to different taxa are shown in Table 2. Data were calculated from the tables recapitulated by Bartnicki-Garcia (4), and were completed with data that appeared in the more recent literature. Some data were originally reported as glucosamine and other as N-acetyl glucosamine. In general, they are regarded as a reflection of the amount of chitin present in the cell walls. Data are expressed as the mean of % glucosamine in the several species examined. Standard deviation and the lowest and highest values reported are included. Mode was calculated only for those groups which had enough representatives to give meaningful results and where data showed modal distribution. Fractional data were taken to the closest integer to calculate mode.

TABLE 2

GLUCOSAMINE CONTENT OF CELL WALLS FROM FUNGI BELONGING TO DIFFERENT TAXONOMIC GROUPS

TAXONOMIC GROUP	GLUCOSAMINE CONTENT (%)				
	MEAN	ST. DEVIATION	LOWER	HIGHER	MODE
Oomycetes	2.3	4.3	0.1	18.2	2
Chytridiomycetes	58.0	-	-	-	-
Zygomycetes	15.0	10.1	2.1	31.0	-
Hemiascomycetes	1.5	0.9	0.05	2.9	1
Euascomycetes	17.6	10.6	5.1	48.0	7.12
Loculoascomycetes	14.8	10.0	4.8	38.0	-
Homobasidiomycetes	12.4	16.7	1.6	61.0	8
Heterobasidiomycetes	4.5	-	3.7	5.4	-
Deuteromycetes	10.5	9.6	0.35	29.7	-

In the case of Oomycetes, the small amounts of glucosamine detected in the cell walls are not supposed to be present in chitin. Only the higher value (18.2%) which corresponds to Apodachlya sp. is considered to be chitin (23).

The only chytridiomycete reported, Allomyces macrogynus (1), contains one of the highest values of chitin, but it may not be representative of the whole class. As a group, Euascomycetes are the fungi with the highest content of chitin. The lowest value (5.1%) corresponds to a particular strain of Neurospora crassa (24); however, other strains of N. crassa have as much as 17.7% chitin in the wall. The highest value reported corresponds to Aspergillus oryzae. Hemiascomycetes are the taxonomical group that contains the lowest amounts of chitin in the cell wall, and,as mentioned above, it is restricted to the bud scars. The lowest value reported is from Hanseniospora uvarum, and the highest value from Saccharomycopsis

gutulata (4). Zygomycetes and Loculoascomycetes contain similar amounts of chitin. The lowest value for Zygomycetes corresponds to conidia from Mucor rouxii (3) and the highest value to the sporangiophore from Phycomyces blakesleeanus (27). Zygomycetes contain chitosan besides chitin in their cell walls. Since chitosan is solubilized by hot acid, the reported values of glucosamine correspond to that released by hydrolysis of the acid-insoluble residue. The lowest value for Loculoascomycetes was reported for the marine fungus Leptosphaeria albopunctata (39), and the highest value corresponds to the phytopathogen Cochliobolus miyabeanus (4).

Homobasidiomycetes contain lower amounts of chitin than Zygomycetes and Loculoascomycetes, but a minor number of genera have been analyzed. Lowest and highest values reported correspond to the same species Selerotium rolfsii, the lowest being for sclerotia and the highest for mycelium (4).

Even more minor amounts of chitin are present in the cell walls of Deuteromycetes; the lowest value was reported for Candida utilis and the highest value for Epidermophyton floccosum (4).

The only member of Heterobasidiomycetes whose cell wall has been analyzed is Tremella mesenterica (30). The yeast form contains lower amounts of chitin than the conjugation tube (see below).

From the collected data it is evident that there are large fluctuations in the chemical composition of the cell wall from different fungi belonging to the same taxonomical group, and even of different strains of the same species. A criticism which can be made of the analyses reported is that fungi were grown under different conditions, in media of variable composition, and for different periods of incubation. The cell wall cannot be considered as a static structure, but it may suffer gross changes in its composition depending on the conditions of growth. It has been demonstrated that synthesis of chitin continues after protein synthesis has been blocked by addition of cycloheximide (38).

Contents of chitin in spores and mycelia

There are only a few reports where the composition of cell walls from conidia has been compared with that from the mycelium or the sporophore. Collected data are shown in Table 3. With one exception, spores contain lower amounts of chitin than mycelium. The most striking differences correspond to Mucor rouxii, whose spores contain only 2% chitin compared to 9% of the mycelium and 18% of the sporophore, and particularly Trichoderma viride whose conidia have no chitin, whereas the mycelium contains 12-22% chitin depending on the age of the culture (7).

TABLE 3

RELATIVE AMOUNTS OF CHITIN IN THE CELL WALL OF FUNGI AT DIFFERENT STAGES

FUNGUS	STAGE	CHITIN (%)
Mucor rouxii	Mycelium	9.4
	Sporangiophore	18.0
	Sporangiospore	2.1
Aspergillus phoenicis	Mycelium	23.7
	Conidia	36.2
Neurospora crassa	Mycelium	8.0-11.9
	Conidia	7.4-9.0
Penicillium chrysogenum	Mycelium	19.5-42
	Conidia	11.4
Trichoderma viride	Mycelium	12-22
	Conidia	0

Comparative amounts of chitin in the cell walls of dimorphic fungi

There are fungi which show different morphology when grown under different conditions. They are called dimorphic fungi. Cell walls from the yeast and mycelial forms of several dimorphic fungi have been analyzed, and the results are summarized in Table 4.

TABLE 4

RELATIVE AMOUNTS OF CHITIN IN THE CELL WALL OF SOME DIMORPHIC FUNGI

FUNGUS	MORPHOLOGICAL STAGE	CHITIN (%)
Mucor rouxii	Mycelium	9.4
	Yeast	8.4
Saccharomycopsis gutulata	Mycelium	2.3
	Yeast	1.7
Blastomyces dermatitidis	Mycelium	13
	Yeast	44
Histoplasma capsulatum	Mycelium	4, 25.8
	Yeast	25, 11.5
Histoplasma farciminosum	Mycelium	40
	Yeast	25.8
Paracoccidioides brasiliensis	Mycelium	11
	Yeast	37
Tremella mesenterica	Yeast	3.7
	Conjugation tube	5.4

There is no correlation between the morphology of the organism and the content of chitin. Mucor rouxii, Saccharomycopsis gutulata and Histoplasma farciminosum contain slightly higher amounts of chitin in the mycelial form than in the yeast form; whereas Paracoccidioides brasiliensis and Blastomyces dermatitidis contain more chitin in the cell wall of the yeast form. Conflicting reports exist on the chemical composition of the cell wall from Histoplasma capsulatum; whereas Domer et al.(12) reported that the yeast form contained more chitin than the mycelial form (25% vs. 4%). Kanetsuna et al.(18) reported that the mycelial form contained 25.8% chitin and the yeast form only 11.5%. Interestingly, the related species Histoplasma farciminosum (36) is the dimorphic fungus which contains the highest amount of chitin in the mycelial form.

Included in Table 4 are data on the composition of the cell wall from the basidiomycete Tremella mesenterica. This organism grows in a yeast-like form, but a long conjugation tube is induced by the presence of specific hormones (2). Analyses of the cell walls from the yeast and the conjugation tubes revealed that the latter contained larger amounts of chitin (30).

From the data presented in Table 4, it becomes apparent that the mere difference in the relative amounts of chitin in the cell wall cannot explain the difference in morphology. Cell shape is probably determined by the pattern of growth of the cell wall. It has been shown (5) that filaments grow only in the apical region, whereas yeasts grow uniformly throughout the cell surface. Alternatively (or simultaneously), differences in the regulation of chitin synthetase may explain the pattern of wall construction (34).

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THE DETECTION AND ESTIMATION OF CHITIN IN INSECT ORGANS

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ABSTRACT

Chitinous membranes are never pure in nature. Usually some chemical purification is necessary before applying a test, and for small and delicate parts the purification is of uncertain validity. Several techniques that are valid with robust samples (e.g., x-ray diffraction) cannot be applied to tiny delicate objects in a heterogeneous medium.

The presentation will cover the working definitions used by various workers. These include (1) simply weighing the residue remaining after prolonged extraction with NaOH; (2) determining the amount of glucosamine or acetylglucosamine after acid or enzymatic hydrolysis of such residues from extraction with alkali; (3) the incorporation of C^{14} labeled glucosamine or acetylglucosamine into an alkali-insoluble component; (4) the classical van Wisselingh chitosan color test with iodine; and (5) the fluorescent chitinase reaction.

INTRODUCTION

My remarks will be of little interest to those whose concern with Arthropods is only as the raw material for the manufacture of chitin and chitosan. As a biologist, I am interested in questions such as: Are all membranes that are dispersed by hot alkali really devoid of chitin? Do chitin percentages range in a continuous series from 60 or 80% down to zero? And, if similar appearing membranes occur with and without chitin, then what is the significance of chitin? These questions cannot be definitely answered today. How close can we come?

METHODS

No pure samples of chitin are known to occur naturally, and to judge from data such as Rudall's x-ray diffraction studies, chitin does not occur free in cuticles but only in association with protein to which it is bound in some manner. In practice, chitin is a substance that is not dispersed by hot 1 N NaOH. Further characterization may or may not be done. The residue from alkaline purification may be quantified by either gravimetric determinations or by estimation of glucosamine (GA) or acetylglucosamine (AGA) following acid or enzymatic hydrolysis. By definition, then, any units which are removed during 'purification' would not be called chitin. The inadequacy of this conclusion will be dealt with in discussing the van Wisselingh chitosan color test.

In 1971 Hackman and Goldberg (2) proposed a semi-micro method of chitin

analysis. This involved dissecting off integuments, cleaning off the epidermal cells by swabbing in 70% ethyl alcohol, extracting with chloroform-methyl alcohol (2:1), drying, and powdering in a Wiley mill. The powder was extracted 2X with 1 N NaOH at 100° for 24 hours, and the supernatants discarded. Without removing the residue from the tube, it was washed successively with water, 2 N HCl to remove adsorbed alkali, 6X with ethyl alcohol and 3X with diethyl ether. Tubes with residues were dried and weighed. Then the 'chitin' was removed and the tube washed and re-weighed. Stated values for chitin were, then, the difference in weight of tube + chitin minus tube alone. Using the above method, the authors concluded that the cuticles of ticks contained 3.8% chitin and the membranes of a bloodsucking bug 11.2%. While the authors did not state it, this technique assumes that all chitin is insoluble in 1 N NaOH and none disperses in any way to become lost (remember that the supernatants were discarded); it also assumes that nothing else is insoluble in 1 N NaOH. Clearly, the method does not employ balance-sheet procedures that account for all components.

For the peculiar 'living fossil' Peripatus, Hackman gave a value of 8% chitin in the cuticle based on calculations from the GA content of acid hydrolysates of alkali-treated cuticles. Many more such values have been given by Jeuniaux from enzymatic hydrolysates of alkali-treated cuticles. Jeuniaux obtained lower values, sometimes much lower values, when the cuticles were not first 'purified' by treatment with alkali. This is the genesis of Jeuniaux's concepts of 'free' and 'bound' chitin which explains higher values from alkali-treated cuticles as being due to the removal of masking by certain chitin-protein associations. GA and AGA are found in the hydrolysates of numerous things other than chitin, but the tacit assumption is made that such alternative sources of GA and AGA have all been removed by treatment with hot 1 N NaOH. Again we encounter the idea that chitin is immune to alkali which, however, allegedly removes everything else (exceptions can be cited for both points in this sentence).

The incorporation of C^{14} labeled GA or AGA into an alkali-insoluble fraction has been reported. While this can be a good approach for studying chitin deposition, it does not seem useful for the identification of chitin as it appears in naturally occurring structures.

This leads to a consideration of the time-honored van Wisselingh chitosan color test. This test involves partial or complete deacetylation of chitin with hot conc KOH to produce chitosan. The more or less deacetylated chitin reacts with iodine at low pH to give a characteristic violet color. Since no other compound resistant to hot concentrated alkali is known to give this color reaction, it is considered specific. Even though the test seems to be reliable when positive, it is still dubious in terms of universal applicability.

In the first place, any object being tested must still be recognizable after heating in conc KOH for some minutes. Since such drastic treatment with alkali destroys most biological structures, the object being tested must not only be durable, but also sufficiently large to be found and identified. Thus, tracheoles (the terminal branches of the respiratory tree in insects) are said to be dispersed, but their size is such that one wonders if they are really dispersed or just no longer recognizable.

More obvious is the case of delicate structures that disintegrate under the normal van Wisselingh procedure, but which if treated less violently

may survive to give a positive chitosan color test. The minute scales on the wings of butterflies and moths are good examples. In many species these contain enough chitin to survive the most violent alkaline treatment. In other species some of the scales will only survive a less hot, less concentrated or less prolonged treatment--and then give a positive color test. Scales of a very few species never survive treatment with hot alkali. Results with scales of more than 100 species are tabulated by Richards (5). These homologous structures from different species of this group of insects form a series in which some give strongly positive test results and hence may be presumed to contain a considerable percentage of chitin. Others contain either less or more readily dispersed chitin, and some seem to be negative--whatever invariable dissolution in hot alkaline solutions means.

The results from these tests, reported 30 years ago, seem clear. Something giving the van Wisselingh color test for chitin can be dispersed by alkali. What, then, is the validity of chitin determinations based simply on alkali-insoluble residues, or GA or AGA determinations from such alkali-insoluble residues? As a biologist, perhaps I can be excused for asking why no qualified chemist has addressed himself to the problem of what is dispersed from such preparations by alkali. Chitins of various molecular weight, such as described by Strout, Lipke and Geoghegan, may be part of the answer; we do not know.

One would think that there must be some minimal concentration of chitin for a structure to remain recognizable after removal of other components. Most chitin-containing integuments are reported to contain 30-40% chitin on a dry-weight basis (3, 6). A few insect species have values as high as 60% (some decapod Crustacea have values up to 85%, if one considers only the organic components), but a few are recorded as having only 2-4%. These low values have sometimes been induced only by gravimetric determinations of the amount of alkali-insoluble residue, but in other cases, they are calculated from AGA content. It would indeed be interesting to know if the component called chitin in these cases with low percentages was really the same as chitin present in high proportions. I wonder if the determinations resulting in reporting such very low percentages are valid. I do not know of any case where a report of very low percentage has been accompanied by identification of all that was removed.

With the uncertainties just itemized, I welcomed the report of the development by Benjaminson (1) of a fluorescent-enzyme technique that seems to hold great promise. He conjugated chitinase with either fluorescein isothiocyanate or lissamine rhodamine B 200 chloride and took advantage of the attachment of an enzyme to its substrate to localize chitin by fluorescence microscopy. This method is comparable to the powerful fluorescent-antibody technique that has been so valuable in immunology. However, the description indicated that only a minimal amount of testing had been done. Benjaminson showed only that the method could work. We bought some chitinase from a commercial source and prepared the fluorescent enzyme only to find that the chitinase was so impure that many things were stained. We attempted to purify the enzyme, and did clean it up by column chromatography to the point that other things were only faintly stained when known chitinous structures were intensely stained. Chemists at the laboratory where Marks & Leopold (4) work did a better job, and nice looking pictures resulted (Figs. 1-6). This technique does not have the ambiguity arising from a preceding treatment with alkali.

There has still been inadequate testing of the fluorescent-enzyme method.

The above authors used only normal cuticles that were not of great delicacy. Presumably these contained the usual concentration of α-chitin. Tests have not yet been made to check the method with all the known crystallographic types of chitin (α, β and γ), with low as well as high molecular weight chitins, with 'free' versus 'bound' chitin (3), with chitosan, with dispersed and regenerated chitins and chitosans, or with membranes which are dispersed by alkali (tracheoles, epicuticle, air sacs of bees, etc.). Presumably, like histochemical methods in general, the fluorescent-enzyme method will be a qualitative test not readily made quantitative. However, qualitative tests are good for identification and localization.

In conclusion, numerous real advances in chitin chemistry have been made in recent decades, but the ability to identify chitin with certainty in small and delicate structures has advanced little. In the 8 years since it was described, the fluorescent-chitinase technique has been used by only one set of authors. It has real promise but needs extensive evaluation.

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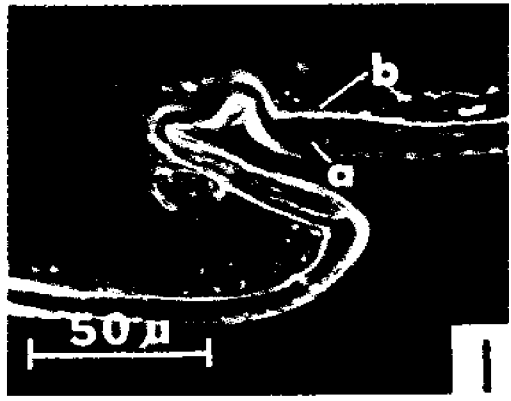

Figure 1. A section through the cuticle of a leg of a freshly molted cockroach as seen in a phase-contrast microscope. a = cuticle; b = epidermis. All photographs from Marks & Leopold (4).

Figure 2. Same specimen stained with fluorescent-chitinase and observed in a fluorescent microscope. Note that only the cuticle is stained.

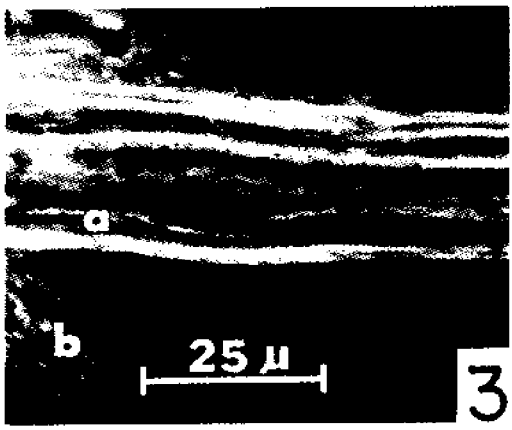

Figure 3. Section through the cuticle of a regenerating leg at 25 days in vitro as seen in a phase-contrast microscope. a = cuticle; b = epidermis.

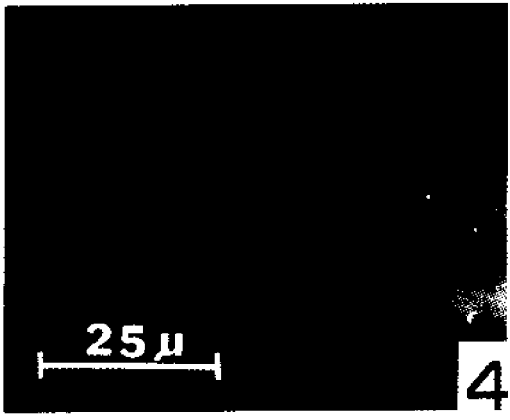

Figure 4. Same specimen stained with fluorescent-chitinase and observed in a fluorescent microscope. Note low intensity of fluoresence in contrast to the normal leg (Fig. 2).

Figure 5. Section through the cuticle of a regenerating leg at 10 days in vitro as seen in a phase-contrast microscope. a = refractile droplets; b = cuticle; c = epidermis.

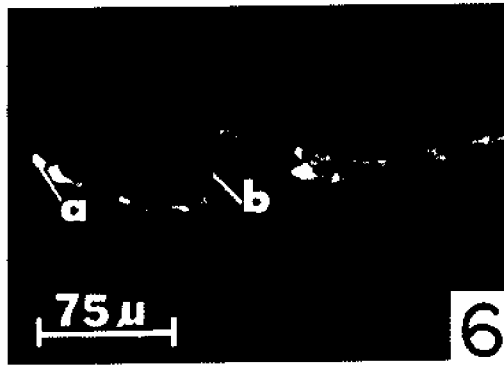

Figure 6. Same specimen stained with fluorescent-chitinase and observed in a fluorescent microscope. The thin cuticle (b) appears as a bright line; the refractile droplets (a) between cuticle and epidermis are also fluorescent and hence assumed to contain chitin.

II. ECONOMIC CONSIDERATIONS OF ALTERNATIVE SOURCES OF CHITIN

APPROACHES TO A PRACTICAL ASSESSMENT OF SUPPLY AND DEMAND FOR CHITIN PRODUCTS IN THE UNITED STATES

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ABSTRACT

Successive waves of interest in chitin development over the past several decades have stimulated a considerable quantity of technical literature and patents for numerous types of applications, but relatively little sustained production and use. We shall discuss a practical analytical framework for making realistic assessments of:

- the likely quantity and cost of chitin producible from the shellfish waste available in the United States;
- the magnitude of likely demand for chitin-derived products at specific prices.

Considering the supply realities, we suggest the general form of a business strategy we feel is likely to succeed in achieving economic viability. Our overall conclusion is that it appears to be commercially feasible to produce between one and four million pounds of chitin/chitosan per year for sale at a price between $1.00 and $2.50 per pound.

INTRODUCTION

Estimates of large quantities of crustacean shell waste available for chitin production have been, in part, responsible for stimulating or enhancing practical interest in chitin. On the other hand, difficulties and uncertainties of supply, as well as uncertainties in market potential, have apparently been a factor in the abandonment of some commercial ventures in this field. Since these problems are crucial to assessing the commercial prospects of chitin, they have received the major emphasis in a study we have recently completed under the sponsorship of the M.I.T. Office of Sea Grant (5). The aim of the study was to consider whether and how chitin might profitably be produced and sold, to obtain reasonable expectations of the possible quantities, and to assess business arrangements by which this might come about. Today, we would like to suggest a sound approach to this type of assessment and to briefly present some results obtained utilizing that approach.

ESTIMATING ACCESSIBLE SHELL WASTE SUPPLY

Impressively large estimates of potential chitin production in the United States have been inferred from statistics on total shellfish landings, or other methods that do not critically consider the scattered distribution or the practical cost of gathering the necessary material into shell waste processing plants. When these factors are considered, virtually all of the lobster shell, and substantial amounts of shrimp and crab shell, must be eliminated as not practical for collection. Our own estimates are based on detailed tabulations of the quantities of different processed shellfish products produced in every state (6). We exclude as practical chitin

sources those products which are marketed still in the shell and others which are produced from imported shellfish in pre-shelled frozen blocks.

Beyond this, it is important to consider that even those products which are landed and shelled in this country produce a shell waste which is highly perishable, of low density, and has a substantial amount of water.* Transportation of this material is, therefore, rather expensive relative to its potential value. Because of these factors, a tentative assumption was made that maximum trucking distance for wet waste would be on the order of 50 miles. The assumption allowed us to define 50-mile radius circles, centered over major processing areas, so as to include the maximum number of waste-producing plants. Circles were applied to all areas containing any substantial supply.

Within each circle available waste was estimated, using appropriate conversion figures for the species and the shelling process involved. For crab, the ratio of solid waste to live weight was taken as 60-75%, while the range of shrimp landed without heads was 9-22%, accounted for by variation in species, size, season of the year and processing method. Shrimp heads were computed separately, based on area-by-area surveys of the frequency with which shrimp are landed with heads on, and taking account of the considerable differences between large and small species.

RESULTS OF THE SUPPLY ASSESSMENT

When waste production was calculated for the 23 encircled areas, the total came to 149 - 192 million pounds, not counting Alaska. Addition of Alaska brought it to 237 - 302 million pounds. This material consists of shrimp body peelings, shrimp head waste and crab waste, each of which has a different chitin content. The chitin yield, if all of this were processed, would be between 7.4 and 11 million pounds, not counting Alaska, or a grand total of between 11.7 and 17.4 million pounds, if Alaskan material were utilized.

Of course, for numerous reasons, it is not in the near future practical to utilize all of this material. Even if there were a ready chitin market, several of the encircled areas do not appear to have enough waste to make chitin production in those areas profitable.

In a chitin plant, certain economics of scale would be necessary for economic viability, and shipment of materials to or from some points, especially in Alaska, can impose unbearable costs. Before many of the areas could be considered as practical sources, special conditions would be necessary.

PROSPECTIVE BUSINESS ARRANGEMENT

Seeking special conditions that could improve the profitability of shell processing in remote or modestly endowed areas, a business strategy involving both chitin and protein from shellfish waste was developed and evaluated. The strategy treats chitin as a by-product of a protein recovery process that, in some cases, may be profitable even in the absence of any

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*The water content of shell waste (60-80%) in particular may have been a source of confusion leading to earlier optimistic estimates of total potential U.S. chitin supply.

market for chitin, and would produce a quantity of dried shell waste sufficient to supply a developing chitin market. The business plan would be for certain strategically located shellfish or food-waste processors to reduce shell waste to two dried components: (1) a mechanically separated protein material for use in animal feed and (2) the remainder of the dried waste for sale to a regional or national producer of chitin derivatives. Such an arrangement would solve many processors' waste problems and allow them a reasonable profit, considering all credits, without requiring them to enter fields far outside their present expertise. It would also provide a larger aggregate supply of chitinous shell residue to a chemical processor whose capabilities are more certain to include appropriate process, product and market development capabilities.

Other options are possible, provided that both supply and marketing potentials are sufficiently assured. Thus, it is feasible for a seafood company, already in command of a substantial supply of waste, to process this material all the way to chitin or its derivatives, or for a chemical company (especially one with food chemical operations) to acquire a large reliable supply of raw waste for conversion to sophisticated products. In both instances, some of the essentials for supply control, financial stability, and product development or marketing capability are already present.

COSTS, PRICES AND QUANTITIES

The cost of any form of shell processing, whether merely drying it or producing chitosan, would vary from region to region, partly because of differences in labor, fuel and chemicals, and partly because the scale of the operation and efficiencies dependent on it will be limited by the quantities of available waste. If the 2-stage processing business arrangement were chosen, the drying state, which is less demanding in scale efficiency, could be feasible in areas that could not support an independent chitin plant. If the output of several of these areas were funneled to one chemical plant, the combined advantages would include the maximum level of chitin production at the lowest overall cost. With this scheme in mind, we have speculatively computed what these numbers might be.

First state processing was assumed to include appropriate steps for collection of mechanically separable proteinaceous tissue superficially attached to the shell, and to result in two dried components: a high-protein by-product and the clean shell-waste residual. Since the protein by-product appears to have a market, the cost of producing the chitinous shell residual was computed as:

CSR = (net cost of collecting shell waste) + (cost of processing steps and associated business costs) − (credits for the sale of separable protein and reduction of waste-disposal costs)

A range of costs was computed for each producing area (defined by the 50-mile circle), based on a range of reasonably expectable circumstances and two alternative levels of production. It was assumed that a modest production level (one third of each area's theoretical potential) could generally be achieved by a single large processing company from wastes generated by its own operations, with no collection cost, and with a saving of $5/ton on waste disposal. For full production in each area, it was assumed that the remaining two-thirds of the available waste would be purchased from other

shellfish processors at $0-20 per ton and trucked up to 50 miles to the plant.

Space does not permit a full elaboration of all aspects of the optimistic and pessimistic sets of assumptions which were used to generate the ranges in estimated production costs for each area and level of production. Briefly, the major uncertainties which gave rise to the ranges were:

- Quantities of total waste available (leading to different economies of scale in both capital and operating costs)
- Composition of the waste (ratio of recoverable protein to other waste components)
- Future long-run selling price of the protein product. The optimistic assumption was that the protein would sell for a price equivalent to 30¢/lb. of 100% protein content. The pessimistic assumption called for a price equivalent to 23.3¢/lb. of 100% protein content. On the basis of fishmeal with 60% protein content, these prices would be equivalent to 18 and 14¢/lb. ($360 and $280 per ton) fishmeal prices.*
- Total labor cost (range: $6.50-9.00 equivalent per hour)
- Necessary before-tax return on investment to attract needed capital (range: 20-40% per year)

It was concluded that for certain areas on the Atlantic, Pacific and Gulf Coasts, and under the most optimistic set of assumptions, a shellfish processor holding at least one-third of the area's waste may be able to earn a sufficient rate of return on the protein alone to justify the required investment, without any need for revenue from sale of the dried shell residual. Under the most pessimistic set of assumptions, there may be no areas around Chesapeake Bay, the South Atlantic Coast and the Gulf Coast where the minimum required return on investment would be earned with shell residual prices of less than 15 cents per pound.

When dried shell residual costs are tabulated for all areas, the focus of study may then shift from the shellfish processor to the firm that will make and sell chitin derivatives. Using these data and other simple tools, it is possible to estimate the cost of various levels of, say, chitosan production and the prices that chitosan would have to bring to induce those production levels. Entering into the calculations are process-based cost estimates which include the purchase and rail transport of dried shell residual, cost of hydrochloric acid for demineralization and the cost of caustic soda for deacetylation. System-based cost estimates were made for wages, utilities and depreciation of capital equipment. By adding to this a minimum required return on investment and appropriate marketing costs, minimum unit prices for various quantities of chitosan were calculated.

For our estimates, several assumptions were helpful. Rail transportation was estimated at two to three cents a ton mile to Augusta, Georgia, where

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*It is conceivable that certain specialized animal feed uses might support higher prices than assumed in our study.

caustic soda and acid should be plentiful and reasonable, and which is well located between the major shellfish centers in the Atlantic and Gulf States. It was also assumed that a chitosan manufacturer will purchase dried shell residual from sources which will allow chitosan production at minimum unit cost. Other assumptions allowed for a doubling of the amount of hydrochloric acid theoretically required, and a ten-fold excess of caustic soda was allowed for maintaining desirable physical properties in the deacetylation mixture. Allowance was also made for the extra acid required for demineralization of crab shells over that required for shrimp shells.

These exercises resulted in a tabulation of the costs of chitosan production from shell residual obtained from each of the different supply areas. Entries were made for each area showing the amount of shell residual available, the cost which that supply would contribute to chitosan produced in Augusta, and the minimum required selling price for the resulting chitosan. Entries were arranged in increasing order of overall cost contribution and a running cumulative total of chitosan production was entered against each. The graphic representation of the supply function which such a table represents is shown in Figure 1. The lower and upper lines of this figure represent, respectively, the optimistic and pessimistic estimates of the minimum price of chitosan plotted against quantity produced. From an examination of this figure, we would conclude that chitosan production of between one and four million pounds per year could be feasible with a 20-40% return on capital investment, at chitosan prices somewhere between $1.00 and $2.50 per pound. What remains to be done is an assessment of the likelihood that the market would absorb those quantities if offered in that price range.

ANALYSIS OF POSSIBLE DEMAND

To estimate the quantities of chitosan that might be sold at various prices we have used two different approaches: First, we have examined the price-quantity histories for selected groups of commercial polymers having uses similar to some suggested for chitosan. By observing the precedent set by functionally related other materials, one may derive similar expectations for chitosan. Second, we have considered the reasonable market potential for some specific applications of chitosan.

Relationships Between Price and Quantity for Selected Groups of Commercial Polymers

Figures 2 to 5 show price-quantity relationships for four quite different polymer groups: general plastics, specialty plastics, cellulosics and water-soluble gums and starches. Each figure shows the cumulative quantity of all the different polymers in that polymer group which sold at or above a given price.* This kind of plot gives us some idea of the distribution of uses** for these materials which have supported given price premiums over the cheapest substances in the category. If a chitin derivative is technically successful in performing some of the functions now carried out by these materials, we can expect that it may, with effort,

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*For example, in the "Gums and Starches" plot, Figure 4, the total quantity of polymer selling at or above $2.40 per pound is the sum of the quantities of tragacanth, agar and pectin sold, or about 12 million pounds.

**It is not, strictly speaking, an economic demand curve.

capture some quantity of the market to the left of each line at some price below the line.

For three of the polymer groups, the general price-quantity relationship appears to be roughly log-linear (linear change in price for logarithmic change in quantity sold) whereas for the gums and starches the relationship appears to be more nearly log-log. We do not have a theoretical framework to explain either type of behavior.

We do find that when adjusted for inflation with the U.S. wholesale price index, these relationships appear to be relatively constant over time. For example, Table 1 shows the prices (corrected to April 1976 dollars) corresponding to one and ten million pound per year quantities of the various polymer groups inferred from price-quantity plots for different years. It can be seen that for constant quantity, the price predicted from the data for different years is essentially the same.

The appropriateness of any polymer group for predicting price-quantity relationships for chitin derivatives must depend on a judgment of whether chitin derivatives are likely to be able to find applications at least as economically valuable as current, successful members of the predictor group. The two groups which, on structural grounds, are most similar to chitin derivatives are cellulosics, gums and starches. It is noteworthy that, for the modest chitin production levels likely to be attainable in the U.S., the two groups suggest quite different potential prices. However, an important caveat must be attached to the use of the gums and starches polymer group as a potential precedent for future chitosan markets. Many of the most expensive members of the gums and starches group find primary usage in food as thickeners, stabilizers, etc. Chitin derivatives cannot be expected to find uses in food for some time because the long-term toxicological studies required for approval as a food additive are not yet available.

Even, however, at the conservative price levels suggested by comparison with the cellulosic polymer group, there appears to be a reasonable expectation that the market can absorb several million pounds of cellulosic polymer at prices between $1.60-1.90 per pound. This range is somewhat below the price needed to induce chitosan production under the most pessimistic set of assumptions on production costs, but it is above the midpoint of the range between the optimistic and pessimistic production assumptions. Thus, there is some indication that prices consistent with realistic producer requirements are also consistent with values associated with similar products.

Potential Demand for Selected Applications

The price-quantity record of polymers with similarity to chitin derivatives allows a very general projection of likely market behavior. The ultimate question, however, which cannot be resolved with the information currently available, is whether or not the specific properties of a chitin derivative will enable it to perform specific economically valuable functions at prices competitive with alternative materials for those functions. Table 2 lists five important properties of chitosan and the major types of economic uses which depend on those properties. In nearly all cases, good comparative cost-effectiveness data indicating the relative technical performance of chitosan and its major competitors for each function are lacking. Because of this, any assessment is incomplete and necessarily

TABLE 1

PRICE-QUANTITY RELATIONSHIPS FOR DIFFERENT POLYMER GROUPS -- DERIVED FROM SALES DATA FOR VARIOUS YEARS

Polymer Group	Year of Data	Indicated Selling Price of the Most Expensive (April 1976 dollars)*	
		1 million pound quantity in group	10 million pound quantity in group
General Plastics	1972	2.40	1.90
	1970	2.30	1.80
	1965	2.70	2.00
	1960	2.50	1.90
	1955	2.30	1.80
Specialty Plastics	1973	5.90	4.10
	1972	7.20	5.00
	1971	6.40	4.40
Cellulosics	1974	1.90	1.50
	1973	1.60	1.20
	1972	1.70	1.30
	1969	1.90	1.50
	1965	1.90	1.50
	1955	1.80	1.40
Gums and Starches	1972	9.10	2.60
	1967	7.40**	2.40**
	1963	6.70**	2.50**

*Adjusted with U.S. wholesale price index for all commodities.

**Very approximate values -- price-quantity relationships not well described as linear, even on log-log plots.

TABLE 2

SUGGESTED APPLICATIONS FOR CHITOSAN

Property	Uses
High charge density and potential binding capacity (relative to other substituted celluloses)	Ion-exchange or chelating solids for chromatography, metal recovery from waste streams, industrial-process water purification for cycling, etc.
Film-forming	Ion-exchange membranes for electro-dialysis
Coagulation	Waste-water treatment
Strong binding to negatively charged polymeric products	Paper-strength additive, dye binder for textiles, binding agent for non-woven fabrics, sausage-casing component, adhesives
Wound-healing promotion, non-thrombogenic	Wound treatments, surgical adjuncts

speculative, but we will summarize our major conclusions about likely markets in two of these areas.

Ion-exchange/chelating uses for chitosan appear to be among the most promising in terms of the economic markets. Current styrene-divinylbenzene ion exchange resins have total annual sales of several tens of millions of pounds. Binding and chelating are functions for which at least some tentative cost-effectiveness comparisons can be made between chitosan's theoretical properties and the properties of commercially available materials on the basis of binding capacity. If we assume that chitosan can match the commercial resins in other important properties, we can ask what the price of an 80% deacetylated (5 meq/gram) chitosan should be on an equal-binding-capacity basis in comparison with available weak base resins. Such a comparison, based on a 1974 price list and property data for various Rohm and Haas resins, suggests the equivalent value of chitosan to be between $2.35-6.41 per pound. More recent resin prices are likely to give somewhat higher values. From this viewpoint, it is not unreasonable to expect that this single market might absorb one or more million pounds of chitosan at prices comfortably above what is necessary to cover production costs and a reasonable return on investment. Such an outcome would, however, depend on developing actual products with packaging properties, stability, uniformity and other characteristics competitive with existing materials.

Recent investigations on coagulation applications provide another example of where some limited comparisons of cost-effectiveness seem to suggest an appreciable market at an acceptable price. In these studies, functional performance of chitosan yielded favorable comparisons with competing polymers selling in the range of $1.50 to $3.00 per pound (2-4). At present, this set of applications is reported to be supporting a price of $3.00 per pound in Japan (1).

Space does not permit full examination of the economic viability of the many other suggested applications of chitosan. Some of these will be the subjects of more specialized discussions by other speakers. We would strongly suggest to researchers, however, that the generation of good comparative cost-effectiveness data for these applications will greatly facilitate commercial exploitation.

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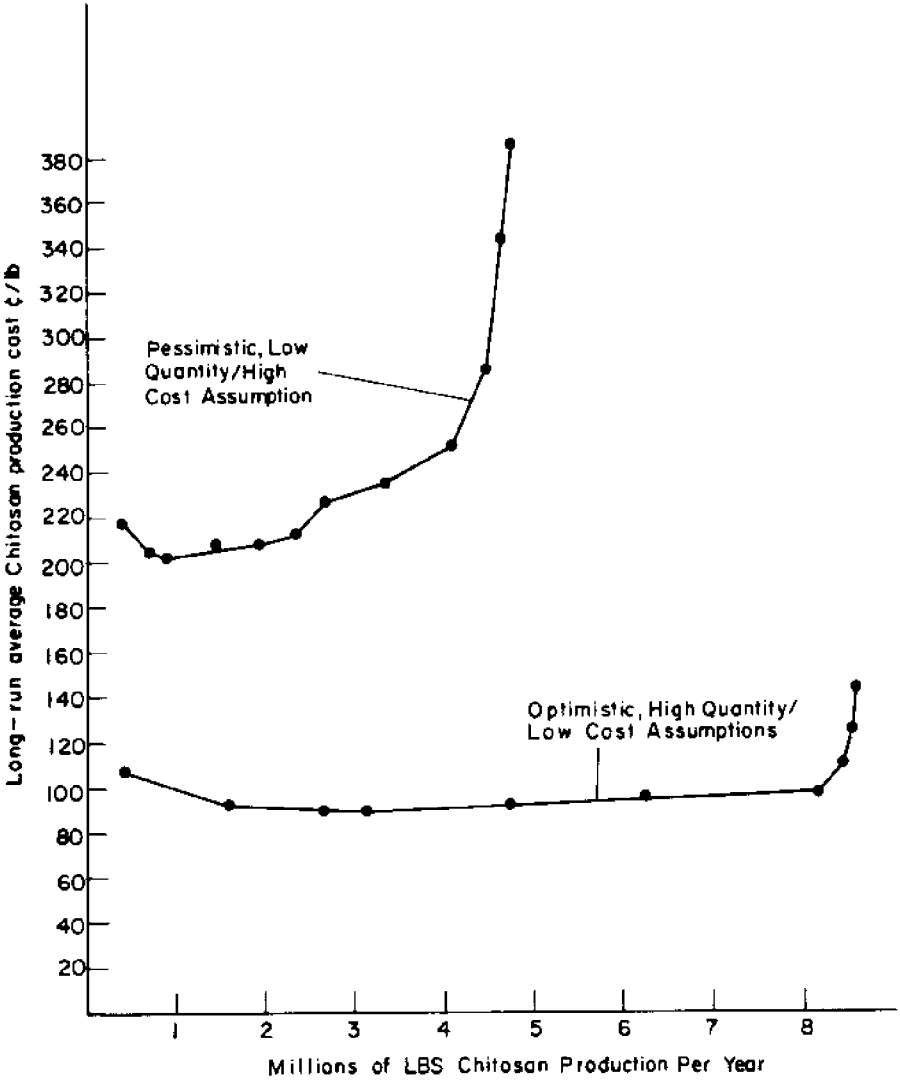
Long-run average Chitosan production cost ¢/lb
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
Pessimistic, Low Quantity/High Cost Assumption
Optimistic, High Quantity/ Low Cost Assumptions
1
2
3
4
5
6
7
8
Millions of LBS Chitosan Production Per Year

Figure 1.

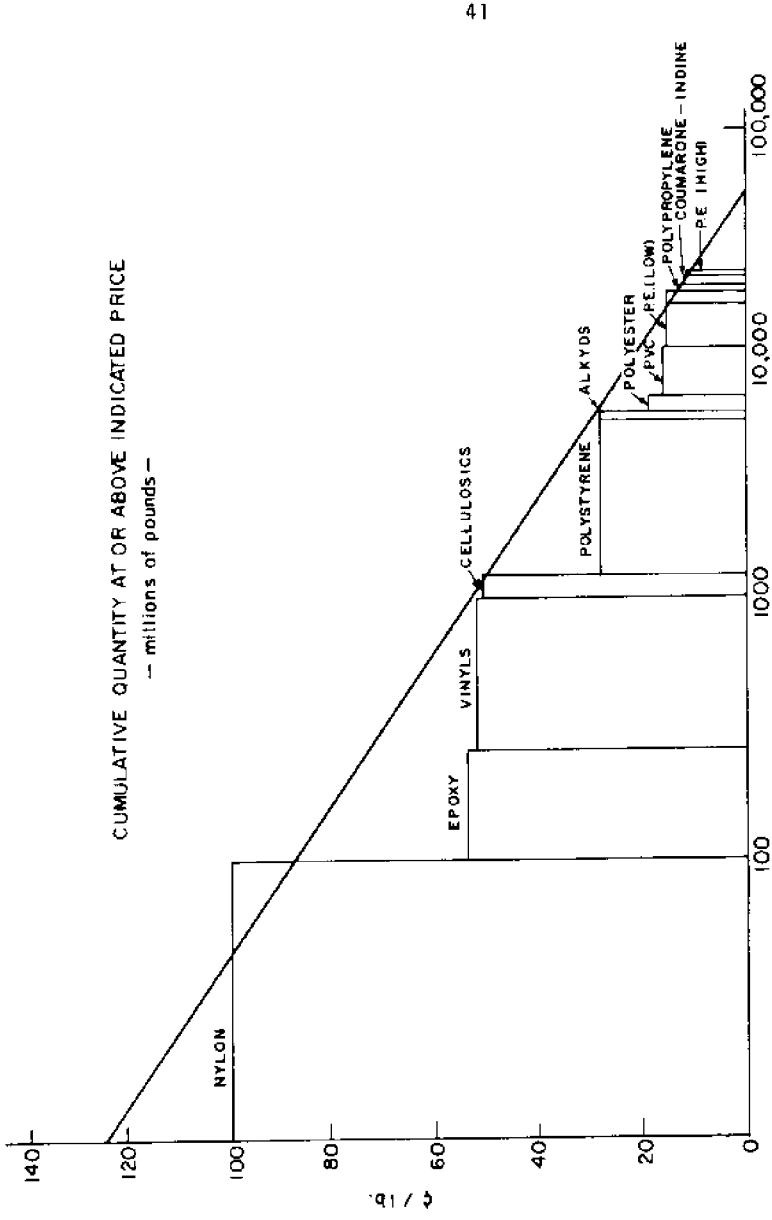

Figure 2. General Plastics 1972

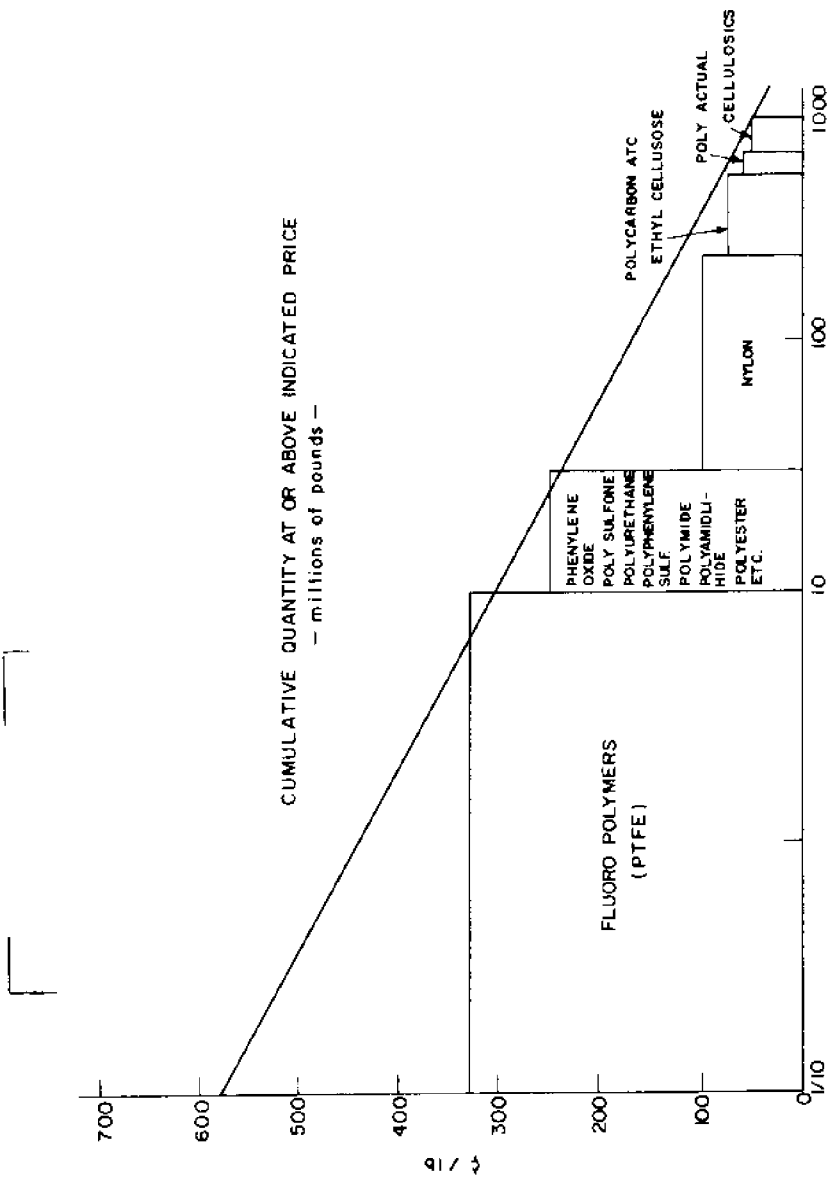

Figure 3. Specialty Polymers 1973

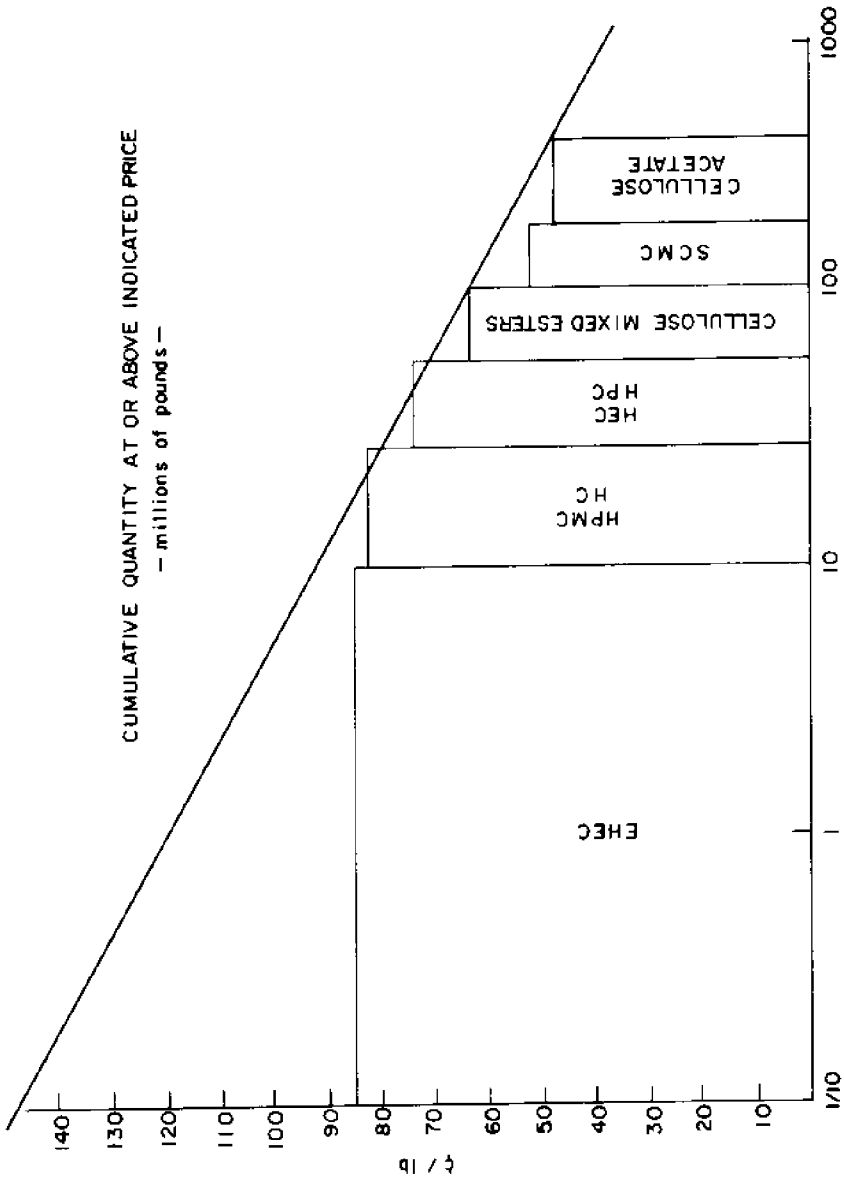

Figure 4. Cellulosics 1973

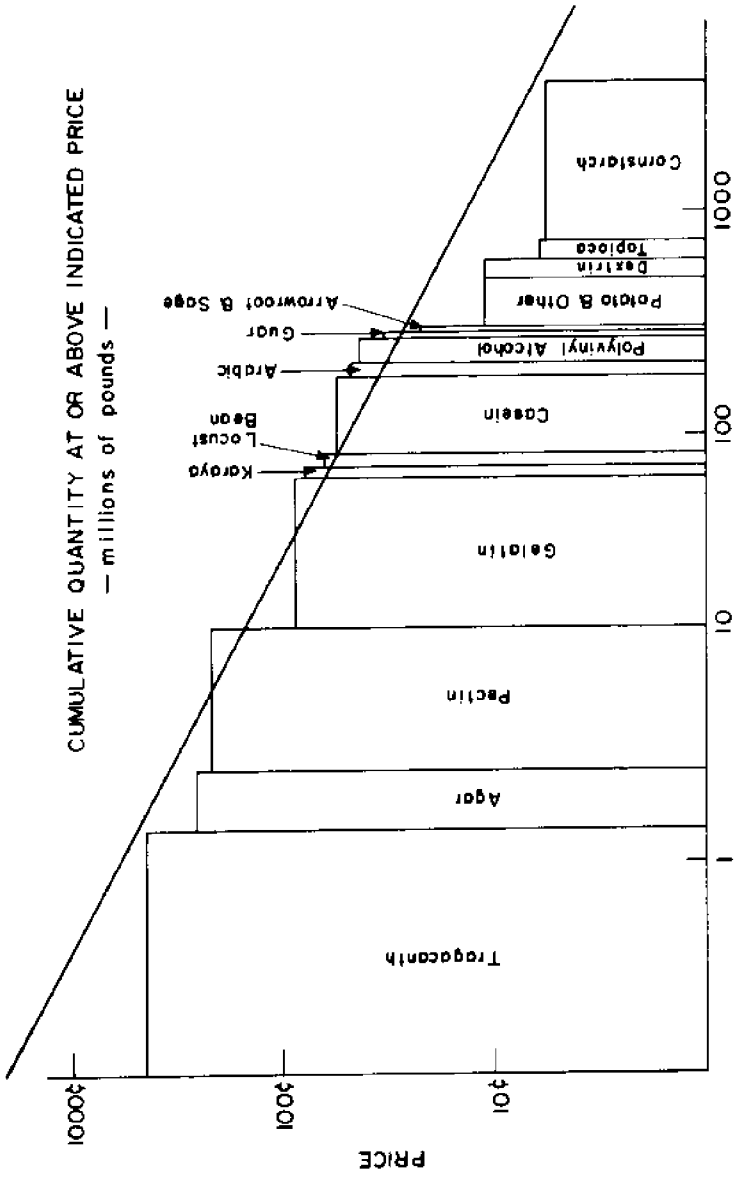

Figure 5. Water-Soluble, Gums and Starches 1972

THE ECONOMICS OF CHITIN RECOVERY AND PRODUCTION

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ABSTRACT

The economic viability of chitin recovery and production, at least at the present time, is mainly dependent upon five factors:

(1) low-cost initial supply of crustacea waste with the capability for expansion without causing inflated raw-material values;

(2) a better understanding by all concerned of any unique properties that already exist in, or can be introduced to, this natural polymer;

(3) production methods which protect high-value market properties;

(4) capability of defraying a significant proportion of production costs by recovery and sale of by-products, especially protein;

(5) quality control sufficient to guarantee minimum standardized product performance.

A plant capable of producing 1,000,000 pounds of chitin a year, or 750,000 pounds of chitosan, should be able to do so at less than $1.00 per pound for chitin and $2.00 per pound for chitosan, even if its only by-product is protein. How far production costs can be reduced below these figures depends upon some of the points covered. How high the market price will be depends upon the remaining factors.

DISCUSSION

The topic "Economics of Chitin Recovery and Production" should really be broadened to include the words "and by-product" because even if chitin were to sell for such a high price that one could ignore the potential revenue from by-products, the tremendous volume involved would create a significant disposal and/or pollution problem. Furthermore, in a world concerned about protein availability, it is unthinkable to waste such a source of superior protein, and most of this protein should contain significant amounts of the important pigment astaxanthin, for which there is a growing demand.

The first and most important factor affecting the economic picture obviously is to obtain a low-cost initial supply of crustacea waste, such that there is the capability of expansion without causing inflated raw-material values. It may sound ridiculous to mention something that is so obvious, but I can assure you, as can others in this room, that without the certainty of expansion of raw materials at reasonable prices your own initial success may put you out of business. Waste materials have little or no value until someone knows you have to have them for expansion. In

the United States, with a few exceptions such as in some parts of the Mexican border area and possibly portions of Alaska, any location that would meet this criterion is likely already to boast a crustacea-meal plant - either crab meal or shrimp meal. This is because large quantities of waste only occur where there is a concentration of processing plants, and such an accumulation of plants doesn't happen overnight. Thus, the concentrations of waste are generally in places that have already had to face the problem of waste disposal by approved methods. Usually their salvation has been to convert it into a relatively low-value meal for poultry feed. This was an entirely logical development. It merely upgraded a material whose only prior utility was as a fertilizer. Admittedly there are locations which are exceptions to what I have just stated, but few if any of these areas have a large enough concentration of waste in a closely defined area to be of primary interest for chitin production. However, those areas may well make good secondary suppliers of raw material.

Such existing utilization of shellfish wastes should not in any way discourage those who are now interested in chitin production. In fact, if you examine the economics of meal production and existing meal-plant operations, you will find that in most cases there is a heaven-sent opportunity to bring about a further upgrading of the same waste. It would be very appropriate if something which was first dumped, then collected as fertilizer, and then converted into poultry feed could now be used to realize the promising applications that have been found for chitin and its derivatives.

The potential chitin-producers' opportunity stems from the meal-producers' problems. With few exceptions, meal plants have been in existence for some time, and with shifting community development patterns many of them now find themselves in areas that have changed from sparse population to commercial or residential areas. The new neighbors don't like the odor; air and water pollution prevention is costly, as is compliance with OSHA and meeting increased fuel and labor costs. An economic study of the shellfish meal industry would reveal the sad fact that the costs of producing the meal continue to rise, but the price that can be obtained is dictated mainly by prices of soy and fish meal, both of which can be produced in tremendous quantities and are therefore not so sensitive to inflated production costs.

I believe you will find that many meal producers would be receptive to some arrangement with a chitin producer. Most importantly they already have established collection systems drawing material from multiple primary producers of the waste. Also, they have a long history of service to their primary producers. Thus chemical companies would not have to get into the difficult matter of negotiating with dozens of different small suppliers or the business of operating potentially smelly garbage trucks.

The second factor which radically affects the economics of chitin recovery and production is to obtain a better understanding by all concerned of any unique properties that already exist in, or can be introduced to, this natural polymer. In the last four years I have heard too many potential customers make the statement, "I don't know enough about chitin or chitosan to really say where it would fit into our line or what qualities I would like to have in the samples we need, but send me some anyway." I believe that by and large the same is true even today. Those of us who have produced or promoted chitin and chitosan still don't know

enough about its unique capabilities to provide potential customers with all the answers they must have. We all know that these chemicals are exciting, or we wouldn't be here in Massachusetts. In our excitement and rush to get a product on the market, we have overlooked much of the basic understanding of "our polymer."

Whatever it is that chitin and chitosan can do that cannot be done by potential competitors should be emphasized. Furthermore, I am convinced that we have only begun to scratch the surface of what there is to know about properties that can be introduced into chitin and chitosan. If the economics of plain chitin and chitosan production are favorable enough to warrant their production, and I believe they are, the economics of other derivatives and combinations should be even more exciting. We have got to understand more about the basic chitin before we can get too far with these other possible markets.

The next factor affecting the economic picture is the production methods which are used at every step along the way. When you produce chitin, let alone chitosan, you are incurring quite a lot of expense, and it would be logical to expect that you will wish to sell your end product for its highest possible value based upon any unique properties. Regardless of which properties you may wish to emphasize, age and previous handling methods for the raw material may have already started working against you. Again you may have succeeded in getting rid of all the calcium, but in continuing acid treatment beyond that point, you risk other damage without any indication of what may be happening until it's too late. Merely showing no ash residue will not tell you this. The same is true for your deproteinization step. It is not hard to get rid of all the protein, but what else did you affect if you used too harsh a treatment? Of course, the same thing applies to deacetylation. From my experience I believe that as much care needs to be used in protecting desirable properties as in disposing of all traces of undesirable ash, protein and acetyl groups.

The fourth factor which is vitally important in considering the economic picture is the matter of maximizing revenue from by-product recovery and sale. Depending upon the raw material used, there are different amounts of protein present and different amounts of calcium to be handled. In the case of chitosan production, obviously there is yet another removal problem in the matter of the acetyl groups. The costs of removing these undesirables can be significant, and what are you going to do with them to avoid being a polluter? The quantities involved are staggering. For example, a plant producing 1,000,000 pounds of chitosan from Gulf of Mexico shrimp waste would have to dispose of about 1.75 million pounds of dry protein annually. A plant working from blue-crab waste and producing 1,000,000 pounds annually of chitin will find itself buried in approximately 3,000,000 pounds of proteinaceous material plus several million pounds of calcium in some form or another.

The calcium presents some interesting disposal and/or recovery problems, but the protein situation is fairly simple. This is excellent protein, which varies according to species and method of extraction from about 60% to 90% protein value. Again, according to species and method of removal, etc., it may contain significant amounts of the important natural pigment astaxanthin. Thus, it has the potential of being marketable both for its protein content and its pigment value. Even if it only sells in competition

with 60% protein fish meal, the revenue producing potential is quite significant.

As it happens, the best way of protecting the natural pigment is to achieve maximum protein removal mechanically rather than chemically. Whatever residual protein still has to be removed chemically is so reduced in quantity that you have the double benefit of lower alkali treatment costs and less danger of possible chitin degradation.

In the ideal situation, the value of the protein alone should be such as to pay for the original raw material plus the costs of its recovery. This can be done in certain situations, and it goes without saying that such a factor affects the economics of chitin production so radically that utilization of some waste materials may be completely uneconomical without maximizing protein recovery and sale.

The final factor which I maintain is significant to this discussion is that of quality control. Enough variables are provided by nature in the form of shell-moulting cycles, sex of the animal, size and age. Even the particular portion of the animal from which the waste originated causes the proportion of chitin to calcium and protein to vary quite widely even if you are using raw material from a highly controlled source. If you now add in the changes that can be wrought by different machines and production methods on the part of the crab, shrimp or lobster processing plant together with the effect of aging of the waste before you get it, it is obvious that your raw material may not be of a very uniform quality. Finally, the extraction of chitin and chitosan begins, and again a host of variables are brought into play. The overall picture can be a quality-control nightmare. Standards of purity and performance must be set which are rigid enough to provide assurance to customers that specific qualities are present while also realistic enough to make it possible for chitin producers to deliver their product at a price which makes it worth their while. There needs to be better understanding on the part of all concerned in this regard, and whatever is resolved may be the most significant of all factors concerned with the economics of chitin production and recovery.

Applying these generalities to a potential chitin location in the lower Chesapeake Bay area, 6,000 tons a year of blue-crab waste is already being processed into meal. Nearby there is sufficient additional waste available to provide an overall initial capability of 1,000,000 pounds of chitin a year. We estimate a per ton price of between $30 and $40 delivered to a chitin plant in the southeastern Virginia area, and we know that there is enough additional raw material in the total Chesapeake Bay/North Carolina area which can be obtained at reasonable prices to permit future expansion to about 2,000,000 pounds of chitin.

Our research indicates that a minimum of 95 pounds of dry chitin is readily attainable per ton of incoming 60% moisture raw material. In the pilot plant we were able to extract mechanically approximately 300 pounds of dry protein per incoming ton of wet waste. This protein was dried in tests by various dryer manufacturers, and it appears to have its maximum value when drum dried as 60% protein flake.

So far, what all this means is that from an economic point of view one would be looking at a raw material cost of between 31-1/2 cents and 42 cents per pound of chitin, but could recover 3.15 pounds of protein

mechanically for every pound of chitin extracted. If you assume even a low value of 15 cents a pound for this by-product protein with some astaxanthin in it, it can yield 47 cents in revenue. However, there is a tremendous amount of calcium material in what is now left of the original blue-crab waste - namely the shell residual. We find that one needs about 9 pounds of 31.45% hydrochloric acid per pound of chitin for demineralization. At a bulk delivered price for HCl of 2 cents/pound and assuming no recovery or re-use of the fluid, acid costs would be at their maximum of 18 cents per pound of chitin.

We further find that turning this demineralized shell into chitin by alkali deproteinization requires close to 1.5 pounds of 50% sodium hydroxide per pound of chitin produced. If we again assumed no recovery of solution and a delivered price in bulk of 7 cents/pound, this would amount to a maximum of 10.5 cents per pound of chitin produced; however, this is just for the chemical needed in the process. If you stop at chitin instead of producing chitosan, raw material and chemical costs would be a maximum of 70.5 cents; however, if you recover the protein from just the mechanical extraction step, it can produce a protein credit of 47.25 cents per pound of chitin. Therefore, a more accurate cost estimate for the chitin would be 23.25 cents per pound. However, don't forget that I am making no allowance for value of any additional chemically extracted protein or possible by-product revenue from the calcium fraction. Nor have I considered the costs of utilities, labor, supervision, overhead and depreciation. Another omission is the factor of whatever savings could be realized by recovery and recycling of acid and alkali.

If one's ultimate goal is the production of chitosan, we find that by using the method which gives greatest control of solution viscosity, we need a maximum of an additional 5.75 pounds of 50% sodium hydroxide per pound of chitosan produced. In actual fact, we start with considerably more than this quantity - so much more that it is impossible not to consider recovery, and we have found that 85% recovery is feasible. This 85% is simply drained off the wet chitosan before it is washed to neutral. If we assume the same bulk delivered price of 7 cents per pound of 50% sodium hydroxide, deacetylation chemical costs would be a maximum of 40 cents. However, the raw material and chemical costs of producing the chitin were 70.5 cents, and allowing for a 25% loss in conversion to chitosan, one would need 1.33 pounds of chitin for every pound of chitosan. This amounts to 93.75 cents of chitin raw material and chemicals before one adds the 40.25 cents of maximum deacetylation cost. Obviously, one now has a figure of $1.34 from which one can subtract the protein credit of 47.25 cents mentioned earlier. The chitosan cost would be now 86.75 cents per pound before adjustment for the costs of utilities, labor, supervision, overhead and depreciation. In this example I am making no allowance for the value of sodium acetate, if indeed it is even worth recovering. I have not reduced the cost of deproteinizing the chitin raw material by using some of the chitosan wash fluid which would obviously contain a considerable amount of sodium hydroxide. In full-scale plant production there would be other cost-saving procedures which might reduce these figures somewhat, and there may be other by-products which could produce incidental revenue. My reason for staying away from estimates of utilities, overhead, supervision, labor and depreciation is that these vary so much from plant to plant and country to country. In most cases I would suspect that industrialists have their own rule-of-thumb multiplier which they would prefer to apply anyway.

From the data I have presented I suggest that a plant capable of an initial production of 1,000,000 pounds of chitin a year - or its equivalent of 750,000 pounds of chitosan - if using blue-crab waste as its raw material, and if it merely recovers the so-called adventitious protein, should be able to do so at a cost of less than $1.00 per pound for the chitin and $2.00 per pound for the chitosan.

How far production costs can be reduced below $1.00 per pound for chitin and $2.00 per pound for chitosan depends upon some of the five factors I have covered. How high above these figures the market price will be depends upon the remaining factors. Provided that all are considered and found present in your equation, the economic picture looks very favorable.

TABLE I

PRINCIPAL FACTORS INVOLVED IN CONSIDERING THE ECONOMICS OF CHITIN RECOVERY AND PRODUCTION

(1) Low-cost initial supply of crustacea waste with the capability for expansion without causing inflated raw-material values

(2) Better understanding by all concerned of any unique properties that already exist in, or can be introduced to, this natural polymer

(3) Production methods which protect high-value market properties

(4) Capability of defraying significant portion of production costs by recovery and sale of by-products, especially protein

(5) Quality control sufficient to guarantee minimum standardized product performance

TABLE II

SOUTHEASTERN VIRGINIA CHITIN PLANT UTILIZING BLUE-CRAB RAW MATERIAL

	Cost per Pound of Chitin		
		¢	
Raw Material Delivered 60% moisture, 95 pounds of dry chitin per input ton. $30 - $40 per ton.	31.5	to	42
Acid for Demineralization 31.45% HCl by weight. 2 cents/pound delivered. Requires maximum of 9 pounds		18	
Alkali for Deproteinization 50% NaOH 7 cents/pound delivered. Requires 1.5 pounds maximum		10.5	
Sub-total Maximum		70.5	
Protein available by mechanical extraction 300 pounds/input ton of raw material or 3.15 pounds/pound of chitin extracted. Assumed sales price = 15 cents/pound			
Protein Credit		47.25	
Unadjusted cost per pound of chitin		23.25	¢

Note:

No allowance for additional chemically extracted protein or possible calcium by-product. Assumes no recovery and recycling of acid or alkali. No costing for utilities, labor, supervision, overhead or depreciation.

TABLE III

SOUTHEASTERN VIRGINIA CHITOSAN PLANT UTILIZING BLUE-CRAB RAW MATERIAL

	Cost per Pound of Chitosan ¢
Transfer cost to chitosan assuming 75% yield of chitosan from chitin.	
Raw Material + Acid for Demineralization + Alkali for Deproteinization Costs from Sub-total on Illustration II = 70.5 cents X 1.333	93.75
Alkali for Deacetylation 50% NaOH 7 cents/pound delivered 5.75 pounds maximum consumed per pound of chitosan produced	40.25
Sub-total Maximum	134.00
Protein Credit (from Illustration II)	47.25
Unadjusted cost per pound of Chitosan	86.75 ¢

Note:

No allowance for additional chemically extracted protein, or any other possible by-products. Assumes no recovery or recycling of acid or alkali in chitin production, or possible re-use in deproteinization of chitosan wash fluid. Allowance is made, however, for 85% NaOH recovery by draining chitosan after deacetylation and before washing.

No costing for utilities, labor, supervision, overhead or depreciation.

ANTARCTIC KRILL (EUPHAUSIA SUPERBA) AS A SOURCE OF CHITIN AND CHITOSAN

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ABSTRACT

Removal of the edible tail of Antarctic krill (Euphausia superba) by mechanical peeling followed by alkaline extraction of the non-tail material results in the production of 14.9% processing waste. The waste material contains 24% chitin, compared to 3.2% chitin in whole krill.

Optimum conditions for deproteinization and demineralization of the processing waste to produce chitin were determined. The deproteinization conditions involved extraction of the waste with 3.5% NaOH solution at 90-95° C for 2 hours at a solids-to-solvent ratio of 1:10, while demineralization was accomplished by extracting the deproteinized waste with 0.6 N HCl at room temperature for 2 hours at a solids-to-solvent ratio of 1:22. An overall recovery of approximately 89% of the chitin present in whole krill was obtained, which was determined to be roughly 92-95% pure. Krill chitin proved to be 2.6 times more porous than crab chitin. The chitin product was highly colored by the presence of small amounts of carotenoid pigments, tentatively identified as astaxanthin, astacin, a series of fatty acid esters of astaxanthin and a protein-bound carotenoid which was not characterized.

Krill chitosan prepared by the method of Broussignac was tan colored and obtained in 90% yield, while crab chitosan was white and obtained in only 60% yield. Krill chitosan was determined to be 2.3 times more porous than crab chitosan. Chitosan from krill contained an average of 7.9% nitrogen and showed an average viscosity as a 1% solution in 5% acetic acid of 60 cps, while the respective values for crab chitosan were determined to be 7.8% nitrogen and 67 cps.

INTRODUCTION

Euphausia superba (E. superba) is a species of krill closely resembling shrimp, but considerably smaller, that is indigenous to the Antarctic Ocean. The Antarctic krill has an average length of four to six centimeters and an average weight of 0.7 to 1.2 grams. Since the demise of whales which feed on krill, this crustacean has experienced a population boom. Conservative estimates suggest that as much as 500 million metric tons of krill, representing approximately 17% of an estimated 3 billion tons that inhabit the Antarctic waters, could be harvested annually without disturbing the stable population. This figure is astonishing when one considers that the world's annual production of conventional fisheries is in the neighborhood of 80 million metric tons.

For the past few years the Fisheries Development Institute in Chile has been studying the Antarctic krill and developing technology for its procurement, processing and utilization as a foodstuff suitable for human consumption. Recently the institute initiated a pilot-plant-scale operation capable of processing whole krill by mechanically removing the edible tails and extracting a majority of the protein, which is easily recovered, from the non-tail material remaining (6). This process, however, results in the production of roughly 15% waste material based on whole krill, and should the fishing industry in Chile develop a full-scale krill program, which it fully intends to do, the amount of waste material generated from processing would be considerable and could present potential disposal and pollution problems. Developing practical uses for the processing waste is, therefore, important in advancing the practical utilization of krill.

With the interest generated in recent years concerning the use of crustacean wastes as a raw material for chitin production, and since krill processing waste was determined to be composed of about 24% chitin compared to 3.2% in whole krill, the institute, in conjunction with the University of Chile, has undertaken a research program directed toward an evaluation of the krill processing waste as a source of chitin and chitosan.

The initial experiments described in this paper deal with the development of laboratory-scale procedures for treatment of the waste that require a minimum investment in time and chemicals and utilize the mildest conditions possible to produce a chitin of reproducible composition. The general properties of krill chitin and commercial crab chitin were determined and compared.

Krill processing waste, when treated using the optimum conditions established in our initial experiments, unfortunately yields a highly colored product. In fact, no conditions could be found employing only acid and base treatments at reasonable concentrations that would remove the color, unless the eyes of the krill were first removed, which proved impractical. Consequently, additional studies were conducted to determine the nature of the colored substances.

A final study discussed here compares several properties of krill and commercial crab chitosan prepared by the method of Broussignac (1) in terms of color, nitrogen content, infrared spectrum, yield, density and bulk density, as well as the viscosities of 1% solutions of the chitosans in 5% acetic acid.

EXPERIMENTAL

Whole krill, processing waste, intermediate products of processing, and chitin were all analyzed for lipids, ash, moisture and crude fiber according to standard methods of the AOAC. The crude-fiber content of all products was used as an estimate of chitin content with the true value possibly being greater by a few percent of the determined value. A standard macro-Kjeldahl procedure was used to determine the nitrogen content of all samples described above in addition to chitosan samples. In the case of chitin samples the value obtained for nitrogen was also used as an estimate of chitin purity.

Approximate densities of chitin samples were determined on 2 g of sample, using a solvent displacement method utilizing toluene. Estimated values for the bulk densities of chitin and chitosan powders from both krill and crab were determined by grinding samples of the above under the same conditions to pass through 100 mesh and placing exactly 1.00 g in a tapered, graduated 10 ml centrifuge tube. The volume occupied by the powders was then measured after vibrating for exactly 1 minute.

Viscosities of 1% solutions of chitosan in 5% acetic acid were determined using a Brookfield LV-Series Viscometer and infrared spectra of both chitin and chitosan samples determined as KBr pellets using a Leitz G III instrument. Results of all analyses are reported on a dry basis, unless otherwise specified.

Isolation of krill chitin

E. superba caught during the months of July and August, 1976, were stored at -56° C until processed. The krill processing waste was obtained by first removing the tails of a large batch of whole krill by mechanical peeling and then extracting the separated, non-tail material after grinding in a colloidal mill, at pH 10.5 with sodium hydroxide solution to remove protein according to a procedure developed in our laboratory (6). The processing waste was separated from the protein solution by continuous centrifugation, and excess moisture was removed from the waste by pressing. A proximate analysis was then performed.

Krill chitin was obtained by deproteinizing the processing waste with dilute sodium hydroxide solution followed by demineralization with dilute hydrochloric acid solution in a manner similar to other methods reported in the past (4). Optimum conditions for purification were established which utilized a minimum of reagents and reaction times and relatively mild conditions. A series of experiments were conducted in which the purification parameters were varied and the nitrogen, ash, fiber and lipid contents analyzed in the resulting chitins. The conditions gave the best value for nitrogen content, highest value for crude fiber and the lowest values for lipid and ash content were considered optimum.

Conditions established to be optimum for deproteinization involved adding enough water and solid sodium hydroxide to the wet processing waste to bring the sodium hydroxide concentration to 3.5% and the solids-to-solvent ratio to 1:10 and heating for 2 hours at 90-95° C with stirring. After deproteinization, the reaction mixture was cooled and filtered through nylon mesh (pore diameter 73μm) and washed to neutrality with a large volume of water. Fifty percent of this material was freed of excess moisture and stored at -56° C for future use.

Optimum demineralization was conducted by stirring the remaining 50% of the residue from above, also freed of excess moisture by pressing, for 2 hours at room temperature with enough added 1 N hydrochloric acid solution to attain a final concentration of 0.6 N and a solids-to-solvent ratio of 1:22. Following demineralization, the product was filtered with the aid of the nylon mesh, washed to neutrality with water, pressed free of excess moisture and 50% of this product stored at -56° C for future use.

The remainder was air dried for 48 hours and ground to pass 60 mesh, yielding a highly colored chitin. The chitin was subjected to analysis according to previously discussed procedures.

Preparation of chitosans

Eight grams each of all chitin samples (ground to 60 mesh) were treated with 53.5 g of KOH dissolved in a solution composed of 48.5 ml of 95% ethanol and 34.5 ml of ethylene glycol at reflux temperature for precisely 20 hours (1). The hot mixture was diluted by the addition of 200 ml of water and the chitosan isolated by suction filtration followed by washing with water to neutrality. Air drying followed by pulverizing to pass 60 mesh yielded the final product. Triplicate samples of both krill chitin and commercial crab chitin (Sigma Chemical Company) were so converted in order to ascertain the amount of variation to be expected in the yield and nitrogen content of the chitosan samples. The samples were analyzed by procedures described earlier.

Characterization of krill pigments

A sample of the dried, colored chitin was exhaustively extracted with acid-free chloroform followed by ethyl ether and finally absolute alcohol. The chitin remained highly colored, and the only lipid soluble pigments present in the sample were extracted by the chloroform. Concentration of the chloroform extract in vacuo followed by three multiple elutions on thin layers of Silica Gel G. using 0.3% ethyl ether in hexane resulted in the tentative identification of astaxanthin (in almost undetectable quantity), astacin and presumably a series of unidentified fatty acid esters of astaxanthin.

The exhaustively extracted colored sample of chitin was then incubated with an excess of NOVA bacterial proteinase for 120 hours at pH 6.5 and a temperature of 55° C. A control experiment was also conducted under the same conditions, but without the enzyme present. Extraction of the enzyme-treated mixture with chloroform resulted in the removal of a considerable amount of color. The chloroform-soluble pigment could not be characterized, but was similar in its chromatographic properties to astacin. In the control experiment no chloroform-soluble pigment was extracted.

RESULTS AND DISCUSSION

A proximate analysis of whole krill (E. superba) and the processing waste obtained after mechanical peeling and protein extraction of the non-tail material is shown in Table 1.

It must be pointed out that the values in this table are subject to seasonal variation (6); they also vary somewhat from batch to batch, with the lipid content exhibiting the greatest batch variation. The protein content was calculated by correcting the nitrogen value for chitin nitrogen and multiplying by 6.25.

Table 1. A Comparison of Proximate Values for Whole Krill and Processing Waste

Sample	N(%)	Chitin(%)*	Lipids(%)	Ash(%)	Protein(%)
Whole krill	12.56	3.2	16.5	1.5	76
Processing waste	8.63	24.0	11.6	23.0	41

*Determined as crude fiber

Table 1 demonstrates that the percent chitin in whole krill is low, representing only 3.2% of the dry weight of the animal. On the other hand, approximately one-fourth the weight of the processing waste is composed of chitin, and since this waste is obtained as a by-product of protein production employing simple, rapid and cheap methodologies, the prospects of using it as a source of chitin appear excellent.

The entire processing scheme for the production of krill chitin starting from whole krill is outlined in Figure 1. This scheme presents a summary of the conditions employed and gives the yields of each individual step of the process. The values for the yields are the average obtained for three separate runs on the same batch of whole krill.

The first two steps of the process produce 14.9% processing waste. Deproteinization removes 53.2% of the total weight of the processing waste as protein and lipids. A large portion of the protein material, incidently, can be recovered by isoelectric precipitation; the food value of the recovered protein, however, remains to be determined. In addition, during the deproteinization step a considerable quantity of ammonia is evolved which can be quantitatively recovered by adsorption into acid. Of the 46.8% deproteinized material remaining, demineralization yields 50.7% chitin and 49.3% minerals plus a small amount of protein and degraded protein material.

When the yield of chitin is calculated based upon whole krill, taking into consideration the purity of the final product (discussed below), a value of 2.8% is obtained (compared to 3.2% theoretical), resulting in an overall recovery of 89%. Based upon processing waste as starting material, a recovery of 93% is realized. These values represent maximum values as shown in Table 2.

An analysis of the three chitins obtained from the individual runs in addition to recovery values is shown in Table 2. If the chitin content in Table 2 is calculated based on a value of 6.89% nitrogen for pure chitin (a simplified assumption), the average chitin content of the samples is calculated to be 94.7%. If the calculation relies upon the data from crude-fiber analysis, the average purity is calculated to be 92%. The true value presumably lies somewhere in this region.

Table 2. Comparison of Analytical Data Obtained for Chitins Produced in Three Separate Runs of the Same Batch of Whole Krill

Sample	N(%)	Chitin(%)*	Chitin(%)**	Lipids(%)	Ash(%)	Recovery(%)***
1	6.58	95.5	92	4.8	0.4	93
2	6.50	94.3	94	5.5	0.5	93
3	6.51	94.5	90	5.4	0.3	93
Mean	6.53	94.7	92	5.1	0.4	93

*Based on a value of 6.89% nitrogen for pure chitin

**Based on crude-fiber analysis

***Based on processing waste; maximum values calculated using the numbers in column three.

The dried, ground, extremely pink-colored krill chitin is similar in many respects to ground commercial crab chitin obtained from Sigma Chemical Company. Commercial chitin, which is an offwhite color, however, was determined to contain 7.18% nitrogen in comparison to an average of 6.53% nitrogen in krill chitin, while the ash content was found to be 2.3%, and the lipid content negligible.

The major bands occurring in the infrared spectra of krill chitin, commercial crab chitin (Sigma Chemical Company) and those reported in the literature by Falk et al. (2) are identical except that the bands occurring in the spectrum of krill chitin are better resolved than the bands occurring in the spectrum of the commercial chitin. This is presumably due to the presence of contaminating protein in the commercial sample, since it was shown to contain more nitrogen than the theoretical value for pure chitin (7.18% compared to 6.89% theoretical) and the fact that the commercial sample possessed about six times the ash content of krill chitin. No conclusion concerning the resolution of Falk's spectrum in comparison to ours can be drawn, since he utilized an instrument of lower resolution than we did to obtain his spectrum.

Aside from color, the sole distinguishing feature between krill and crab chitin that we have discovered to date is the difference in the values for their bulk densities. Krill chitin demonstrates a bulk density (100 mesh powder) of 0.11 g ml^{-1}, while commercial chitin of the same particle diameter gives a value of 0.29 g ml^{-1}. Thus, krill chitin occupies 2.6 times more volume than the same weight of crab chitin with the same average particle diameter, while the densities of the two are approximately the same and equal to 1.4 g ml^{-1}. The overall structure of krill chitin must certainly be much more porous than that of crab chitin, judging from these values.

In the case of the chitosans produced from krill and crab chitins under identical conditions by Broussignac's method (1), a similar situation exists. That is, krill chitosan exhibits a bulk density of 0.18 g ml^{-1} compared to 0.42 g ml^{-1} for crab chitosan, while their densities are approximately equal, demonstrating that the former occupies 2.3 times the volume of the same weight of the latter.

Absolutely no differences exist, however, in the infra-red spectra of the two chitosans, and they are identical, within the limits of experimental error, to a spectrum obtained by Muzzarelli (5) of crab chitosan.

Table 3 shows the nitrogen-content yields corrected for degree of deacetylation, viscosities of 1% solutions in 5% acetic acid, and colors for triplicate samples each of krill and crab chitosans prepared by Broussignac's method. The average content of the krill chitosan samples is shown to be 7.88% nitrogen, while it is 7.80% for the crab chitosan samples prepared under identical conditions, indicating a slightly greater degree of deacetylation in the former sample. This small difference might be attributable to the difference in porosities between the krill and crab products but at this stage of research this can only be conjecture.

A comparison of the average viscosities for krill and crab chitosan solutions in acetic acid in Table 3 shows that the values are similar, but that the value for krill chitosan is lower by 7 cps (67 cps for crab chitosan compared to 60 cps for krill chitosan). The difference in the viscosities may or may not be significant. The lower viscosity for the krill chitosan solution is, however, in accord with the higher nitrogen content of the chitosan (1).

Table 3. Comparison of Analytical Data Obtained for Chitosans (From triplicate conversions of 8 g each of krill and crab chitin samples)

Sample	Nitrogen(%)*	Yield(%)**	Viscosity(cps)***	Color
Crab chitosan	7.78	60	70	white
Crab chitosan	7.80	64	64	white
Crab chitosan	7.81	63	67	white
Mean	7.80	62	67	--
Krill chitosan	7.84	90	57	tan
Krill chitosan	7.87	89	62	tan
Krill chitosan	7.92	91	61	tan
Mean	7.88	90	60	--

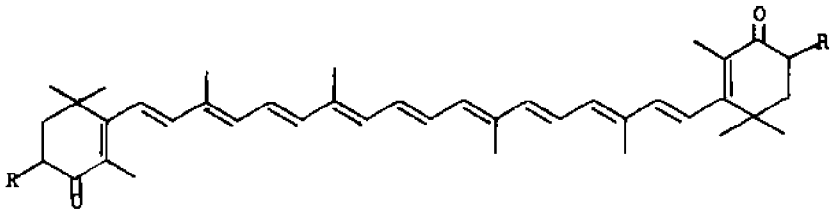
*Corrected for ash content

**Corrected for degree of deacetylation

***1% solution in 5% acetic acid

Certainly the most striking feature of Table 3 is the large difference between the mean yields of chitosan obtained from krill and from crab chitins. Krill chitosan is obtained in an average yield of 90%, while crab chitosan is produced in only 60% yield. At the present time the authors have no explanation for this difference. One possible explanation is that the process utilized by Sigma Chemical Company to prepare their chitin involves a bleaching step. We have determined that in most cases chitin treated with bleaching agents yields less chitosan than does chitin that is untreated (unpublished results).

A second major difference between the crab and krill chitosans shown in Table 3 is that the krill chitosan is tan in color as compared to the white crab chitosan. It was suspected that the tan color in the krill chitosan was produced by the degradation of pigments present in the krill chitin during the deacetylation step. The structures of some of the krill chitin pigments, therefore, were established using the methods described earlier. Two types of pigment were found to be present. The first type was shown to consist of lipophylic carotenoid pigments tentatively identified as astaxanthin, astacin and a series of fatty acid esters of astaxanthin shown below.



Astaxanthin, R = OH

Astacin, R = =O

Astaxanthin fatty acid esters, R = R'COO

These pigments occur in quantities that are otherwise insignificant, but that are sufficient to contribute a small amount of color to the chitin. The possibility was considered that the second type of pigment, which was non-lipophylic and the major pigment contaminating krill chitin, might be a protein complex of astaxanthin, since it is one of the few carotenoid pigments known to form organic solvent-insoluble protein complexes (3). Our suspicions concerning the protein nature of the pigment were confirmed when it was shown that the pigment was attacked by a proteolytic enzyme, releasing a lipophylic carotenoid whose identity remains undetermined.

The source of the protein-carotenoid complex was shown to be the eyes of the krill, since krill chitin manufactured from krill whose eyes had been carefully removed by hand, was practically pure white, and the chitosan produced from that material was pure white.

CONCLUSIONS

It was demonstrated that the processing waste obtained from whole Antarctic krill after tail removal by mechanical means and extraction of protein from the non-tail material remaining, utilizing mild conditions, contains significant quantities of chitin (24%). Since this waste is obtained as a by-product of protein production employing simple, rapid and cheap methodologies, the prospects of using it as a source of chitin appear excellent.

Chitin from the krill processing waste can be obtained in 93% yield by a method discussed in this paper, and, except that it is highly colored by carotenoid pigments and much more porous than commercially produced crab chitin, it appears very similar to the latter in its properties.

Chitosan is obtained from krill chitin by the method of Broussignac (1) in excellent yields (90%), while commercially produced crab chitin is converted to the extent of only 60%--a significant difference. Again, with the exceptions noted of color and greater porosity, the two chitosans appear similar in their properties.

ACKNOWLEDGMENTS

The authors would like to thank the United States Peace Corps for presenting Dr. Anderson with the opportunity to participate as a Peace Corps Volunteer in the development of the Krill Project in Chile. The authors sincerely thank Teresa Barbato for technical assistance in the final stages of this work. This work was partially supported by financial aid from the University of Chile, Institute of Nutrition and Food Technology, and the Agency for International Development (AID).

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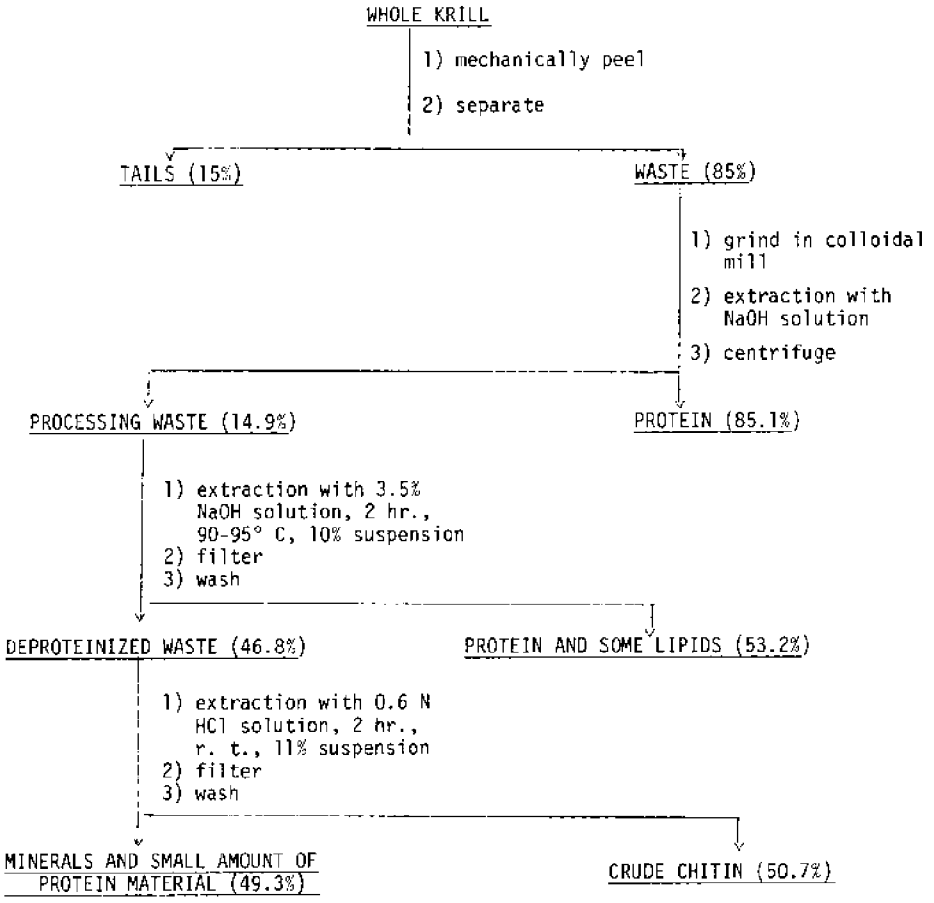

Figure 1. Processing scheme for production of krill chitin. Yields in parenthesis represent the average of three runs.

A CRITICAL EVALUATION OF THE POTENTIAL SOURCES OF CHITIN AND CHITOSAN*

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ABSTRACT

To provide a basis for the expansion of the use of chitin and chitosan, a critical survey of possible sources was undertaken. Attention was focused on chitin- and chitosan-yielding Crustacea, Insecta, Mollusca and Thallophyta, which could either be harvested from the natural environment, artificially cultured or derived from existing waste streams.

It is concluded that in the immediate future the principal source of chitin and chitosan will remain shrimp and crab waste. Cultured fungi capable of synthesizing chitin alone or in association with chitosan will probably assume the major supply role thereafter, although the rearing of insects as a chitin source cannot be unequivocally ruled out. In the longer term, Antarctic krill or Californian red crab may become important supplemental chitin sources, but abundant clam and oyster shells will probably never be processed for the polymer except as part of an acid-absorption system. Squid and diatoms can only be regarded as sources of small quantities of high-quality chitin.

INTRODUCTION

Chitin and chitosan prepared from crab and shrimp shells are now commercial products both in the United States and overseas (39). The current marketing strategy for these marine polymers seems to be focused on the development of high-profit–low-volume uses exemplified by pharmaceuticals, glucosamine production, water-purification aids and chromatographic media (43). These markets can all support the current relatively high cost of $4.40-11/kg (from Food, Chemical, and Research Laboratories, Seattle) of the nitrogeneous polysaccharides. Moreover, if this marketing philosophy is maintained, the present potential world supply (Table 1) of chitin from shellfish wastes will probably be adequate for the foreseeable future. In this event, the continuing contribution of the U.S. National Sea Grant Program in catalyzing the commercial utilization of a fishery waste will have been noteworthy (5). However, these spinoff benefits to the nation of the Sea Grant Program could be multiplied many times over if chitin and chitosan could be made available at a cost that would facilitate penetration of large-volume markets. Among the several possibilities in the areas of nonwovens (18), fibers (17), films (28), textile sizes (27), tobacco substitutes (6) and adhesives (19), the greatest volume opportunity, perhaps, lies in the utilization of chitosan as a paper additive (7). In this application the

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* Part 8 in the series "Marine Polymers" (1)

Table 1. Global Estimates of Annually Accessible Chitinaceous Materials as Potential Chitin Sources[a]

Chitin Resource	Quantity Harvested[b]	Chitinaceous Waste: As Fraction of Harvest (percent)	Chitinaceous Waste: Wet Weight	Chitinaceous Waste: Solids Content (percent)	Chitinaceous Waste: Dry Weight	Chitinaceous Waste: Chitin Potential[c]	References
Shellfish[d,e]	1,700	50-60	468	30-35	154	39	(24,37)
Krill[f]	18,200	40	3,640	22	801	56	(23,31,36)
Clam/Oysters[e]	1,390	65-85	521	90-95	482	22	(8,24,30)
Squid[e,g]	660	20-40	99	21	21	1	(3,11,24,30)
Fungi[h]	790	100	790	20-26	182	32	(12,21,26,33)
Insects	negligible	---	---	21-56	---	--	(52)
Total	22,740		5,118		1,640	150	

[a]Estimates are based upon mean values and are given in kilotons.

[b]It is assumed that only half of the harvested organisms are processed, except for fungi where the entire production is already processed for the principal product of the fermentation.

[c]Calculated using mean chitin content values listed in Table 2.

[d]Shellfish comprises crab, shrimp, prawn, lobster and crayfish.

[e]Five-year (1970-1974) average landings.

[f]FAO (Rome) projection of potential landings (23).

[g]Calculated on the assumption that the pen is 50% water.

[h]By-products of citric acid and antibiotic manufacture; it is assumed that the quantity of citric acid and its mycelium are equivalent (26), antibiotic waste is 50% nonfungal (21) and world production is twice that of the U.S. (21).

cationicity of this chitin derivative can improve both pulp drainage and fiber retention during sheet formation as well as the printability and wet and dry strengths of the finished paper (1). However, an optimistic estimate of the potential worldwide chitin production capacity based on shellfish wastes would amount to only 39,000 tons per year. In contrast, the 1976 world production of newsprint alone was 27,610,000 tons, and other types of paper made up an additional 47,613,000 tons (20), more than a thousand times this quantity. Clearly, considering paper alone, there is a gross imbalance between the supply and demand for chitosan, even at the 1% addition level. As a prelude to remedying this imbalance, a critical survey of all possible sources of chitin and chitosan was undertaken with a focus on representative chitinaceous organisms from Crustacea, Insecta, Mollusca, or Thallophyta genera which either could be derived from existing waste streams, harvested from the natural environment or artificially cultured.

CRUSTACEA

Within this genera, because of their apparent abundance, shellfish wastes have historically been the choice for commercial production of chitin and chitosan. Typically, the total wastes generated by shellfish processing fluctuate around 65 to 85% of the landed weight, depending upon the species processed and whether hand or mechanical cleaning is employed (37). Of this total waste only about 60% are recoverable solids. Its precise composition again is dependent upon both the species processed and the methods of manipulation. Representative analyses usually report (Table 2) values for chitin (14-35%), protein/fat (25-50%), and ash (25-50%). Thus, a simplified general dry-weight composition for the solid waste associated with shellfish processing can be reasonably taken as chitin (25%), protein/fat (25%), ash (50%), for the purposes of estimating the total availability of chitin and chitosan from shellfish wastes. However, it is practically impossible, especially on a worldwide basis, to determine the actual amount of shellfish processed in relation to the quantity discarded at sea or sold whole after landing. For these reasons, the conservative estimate can be made that 50% of the shellfish landings could be available for chitin or chitosan production. On this basis, the present annual world shellfish-waste availability is about 468,000 tons.

With the maintenance of current fishery practices, this quantity cannot be expected to increase significantly in the future. Indeed, world production of seafood is forecast to increase only about 8% over the next decade (2) and could quite possibly decline in many locales (41). For example, member nations of the International Commission for the Northwest Atlantic Fisheries have agreed to reduce the overall catch of all marine oranisms by about 25% for a seven-year period in order to promote the recovery of this fishing area. This reduction will amount to about 200,000 tons per year.

Nonetheless, although the fishery resource is not increasing, a better utilization of what is harvested could augment the supply of chitin and chitosan. Specifically, it is common practice to behead and devein shrimp at sea and to dump the waste overboard. The material discharged

amounts to about 40% of the shrimp weight caught. In addition, the discarded heads contain significantly more chitin than the beheaded body (5). Thus, in the Gulf states of the United States alone, the collection of this dumped material could effectively double the national supply of chitin from shellfish waste.

On the other hand, the reliability of natural shellfish catches is not great for a variety of reasons, and the noncontinuous fishing seasons (6 months for king crab and 8 months for shrimp per year) are not conducive to the maintenance of a steady supply of raw material for chitin manufacture. Both of these factors are strong negatives from the point of view of a chemical manufacturer. Aquaculture theoretically could, of course, produce a nearly unlimited supply of shellfish, and hence raw material, for chitin manufacture. Shrimp culture is already well established in Japan and is under active investigation on the southeastern coast of the United States (14). However, this production of a luxury food item does not seem likely to provide important amounts of chitin-yielding wastes in the near future. The situation with other crustaceans such as crab and lobster is even less promising because these will probably be sold in their entirety.

ZOOPLANKTON

While the well-known shellfish do not offer any immediate prospect for increasing the supply of chitin, zooplankton may constitute a viable alternative source. These oceanic organisms are lower on the aquatic chain than shrimp, crab and lobster and are estimated to account for the annual biosynthesis of more than one billion tons of chitin (51). Nonetheless, it is important to appreciate that in spite of their immense number, the small size and low local concentration in the oceans make it generally uneconomic to harvest zooplankton using existing technology. This conclusion may not be valid for Antarctic krill (Euphausia superba) or red crab (Pleuroncodes planipes) which, during certain times in their life cycles, congregate in huge fishable local concentrations. Both of these organisms are of particular interest as chitin raw material sources because of the compositional similarity to the crustaceans shown in Table 2. Thus, it is estimated that the annual sustainable harvest of Antarctic krill, a reddish shrimp-like creature 40 to 70 mm in length, could reach over 18 million tons in the vast region of upwelling between the Antarctic and tropical oceanic currents called the "Antarctic convergence" (23). Both the Russians and the Japanese have carried out a considerable amount of research on krill (2) and have begun commercial harvesting to a limited extent. The Japanese are reported (24) to have collected over 4,000 tons per year between 1970 and 1974.

The primary step in the processing of the krill is to express the body fluids to obtain the soluble proteins, which are subsequently coagulated to a shrimp-like paste. The expressed residue comprises about 40% of the catch.

Since the chitin content of the expressed residue is low in comparison with crustacean wastes (Table 2) the amount of material handled per unit of product chitin would be increased approximately fourfold. However, the total yield of chitin could be as much as 56,000 tons. In spite of the apparent attractiveness of the volume of this source, the considerable practical difficulties associated with its collection should not be underestimated. These include the extreme remoteness of the fishery, adverse weather, and a short fishing season. It should also be emphasized that the primary product from the harvest of krill will be protein, and this must be saleable as such on the world market. For all of these reasons, even though the supply of chitin from krill could be very large, this source could not be seriously counted upon for at least several years.

Table 2. Characteristic Compositions of Chitinaceous Wastes

Origin of Waste	Dry-Weight Composition			References
	Inorganic (percent)	Protein/Fats (percent)	Chitin (percent)	
Shellfish	25-50	25-50	14-35	(37)
Krill	24	61	7	(31)
Clams/Oysters	85-90	negligible	3-6	(30)
Squid	negligible	76-95	1-2	(3, 11, 30)
Fungi	negligible	25-50	10-25	(12, 48, 16)
Insects	negligible	60-80	0-8	(45, 46, 49, 52)

Many of these difficulties are not so serious when the harvest of red crab is considered. This particular zooplankton is small in size and inhabits the temperate coastal waters of Chile, Mexico and Southern California. Using conventional shrimp gear, Chile apparently landed over 10,000 tons in 1964, but data on the current annual production are not now being published (34). Longhurst, however, has estimated that the annual red-crab catch in Mexican and Southern Californian coastal waters could be increased to as much as 27,300 tons. Using the assumption that this material is 80% water and that the associated solid contains 9% chitin (47) the potential yield of chitin could be 490 tons per year. This would certainly constitute a significant addition to the North American chitin supply. Of course, red crab can be eaten whole (34) or used in its entirety in commercial aquaculture as a fish-food supplement for flesh pigmentation (47). Under these circumstances, the waste necessary for chitin and chitosan production is eliminated. Thus, like krill, red crab cannot be confidently regarded as a viable chitin source at this time.

MOLLUSCA

Clam and oyster shells

More certain marine sources of chitin are provided by clam and oyster shells, which contain significant quantities of the desired polymer. On a worldwide basis the sea yielded an average of 1,390,000 tons of unprocessed clams and oysters annually during the period 1970-1974 (24). Since about 65% of the whole clam and 85% of the whole oyster (8) consists of shell, these landings could in principle yield 1,043,000 tons of stable chitinaceous raw material.

While the presence of chitin in these shells is qualitatively well documented (44), quantitative data on the amount present is notably missing in the literature. In the laboratory of the authors, local clam and oyster shells were found to contain 6 and 4% of chitin and 90 and 85% of ash respectively (30).

Certainly all clam and oyster shells could not be retrieved for chitin processing, but currently about 190,000 tons are sold annually in the United States alone at about 1¢/lb (40). Current uses include soil liming (54), animal-feed additives (35) and road building (50). These applications demonstrate the relative stability of this chitin source, which could therefore serve to even out the fluctuations associated with the harvesting of some of the other marine organisms. Clearly, however, these shells could not be directly substituted for crustacean waste in a chemical plant, since the additional inorganic material represents a formidable processing challenge. This difficulty has allegedly been overcome in Japan (42) by decalcification of oyster shells with acetic acid. The chitin is recovered by filtration, and the acetic acid is regenerated for recycling by treatment of the filtrate with sulfur dioxide from waste-gas streams. The large quantities of acids which have to be handled per unit of weight of chitin suggest that this shell treatment process could only be economically attractive as an adjunct to a management problem involving acidic pollutants. The total annual potential availability of chitin from clam and oyster shells might then total 22,000 tons.

Squid skeletons

In contrast to the bivalve shells, which constitute the most contaminated source of chitin, the backbone or pen of squid is among the purer forms. This skeletal tissue accounts for about 1% of the whole body weight in the case of squid and can relatively easily be separated from the carcass. The pen on a dry basis contains about 40% chitin (30) which is free of calcium salts. The customary acid demineralization step, which can depolymerize chitin rapidly, can therefore be eliminated from the isolation scheme.

At this time current world landings of squid are about 660,000 tons annually. This would correspond only to a source of approximately 625 tons of chitin per year (Table 1). Squid, however, is not extensively consumed in Western countries, and the catch could be greatly

increased if a gastronomic demand could be created (3). Thus, Voss has estimated (53) that the continental-shelf regions of the world could yield a catch of 7.4 million tons of squid per year. This, in turn, would correspond to a source of about 7,000 tons of chitin. In addition, the estimated potential for oceanic squid landings, based on sperm-whale populations, range from 90 to 450 million tons. These huge quantities of squid would assuredly provide a substantial contribution to the supply of chitin, but, as in the krill situation, the primary product of this fishery would be protein, which again must be saleable in its own right.

INSECTA

Another potentially large source of chitin with associated protein is provided by insects, since most have a chitinaceous exoskeleton (Table 3). This is advantageously free of calcium carbonate, which means that the isolation of insect chitin appears only to involve simple alkaline extractions for protein removal (62). However, very few species of insects carry more than 10% of their weight in chitin. Moreover, even including the commercially grown silkworm, Bombyx mori (55), no significant quantities of insect wastes are available. Nonetheless, the mass rearing of insects has been tried in several cases.

Table 3. Whole Body Composition of Some Common Insects (28)

Insect Species	Body Solids (percent)	Composition of Body Solids Cuticle (percent)	Chitin (percent)
Blatta orientalis (Flour beetle)	24.8	15.5	4.9
Tribolium confusum (Yellow mealworm)	55.5	11.7	2.7
Phormia regina (Blowfly)	21.3	13.3	6.5
Tenebrio molitor (Wax moth)	34.2	--	7.0

For example, the production of fly larvae for animal feed was studied by the U.S. Dept. of Agriculture (USDA) using animal manure as the growth medium (38). It was found that the manure from a chicken ranch with 100,000 birds could support the daily production of 250-500 kg of fly larvae. Even so, the concept had to be abandoned because of the low yields and high production costs. Economical mass insect raising is, however, still continuing in the screwfly sterilization programs, also under the aegis of the USDA. In this endeavor more than 12 tons of flies are grown weekly at a single location, using manure and sawdust as the support media (49).

Of course, in this situation, it must be remembered, the flies are being produced as a high-value biological control device. Despite the existence of these examples, proposals to rear cockroaches (49) or water beetles on sewage lagoon algae (9) as a source of chitin always seem to engender flippant responses. Setting aside witticisms, the main drawbacks to culturing insects seem to be primarily psychological, coupled with a handling problem. The trapping of natural concentrations of insects, such as locusts, as a source of chitin is considered by the authors to be impractical. Even though locust swarms may contain as many as 400 billion individual insects (49), their frequency of occurrence and location would necessitate a collection and transportation system of extreme size and complexity, not to mention the decay problem associated with the protein content of the dead insects.

THALLOPHYTA

Marine diatoms

The constant intrusion of the question of massive protein marketability into chitin manufacture can be minimized to some extent by focusing on two marine diatoms, Cyclotella cryptica and Thalassiosira fluviatilis. The extracellular fibers of these organisms constitute the only recognized source of pure, unassociated chitin found in nature (32). The fibers make up 10 to 15% of the dry weight of the diatoms and can be mechanically separated from the parent cells in 80% or greater yield by sequential application of a shear force, differential centrifugation and ultrafiltration (Table 4). These facts suggested that in artificial monoculture these marine diatoms could possibly be a source of very pure chitin. Unfortunately, in both batch and continuous culture, C. cryptica proved to be relatively slow growing and produced low-density cultures even though the growth was more abundant than that observed with T. fluviatilis. Thus, the maximum cell concentration reached in the continuous culture equipment with C. cryptica was approximately 250 mg/liter. This quantity of cells would yield only 20 mg/liter of chitin: that is, 50,000 liters of culture would have to be processed to isolate 1000 g of algal chitin. Moreover, the prospects of increasing this yield significantly are dim because the cell concentration values fall within the range normally observed in many other autotrophic mass algal culture experiments. Therefore, barring a major engineering breakthrough in algae culture, algae can only be considered as a viable source of chitin in high-profit, low-volume situations where a high-quality product is mandatory.

Chitinaceous filametous fungi

The apparent limitations of controlled culture largely disappear when the organism to be grown is a fungus rather than an alga. That such culture is practical is attested to by the large-volume commercial production of citric acid and of antibiotics. The estimated volume of waste mycelia annually generated in the world is now about 790,000 tons.

Table 4. Comparison of Batch Cultures[a] of Cyclotella cryptica and Thalassiosira fluviatilis

	Cyclotella cryptica	Thalassiosira fluviatilis
Yield of diatoms	160 mg/l	143 mg/l
Chitin content of diatoms	15.2%	10.8%
Fiber yield from diatoms	13.0%	10.0%
Fiber collection efficiency	85.5%	92.5%
Chitin yield from culture	20.8 mg/l	14.3 mg/l

[a]Aerated and grown for nine days.

This constant flow of waste contains about 15-30% (Table 2) by weight of chitin, with the precise level being dependent upon the fungal species as well as culture age and growth conditions. Processing waste mycelia for fungal chitin does not pose any unique difficulties, although it has been reported that the chitin is chemically bonded to an alkali-resistant glucan, possibly crosslinked or branched (9). The conventional isolation procedures would, therefore, yield a mixture of the chitin plus glucan. For many of the possible applications of chitin such a mixture could be perfectly usable. In limited testing, for example, fungal chitosan from Aspergillus niger performed comparably to crab-derived chitosan as a strength additive for paper made from an unbeaten sulfite pulp (30).

Furthermore, it is not a sine qua non that fungal chitin be produced only as a fortuitous by-product of the manufacture of some exotic mold metabolite. Filamentous fungi could be grown directly on any of a number of substrates. In Finland, the sugars in pulp-mill spent sulfite liquors are already being utilized to generate a fibrous fungal animal feed (25). This type of operation could afford an optimized reliable source of chitin which would be largely free of the troublesome ties to large-scale protein marketing.

Chitosanaceous fungi

Although the production of chitin from fungi has many attractive features for many applications, it is probably really deacetylated chitin that offers the greatest polymer marketing opportunity (5). This deacetylation reaction, which converts chitin to chitosan, is difficult and costly to carry out from a chemical engineering standpoint, since it involves the use of concentrated alkali at an elevated temperature for extended periods of time. Clearly, the avoidance of this deacetylation step would be desirable and could lead to a lower cost chitosan able to penetrate more markets. This goal may be directly attainable by the culture of fungi that yield chitosan. Quite a variety of such chitosanaceous organisms are members of the order Mucorales, many of which are common saprophytic soil fungi (Table 5). The chitosan is located within the cell

Table 5. Chitin and Chitosan Content of Some Common Microorganisms[a]

Microorganisms[b]	Composition of whole dry body			Dry Cell Wall Content		References
	Cell Wall (percent)	Chitin (percent)	Chitosan (percent)	Chitin (percent)	Chitosan (percent)	
Filamentous Fungi						
Allomyces macrogynus	25-50	15-30	0	60	0	(4)
Aspergillus niger	64	15	0	24	0	(48)
Penicillium chrysogenum	43	15	0	35	0	(48)
Penicillium notatum	--	25	0	--	0	(12)
Mucor rouxii[c]						
mycelial form	50	5	17	9	33	(10)
yeast form	50	4	14	8	28	(10)
Phycomyces blakesleeanus	--	--	--	27	10	(16)
Choanephora cucurbitarum	--	--	--	17	28	(32)
Yeast						
Candida utilis	--	0	0	2.2	0	(15)

[a]Data are given for a specific culture but can vary widely under different conditions.

[b]Bacteria are not listed due to absence of chitin in cell wall (13).

[c]Each form is assumed to be capable of generating 50% cell water under optimal growth conditions.

wall of the organisms, where it apparently acts as an acid-extractable (32) interchitin fiber cement replacing the polymeric glucans found in other fungal orders. Thus these fungi can act as a source of both chitin and chitosan. Among the several candidates for detailed investigation, Mucor rouxii and Pycomyces blakesleeanus have been found (Table 5) to contain significant quantities of chitosan, and, as such, constitute an attractive direct source of chitin's most important derivative.

CONCLUSIONS

This evaluation of new potential sources of chitin and chitosan discloses that, while there are two promising prospects in nature for the large-scale supply of chitin represented by Californian red crab and Antarctic krill, these will not likely be brought to reality within the next decade. In contrast, the processing of clam or oyster shells for their chitin content will probably never be implemented because of the cost of demineralization. Squid, likewise, will not become a major source of chitin until the eating habits of the Western world change substantially. Insect-based factories for chitin manufacture are also only likely to be given adequate consideration when the humor value of such proposals has become passé. On the other hand, a technical engineering breakthrough will be needed before the culture of chitin-yielding diatoms can go forward on a substantial scale. This is not the case for the culture of fungi, where the knowledge and technology needed to move speedily ahead to produce either chitin or chitosan are already available.

ACKNOWLEDGMENTS

The research reported in this publication was supported by the Washington Sea Grant Program funded, under Grant no. 04-7-158-44021, by the National Oceanic and Atmospheric Administration of the U.S. Department of Commerce.

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III. TECHNOLOGY OF CHITIN-CHITOSAN PROCESSING

THE PRODUCTION OF CHITIN AND CHITOSAN

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ABSTRACT

Factors of economic and functional importance in the location, design and operation of a manufacturing facility to produce chitin, chitosan, protein and possibly other products from crustacean waste are discussed. These include problems of shell supply, chemical and energy requirements, elements of manufacturing cost, alternatives of plant design as a function of plant size and differing physical characteristics of shells from various crustacean species, quality-control requirements, disposal of plant effluents, possibilities for by-product recovery and estimates of required plant investment.

From the above considerations, and possible price ranges for products derived from market evaluation studes, estimates of possible profitability of a chitosan enterprise are projected.

INTRODUCTION

In the past ten years there has been a revival of interest in the possibilities of producing chitin and its derivatives as new materials for use in chemical and allied industries. (1). This new interest has been generated and stimulated by technological developments in several fields. One of the most important of these is the marked expansion of the shellfish industry in the last 20 years making supplies of raw material (crustacean shell) available in concentrated areas and in much larger quantities than formerly. This is a direct result of the phenomenal growth of the frozen-food industry and our food-distribution system. In the 1940s and before, shellfish production was generally a small local enterprise with markets, mostly for fresh products lying within a small radius. Now shellfish is processed in large centers, each with a production in the order of several million pounds per year. Markets for frozen and canned products are located around the world. Frozen shrimp constitute the largest volume of frozen food marketed today.

This expansion and centralization of the shellfish industry has made raw material for chitin-chitosan enterprises available in much larger quantities and with less seasonal fluctuation in supply than in previous years, because different shellfish species have overlapping production seasons in different production areas. There are still, however, problems in ensuring an adequate supply of raw material of suitable quality to support a chitin-chitosan facility on a steady year-round basis. These problems concern the preservation, transportation, storage and quality control of the raw material.

Another factor that has promoted interest in chitin and its derivatives is the large and diversified volume of basic and applied chitin-related research which has been conducted in universities and private industries in recent years. To mention a few developments:

1. Work at the University of Washington, in the College of Forest Resources, in uses for chitosan in the pulp and paper industry.

2. Studies at the University of Georgia by Dr. Wayne Bough on the recovery of proteins from food-processing wastes by flocculation with chitosan and the use of the recovered products as animal feed.

3. Studies at Rutgers University by Dr. Eveleigh on the occurrence of chitin and chitosan in fungi, the production of chitinase and chitosanase, and the use of chitin as a substrate for the growth of microorganisms.

4. Studies at the University of Delaware by Dr. Paul R. Austin and associates on solvents for chitin and the production of fibers and films.

5. Work by Professor R. A. A. Muzzarelli at the University of Ancona on the chelating properties of chitosan and its potential use for the removal of heavy metals from industrial wastes.

6. Work by the Food, Chemical and Research Laboratories and the Kypro Company of Seattle, Washington, on the use of chitosan as a flucculant and flocculant aid.

Much of the above activity has been stimulated and sponsored by the Sea Grant Program of the National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

BASIC ECONOMICS OF CHITIN-CHITOSAN PRODUCTION

In spite of the promising new markets for chitin and chitosan and improvements in the availability of raw materials, obstacles have been encountered in launching commercial enterprises. In this report an attempt is made to analyze difficulties and to determine ways in which they may be overcome. Essentially the problem is to prove the cost-effectiveness of chitin and chitosan in market areas that appear to have volume potentials in balance with production capabilities and to determine production costs and probable market values of products with sufficient accuracy to ensure the profitability of a proposed business venture.

The principal direct costs in the manufacture of chitin and chitosan are for raw materials, labor, chemicals, utilities and retirement of plant investment. A brief look at each of these should serve to define a range of production costs. For this purpose, let us assume a facility designed for production of one million pounds of chitosan per year, or 1.25 million pounds of chitin per year, and probably an equal amount of a highly nutritional feed grade protein.

Raw-material costs

Recoverable waste from shellfish processing will generally fall within the composition ranges indicated in Table 1.

Table 1. Composition Ranges for Shellfish Waste

	Percent
Solids as received	30-35
Chitin: dry basis	15-30
Protein: dry basis	15-40
Mineral matter ($CaCO_3$ + $Ca_3(PO_4)_2$	35-55
Fats	0 - 5

The chitin content in shrimp waste is lower in most species of crab, crayfish and lobster. The protein content is partially unrecovered protein contained in the flesh but also protein contained within the shell matrix. Its content can vary widely, depending on species and type of primary shellfish processing. For example, Chesapeake Bay blue crab is commonly processed by hand picking, leaving considerable unrecovered flesh in the waste material. Shrimp in Atlantic and Gulf fisheries are beheaded at sea at a loss of 40% of the live weight and perhaps 55% of the total waste. In other localities crab butchering and extraction are separate operations, so that butchering wastes are not recovered.

The mineral content of the waste material depends on the species, shell maturity after molting, and amount of non-shell components in the waste. Mineral matter usually consists of 90% or more calcium carbonate, with the balance as calcium phosphate. Fats are largely derived from visceral material and vary from 0% in clean crab-leg shells to perhaps 5% or more in some shrimp and lobster species.

An average shell composition for discussion purposes is shown in Table 2.

Table 2. Assumed Waste Composition

	Percent
Solids as received	33
Chitin: dry basis	25
Protein	25
Calcium carbonate	50
Shell required for 1.25 million pounds of chitin: 5 million pounds of shell (dry basis) or 15 million pounds of shell as received.	

In crab processing, meat recovery is generally 20-25% of live weight with cooking losses on the order of 25%, so that recoverable waste is generally 50-55% of live weight. Fifteen million pounds of waste is thus equivalent to 27-30 million pounds of live weight crab, or to 5.4-7.5 million pounds of primary product. The chitin yield is 8.33% of recovered waste, or 4.40% of live weight. It can be seen that a fairly large shellfish operation is necessary to support a plant that is manufacturing a million pounds of chitosan per year.

As to raw material cost, this can vary widely depending on the practicalities of shell supply. In many localities harbor pollution from shellfish waste is a serious problem, and in such situations processors generally are faced with waste-disposal expense. A chitosan plant might thus have a negligible, or even negative, cost for most of its raw material. There are means of stabilizing fresh shellfish waste for one to three weeks against bacterial spoilage, so that short-term fluctuations in shell supply can be ironed out to permit a constant production rate. Seasonal fluctuations, such as closed periods for the fishery, are, however, a more serious matter. To provide a constant production rate on an annual basis and thus minimize plant investment costs, it may be necessary to import shell from other ares or to store excess shell from peak periods. This will require long-term stabilization measures, such as drying or freezing.

Assuming a cost for heat of $4 per million BTUs with 50% thermal efficiency, the cost of heat per pound of dry shell would be (67/33) x 1,000 x 2 x ($400/10^6$) = 1.62¢ or 6.5¢ per pound of chitin. Added to this will be fixed charges on the drier, labor, utilities, transportation, storage and overhead, so that the total raw material charge might be 20¢ per pound of chitin. Obviously a chitosan plant would want to minimize its dependence on dried shell. In addition, excessive temperatures in drying must be avoided, as it causes serious damage to chitin and results in an unsatisfactory product.

Freezing is less expensive than drying from the standpoint of fuel costs, but from the point of view of transportation and storage costs it will be higher. In short, raw-material cost is entirely dependent on each individual plant's circumstances. If most of the shell can be supplied from local sources, overall raw material cost might be reduced to 8¢ per pound of chitin.

Labor costs

A production of 1.25 million pounds of chitin or one million pounds of chitosan per year, assuming 300 days operation, 24 hours per day, is equivalent to a production rate of:

$$1.25 \times 10^6/7{,}200 \text{ or } 173.6 \text{ lbs of chitin per hour.}$$

This could probably be handled by four men per shift at about $5 each per hour for a total cost of 2,000/173.6 = 11.5¢ per pound of chitin. It is possible to design a continuous plant in which labor would be reduced, but for the production rate projected, the savings would probably be offset by greatly increased investment costs.

Chemical costs

The principal chemical cost is for the acid required to dissolve mineral matter from the shell. Assuming the use of 23% hydrochloric acid at $55 per ton, this cost would be

2 x (73/100) x (1/.32) = 4.56 lbs 32% HCl

or 4.56 x (55/2,000) = 12.55¢ per lb of chitin.

There are ways in which this cost might be reduced: for example, calcium chloride might be marketed as a by-product. This procedure, however, would require added investment costs and labor so that savings might not be significant, especially in a small production plant.

Another need for acid is in neutralization of the sodium proteinate liquor to the isoelectric point for protein precipitation. Sodium hydroxide required for protein extraction is generally about 15% of the weight of protein, with an equivalent amount of acid needed for precipitation.

Using the example shell analysis, the sodium hydroxide required would be 150 lbs per 1,000 lbs of chitin or protein. At 8¢ per lb for caustic soda purchased as 50% solution, this would be 1.20¢ per lb of chitin. The acid requirement would be 428 lbs at $55 per ton, or $11.76 per 1,000 lbs, or 1.18¢ per lb of chitin.

Total chemical costs per pound of chitin including protein precipitation are thus:

Hydrochloric acid	13.73¢
Sodium hydroxide	1.20
Total	14.93¢

Conversion of chitin to chitosan requires removal of the N-acetyl group with sodium hydroxide. Stoichiometrically the NaOH consumption would be 197 lbs per 1,000 lbs of chitin which amounts to $15.76 or 1.58¢ per lb of chitin.

In practice, chitin is treated with a 50% sodium-hydroxide solution at an elevated temperature, and a liquor to solids ratio of at least five is required so that 2,500 lbs of NaOH per 1,000 lbs of chitin is actually used in the deacetylation process. This would be 20¢ per lb of chitin. By careful control of reaction conditions and countercurrent washing of the chitosan, much of the alkali can be recycled, and dilute washings can be used for protein extraction. Sodium acetate can also be recovered and marketed as a by-product. How close the alkali consumption can be brought to the stoichiometric limits will depend on plant design, investment and process control. Actual cost for alkali in conversion could thus vary over wide limits. A cost of 10¢ per pound of chitin appears reasonable for the projected production.

Heat requirements

Energy requirements for chitosan production are moderate. Protein extraction can be conducted using countercurrent apparatus to minimize consumption of heat and water and produce a sodium proteinate extract at high concentration. Demineralization is at room temperature or lower, and deacetylation is at a moderate temperature using 20% chitin concentration.

Principal heat requirements are for drying products, either chitin, chitosan or protein. Chitin can be centrifuged to about 40% solids before drying so that evaporation is only 1.5 lb of water per lb of product. Assuming $4 per million BTUs and 50% drier efficiency, this would amount to 1.2¢ per lb of chitin.

Protein can be roll dried from a slurry at about 25% solids, requiring three pounds of evaporation per pound of product, or 2.4¢ per pound of protein.

Total heat costs would amount to:

For	per Pound of Chitin
Protein extraction	2.4¢
Deacetylation	2.4¢
Chitin-chitosan drying	1.2¢
Protein drying	2.4¢
Total heat cost	8.4¢

Plant investment

For a plant producing only 1.25 million pounds of chitin per year or 173 pounds per hour, the most practical plant design from our experience is one that uses batch operations. This design minimizes equipment costs, and, while operating labor costs may be somewhat greater than for a fully continuous plant, maintenance labor costs would probably be less.

Extrapolating plant investment at our Tukwila pilot plant for the increased production, a factor of 5.77 times the present rate is obtained. Equipment cost is estimated at about $350,000. Costs for a building, steam boiler and installation should be added to this for a total of about $600,000. Amortizing at 10% yearly, this would amount to 4.8¢ per pound of chitin.

Other costs

Water and power can be estimated at about 3.25¢ per pound of chitin. Plant supervision and quality control would amount to about $50,000 per year, or 4¢ per pound of chitin. Maintenance can be estimated at 5% of equipment cost per year, or 1.6¢ per pound of chitin. Plant overhead at $50,000 per year would add 4.0¢ per pound of chitin.

Table 3. Total Manufacturing Cost

		Cents per Pound/Chitin
Raw material		8.00
Chemicals (HCl)	13.73	
(NaOH)	1.20	14.93
Production labor		11.50
Steam		8.40
Amortization		4.80
Water and electricity		3.25
Supervision and quality control		4.00
Maintenance		1.60
Plant overhead		4.00
Total manufacturing cost		50.48

If chitosan is to be the main product, an additional alkali cost of 8.2¢ per pound of chitin will be encountered and about 25% additional labor and utility costs should be added. A 20% shrinkage in yield can also be expected. Total manufacturing costs for chitosan would thus be about 82¢ per pound.

Potential sales

There are many possible markets for chitosan which can give it widely different market values depending on use. The use that appears to offer the most promise for rapid development is wastewater treatment, both as a flocculant for suspended solids and for heavy-metal removal. For these purposes, chitosan should be cost effective in competition with existing products at about $2.00 per pound. Recovered protein has been found to be equivalent to casein in animal-feeding tests when approximately 0.5% of methionine is added. It is believed that a price of 35¢ per pound is realistic for this material when used as an additive for pet foods and specialty feeds.

Using the above estimates, profitability can be projected as in Table 4. The variance in profit figures can obviously be considerable, and if inflation trends in plant construction and labor costs continue as they have been in recent years, return on investments might not be as attractive as indicated. Nonetheless, it is still likely that chitosan from shellfish waste could prove to be a worthwhile endeavor.

It is probable that 10 or more plants of the projected size or larger could be operated in the United States, but it does not appear likely that chitosan production will exceed 20 million pounds per year in the near future.

Table 4. Projected Profitability of a Chitosan Enterprise

Sales	Dollars per year
Chitosan, 1,000,000 lbs @ $2.00/b	2,000,000
Protein, 1,250,000 lbs @ $0.35/lb	437,500
Total sales	2,437,500
Costs	
Manufacturing cost, 1,000,000 lbs @ $0.82/lb	820,000
General, administrative and sales expense @ 15% of sales	365,000
Total cost of sales	1,185,000
Profit	
Gross profit	1,252,500
Federal income tax @ 50%	626,250
Net profit	626,250
Percent of sales	25
Percent of investment	104

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A STUDY OF VARIABLES IN THE CHITOSAN MANUFACTURING PROCESS IN RELATION TO MOLECULAR-WEIGHT DISTRIBUTION, CHEMICAL CHARACTERISTICS AND WASTE-TREATMENT EFFECTIVENESS

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ABSTRACT

Manipulation of variables in the chitosan manufacturing process produced chitosans with varying chemical characteristics and molecular-weight distributions (MWD). These products also differed in effectiveness as waste-treatment agents for conditioning of activated sludge and coagulation of cheese whey. Viscosity was not as reliable as MWD distribution in correlating with waste-treatment effectiveness. For a graded series of chitosan products made from a single batch of chitin differing only by time of deacetylation, one time of hydrolysis was shown to be optimum for giving the most effective product. Its MWD was neither the highest nor the lowest of the series. High-viscosity products which were more effective for sludge conditioning were less effective for coagulation of cheese whey.

Times of hydrolysis and concentrations of alkali were manipulated to produce chitosan products having various characteristics and MWD, allowing choices between labor, reagents, and equipment to be balanced by the manufacturer.

High-pressure liquid chromatography (HPLC) in the size exclusion mode has proved to be a useful method for estimation of the MWD of chitosan products. Analysis time is approximately 20 minutes per sample. Chitosan molecules dissolved in 2% acetic acid containing 0.2M sodium acetate are separated according to size by passage through a sequential combination of columns packed with coated glass beads having controlled pore sizes of 2500 Å, 1500 Å, 250 Å, 100 Å, and 40 Å. A survey of commercially manufactured samples showed MWD values of 0.1-4 million for the weight average, 0.05-0.08 million for the number average, and 2-9.8 for the dispersity or ratio of weight and number averages.

INTRODUCTION

The manufacture of chitosan from the exoskeletons of crustaceans is well documented in the literature (12, 13), and some other methods have been extensively reviewed by Muzzarelli (10). Three major steps are generally involved: demineralization with dilute acid, deproteinization with dilute alkali and moderate heating to purify chitin, and deacetylation with concentrated alkali and high temperature to convert chitin to chitosan. Variations in the reagent used and its concentration, as well as time and temperature of the treatment, determine the quality and performance of the product.

As a result of increasing problems in the treatment of waste effluents from industries, the use of chitosan as a coagulating agent has been extensively

studied in the laboratory. The high effectiveness of chitosan for this purpose has been demonstrated (2, 3). Although measurement of viscosity has been commonly used for estimating the quality of chitosan, it is not satisfactory for estimating the effectiveness of chitosan as a coagulant (5, 16). Since estimation of molecular-weight distribution (MWD) of chitosan by high-pressure liquid chromatography (HPLC) has been found effective (15), the use of this method, together with the information on charge density estimated by the titration method (7) to correlate the performance of chitosan, has been investigated in this laboratory.

This presentation includes our findings in the evaluation of characterization methods, using them to estimate the effectiveness of chitosan and to survey the MWD values of some commercially prepared chitosan samples. Studies on the effects of variables in the deacetylation process, such as the concentration of alkali and time of deacetylation, on the quality and characteristics of the products are also reported.

EXPERIMENTAL

Raw materials for producing chitosan were shrimp hulls collected from a processing plant, iced during transport, and dried the next day in a forced-air oven at 103° C for 24 hours. Dried hulls were ground to 1 mm particle size. Activated sludge was obtained from a biological treatment plant at a commercial vegetable-processing plant. The turbidity of the sludge was adjusted to a reading of 1250 Formazine Turbidity Units (FTU) before use.

Manufacturing of chitin

A large quantity of chitin with uniform quality was needed for conducting the experiment under different deacetylation conditions. The reaction tank for demineralization had a capacity sufficient for using 900 g dry shrimp hulls as starting material. Ten liters of 0.5N HCl was required for this process, which represented a 10% excess of HCl over the stoichiometric amount of ash in shrimp hulls as calcium carbonate. The demineralized material was collected on 60 and 200 mesh screens, and washed to neutrality with deionized water. The residue was then deproteinized with nine liters of 1% (w/v) NaOH for one hour at 65° C with constant stirring. Thereafter the residue was collected and washed as above.

The chitin product was dried at 85° C in a forced-air oven for 18 hours. Ash content was determined.

Deacetylation of chitin

A four-liter reaction kettle jacketed with a heating mantle controlled at 100° C by a variable transformer was used in this step. The four-hole kettle cover provided mountings for an overhead stirrer, a nitrogen purging tubing, a thermometer and a sampling port. Conditions other than variables to be studied were maintained consistent for every experiment. Temperature was kept at 100° C for all studies in the deacetylation step. Alkali concentration was kept at 50% in the time-variable study, and was varied at 35, 40, and 50% in the concentration study.

Moisture and ash

AOAC. (1) methods were followed to determine the degree of deacetylation expressed in percentage of repeating units deacetylated among total number of repeating units in a chitosan polymer. One was based on the determination of acetylated residues by Lemieux and Purves (8), the other was based on the measurement of free amino residues by Broussignac (7). These two methods have been shown to agree with each other by Bough et al. (6), and the Broussignac method was used for most samples because of its simplicity.

Viscosity measurement

A Brookfield viscometer, Model RVT spindle-type, was used for this purpose. In all cases, 500 ml of chitosan solution was prepared in 2% acetic acid at 10 g/l concentration on a moisture-and ash-free basis. Measurements were made in triplicate at four different speeds on solutions at 20° C. The viscosity value was taken from the condition requiring the lowest conversion factor.

Molecular-weight determination

MWD values of chitosan samples were determined by HPLC method. MWD values included weight average MW ($\bar{M}_w$), number average MW ($\bar{M}_n$), and dispersity (D). Conditions used were basically as described by Wu et al. (15) and modified by Bough et al. (4). Samples were prepared in 2% (0.33M) acetic acid with 0.1M or 0.2M sodium acetate. The presence of salt was necessary to minimize the absorption phenomena (4). The column materials were Glycophase-CPG ranging from 40 to 2500 Å pore sizes. The standard curve calibrated by dextran standards was $\text{Ln } M_i = 24.8041\text{-}0.8520\ V_e$, where M_i was the molecular weight of ith species eluted at V_e in ml (6)

Effectiveness as a coagulant

Effectiveness was measured by the specific resistance (r) measurement using the Buchner funnel filtration test (5, 6). The r values determined on increasing chitosan dosages (x) added to activated sludge were fitted into a best quadratic equation for each chitosan sample. The x and r values at the inflection point of the equation were obtained by letting the first derivative of the equation be zero. The product of x and r for each sample was termed the optimum equivalent dosage (OED), which was a compound parameter for comparison with other samples (6). The lower the OED value, the more effective the product.

The overall filtrate volume obtainable in 30 seconds with the Buchner funnel test was used in one study to represent the chitosan effectiveness (5).

In other cases, when the waste effluent such as cheese whey was tested, the jar test measuring the reduction of turbidity was used to determine the effectiveness (16).

RESULTS AND DISCUSSION

Evaluation of chemical analyses

Using a series of chitosan samples varied only by the length of time in the deacetylation process, several methods were performed and compared for measuring the degree of deacetylation, molecular size and effectiveness of the product. For determining the degree of deacetylation, the Broussignac method was highly correlated with the Lemieux and Purves method (8), but the Broussignac method is simpler to perform.

Tested on the same series of samples, correlations between MWD values and viscosity data followed Staudinger's equation (9), although the coefficients of correlation were not high (6). When comparison was based on random chitosan samples, the correlation was not even as high as above. The study included commercially prepared samples. Viscosities of samples that varied in some ways in addition to the difference in the molecular size are apparently no longer following the Staudinger equation. Besides, as observed by Bough et al. (4), undissolved or swollen chitosan particles in the solution would dramatically affect the viscosity reading which could be eliminated through filtration prior to HPLC determination. Thus, the HPLC method was preferred to the viscosity measurement in estimating the molecular size of the sample.

For determining the effectiveness of chitosan as a coagulant for activated sludge, the Buchner funnel test is often used for testing the dewatering efficiency of the treated sludge. Although the overall filtrate volume was found workable (5), the filterability rate estimated by the specific resistance gave better precision based on the coefficient of determination in the polynomial regression analysis (6).

Effect of different waste-effluent systems

Two wastewater systems, activated sludge from a vegetable-processing plant and cheese whey from a dairy plant, were tested with a group of ten different chitosan samples manufactured in this laboratory (5). The Buchner funnel test was used for the activated sludge study and the jar test for the cheese-whey study. Results showed that effectiveness of chitosan samples for the two systems were virtually opposite (5, 16). We postulate that this is primarily due to the difference in particle sizes and/or charge characteristics of the two wastewater systems (14), suggesting that different chitosan samples can be produced to suit different wastewater systems.

Stability of chitosan solutions

A commercial sample was prepared in 2% acetic acid and stored at room temperature for different periods of time. Analysis of these preparations with HPLC indicated that MWD values of the sample decreased slowly but continuously. $\bar{M}_w$ and $\bar{M}_n$ values decreased 20 to 25% in one year. This result indicates that the industry needs to be more aware of shelf life, if the sample is to be sold in a solution form. Whether this is due to the degradation of the polymer or the change in the molecular configuration or other causes is undetermined.

Concentration of alkali in deacetylation

One study with 50% NaOH having three replications demonstrated that the precision of the experiment was excellent, judging from the degree of the deacetylation data in Fig. 1 and MWD data in Table 1 (6). As shown in Fig. 1 and Fig. 2, both percent deacetylation and MWD data decreased very fast in the first hour and slowed down afterward. Another study with 35, 40, and 50% NaOH showed that, as the alkali concentration decreased, the rates of decrease in both viscosity (Fig. 3) and MWD data (Fig. 4) slowed down. The transition phenomena which appeared for the 35% NaOH indicated that the viscosity increased up to 21 hours and decreased afterward. It demonstrates the conversion of insoluble chitin material to soluble chitosan material due to the deacetylation action of the concentrated sodium hydroxide. The MWD values increased up to 27 hours, decreased afterward, but stayed the same after 30 hours. This is probably because the deacetylation under mild alkali concentration can completely solubilize chitin only after being deacetylated for 27 hours. Up to 27 hours, products are only partially soluble.

Table 1. Molecular-Weight Distributions of Chitosan Samples Prepared by Different Deacetylation Times Dissolved in Acetic Acid-Sodium Acetate (0.33 M-0.1 M) and Distilled Water at pH 4.15

Sample	Time (hrs)	$\bar{M}_w$ ($\times 10^3$)	$\bar{M}_n$ ($\times 10^3$)	D[1]
A	0.5	1487 ± 98[3]	322 ± 27	4.63 ± 0.18
B	1.0	1142 ± 94	232 ± 21	4.93 ± 0.25
C	2.0	925 ± 63	186 ± 8	4.98 ± 0.19
D	3.0	846 ± 24	177 ± 7	4.79 ± 0.15
E	4.0	775 ± 61	161 ± 12	4.81 ± 0.09
F	5.0	667 ± 26	139 ± 8	4.80 ± 0.16
4-74[2]	---	682 ± 22	129 ± 3	5.28 ± 0.2

[1] D is dispersity ($\bar{M}_w/\bar{M}_n$)

[2] Sample 4-74 is a commercial product (12).

[3] Averages and standard deviations of two replicant analyses of three batches of chitosan.

Between 21 and 27 hours of deacetylation, partially dissolved residual particles may be responsible for the high viscosity of the solution, which is known as the "fish-eye effect" (4). After 30 hours of deacetylation, the molecular size of chitosan may remain constant, while the degree of deacetylation keeps on increasing, and this decreases the viscosity continuously after 30 hours. This speculation awaits proof of the data on the degree of deacetylation of these samples.

Turbidity of these chitosan solutions made from different concentrations was also measured. The plots of turbidity vs. time of deacetylation indicate that the turbidity readings level off when the sample is completely soluble, implying that turbidity measurement could possibly be a quick and qualitative method for monitoring the solubility of the product.

Finally, this study indicates that, to obtain a product with similar quality, different manufacturing processes can be chosen, based on the need to save either the reagent or the labor costs.

Effect of deacetylation time on the effectiveness

The first set of samples deacetylated at 100° C for 0.5 to 5 hours was used to test effectiveness in relation to chemical and molecular-weight characteristics. Activated sludge was used in this experiment. Effectiveness measured by the OED values is shown in Fig. 5. These OED values were analyzed by Duncan's multiple range test. It concluded that the effectiveness increased in the first two hours of deacetylation, and no significant change in effectiveness measured by OED was shown afterwards. This coincides with the study on synthetic polymers (14), which shows that each polymer has an optimum MW for a particular waste-treatment application. The optimum MW is not necessarily the highest possible value. If different wastewater systems had been used for the testing, these chitosan samples might perform differently, as we observed previously when one chitosan product was most effective for sludge and another for cheese whey. Thus, different grades of chitosan samples may be produced that will be useful in different waste-treatment applications (5, 16).

Correlation between the performance and chemical characteristics

With the same group of samples (Fig. 2), correlations among these parameters were carried out in an attempt to determine which chemical characteristics were useful for predicting the performance. Multiple linear regression with the logarithmic transformation was used to correlate data. Only the samples deacetylated for 0.5, 1, and 2 hours were used, based on their effectiveness data shown in Fig. 5. Results, as shown in Table 2, indicate that MWD data correlated better with effectiveness than with viscosity. When viscosity data was used, the weight of percent deacetylation was almost negligible judged from the beta prime values. This may be due to the fact that the effect of percent deacetylation is already included in the viscosity measurement. Both $\bar{M}_w$ and $\bar{M}_n$ correlated similarly with effectiveness, while $\bar{M}_w$ was weighed more in the correlation than $\bar{M}_n$. Thus, it is logically concluded that $\bar{M}_w$ and percent-deacetylation data are the two most useful parameters in predicting the performance of chitosan as a coagulant.

Since the possibility of experimental error is fairly great in the effectiveness measurement with activated sludge (Fig. 5), an experiment with greater sample size may be needed to confirm this conclusion. A similar experiment might be performed to investigate the correlation of these chemical characteristics with other functionalities of a chitosan product, such as film forming or medical applications. This information is useful in producing the desired product for a specific application.

Table 2. Correlations between the Chemical Characteristics and Effectiveness of Chitosan Samples[1]

MODEL: $(OED) = b_0 + b_1 \cdot \text{Ln } X_1 + b_2 \cdot \text{Ln } \underline{\%\text{Deacetylation}}$

Variables	Coefficients			Beta Prime		
(X_1)	b_0	b_1	b_2	b_1'	b_2'	R^2
Viscosity	-51.27	16.22	0.02	0.74	0.03	0.53
$\bar{M}_w$	-2579.20	120.66	223.85	1.36	0.65	0.74
$\bar{M}_n$	328.96	53.65	-217.71	0.69	-0.64	0.75

[1]Units used in the formula are OED in 10^6, viscosity in cps, and MWD data in daltons.

Survey on MWD values of commercially prepared samples

Table 3 shows some examples of MWD values for several commercially prepared chitosan samples sent to this laboratory. Solutions prepared in 0.33 M (2%) acetic acid with 0.2 M sodium acetate have pH 4.45. The $\bar{M}_w$ values ranged from 110,000 to 3,999,000, $\bar{M}_n$ values from 51,000 to 839,000, and dispersity from 2.18 to 9.81. Values are averages of two measurements. The precision of the replication was usually within 10%. The determination of MWD by size-exclusion mode as in the HPLC method was based on the molecular size and shape in the solution used. Although the column material was the Glycophase-CPG having the minimal absorption effect (15), the 0.2 sodium acetate was still needed to eliminate the residual interactions between the columns and chitosan and to minimize the polyelectrolyte effect (4, 19). The molecular-weight values of some products fell in the 120,000 range, as reported by Muzzarelli (10), while many other products had both $\bar{M}_w$ and $\bar{M}_n$ values higher than this range. The value reported by Muzzarelli was determined by the light-scattering technique; thus it is considered equivalent to $\bar{M}_w$ values. It is assumed that milder manufacturing conditions in this study were used for the products being surveyed. Many other MW values have been reported based on the viscosity measurement, which would be equivalent to the viscosity average MW ($\bar{M}_v$) and related to a "complicated average falling somewhere between $\bar{M}_w$ and $\bar{M}_n$" (9). The complexity of this value would increase as the dispersity of the MW of the polymer increases, as is true of many chitosan products (Table 3). $\bar{M}_v$ values of chitosan samples have been reported ranging from 216,000 to 365,000 (Brine, personal communication; 11).

So-called chitosan is in any case not a very specifically defined substance. Molecular size and charge density can vary according to the manufacturing conditions used. The MW values also depend on the method of measurement, either direct or indirect, or the standard used for calibration in the indirect measurement. In those cases where $\bar{M}_w$ values of over 1 million are observed, association of monomer units to form large oligomer complexes may be involved. Such high $\bar{M}_w$ values have been observed upon analysis of certain commercial chitosan samples, as well as products made in our laboratory under mild conditions. At this point, we are not certain if the high $\bar{M}_w$ values we observed represent a natural or an artificial state.

Table 3. MWD Values for Chitosan Samples Manufactured Commercially and Prepared in 0.33M Acetic Acid with 0.2M Sodium Acetate

	M_{w_3} (10^3)	M_{n_3} (10^3)	D
Kytex H	1302	198	6.60
Kytex L	110	51	2.18
J-I	1520	223	6.83
J-II-1	1685	203	8.32
J-II-2	1449	149	9.81
J-III	1750	289	6.06
RST-Co	315	80	3.96
MCI-17	3999	839	4.77
MCI-18	489	151	3.26
MCI-32	1871	414	4.53
Flonac-N-A	3686	101	3.68
Flonac-N-D	1333	207	6.45
FCRL 16-73	807	171	4.99
FCRL 4-74	233	67	3.48
FCRL 5-76	710	111	6.39

The waste-treatment effectiveness of chitosan compared to synthetic polymers having molecular weights of over 1 million, the viscosity of chitosan solutions and our HPLC results all support our belief that, at least in dilute acetic-acid solutions, chitosan behaves as a high-molecular-weight polymer. The forces or bonds that hold the complex together are unknown to us at this point. Further studies on dissociation of the complex and analysis of sub-units are planned. Joint experiments are in progress to correlate our results by HPLC analysis with x-ray diffraction and light scattering in Professor Averbach's laboratory at M.I.T.

A conclusion of our study is that HPLC is a better tool than viscosity measurement for determining the quality of chitosan samples as a coagulant. Information on the percent deacetylation of samples is also needed. With the selected characterizing methods, products can be well monitored. Series of different grades of chitosan samples for different applications can be produced merely by varying the manufacturing conditions, especially the deacetylation steps. Because performance varies depending on the wastewater systems used, a case-by-case survey is needed for each type of application, unless a well-designed experiment is conducted and the relationship between the characteristics of chitosan samples and the nature of the wastewater system can be revealed.

ACKNOWLEDGMENTS

This work is a result of research sponsored in part by the Georgia Sea Grant Program, supported by NOAA Office of Sea Grant, Department of Commerce, under Grants nos. 04-6-158-44115 and 04-6-158-44017. The U.S. government is authorized to produce and distribute reprints for governmental purposes, notwithstanding any copyright notation that may appear hereon.

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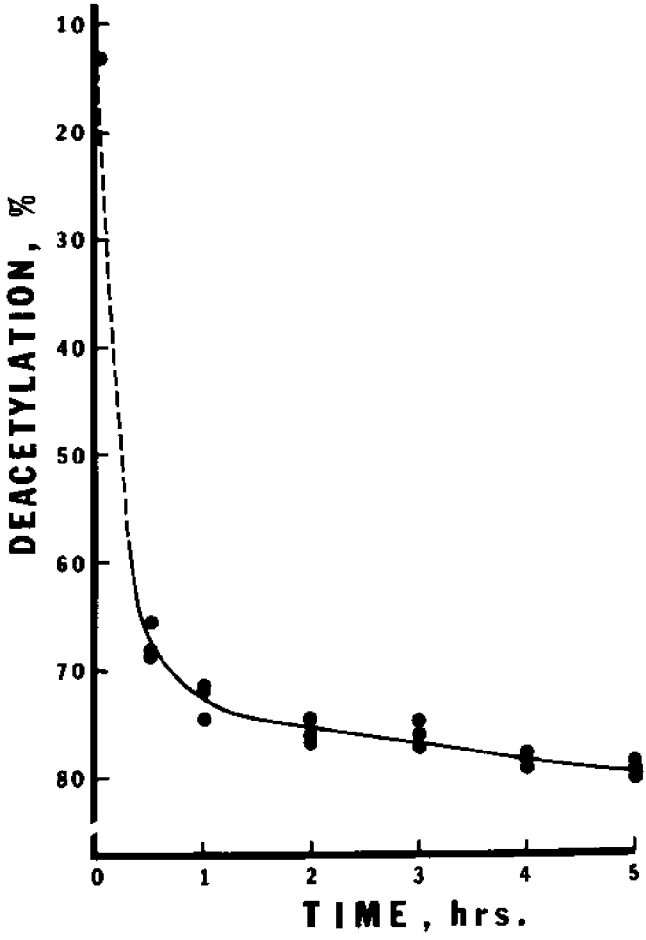

Figure 1. Deacetylation of Chitin with 50% NaOH.

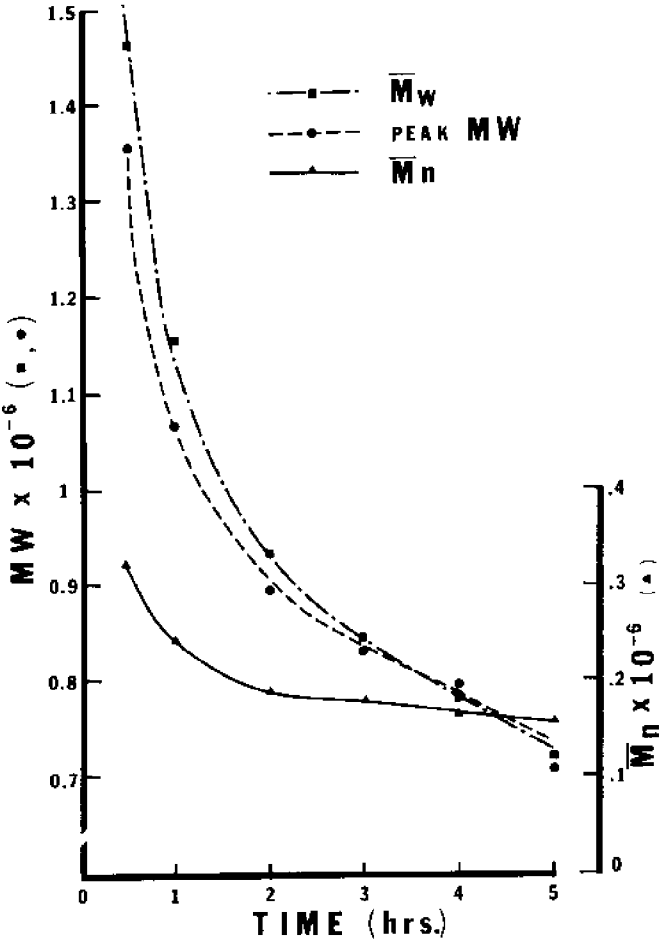

Figure 2. Changes in Molecular Weight Average (M_w), Peak Molecular Weight (MW) and Molecular Number Averag s (M_n) during the Deacetylation of Chitin with Alkali.

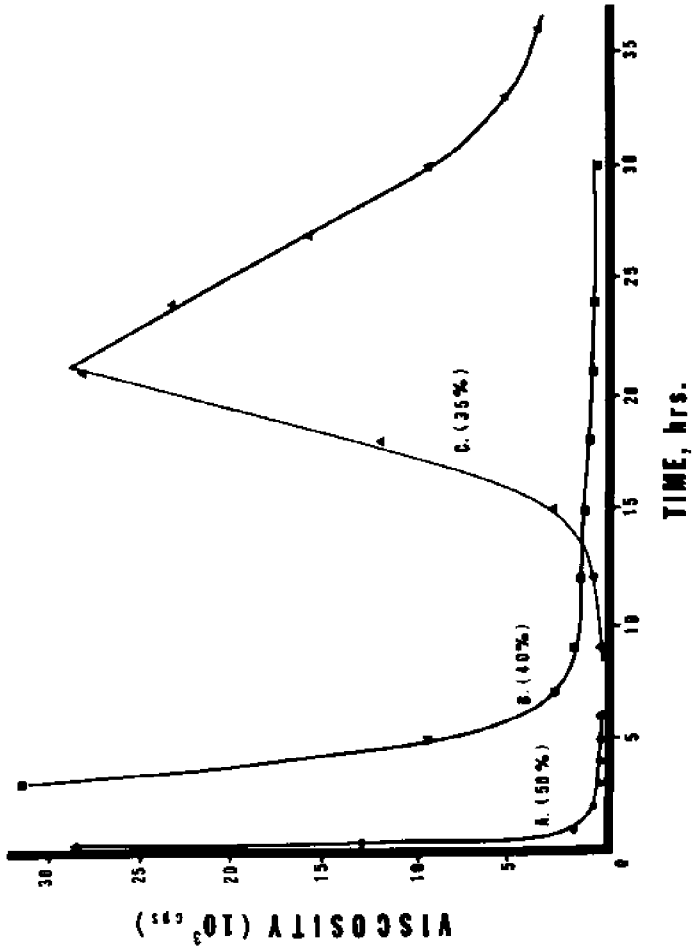

Figure 3. Viscosity Changes during the Deacetylation of Chitin with different Concentrations of NaOH.

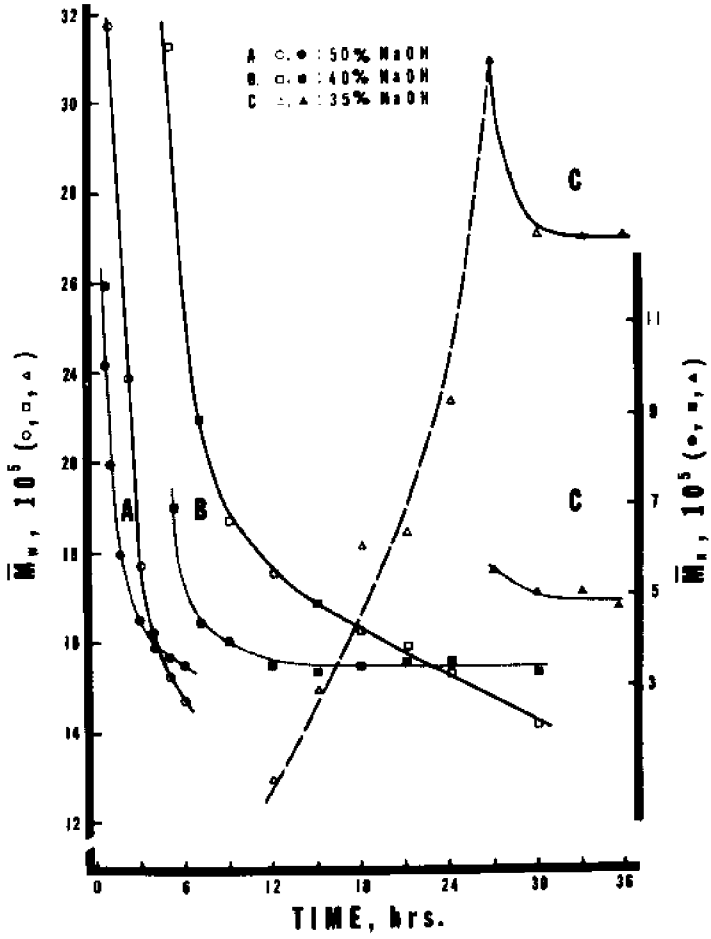

Figure 4. Changes in Molecular Weight and Number during the Deacetylation of Chitin with different Concentrations of NaOH.

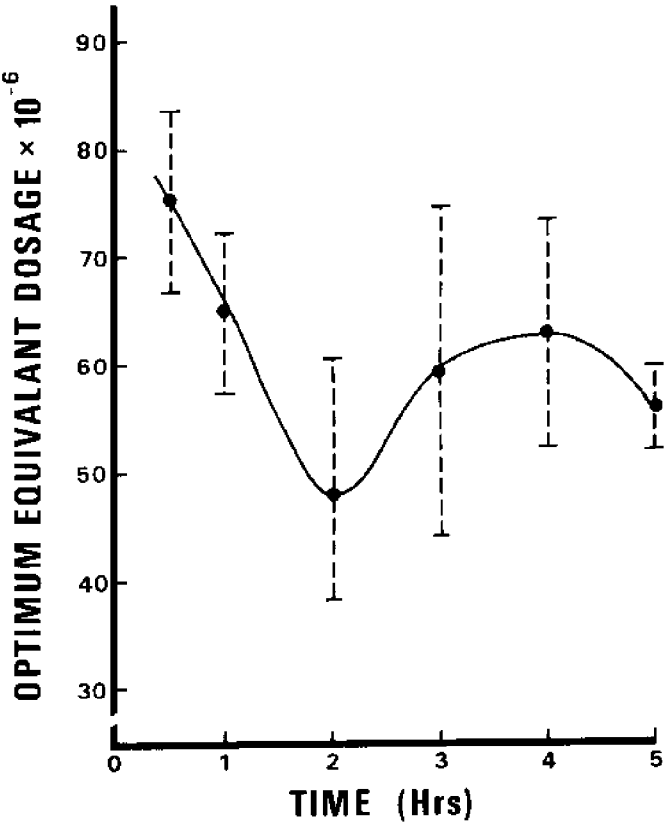

Figure 5. Effectiveness of Alkali Deacetylation of Chitin at 100°C for 0.5 to 5.0 Hours.

ORGANIC ACID SOLVENT SYSTEMS FOR CHITOSAN

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ABSTRACT

A number of organic systems have been studied as possible solvents for chitosan. They are divided into four categories based on their viscosity versus concentration curves.

INTRODUCTION

Muzzarelli (3) has reported that chitosan is soluble in formic, acetic, 10% citric, pyruvic and lactic acids and that other organic acids fail to dissolve it. Because of the diverse nature of these acids (carboxylic, tricarboxylic, alpha keto, and hydroxy) and because of the reported absence of a systematic survey of chitosan's solubility in organic acids (3), it is very likely that additional solvents for chitosan exist, and it is important that these additional solvents be identified because chitosan is a polyelectrolyte that has potential use as a flocculant to remove impurities from aqueous systems (1, 2, 3). Each solvent will have a different cost and possibly a different effect upon the environment.

EXPERIMENTAL

The chitosan used in most of these studies was produced by the Food, Chemicals and Research Laboratory Ltd., 4900 Ninth Ave., Seattle, Washington and was supplied by the Office of Sea Grant, National Oceanic and Atmospheric Administration, Department of Commerce, Rockville, Maryland. Some of the later experiments used chitosan purchased from the Kypro Company, which has incorporated the Food, Chemicals and Research Laboratory Ltd. and has the same address.

The solutions were prepared by stirring the solvent with chitosan for at least 24 hours or by homogenizing the system with a Polytron homogenizer; the latter procedure eliminated the long stirring times. Because of the high viscosities of the systems in which chitosan is soluble, viscosity measurements proved to be the only practicable method for obtaining a measure of the solubility. The viscosities were determined with either a Brookfield LVT or RVT viscometer.

RESULTS AND DISCUSSION

The solvent systems are divided into four categories. Category 1 consists of solvents that produce slightly non-Newtonian solutions which have no clearly defined solubility limit. As the concentration of chitosan is increased, the solution becomes more viscous until a paste or a plastic-like

solid is formed. The viscosities of all these solutions lie within a narrow band (see Fig. 1) and increase slightly with time. The straight-line relationship was unexpected. The color of the solutions varied from light yellow to dark brown. Solvents in this category are 2M aqueous solutions of acetic, citric, formic, glycolic, lactic, maleic, malic, malonic, pyruvic, and tartaric acids.

Category 2 includes those solvents that produce solutions whose viscosities fall within the limits shown for category 1, but the solutions are very non-Newtonian. The viscosity decreases as the rate of sheer increases and, for a 5-percent solution of chitosan in oxalic acid, the rate of sheer versus sheer-stress curve (see Fig. 2) passes through the origin and has a slope of 1.2×10^{-3} cP^{-1} (the slope of a similar curve for a 5-percent solution of chitosan in acetic acid is 0.54 cP^{-1}). No hysteresis was observed in these curves. This type of behavior is characteristic of pseudo-plastic materials and many emulsions. When the solutions are allowed to stand for long periods, gels are formed. The two acids in this category are 2M dichloracetic and 10% oxalic acid.

Category 3 contains three acids: 0.041 M benzoic, 0.36 M salicylic and 0.052 M sulphamilic. Solutions in these acids show an initial increase in viscosity and therefore some solubility (see Fig. 1). These acids may properly belong in category 1, since the low solubility of chitosan is probably due to the low concentration of these acids in the solution.

Category 4 includes solvent-chitosan systems for which the viscosity is independent of the concentration of chitosan (see Fig. 1). These systems were colorless, and there was no indication of any solubility. Solvents in this category include aqueous solutions (generally 2M) of dimethylformamide, dimethylsulphoxide, ethylamine, glycine, methylamine, nitrilotriacetic acid, iso-propylamine, pyridine, salicylic acid, trichloracetic acid, and urea, and a 2 M solution of benzoic acid in ethanol.

ACKNOWLEDGMENTS

We are grateful to the Fisheries and Marine Service of Environment Canada for an operating grant and to Susan Murphy and Barbara Stanford who assisted in the experimental work.

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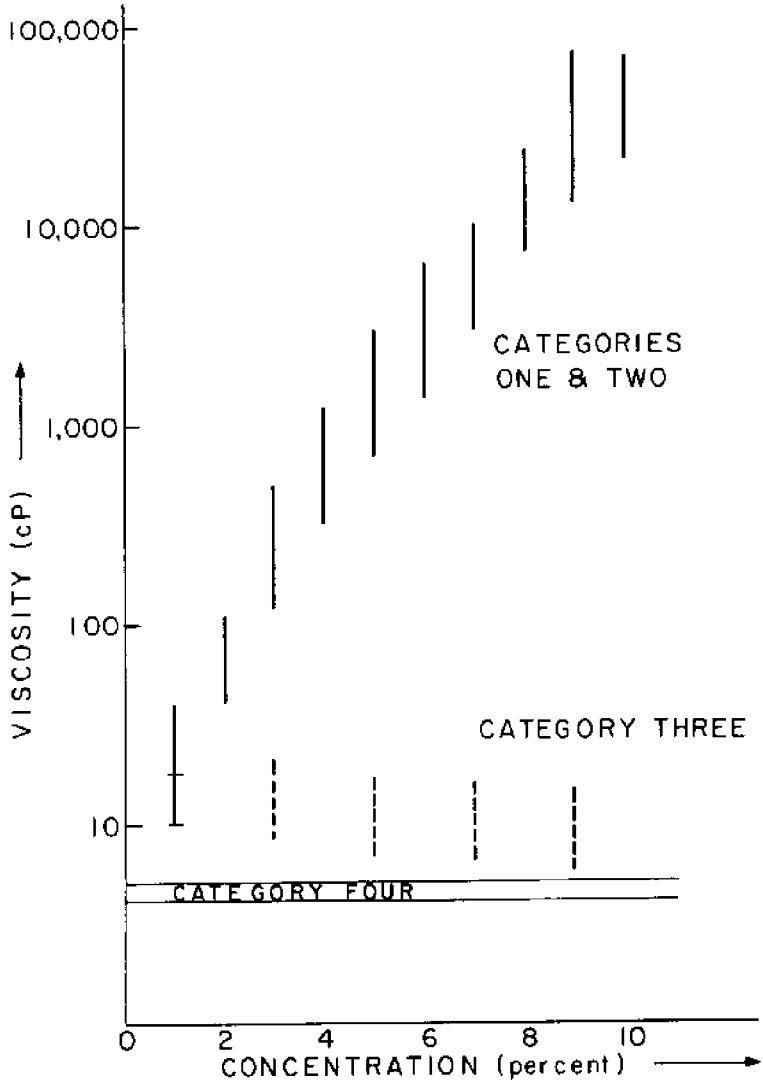

Figure 1: The viscosity of chitosan - solvent systems as a function of the concentration of chitosan.

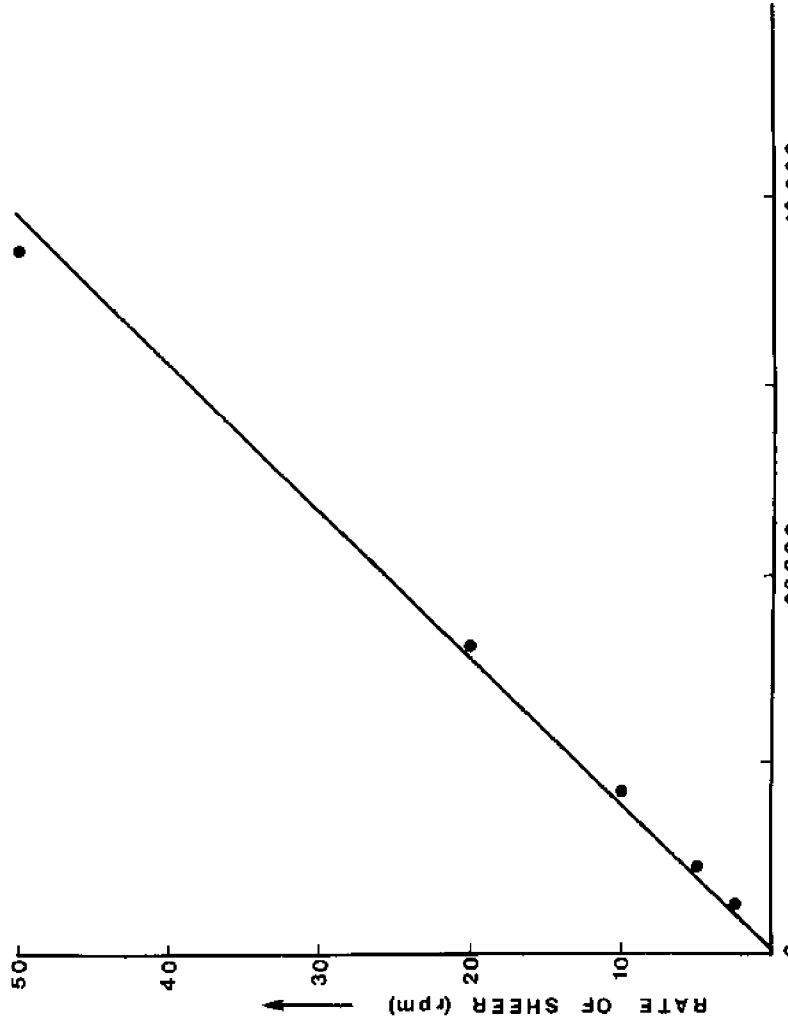

Figure 2: The rate of sheer versus sheer stress curves for a 5% solution of chitosan in 10% aqueous oxalic acid determined with a Brookfield RVT viscometer using spindle #3.

IV. CHARACTERISTICS OF CHITIN-CHITOSAN

DETERMINATION OF THE STRUCTURES OF α- AND β-CHITINS BY X-RAY DIFFRACTION

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ABSTRACT

The structures of α- and β-chitins have been determined by x-ray diffraction, using rigid-body least-squares refinement methods. The polarities of neighboring chains: antiparallel in α-chitin and parallel in β-chitin, have been confirmed, and the hydrogen-bonding network has been determined. α-Chitin has an orthorhombic unit cell with dimensions: a=4.74Å, b=18.86Å, and c=10.32Å (fiber axis). The space group is $P2_12_12_1$, and the cell contains disaccharide sections of two chains. The chain possesses an intramolecular O3'-H···O5 hydrogen bond, as in cellulose, and successive chains along the a axis form sheets linked by C=O···H-N hydrogen bonds. The CH_2OH side chains have different conformations on the two chains in the unit cell. On one chain the CH_2OH forms a second intramolecular hydrogen bond to the carbonyl group of the next residue, O6-H···O7'. That on the second chain forms an intermolecular (intersheet) hydrogen bond O6-H···O6'. The data indicate that the structure contains a statistical mixture of these two types of bonding, equivalent to half oxygens at both O6 positions for each residue, such that the $P2_12_12_1$ symmetry is maintained. All the hydroxyl groups are hydrogen bonded, and the intersheet bonding accounts for the inability of α-chitin to swell in water. In addition the structure contains two types of amide group, which have different hydrogen bonding. This feature explains the splitting of the amide I band in the infrared spectrum.

For β-chitin, the unit cell is monoclinic, with dimensions a=4.85Å, b=9.26Å, c=10.38Å (fiber axis) and β=97.5°. The space group is $P2_1$, and the unit cell contains the disaccharide repeat of one chain, necessitating a parallel chain structure. The chain conformation is the same as in α-chitin, and the chains form sheets linked by C=O···H-N hydrogen bonds. The CH_2OH groups form intrasheet hydrogen bonds to the carbonyl of the next chain along the a axis. The absence of hydrogen bonding between the sheets explains the ease with which β-chitin can be swollen in water to produce hydrates.

INTRODUCTION

This paper describes recent determinations of the structures of α- and β-chitin in our laboratory, using x-ray diffraction methods (13, 23). Chitin occurs naturally in several crystalline polymorphic forms (30), in which the poly(N-acetyl-D-glucosamine) chain adopts a 2_1 screw axis, with two residues repeating in 10.3-10.4Å, as in cellulose. The polymorphs differ in the packing and polarities of the adjacent chains. The so-called α-chitin is by far the most common form, and it is abundantly present in nature, e.g., in the arthropods (27). β-chitin has been identified in *Loligo* pen (18), *Aphrodite chaetae* (28), pogonophore tubes (4), and the spines of certain marine diatoms (5). A third form, γ-chitin, which is less well characterized, has been reported to occur in *Loligo* stomach lining (29).

X-ray diffraction studies of the structures of chitin date from the 1920s. Early work (14, 20, 22, 7) on the α-form established the unit cell and probable space group, and was followed by the detailed structure proposed by Carlstrom (6) in 1957. This structure had an orthorhombic unit cell with dimensions: $\underline{a}$=4.76Å, $\underline{b}$=18.85Å, and $\underline{c}$=10.28Å (fiber axis), and space group $P2_12_12_1$. The cell contains disaccharide sections of two chitin chains which are required by the space group to have alternating sense, i.e., the chains are antiparallel. Successive chains of the same sense along the $\underline{a}$ axis are linked by C=O···H-N hydrogen bonds, and the structure can be seen as a series of hydrogen-bonded sheets of chains.

The Carlstrom structure gives reasonable agreement between the observed and calculated x-ray intensities (19), but is subject to the following criticisms and drawbacks.

1. The $-CH_2OH$ groups are not hydrogen bonded, whereas the infrared spectrum indicates all the hydroxyl groups form donor hydrogen bonds.
2. The evidence for the $P2_12_12_1$ space group is questionable, especially since an 001 reflection is observed. A less symmetrical structure could contain parallel rather than antiparallel chains.
3. There is no hydrogen bonding between the sheets of chains, i.e., along the $\underline{b}$ axis. α-chitin does not swell on soaking in water, unlike β-chitin, where water penetrates between the sheets of chains. This resistance to swelling suggests the presence of tight intersheet bonding in the α-form.
4. The amide I band in the infrared spectrum is a doublet, with frequencies at 1621 cm^{-1} and 1656 cm^{-1}, whereas a singlet band is predicted for the Carlstrom structure.

There have been numerous attempts to modify the Carlstrom structure in order to eliminate the above criticisms. Pearson et al. (25) proposed that the structure contained a 50/50 mixture of $\underline{cis}$ and $\underline{trans}$ amide groups, to account for the amide I doublet. Alternatively, Parker (24) suggested that the doublet was due to water of crystallization. However, both of these modifications are stereochemically unacceptable. An intersheet hydrogen bond was proposed by Dweltz (8), but this was based on the unacceptable chain conformation used by Meyer and Misch (21) for cellulose, and the proposed hydrogen-bonding network in Dweltz's structure could not be transferred to the Carlstrom structure. More recently, Ramakrishnan and Prasad (26) and Haleem and Parker (15) have applied least-squares refinement methods in efforts to determine the structure, but both groups have been unable to resolve the problems listed above.

β-chitin was first distinguished from α-chitin by Lotman and Picken (18), who observed a new x-ray pattern for the deproteinized $\underline{Loligo}$ pen. Dweltz (9) indexed this pattern in terms of a unit cell containing only one chitin chain, which necessitates a parallel chain structure. In later work, highly crystalline β-chitin was found in pogonophore tubes (4), and

the spines of a few species of marine diatoms (5). The diatom chitin was first isolated by Falk et al. (11), who showed that this material had no non-acetylated residues. The unit cell of diatom chitin is monoclinic, with dimensions (4): a=4.85Å, b=9.26Å, c=10.38Å (fiber axis) and γ=97.5°, and space group $P2_1$. The structure was refined by Blackwell (3), who showed that the chitin chains are linked together in sheets through C-O···H-N hydrogen bonds, very similar to those in α-chitin. However, the bonding of the CH_2OH groups was not determined, and these hydroxyls were left unbonded. Dweltz et al. (1) proposed that the CH_2OH groups formed intersheet hydrogen bonds to the carbonyl of the next chain along the ab diagonal. This structure, however, is not compatible with the ease with which the β-structure is swollen when water penetrates between the sheets, and would give rise to a band with perpendicular dichroism in the O-H stretching region of the infrared spectrum, whereas only parallel dichroism is observed.

The β-chitins of pogonophore tube, Loligo pen and Aphrodite appear to be more complex and contain a mixture of two structures which have been designated β-chitin A and B (3). The β-chitin A structure is that found in diatom chitin and was obtained exclusively in pogonophore chitin after bleaching with diaphanol (chlorine dioxide in acetic acid). The β-chitin B structure is less well defined. However, both the A and B structures form a series of hydrates, where water penetrates between the sheets of chains and increases the b dimension of the unit cell. The β-chitin A structure described above is the only known anhydrous β-structure.

The foregoing discussion describes the knowledge of the structures of α- and β-chitin at the time we commenced our reexamination of these polymorphs. We have refined the structures using rigid-body least-squares methods, in order to determine the packing of the chains and the hydrogen-bonding network. We had previously applied these methods to determine the structure of cellulose I (12) and II (17), where we were able to determine the polarity of the chains and the hydrogen-bonding networks. The methods and results for α- and β-chitins are summarized below; a detailed description of the refinement is given elsewhere (13,23).

EXPERIMENTAL

Specimens of α- and β-chitin were obtained respectively from lobster tendon (Homarus americanus) and pogonophore tubes (Oligobrachia ivanovi). These materials were deproteinized by boiling in 5% potassium hydroxide for 24 hrs, rinsed, soaked in cold 3N hydrochloric acid for 3 hrs, and washed in water. The lobster tendon was subjected to a further treatment with 6N hydrochloric acid for 10 minutes to increase the crystalline order, followed by thorough washing. The pogonophore tube was bleached in diaphanol to remove the black pigment; the diaphanol was allowed to evaporate to dryness, and the specimen was washed thoroughly.

The specimen of α-chitin used to obtain the x-ray data was in the form of a thin sheet peeled from the purified tendon. The pogonophore β-chitin (β-chitin A) was ultrasonically dispersed in water and mixed with fibrinogen solution. This mixture was clotted by addition of thrombin, and the clot was stretched into a fiber containing oriented β-chitin

fibrils (3). The specimen used for diffraction was an oriented bundle of sections of this fiber. The x-ray patterns were recorded on Kodak No-Screen Film using a vacuum camera with pinhole collimation and CuKα radiation.

The X-ray intensities were measured using an optical densitometer, except for the very weak reflections; these were estimated by eye. Forty-five observed non-meridional reflections were recorded for α-chitin, and sixty-one for β-chitin. These data were corrected for Lorentz and polarization effects, and converted to the observed structure amplitudes, F(obs). Within the range of the observed data for α-chitin, twenty-four additional reflections which have intensities too weak to be detected are predicted. Each of these reflections was assigned a structure amplitude equal to two-thirds of F(thr), the threshold or lowest F(obs) that could be detected in the region of the predicted reflection. For β-chitin, only five unobserved reflections were predicted, and these were assigned zero F(obs).

UNIT CELL DETERMINATION

The observed d-spacings for α-chitin are indexed by an orthorhombic unit cell with dimensions: $\underline{a}$=4.74Å, $\underline{b}$=18.86Å, and $\underline{c}$=10.32Å. Odd order 00ℓ reflections are absent except for 001, which is weak. Comparison of x-ray photographs of α-chitin specimens of different degrees of crystalline order shows that this 001 intensity decreases as the crystallite size increases. Hence there is a 2_1 screw axis parallel to $\underline{c}$, and the 001 reflection is probably due to lack of perfection of this 2_1 symmetry in small crystallites, which is eliminated as these crystallites coalesce.

Previous work and our own refinements below indicate that the space group is at least very close to $P2_12_12_1$. However, it is not possible to hydrogen bond all the hydroxyl groups in a $P2_12_12_1$ structure, and deviations which reduce the symmetry to $P2_1$ must be considered. It is not possible to be definite about absences for the h00 and 0k0 reflections. Odd order 0k0 reflections are not detected, although the possibility of weak 010 and 030 reflections cannot be eliminated, due to an equatorial streak. The 100 reflection would be coincident with 040, and a strong reflection is observed in this region.

For β-chitin, the observed d-spacings are indexed by a monoclinic unit cell with dimensions: $\underline{a}$=4.85Å, $\underline{b}$=9.26Å, $\underline{c}$=10.38Å (fiber axis), γ=97.5°. The only systematic absences are for odd order 00ℓ reflections, and the space group is $P2_1$.

CHAIN CONFORMATION

The model for the chitin chain was based on that used in our refinement of cellulose I(12), where averaged D-glucose residues (1) are arranged with β-(1→4)-glycosidic linkages repeating in 10.38Å. For chitin, the O2-H group was replaced by the trans $NHCOCH_3$ group of N-acetyl glucosamine (16). A projection of this chain showing the numbering of the atoms is shown in Figure 1.

As described, the chain has the same fiber repeat as β-chitin and possesses an intramolecular O3-H...O5' hydrogen bond. To accommodate the apparent shorter fiber repeat for α-chitin, the C1-O1 bond length was decreased from 1.42Å to 1.39Å, and small changes were made in the residue rotations about the C1-O1 and C4-O1' bonds. X-ray refinements based on fiber diffraction data are not sensitive to such small changes.

The chitin chain so described is completely rigid except for rotational freedom for the side chains. The orientations of the CH_2OH and planar amide groups are defined as χ and χ' respectively (see Figure 1). χ=0 when C6-O6 is cis to C4-C5; χ' =0 when N-C7 is trans to C1-C2; positive χ and χ' correspond to anticlockwise rotations of the groups when viewed along C5 and C2-N, respectively. [A further parameter χ'' defines the rotation of C8 and O7 about N-C7, i.e., it allows for non-planarity of the amide group. Our refinement work for β-chitin yielded a value of χ'' not significantly different from zero, and planar amide groups are assumed in the work described below.]

REFINEMENT OF THE STRUCTURES

α-Chitin

Models were considered with both $P2_1$ and $P2_12_12_1$ symmetry. In the former case, the space group requires consideration of both parallel and antiparallel chain systems. Models were set up with chains passing through the corner and center of the ab projection. [Corner and center chains are designated (1) and (2). Thus χ (1), for example, defines the orientation of the CH_2OH group on the corner chain]. Parallel and anti-parallel $P2_1$ models are designated p1 and a1, respectively. The refinable packing parameters in these models are PHI (1) and PHI (2), the orientation of chains 1 and 2 about their axes, and SHIFT, the stagger of chain 2 along c with respect to chain 1. The $P2_12_12_1$ model is designated a_2, and the refinable packing parameters are PHI (1) and SHIFT [since PHI (2) =180°-PHI (1)].

The structures are refined by adjusting the refinable parameters in a least-squares procedure based on those used by Arnott and Wonacott (2) to give the best fit between the observed and calculated structure amplitudes. The refinement will be discussed in terms of the crystallographic residuals:

$$R = \frac{\Sigma||F(obs)|-|F(calc)||}{\Sigma F(obs)}; \qquad R' = \frac{\Sigma w||F(obs)|-|F(calc)||}{\Sigma w|F(obs)|}$$

$$R'' = \frac{\Sigma w(F(obs)-F(calc))^2}{\Sigma wF(obs)^2}$$

F (obs) and F (calc) are the observed and calculated structure amplitudes, and w is a weight assigned to each observation; we used w=1 for the observed reflections and w=1/2 for the thresholds assigned to the unobserved reflections. The latter were incorporated only where F(calc)> F (thr).

$P2_12_12_1$ model

Model a_2 was refined in terms of six variables: PHI(1), SHIFT, χ(1) and χ' (1), an isotropic temperature factor B, and a scale factor K. The data were not sufficiently sensitive to allow for simultaneous refinement

from a general starting position, and the refinement was performed starting with one variable and adding further variables in successive cycles in the order: K, PHI(1), SHIFT, $\chi(1)$, $\chi'(1)$, B. This led to a final model a_2 with residuals R=0.184, R"=0.161 for the observed data only, and R=0.232, R'=0.218 and R"=0.181 for the observed plus unobserved data. This model is very similar to that proposed by Carlstrom. The agreement between the observed and calculated data is very good, but the structure is subject to the same criticisms as given above, particularly that the O6-H groups cannot form hydrogen bonds, and is thus unacceptable.

Study of the a_2 model shows that two possible hydrogen bonding schemes are possible for a $P2_12_12_1$ model. Shift of the CH_2OH groups from χ=149.7° in model a_2 to χ=110° would allow for an O6-H···O7' intramolecular bond, but increases the residual to R"=0.26. Alternatively, with $\chi(1)$= -30°, an intersheet O6-H···O7' bond is formed, which increases the residual to R"= 0.31. Both of these possibilities can be eliminated at a high significance level in favor of the unbonded model for which R"=0.16. Thus an acceptable hydrogen-bonded $P2_12_12_1$ model cannot be found and $P2_1$ symmetry must be considered.

$P2_1$ models

Models p_1 and a_1 are defined in terms of nine parameters: PHI(1), PHI(2), SHIFT, $\chi(1)$, $\chi(2)$, $\chi'(1)$, $\chi'(2)$, K, and B. As for model a_2 it was not possible to refine all these parameters simultaneously. Rather, PHI(1), PHI(2), SHIFT, K and B were refined initially, and then the χ and χ' angles were refined (along with K) while the other parameters were held constant. The final p_1 model had a residual R"=0.25, which is significantly higher than can be achieved for antiparallel chains (R"=0.16), and was stereochemically unacceptable due to bad contacts between the amide and CH_2OH groups on adjacent chains. As a result parallel chain models can be eliminated.

Prior to refinement of the χ and χ' angles, model a_1 had packing parameters that were not significantly different from those in the $P2_12_12_1$ model a_2. It appears, then, that the structure will be similar to a_2, except that χ and χ' may have different values on the two chains. Examination of the effect of variation of $\chi(1)$ and $\chi(2)$ on R" shows that it is possible to find positions for the CH_2OH groups that give reasonable values of R" and allow for hydrogen bonding. At ($\chi(1)$, $\chi(2)$) = (12-°, 180°) and (180°, 120°), R" is increased by less than 0.003, and it is possible to form an intramolecular O6-H···O7' bond for one chain and an intermolecular O6-H···O6' bond for the other chain (between center and corner chains). There are marginal bad contacts for the chain with the intramolecular bond, but these can be eliminated with non-bonded constraints on the refinement.

Statistical $P2_12_12_1$ model

In the above refinement, the CH_2OH orientation was constrained in the refinement to produce an intra-bond for the corner chain and an inter-bond for the center chain. The reverse bonding pattern--inter- on the corner and intra- on the center--is equally acceptable, however,

as is an arrangement of half oxygens at both positions for each residue, corresponding to a statistical mixture of both bonding patterns. The bonding in one unit cell is completely independent of that in neighboring cells, and thus the statistical mixture is the probable structure, which corresponds to $P2_12_12_1$ symmetry.

This statistical structure was modeled by placing half oxygen atoms at both χ=112.1° and χ=187.2° for each residue and refining PHI(1), PHI(2), SHIFT, B and K. This resulted in the final model for α-chitin shown in Figure 2, where the half oxygens are indicated by the shading. The final residuals were R=0.223 and R"=0.188 for the observed data, and R=0.279, R'=0.265, and R"=0.205 for the full data. An exact 50/50 mixture of the two hydrogen-bonding options, however, can only be expected for large crystallites. The $P2_12_12_1$ symmetry will break down for small fibrillar crystallites, and hence a 001 reflection is observed, which decreases in intensity as the crystallite size increases.

The proposed structure also gives a simple explanation of the splitting of the amide I mode in the infrared spectrum. The structure contains two types of amide group: all the amides are involved in C=O···H-N bonding, but half of them also act as acceptors for CH_2-O-H···O (carbonyl) bonds. As will be shown below, all the amides in β-chitin are involved in C=O···H-N and CH_2-O-H···O (carbonyl) bonds, and the infrared spectrum of this polymorph (4, 24) contains a single amide I band at 1631 cm^{-1}. Thus the 1621 cm^{-1} band for α-chitin is assigned to the amide I mode of the doubly bonded groups, and the 1656 cm^{-1} band is assigned to the singly bonded groups. There is no need to invoke coupling of the amide vibrations or the presence of water of crystallization.

β-Chitin

Determination of the structure of β-chitin is simpler since the unit cell contains only one chain. The structure was refined in terms of five parameters: PHI, χ, χ', B and K, which are defined in the same way as above for α-chitin. These parameters were refined simultaneously, resulting in a structure which had R=0.244 and R" 0.274. The refined value of χ(=81.7°) resulted in a marginally short contact (O6···O5=2.61Å). This was eliminated by incorporating a non-bonded constraint, and gave a stereochemically acceptable structure with R=0.250 and R"=0.288 for which χ=-60.7°. The increase in the residuals is not statistically significant. The structure is still unacceptable, however, since the CH_2OH groups are not hydrogen bonded.

Examination of the refined model indicates that two possible hydrogen-bonding schemes exist for the CH_2OH groups: an intrasheet bond to the carbonyl of the next chain in the 100 plane, O6-H···O7', and an intersheet bond to the carbonyl of the next chain in the 110 plane, as was proposed by Dweltz et al. Each of these possibilities was considered by inclusion of constraints in the refinement to require formation of the particular hydrogen bonds. When constrained to form the intrasheet bond, the final residuals were R=0.267 and R"=0.302, and the final model is stereochemically acceptable. For the constrained formation of the intersheet bond, the

refined model is acceptable except for marginal bad contacts between O6 and both C7 and C8 of the bonded amide. These can be eliminated by further constaints, and the final residuals are R=0.347 and R" 0.378. Thus the model with the intersheet hydrogen bond can be rejected at a high significance level, and the model with the intrasheet bond is that proposed for β-chitin.

The proposed structure for β-chitin is shown in Figure 3. The chains are arranged in sheets rather similar to those in α-chitin. The amide group orientation χ' is -103.0° (cf. -102.6° for α-chitin) and the O7-H···O7' intrasheet hydrogen-bond length of 2.89Å.

In the proposed structure, both of the O-H groups are oriented along the chain and would give rise to parallel dichroism for the bands in the O-H stretching region, as observed. The intersheet-bond model would be expected to show some parallel dichroism. Thus there is no bonding between the sheets of chains, and easy penetration by water on swelling is to be expected.

DISCUSSION

α- and β-chitins both consist of arrays of sheets of chains. These are stabilized by C=O···H-N bonds, as well as by the nonbonded interactions between the pyranose rings. The most obvious difference between the two structures is the absence of bonding between the sheets of the parallel β-structures. This lack of bonding probably accounts for the swelling properties of β-chitin.

When the structures of cellulose and chitin are compared, obvious analogies emerge between the parallel chain structures determined for cellulose I and β-chitin and between the antiparallel chain structures of cellulose II and α-chitin. In both cellulose I and β-chitin the O3-H and O6-H groups form hydrogen bonds along the chain axis. The O6-H group forms an intramolecular O6···O2' bond in cellulose I. The analogous O6···N bond is not possible in β-chitin, and there is a small rotation of the CH_2OH to allow for an intrasheet bond to the protruding carbonyl of the chain above. In cellulose II and α-chitin, the change in chain polarity has resulted in additional interchain bonding, and these polymorphs are the more stable: the β→α-chitin and cellulose I→II transformations cannot be reversed.

However, neither cellulose I nor cellulose II can be swollen in water. Of the four structures, all but β-chitin have intermolecular hydrogen bonding in the plane of the sugar rings. This swelling property probably makes β-chitin unsatisfactory as a structural material, and may explain why the antiparallel chain α-structure is the abundant native structure, in contrast to the parallel chain system in native cellulose.

ACKNOWLEDGMENTS

This work was supported by NSF Grant no. DMR 77-82668 and N.I.H. Research Career Development Award No. AM 70642 (to J.B.).

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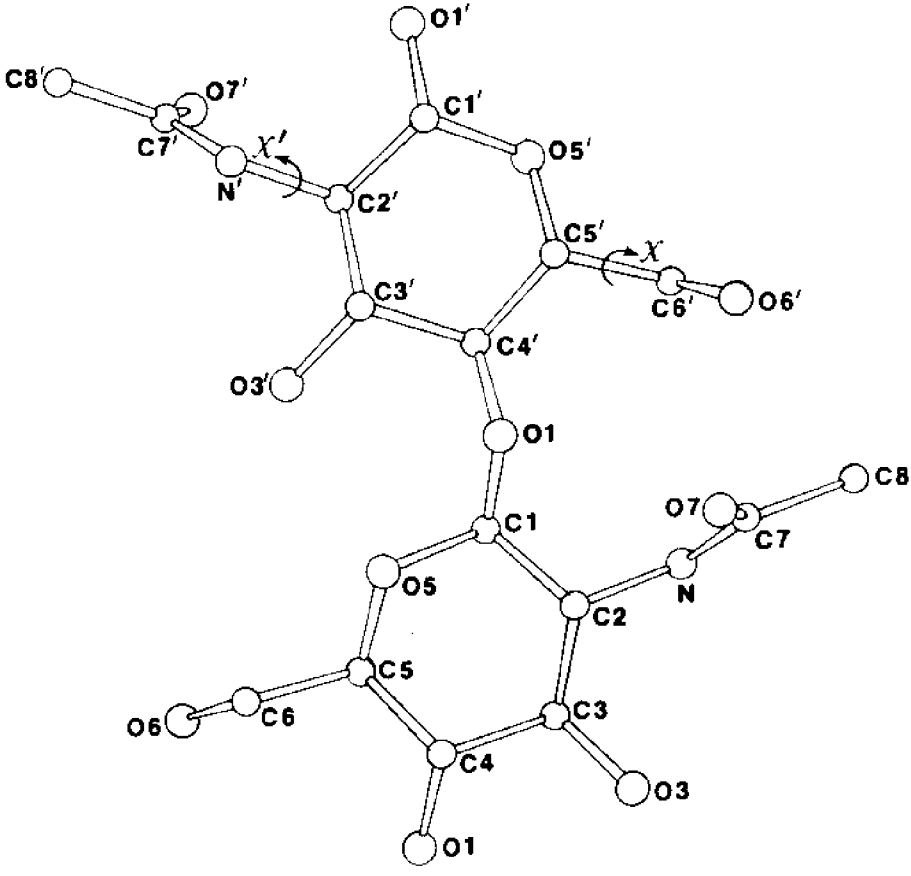

Fig. 1: Disaccharide repeating residue for chitin, showing the numbering of the atoms and the refinable parameters χ and χ'.

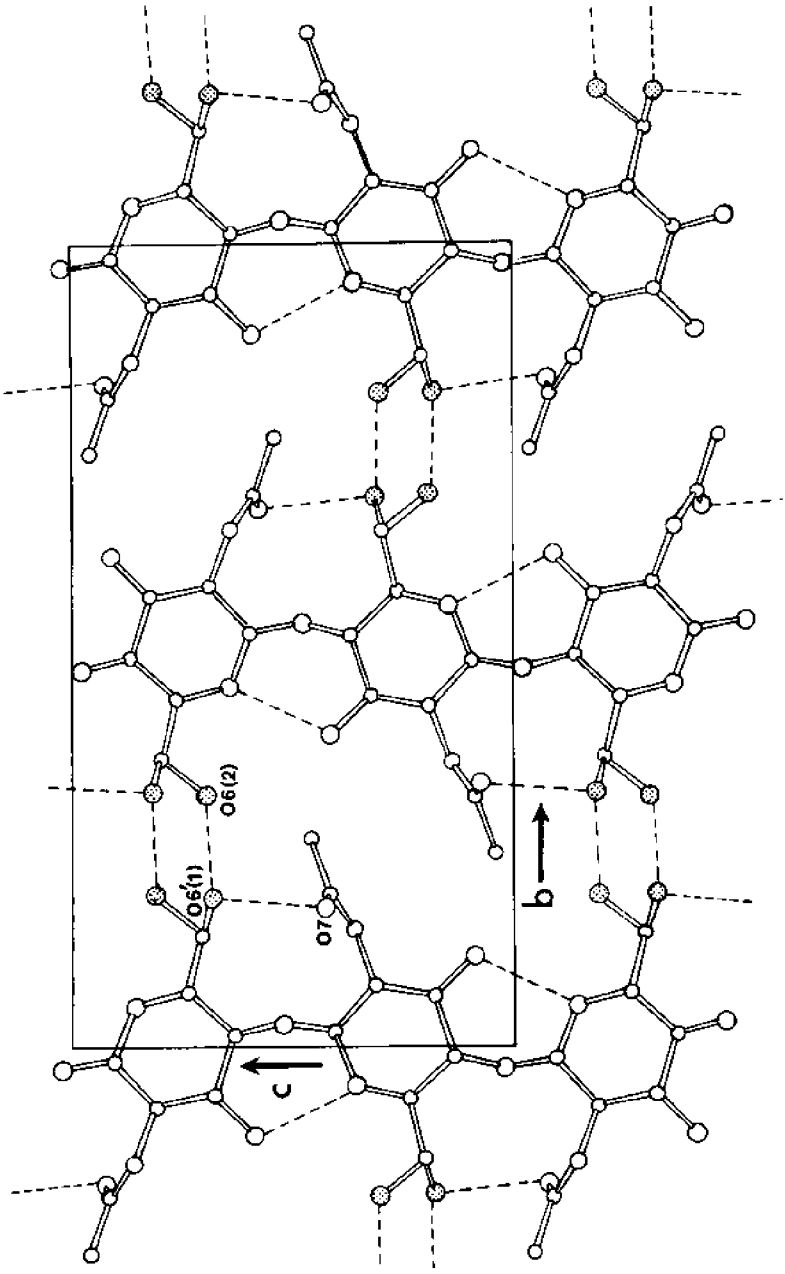

Fig. 2: The structure of α-chitin (model a_1)
a) bc projection The CH_2OH group on the corner chain forms an intramolecular O6'(1)-H···O7 bond and the CH_2OH of the center chain forms an intermolecular O6(2)-H···O6'(1) bond.

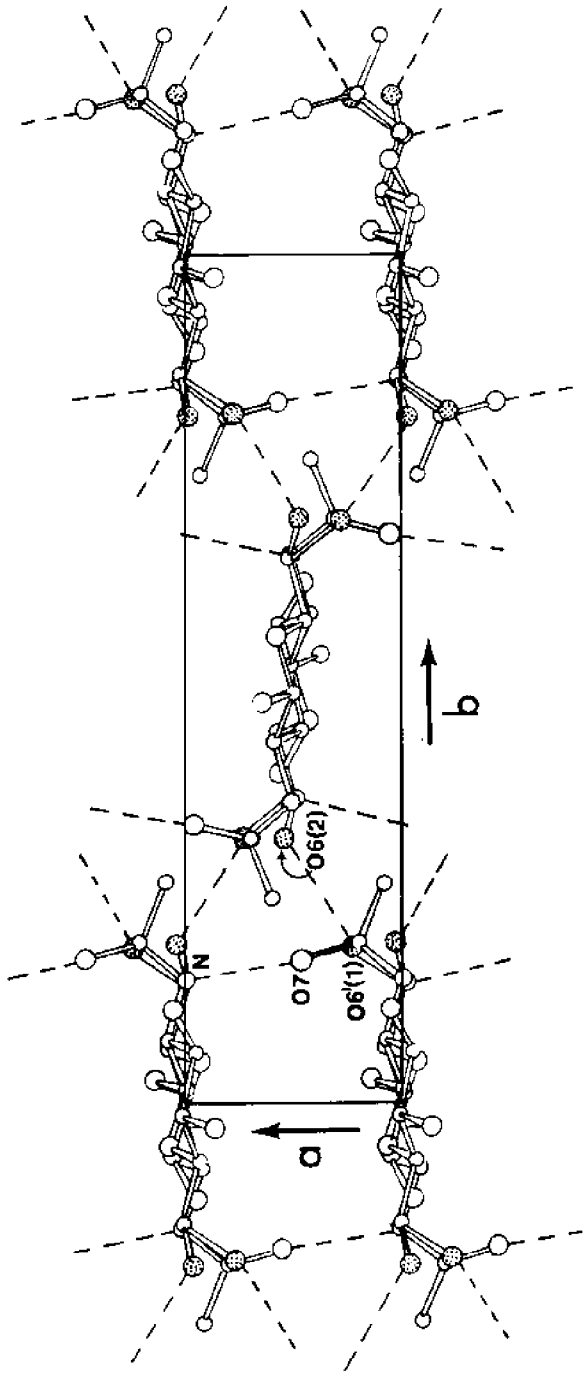

Fig. 2: The structure of α-chitin (model a_1)
b) ab projection showing the C7=O7...H-N and O6(2)-H...O6' (1) intermolecular bonds.

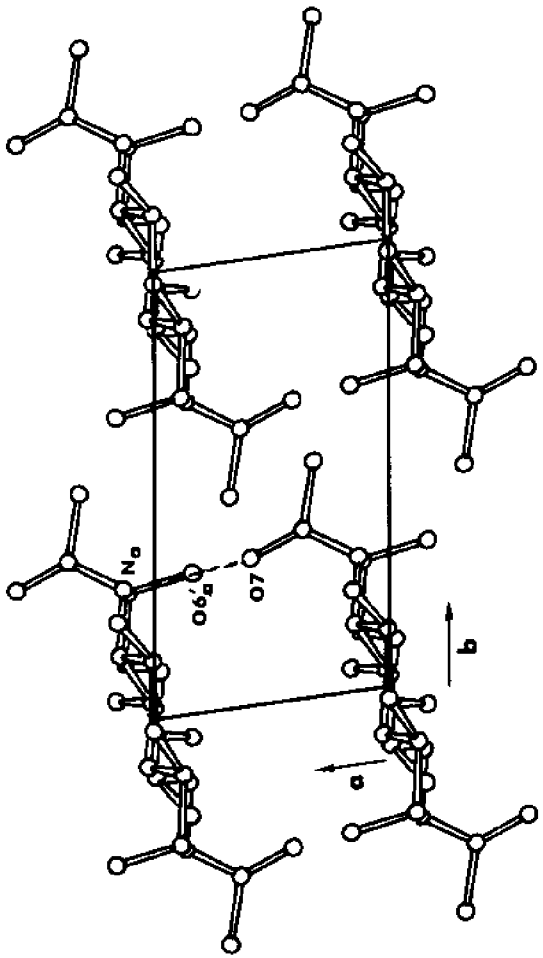

Fig. 3: The structure of anhydrous β-chitin
a) ab projection showing the C7=O7···H-N intermolecular bond.

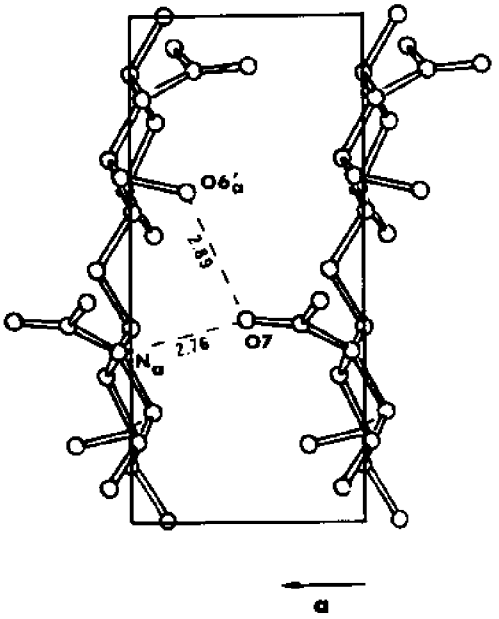

Fig. 3: The structure of anhydrous β-chitin
b) ac projection showing the O6'-H···O7 and $\overline{C7}$=O7···H-N intermolecular bonds.

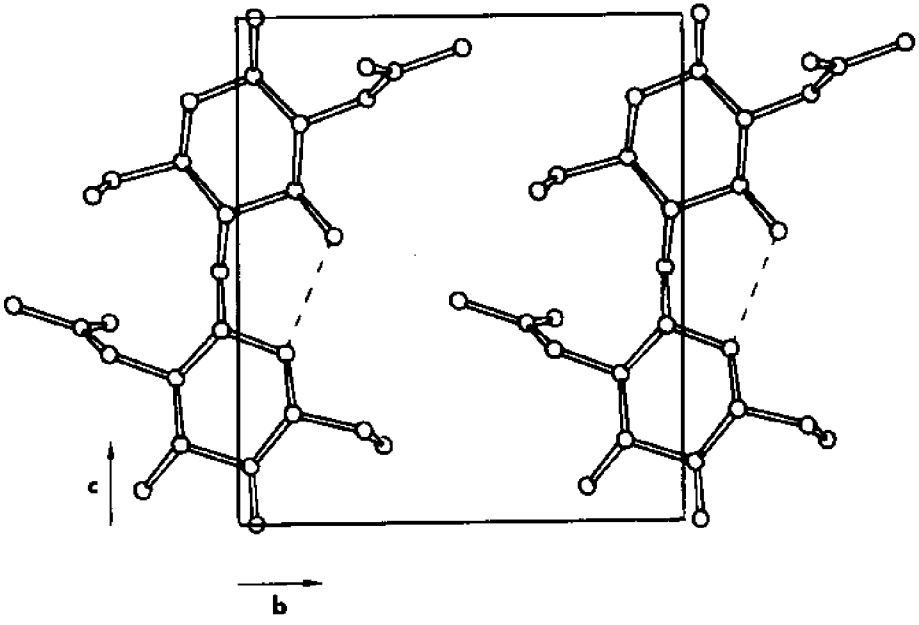

Fig. 3: The structure of anhydrous β-chitin
c) ac projection.

TENSILE MECHANICAL PROPERTIES AND TRANSCONFORMATIONAL CHANGES OF CHITINS

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ABSTRACT

Tensile mechanical properties of naturally occurring α-, β- and γ-chitin and of chitosan fibers and films regenerated by three different processes are described. All of these materials are strain-rate dependent in both the wet and dry states. As such, they are typically visco-elastic polymers in which the elastic modulus is time dependent.

The tensile properties within and among chitin polymorphs vary in stiffness, strength and extensibility by several orders of magnitude. These differences are ostensibly related to crystal structure, packing order and degree of orientation. These properties in native chitin, as well as the extent of stress relaxation, are sensitive to deformation rate and solvation in various media. The mechanical properties of renatured chitosan fibers vary enormously with the method of manufacture.

Although all of these materials are visco-elastic, chemical-mechanical manipulation of their basic properties is possible, and it would appear that different modes of viscous behavior occur: α-chitin apparently deforms by chain slipping, while β-and γ-chitin and the renatured chitosans exhibit viscous behavior consistent with basic molecular conformational changes.

INTRODUCTION

Some accounts, mostly of historical interest, of the mechanical properties of chitin and chitosan are available from earlier decades (14, 26, 32, 33); however, only recently has there been any attempt at systematic measurement and description of the properties of naturally occurring chitins (11, 12). Owing to the complex structural arrangements of chitin fibers in animal skeletons (1, 23), test data now available (11, 12) from these sources are not ideally suited for estimating the tensile behavior of isolated chitin fibers, much less that of possibly mercantable regenerated chitosan fibers and films. For these reasons, more basic characterizations of the tensile mechanical properties of naturally occurring α-, β- and γ-chitin fibers and derivative chitosans have been made. Finally, by means of chemical manipulation, fundamental differences in the viscous elements of these materials have been explored.

EXPERIMENTAL

Material was obtained as follows: α-chitin - locust (*Locusta*) hind leg apodemes, squid (*Loligo*) esophagus and prawn (*Penaeus*) carapace; β-chitin - the pen of the squid; γ-chitin - beetle (*Ptinus*) cocoon fibers and squid stomach. Specimens were cleaned by steeping them in hot 5% KOH for several hours and then washing them in distilled water. Renatured fibers and films were prepared from squid pens using the xanthate dispersion (32), formic acid (22) and trichloroacetic acid (4) methods.

All testing was performed in the tensile mode on a previously described instrument (16) at ambient room temperature and relative humidity for dry specimens and in distilled water for wet ones. Each material was tested until an average of about 6 entirely reproducible curves were obtained. Zero points were chosen by extrapolating back along the first linear regions of the curves to the x-axis intercept. All strains are calculated from this corrected point (justified below). Strain-rate tests over three orders of magnitude were also made. The elastic limit is given as that stress below which recycling results in curves of slope similar to that obtained on the initial extension and above which stress relaxation occurs when crosshead movement is stopped. In practice, the elastic limit is actually determined to lie between a very narrow range of stress values.

The tensile stress-strain curves were resolved into the following components: E_0-elastic modulus (slope of first linear region, σ_0-proportional limit stress and ε_0 strain; E_1-slope of second linear region, σ_1 and ε_1 largest respective stress and strain associated with E_1; σ_{max} - maximum or ultimate tensile strength, ε_{max} associated strain.

RESULTS AND DISCUSSION

The Tensile Stress-Strain Curve

A generalized tensile stress-strain curve for native polymorphic chitin and regenerated chitosan fibers is shown in Fig. 1. It can be seen that there is an initial concave region in the curve, the occurrence of which is of great importance; if it is a true property of the material, then an elastic modulus is precluded and one can only calculate an approximate stiffness, the tangent modulus. The first samples of chitin for which such curves have been published (10) were, on the contrary, entirely linear for this same line segment. Moreover, subsequent studies on various arthropod chitins (12, 13, 15) revealed mixed behavior in this regard: some were Hookean, some not, while others alternately exhibited both kinds of behavior on different occasions. Careful reinvestigation of this point on various chitins (including those presently under discussion) has established that this concavity is an artifactual element of the tensile test. It is entirely associated with "bedding-in" phenomena with respect to loading: taking up slack, co-axial alignment of skewed fibers, surface-mating effects between specimen and grips, and state of hydration. Therefore, it is entirely correct to ignore this concavity in zero-point selection and, hence, in calculating the initial slope of the curve. This done, an entirely linear relationship between stress and strain is obtained, the slope of which, E_0, constitutes Young's or the elastic modulus of the material. Consequently, previously published values (summarized in 12) for the tangent modulus of chitin should also be considered as elastic moduli.

The initial linear region of the curve in Fig. 1 is seen to terminate in a clearly defined deviation from linearity between stress and strain. This point is the proportional limit characterized by the coordinates σ_0, ε_0. Conventionally, the material under test is said to be within the elastic domain from the origin of the curve to the proportional limit. However, non-linear elastic behavior is commonly encountered in polymers (35), necessitating an experimental determination of the relationship, if any, between the true elastic limit and the proportional limit. That the proportional limit is, in fact, just about coincident with the elastic limit for chitin tested under uniform conditions of temperature, hydration state and strain rate was ascertained in the following way. Specimens were

axially loaded in small increments, the crosshead movement was stopped and reversed,thus returning the specimen to zero load. On subsequent extensions the load was gradually increased until deviations from linearity occurred vis-à-vis stress-relaxation on stopping crosshead movement. For all three of the chitin polymorphs and for regenerated chitosan fibers, the onset of stress-relaxation approximately coincides with the first deviation from linearity,thus establishing equality between the proportional and elastic limits. The elastic domain, then, extends from the origin of the tensile stress-strain curve to the proportional (elastic) limit.

Extensions beyond the proportional limit are associated with varying degrees of permanent deformation, and the region between this limit and complete failure constitutes the plastic domain of the material. The versatility of a material often depends upon the kind and degree of plastic behavior that can be utilized prior to failure. In this regard, the plastic domains of the chitins are highly variable (11, 12). For example, in α-chitin two linear plastic regions occur in wet samples at slow strain rates,and these can be "compressed" into a single linear region if the strain rate is greatly increased or dried specimens used. Alternatively, γ-chitin exhibits but one linear region in the plastic domain irrespective of hydration and strain rate. Plastic deformation of chitin is almost invariably associated with regions of positive slope and this behavior is defined as work-hardening.

In the case of native chitin, this work-hardening element can be interpreted as a strain-induced crystallization that is indicated by increasing birefringence of test fibers on stretching, and by the observation that crystallographers almost invariably stretch their specimens prior to x-ray diffraction in order to enhance the crystallinity of their samples. It is no accident that the ultimate tensile strengths (σ_{max}) of chitins and chitosans always occur within the plastic domain, that is, after the materials have already sustained irreversible change because maximum crystallinity and crystal orientation are associated with stretching and, ultimately, with the propagation of crystal defects. This seeming anomaly evokes the old engineering adage that defects are both the strength and weakness of a material. Crystal defects that could theoretically be operative in the deformation of chitin are discussed in a companion paper immediately following this one.

Tensile properties of α-, β-and γ-chitins

From a consideration of the tensile mechanical data given in Table 1, a number of comparisons can be made. However, in view of vast differences reported for a wide variety of putative α-chitins (11, 12) it must be emphasized that this account primarily concerns those chitins under present discussion. α-, β-and γ-chitins share a number of features in common which can, therefore, be considered as intrinsic to the basic polymer. For example, the main elastic elements of β-and γ-chitin are strain-rate sensitive,and the general trend is that of increasing stiffness (E_0) and stress (σ_0) while the elastic-limit strain (ε_0) is decreased. From the particularly high modulus given for the locust α-chitin, it can be expected (although not determined experimentally) that this particular α-chitin is less sensitive to deformation rate than are the β and γ-chitin fibers. However, that α-chitin is strain-rate dependent has been well documented for both wet and dry samples (11, 12).

All three polymorphs show marked hydration sensitivity, the effects of which are that E_0, σ_0 and σ_{max} all increase while the corresponding values of ε_0 and ε_{max} obviously decrease on dehydration. This relationship points to very basic properties of the chitin macromolecule. It is of particular interest to note that β-chitin, which is the most hydration dependent, exists in hydrated crystal forms (2, 7, 8, 28), that γ-chitin forms hydrates (30), and that both β-and γ-chitin revert to crystalline α-chitin on solvation in hydrochloric acid and lithium thiocyanate respectively (27, 31). By the same token, it should be recalled that the ultimate strengths (σ_{max}) of α, β-and γ-chitin are quite similar when tested dry at comparable strain rates vis-à-vis intra- and inter-polymorphic variation when wet. When dry, α-chitin is the stiffest fiber (highest E_0) followed in turn by β-and γ-chitin. Similarly, the highest maximum strain value occurs in γ-chitin. These observations are consistent with the raw tensile data on α, β-and γ-chitin for which the stress-strain curves were quasi-linear for up to 90% in α, but only 50% in β and γ-chitin.

Certain structural inferences can be made from these data. It would appear that locust-tendon α-chitin is of the highly oriented crystalline form having only small amorphous regions. The high degree of oriented micelles is evidenced by birefringence in this tendon and the sharpness of x-ray diffraction photographs of other α-chitin locust tendons (9, 23, 30), while a reduction in the size of amorphous regions is evidenced by the insensitivity of this material to hydration state. Highly oriented material is also suggested by the high stiffness (E_0), low ε_{max} and brittle failure of the tendons. Squid esophagus, like locust tendon, is an α-chitin (28), but it is orders of magnitude less stiff and over ten-fold less strong than the latter. Coupled with the facts that this material is greatly distensible, and that it is non-birefringent, even when this effect is enhanced with a first-order gypsum filter, it is concluded that squid oesophageal chitin consists mainly of fibers which are largely amorphous and which contain few, non-oriented micelles.

On the basis of a relatively low hydration sensitivity, the beetle-cocoon fibers must also be crystalline (indeed, they are strongly birefringent), but the amount of oriented material is probably less than that of locust tendon because of a correspondingly lower elastic modulus and higher extensibility in the cocoon fibers. The fact that the cocoon fibers are nonetheless somewhat hydration sensitive points to the occurrence of some amorphous regions. The intra-polymorphic differences seen in the α-chitins extends to γ-chitin as well. For example, squid stomach is of very low modulus and strength and, like squid esophagus, is also non-birefringent and greatly distensible, hence it is mainly amorphous and non-oriented. Finally, squid pen β-chitin is probably largely amorphous (it is also greatly hydration dependent) but, owing to a high modulus and low strain when tested dry, those micelles present must be in the oriented state. Microstructural variation in the three chitin polymorphs therefore includes those fibers which are: (1) highly crystalline and oriented - locust tendon and beetle cocoon fibers; (2) highly amorphous and oriented - squid pen; (3) highly amorphous but not oriented - squid esophagus and squid stomach. This packing-order variation is consistent with all of the observed mechanical (above) and chemical (9) properties, and suggests that chemical and mechanical manipulation of all three polymorphs, especially that of β and γ-chitin, is possible (discussion follows).

Tensile properties of the chitosans

Reference to the data in Table 1 shows that, like the chitins, all three chitosans share a number of properties in common. They are markedly strain-rate dependent and characteristically display higher stresses, greater stiffness and lower strain with increasing strain rate. Chitosans are also extremely hydration sensitive and are just as typically visco-elastic as are the chitins. The chitosans remain elastic for up to 50% of the maximum load which they can sustain; of the three tested, the chitosan acetates appear to be stronger when dry than either the formate or xanthate preparations. The acetate and xanthate chitosans were, however, tested in fiber form,and it might be expected that the polymerization process would confer some degree of chain orientation in an otherwise relatively non-birefringent amorphous material.

The natural visco-elasticity of the chitosans is a property which offers future possibilities for chemical modification of mechanical behavior; but at present, little is known of the sensitivity of different source materials to formation processes. For example, the samples of chitosan formate studied were films,and it might be expected that fibers would be of higher stiffness and strength than films. But, it was not possible to produce chitosan-formate fibers because the formate solution was insufficiently viscous to maintain fiber integrity during the lengthy polymerization process. Similarly, an attempt to cold-draw chitosan acetate fibers from solution was only partially successful, though this has reportedly been achieved by others (4), because presumptive forming fibers thinned and broke under their self-weights at slow extrusion rates. At higher flow rates, the fibers "fell" through the polymerizing acetone for a short distance before reaching the bottom of the vessel. These slightly drawn fibers were the ones used in this study. The flow rate required to keep the forming fiber near to the breaking point was too critical to be determined and further attempts at cold-drawing chitosan acetate fibers were abandoned.

Comparisons of chitin, cellulose and their derivatives

It has been stated that regenerated or renatured chitosan fibers display physical characteristics, particularly in regard to optical and x-ray diffractional properties, that are very similar to those of native α-chitins (3,4). A comparison of the tensile mechanical properties of α, β and γ-chitin with those of the three chitosan preparations provides much of interest that bears on this point (Table 1).

Generally, the mechanical properties of the renatured chitosan preparations are quite different from those of α, β and γ-chitin (also, see ref. 30). With the single exception of the dry chitosan acetate fibers tested at the highest strain rate, the chitosan samples are more elastic, hence less stiff,than any of the native chitins at comparable rates of deformation and hydration state. Moreover, α, β and γ-chitin exhibit considerably greater ultimate tensile strengths (σ_{max}) than do the chitosans which, at best, sustain only half the average σ_{max} of the native chitins. The elastic-limit stress values (σ_0) of α, β and γ-chitin are also very much higher than those of chitosan acetate, xanthate or formate. The corresponding proportional-limit strains and ultimate strains of the native chitins are, as must be expected, lower than those of the chitosans. Finally, the dry to wet σ_{max} ratio, which is an extremely important property of textile

Table 1. Mechanical Properties of Chitins and Chitosans

Materials	Hydration state	Strain rate %/second	E_0 (N/mm^2)	σ_0 (N/mm^2)	ε_0	E_1 (N/mm^2)	σ_{max} (N/mm^2)	ε_{max}
α-chitin								
Locusta tendon	wet	.16	3232	187	.06	-	208	.1
	dry	.18	5068	303	.06	-	-	-
Loligo oesophagus	wet	.16	35	13	.41	-	-	-
β-chitin								
Loligo pen	wet	1.03	91	5	.06	39	8	.15
	wet	.11	116	6	.05	48	8	.12
	wet	.01	118	4	.03	52	7	.12
	dry	1.03	3919	119	.03	2735	207	.07
	dry	.11	3847	114	.03	2666	261	.08
	dry	.01	3974	90	.02	2674	258	.08
γ-chitin								
Ptinus fiber	wet	1.90	1069	54	.05	-	-	-
	wet	.33	1129	22	.02	350	31	.06
	wet	.03	913	44	.05	-	-	-

Table 1. (Continued)

Materials	Hydration state	Strain rate %/second	$E_o N/mm^2$	$\sigma_o (N/mm^2)$	ε_o	$E_1 (N/mm^2)$	$\sigma_{max} (N/mm^2)$	ε_{max}
γ-chitin Ptinus fiber	dry	2.62	1883	184	.08	-	-	-
	dry	.27	1940	151	.06	771	207	.10
	dry	.02	1683	86	.04	706	165	.09
Chitosan acetate	wet	1.41	4.4	.3	.08	3	.8	.25
	wet	.14	3.4	.4	.12	2.6	.7	.26
	wet	.01	2.6	.3	.12	2.2	.7	.30
	dry	1.67	10633	90	.01	342	179	.08
	dry	.17	7031	60	.01	-	113	.04
	dry	.02	6286	61	.01	217	109	.07
Chitosan xanthate	wet	.09	.4	.1	.18	.7	.2	.38
	dry	.09	1457	25	.02	1152	39	.16
Chitosan formate	wet	.17	11.8	6.1	.53	21.7	14.4	.98
	dry	.08	2665	52	.02	149	101	.18

fibers, of native chitin is on the average ten-fold more favorable than that of the chitosans.

Since it is well known that chitin is chemically closely allied to cellulose (6, 8, 20) and that a number of regenerated celluloses, particularly viscose and acetate rayon, have proved to be commercially practicable, it is worth briefly comparing the relative properties of chitins and chitosans vis-à-vis those of cellulose and its derivatives. From Table 2 it can be seen that the maximum tensile strength and elasticity of the highly oriented α-chitin compares favorably with cotton when both are dry. However, flax, which is known to have an expecially highly oriented molecular structure, is much stronger and stiffer than any of the chitins. It may be that cellular materials can achieve a more highly oriented crystalline content than can the extra-cellular chitins (34), but systematic investigations of the crystalline/amorphous ratios of native chitins have not been done.

A comparison of chitosan formate with rayon acetate shows that the latter is the stiffer of the two when wet. However, on the basis of dry strengths, the chitosan formate is clearly comparable to the rayon but is more elastic than the acetate in water. It would appear, then, that the native celluloses are stiffer and stronger than the native chitins, while the dry regenerated derivatives of cellulose and chitin are comparable. Furthermore, both groups of derivatives are markedly less stiff and strong and more extensible than the primary compounds. The main failing of the regenerated chitosans vis-à-vis the rayons is that the former are inordinately susceptible to stiffness and strength reduction in the hydrated state. Because of poor wet strength, chitosan acetate and chitosan xanthate do not appear to be of practical use at present. Chitosan formate, however, might be, if it were either mixed with another fibrous material or chemically stabilized (indeed, native chitins are always associated with protein [28, 29, 30, 31]) to decrease its extensibility when wet (pure chitosan-formate fabrics would stretch very badly when wet).

In the present paper the view is taken that regenerated chitosans hold promise for future textile application if some of their more undesirable characteristics can be controlled, but this presupposes a greater understanding of the basic processes of deformation for both chitins and chitosans than has been available up to the present. In much the same way that the derivatives of cellulose require modification of properties inherent to the polymer (17), it is extremely pertinent to consider a molecular basis for the fundamental mechanical properties of the native α, β and γ-chitins.

Deformation and transconformations in chitins

α, β and γ-chitin, as well as the regenerated materials discussed above, are visco-elastic and so lack unique elastic moduli. Nonetheless, if these fibers are deformed in a non-destructive mode, it is possible that loads can be sustained in an elastic and/or viscous manner, depending on the rate of loading, extent of strain and time given for recovery. The following observations illustrate the extent to which the tensile mechanical behavior of α, β and γ-chitin can be modified with suitable chemical and mechanical manipulation.

α-chitin

Air-dried prawn and locust specimens were loaded slowly to a force near

Table 2. Mechanical Properties of Chitin, Cellulose and Their Derivatives

Material	Strain rate, % / second	Elastic modulus, E N/mm^2	Ultimate tensile strength, σ_{max} N/mm^2	Associated strain, ϵ_{max}	Source
Chitin acetate					
Dry	.17	7031	113	.04	This work
Wet	.14	3.4	.75	.26	This work
Chitosan xanthate					
Dry	.09	1457	38	.16	This work
Wet	.09	.41	.23	.38	This work
Chitosan formate					
Dry	.08	2665	101	.18	This work
Wet	.17	11.6	14.4	.98	This work
Cotton					
Dry	-	5900	540	.07	(1)
Flax					
Dry	-	49000	784	.02	(1)
Viscose Rayon					
Dry	-	14700	169	.01	(1)
Wet	-	1320	67	.05	(1)
Acetate Rayon					
Dry	-	6100	122	-	(1)
Wet	-	1720	68	-	(1)

but below that of the elastic limit and the crosshead movement stopped at time t_1 (Fig. 2). The specimens were held in this way for a short period of time, t_1-t_2, over which the time-dependent stress remained constant, thus precluding viscous effects over the interval considered. When distilled water is applied to the specimen at time t_2, stress relaxation occurs and the previously sustained force falls to zero, t_3. At time t_4, the now wet specimen is further extended to the force originally sustained at t_1 and crosshead movement is stopped, t_5. The specimen is observed to maintain this stress. If the water in the test chamber is replaced at t_6 with 6N HCl, a partial stress relaxation occurs. Now, although precisely what physico-chemical effects transpire on HCl vs. water solvation remain undetermined, it is at least possible to distinguish between the two modes of viscous flow that might be associated with the observed behavior.

In the case of protein polymers such as wool or cross-β Chrysopa silks, it is well established that the large viscous component of a typical tensile stress-strain curve is positively associated with the α to β and X-β to parallel β conformational change in the respective materials. This kind of change can be induced chemically or mechanically as has been confirmed by x-ray diffraction methods (19, 24). Similarly, a conformational change occurs when parallel β-chitin is solvated in nitric (18) or hydrochloric acid (27, 28). If, for example, a fiber of β-chitin is held taut and so prevented from contraction, the application of HCl results in energetic effects which are readily observed as force changes (Fig. 4). However, if a sample of prawn or locust α-chitin is simply held taut (not stretched) and the time base recorded, no x-axis force changes occur on the addition of water, HCl or LiCNS; that is, the energetic effects associated with conformational changes are absent. Therefore, the viscous (stress-relaxation) effect seen in Fig. 2 for α-chitin is very likely to be associated with chain-slipping of neighboring molecules. This would be entirely consistent with the fact that HCl is known to disrupt secondary forces such as interchain van der Waals forces and hydrogen bonding. Entirely different behavior is observed for β and γ-chitin.

β-chitin

Squid pen specimens were loaded in a tensile manner as were the α-chitins just described, except that the pens were extended in a water-filled chamber. When such a specimen is extended to a point near the elastic limit and the crosshead stopped (Fig. 3, t_1), there is an immediate stress-relaxation effect that, as in α-chitin, can be associated with chain-slipping or swelling. The force sustained is now t_2, only 30% of the original load, but equilibrium has occurred. When the water in the test chamber is replaced with 6N HCl at time t_2, there is a slight decrease in the load followed by a very rapid rise in force to a level exceeding that obtained on the initial, t_1, deformation. It must be remembered that the crosshead has remained in the off position since t_1, so that the rise in force is solely the result of HCl addition. The implication of this is that the now partially strained pen specimen has sufficient internal slack to allow for at least partial chain-folding in its attempt to complete the conformational change to α-chitin. Interestingly enough, if the specimen is allowed to equilibrate at its new HCl-induced stress and this level shifted to the zero base line, t_3, and the HCl in the test chamber replaced with water, a force-elongation curve in all essentials similar to that seen between t_0 and t_1 is obtained (Fig. 3). These observations complement those of Lotmar and Picken (18) and of Rudall (28) that on conversion from

β to α-chitin squid pen samples characteristically contract by as much as one half of their original lengths in the course of transconformation.

The question inevitably emerges as to whether or not the squid pen test sample shown in Fig. 3 has in fact completed conformational change between t_2 and t_3. Because the specimen is constrained and not allowed to contract, it is very likely that transconformation cannot go to completion as shown by the results of the following experiment. When a pen specimen is simply held taut (no load applied) and the zero base line adjusted to record positive or negative change, addition of 6N HCl results in an immediate positive force which reaches equilibrium (Fig. 4) and remains constant until experimentally perturbed. If the HCl in the test chamber is replaced by water, there is an immediate stress-relaxation,and the force drops to zero load. Moreover, when the water is again replaced with 6N HCl the initial behavior is repeated: there is the immediate development of a positive force, equilibrium occurs, and when the HCl is once again replaced with water the load returns to zero. This kind of HCl-induced mechanical behavior is exactly reproducible,and the hysteresis is entirely consistent with the material attempting a transconformation which is precluded because the specimen is held taut and cannot contract.

More conclusive evidence for the above interpretation would be to test pen specimens which had been allowed to complete the transconformation to α-chitin in 6N HCl prior to any testing. When this is done a result typical of many others obtained in the same way is obtained (Fig. 5). The specimen is stretched to a force just below the elastic limit and the crosshead is stopped, t_1. The specimen reaches equilibrium whereupon, t_2, the water is replaced by 6N HCl. There is then an immediate stress relaxation of the specimen and this entire acid-related sequence of behavior is similar to that of α-chitin. The sequences illustrated in Figs. 3 and 4 are exactly opposite of those in Fig. 5 with respect to HCl (remembering that constrained samples of squid pen β-chitin develop a positive force on addition of HCl).

Ordinarily, one might have expected a zero force over the range of conformational change as occurs in the α→β transconformation of wool (19); however, the transconformation of β- to α-chitin is in a sense exactly the obverse of that in the proteins. That is, the extended parallel β form of chitin is required to contract in order to assume the folded anti-parallel form of α-chitin, while in the protein a folded helical form is extended into sheet form. In this connection, a note of caution ought to be introduced; it might be that β-chitins treated with HCl give rise to a folded pattern, which on x-ray diffraction resembles α-chitin but which might possibly be only a "pseudo α-chitin," since it has not been unequivocally established that α-chitin is folded even if it is an anti-parallel structure.

There are two important reasons for the above caution. In the first instance, that α-chitin is anti-parallel in arrangement is evident from crystallographic studies (5, 7, 25, 27, 28); however, the suggestion that α-chitin is folded in the native state is only an inference, based on the observation that β-chitin contracts from an extended form to a folded one when chemically treated (34). The second point is a set of experimental observations; samples of squid pen were soaked in 6N HCl and were observed to contract to about one-half of their original lengths. They were then stretched (but not broken) beyond their elastic limits so that permanent plastic deformation had occurred. The specimens were then removed from the

extensometer, their new lengths measured and left unrestrained in distilled water overnight. On the next morning no length change had occurred. When these specimens were then placed in 6N HCl, contraction occurred. This implies at least partial reversibility of an HCl-induced β→α transconformation especially in light of the fact that native α-chitin does not contract in HCl.

γ-chitin

Beetle-cocoon fibers were tested in the same manner as were those just described for α- and β-chitin. Air-dried fibers were extended to just near the elastic limit and the crosshead stopped,whereupon the fibers maintained the load. The viscosity of these γ-chitin fibers is apparent on addition of water, as there is a rapid partial stress relaxation as in α and β-chitin. If, on the other hand, the extended fiber is treated with LiCNS there is complete stress relaxation to zero load,and this effect is not changed on substitution of water or HCl. Similarly, the application of HCl to extended fibers results in partial stress relaxation as observed in α-chitin.

γ-chitin fibers were also analyzed for chemical hysteresis effects (as in the squid pen samples) by holding them taut but not loaded. The application of LiCNS at room temperature to such fibers results in a positive force which can be maintained in equilibrium. If the LiCNS is replaced with water, the force is dissipated and falls to zero. If the procedure is repeated, one observes a LiCNS water hysteresis exactly like that shown in Fig. 4 for the β-chitin HCl-water hysteresis. This is entirely reproducible and, as in β-chitin, points to an arrested transconformation.

Cocoon fibers were also treated in LiCNS, a solvent known to induce a γ→α transconformation (28), and then subjected to partial loading and treatment with various solvents. When this is done, LiCNS, HCl and water all produce varying degrees of stress relaxation, i.e., result in negative force, just as occurred in α-chitin subjected to these solvents. Likewise, the results associated with LiCNS addition to untreated and then treated fibers are consistent with a γ→α transconformation in just the same way as HCl is associated with the β→α transconformation.

There was one important remaining difficulty in accounting for the behavior of the γ-chitin fibers. If it is indeed true that one obtains a LiCNS-water hysteresis (which can be eliminated if the fibers are treated in LiCNS prior to testing), why is it that no macroscopic changes in the fibers were observed when restrained dry fibers were placed freely in a solution of saturated LiCNS? The development of a positive force in the taut specimens suggests that the γ→α transconformation involves contraction, yet no contractions were observed when dry specimens were put into LiCNS. A partial explanation for these observations might be that the crystallites of the beetle-cocoon fibers are too closely packed to allow LiCNS solvation of dry fibers. The reason, also, why one obtains a LiCNS-water hysteresis is the fact that the water sufficiently opens the structure to allow LiCNS solvation. Indeed, if air-dried cocoon fibers are soaked in water for a few minutes and then placed in saturated LiCNS, a very rapid contraction of the fibers occurs.

Although the different kinds of viscous behavior explained as chain-slipping in α-chitin and transconformation in β and γ-chitin are consistent with all of the mechanical-chemical data, an unresolved problem

remains: Why is it that taut specimens of β- and γ-chitin display what are undoubtedly attempted conformational changes when suitably treated chemically, while the same materials (under conditions of partial loading) accommodate the stress partially by viscous stress relaxation? It is possible that the untreated but taut specimens undergo the partial axial realignment required of transconformation as well as by partial distension of the amorphous regions, while in extended specimens the decline in force might primarily be the result of the amorphous regions flowing in a viscous manner. The solution to this and other problems in the visco-elastic behavior of chitins requires detailed information on the relationships between oriented and amorphous regions in chitin. The realization of this knowledge coupled with the necessary theoretical treatment of the actual modes of deformation is the key to making the seemingly intractable chitins of today valuable and useful textiles of tomorrow.

ACKNOWLEDGMENT

Partial financial support for this work was provided by the Council for Scientific and Industrial Research, Pretoria.

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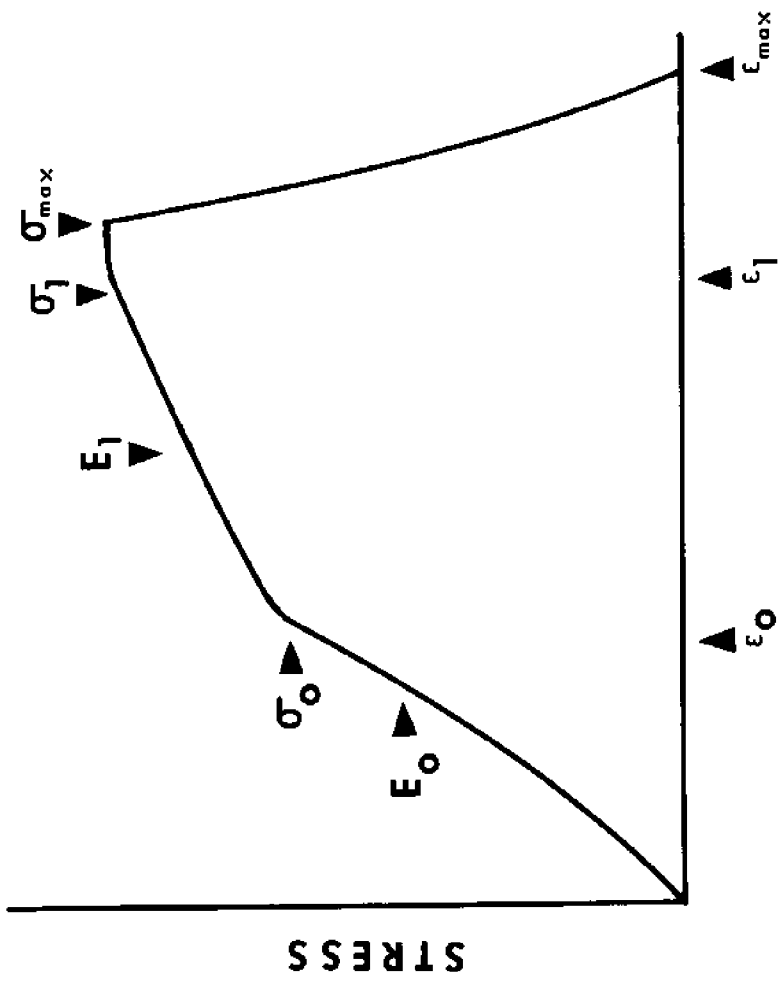

Figure 1. Typical tensile stress-strain curve for chitins and chitosans.

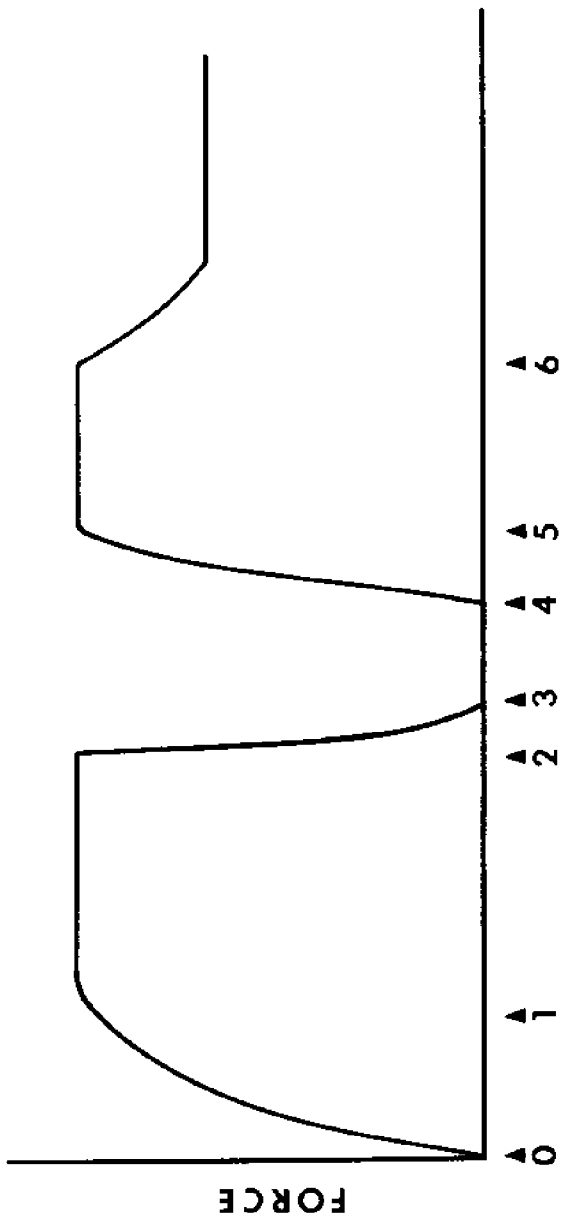

Figure 2. Force-extension and stress-relaxation curves for crystalline α-chitin.

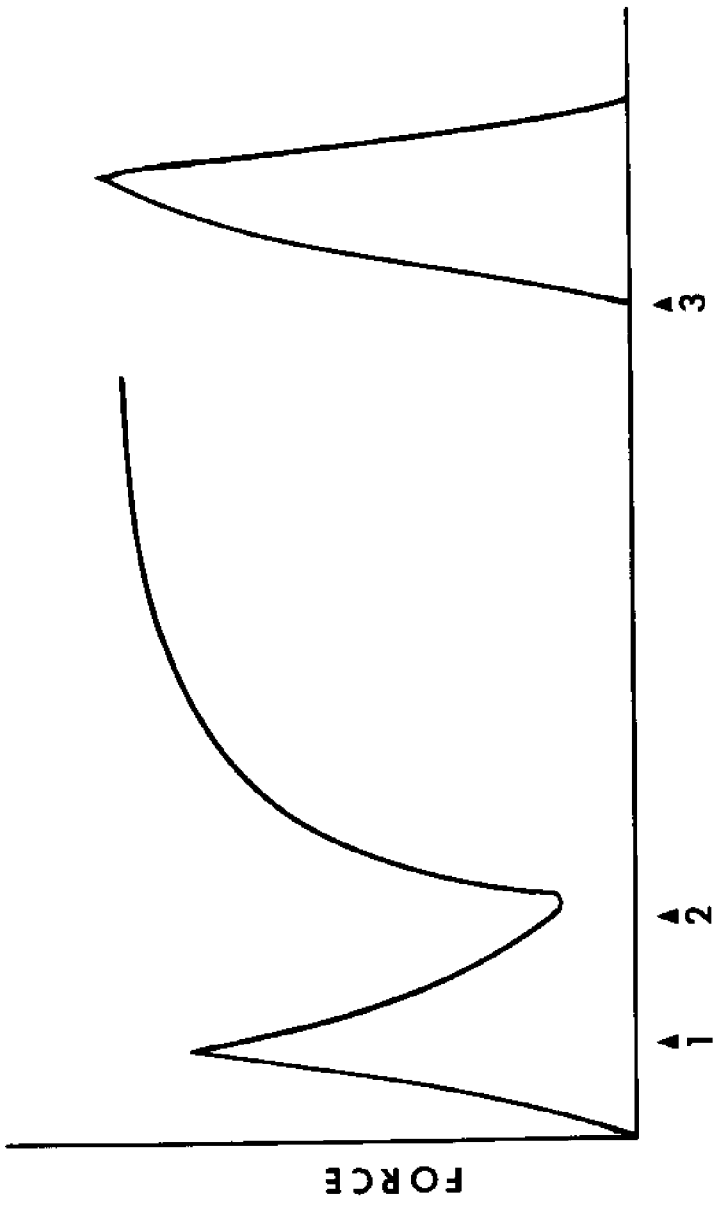

Figure 3. Force-extension and stress-relaxation curves for β-chitin.

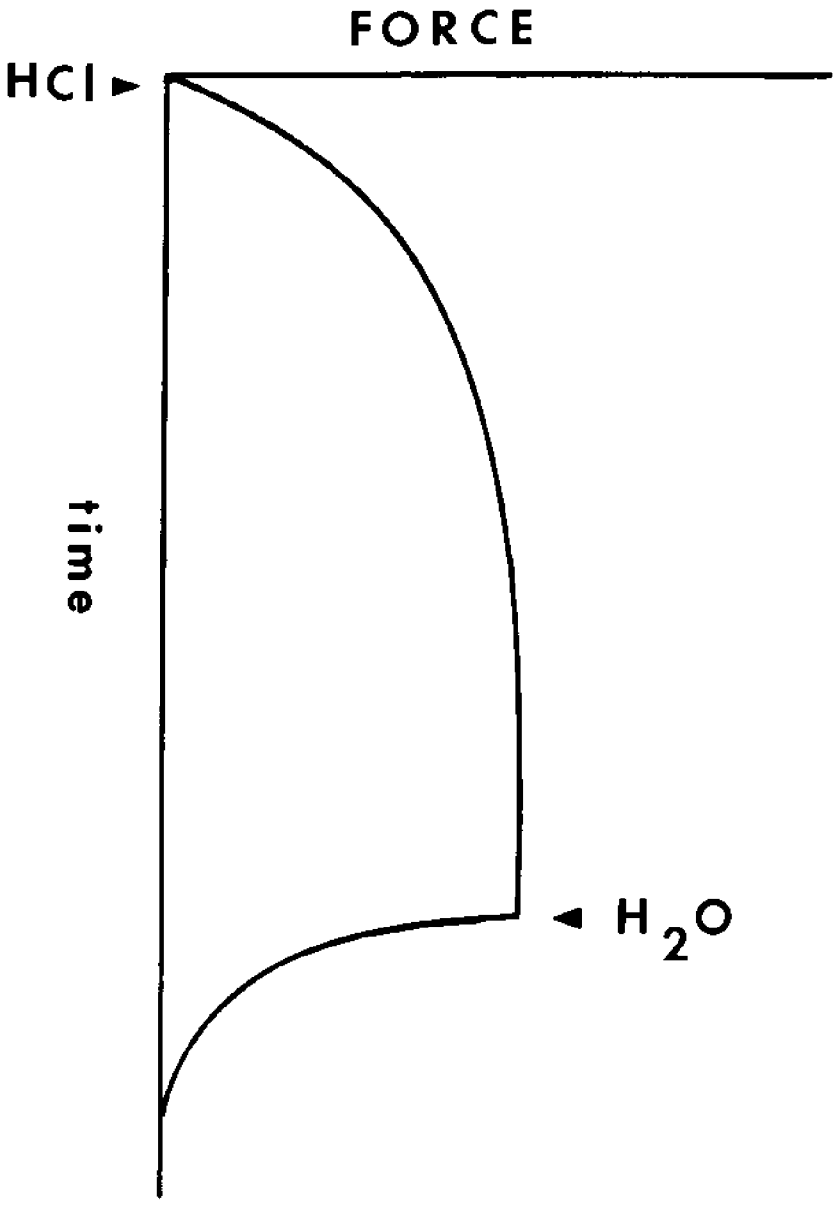

Figure 4. Hydrochloric acid/water hysteresis curves for β-chitin.

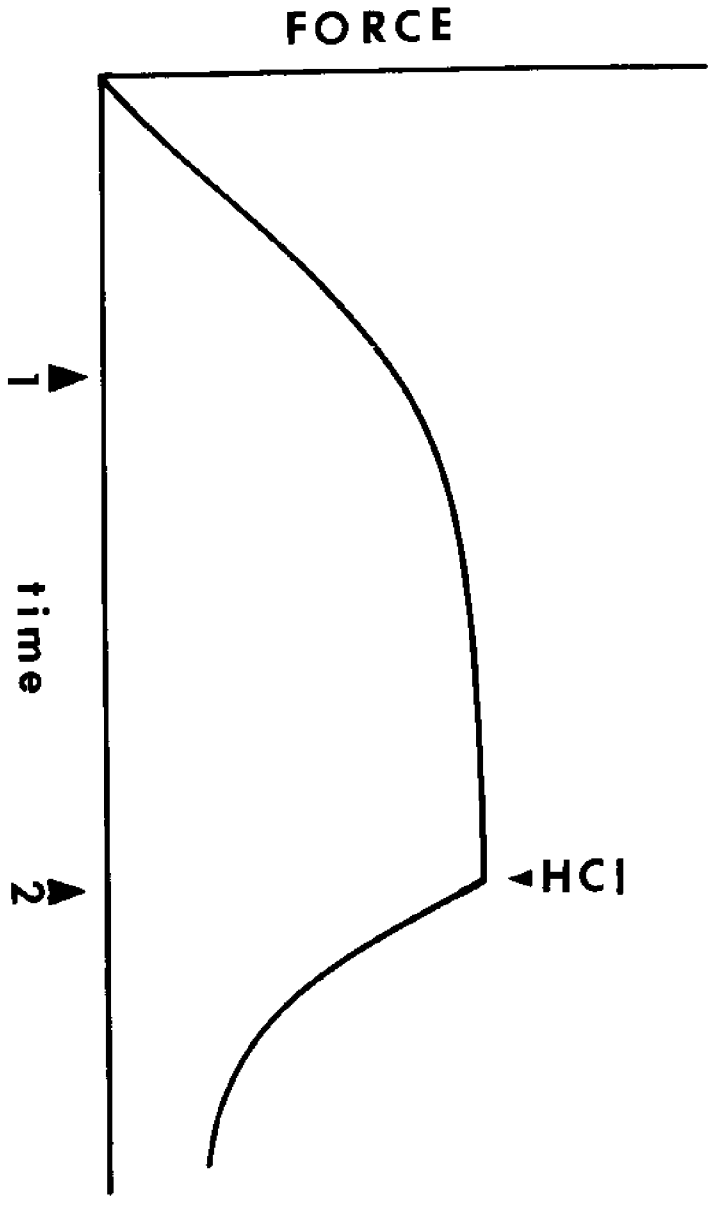

Figure 5. Acid-induced stress-relaxation curve for β-chitin.

CRYSTAL-DEFECT MECHANISMS AND THE TRANSCONFORMATIONAL CHANGES OF CHITIN

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ABSTRACT

The possible structures of dislocations and dislocation-like defects that might occur in polysaccharide polymers such as chitins and their possible roles in deformation mechanisms such as the inter-conversions between α-, β- and γ-chitins are discussed.

Crystal defects in inter-molecular bonding, such as edge and screw dislocations and disparations, could allow chains to slip over one another with only localized disruptions of the hydrogen bonds or van der Waals forces. Deacetylation, for example, results in inter-chain bonding discontinuities which can act as defect sources for the glide of mobile dislocations through the chitin crystals. This can partially explain why the mechanical properties associated with deacetylation thus result from the disruption of a large number of hydrogen bond-sites on repolymerization.

The structure of the bends thought to occur in the molecular chains of α- and γ-chitin and the possible mechanisms whereby β- and γ-chitin are converted to α-chitin are discussed. A bend structure, formally equivalent to two-edge dislocations in the intra-chain hydrogen-bond network, is proposed to account for the folding and unfolding of chains into specific crystallographic forms of chitin. The reversibility of the β→α chitin transconformation is also discussed.

INTRODUCTION

Many physical properties of materials are governed, not by the structure of the perfect crystal lattice, but by the presence and behavior of defects in the structure. The most studied of defect-controlled processes is, perhaps, the phenomenon of metal plasticity, in which irreversible flow of the material under stress occurs when dislocation defects glide through the lattice (9,21). During the last 15 years or so, similar concepts have been applied in helping to understand the flow behavior of macromolecules (1,6, 11,24,26). However, most of the work so far has discussed the possible role of defects in the deformation of simpler materials such as polyethylene and nylon. More recently, the defect concept has been extended to the behavior of helical polypeptides, and the well-known α→β transformation that occurs on stretching wool fibers was ascribed to the glide of edge dislocation defects (14). The purpose of the present paper is to discuss possible kinds of defects that could occur in polysaccharide materials, with special reference to chitins and chitosans, and to show how these might play a role in the deformation and transconformation change effects that are observed in these materials.

Various defects are known to exist in chitins and chitosans. For example, there is a possibility that some residues in native chitins are de-acetylated (30), and, in the production of re-natured chitosans, de-acetylation can take place to a considerable extent. De-acetylated sites

are, in effect, point defects in the chain and, although themselves immobile, might act as nucleation sites for mobile defects. Chain folding has been discussed extensively with reference to chitins, chitosans and celluloses (20,29); such folds, as will be discussed below, are capable of description in terms of edge dislocation defects. In addition to these, it has been suggested that many effects, generally ascribed to lack of crystallinity, could be due to defects at the molecular level (3). Also, some of the pyranose rings can adopt higher energy conformations, such as some of the boat forms rather than the favored C1 chair conformation. Such defects could lead to greater chain flexibility with consequent effects on the physical properties (7).

Experimental evidence for the existence of crystal defects in chitins is, so far, rather indirect. On the microscopic scale, defects have been observed in the chitin fiber architecture of Arthropods (5), and it has been hypothesized that arthropod cuticle solidifies from a cholesteric liquid crystal phase (22). Such phases often contain dislocation and disclination defects (6). On the sub-microscopic scale, it has been pointed out that many chitin preparations give anomalous x-ray reflections (30), and it has been suggested that similar reflections observed in nylon could be due to the presence of crystal dislocations (25). Indirect evidence is also provided by mechanical test data (15,16). Even in highly crystalline and well-orientated chitin preparations, there is evidence for fairly well-defined yield points followed by irreversible plastic flow, phenomena which, in many materials, can be associated with the mobility of crystal defects.

Chitin consists of long chain molecules, hydrogen bonded together to form sheets as illustrated schematically in Fig. 1. Sheets are held together by van der Waals forces to form the crystalline structure. Refinements of the structure have indicated that other inter-chain intra-sheet hydrogen bonds may be formed (10). For the purposes of simplification, the hydrogen-bond arrangement of Fig. 1 will be used for the following qualitative arguments, since these will be unaffected by any additional inter-chain hydrogen bonding. There are three crystalline forms of chitin, depending upon how the chains are aggregated (30). In the most usual form, α-chitin, neighboring chains are anti-parallel, in β-chitin, chains are parallel and, in γ-chitin, chains are arranged with two parallel and one anti-parallel. From the unit-cell dimensions of chitin (3,8), it would seem difficult for chitin to deform simply by adjacent sheets sliding past one another, since interference between acetyl groups on neighboring chains would tend to occur. In introducing the notion that the deformation of chitin and chitosan is a defect-controlled process, it is suggested that defect propagation provides a lower energy means by which chains may slide over one another.

TYPES OF LATTICE DEFECT

Lattice defects are described in terms of the symmetry operations that they perform on the regular unstrained lattice. Three types of symmetry operations may be performed; translational, rotational and screw operations. Defects associated with these operations are dislocations associated with translational, disclinations associated with rotational and dispirations associated with screw symmetry operations.

Dislocations

There are two basic types of dislocation, the edge and the screw dislocation. These may be illustrated as in Fig. 2, which shows a cylinder of material with a single cut along its length and the cut faces displaced relative to one another. The edge dislocation (Fig. 2a.) is formed by displacing the cut faces in a direction perpendicular to the axis of the cylinder, the dislocation line. The screw dislocation (Fig. 2b.) is formed by displacing the cut faces parallel to the dislocation line. A dislocation is specified by its dislocation line and its Burgers vector, denoted $\underline{b}$, which specifies the magnitude of the displacement and its direction relative to the dislocation line. In three-dimensional crystals, dislocations are line defects, and in two-dimensional crystals are point defects.

Disclinations

There are two basic types of disclination, the twist disclination and the wedge disclination, as illustrated in Fig. 3. In the twist disclination (Fig. 3a.), the rotation axis $\underline{\omega}$ is perpendicular to the disclination line. In the wedge disclination, which is formed by adding or removing a wedge of material from the lattice, the rotation axis is parallel to the disclination line.

The wedge disclination is generally only of interest in two-dimensional structures such as membranes, as the strain energy can be very high (12). Twist disclinations of small angle have been discussed with reference to polymer molecules (19) and can be applied to chitins.

Dispirations

Several types of dispirations are possible, of which the twist co-dispiration illustrated in Fig. 4 is the type most likely to be of interest in polymer deformation. The defect is specified by a rotation about an axis $\underline{\omega}$ perpendicular to the dispiration line and a Burgers vector $\underline{b}$ parallel to $\underline{\omega}$. Kink models of defects in polyethylene chains are essentially defects of this type (23).

It is possible for all three types of defect to exist in chitins, chitosans and other polysaccharide molecules. In such molecules it is convenient to consider two further classes of defect:

a) Defects affecting inter-molecular forces and bonds.
b) Defects affecting intra-molecular bonds.

Defects in the inter-molecular arrangement are important because they could affect the packing, the degree of crystallinity of a given preparation and, in addition, could allow chains to slide over one another more easily, hence influence the mechanical properties. Intra-molecular bond defects can affect the morphology of individual chains and give rise to bends in the chain.

INTER-MOLECULAR DEFECTS

Dislocations

For crystalline polyethylene, four types of dislocation have been suggested (17), which are illustrated in Fig. 5. Types a and b are screw and

edge dislocations respectively, with their Burgers vectors normal to the chain direction. Therefore, for a well-orientated fiber system with a tensile load applied in the fiber direction, these particular defects would not account for any elongation. If the fiber were twisted, however, such defects could contribute to torsional flow. Types c and d are screw and edge dislocations respectively, with Burgers vectors parallel to the chain axis; propagation of these could cause chains to slip over each other and contribute to the elongation of the fiber.

Similar kinds of defects have been discussed with reference to the deformation of nylon (25), which is similar to chitin in that it has a structure composed of aggregates of hydrogen-bonded sheets held by van der Waals forces. It has been shown that such defects can be generated by chain ends in the crystal, an example of which is shown in Fig. 6. An array of such defects can lead to considerable distortion of the crystalline lattice. Such defects could easily be accommodated in chitins and chitosans. The chain-end defect, of Fig. 6, represents a chain termination within a hydrogen-bonded sheet. Another form could be a sheet terminating within an aggregate, which would give rise to stacking faults in an anti-parallel arrangement, as illustrated in Fig. 7. The possibility of such faults in chitin-sheet arrangements has been discussed (30).

Besides defects affecting the van der Waals bonding arrangement, other forms could affect the intra-sheet hydrogen bonding. The most probable forms are the screw dislocation similar to Fig. 5a and the edge dislocation similar to Fig. 5d. The screw dislocation would be more likely in parallel chain structures because in anti-parallel structures, the Burgers vector would be large, and the defect would have to span two chains to be a symmetry operation. The edge dislocation is of more interest, however, as its presence would cause a disruption in crystalline order and its propagation could allow chains to slide over one another. The defect is illustrated in Fig. 8 and, if it occurs in a single pile of chains, is a point defect. To accommodate such a defect, the normal structure would have to be deformed as shown in Fig. 8, and it is easier to accommodate if some of the residues are in conformations other than the favored C^1 conformation (labelled B in Fig. 8). If some residues were to crystallize in alternative conformations during the solidification process, then it is quite possible that some of these edge-dislocation defects could be formed. Slip of this type of defect and hence chain sliding could occur by breaking only two hydrogen bonds at a time; this process would require far smaller forces than would be required to slide chains over each other bodily.

Defects, such as those shown in Fig. 8, could occur readily in materials such as chitins and chitosans which consist of stacked hydrogen-bonded chains, but less readily in other polysaccharides, such as cellulose, in which the strain energies would be much greater.

Aggregations of edge dislocations could lead to the formation of low-angle grain boundaries or small-angle wedge disclinations, in much the same way as such defects can be formed in metals, Fig. 9 (21). The presence of such aggregations could, therefore, influence the crystalline order.

Disclinations

It is improbable that large-angle disclinations will be present in cellulose or chitin preparations, since their strain energies are likely to be

prohibitively large; but small-angle wedge disclinations such as those of Fig. 9, and small-angle twist disclinations could exist.

Small-angle twist disclinations are more likely to influence the mechanical properties of chitins and chitosans through their influence on the crystallinity of the material and as sources of mobile defects, rather than through their own mobility. From potential energy maps of β-linked polysaccharides (27) it appears that the lowest energy conformations for chitins have a slight right-handed twist (31). This implies that the normal crystalline form of chitins and chitosans, in which the chains are not twisted, contain small-angle twist disclinations. It would be expected, therefore, that high-chain-density preparations would be more crystalline than low-density preparations since, in the latter, the chains could tend to adopt a lower energy twisted form. Many naturally occurring protein/chitin complexes such as soft cuticles in which the chitin-volume fraction is relatively low are, in fact, much less crystalline than hard cuticle. Chitin extracted from soft cuticle can be made to appear more crystalline and produce clear x-ray diffraction patterns by stretching it (30). This is a fairly standard procedure for preparing such cuticle for diffraction work. The effect of stretching produces strain-induced crystallization which probably involves aggregation of chains into sheets and the removal of the twist.

Dispirations

From Fig. 1 it can be seen that a symmetry operation of a chitin chain within a hydrogen-bonded sheet is a translation of one residue in the b direction followed by a rotation of radians about the b axis. The defect which involves this operation is the dispiration similar to that in Fig. 4. Such a defect in a stack of chitin chains is illustrated in Fig. 10. The defect involves the disruption of two hydrogen bonds as does the edge dislocation of Fig. 8. Again, some lattice strain is produced by the defect, but this is less than that produced by the edge dislocation. It would be expected that this kind of dispiration defect is mobile, and its propagation would cause sliding of the chain containing the defect past the others in the stack.

It is possible to accommodate such defects in other polysaccharides such as celluloses. However, as each chain in cellulose is hydrogen bonded to four others, rather than two as in the case of chitins, much more distortion of the surrounding structure would be produced, and, consequently, its ease of propagation would be reduced.

INTRA-CHAIN DEFECTS

The intra-chain hydrogen-bond defect of greatest interest is the point-defect-edge dislocation, which is capable of changing the topology of an individual polysaccharide chain in much the same way as has been suggested for polypeptide fibers (13). This kind of defect is best described by considering the polysaccharide chain to be a helical cylindrical crystal. Conformational energy maps of β-linked polysaccharides (27) suggest that three helical forms are possible: a helix with two residues per turn, one with five residues in two turns, and one with three residues per turn. In the native state, the helix with two residues per turn is the most abundant, but poly-D-1,4'-xylan has been found to exist in the three-residues-per-turn form (32). It has been suggested that the addition compound of chitin with NaOH also exists in this form (31). It will be shown on the following page that the different helical forms are related and may be

inter-converted by the passage of intra-chain hydrogen-bond edge dislocations. It will be further suggested that bends in polysaccharide chains are formed by short regions of higher order helices.

The formal geometry of cylindrical crystals has been developed and applied to crystals such as viral tail sheaths (13). Similar conventions and notations will be used to describe polysaccharide helices. Any cylindrical crystal can be represented on a 2-dimensional plane. The plane representation is developed by imagining the crystal to be a roller, inking in equivalent lattice points and printing these onto the plane. Fig. 11 shows the equivalent 2-D representation for a polysaccharide chain using the C_5 atoms as the reference points. The basic plane lattice is represented by the vectors $\underline{a_1}$ & $\underline{a_2}$, $\underline{a_1}$ representing the hydrogen bonding sense of the lattice and $\underline{a_2}$ representing the covalent bonding. Within the basic lattice framework, particular topological forms are described by the vectors $\underline{C_1}$, $\underline{C_2}$ ...$\underline{C_x}$ where:

$$\underline{C_x} = n_1 \underline{a_1} + n_2 \underline{a_2}$$

n_1 and n_2 being integers.

A particular topological form can be made from the reference lattice by rolling a portion of the lattice into a cylinder such that $\underline{C_x}$ is a circumference of the cylinder. Lattice points connected by $\underline{C_x}$ are thus identical points in the cylindrical crystal. A further convention that is adopted is to form the cylindrical crystal from the plane such that the edges to be joined are folded away from the observer, i.e., a crystal with characteristic vector $\underline{C_3}$, when formed from the plane lattice, has $\underline{C_3}$ as a clockwise directed circle and is a right-handed helix, the lattice with characteristic vector $\underline{C_{-3}}$ being a left-handed helix.

For a particular lattice characterized by its base vectors, the topological form of a particular crystal is specified by the integers n_1 and n_2. From Fig. 11 for a polysaccharide helix, n_1 refers to the number of chains in the helix and n_2 to the hydrogen-bond topology. For the materials being discussed, each helix contains only one chain, and n_1 is thus unity and the helical forms can be described by n_2 alone. Thus, the right-handed helix with 5 residues in two turns, described by the characteristic vector $\underline{C_2}$ in Fig. 11, is a (2)-helix. The helix with three residues per turn, represented by $\underline{C_3}$, is the (3)-helix, and the equivalent left-handed forms are denoted $(\overline{2})$- and (-3)-helices respectively. The usual form with two residues per turn is the (± 1)-helix, as right-and left-handed forms are equivalent. Polysaccharide helices of the (3), (-2) and (1)-forms are illustrated in Fig. 12.

Two main kinds of lattice deformation may occur: metrical contractions and topological contractions. In a metrical contraction, the vectors $\underline{a_1}$, $\underline{a_2}$ and $\underline{C_x}$ are changed, leaving n_2 and, hence, the order of the helix unchanged. Helical forms, such as the seven-residue-per-turn helical structure that has been proposed for cellulose (35), are metrical

contractions of the (1)-helix. Topological contractions of the lattice leave the base vectors unchanged but alter C_x and n_2 and, hence, change the topology of the crystal. Topological contractions can be brought about by the glide of edge dislocations with Burgers vectors, such as $\underline{b_2}$ and b_3 in Fig. 11. Fig. 13 shows the structure of an edge dislocation at the junction between a (-2) and (-3)-helix, and is, in effect, a non-hydrogen-bonded H_1 atom. The defect is mobile and may propagate through the lattice by a process of breaking and re-forming hydrogen bonds one at a time. If the defect propagates in the direction A, the whole helix will be transformed to a (-2)-helix, and if it propagates in the direction B, the remaining (-2)-helical regions will be converted to (-3)-helix.

Bend structures in polysaccharide chains may be described in terms of this kind of defect. Fig. 14 illustrates a bend formed by a short section of (-2)-helix, and Fig. 15 a bend formed by a section of (-3)-helix. Both these structures are analogous to corresponding structures in proteins (18,34). Formally, the (-2) bend contained two edge dislocations of opposite sign, one negatively charged formed by a non-hydrogen bonded O^5 and the other positively charged due to a non-bonded H_3 atom. The (-3) bend of Fig. 15 contains four edge dislocations, two positively and two negatively charged.

As with the single-edge dislocation of Fig. 13, the edge dislocations forming bends are capable of movement by glide. This property of structures containing bends could thus partially, at least, account for the relatively low strength of preparations of polysaccharides containing bends as distinct from straight chain structures, since glide of dislocations and movement of the bends could give rise to the yield point. In regenerated celluloses such as rayon, bends have been observed (20), and these materials characteristically exhibit yield points in tension, have lower tensile strengths and greater elongations to failure than the native materials.

It is likely that regenerated chitins, although they are allegedly in the crystallographic α form (33), are essentially structures containing bends, whereas native chitins which have much greater strengths (15) are straight chain materials. This is probably a further reason why chitosans formed from lower viscosity solutions are weaker than those formed from high viscosity solutions (33), since there is more chance for bend formation to take place in the former.

DISCUSSION

Some of the types of defects to be expected in polysaccharide molecules have been described above, together with their possible role in deformation processes. The overall process of deformation is, however, likely to be far more complex and incapable of simple explanation in defect terms. Overall gross deformation of α chitin probably involves the creation and mobility of a number of different types of defects and interactions between them. It is unlikely, therefore, that any quantitative estimates of behavior can be obtained by only considering defect processes in these materials. However, a knowledge of the kinds of likely defects present in these materials may be useful and may suggest methods by which strength may be improved. The strongest materials are the native materials. These most likely are straight chain materials containing very few bends or mobile defects. Comparison of the mechanical properties of native chitins and celluloses, however, indicates that although the elastic moduli are fairly similar, the

strength of the chitin is less, since they show well-defined yield and plastic flow regions. This can probably be explained in terms of the dislocation and disclination defects described previously, since these defects are relatively easy to form in chitin structures but less easy to form in celluloses, where each chain is hydrogen bonded to four near neighbors rather than the two of chitin, which renders chitin sheets inherently more flexible.

The bend-type defects probably play a large role in the crystallization of regenerated materials from solution and on the subsequent mechanical properties. A good illustration of the effect of bends in the structure is provided by the mechanical strength of chitin formed from β-chitin treated in 6N HCl. Although the material is crystallographically α-chitin, bends must form during the 50% length contraction that occurs on transformation. The resultant material is mechanically much weaker than the original β-chitin or native α-chitin, and the HCl material shows yield and flow behavior almost as soon as a tensile stress is applied.

To strengthen materials, either all the defects should be removed or the defects should be rendered immobile. The first solution is probably not really feasible as defects are almost certain to be formed during any crystallization procedure, although with careful solidification from suitable solvents their number could be significantly reduced. Rendering defects immobile is probably more feasible. Most of the defects described above characteristically involve the presence of non-hydrogen-bonded atoms that are normally hydrogen bonded and, consequently, many of the defects possess a small charge. This indicates that it might be possible to bind other larger charged groups to defect sites. If these were sufficiently tightly bound, such groups could block defect movement and raise the yield points of the materials. Further experimental work along these lines is therefore indicated before the relatively weak chitosans can be used commercially as load-bearing fibers.

ACKNOWLEDGMENT

Partial financial support for this work was provided by the Council for Scientific and Industrial Research, Pretoria.

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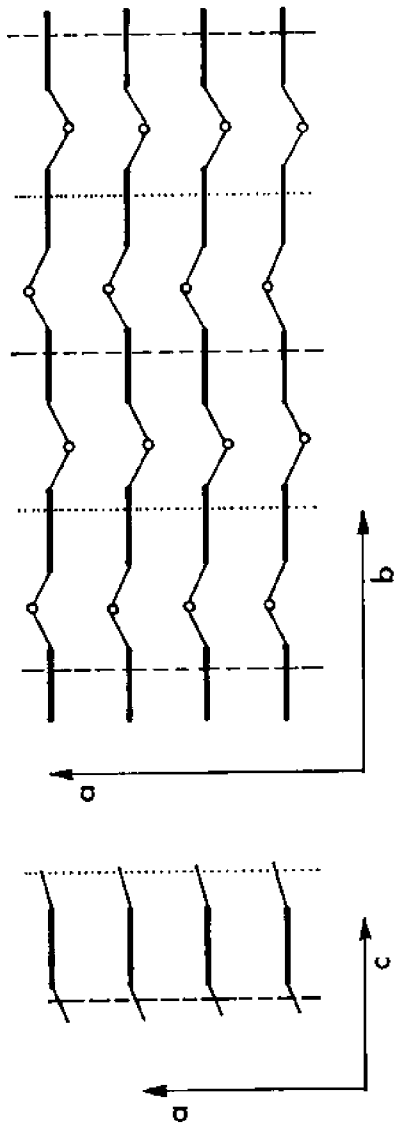

Figure 1. Schematic illustration of a chitin sheet. The dashed lines represent hydrogen bonds in front of the sheet and the dotted lines those behind.

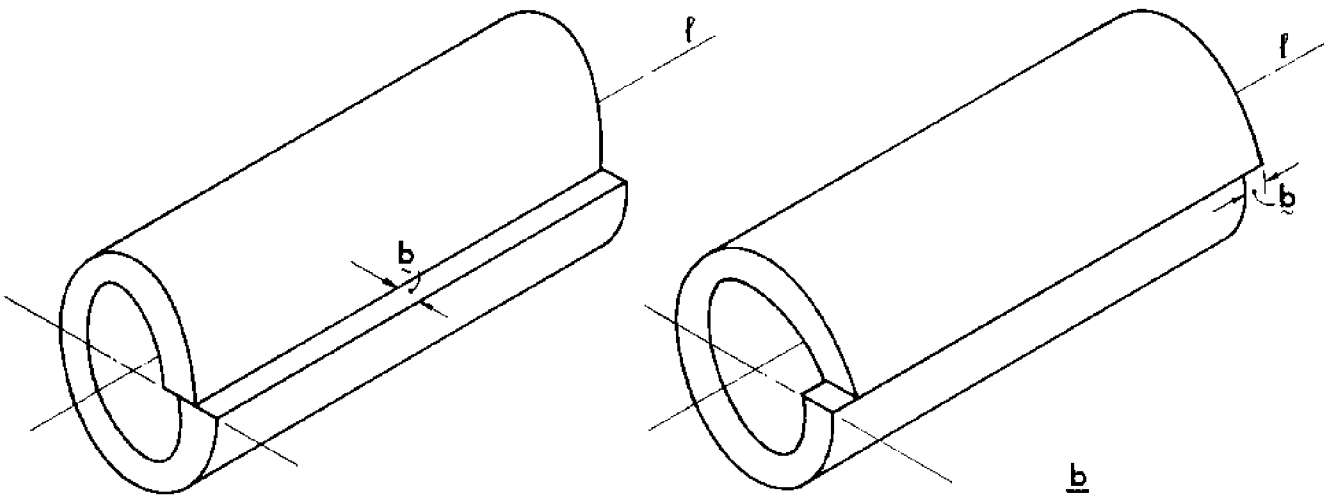

Figure 2. a) Edge dislocation formed by cutting a cylinder of material.
b) Screw dislocation.

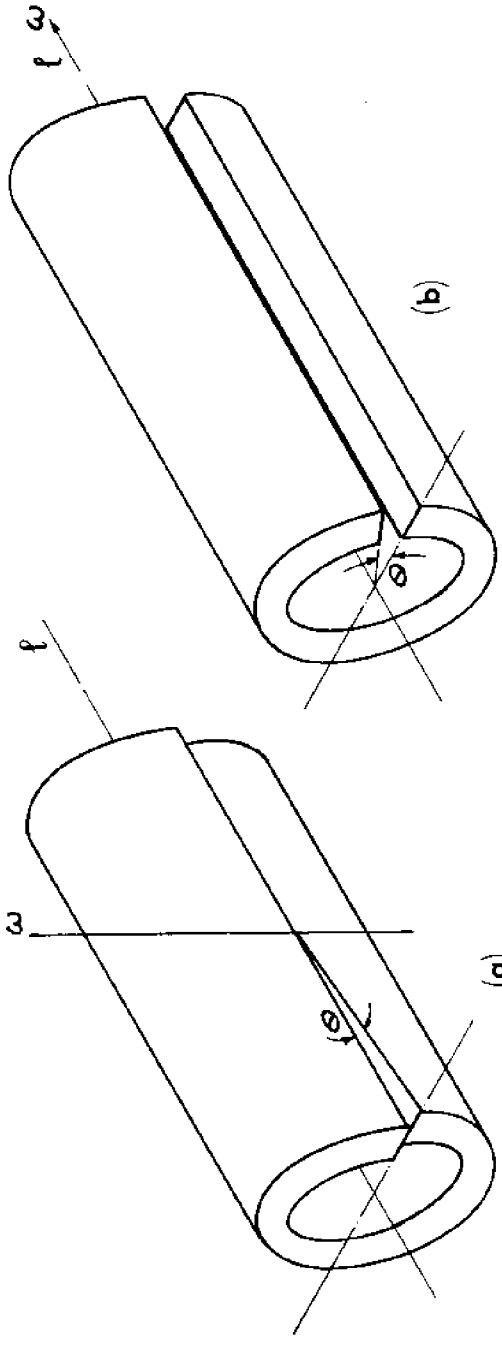

Figure 3. a) Twist disclination. b) Wedge disclination.

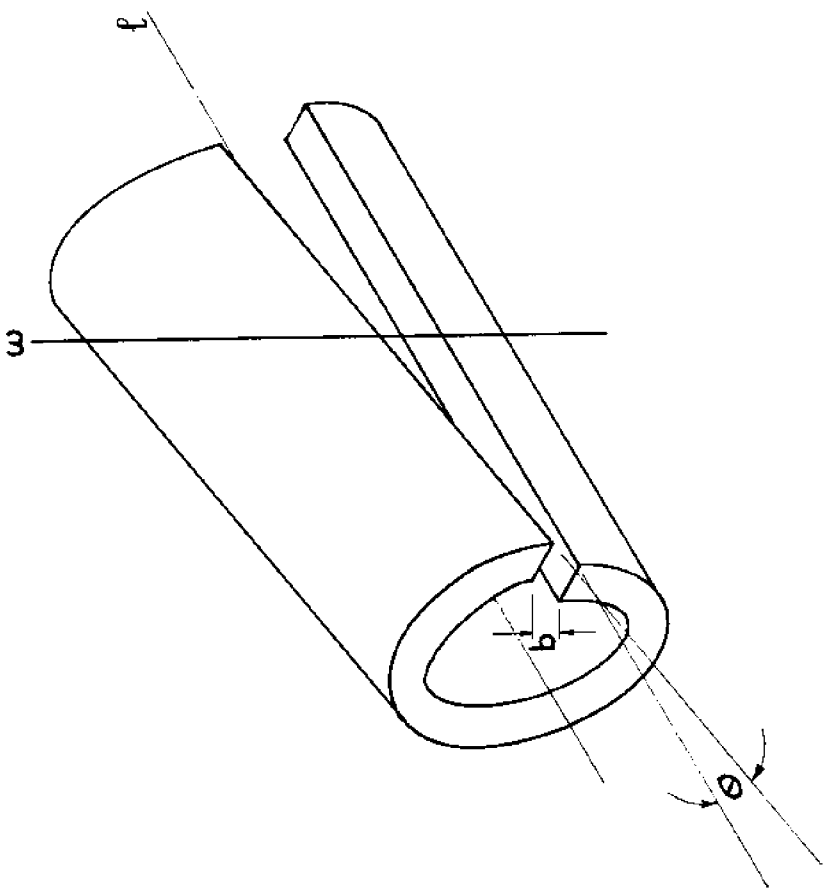

Figure 4. Twist co-dispiration.

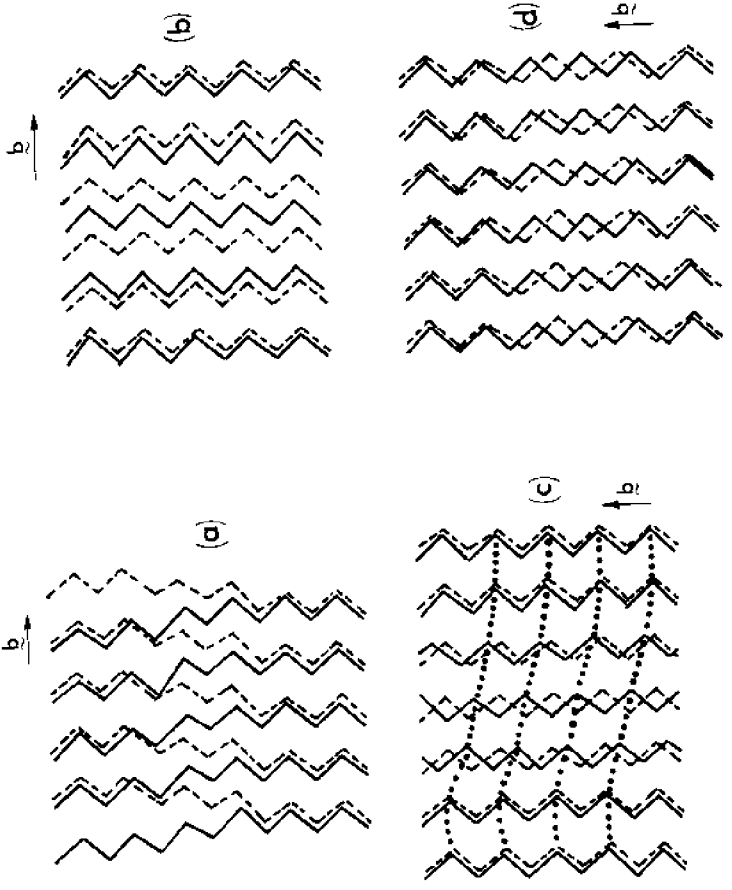

Figure 5. Four types of dislocation in polymer chains as proposed by Keith and Passaglia (1964).

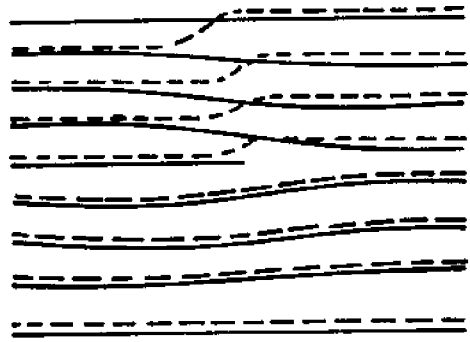

Figure 6. Screw dislocation formed by a chain end in a crystalline polymer.

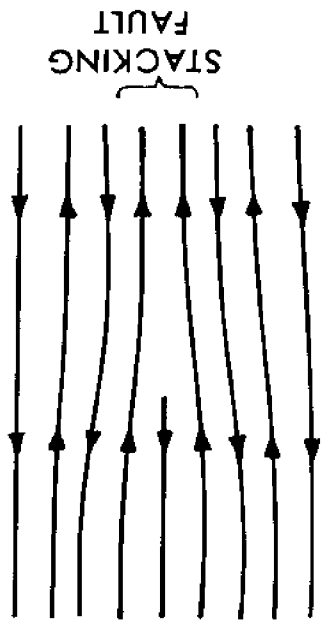

Figure 7. Stacking fault in α-chitin formed by a terminating sheet.

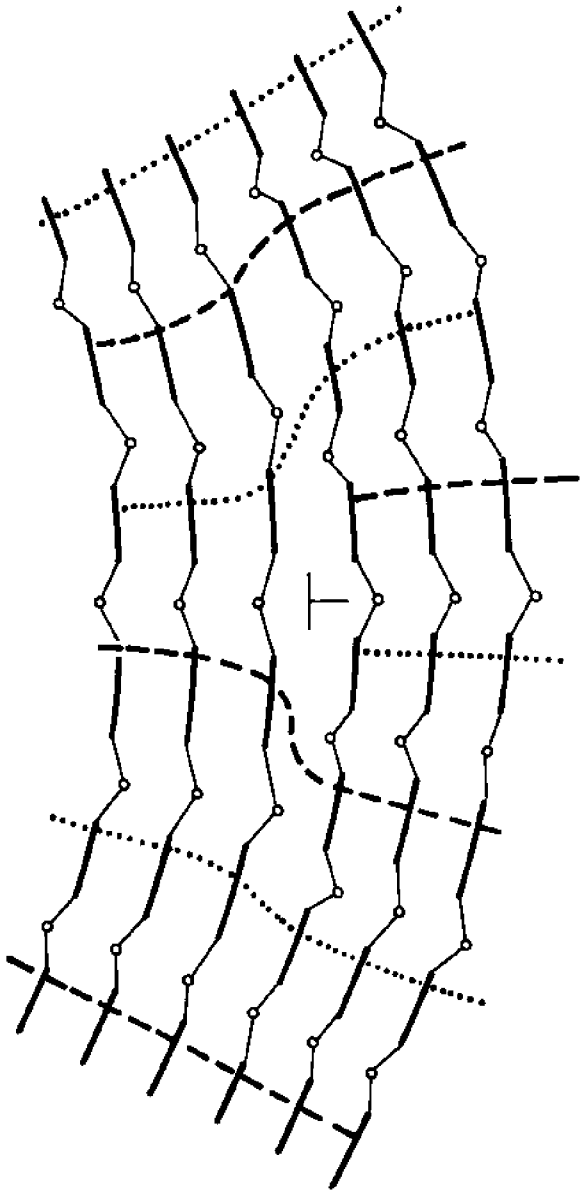

Figure 8. Schematic illustration of an intra-chain hydrogen bond dislocation in a chitin sheet. The dislocation is indicated by the usual symbol ⊥.

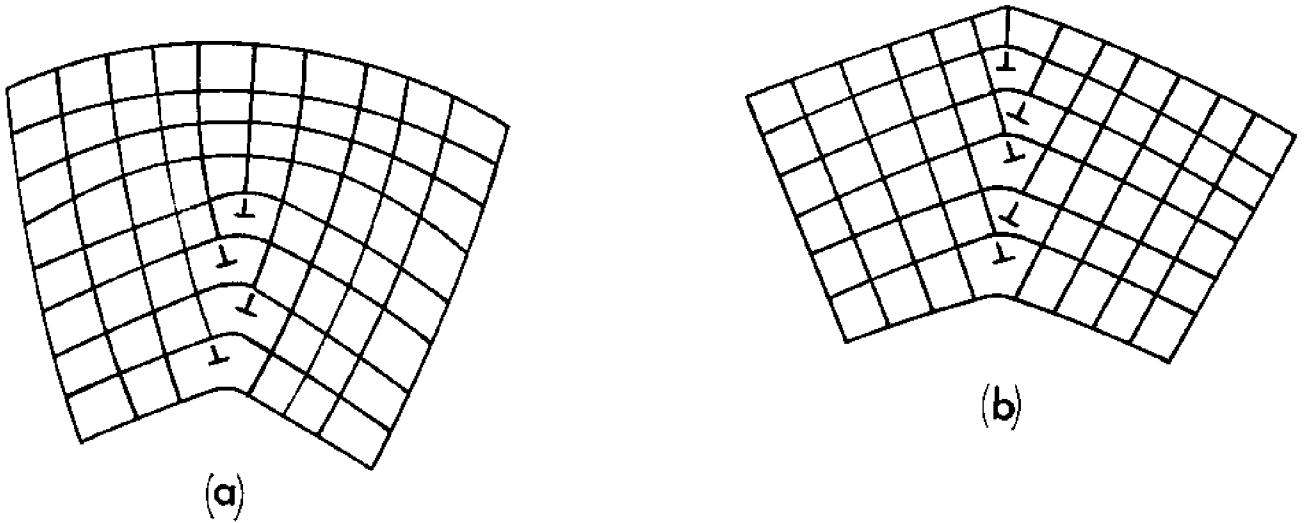

Figure 9. a) Small angle wedge disclinat on and b) low angle grain boundary formed by dislocation arrays.

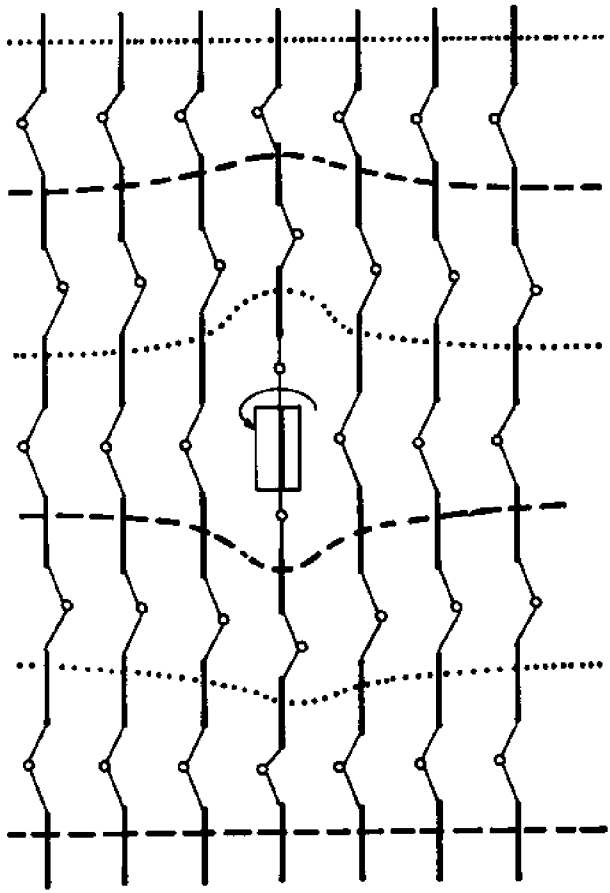

Figure 10. Schematic illustration of a dispiration formed in a chitin sheet.

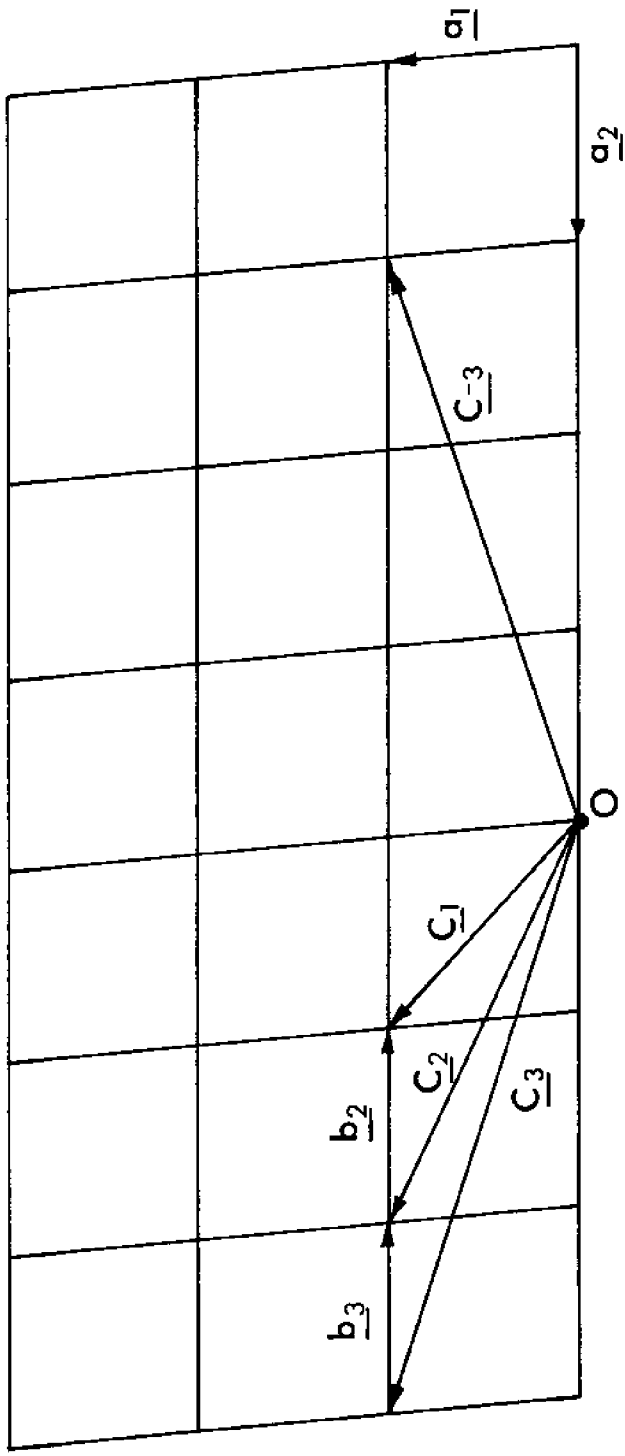

Figure 11. Two dimensional lattice representation of a polysaccharide chain.

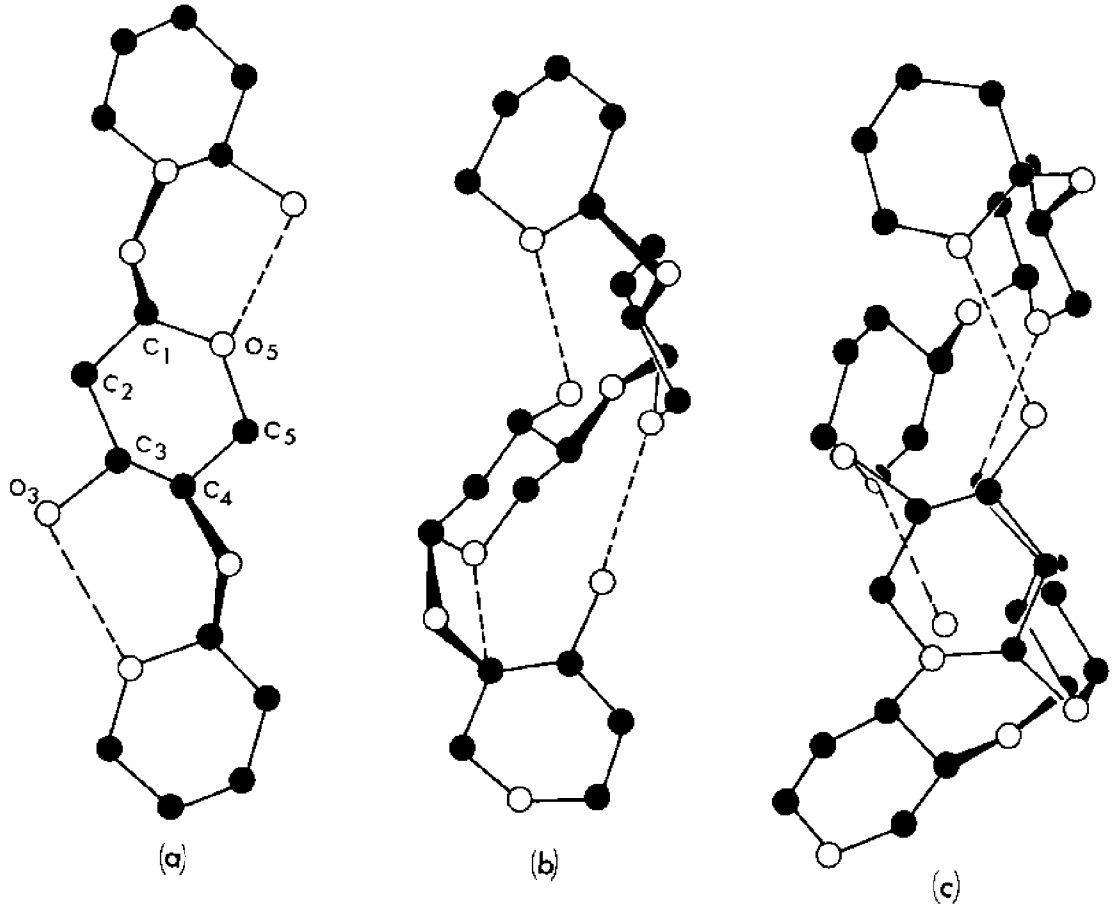

Figure 12. Polysaccharide helices a). (1)-helix, b). (-2)-helix and c). (-3)-helix.

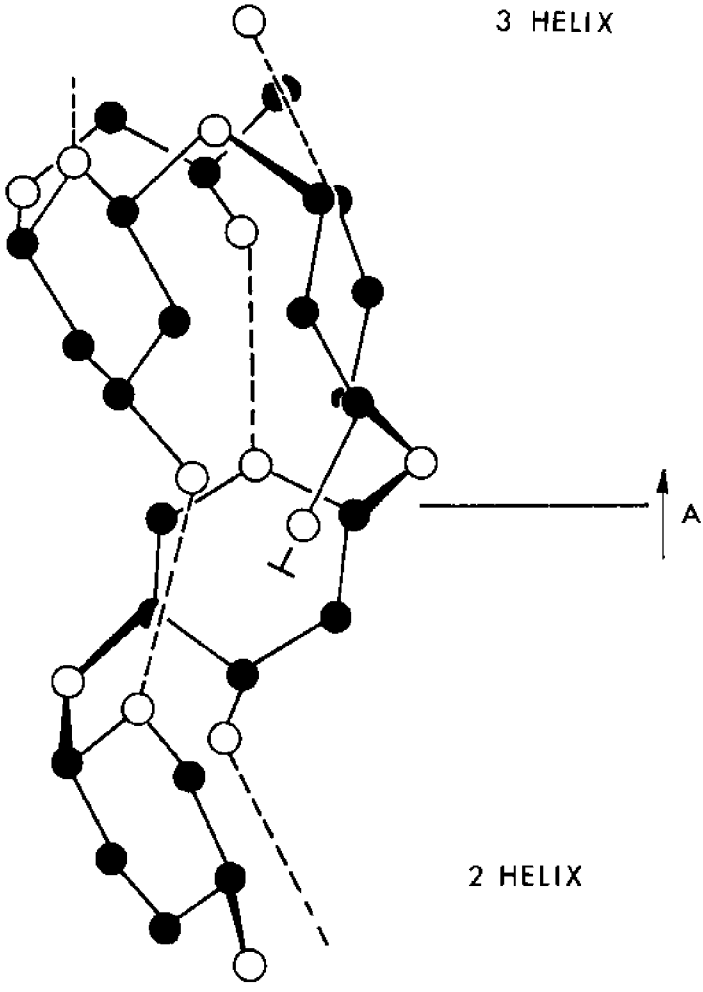

Figure 13. Edge dislocation at the junction between a (-3)-helix and (-2) helix.

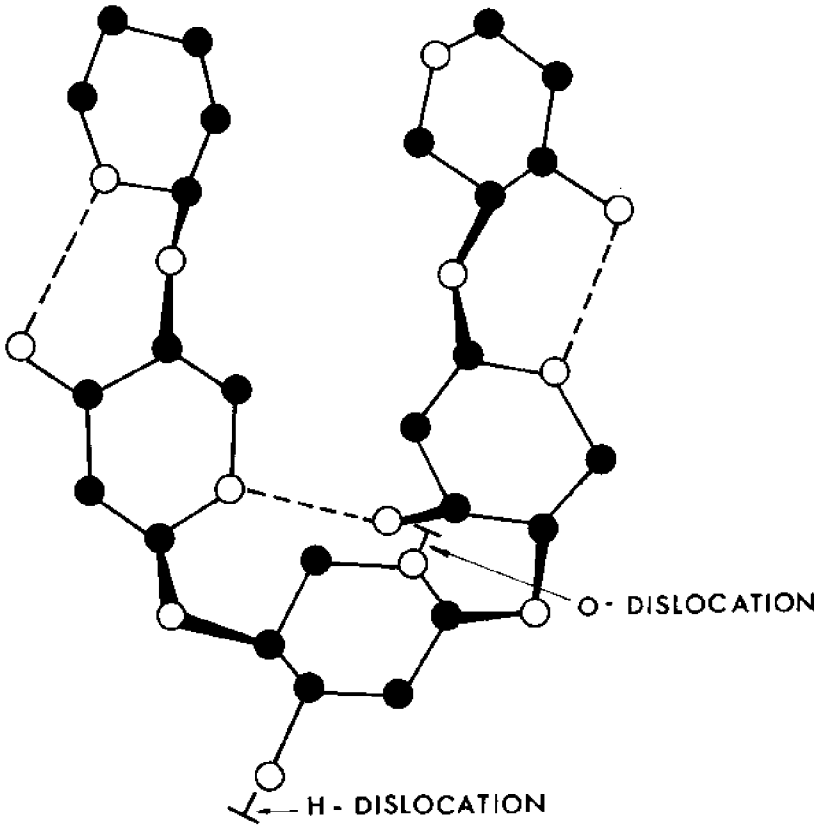

Figure 14. Bend in a polysaccharide chain formed by residues in the (-2)-helix conformation.

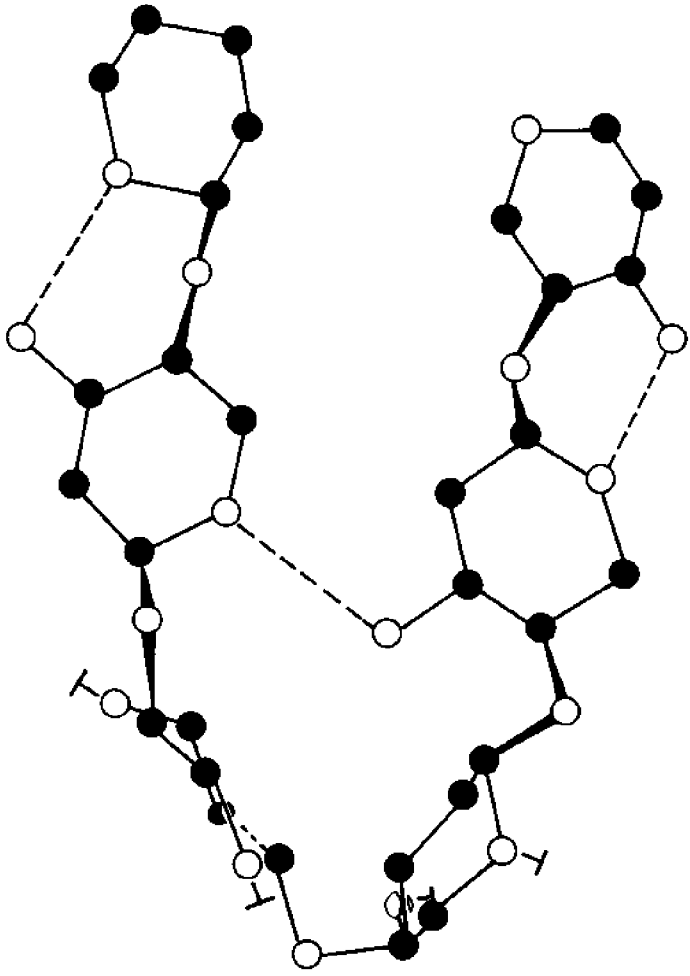

Figure 15. Bend in a polysaccharide chain formed by residues in the (-3)-helix conformation.

BULK AND SOLUTION PROPERTIES OF CHITOSAN*

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ABSTRACT

Chitosan is a collective term applied to deacetylated chitins in various stages of deacetylation and depolymerization. If chitin is considered a polymer of N-acetylglucosamine residues exclusively, its degree of deacetylation can be estimated from simple analytical data. Although a specific degree of deacetylation above which the polymer is completely soluble is difficult to define, chitosan samples 75% or more deacetylated dissolve readily in dilute organic acids to give solutions containing few, if any, swollen gel particles. Such solutions are clear, homogeneous and viscous, the viscosity being a function of the molecular weight of the polymer, its concentration and the particular acid--and its concentration--used as a solvent.

Much of the commercial potential of chitosan stems from its solubility behavior and from the properties of aqueous solutions of the polymer. In selected applications, it may compete with established water-soluble polymers, such as cellulose derivatives, natural gums and microbial polysaccharides. As a basis for selecting specific applications in which to investigate the possible commercial uses for chitosan, a number of its properties in bulk and in solution have been investigated. Specific properties covered in this paper include the following.

1. Equilibrium Moisture Content as a Function of Relative Humidity
2. Solubility
3. Organic Solvent Compatibility
4. Salt Tolerance
5. Viscosity as a Function of Concentration
6. Viscosity as a Function of Temperature

INTRODUCTION

Although chitosan and some of its intriguing properties have been known for many years (1,2,5), the polymer has remained until recently little more than a laboratory curiosity. The reasons for the chemical industry's inability or unwillingness to exploit the polymer have been primarily economic, especially as they are affected by the logistics of gathering chitin, the precursor of chitosan, in sufficient quantity at a cost that is not prohibitive. Chitin is widely distributed in nature in crustaceans, in insects and in certain fungi, but it is only those organisms which offer an incentive for commercial harvesting that can provide the volume of chitin needed to make chitosan a product of industrial importance. Thus only the shellfishing industry, through its catch of such species as shrimp, blue crab and king crab, affords the potential for economical gathering of commercial quantities of chitin in the form of the largely unwanted shells of these

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*Hercules Research Center Contribution No. 1697.

marine delicacies. Even here, however, we have had to wait for the evolution of at least a partial centralization of the industry in the form of processing plants, so that large quantities of shell are available at relatively few sites. More recently, ecological concern about the disposition of huge amounts of shell and the growing desire to use most effectively all the resources available from the sea have added incentive to use chitin or its derivatives to the best advantage.

Chitosan might be expected to perform functions in aqueous systems identical to those performed by such already established polymers as carboxymethyl cellulose (CMC), hydroxyethyl or hydroxypropyl cellulose, and methyl cellulose. Because it is insoluble at pH above about 5.5, chitosan functions only in acid systems to show possible utility as a thickener, stabilizer, suspending agent or film former.

Although there may be some question about whether all the amino groups are acetylated, chitin is generally accepted to be a linear polymer comprised of 2-acetylamino-D glucose units. Chitosan, however, is less easily defined in terms of its exact chemical composition. The term "chitosan" may be considered as referring to a family of polymers derived from chitin that has been deacetylated to provide sufficient free amino groups to render the polymer soluble in certain aqueous acid systems. The exact degree of deacetylation required to render a polymer soluble is not readily determined, and it undoubtedly varies with such factors as polymer molecular weight, temperature, and concentration and nature of the acid species. For the purposes of this paper, we are defining chitosan as chitin that has been 80-85% deacetylated, or which contains about 7.5-8.2% by weight of free amine functionality.

Measurement of primary amine has several advantages over measurement of residual acetyl: it is simply and accurately measured by titration; it is a direct measure of the functionality of the polymer; and, as shown in Figure 1, it is a much more sensitive measure of the degree of deacetylation than is analysis for nitrogen.

For this work, we have selected three molecular-weight ranges of chitosan, defined in terms of solution viscosity. These viscosity types were selected as representative of ranges we consider most generally useful in various applications and which could readily be produced on a commercial scale from shrimp shell. The viscosity ranges are:

High--1000 cps. minimum, 1% polymer in 1% acetic acid.
Medium--100-250 cps., 1% polymer in 1% acetic acid.
Low--25-70 cps., 2% polymer in 2% acetic acid.

Intermediate viscosity types are possible, if there is a demonstrated need.

Detailed analytical data, typical of the samples used in this work, are given in Table 1.

Table 1. Composition of Chitosan Samples

Viscosity Grade	High	Medium	Low
Moisture, %	9.8	2.3	8.1
Analytical Data (Dry Basis)			
% N	8.17	7.79	8.41
% Ash (as $CaCO_3$)	1.9	0.81	1.2
% NH_2	7.34	7.76	8.13
% Acetyl	4.66	-	3.89
% NH_2 (Ash free)	7.48	7.82	8.23
Est. % Deacetylated	79.5	82.5	86
Solution Properties			
Viscosity, cps.	2780[a]	180[a]	50[b]

[a] 1% polymer in 1% acetic acid.
[b] 2% polymer in 2% acetic acid.

EXPERIMENTAL

Solutions of polymer were prepared by slurrying a weighed amount in a volume of distilled water equal to half that of the desired volume of polymer solution. The same volume of acid solution of twice the concentration finally desired was then added, and the mixture was stirred for about 30 minutes.

Viscosities of polymer solutions were determined with a Brookfield LVT viscometer.

Amine content was measured on solutions of the polymer in 0.03 M HCl. The solutions were titrated with 0.5 M NaOH, and amine content was calculated from the volume of standard base consumed between inflection points at pH 4.4 and 8.5 (3).

Acetyl content was measured by the terminal methyl method (4).

RESULTS

Solubility

Chitosan is insoluble in water, alkalis and organic solvents but soluble in many dilute aqueous organic acids at concentrations in the range of 0.25 to 10% (at pH levels below 6). These acids include formic, acetic,

propionic, oxalic, malonic, succinic, adipic, lactic, pyruvic, malic, tartaric and citric. Chitosan is also soluble in dilute (1% concentration or less) nitric and hydrochloric acids, marginally soluble in 0.5% H_3PO_4, and insoluble in H_2SO_4 at any concentration at room temperature. Water saturated with CO_2 is not sufficiently acidic to dissolve, or even swell, this polymer. Formic acid is the best solvent overall; good solutions are obtained in aqueous systems containing 0.2 to 100% of this acid.

The limited acid-concentration range in which chitosan is soluble in cheap, widely used mineral acids such as hydrochloric and sulfuric will restrict the utility of the polymer in certain important industrial applications. For instance, the petroleum industry uses large quantities of polymer in acid fracturing, where 15% HCl is the dissolving medium. The insolubility of chitosan at HCl concentrations greater than about 1% would appear to eliminate this polymer as a potential candidate in this important market.

Acetic acid has been selected as the standard solvent for solution property measurements. Except for very low viscosity chitosans, solutions are prepared at a concentration of 1% in 1% acid. To facilitate dissolution, the polymer is first dispersed in an amount of water equal to one-half the final solution volume, and then an equal volume of 2% acetic acid is added with good agitation. Normally such a solution has a pH of 4.0 ± 0.3. Since the viscosity of acetic-acid solutions of chitosan varies with acid concentration, viscosity comparisons are made at pH ~4. Thus, in the case of a low viscosity polymer, 2% solutions are prepared in 2% acetic acid to achieve this approximate pH level.

Solvent compatibility

As previously mentioned, chitosan is not soluble in any common organic solvents (including dimethyl formamide and dimethyl sulfoxide), but it does swell in acidified polyols. It dissolves readily in 3:1 glycerol:water when the mixture contains 1% acetic acid; the resulting solution is clear, colorless and very viscous. Ethylene glycol behaves similarly, except that the solution produced is less viscous. A blend of 29% water, 1% HOAc, and 70% sorbitol with 1% chitosan gels when agitated. Aqueous chitosan solutions, properly acidified, will tolerate appreciable quantities of polar organic solvents, as indicated by the data in Table 2. The chitosan used in these tests was high viscosity, and the polymer concentration was 1%.

Organic solvents appear to exert very little effect on chitosan-solution viscosity except in the case of polyols. The composition containing glycerol can be described as a jelly, while that containing sorbitol is a semirigid gel. Glycerol acidified with HOAc (99% glycerine plus 1% glacial acetic acid, by volume) functions as a near-solvent without any water.

Table 2. Chitosan-Solution Tolerance for Common Water-Miscible Solvents

Solvent	Maximum % Solvent for Complete Compatibility	% Acetic Acid (by Volume)	Solution Properties Viscosity, cp.	pH
Methanol	50	5	2480	4.1
Ethanol	50	5	2400	4.1
Isopropanol	40	3	3440	4.15
Acetone	40	3	2020	4.2
Ethylene Glycol	75	5	7600	4.0
Glycerol	80	3	60000	4.1
Sorbitol	70	1	146000	4.1
Water (for comparison)	-	1	2780	4.1

Salt tolerance

The solution properties of chitosan are normally determined in 1% acetic acid at a polymer concentration of 1%; under these conditions, as previously indicated, solution pH is approximately 4. For this reason, salt compatibility studies were also made at pH 4. Salt solutions were adjusted to this level of acidity with acetic acid at a final salt concentration of 10%. A 10-ml. portion of each of these solutions was then mixed with 1 ml. of a standard 1% chitosan solution.

Since chitosan behaves as a cationic polymer in acid solution, sodium salts were used--almost exclusively--in compatibility studies to explore the effect of various anions. A few nitrates of certain metals recognized as complexing agents were also included to examine the effect of these multivalent cations. Of all the salts considered, only sodium sulfate precipitated chitosan. The following salts were all compatible at the 10% concentration level:

Sodium
- Acetate
- Bromide
- Chloride
- Citrate
- Formate
- Nitrate
- Nitrite
- Phosphate, Dibasic

Aluminum, Calcium, Chromic, Cupric, Ferric · Nitrate

Viscosity as a function of concentration

The effect of polymer concentration on solution viscosity is shown on Figure 2. Solution pH was adjusted to ~4 with acetic acid. The slopes of the curves for the different viscosity types are similar to those of comparable types of cellulose ethers.

Viscosity as a function of temperature

The same chitosan solutions that were prepared for viscosity-concentration measurements were used in viscosity-temperature studies. Viscosity was determined at several concentration levels for each chitosan over the temperature range 25 to 60°C., as shown in Figure 3. All solutions appeared to undergo viscosity loss at about the same relative rate with increasing temperature, regardless of concentration. Again, the solution-viscosity behavior of chitosan resembles that of the cellulose ethers, rather than that of certain other polysaccharides such as xanthan gum.

Effect of pH and salt addition on solution viscosity

The viscosity of chitosan solutions in acetic acid varies with acid concentration. This phenomenon is undoubtedly due to a change in molecular configuration. In a low density ionic environment, as pH is reduced, chitosan molecules uncoil and assume a more elongated or rod-like shape. The equilibrium $-NH_2 + H^+ \leftrightarrows -NH_3^+$ is driven to the right, and the mutual repulsion of the charged groups supplies the uncoiling force. The magnitude of the viscosity change can be reduced either by using a more highly ionized acid as a solvent or by adding a salt to an acetic-acid solution.

The effect of salt addition is indicated by the difference between the two curves in Figure 4. The salt increases pH and minimizes viscosity variation, especially for acid concentrations above 2% (i.e., below a pH of approximately 4). The same effect may be achieved by using a dilute, highly ionized acid--such as HCl--as a solvent.

Equilibrium moisture content as a function of relative humidity

Duplicate samples of chitosan were vacuum-dried and exposed to atmospheres of 20, 51 and 79% RH at 25°C. (water vapor in equilibrium with saturated solutions of $KC_2H_3O_2$, $Ca(NO_3)_2.4\ H_2O$, and NH_4Cl, respectively).

After sample weight equilibrium was established, dry weights were determined following a 3-hour heating period at 105°C. Results are depicted graphically in Figure 5.

Obviously the equilibrium moisture content (from 7.6-10.2 to 21-22% for the humidity range considered) of chitosan is a function of relative humidity, but it is not affected appreciably by sample molecular weight (viscosity grade). L-grade material does absorb more moisture than the higher viscosity grades, but the difference is significant only at the lower humidity levels. The greater affinity of the low viscosity sample for moisture can be rationalized in terms of lower average molecular chain length and higher degree of deacetylation (higher concentration of NH_2 groups). The reason for the higher moisture absorbance of H-grade compared to M-grade chitosan at 20% RH is not clear.

The equilibrium moisture content of these chitosans is comparable with that of carboxymethyl cellulose of D.S. 4.

Viscosity stability of solutions at pH 3 and 4

The viscosity stability of chitosan and CMC solutions was compared at pH 3 and 4 under accelerated aging conditions. Duplicate 1 percent solutions were adjusted to the proper pH with acetic acid and then oven-aged for 5 days at 60°C. The average viscosity retention of these solutions under these conditions is listed in Table 3.

Table 3. Summary of Viscosity Data on Solutions Aged 5 Days at 60°C.

At pH 4 No.	Sample	Est. % Deacetylated	Viscosity, cp. Original	Final	% Retained
1	Shrimp chitosan	79.5	2780	170	6.1
2	CMC-7HOF	-	1600	66	4.1
3	King-crab chitosan	70.5	1460	460	31.5
At pH 3					
1	Polymer samples		2640	385	14.6
2			3600	40	1.1
3	Same as above		1460	570	39.0

The king-crab chitosan has the best viscosity stability of the samples considered in this study. The estimated degree of deacetylation (from its $-NH_2$ content) of this sample is 70.5%--nearly 10% lower than that of the other sample. This compositional difference may account for its better performance in this comparison, or its stability may be inherent in some subtle difference between shrimp and crab chitins.

In a separate experiment, a 1% solution of chitosan in 1% acetic acid (pH 4.0) was shelf-aged at room temperature (~25°C.) for 16 weeks. At the end of this period, its viscosity had dropped from 2900 to 1000 cp., a loss of 65%.

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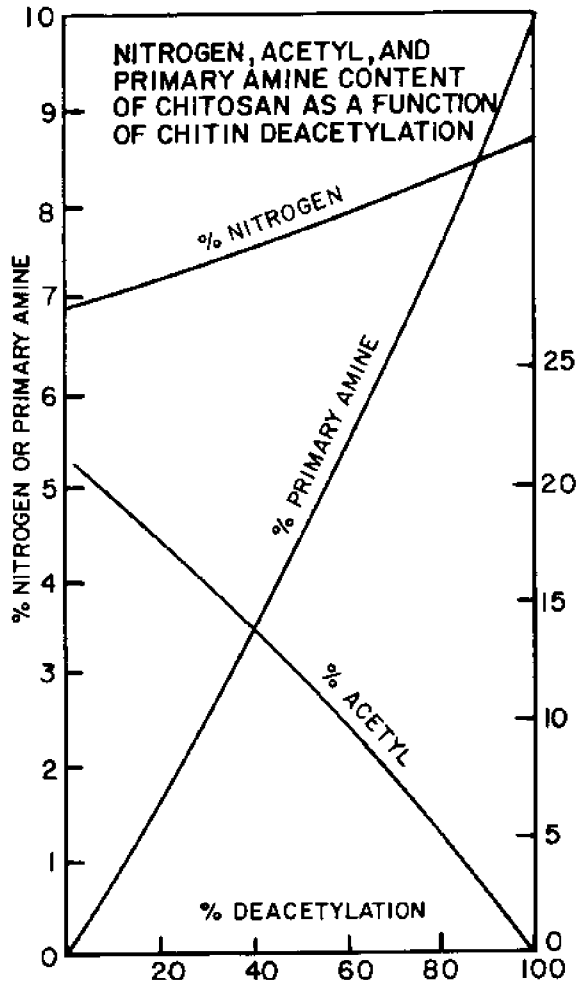
NITROGEN, ACETYL, AND
PRIMARY AMINE CONTENT
OF CHITOSAN AS A FUNCTION
OF CHITIN DEACETYLATION
% NITROGEN
% PRIMARY AMINE
% ACETYL
% DEACETYLATION
% NITROGEN OR PRIMARY AMINE
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
20
40
60
80
100

Figure 1.

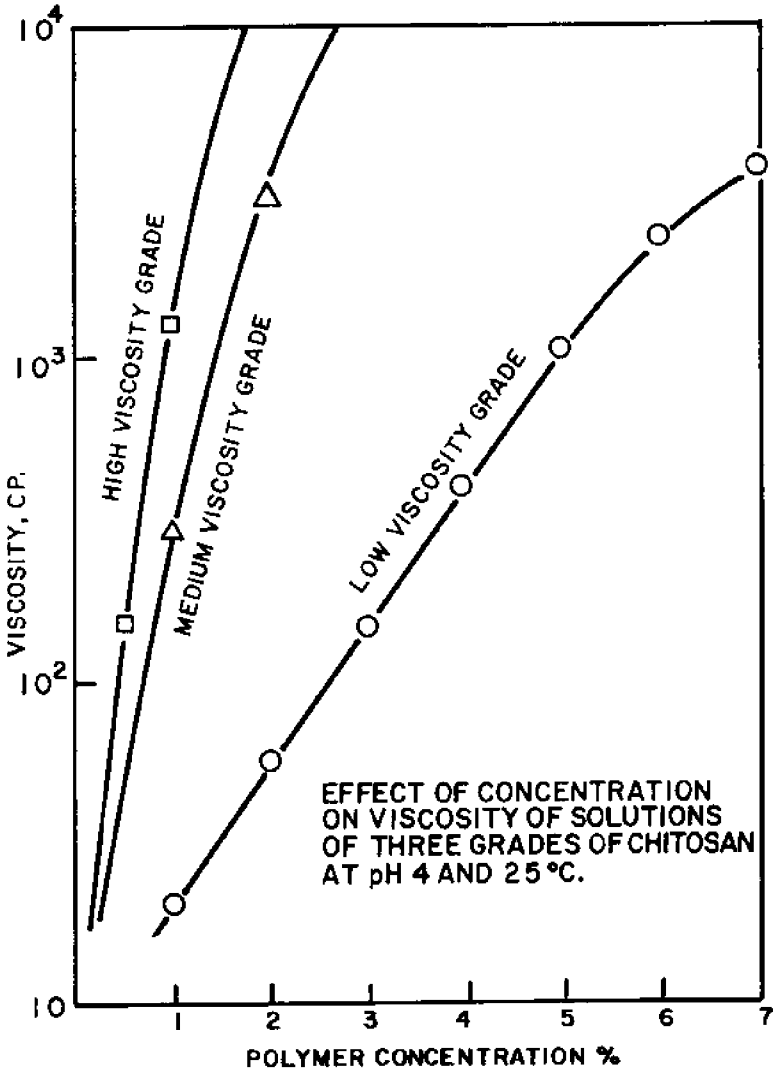
10^4
10^3
10^2
10
VISCOSITY, CP.
HIGH VISCOSITY GRADE
MEDIUM VISCOSITY GRADE
LOW VISCOSITY GRADE
EFFECT OF CONCENTRATION ON VISCOSITY OF SOLUTIONS OF THREE GRADES OF CHITOSAN AT pH 4 AND 25 °C.
1
2
3
4
5
6
7
POLYMER CONCENTRATION %

Figure 2.

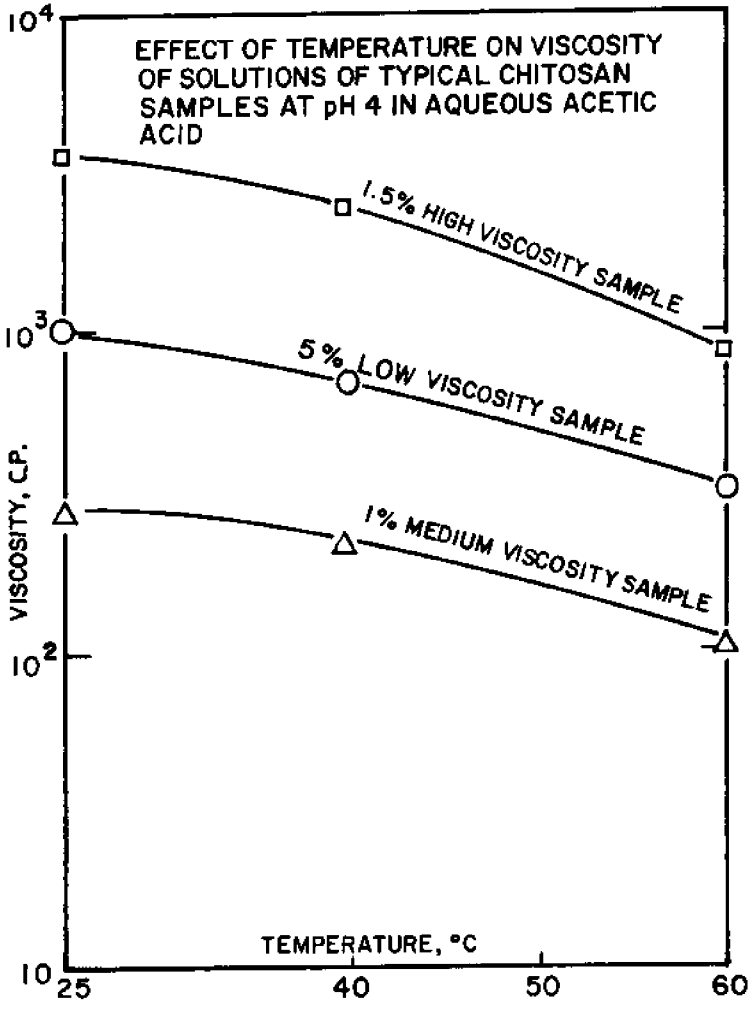
EFFECT OF TEMPERATURE ON VISCOSITY OF SOLUTIONS OF TYPICAL CHITOSAN SAMPLES AT pH 4 IN AQUEOUS ACETIC ACID
1.5% HIGH VISCOSITY SAMPLE
5% LOW VISCOSITY SAMPLE
1% MEDIUM VISCOSITY SAMPLE
VISCOSITY, C.P.
10^4
10^3
10^2
10
TEMPERATURE, °C
25
40
50
60

Figure 3.

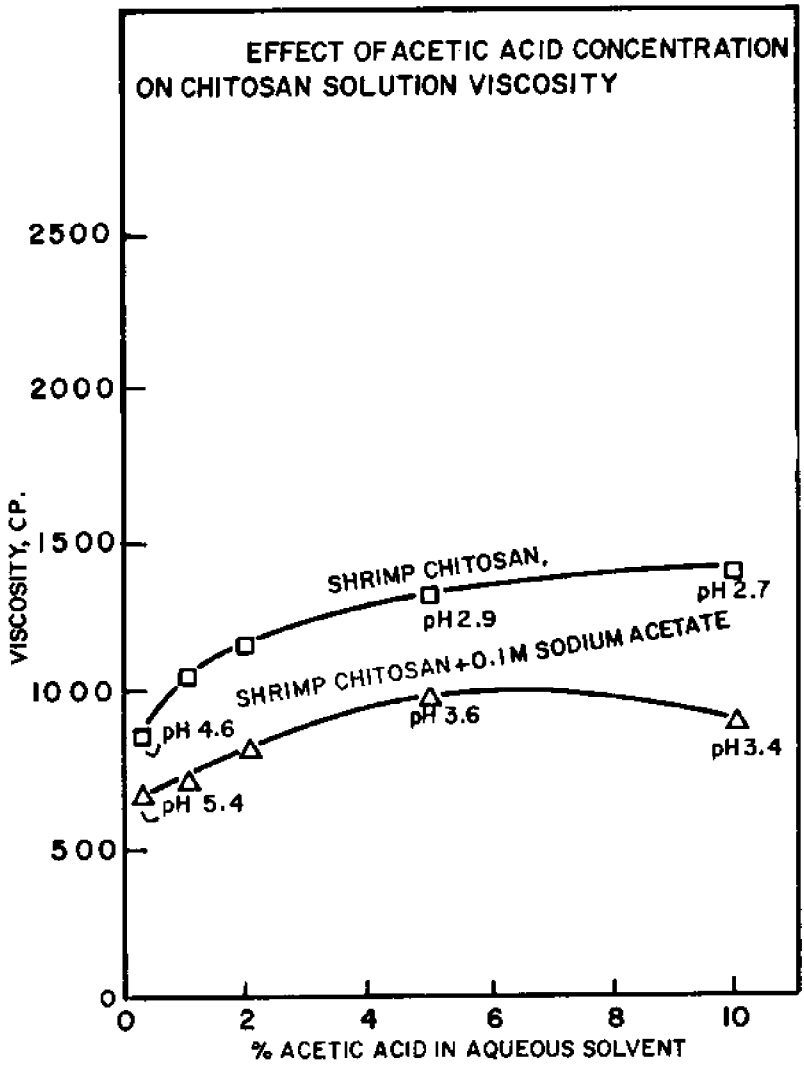
EFFECT OF ACETIC ACID CONCENTRATION
ON CHITOSAN SOLUTION VISCOSITY
VISCOSITY, CP.
2500
2000
1500
1000
500
0
SHRIMP CHITOSAN,
pH 2.7
pH 2.9
SHRIMP CHITOSAN + 0.1 M SODIUM ACETATE
pH 4.6
pH 3.6
pH 3.4
pH 5.4
0
2
4
6
8
10
% ACETIC ACID IN AQUEOUS SOLVENT

Figure 4.

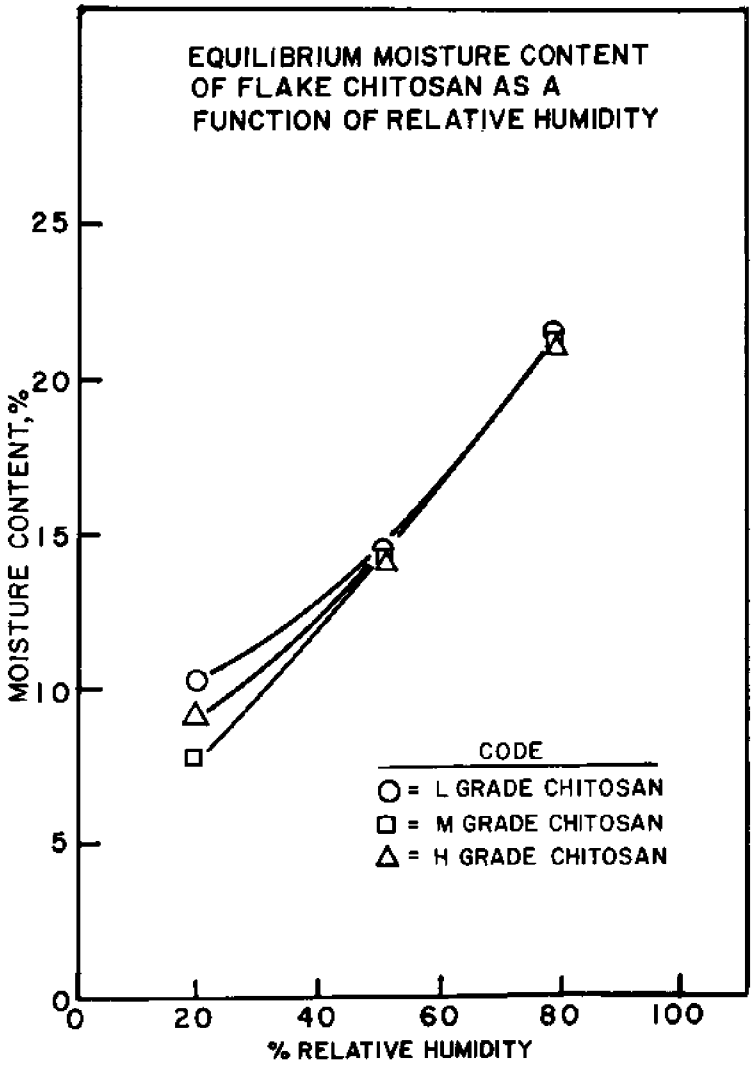
EQUILIBRIUM MOISTURE CONTENT
OF FLAKE CHITOSAN AS A
FUNCTION OF RELATIVE HUMIDITY
MOISTURE CONTENT, %
25
20
15
10
5
0
0
20
40
60
80
100
% RELATIVE HUMIDITY
CODE
○ = L GRADE CHITOSAN
□ = M GRADE CHITOSAN
△ = H GRADE CHITOSAN

Figure 5.

MARINE CHITIN PROPERTIES AND SOLVENTS

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ABSTRACT

Chitin, a cellulose-like polymer becoming available from crab and shrimp shell waste, is being investigated as a potential source of food-wrapping film and specialty filaments, to capitalize on its toughness and bioacceptability. In exploratory studies, a new group of inert tertiary amide solvent systems for chitin has been discovered, which has facilitated the solution, purification and characterization of this intractable material.

One of the novel chitin solvents, dimethylacetamide containing 5% lithium chloride, has been used in the comparison of six chitins from marine sources, including the non-calcified horseshoe crab, with respect to solubility, solution viscosity and optical activity. The latter is a very sensitive indicator of sample history. All of the chitins were of high molecular weight (over 400,000), but mild conditions of acid and alkali treatments and of drying are believed requisite for high quality chitin. Good solubility, the levo optical rotation of natural chitin, and minimal deacetylation appear important for the preparation of strong films and filaments.

INTRODUCTION

Possible applications of chitin include films and filaments (9), tobacco extender (3,30), nutrient for crayfish and cattle (17,25,26), and wound-healing accelerator (27). Chitin (poly-N-acetylglucosamine) can be deacetylated readily to form chitosan, an effective cation-active binder and coagulant (6,23). However, the investigation of chitin has long been plagued by its intractability. Common solvents are the strong mineral acids and concentrated salt solutions, which may degrade or denature the chitin or are difficult to handle.

In our initial studies on chitin utilization, an effort was made to find a better solvent that would lend itself to the ready purification of chitin, to the meaurement of its physical properties, and to the preparation of continuous films and filaments. Several acid systems modified with organic solvents, such as chloroethanol and sulfuric acid (1) and mixtures containing trichloracetic acid (TCA) (2,9) were found useful. With anhydrous systems involving TCA and chloral, the ability of chitin to be dissolved, reprecipitated and converted into its natural fibrous form (renatured) was established, and high-strength, cold-drawn filaments were prepared for the first time (9). Both natural and renatured chitin show a high degree of crystalline order. Certain fluorinated compounds, hexafluoro-2 propanol and hexa-fluoroacetone sesquihydrate have also been found to dissolve chitin (8,29). However, all of the above solvents leave much to be desired, and the search for an inert organic medium has been continued.

From the investigation of over 200 mixtures, two tertiary amide systems N,N-dimethylacetamide (DMAc)-LiCl and N-methyl-2-pyrrolidone (NMP)-LiCl, were found to be the best inert solvents for chitin. The solvent's power is derived from the addition of LiCl, as the two liquids alone are only swelling agents for chitin. Tertiary amide solvents with salts have been used for the dissolution of highly crystalline poly-amides (19). The LiCl apparently reduces or breaks the crystalline forces, such as hydrogen bonding, by association with the polymer and solvent (24).

The choice of a representative chitin for property determination in the inert solvent system also presents a problem, as chitin is not a chemical entity, but a product defined by its source and method of isolation. It is readily susceptible to degradation by hydrolysis of its glycoside linkages or acetyl groups, or by denaturing from over-heating or other harsh treatment. There is no well-established, standard chitin. Accordingly, the horseshoe crab, Limulus polyphemus, was selected for chitin preparation and characterization tests because it lacks calcium carbonate, and acid treatment can be avoided. In addition, five crustacean chitins isolated by alternate acidic and alkaline treatments were chosen for comparison because of their species differences and variation in ability to form renatured films or fibers (9). A range of chemical and physical properties was selected on which to base critical comparisons, including solubility, molecular weight, optical activity, acetyl value and the tensile strength of films and fibers prepared from the several chitins.

MATERIALS AND METHODS

Chitin sources and isolation

Limulus polyphemus (Horseshoe Crab). Dead Limulus were collected at Lewes, Delaware; carapaces were separated, ground to chips and washed. The shell material (20 g) was placed in 500 ml of 5% NaOH at 25°C for five hours to hydrolyze and dissolve the associated protein. The chitin was filtered, rinsed, resuspended in 200 ml of 5% NaOH and stirred for twelve hours. It was again filtered, rinsed until neutral and air dried. The yield of chitin was 5.5 g or 27.5%.

Callinectes sapidus (Blue Crab). Live crabs were caught at Lewes, Delaware, and killed by immersion in 2-propanol. The carapaces were immersed in 5% NaOH for eighteen hours to remove the tissue remains, rinsed until neutral and then ground. To 500 ml of 5% acetic acid (pH 2.5), 7 g of the ground material were added, the mixture was stirred for five hours, filtered, and the product rinsed. The chitin was placed in 500 ml of fresh 5% acetic acid for an additional five hours at room temperature, then filtered and washed until neutral. The chitin still contained a pink color so the chitin was resuspended in 2N HCl for two hours at room temperature, filtered, washed until neutral and air dried. The yield was 1 g or 14.3%.

Chionecetes opilio (Japanese Red Crab). This chitin was obtained from Eastman Kodak Company (Lot #72-S). Cancer magister (Dungeness crab) and Pendalis borealis (Alaskan Pink Shrimp) chitins were obtained through the courtesy of the Food, Chemical and Research Laboratory (FCRL), Seattle, Washington. The isolation procedure involved a caustic treatment to remove the protein, followed by aqueous HCl and hot-air drying (23). Penaeus aztecus (Brown Shrimp) chitin was kindly provided by Hercules, Inc. The

material was prepared by successive treatments of shell with 1.5% HCl at room temperature and 2% NaOH at 60°C, with drying under ambient conditions.

Analytical methods

Nitrogen was determined by Micro-Analysis, Inc., Wilmington, Delaware, using the Kjeldahl method. Analyses for ash were made by heating samples at 700°C for six hours. For moisture determination, a sample of each chitin (about 0.3 g) was conditioned in a desiccator over silica gel overnight. The residual moisture was then determined by the weight loss of the sample after drying in a vacuum oven (100°C, 23" Hg vac) for seven hours. A fine stream of air, dried by silica gel, was allowed to flow through the oven to remove the moisture. For solubilities, a 0.25 g sample of chitin was stirred in 50 ml DMAc-5% LiCl for 1.5 hour at room temperature, filtered through wool felt, and the dissolved chitin precipitated with acetone, washed four times with water and dried.

Acetyl value was based on hydrolysis of the acetyl groups by strong alkali, and their conversion to acetic acid. The acetic acid was distilled off as an azeotrope with water and titrated. The sample of chitin (0.1 g) and 40 ml of 50% NaOH was refluxed for 1.5 hours; 25 ml of H_3PO_4 (conc. 85%) was added carefully to the flask. The mixture was then fractionally distilled using a Vigreux column. As the distilling flask began to go dry, 15 ml of hot distilled water was added to the flask (care!). This step was repeated until 250 ml of distillate was collected. Aliquots (25 ml) of the distillate were titrated with 0.01 N NaOH using phenolpthalein as an indicator, and the value extrapolated to the total volume of the distillate. Finely divided filter paper (cellulose) was used as a blank. The percent acetyl of chitin was determined by the following equation (16):

$$\frac{\text{ml 0.01N NaOH, corrected} \times 0.4305 \times 10^{-3} \times 100}{\text{weight of sample}} = \% \ CH_3CO$$

For optical activity, the observed rotation, θ, of each chitin was measured in the N,N-dimethylacetamide (DMAc)-5% LiCl solution using a Polyscience Polarimeter Model SR 6. The solutions were filtered through felt and centrifuged before the rotations were observed. The rotations (θ) were measured to the nearest 0.1° and converted to specific rotations by the equation:

$$[\alpha]_D^{25^\circ} = \theta/lc \text{ where } c = \text{conc. in g/ml and } l = \text{cell length in decimeters}$$

The concentration was determined by precipitation of the chitin from an aliquot of the solution with acetone, filtration and rinsing with acetone, and drying. Several water washes were used to remove the LiCl from the precipitate.

The intrinsic viscosities and molecular weights were determined using a Cannon-Fenske Viscometer (size 100) giving a solvent efflex time greater than 100 seconds. The temperature was maintained at 30.0°C ($\pm$0.02). Either N,N-dimethylacetamide (DMAc)-5% LiCl or N-methyl-pyrrolidone (NMP)-5% LiCl was used as a solvent. For the viscosity determinations, 0.23 g of chitin was stirred in 50 ml of solvent for 1.5 hours. The mixture

was filtered through wool felt and then filter paper (Whatman #1). Seven ml of this solution were placed in the viscometer and equilibrated to the temperature of the bath for 15 minutes. Three efflux times were determined, which agreed within 1.0% of their mean. The concentration of the solution was determined by the same method as for optical activity. The solution was removed from the tube and diluted for the next run. The procedure was repeated for three different concentrations.

The ratio of the efflux time for the solution, t, to that of the solvent, t_o, provides a means of converting time to relative viscosity ($\eta_{rel} = t/t_o$), specific viscosity, $\eta_{sp} = (t-t_o)/t_o$, and the inherent viscosity, $\eta_{inh} = (\ln \eta_{rel})/c$ (11). To determine the intrinsic viscosity, plots of $\eta_{sp/c}$ versus c (concentration) and η_{inh} versus c are made on the same graph. The common intercept at c = 0 of the best straight lines through the two sets of points is the intrinsic viscosity [η]. The best straight lines were determined by regression analysis.

The intrinsic viscosity is related to molecular weight by a modified Staudinger equation (5)

$$[\eta] = KM_{vol}^{a}$$

K, a = constants; $M_{vol} = M_w$ = average molecular weight. The constants in the equation for the molecular weight must be determined by an independent method, in conjunction with measuring the viscosity of those solutions. This has not been done for chitin, but Lee (16) obtained the constants for a chitosan (acetyl 9.1%) in 0.2M acetic acid/0.1 M sodium chloride/4 M urea. Assuming that these are close to the values of chitin, a normal assumption for related classes of polymers, the constants are: $K = 8.93 \times 10^{-4}$; a = 0.71.

Tensile-strength measurements were made on fibers prepared using the DMAc-5% LiCl system. To 60 ml of the solvent, 3.0 g of chitin were added and stirred for two hours. The solution was filtered through wool felt, and extruded from a syringe through a 15-gauge needle into an acetone bath. After 15 minutes, the fiber was placed into a fresh bath. One-half hour from the extrusion time, the fiber was gently pulled to stretch it and aid in the acetone washes which were required to harden the fiber. After the acetone washes the fiber was dried in air for 15 minutes. During this time the fiber was cold drawn by hand to orient the material along the fiber axis. After the drying and orienting step, the fiber was washed with de-ionized water several times to remote the LiCl.

To determine the tensile strength of the fibers, 3 cm sections were mounted with epoxy glue between emery paper tabs. The samples were conditioned in a 60% humidity chamber at room temperature for 48 hours. The samples were run on an Instron TT-CM Tensile Testing machine using a 2000 g load cell and the stress being applied at 0.1 cm/min.

RESULTS AND DISCUSSION

Solubility of chitin

Two tertiary amide systems, N,N-dimethylacetamide (DMAc-5% LiCl) and N-methyl-2-pyrrolidone (NMP)-5% LiCl, appeared to be the best inert

solvents for chitin and were used throughout the study. Chitins from different sources show variations in their solubilities and other properties in DMAc-5% LiCl (Table 1). Variations in molecular weight usually produce differences in solubility, with high molecular-weight fractions being less soluble (5). The solubility of chitin does not appear limited by molecular weight, as that from Limulus, with the highest molecular weight, is also quite soluble.

Chitosan (partially deacetylated Dungeness-crab chitin which is soluble in dilute acetic acid) from Food, Chemical and Research Laboratories (FCRL) was found to be insoluble in the DMAc-5% LiCl. A major difference, chemically, between chitin and chitosan is the degree of acetylation of the polymer. The acetyl group may be the site of interaction with the solvent (24) and may control the solubility. The more soluble chitins, from brown shrimp, Limulus and red crab, have higher acetyl values, while the two less soluble, except Dungeness crab, have lower acetyl values. The low solubility of Dungeness-crab chitin was due to the formation of swollen gel particles, which were removed by filtration. Such particles are formed when a polymer becomes crosslinked, in this case perhaps by high drying temperature.

Table 1. Solution Properties of Chitins in DMAc-5% LiCl

Chitin	% Sol. Material	$[\eta]$ ml/g*	Mol. Wt. (x 10^6)	Opt: Act. $[\alpha]$
Limulus	82	25.6	1.8	-56
Blue Crab	58	23.0	1.6	+33
Red Crab	76	22.3	1.3	+23
Dungeness Crab	30	12.5	0.6	NA
Pink Shrimp	62	9.2	0.4	+75
Brown Shrimp	92	13.2	0.8	-39

*Linear regression, ln η_{rel}/concentration

Both of the above systems were found to be superior to other solvents because of the stability of the dissolved chitin. After 48 days, a red-crab-chitin solution retained its high viscosity essentially unchanged.

Molecular weight

The molecular weights derived from the intrinsic viscosities of the six chitins in DMAc-5% LiCl gave a wide range of values. The Limulus chitin (MW 1.8 x 10^6), having been isolated by an alkaline treatment only, produced the highest molecular weight or the least depolymerization. The blue-crab chitin (MW 1.6 x 10^6) was isolated by a relatively mild acid treatment, and it, too, has a substantial molecular weight. The three chitins from the semi-commercial processes: pink shrimp (MW 0.4 x 10^6), Dungeness crab (MW 0.6 x 10^6) and brown shrimp (MW 0.8 x 10^6) are lower in molecular weight probably due to a harsher acidic isolation process. Similar intrinsic viscosities for the pink-shrimp and Dungeness-crab chitins were obtained using NMP-5% LiCl, verifying the low values. The molecular weights of the six chitin sources are all sufficiently high to produce renatured films and fibers.

The red-crab chitin had an intermediate molecular weight (1.3×10^6), but scattered results were obtained for every intrinsic viscosity determination with this chitin. The scattered results may be due to the shearing effect of the polymer on the walls of the viscometer, as the polymer flows through the capillary tube. For polymers of high molecular weight ($>1 \times 10^6$), this can be corrected for, but was not in this study.

The molecular weights obtained are comparable to those of the literature. Lee (16), using formic acid as a solvent in the viscosity measurements, determined the molecular weight of squid-pen chitin to be 2.5×10^6. Lee's value was obtained by measuring the reduction of the intrinsic viscosity over time, and extrapolating back to zero time. Hackman and Goldberg (14) found a molecular weight of 1.0×10^6 for crab (Scylla serrata) chitin, using light scattering with the chitin dispersed in an aqueous salt solution.

Optical activity

The optical activity for chitin has been reported as $[\alpha]_D^{25} = -14°$ in hydrochloric acid but, due to glycoside hydrolysis to glucosamine hydrochloride, slowly changed to +56° (15). With the use of the inert solvent (DMAc-5% LiCl) that does not hydrolyze the glycoside linkages, a wide range of optical activities was found (Table 1), varying from $[\alpha]_D^{25} = -56°$ for Limulus chitin to +75° for that from pink shrimp. This behavior suggests that, depending upon the chitin isolation method, there may be epimerization of glycoside linkages, loss of helical structure, possibly with deacetylation and reduced hydrogen bonding, or a combination of both (18,20,22,28).

This conformation change may be very significant to film and fiber formation and to biological activity, all of which may require the native levo form for optimum efficacy. Optical activity thus affords a very sensitive test of sample history and perhaps a requisite specification for these applications.

Of corollary interest, a sample of chitosan (Lot #1, FCRL) tested in 2% acetic-acid solution had an optical rotation value of approximately $[\alpha]_D^{25} = +30°$.

Acetyl value

The acetyl group provides the site for the interchain hydrogen bonding which imparts strength to chitin products. This is evidenced by the superior strength of chitin over chitosan (4). The acetyl values (Table 2) indicate that all the chitins have been deacetylated to varying degrees. Even the Limulus chitin, which was isolated under mild conditions, has lost approximately one acetyl group per five monomer units. Chitin from brown shrimp had an even higher acetyl value (20.7); the reason is unknown, but may be related to a species difference or to the details of the mild conditions employed for its isolation. The literature indicates that the acetyl group is susceptible to strong acids and alkali (22), but is unaffected by 5% alkali (16). From the degree of deacetylation which occurred in the Limulus sample, even 5% alkali may deacetylate the chitin.

The ratio of one deacetylated monomer for every five or six acetylated monomers is a common phenomenon with chitin (21). Hackman and Goldberg (13) believe that it is a natural occurrence, possibly the site of protein bonding. The true nature of this ratio is difficult to confirm due to the

deacetylation which occurs during the isolation, as was seen with Limulus. Chitin that was isolated from crayfish without acids or alkali has yielded an acetyl value of 21.9 (12), which refutes the existence of the natural one-to-six ratio (acetyl 18.3%), but it does support the supposition that even dilute alkali may cause deacetylation. Note also that brown-shrimp chitin showed only minimal deacetylation (acetyl 20.7) (Table 2).

The one-to-six ratio may be a function of the position of the acetyl group on every sixth monomer group. The chains of chitin, still associated with protein, are helical, and every sixth monomer unit produces a distinct x-ray periodicity (28). Upon treatment with dilute alkali (5%), this periodicity is lost, possibly due to the removal of the acetyl group on every sixth monomer unit, which probably projects out away from the chain, making it more susceptible to chemical attack.

The acetyl value helps to distinguish between chitin and chitosan, which is soluble in dilute acetic acid (1-3%). Lee (16) reported a chitosan with an acetyl value of 12.4, which is close to the acetyl value for the pink-shrimp chitin (13.8). The pink-shrimp chitin is not soluble in dilute acetic acid; yet, at some point between these two samples is the boundary between chitin and chitosan.

Nitrogen content

A value for nitrogen higher than the theoretical 6.9 is an indication of deacetylation, whereas a lower value is an indication of hydrolytic deamination or contamination in the product. Protein not removed during isolation of the chitin could also account for a nitrogen value higher than the theoretical.

Table 2. Analyses of the Various Chitins

Chitin	Percent			
	N[a]	Moisture[b]	Ash	Acetyl[a,c]
Limulus	7.2	2.8	1.9	17.2
Blue Crab	6.7	2.9	4.0	15.0
Red Crab	7.1	2.4	0.7	16.5
Dungeness Crab	8.3	3.6	4.0	15.8
Pink Shrimp	7.6	2.9	0.8	13.8
Brown Shrimp[d]	6.8	7.6	0.6	20.7
Theoretical	6.9	-	-	21.2

[a]Calc'd. on ash- and moisture-free product

[b]Ave. of 3 determinations

[c]Ave. of 2 determinations

[d]Values provided by supplier

The nitrogen values for the examined chitins complement the acetyl values (Table 2). Specific structural units can be assigned to the Limulus and pink-shrimp chitins based on the nitrogen and acetyl values. The Limulus fits a structure with one glucosamine to 5 N-acetyl glucosamine units, and the pink shrimp fits one with a 2:5 ratio.

Moisture and ash values

The ash content of chitin gives an indication of the effectiveness of the isolation method in removing carbonates (Table 2). The Dungeness crab, Cancer magister, chitin contained the highest amount of ash, which led to some problems in determining the optical activity of the samples by the appearance of a cloudy precipitate, removed by centrifuging. The Li ion appears to react with the remaining carbonates to form insoluble Li_2CO_3.

The residual moisture of the five chitin samples was similar. The values range from 2-4% and are low compared to the usual literature values of 5-10% (7,16,31). The difference was possibly due to the isolation methods affecting the crystallinity of the material. The water residing in the amorphous regions of the polymer is easily exchanged, as opposed to that in the crystalline region (13). The methods of the literature often use higher temperatures (>70°C) for isolation of the chitin. The heating of chitin may cause denaturation by intertwining the chains and destroying the crystallinity. The Dungeness crab chitin exhibited insoluble, swollen particles resulting in the low percentage of soluble material, and the higher residual moisture.

Tensile strength

The tensile strength of the chitins was tested to determine if the properties investigated had any relationship to the strength of the renatured products. High-quality crystalline polymers should have greater strength due to the more regular structure which allows for greater interchain bonding.

Chitin has a high degree of crystallinity which is required to produce films and fibers with the capability of being oriented or cold drawn (5). The cold drawing is an orientation process by an external force, and the orientation increases the strength of the material. The fibers from each chitin source could be cold drawn, and the degree of drawing was reflected in the strength of the renatured films and fibers (Table 3).

To produce fibers with high tensile strength, the chitin must be isolated with minimal hydrolysis of glycosidic or acetyl groups, as indicated by the Limulus values. However, difficulty arises if one tries to rank the other chitins as to their total degradation. Each type of degradation affects the polymer, and the combination of these effects causes the variability of the tensile strengths. It is recognized also that fiber properties are highly dependent upon the details of spinning, renaturing and drying conditions, and that development of optimum values will require much further study.

Table 3. Tensile Strength of Renatured Chitin Films and Fibers

Chitin	Cold Drawn (%)	Tensile Strength (kg/mm^2)
Limulus	NA	60
Blue Crab	83, 45	22, 17
Red Crab	114, 154, 116	35, 31
Dungeness Crab	52, 52	32
Pink Shrimp	50, 60, 55	24
Brown Shrimp	123, 123	24

NA: Not Available

Table 4. Property Correlation of the Chitins

Chitin	Sol. (%)	Acetyl (%)	$[\alpha]_D^{25}$	Strength (kg/mm^2)
Limulus	82	17.2	-56	60
Blue Crab	58	15.0	+33	21
Red Crab	76	16.6	+23	35
Dungeness Crab	30	15.8	NA	32
Pink Shrimp	62	13.8	+75	24
Brown Shrimp	92	20.7	-39	24

CONCLUSIONS

1. The two tertiary amide solvent systems, DMAc-5% LiCl and NMP-5% LiCl, provide media in which chitin is not hydrolyzed. Comparative solution measurements of solubility, intrinsic viscosity and optical activity are all indicative of functional properties important for chitin films and fibers.

2. All chitins renatured from this system formed films and fibers that could be cold drawn, indicating a good degree of crystalline order. The molecular weights of the chitins are so high that appreciable depolymerization can be tolerated for film and fiber preparation if other conditions are mild; denaturing is avoided and solubility maintained.

3. Chitin is a sensitive material and may be degraded in several ways: by hydrolytic depolymerization, deacetylation, and heat denaturing with loss of physical properties. The degradation may be minimized by isolation of the polymer under mild conditions of pH and temperature.

4. The treatment of chitin with strong acids causes a change in the optical activity of solutions from the natural levo rotation toward a dextrorotatory value. With the careful preparation of chitin under mild conditions, the native structure can be retained. This structural

conformation is believed important in such applications for chitin as wound healing and the preparation of films and filaments.

5. The horseshoe-crab carapace provides a source of superior chitin for renaturing. A commercial sample of chitin from brown shrimp isolated under mild conditions also appears of high quality as judged by comparative analytical values. However, the persistent variation in properties observed among isolates gives further evidence that there is a family of chitins; each chitin sample must be characterized by its source and critical physical properties, including solubility, acetyl value, solution viscosity and optical rotation.

ACKNOWLEDGMENT

This investigation was sponsored in part by NOAA, Office of Sea Grant, Department of Commerce, under Grant No. 04-3-158-30.

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CHARACTERIZATION OF CHITOSAN. I:THERMOREVERSIBLE CHITOSAN GELS

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ABSTRACT

Chitosan forms non-Newtonian solutions in aqueous oxalic acid which, when allowed to stand at room temperature, become thermoreversible gels. Similar gel formation has not been observed with chitosan in any other solvent except dichloroacetic acid, in which the gel formation occurs but is much weaker than in oxalic acid (Hayes and Davies, these Proceedings). Gel-sol transition points are reported and discussed in terms of a theory proposed by Eldridge and Ferry (7) as modified by Harrison et al. (9). If two chains are considered to be involved in each junction point, the heat absorbed in forming one mole of junction points is -29 kJ. It is proposed that chitosan molecules in solution normally exist in a random coil configuration, but that in the presence of oxalic acid they form double helices, thus creating crosslinks which eventually lead to gel formation.

INTRODUCTION

Hirano (11,12,13) has reported that chitosan forms thermally stable gels when it is dissolved in a carboxylic acid and treated with the corresponding anhydride. These gels presumably consist of the polyhydrates of the partially acetylated chitosan, and they are considered to be similar to certain cellulose gels. In this paper, we report the formation of a thermally reversible gel in aqueous oxalic acid and suggest a possible structure for it.

EXPERIMENTAL

The chitosan used in most of these studies is commercial chitosan (this is sample K2 in Hayes and Davies, these Proceedings) purchased from the Kypro Company of Seattle (which has incorporated the Food, Chemicals and Research Laboratory). A sample of chitosan was also prepared by refluxing 50 g of chitin purchased from the Kypro Company in 2.4 l of 40% (wt/wt) sodium hydroxide for six hours. Both of these samples had identical mass spectra.

Samples of chitosan were homogenized in 10% (wt/vol) aqueous oxalic acid with a Polytron homogenizer and allowed to sit until a gel formed. The gel was melted in a sealed flask, and the solutions were poured into 25 ml graduated cylinders which were set aside until the gel had reformed.

A modified form of the procedure proposed by Paul (16) was employed to determine the gel-sol transition points. Because the mercury drops he suggested proved too dense for our samples, steel ball bearings were

inserted under the surface of the gels. The graduated cylinders containing the gels were sealed and placed in an oil bath to equilibrate at a temperature approximately 10 degrees below the transition temperature. The temperature of the bath was then increased, and the position of the ball bearing was determined every 3 minutes as a function of temperature. The transition temperature of the sample was taken to be at the break in the position-temperature curve. Several ball bearings of different weights were employed, and the gel-sol transition temperature was obtained by extrapolating to zero weight.

RESULTS

Chitosan forms non-Newtonian solutions in 10% (wt/vol) aqueous oxalic acid (Hayes, Davies, Munroe, these Proceedings). When these solutions are allowed to stand at room temperature, thermoreversible gels are formed. A solution containing more than 7% chitosan will gel in less than a day, and a 3% solution will gel in about three weeks. As the gel forms, its color changes from gold to beige. The gels reform much faster after they have been melted, and the new gels are more grayish in color. In no case are the gels completely homogeneous, since some small flakes do not dissolve, but the reformed gels are much more homogeneous than the original ones. The addition of iodine to the solution produces a thermoreversible blue color at a rate comparable to the rate of gel formation. Similar results have been reported for polyvinyl-alcohol–Congo-red gels (3).

It is difficult to obtain accurate gel-sol transition points because the gels are not homogeneous and because the rate at which the oil bath heated varied somewhat. Since only relative melting points are required, however, we have accepted those results for which the slope of the melting point versus weight of the ball-bearing line was 2.0 ± 0.4 deg. g^{-1}. These results for commercial chitosan are shown in Figure 1 together with the line obtained from a least-squares fit. The average deviation of a point from the line is 0.5°, and the maximum deviation is 1°. Similar results were obtained by using the average of a number of determinations of the transition temperature. The gel-sol transition temperatures for 10% solutions of commercial chitosan and for the chitosan which we prepared are 93.2°C and 104°C respectively.

Intrinsic viscosities are obtained by plotting (C/nsp) against $\sqrt{c}$ and extrapolating to zero concentration, i.e., by employing the equation

$$\frac{nsp}{C} = \frac{A}{1 + B\sqrt{c}} \qquad (1)$$

which is generally valid for polyelectrolyte solutions. The intrinsic viscosities for commercial chitosan and for the sample which we prepared are 10.8 and 13.2 ml/g, respectively, in 2% acetic acid.

DISCUSSION

Harrison and his co-workers (9) have extended the previous treatment of gels (7) to a situation in which each network point in the gel involves n chains. Under these conditions,

$$\left(\frac{\delta \ln c}{\delta (1/T_m)}\right)_M = \frac{\Delta H}{(n - 1) R} \qquad (2)$$

where c is the concentration of chitosan in the gel, T_m the gel-sol transition temperature, M the molecular weight of the polymer, and ΔH the enthalpy involved in the formation of one mole of junction points. For commercial chitosan, ΔH is equal to -29 kJ mol^{-1} if n=2. This value is comparable with the results obtained for polyvinylchloride gels of various molecular weights for which ΔH varied from -26 to -45 kJ mol^{-1} (9) and is very close in magnitude to the activation energy of 23 kJ mol^{-1} reported for the unfolding process of α-gelatin (6).

At constant concentration, the variation of the gel-sol transition temperature with molecular weight is given by the equation (9)

$$\left(\frac{\delta \ln M}{\delta (1/T_m)}\right)_c = \frac{\Delta H}{R} \qquad (3)$$

where the symbols have the same significance as in equation 2. On the basis of equation 3, the chitosan that we prepared should have a molecular weight about 30% greater than that of our sample of commercial chitosan. This difference in molecular weight could easily result from differences in the preparations, since it is known that, if the treatment with sodium hydroxide is extended after the degree of acetylation has reached a constant value, the viscosity of the chitosan solution continues to decrease with time (15). We have also observed that different samples of commercial chitosan have markedly different properties (Hayes and Davies, these Proceedings). Based on this result, the constant "α" in the equation

$$[\eta] = K. \ M^{\alpha} \qquad (4)$$

has a value of 0.8 for chitosan in 2% acetic acid. Since the value of α varies from zero to unity as the polymer chain changes from being coiled into a ball to being kinked in a random manner, and since one would expect a polyelectrolyte in a dilute solution to be rather extended, this value seems to be reasonable. As it is based on only one measurement, however, it must be considered as a preliminary result.

Many polysaccharides have very striking properties as gels *in vitro*, and studies of gel formation have been reported for the κ and ι-Carrageenans (1,4) and for hyaluronic acid (a linear polydisaccharide of the form -G-N-, in which G is glucuronic acid and N is N-acetylglucosamine [5]). In all these cases, the spectroscopic data and/or the x-ray diffraction data suggest a double helical model for the gel. Beltman and Lyklema (3) have also proposed a helical structure for polyvinyl-alcohol–Congo-red gels since, when iodine is added to the solutions, a thermoreversible blue color is produced at a rate comparable to the rate of gelation. This phenomenon is also observed with thermoreversible chitosan gels. Dea et al. (5) have postulated that the hyaluronic-acid molecules exist mainly in the random-coil form in solution with a slight degree of crosslinking by double helices and that gels form when the number of crosslinks is increased. This postulate is supported by the observation that there is no evidence of heat absorption due to the melting of gelatin gels (6).

The acetylamino groups play a very important role in the bonding of chitin, and the properties of chitosan are very dependent on the degree of deacetylation (2). The presence of these residual groups is also essential for gel formation, because in principle it is impossible for a true homopolymer to form a thermoreversible gel (17). We have no information on the

types of bonding present in the gel, nor do we know the role of the oxalic acid. While it has been postulated that the main function of gelling agents is to promote the formation of a superstructure (3), the oxalic acid may be more intimately involved in the helix, since gels are not formed in the presence of other carboxylic acids.

We propose that chitosan molecules in solution normally exist in a random-coil configuration, but that in the presence of oxalic acid they form double helices, thus creating crosslinks that lead to the formation of a thermally reversible gel.

SUGGESTIONS FOR FURTHER WORK

There is evidence that prolonged treatment with sodium hydroxide (15), the formation of derivations (8), and even solution in acids (8,14) cause chain scission in chitosan. Recent work by Hirano et al. (10) suggests that extensive chain scission occurs in the cycle chitin-chitosan-N-acetylchitosan, since, while they have identical IR spectra, the N-acetylchitosan is soluble in formic acid while the original chitin is not. It should therefore be possible to prepare samples of "chitosan" having various molecular weights and degrees of acetylation. We hope to improve our techniques and to study gel formation as a function of these parameters by determining gel-sol transition temperatures, intrinsic viscosities [^{13}C]-NMR spectra (4), optical rotations (4,10), mass spectra, degrees of deacetylation (Hayes and Davies, these Proceedings), and the rates of gel formation.

ACKNOWLEDGMENT

We are grateful to the Fisheries and Marine Services of Environment Canada for an operating grant and to Barbara Stanford, who assisted with the experimental work.

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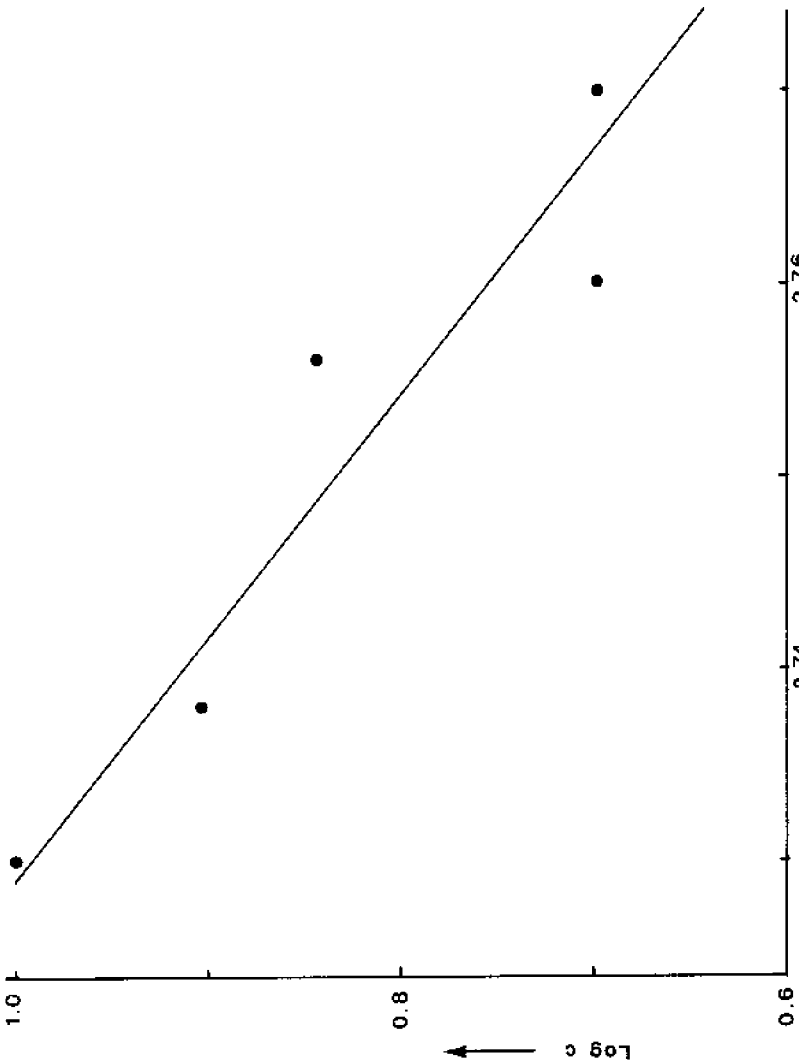

Figure 1: Gel-sol transition points for commercial chitosan in 10% aqueous oxalic acid.

FILM-FORMING CAPABILITY OF CHITOSAN

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ABSTRACT

The formation of films and fibers from chitosan is dependent on the structure of the bulk chitosan from which it is cast. The structure of the bulk chitosan is related, in turn, to the processing steps used in preparing chitin and chitosan from the shell, and it may be influenced to some extent by the species of crustacea used as the starting material. The deacetylation procedure appears to be a critical step, in that sufficient acetyl groups must be removed to allow the chitosan to dissolve in dilute organic acids, but the process must not be carried far enough to reduce the polymer chain length excessively. Viscosity measurements of a standard dilute solution of the polymer in acetic acid can be used as a rough indication of the degree of polymer degradation, if the raw materials and the processing steps are closely controlled. It has been found, however, that viscosity alone is not a good criterion for film formation, because the inclusion of small inhomogeneities from the starting material can greatly influence the viscosity. The film-forming qualities appear to correlate well with the structure as defined by x-ray diffraction, which is indicative of the molecular structure of the polymer.

Tough flexible films, with a tensile strength of 20,000 psi and an elongation of 6 percent, have been cast from dilute acetic or formic acid solutions. These films are virtually impervious to air and water.

INTRODUCTION

The film-forming qualities of chitosan were recognized early, and the basic technique for the casting of films and fibers was outlined by Rigby (4,5). Two patents were granted to Rigby simultaneously, and the second of these (5) describes the following procedure for making films and fibers. The chitosan is dissolved in a weak organic acid, typically acetic or formic acid at about 2% concentration by weight. This forms a suitably viscous solution, and the film is cast onto a smooth surface. At this point the chitosan is in the form of a complex salt, which has been formed with the dissolving acid, and the anion must be removed if the chitosan is to exhibit any resistance to water. Two procedures for doing this are given. In the first, the film is heated to about 90°C to remove the volatile acid component. In the second, the film is first dried at a lower temperature and then immersed in a weak caustic solution; the excess acid is neutralized and the soluble products are washed out by rinsing in water. The resultant films are described as flexible, tough, transparent and clear with a tensile strength of about 9000 psi.

Very similar procedures are described by Muzzarelli (2) for the casting of chitosan films. In the latter procedure, however, the chitosan was dissolved in 65% formic acid, cast, and neutralized with 1 N NaOH. These films were also clear and flexible, with a tensile strength of 10,000 psi (7 kg/mm^2), but without any appreciable elongation. There have been other reports of the fiber and film formation by Rutherford and Austin (see these Proceedings), but these were formed by the direct dissolution of

chitin in dimethylacetamide containing 5% lithium chloride. Fibers have also been prepared from a chitin viscose (3) which was prepared by immersing chitin in a 40% (w/w) aqueous solution of sodium hydroxide for two hours at 11-13°C. The resultant material was dried, pulverized and frozen at -20°C for 10 hours. Carbon disulfide was then poured in and the xanthogenate reaction proceeded for 15 hours at 30°C. The contents were then dissolved in 4-5% sodium hydroxide at 0°C, and then frozen at -20°C for 5 hours. The solution was then warmed to 0-5°C and spun into fiber. In some instances, urea was added to the viscose solution to improve the spinning. The resultant chitin fibers had a high modulus and exhibited a ramie-like feeling which was considered quite desirable. These fibers appear to be very promising and could lead to a variety of new applications which would take advantage of the unique properties of chitin.

There were some early indications, however, that the process used in making the chitosan could have a significant effect on the film-forming properties. Rigby (4) cautions that the deacetylation step should be continued until a product at least swollen by, but preferably soluble in, dilute acetic acid is obtained, but it should not be continued to the point where the material becomes degraded. Since several of the other processing steps can also cause degradation, it is evident that care must be exercised in the production of chitosan in order to achieve reproducible film-forming characteristics. It appears that only a partial deacetylation can be carried out if polymer degradation is to be avoided, and this step in the process must be subjected to very careful control. Enough acetyl groups must be removed so that the material will be dissolved in the acid used for casting, yet it must not be carried to the point where the polymer is degraded sufficiently to make film and fiber formation difficult.

Deciding whether chitosan has gone into solution can also be troublesome. If a high-speed blender or violent agitation is used, the apparent solubility will be enhanced over the same material under mild stirring conditions. There are indications that the polymer may be degraded by shear and the resultant solution has different properties than the original material. It has also been noted that a solution which is apparently homogeneous on solution may separate on standing into layers with substantially different viscosities. In most cases it appears that these materials were not homogeneous, probably because of uneven deacetylation.

It has become evident in the course of our work on film formation that chitosan can be made in a variety of ways and that only some of these products are suitable for film and fiber casting. We attempted to correlate the film formation with the structure of the starting material by means of x-ray diffraction patterns, by viscosity measurements and by molecular weight determinations. No single method was definitive, but a combination of data provided considerable insight into the requirements for film formation. The requirements outlined by Rigby are still valid, but the processing must be very carefully controlled in order to achieve solubility without degradation of the polymer.

Viscosity

Approximately 20 samples of chitosan were obtained from various sources. In some instances, these were samples of production lots or pilot-plant runs, and in others, small batches were made in the laboratory. Most of the material was produced from various species of crab, but a few were derived from shrimp shells. Some of the samples retained considerable red

coloration, some were almost white, and a few were brownish. The resultant films, however, were most always clear, but in a few cases there was a color tint to the film. It should be emphasized that many of the batches were made specifically for this program, and no inference should be drawn on the quality of a particular chitosan reported here since it may not be representative of the normal output.

Viscosity measurements were made with a Brookfield viscosimeter using the No. 1 and 2 spindles at various speeds. The results were generally consistent, but in some of the viscous solutions there was considerable scatter. Solutions containing 1 gm of chitosan, 2 g of either formic or acetic acid and distilled water to make 100 ml of solution were used for most measurements. This solution has been adopted as a standard for viscosity measurements by several investigators in the U.S. In Japan, however, a solution containing 0.5 g chitosan and 0.5 g acid is more common, and measurements were made of these solutions for comparison. Each solution was stirred lightly with a small mechanical mixer and left overnight.

The samples exhibited a very large range of viscosities from a low of 19 to a high of 13,200 in formic acid (Table 1) and from 13 to 8950 in acetic acid. In general, the samples were ranked in about the same order in formic acid and in acetic acid. The viscosity readings were consistently high in formic acid, and this is ascribed to the greater ease of solution of the polymer in the formic acid. It was also noted that several of the solutions were not homogeneous, and this appeared to be caused by the presence of very small lumps of material which were only partially dissolved. This was probably the result of incomplete deacetylation, although the inhomogeneous solutions were not always the most viscous.

The viscosity data did not correlate well with the film-forming characteristics. Apparently, high viscosity did not always indicate long polymer chain lengths, which should have enhanced film formation. In some cases, the x-ray diffraction patterns indicated that high viscosities were produced by material which had a component with a structure close to that of the original chitin. In other cases, high viscosities were obtained without these crystallite peaks, but the solutions were not homogeneous and contained translucent gel-like particles. In some instances the solutions were clear and homogeneous, and in these instances we assumed that high viscosities corresponded to long polymer chain lengths.

The chitosans represented in Table 1 were experimental lots prepared for a variety of additional tests. For example, M-1, -2 and -3 were used in a study of molecular weight distribution by Bough and coworkers (6). The FCRL lots were used in processing studies, and J-1 and J-2 were produced in special batches. These data illustrate, however, the difficulties of obtaining and characterizing a standard chitosan.

Film formation

Films were cast from these materials, but it was soon evident that the casting techniques were dependent on the nature of the chitosan. Samples J-1 and J-2 produced films of good quality by the following procedure. Four grams of chitosan were dissolved in 4 gms of formic or acetic acid in 100 ml of solution. The solutions were stored and allowed to stand overnight. The viscosities of these solutions were approximately 1500-2000 cps. Films were cast on polyethylene or glass and allowed to dry at 35-40°C. These films were then spread onto polyethylene or stretched over a frame, and dried at 65°C. These films were tough, clear and very flexible.

Table 1

Viscosity of Chitosan Solutions

1 g chitosan, 2 g acid, 100 ml water

Brookfield Viscosimeter

	formic acid		acetic acid	
Batch	Viscosity cps	Observations	Viscosity cps	Observations
J-1	1,050	homogeneous	280	homogeneous
J-2	4,260	homogeneous	1,500	homogeneous
R-1	30	homogeneous	140	homogeneous
FCRL-7	19	homogeneous	27	homogeneous
M-1	60	homogeneous	75	homogeneous
M-2	62	homogeneous	300	homogeneous
M-3	142	inhomogeneous	5,100	inhomogeneous
shrimp-1	50	homogeneous	58	homogeneous
shrimp L-2	13,200	inhomogeneous	8,950	inhomogeneous
blue crab 1	177	homogeneous	260	inhomogeneous
blue crab 9	225	inhomogeneous	182	inhomogeneous
king crab, T-1	2,167	inhomogeneous	1,260	inhomogeneous
squid	---	---	935	homogeneous

Sources of chitosan: J-1 and J-2 from Kyowa Oil and Fat Co.; R-1 from Rousselot; FCRL-7, blue crab 1 and 9, king crab T-1 from Food Chemical and Research Laboratory; shrimp-1 and L-2 from Marine Commodities; M-1, -3 and -4 from Dr. Wayne Bough, Sea Grant Program, University of Georgia.

The same casting procedure was not successful with R-1 and F-7. We were eventually able to dissolve these materials in 40 pct formic acid by heating gently at about 35-40°C. This material was then cast, dried, and treated with NaOH as in the procedure for the other samples. The resultant films were clear but not quite as flexible as the J-series. We concluded that these materials had been deacetylated somewhat less than the others and that the higher strength acid was required to dissolve the residual chitinous structure. The resultant polymer may have thus had a shorter chain length, and the film forming characteristics were thus different.

The samples M-1, -2 and -3 were studied in detail by Bough and coworkers. The samples were made from dry shrimp hulls. Deproteinization was carried out in 1% NaOH (w/w) solutions for 30 min. at 100°C. M-1 was not demineralized; deacetylation was carried out in a 50% NaOH (w/w) solution at 143°C for 15. min. in a ratio of 10 parts of 50% (w/w) solution to 1 part chitin on a dry-weight basis. The molecular weights and nitrogen contents are given in Table 2. This sample could not be cast into films, and it appeared that the high mineral content interfered with the film formation. Samples M-2 and -3 differed only in the time of deacetylation. The shrimp shells were deproteinized as above in 1% NaOH for 30 min. at 80°C. Demineralization was accomplished with 0.5 N HCl for 15 minutes at room temperature. The mineral content is mainly in the form of calcium carbonate, and a three-fold excess to the stoichiometric amount of calcium carbonate was used. The shells were washed to neutrality and dried at 103°C for 3-4 hours. Removal of the acetyl group was accomplished by boiling in 50% (w/w) NaOH at 143°C as described above. Sample M-2 was deacetylated for 15 minutes, and sample M-3 was deacetylated for 5 minutes. The nitrogen and ash contents were determined by Bough, and these are given in Table 2. The molecular weights were also determined by high pressure liquid chromatography (HPLC), and these data are also listed in Table 2.*

It is interesting to note that the nitrogen and ash contents for M-2 and -3 were not very different. Both the weight average, $\bar{M}_w$, and the number average, $\bar{M}_n$, molecular weights were lower for the longer deacetylation times. There was a large difference in viscosity, however, and the sample with the longer deacetylation time was considerably less viscous than the other.

As indicated earlier, sample M-1 could not be cast into films, probably because of the high mineral content. M-3 was also a poor film former. The material gelled but also formed lumps which eventually dried into a hard brittle mass. M-2 formed films which were transparent, flexible and free of lumps. However, these films were not as ductile as those formed from sample J-2. Films from M-2 exhibited an elongation of about 3 pct, whereas those from J-2 exhibited about 6-pct elongation.

The best films were obtained from the J-2 chitosan. This was made by Kyowa Fat and Oil Co., Ltd. from a single species, the scarlet queen crab, as a special batch. The usual material, represented by J-1, is a blend of chitosans produced from several species of crabs. Although reasonably good films were obtained from J-1, there was some tendency for the formation of lumps around some translucent gel-like particles.

The R-1 chitosan formed good films, but these tended to be somewhat weaker. The remaining samples were not good film formers and tended to form brittle masses.

* These data were obtained by W.A. Bough and A. Wu in the course of their work under Sea Grant Program No. 04-6-158-44115.

Table 2

Physical and Chemical Characteristics of Chitosans

Bough et al. (6)

Sample	Ash %	Nitrogen %	Viscosity cps	$\bar{M}_w$ (10^3)	$\bar{M}_n$ (10^3)
M-1*	14.3	6.24	60	1217	711
M-2	0.27	7.33	62	1663	518
M-3	0.09	7.48	142	2909	584
J-1	–	–	1052	1520	223
J-2	–	–	4260	1685	203
R-1	–	–	30	315	80

* This sample was not demineralized, and contained about 31 pct less chitosan than the other samples.

The conditions for good film formation cannot be deduced from viscosity data alone. In some instances, where incomplete deacetylation occurs because of the added thickness of some of the chitin flakes, the chitosan may be deacetylated on the exterior and only partially deacetylated at the interior. When such particles are dissolved in weak acid a suspension of particles will occur, with the swollen cores of these particles suspended in a colloidal system. This suspension will exhibit a very high viscosity, but this high viscosity will not be indicative of a high molecular weight or a long polymer chain length. The presence of these swollen and microcrystalline particles adversely affects the film-forming properties, and they must be avoided if good films and fibers are to be formed. At the other extreme, the deacetylation of chitosan can be carried to the point where all of the material goes into solution readily, forming a solution of very low viscosity. These materials are also poor film formers and form brittle masses or fragile films on drying. The low viscosities in these cases appear to be indicative that the molecular weight is also low. The best films were formed from clear solutions with a viscosity of about 1500 cps. These films were clear, tough and flexible (Table 3) and were very resistant to moisture.

The mechanical properties of the chitosan films are shown in Table 3. The films were .002-.004 in. thick and were tested in the form of strip 1 in. wide and 6 in. long. The relative humidity at the time of testing was approximately 50 pct. The strongest and most ductile films were formed from the J-2 chitosan. Films from low-viscosity chitosans tended to be brittle, and chitosans with intermediate viscosities fell in between.

X-ray diffraction

X-ray diffraction patterns were obtained on samples of the flake chitosan used in the film-casting experiments. Monochromatic CrKα radiation was used, and the samples were lightly pressed into briquettes 1 x 1/2 x 1/4 in. The patterns for J-1 and J-2 are shown in Figure 1. The patterns exhibit two amorphous peaks, but it is evident from the position of these peaks that the structures are different. These chitosans were made at the same plant from crab shells; J-2 was made from a single species, J-1 is a blend of several chitosans. The structural differences are unresolved at this point, but it is evident that there is a significant difference.

Figure 2 shows the series M-1, -2 and -3. Sample M-1 exhibits a crystalline peak, probably from the calcium carbonate which is present in this sample. M-2 and -3 are quite similar, except that the second peak for M-3 is shifted to lower angles. This is a characteristic change correlated with increased deacetylation.

Earlier work on the structure of amorphous chitosan (1) had indicated that the glucosamine rings were retained in these amorphous structures. The variations exhibited in these samples show that the chain configuration may be quite complex, and this remains to be resolved. These patterns indicate, however, that chitosan does not have a well-defined unique structure.

DISCUSSION OF RESULTS

Our results on these samples of chitosan indicate that chitosan can be a very variable material. The viscosities, the structures and the film forming characteristics can vary widely.

Viscosity measurements in themselves are not a good guide to polymer chain length, since high viscosities can be achieved by a minimal deacetylation of the chitin, which in turn produces suspension of partially

Table 3

Properties of Chitosan Films

Chitosan Sample*	Tensile Strength psi	Tensile Strength MN/m^2	Elongation %
J-2	22,000	152	6.5
J-1	12,200	84	2.6
M-2	12,500	86	3.0
M-3	8,000	55	1.0
R-1	7,600	52	1.2

*Film thicknesses were .002-.004 in.

deacetylated particles. On the other hand, it is possible to deacetylate sufficiently to remove the chitin structure without destroying the polymer. The material is easily dissolved in weak acid solutions and casts excellent films.

Careful control is required in the processing of the polymer, and each step - raw material selection, demineralization, deproteinization and deacetylation - must be standardized. There is also evidence to suggest that each species of shrimp or crab may require somewhat different processing to achieve a uniform product. Nevertheless, uniform products are being produced on a large scale, but care should be exercised in documenting sources and structures of these materials before extensive work on applications is undertaken.

ACKNOWLEDGMENTS

This publication is based on research carried out as part of the MIT Sea Grant Program with support from the Office of Sea Grant, a division of the National Oceanic and Atmospheric Administration in the U.S. Department of Commerce, under grant number 04-6/158-44081 and from Massachusetts Institute of Technology.

Much of this work was performed by the students who have been associated with this program, Carmen Egido, Harry Gearhart and Randall Clark. The author is also indebted to Dr. E. R. Pariser for many interesting discussions.

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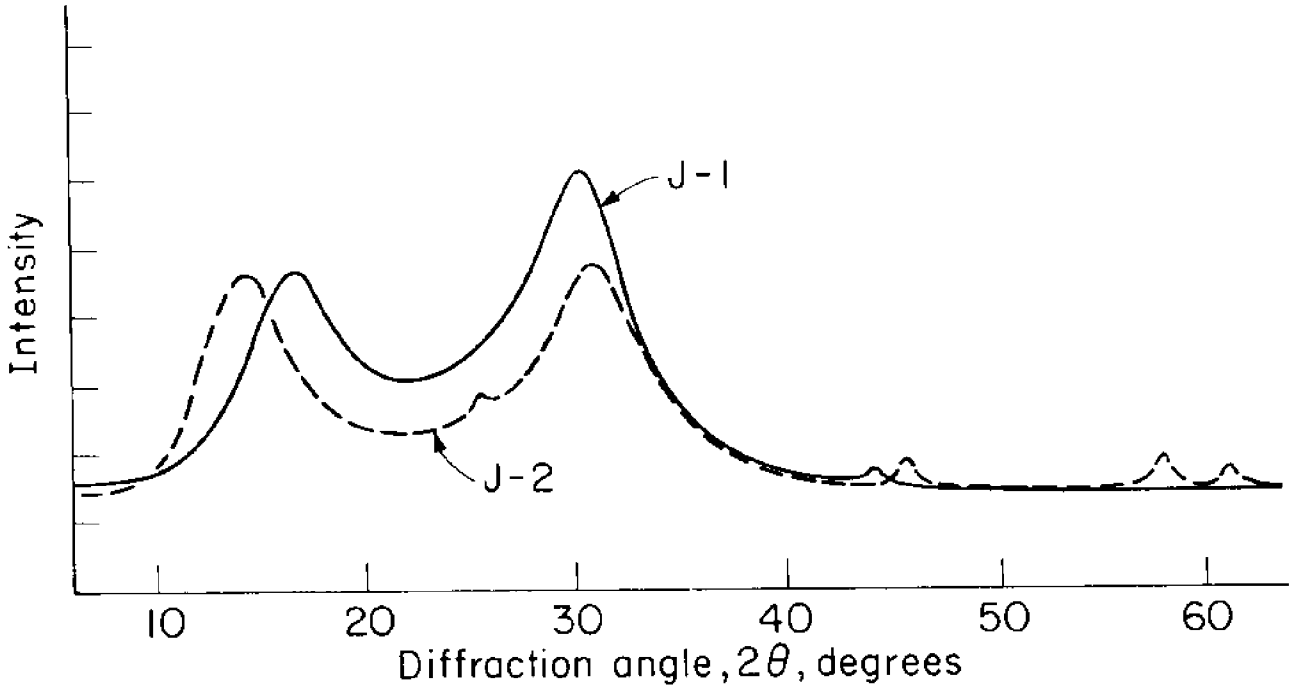

Figure 1. X-ray diffraction patterns from J-1 and J-2 chitosans. CrKα radiation.

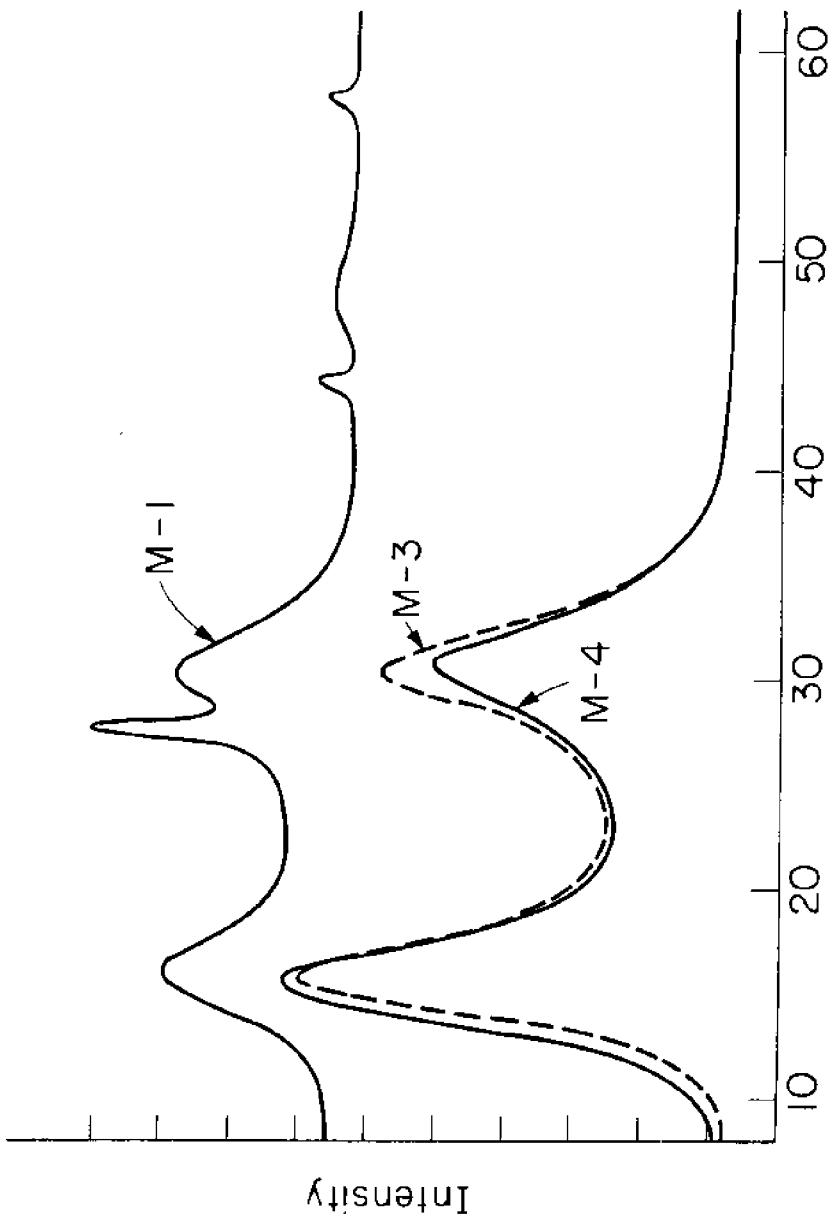

Figure 2. X-ray diffraction patterns from chitosans. Cr Kα radiation.

CHITOSAN OF HIGH VISCOSITY AND PROTEIN AS A VALUABLE BY-PRODUCT FROM SQUILLA

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ABSTRACT

Huge quantities of squilla (50,000 tons) and shrimp/prawn waste (25,000 tons) from freezing plants could be effectively utilized to produce chitosan and protein as very valuable by-products. Bench-scale trials processing 100-200 kgs of squilla at a time for chitosan extraction have been completed. They showed that chitosan of high viscosity can be obtained from deacetylation of chitin under optimum conditions. A 1.25% solution of that chitosan in dilute acetic acid had a viscosity of 4000-8000 centipoises or more. Further leaching of the product to obtain a white substance affected its viscosity, however, as did prolonged storage at room temperature. Consequently, for many industrial applications, chitosan solutions should be freshly prepared.

The experimental use of chitosan in the purification of water has yielded very encouraging results.

INTRODUCTION

Among the crustacea species, such as shrimp, crabs, prawns, lobsters and cray fish that are harvested commercially, squilla (order Stomatopoda) are of considerable importance because of their high chitin content. Large quantities of unusable squilla or "Puchee" are caught along with commercially usable prawns, and their disposal poses a problem. The total catch by trawlers fishing along the Mangalore coast alone is estimated to be around 2 tons daily. The spines around the shell, i.e., the exoskeleton, are composed primarily of chitin; there is very little muscle inside the body as compared to prawn render. Stomatopoda is useless as food. Most of it is thrown overboard when caught; only that part too closely mixed with fish and prawns is brought ashore to be sorted out and disposed of. This indiscriminate disposal at the landing sites poses problems of pollution as the discarded waste rots.

The three important commercial varieties of squilla are Orato squilla nepa, Orato squilla holoschista and Harpio squilla raphidia, the last one being the largest of the species caught along Maharashtra. It is estimated that the total annual catch of squilla off the coast of India is in the order of 50,000 tons. As methods of fishing by trawler improve, the possibilities of even larger catches increase, and this could provide an excellent source of material for the processing of chitosan.

EXPERIMENTAL

A number of earlier attempts have been made to process chitosan from the shells of lobster, crab and shrimp (2-4,6,9). At the Food Technological Institute we have utilized squilla of the varieties Orato squilla nepa and Orato squilla holoschista for obtaining chitosan with a very high

viscosity. The approximate composition of squilla is indicated in Table 1. The results show that the exoskeleton comprises the largest part of the squilla, followed by protein, minerals (largely calcium carbonate) and minor amounts of lipids, phosphates and carotenoid pigments. Among the various steps in processing, i.e., demineralization, deproteinization, deacetylation and bleaching, the most important is deacetylation. The concentration of caustic alkali solution, the ratio chitin:alkali solution and the temperature of deacetylation play the most significant roles in determining the quality of chitosan, based on viscosity determination.

Under optimum conditions, chitosan with an intrinsic viscosity of 50-60 poises can be obtained. A 1.25% solution of that chitosan in dilute acetic acid was measured by a Brookfield Synchrolectric viscometer, giving a value in the range of 4000-8000 centipoises or more. It was found that a good quality deacetylated chitin at a concentration of 1.25% in dilute acetic acid has a viscosity of ~1200 cps; a medium grade has a viscosity of ~160 cps; a low grade has a viscosity of ~15 cps (11). Optimum conditions during demineralization and particularly during deacetylation have to be strictly maintained to obtain chitosan with a high viscosity. Further bleaching to yield a white product considerably affects the viscosity.

Laboratory-scale trials have been successfully translated into bench-scale trials that can process 100-200 kgs of squilla per batch. The yields and the properties of the produced chitosan are reported in Table 2.

The results of purifying water with chitosan and with alum (Table 3) indicate that, even at 300 mg/l of alum, settled-water quality does not improve, while 0.6 mg/l of chitosan reduces turbidity to 18 units, and 0.15 mg/l of chitosan combined with 10 mg/l of alum reduces the settled-water turbidity to 15 units.

Chitosan dissolved in 1% acetic acid shows that viscosity decreases with length of storage (Fig. 1); it is therefore recommended that for some industrial applications chitosan solutions be freshly prepared if good results are to be obtained.

At the height of the season large quantities of chitosan are left to dry in the sun. Attempts were made to process chitosan from this dry material. The dried squilla were coarsely ground to separate out an appreciable quantity of protein in powdered form. The dried material was demineralized, deproteinized and deacetylated by a process identical to that used for the fresh squilla, but the time needed for deacetylating the dry material was less than for the wet squilla. The viscosity of chitosan from the dry material was, however, much lower. This indicates that the exoskeleton was affected by bacterial action during the drying because of its high protein and moisture content.

Since the availability of squilla is limited to 2 to 3 months a year, the intermediate product (chitin) can be stocked by demineralizing and deproteinizing the raw material. Crude chitin separated from squilla can then be stored for subsequent deacetylation to obtain chitosan.

One valuable by-product--protein--has been recovered from squilla by passing the material through meat-separating machines, precipitating it at its isoelectric point, treating it with heat and then drying it. The average yield is 5 kg from every 100 kg of fresh squilla. Experiments are

Table 1. Approximate Composition of Squilla

(figures in percentage on moisture-free basis. Moisture content of the starting material was 54.2 percent.)

Total N	8.0
Chitin N	1.1
Chitin	9.8
Fat	2.6
Ash	28.4

Table 2. Yield of Chitosan and Its Properties

	Weight (kg)
Starting material, squilla (wet) produced:	100.0
Chitin (dry)	4.5
Chitosan	3.5
Protein (as a by-product) (dry)	5.0

Viscosity of 1% chitosan solution in 1% acetic acid:

Intrinsic Viscosity: Plots of η_{sp}/C vs C (range 50-60)

η_{sp} = Specific Viscosity = $\eta/\eta_0 - 1$

C = Concentration

Table 3. Chitosan as an Aid for Purification of Water

Raw-water turbidity		3200 units
Flash mixture		1 min. @ 100 rpm
Flocculation		9 min. @ 40 rpm
Settling		10 min.
Quantity of chemicals (mg/liter):		Settled-water turbidity
Alum	300.	90
Chitosan	0.6	18
Chitosan + Alum	0.15 10.0	15

in progress to determine whether it has uses in the textile and plywood industries, for fire extinguishing and for other technologies.

Another useful source of chitosan is prawn/shrimp waste. India exports an enormous amount of frozen shrimp (about 1250 million rupees' worth in 1976); about 50% of its catch ends up as frozen shrimp for export, and that industry could supply large quantities of waste in the form of shrimp heads, shells, etc. The viscosity of chitosan from shrimp waste is lower than that from squilla. Nevertheless, such a huge quantity of shrimp waste can be made available for diversion into the production of chitosan and its by-products that deriving chitosan from this source would certainly be worth considering. Chitosan is used in a number of industries, including paper, textiles, water purification, fiberglass, ion exchange and photographic film. Its by-products (such as protein) are also essential. Commercial production of chitosan would increase the profits of fish processors and considerably improve the lot of the poor fishermen, and this would mean a great deal to India, with its abundant supplies of squilla and prawn/shrimp waste. Many countries have already evinced a very keen interest in the industrial application of chitosan and its by-products (1,5,7,8,10).

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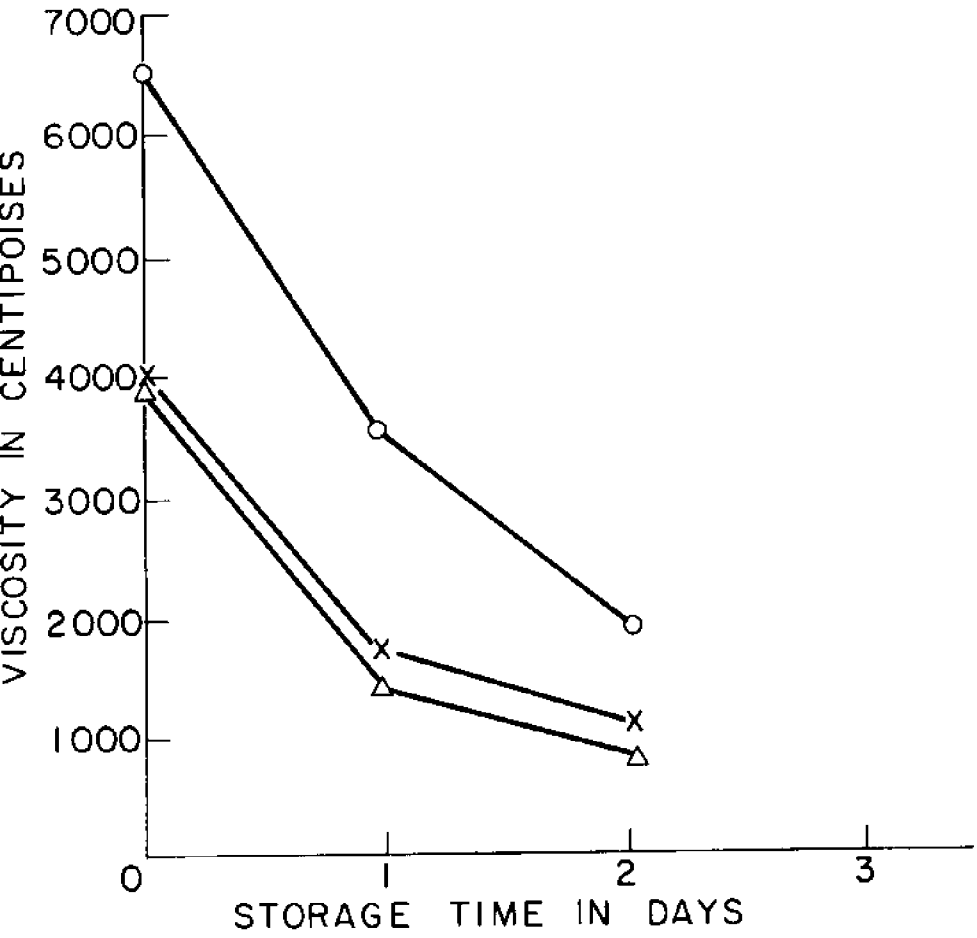

Fig. 1: Decrease in Viscosity of 1% Chitosan (three samples) in 1% acetic acid solution on storage at room temperature.

V. ORGANIC AND MINERAL RECOVERY

TREATMENT OF FOOD-PROCESSING WASTES WITH CHITOSAN AND NUTRITIONAL EVALUATION OF COAGULATED BY-PRODUCTS

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ABSTRACT

Chitosan has been shown to be an effective agent for coagulation of suspended solids in various food-processing wastes, including poultry, egg, meat, shrimp, cheese and vegetable processing wastes. It is also effective for dewatering of activated sludge suspensions. Treatment with chitosan and removal of the coagulated solids by gravity settling, dissolved air flotation, or centrifugation, as required for specific applications, typically resulted in suspended-solids reductions of over 90%. In certain cases, such as poultry, egg, meat and shrimp wastes, reductions in the chemical oxygen demand of 60% to 80% have been obtained.

In general, the by-products recovered with the aid of chitosan from food-processing waste contain significant amounts of protein (30-70%), and in certain cases, such as poultry and egg wastes, 30-50% fat. Results on the physiological effects of free chitosan and feed products coagulated with chitosan when fed to young white rats show no adverse effects at chitosan levels below 5% of the diet. This is 10-20 times the levels expected in feeding coagulated by-products to animals. When free solid chitosan (ground to 1 mm) was mixed into diets at levels of 0.1, 2.5, 5, 10 and 15% of the diet, no adverse effects on growth rate, blood, or liver composition were observed at levels of 5% chitosan or less.

Chitosan in solution was mixed with a suspension of starch and casein, neutralized, centrifuged and dried. This product was fed for six weeks to rats at levels to contain 0, 1, 2, 5, 7.5, and 10% chitosan in the diets. Only those animals receiving the two highest levels showed significant differences from the control group.

Proteinaceous solids recovered from cheese whey, with and without chitosan as a coagulating agent, were found to have protein-efficiency ratios equivalent to the casein control.

INTRODUCTION

Food-processing wastes have been characterized on a unit basis by going into the plant and determining waste loads and flow rates at each step of the processing operation and relating each step to the total waste load. In this

way we could identify concentrated sources of waste and see how best to modify the process to reduce the waste load. Much of our research on waste treatment has stemmed from similar characterization studies where we have identified concentrated unit effluents (3). We have proceeded on the theory that (a) it is desirable to segregate concentrated unit wastes and treat them separately rather than allowing them to mix with more dilute wastewaters and (b) that by-products recovered from food-processing wastes can be recycled into animal feeds.

The yield of chitosan from dry shrimp hulls is approximately 20%. Thus approximately 400 pounds of chitosan can be derived from a ton of shrimp meal, which at $2 a pound is worth approximately $800. The value of the original ton of shrimp meal is thereby increased by a factor of 8 over the value of shrimp meal sold at $100 per ton for use as an animal feed supplement.

EXPERIMENTAL

Chitosan has been evaluated as a coagulating agent for treatment of various food-processing wastes. Different separation methods for recovering coagulated solids have been evaluated, including coagulation and settling (3, 6), dissolved air flotation (8), and centrifugation (7). Experiments were initially conducted on a laboratory scale, using standard jar tests on 500 ml portions of wastewater (9). In addition to the effects of different chitosan concentrations, different conditions of pH and combinations of inorganic salts and other polyelectrolytes were tested. For screening purposes, the reduction in turbidity measured as formazin turbidity units was typically measured (10). Suspended solids and chemical oxygen demand (COD) were measured by standard methods (1).

Following laboratory screening trials, pilot-scale studies were conducted on 10-gallon batches of wastewater. In general, the methodology involved coagulation and settling in an apparatus constructed for physical-chemical treatment studies (3). Dissolved air flotation experiments were conducted in a modified canning retort (8).

Field experiments were conducted in conjunction with industrial cooperators and consulting engineers. In one case, a pilot-scale basket centrifuge was evaluated; in another, a commercial horizontal bowl centrifuge was being tested (7). In other studies, large vats, tanks, or barrels were used to conduct coagulation and settling experiments (4, 6).

RESULTS AND DISCUSSION

In characterization studies on vegetable wastes, we found, as expected, that peeling and blanching effluents were large contributors to the total waste load. Somewhat unexpectedly, we found that choppers and fillers can contribute up to 40% of the total load of suspended solids in processing wastes from the canning of leafy green vegetables (3). In poultry processing, the killing, eviscerating, and scalding operations contribute heavily to the waste load. Chilling does too, especially giblet chillers. In the egg-breaking industry, the washing machines and clean-up operations contribute the majority of the waste load. In the manufacture of cheese, whey is the major component of the waste load. Activated sludge is another concentrated waste that results from secondary biological treatment of wastewaters. An excess of sludge is produced, approximately 0.3 lb. sludge per pound biochemical oxygen demand (BOD) removed, and this sludge must be either disposed of or utilized as a by-product. In a typical activated sludge system for treatment of food-processing wastes, the mixed liquor is pumped to a clarifier where the sludge solids settle out and are either returned to the aeration basin or pumped into a tank truck to be hauled away for disposal. We were involved in feasibility studies at two plants investigating the use of centrifugation to dewater the sludge. In one study at a vegetable-canning plant, the clarifier underflow was pumped to a pilot-scale basket centrifuge which was fitted with a polymer delivery system. We tested several different polymers and were able to reduce the susepended solids by over 98%, as shown in Fig. 1, using a ratio of chitosan to suspended solids of 0.2-0.4% (7). The machine could be operated satisfactorily without polymer, but it could be fed at a higher throughput rate, as shown in Figure 2, by using a polymer to condition the sludge. We will comment later on the protein content and amino-acid composition of these vegetable-sludge solids.

In another study involving sludge grown on brewery wastes, we tested the use of a polymer and a commercial centrifuge in different operating parameters. With the aid of a polymer, a cake was built up in the solids-discharge chute that was so thick it had to be washed off with a hose. Suspended solids in the centrate were reduced approximately 95%, from 2000 mg/l to less than 400 mg/l (as shown in Fig. 3), by using a ratio of chitosan to suspended solids of 0.6% to 0.8%. The total solids content in the sludge cake was about 7.5% (7).

In all the studies described we were investigating the use of chitosan for coagulation of suspended solids. Table 1 summarizes data from several studies on the composition of suspended solids and COD in various food-processing wastewaters both before and after treatment with chitosan.

We believe that because chitosan is derived from a natural product and is composed of biodegradable structures, it may prove useful in the recovery of coagulated by-products from food-processing wastes, where these by-products will be recycled into animal feeds. We have done some studies, discussed below, on the incorporation of chitosan into the diets of small animals, and we have compared the protein-efficiency ratios of a coagulated by-product from whey with a control containing no polymer. In the case of vegetable sludge, brewery sludge, and the coagulated solids from egg-breaking wastes, we have determined the amino-acid composition of the dry solids. All three of these materials contain adequate amounts of the

essential amino acids, with the possible exception of cystein, which was destroyed by the acid-hydrolysis procedure used to hydrolyze the proteins. Brewery sludge was analyzed for tryptophan by a microbiological method and found to contain adequate amounts of this essential amino acid (7). Table 2 summarizes the proximate composition, on a dry-weight basis, of protein, fat and ash in the various by-product solids recovered with the aid of chitosan as a coagulating agent.

Coagulated brewery sludge was found to contain 37% protein based upon the amino acid content, 2% fat and 16% ash. The vegetable-sludge solids contained 28% protein, 1% fat and 20% ash (7). Assuming 75% recovery of the suspended solids, when in fact we achieved 95 to 98% reduction, and assuming a value of 10¢ per dry pound for the value of the coagulated solids, the value of the coagulated solids from brewery sludge would be approximately $7.50 per thousand gallons. Chitosan was added, 75 mg/l, at an estimated cost of $1.26 per thousand gallons, based on a chitosan price of $2 per dry pound. Obviously, there are many other important costs besides polymer costs, but we are only attempting to determine whether or not the value of coagulated solids recovered from a waste effluent would even cover the cost of the polymer. In the case of brewery sludge and vegetable sludge, it appears that the value of the solids recovered from the sludge would be 5 to 10 times greater than the cost of the polymer applied. The Coors Brewery in Golden, Colorado, will soon begin commercial production of dried sludge for use as a feed supplement.

In studies on poultry wastes, treatment with chitosan was applied to the final or composite effluent, and separate treatments were applied to the chiller and scalder effluents (8). These were laboratory and pilot-plant studies performed on 10-gallon batches of effluent. When coagulation and gravity settling were applied to the composite effluent, the dry coagulated by-product contained 54% protein and 29% fat. When dissolved air flotation was used, a product containing less protein and more fat was obtained. The chiller effluent yielded a by-product containing 36% protein and 54% fat. The scalder effluent produced a by-product high in protein (68%) and relatively high in ash (15%). The most promising result to come out of this study was the indication that the chiller effluent could be treated separately. After the suspended solids are removed, the treated effluent could perhaps be recycled in the chilling operation, if sanitation standards were met. Such a recycling process would be saving of both water and energy because of the saving on ice required to chill the water.

In the case of egg-breaking wastes, chitosan quite effectively coagulated suspended solids both in the composite effluent and the concentrated effluent from the egg washers. Suspended solids were reduced by 70-90% and the COD by 55-75% (4). The amino-acid compositions of five coagulated egg by-products were similar to the composition of whole eggs. The feed value of the coagulated solids and the potential savings in waste treatment charges make treatment with chitosan an attractive option for waste management.

In studies on meat wastes, we found that the composite effluent from a meat-packing operation yielded a coagulated by-product containing 41% protein, 17% fat and 11% ash (5). As would be expected, the by-product recovered from a plant engaged in further processing and curing was lower in protein than that recovered from a plant used for killing and packing; it

Table 1. Reduction of Suspended Solids and Chemical Oxygen Demand in Food-Processing Waste Effluents by Coagulation with Chitosan

Effluent	Chitosan mg/l	Separation	Suspended Solids Raw mg/l	Suspended Solids Effluent mg/l	Suspended Solids Red'n(%)	Chemical Oxygen Demand Raw mg/l	Chemical Oxygen Demand Effluent mg/l	Chemical Oxygen Demand Red'n(%)	Ref
Greens washer	10[a]	GS[j]	1624	6	99	2394	915	62	(3)
filler	5[b]	GS	1747	125	93	1700	1530	10	
composite	10	GS	143	15	90	-[i]	-	-	(3)
Spinach	20	GS	298	29	90	-	-	-	(3)
Pimiento peeling	40	GS	248	10	96	-	-	-	(3)
coring	10	GS	32	5	84	-	-	-	(3)
composite	30	GS	75	8	89	-	-	-	(3)
Green-bean blancher	5[c]	GS	116	6	95	-	-	-	(3)
Brewery act. sludge	75	Cent.[k]	12000	600	95	-	-	-	(7)
Vegetable act. sludge	40	Cent.	16000	200	99	-	-	-	(7)
Poultry composite	5	GS	361	20	94	1240	1080	13	(8)
	5	DAF[l]	503	210	58	1400	880	37	
	0	DAF[l]	451	297	34	1052	902	14	
Poultry chiller	6	GS	260	65	75	942	362	62	(8)
	6	DAF	212	28	82	740	280	62	
	0	DAF	212	70	67	740	300	57	
Poultry scalder	30	GS	428	52	88	1220	620	49	(8)
	30	DAF	456	103	77	1320	720	46	
	15	DAF	443	157	64	1280	820	36	
	0	DAF	483	423	12	1280	1120	12	
Egg-breaking composite	150[d]	GS	1610	450	72	29000	7000	76	(4)
	150[d]	DAF	1930	177	91	29000	11000	62	
	0	DAF	1070	557	48	-	-	-	
	200[e]	GS	1005	256	74	20900	8900	57	
Egg-washer waste	100[f]	GS	5027	287	94	35500	14250	60	(4)
Meat packing	30	GS	465	49	89	1800	800	55	(5)
Meat processing & curing	10[g]	GS	169	8	95	865	240	72	(5)
	5	GS	416	35	92	1596	341	79	
Shrimp composite	10[h]	GS	2808	178	94	6500	1560	76	(5)
	10[h]	DAF	1900	33	98	3200	280	92	
Fruit-cake waste	2	GS	522	33	94	3150	1660	47	(5)
	2	DAF	158	98	38	1134	934	18	
Cheese whey	53	GS	2470	198	92	68500	65760	4	(6)

[a] Plus 15 mg/l NJAL-240
[b] Plus 10 mg/l NJAL-240 and 40 mg/l alum
[c] Plus 80 mg/l $CaCl_2$
[d] Plus 10 mg/l Betz 1130
[e] Plus 15 mg/l Betz 1130
[f] Plus 20 mg/l Betz 1130
[g] Plus 40 mg/l $FeCl_3$
[h] Plus 5 mg/l WT-3000

[i] Not determined
[j] Coagulated solids separated by gravity settling
[k] Coagulated solids separated by centrifugation
[l] Coagulated solids separated by dissolved air flotation

Table 2. Proximate Composition of Coagulated Solids Recovered from Food-Processing Waste Effluents by Coagulation with Chitosan

Effluent	Solids Composition %		
	Protein	Fat	Ash
Brewery activated sludge	37[b]	2	16
Vegetable activated sludge	28[b]	1	20
Poultry composite[c]	54[a]	29	4
Poultry chiller	36[a]	54	1
Poultry scalder	68[a]	1	15
Egg-breaking composite	40[b]	40	6
Egg-washer waste	44[b]	38	4
Meat packing	41[a]	17	11
Meat processing and curing	14[d]	-[f]	-
Shrimp composite	32[a]	-	-
Fruit cake	13[a]	-	2
Cheese whey	75[e]	0.2	10

[a]Based upon Kjeldahl N X 6.25

[b]Based upon total amino acid content

[c]Solids recovered by DAF contained 35% protein, 49% fat and 3% ash

[d]Based upon biuret analysis

[e]Based upon Kjeldahl X 6.4

[f]Not determined

contained only 14% protein. The composite effluent from a shrimp breading and freezing operation treated with chitosan yielded a by-product containing 32% protein. This protein content would have been higher except for a significant amount of breading in the effluent which was also coagulated and recovered. Fruit-cake wastes yielded a by-product containing only 13% protein, approximately the same protein content that we observed on by-products recovered from vegetable-processing wastes (5).

In the case of cheese whey, the coagulated by-product contained 72% protein, 0.2% fat, 10% ash and 6% lactose (6). Cheese solds are settled out in large vats, and the whey is drained off and discharged or otherwise disposed of. It is a concentrated source of wastes containing approximately 30,000-50,000 mg/l BOD and 1000-5000 mg/l suspended solids. In some cases, the whey is discharged down a drain that goes to the municipal waste-treatment system. The municipal surcharge was 5.3¢ per pound for any excess over 250 mg/l suspended solids and/or BOD. Thus, the surcharge for discharging this whey amounted to approximately $3 per day for suspended solids and $52 per day for BOD. Treatment of this whey by coagulation and settling, using a ratio of chitosan to suspended solids of 2.5%, reduced suspended solids by 92%, but the BOD was only reduced by 4%. The coagulated solids were recovered by centrifugation and were freeze-dried. Because of the concentrated nature of cheese whey and the large particle size of the suspended solids in whey, we were also able to recover some of the whey solids by centrifugation without the aid of any polymer. These solids were also freeze-dried for comparison to the solids containing chitosan. In a comparative study using rats to determine the protein-efficiency ratio or PER values of whey solids, no statistically significant differences were found between the PER values of coagulated whey solids containing chitosan, whey solids containing no polymer, and a casein control. These results show that the utilization of the protein in the coagulated by-product was not diminished by the presence of such a small amount of chitosan. They also show that coagulated whey solids would be an excellent protein supplement. While it really would not save the plant much money on surcharges for suspended solids and BOD, it does appear that the value of the cheese-whey solids would exceed the cost of the polymer.

We referred earlier to the studies that we have done on incorporation of chitosan into the diets of experimental animals (6, 7, 11). This work was directed by Dr. D. R. Landes of the Food Science Department at the Georgia Experiment Station. In one study, chitosan, ground to 20 mesh, was incorporated to constitute up to 15% of the total diet. During the course of an 8-week feeding study, chitosan intake, rat weight gain and feed efficiency were monitored. There were no significant differences, at the 5% level, of confidence between diets containing 0, 1.0, 2.5 and 5.0% chitosan (11). At higher levels, of 10 and 15% chitosan, feed efficiency did decrease. Similar trends were observed when measuring the effect of chitosan in the diet on the weights of the rat livers, kidneys and spleens. At 5 to 10% chitosan and below, no significant differences were observed. Only at the highest level of 15% were differences observed in the case of all three organs. Analyses performed on the rat blood and serum showed the same trends as did the organ weights. These results are similar to those of a Japanese study, which showed no harmful effects in mice fed chitosan levels up to 18 g per kg body weight per day (2). This corresponds to our highest level of 15%, which was 16 g intake per kg body weight per day.

Because chitosan had been incorporated as dry, finely ground particles into the rat diets in the previous study, an experiment was designed to test exaggerated levels of chitosan where the coagulated solids were formed by chitosan in solution combining with the test proteins. Chitosan in solution was mixed with a suspension of starch and casein, neutralized, centrifuged and dried. This product was fed for six weeks to rats at levels to contain 0, 1, 2, 5, 7.5 and 10% chitosan in the diets. Only those animals receiving the two highest levels showed significant differences from the control group (unpublished results).

CONCLUSIONS

We feel that these results indicate that amounts of chitosan in the diet of 5% or less would have no adverse effect upon animals. This is 10-20 times the levels expected in feeding coagulated by-products to animals. Of course, our experiments are limited in that we have only worked with rats. Larger-scale studies will have to be performed.

Without a doubt, chitosan is an effective coagulating agent--as effective as the top 10% of the dozens of polymers we have evaluated for treatment of food-processing wastes. But that is also saying that synthetic polymers can be found that will perform as well as chitosan. What would make chitosan uniquely different in qualification would be for it to obtain FDA approval as a feed ingredient, allowing coagulated by-products recovered from food-processing wastes to be used for feed.

RECOMMENDATIONS FOR FUTURE WORK

While the FDA has many requirements that relate to approval of a "feed additive," which was their ruling on chitosan (13), the most expensive are these that require experimental evidence of safety. The FDA communication in response to our preliminary proposal for approval of chitosan as a feed additive, when commenting on safety tests, stated: "Rat data are not adequate to demonstrate animal safety. We require that animal safety studies be conducted in the target species, i.e., chickens, swine, cattle, sheep, etc. If residues of the additive occur in meat, milk, or eggs of animals consuming the additive, then rat data may be useful in satisfying some of the human safety requirements" (13). Of course, the human safety division within the FDA is different from the animal safety division, and this compounds the requirements for obtaining FDA approval.

Still, the need for such a coagulating agent as chitosan remains. Stringent effluent limitations and costly municipal surcharges are forcing more and more food processors into pretreatment of their wastewater prior to discharge for secondary treatment. Most are segregating concentrated effluents for separate treatment, and a polymer is often needed. What a pity for the coagulated solids that are recovered to be buried or burned! But before these solids can be recycled into animal feeds, a polymer that has FDA approval as a feed additive is needed.

There are polymers approved for use in treatment of potable water, in juice clarification during sugar manufacture, and as a thickener and suspending agent in nonmedicated aqueous suspensions intended as animal-feed additives (21CFR 121.288). However, according to an FDA communication (12) this approval "does not relate to the use of the polymycin as a coagulation agent. We are unaware of any documents which support the use of this polymer as a coagulating agent for food processing wastes, and 21CFR 121.288 does not provide for this use." (12) Thus, a polymer is needed that can be used for recovering suspended solids from food-processing wastes, which can then be used in animal feeds. Some industry with interests in future profits must take up the challenge and the expense of developing a product and obtaining FDA approval for this speciality use. Chitosan could be that product.

More research is needed on the effects of manufacturing variables on the characteristics and performance of chitosan products. To use a single word - chitosan - to define such an array of products is misleading. In the cooperative work completed to date, Professor Averbach, at M.I.T., and we agree in our observations that the commercial and academic sources of chitosan, including our laboratory, produce different products. We see differences in size and texture of the dry products, molecular weight distribution, viscosity of solutions and waste-treatment effectiveness. Professor Averbach's laboratory has observed differences in film-forming characteristics, x-ray diffraction patterns and light scattering. We are exchanging samples in an attempt to understand the complex effects of raw-material sources, manufacturing variables and other experimental variables.

The range of chitosan products available can be an advantage to chitosan users and manufacturers, if the variants are known and controlled. The ability to tailor-make a chitosan product for a particular application would be a good reason for a user to choose chitosan over a competing product. If he does, the user will expect a consistently effective product, and this would require stringent quality control by the chitosan manufacturer who, in turn, will need a thorough knowledge of the chemistry of his process and the raw materials that go into it. It is this basic information that we need to extract from our research projects.

ACKNOWLEDGMENTS

Chitosan samples were obtained from Food, Chemical, and Research Laboratories, Inc., Seattle, Washington. This work is a result of research sponsored by the University of Georgia College of Agriculture Experiment Stations and by the Georgia Sea Grant Program, supported by NOAA Office of Sea Grant, Department of Commerce, under grants nos. 04-3-158-4, 04-5-158-4, 04-6-158-44017 and 04-60158-44115. The U.S. Government is authorized to produce and distribute reprints for governmental purposes, notwithstanding any copyright notation that may appear hereon.

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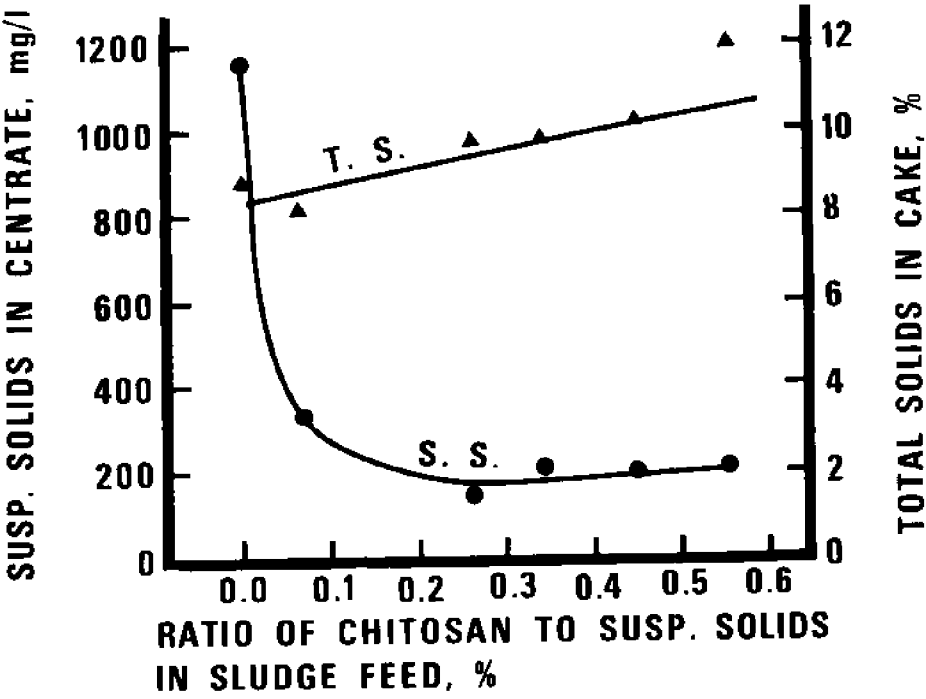

Fig. 1. Effects of Chitosan Concentration and Suspended Solids in Centrate and Total Solids in Sludge Cake Obtained by Batch Centrifugation of Vegetable Cannery Activated Sludge (8)
(Sludge feed rate was 8-9 l/min; centrifuge speed was 3,2000 rpm.)

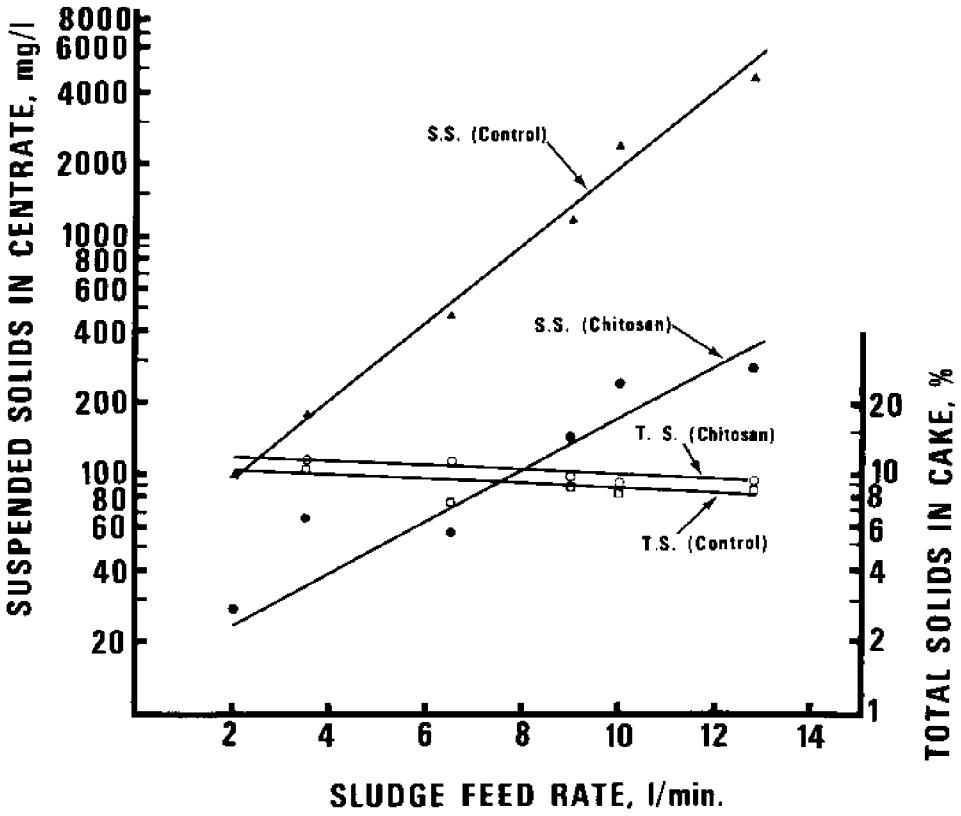

Fig. 2. Effects of Sludge Feed Rate With and Without 40 mg/l Chitosan on Suspended Solids in Centrate and Total Solids in Sludge Cake from Batch Centrifugation of Vegetable Cannery Activated Sludge (8)

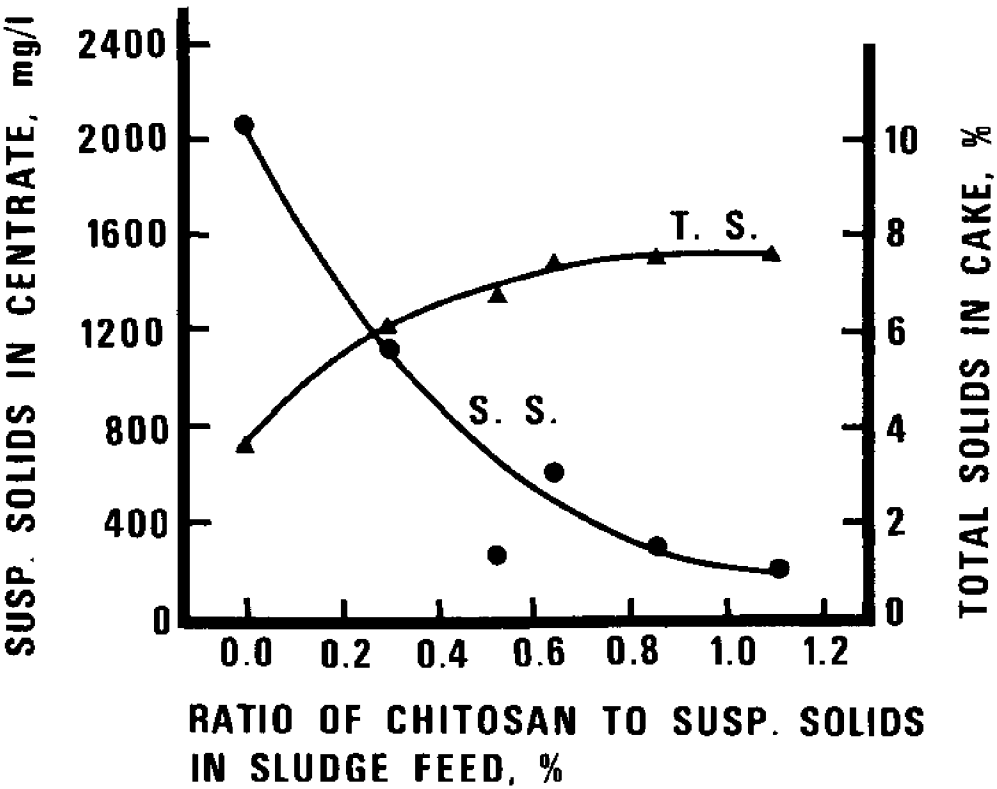

Fig. 3. Effects of Chitosan Concentration on Suspended Solids in Centrate and Total Solids in Sludge Cake Obtained by Continuous Centrifugation of Brewery Activated Sludge (8)
(Centrifuge bowl speed was 3,300 rpm and differential was 17 rpm.)

CHITOSAN APPLICATIONS IN WASTEWATER SLUDGE TREATMENT

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ABSTRACT

The primary objective of any dewatering operation in municipal and industrial wastewater treatment is to reduce the sludge moisture content to a degree that allows the ultimate disposal of sludges by incineration, landfill, or other means. Dewatering differs from sludge thickening in that the sludge is processed into a non-fluid form in which the suspended-solids concentration is increased to within the range of 10 to 30%.

In modern dewatering operations, solid-liquid separation is commonly carried out by mechanical means such as centrifugation and vacuum filtration with or without an addition of polyelectrolytes. Centrifugation has some inherent advantages over vacuum filtration and other means of sludge dewatering. It is simple, compact, clean, totally enclosed and flexible, and its costs are comparable to those of other means of mechanical dewatering.

Here centrifugal sludge dewatering by the use of the chitin-chitosan derived polymer commonly known as Flonac will be reviewed. A brief description of the manufacturing process of Flonac, of its characteristics, and of the application of Flonac in the sludge dewatering of municipal and industrial wastewater and its dewatering performance by centrifugation is given. Sludge-cake disposal, toxicity and the decomposition properties of Flonac in soil environments are also discussed.

INTRODUCTION

The primary objective of any dewatering operation in municipal and industrial wastewater treatment is to reduce the sludge moisture content to a degree that allows the ultimate disposal of sludge by incineration, landfill or other means. The dewatering operation differs from sludge thickening in that the sludge is processed into a non-fluid form in which the suspended solid concentration is measured from approximately 4% to within the range of 10 to 30%, depending on the characteristics of the sludge. In modern sludge dewatering operations, solid-liquid separation is commonly accomplished by mechanical means such as centrifugation and vacuum filtration with or without an addition of polyelectrolytes. The centrifuge has some inherent advantages over the vacuum filter and

other means of sludge dewatering. It is clean, compact, totally enclosed and flexible, and its costs are comparable to those of other means of mechanical sludge dewatering.

The objectives of sludge centrifugation are, however, similar to other dewatering operations and, in the case of centrifugation, it is necessary to obtain a dry cake, clean centrate (effluent) and a reasonable throughput (centrifugal yield). In the last decade, the most significant addition to sludge-conditioning technology has been the practical application of polyelectrolytes or polymers. Similar to earlier applications of inorganic sludge-conditioning agents, investigations of a wide variety of polymers have indicated that a selection of the required dosage for optimum sludge dewatering is highly dependent on the specific sludge and the accompanying physical condition.

Among various polyelectrolytes, manufactured from both petrochemical bases and natural polymers, the chitin-chitosan derived polymers known as Flonac in Japan have been widely used for sludge-dewatering applications. This is due mainly to their effectiveness in sludge conditioning, rapid biodegradability in soil environments, and reasonable economic advantages in centrifugal sludge dewatering.

The purpose of this paper is to present the state-of-the-art review of the applications of chitin-chitosan–derived polymers in the centrifugal dewatering of municipal and industrial sludges. Brief descriptions of the manufacturing process of Flonac and of its characteristics are presented. The paper further discusses the effects of Flonac application in centrifugal sludge dewatering from the standpoints of cake disposal, toxicity to animals and plants and decomposition in soil environments.

SOURCES OF SLUDGE PRODUCTION IN WASTEWATER TREATMENT SYSTEMS

Many of the problems associated with water quality control are due to the presence of dissolved, suspended, and colloidal organic matter from natural sources or from wastewater discharges. This organic matter is normally stabilized biologically, and the microorganisms involved utilize either aerobic or anaerobic oxidation systems. Many of the pollutants in water and wastewater are present as suspended particles which are carried along in flowing liquids but will settle out in quiescent conditions, such as in clarifiers. When removed, these solids are generally referred to as sludge, but they represent a variety of characteristics and quantities. The amount of sludge produced obviously depends on the origin of the wastewater, the type of treatment facility used and, more important, on the operation and control of the treatment facilities.

Figure 1 shows a typical wastewater treatment system with its various sources of sludge production. The activated sludge process most commonly used in modern wastewater-treatment facilities is depicted. As noted from Table 1, the various wastewater treatment processes produce different amounts and types of sludge; the table presents data on typical volumes of sludges produced in several conventional wastewater treatment processes. One will note the striking increase in the volume of sludges to be processed when a plant is upgraded to activated sludge treatment. Table 2 illustrates typical masses or weights of sludges produced by various conventional methods of wastewater treatment.

The types and quantities of sludges produced in the wastewater treatment system can vary from plant to plant, and their dewaterability can also vary significantly. In general, both primary and anaerobically treated sludges are relatively easy to dewater in a mechanical dewatering device such as a sludge centrifuge; however, aerobically treated sludges, such as activated sludge, are notoriously difficult to dewater (2). Application of organic polyelectrolytes (polymers), such as chitin-chitosan derived polymers, may improve sludge dewaterability significantly. While noting that sludge handling is responsible for about 30 to 40% of the capital cost of a wastewater treatment plant and about 50% of the operating cost, most treatment-plant operators will agree that, in terms of the headaches and trouble caused, sludge handling is worth 90% of the wastewater treatment system (10). As a result, application of sludge-conditioning chemicals for liquid-solid separation in clarifiers and in sludge dewatering has been practiced for many years. Furthermore, the use of organic polymers such as Flonac will be increased when more stringent water-quality standards are enforced to abate water pollution.

CHITIN-CHITOSAN-DERIVED POLYMER AS A SLUDGE DEWATERING AGENT

The sludge dewatering agent (coagulant) known as Flonac, which is derived from the chitin-chitosan base is manufactured by Kyowa Oil & Fat Co., Ltd., Tokyo, Japan. Flonac N is a chitosan product without acid treatment and must be mixed with various acid solutions such as formic, acetic, hydrochloric and sulfamic acids before its application to sludge dewatering. Flonac 250 is treated with formic acid and can be readily dissolved in an aqueous solution.

Manufacturing process

A schematic diagram of Flonac production from crab shells is shown in Figure 2. Major sources of crab shells used include the king crab (*Paraliphodes camtschaticus*), the tanner crab (*Chinoecepes opilio*) and the Korean horsehair crab (*Erimaceus isenbeckii*). As shown in Figure 2, chitin is produced by extracting protein- and alkali-soluble material in a protein-extraction process and by a demineralization process which removes inorganics such as calcium by the addition of hydrochloric acid. Further treatment of chitin in the deacetylation process by the addition of sodium hydroxide produces chitosan. The product quality will obviously depend on these manufacturing processes, particularly with the deproteinization, demineralization and deacetylation processes; it appears that the product quality also depends on the kind and age of the crab.

Characteristics

One of the useful characteristics of chitosan as a cationic polyelectrolyte for sludge dewatering is its high solubility in an aqueous solution. Since an ionic functional group of the chitosan arises from $-NH_2$ base, it is expected that, with a higher chitosan content in Flonac products, a more effective coagulation of sludge will take place. In the coagulation of wastewater sludge with a polyelectrolyte, the molecular weight of the polymer will affect the size of floc formation. It therefore is believed to be an important operational parameter in solid-

liquid separation such as wastewater sludge centrifugation. Since the molecular weight of chitosan will affect the solution viscosity, general correlation between the extent of deacetylation and the solution viscosity is observed, as shown in Figure 3.

Production control of Flonac is such that both alkali solubles (residue from the protein extraction process) and ash contents (residue from the demineralization process) are maintained at less than 5% of the product, and when dissolved in water, complete dissociation and a clear aqueous solution result. Table 3 shows the general characteristics of Flonac N, which was directly derived from chitosan without acid treatment. An average molecular weight of Flonac N is in the range of 100,000 to 300,000 and its 1/2% aqueous solution exhibits a viscosity of 200 to 500 cp at 20° C.

Table 1. Sludge Volumes Produced in Conventional Wastewater Treatment Processes

Wastewater Treatment Process	Gallons of Sludge Produced per Million Gallons Wastewater Treated			
	Keefer (5)	Fair and Imhoff (4)	Babbit (3)	McCabe and Eckenfelder (6)
Primary sedimentation	2,950	3,530	2,440	3,000
Trickling filter	745	530	750	700
Activated sludge	19,400	14,600	18,700	19,400

Table 2. Sludge Masses Produced in Conventional Wastewater-Treatment Processes (9)

Wastewater Treatment Process	Percent Suspended Solids Removed by Process	Pounds of Solids Generated by Process per Million Gallons Treated	Specific Gravity of Suspended Solids
Primary sedimentation	60	1,020	1.33
Trickling filter	30	510	1.52
Primary plus activated sludge	92	1,563	1.33

Table 3. General Characteristics of Flonac N (Chitosan)

Appearance:	Off-white powder
Grain size:	Less than 3 mm
Apparent specific weight:	0.15 ± 0.05
Moisture content:	Less than 10%
Average molecular weight:	100,000-300,000
$-NH_2$ Content:	7-10%

Preparation

In order to dissolve chitosan into aqueous solution for its use as a sludge-dewatering agent, it is necessary to add a proper amount of acid to the solution. Table 4 shows the preparation of Flonac N solution and the pH of the 0/5% solution.

As discussed earlier, in Flonac 250 the chitosan is treated with formic acid; therefore, unlike Flonac N, the solution preparation is unnecessary. Storage of Flonac 250 for more than 3 months should be avoided, however, due to the deterioration of its solubility caused by acid-amide formation and evaporation of formic acid from the product.

Since chitosan dissolves readily in acidic solution but is insoluble in alkaline solution, as shown in Figure 4, effective sludge coagulation will not take place if the pH of the sludge solution is above 7. In these cases, controlling the pH by adding either a low-pH Flonac solution or an excessive amount of acid is necessary for an effective sludge-dewatering operation.

Table 4. Preparation of Flonac N Solution with Acid (product weight basis)

Acid	Flonac N	pH of 0.5% Solution (20°C)
Acetic acid (purity 98% or above)	1 to 1	4.3
Sulfamic acid (purity 99.5% or above)	0.6 to 1	2.6
Concentrated hydrochloric acid	1 to 1	1.8

COAGULANT APPLICATION IN SLUDGE CENTRIFUGATION

The purpose of this section is to discuss the results of the centrifugal sludge-dewatering experiments and to compare the performance of Flonac as a sludge-conditioning agent with other synthetic polyelectrolytes. The Nishihara SD sludge centrifuge, shown in Figure 5, was used for the experiments.

Dewatering performance of municipal wastewater sludge

Figures 6, 7 and 8 show the data plots of various sludge centrifugal experiments performed with the application of Flonac 250. Similar results can be expected by using Flonac N, although dissolution of Flonac N is somewhat tedious because it requires the addition of an acid to form its aqueous solution.

Figure 6 shows the experimental results of dewatering anaerobically digested sludge. The Flonac dosage was in excess of 0.7 to 1.5% (by dry weight). Suspended solids captured by the centrifuge exhibited 96%, or more, efficiency and resulted in a sludge cake containing 65 to 75% moisture. The sludge cake had a flaky, dry appearance. Figure 7 shows the sludge centrifuge performance when dewatering mixed sludges of primary sludge and excess activated sludge. The experimental results indicated that when Flonac was added in the range of 0.6 to 1.4% or more, suspended-solids capture was over 96%, but the moisture content of sludge cake was in the neighborhood of 75 to 83%. Figure 8 depicts the experimental results of the sludge that is most difficult to dewater, i.e., undigested excess activated sludge. When the Flonac dosage was 0.8 to 2.2%, solids capture was over 96%, but sludge-cake moisture was from 80 to 87%, resulting in rather wet cakes. It is generally observed that larger loosely bound sludge cake is formed when the cake moisture content exceeds 75%.

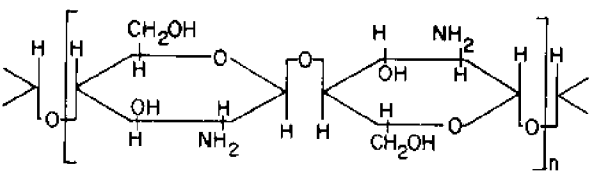
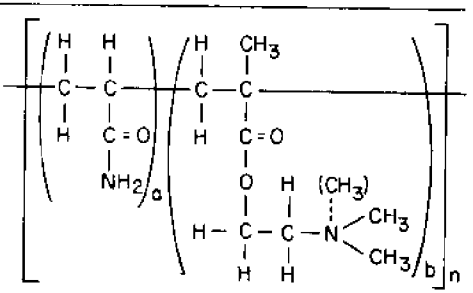
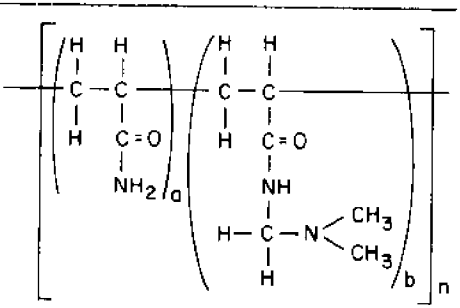
Comparison of Flonac to other synthetic polyelectrolytes

Several synthetic polyelectrolytes were used as sludge dewatering agents in the centrifugal sludge dewatering experiments to compare their performances to that of Flonac. Table 5 shows their structural formulas, substance names, trade names and manufacturers. In particular, the copolymer of acrylamide and methacrylate ester (e.g., Praestol and Diafloc) is widely used in sludge dewatering because of its effectiveness in a wide range of product applications. In Table 5, the ratio of subscripts a and b in the copolymers can be varied so that the cationic strength of the copolymer is adjusted. Since subscript a relates to the nonanionic group and subscript b relates to the cationic group, a high cationic copolymer can be produced by increasing the ratio of subscript b to subscript a in the product. As a result, polymers with a varying degree of cationic strength can be manufactured in accordance with their intended application, depending on the characteristics of the sludges.

Figures 9, 10 and 11 show the comparative results of Flonac applications and of applications of other synthetic polyelectrolytes in centrifugal sludge dewatering. One of the advantages of the synthetic polyelectrolytes is their effectiveness in a wide range of the sludge pH (notably in the pH range of 4 to 10). Thus the application of synthetic polyelectrolytes is particularly effective in the high-pH and high-alkalinity sludges such as the anaerobically digested sludges. Flonac will not be effective in this case.

Excellent treatment by the application of Flonac has been found in the centrifugal dewatering of raw sludges, such as excess activated

Table 5. Commonly Used Polyelectrolytes in Centrifugal Sludge Dewatering

Compound Name	Trade Name (Manufacturer)	Structural Formula
Chitosan	Flonac (Kyowa Oil & Fat Co., Ltd., Japan)	
Copolymer of acrylamide and methacrylate ester	Praestol (Stockhausen, West Germany) Diafloc (Diafloc Co., Ltd., Japan)	
Polyacrylamide Mannich modifying agent	Himoloc (Kyoritsu Yuuki Co., Ltd., Japan)	

sludge, and mixed sludges whose pH's are relatively low. On the other hand, excessive amounts of Flonac and acid are required for anaerobically digested sludge, because of the relatively high sludge pH and alkalinity. It is difficult, therefore, economically to justify the use of Flonac in this case.

Unit product prices of the various polyelectrolytes that have been used in centrifugal sludge dewatering are shown in Table 6.

Table 6. Unit Product Prices of Various Polyelectrolytes (current Japanese market price)

Polyelectrolyte	Unit Price $/kg Product
Flonac 250	4.33
Flonac N with acid	5.00
Synthetic polyelectrolyte (100% cation)	8.00
Synthetic polyelectrolyte (10% cation)	4.33

When Flonac is applied to raw sludges, such as excess activated sludge, and mixed sludges, its unit product price is between $4.33/kg and $5.00/kg in comparison with $5.00/kg to $6.67/kg for the synthetic polyelectrolytes. Economical application of Flonac can therefore be expected. It was found, however, that synthetic polyelectrolytes of relatively low cationic content and priced at $4.33/kg are quite effective for treating anaerobically digested sludge. Thus, the application of synthetic polyelectrolytes has a definite economic advantage in this aspect.

DISPOSAL OF DEWATERED CAKE

One of the important requirements for chemical coagulants used in sludge conditioning is their biodegradability in a soil environment, since the ultimate disposal of dewatered sludges often involves a land treatment and disposal scheme (7). It is therefore important to establish the limit of toxicity under the various application conditions in the environment. Several bioassay results have been reported with Flonac and other synthetic polyelectrolytes. Table 7 summarizes the bioassay results reported by Arai et al. (1). The test animals were laboratory-grown mice (Mus musculus), and the toxicity levels were reported in LD_{50}. It is apparent from Table 7 that LD_{50} values for Flonac are somewhat less than one-tenth of those for copolymers of acrylamide and methacrylate ester and of the same order of magnitude as lethal doses of sugar or salt. Consequently, Flonac does not seem to be in the category of toxic substances so far as acute toxicity is concerned (1).

To an environmental engineer, another aspect of interest in coagulant applications is their ultimate degradability in soil environments. Table 8 shows the experimental results reported by Takahashi (8). The production of carbon dioxide was determined at 28° C over various time intervals.

Table 7. Toxicity Bioassay of Sludge Dewatering Agents (test animal: laboratory-grown mice (Mus Musculus) (1)

Substance	Lethal Dose LD_{50}, g/kg of body weight	Trade Name
Chitosan	Greater than 16	Flonac 250
Chitosan acetate	Greater than 14	Flonac N
Copolymers of acrylamide and methacrylate ester	Greater than 1.5	Diafloc KP-001 (high cationic)
	Greater than 4.5	Diafloc KP-007 (low cationic)

Flonac: Products of Kyowa Oil & Fat Co., Ltd., Japan.
Diafloc: Products of Diafloc Co., Ltd., Japan.

The coagulants used in the degradability tests in soil environments were Flonac 250 (chitosan), Diafloc KP-007 (low cationic copolymer of acrylamide and methacrylate ester) and Prestrol 444K (high cationic copolymer of acrylamide and methacrylate ester), respectively.

Test results in Table 8 indicate that Flonac 250 (chitosan) showed substantially greater biodegradation as measured in comparison with other synthetic polyelectrolytes by the generation of carbon dioxide. It can be concluded that in cultivated soil approximately 70% of the carbon in the Flonac molecules was oxidized to carbon dioxide in 28 days. Rate of degradation of Flonac 250 was somewhat slower in uncultivated soil; it was approximately one-third of that in cultivated soil. When the synthetic polyelectrolytes were tested under the same experimental conditions, it appeared that carbon-dioxide generation was somewhat suppressed, particularly in the high cationic copolymer applications. This phenomenon was considered to be due mainly to the coagulation effects on the physical properties of the test soilds. According to Takahashi (8), nitrogen data indicated that similar trends were observed for Flonac 250. Inorganic nitrogen produced by the degradation of polyelectrolytes in the soil environment is taken up by plants as a nitrogen source. It was a common observation that the soils containing Flonac often exhibited enhanced plant growth.

SUMMARY AND CONCLUSIONS

Comparative studies of centrifugal sludge dewatering of wastewaters were conducted with the addition of Flonac (chitin-chitosan derived polymers) and synthetic polyelectrolytes (copolymers of acrylamide and methacrylate ester).

Table 8. Carbon Dioxide Generation Due to the Degradation of Coagulants in Soil Environment (8)

(CO_2 - C mg/50 grams soil + 0.1 grams coagulant)

Soil	Coagulant	Elapsed Time				
		1 Day	4 Days	9 Days	16 Days	28 Days
Cultivated	No coagulant	2.68	5.66	8.65	12.07	17.37
	Flonac 250	4.75	11.00	19.96	29.32	39.03
	Diafloc KP-007	2.39	5.09	8.07	11.49	16.88
	Praestol	2.15	4.07	6.17	8.65	12.62
Uncultivated	No coagulant	0.98	1.71	2.20	2.74	3.71
	Flonac 250	1.52	4.41	5.44	6.98	9.63
	Diafloc KP-007	0.86	1.64	2.31	3.09	4.67
	Praestol	0.96	1.81	2.37	2.81	3.58

The Nishihara SD sludge centrifuge was used for dewaterability studies on anaerobically digested sludge, primary sludge plus excess activated sludge, and excess activated sludge only. The most effective applications of the chitin-chitosan-derived polymer were found in relatively low pH sludges; on the other hand, the synthetic polyelectrolytes exhibited a wider application range, even in high-pH and high-alkalinity sludges such as anaerobically digested sludge.

Flonac N and Flonac 250 (chitosan-derived polymers) are manufactured from crab shells; thus, their quality tends to vary with the species and age of the crabs. Production control of Flonac is therefore important for its consistency in quality, because quality affects its sludge-dewatering performance.

The toxicity and biodegradability tests indicated that Flonac exhibited less toxicity than other synthetic polyelectrolytes and that rapid decomposition of Flonac will take place in soil environments when compared with the degradation of other synthetic polyelectrolytes. Thus the ultimate disposal of dewatered sludge with Flonac poses no detrimental effects on soils and crops.

Resource recovery of crab shells, which cause pollution of marine environments and disposal problems on land, has been an added advantage in successful utilization of the chitin-chitosan-derived polymer as a wastewater-sludge conditioning agent.

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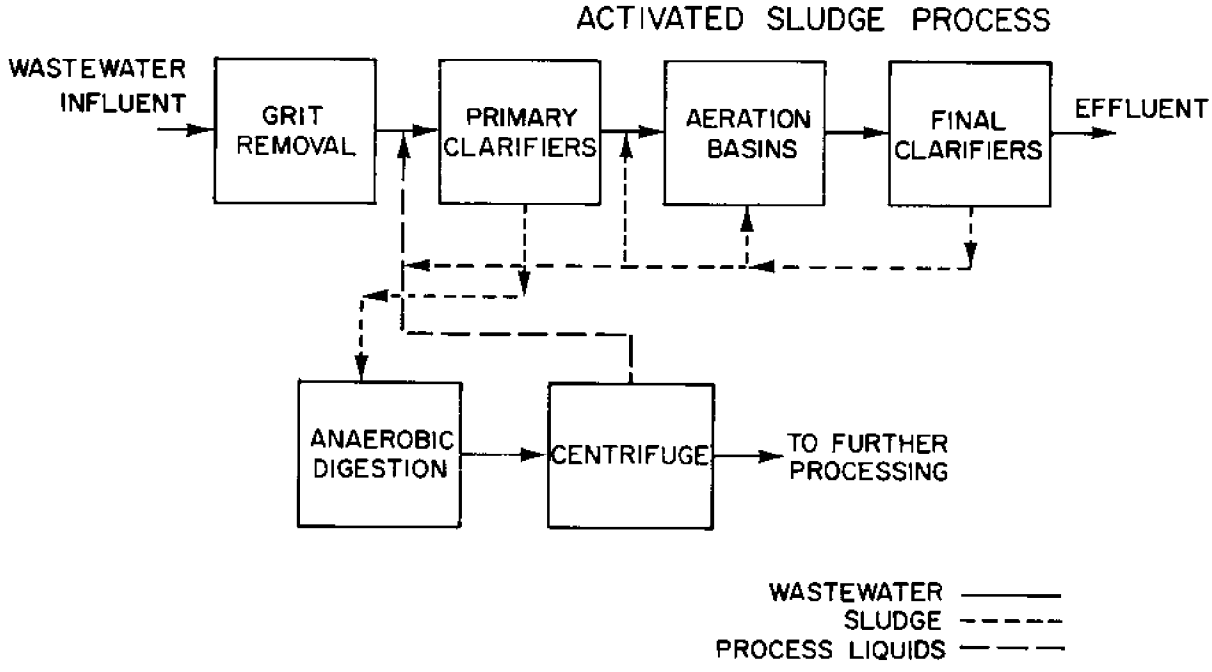


Figure 1. Typical Wastewater Treatment System with Sources of Sludge Production

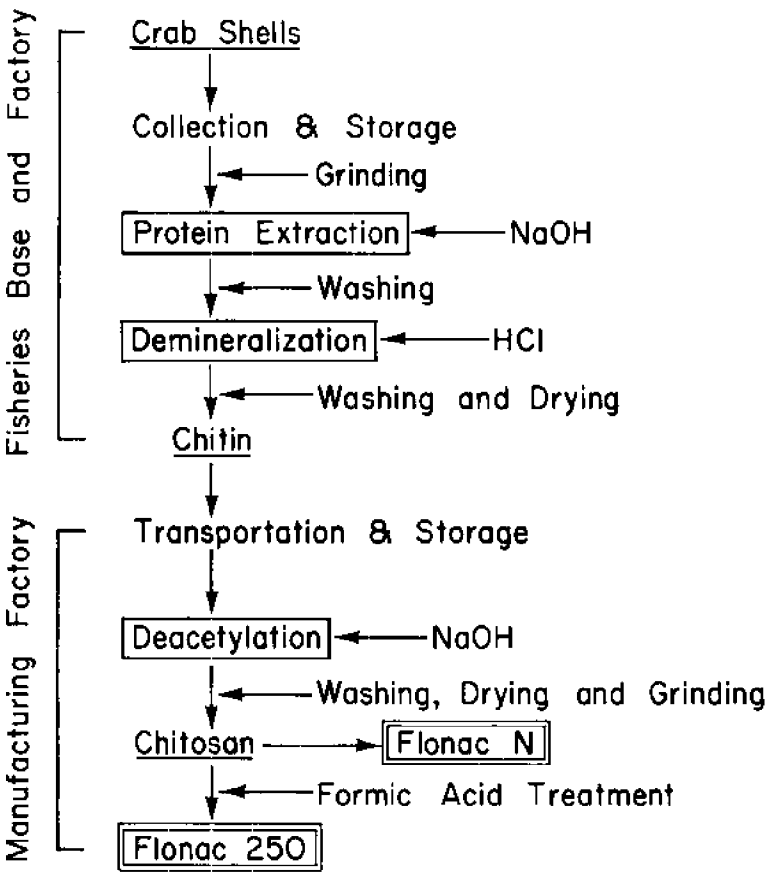


Figure 2. Manufacturing Diagram of Flonac N and Flonac 250

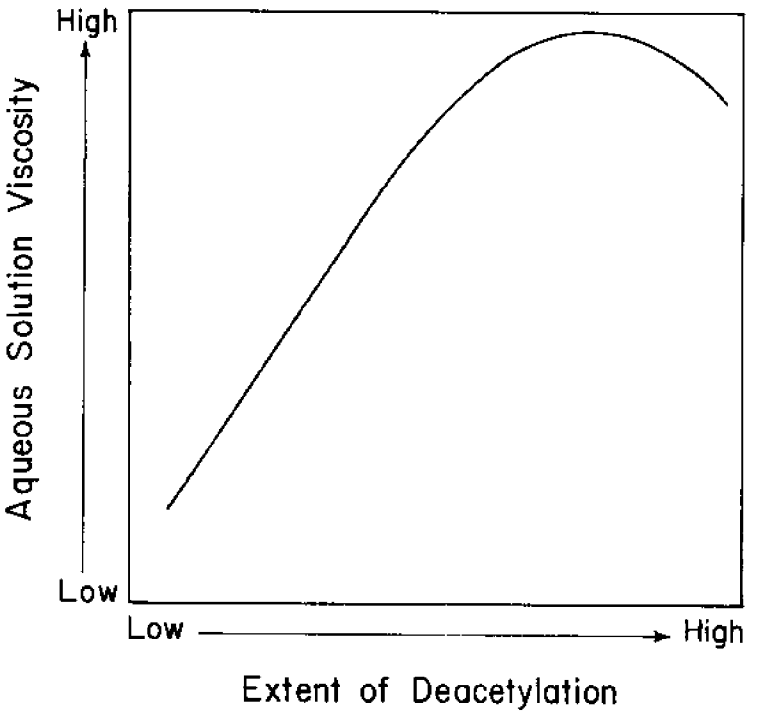


Figure 3. Relationships between Chitosan Solution Viscosity and Extent of Deacetylation

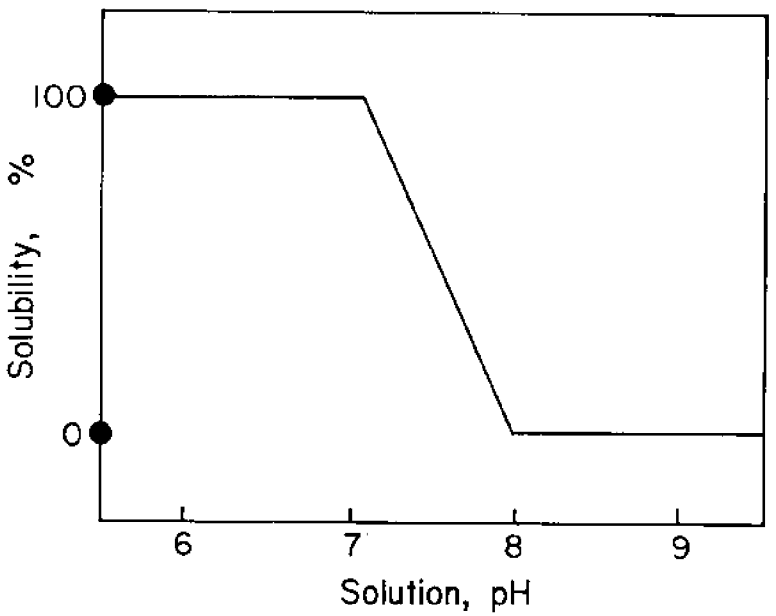


Figure 4. Solubility of Chitosan in an Aqueous Solution as a Function of pH

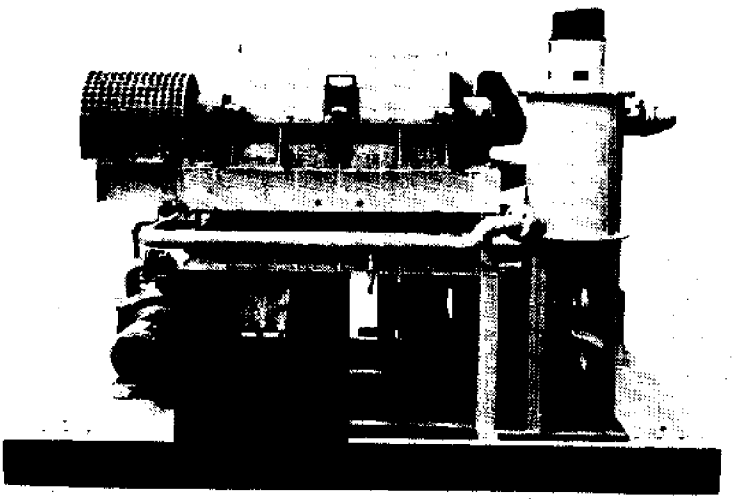
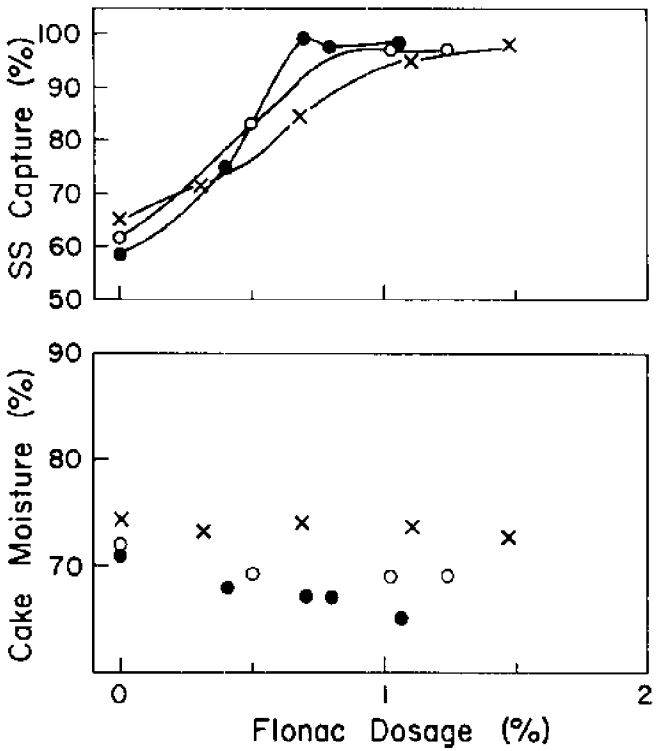
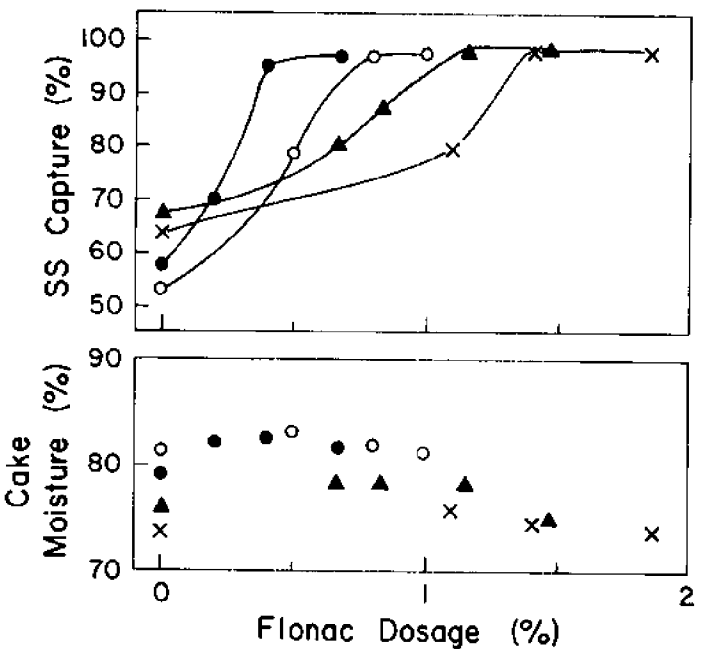


Figure 5. Uncovered SD Sludge Centrifuge, Model 700P



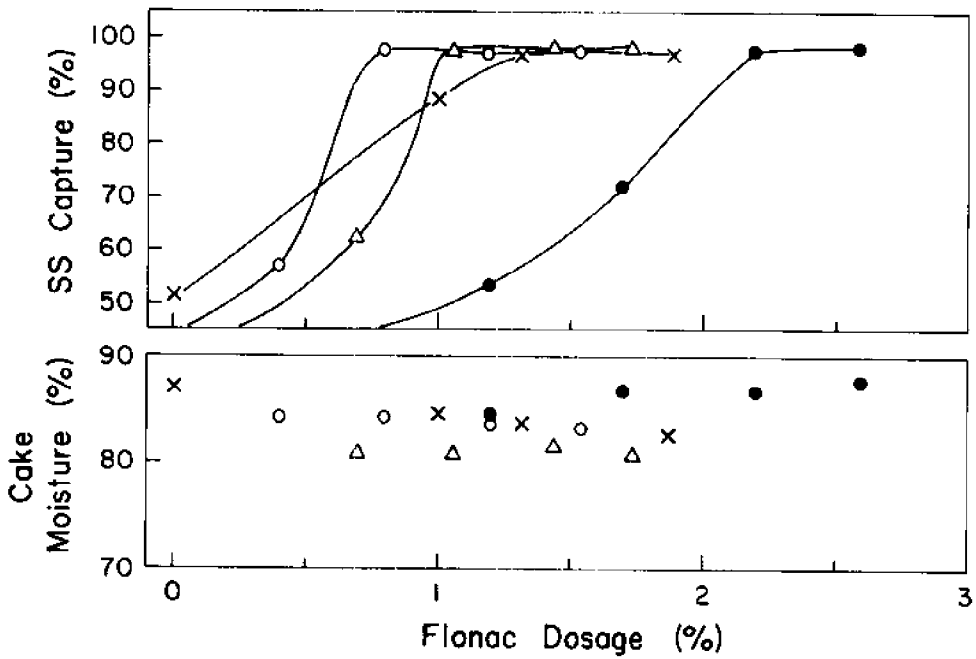
	Plant	pH	TS (%)	SS (%)	VTS (%)	Flonac
● Y	Plant	7.7	3.67	3.58	32.6	250
○ Y	Plant	7.5	5.04	4.82	39.7	250
× T	Plant	7.3	3.05	2.86	43.5	250

Figure 6. Effects of Flonac 250 on the Dewatering of Anaerobically Digested Sludge



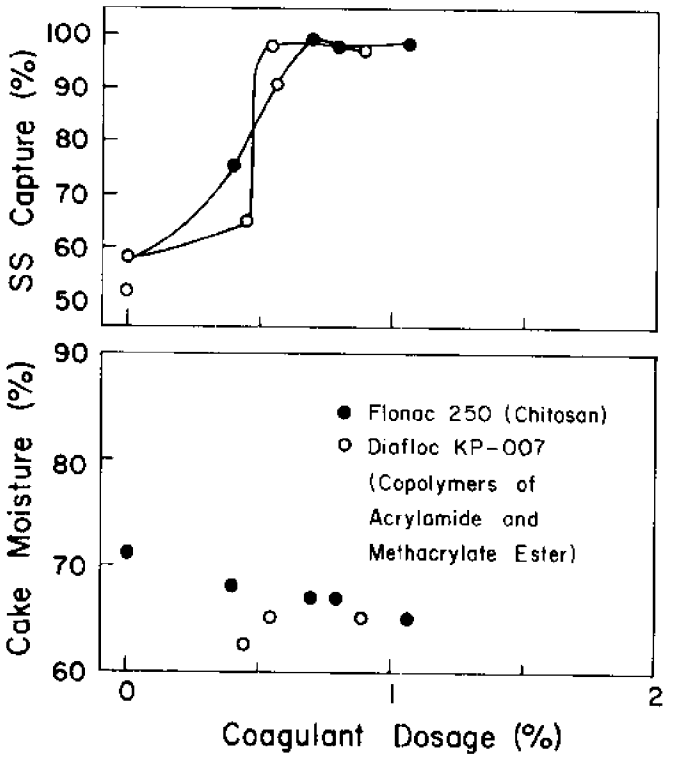
	Plant	pH	TS (%)	SS (%)	VTS (%)	Flonac
● M	Plant	5.5	2.40	2.18	64.5	250
○ A	Plant	6.2	2.07	1.99	66.7	250
× H	Plant	6.3	2.85	2.48	58.4	250
▲ O	Plant	6.0	4.47	4.37	60.2	250

Figure 7. Effects of Flonac 250 on the Dewatering of Mixed Raw Sludges (Primary + Excess Activated Sludge)



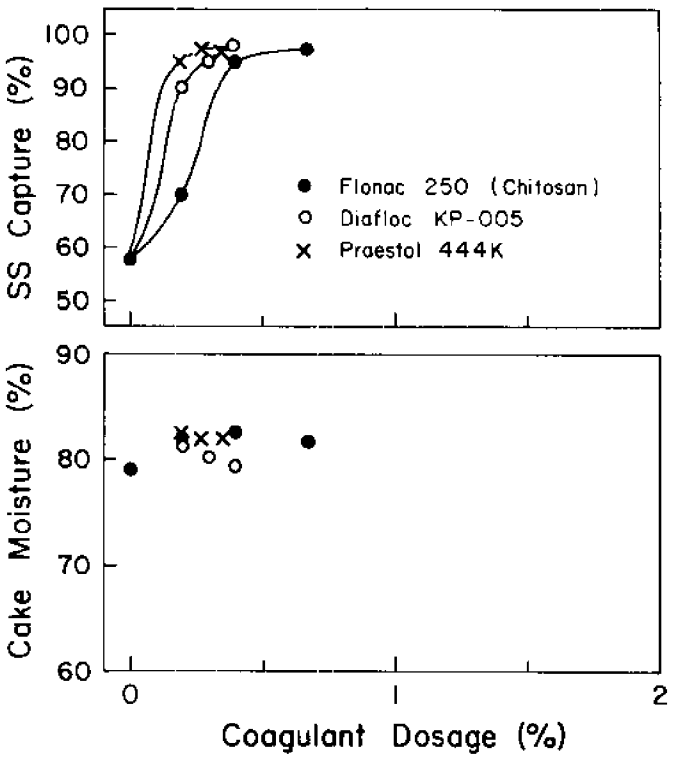
		Plant	pH	TS (%)	SS (%)	VTS (%)	Flonac
●	T	Plant	6.7	1.50	1.47	85.0	250
○	M	Plant	6.5	1.78	1.70	78.0	250
×	C	Plant	6.4	1.09	1.03	82.6	250
△	P	Plant	6.1	1.85	1.81	81.5	250

Figure 8. Effects of Flonac 250 on the Dewatering of Excess Activated Sludge



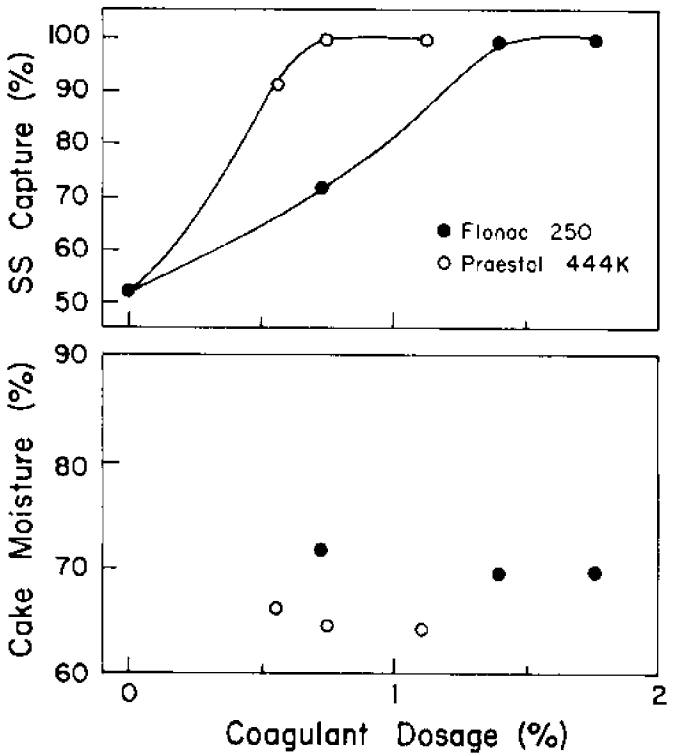
Y Plant	pH	TS (%)	SS (%)	VTS (%)
	7.4	4.83	4.60	33.0

Figure 9. Dewatering Performance of Flonac 250 and Diafloc KP-007 on Anaerobically Digested Sludge



M Plant	pH	TS (%)	SS (%)	VTS (%)
	6.3	2.35	2.12	64.5

Figure 10. Dewatering Performance of Flonac 250 and Other Synthetic Polyelectrolytes on Mixed Sludges (Primary + Excess Activated Sludge)



K Plant	pH	TS (%)	SS (%)	VTS (%)
	6.6	3.52	3.43	56.2

Figure 11. Dewatering Performance of Flonac 250 and Praestol 444K on Mixed Sludges (Primary + Excess Activated Sludge)

THE RECOVERY OF PROTEIN AND PIGMENTS FROM SHRIMP AND CRAB MEALS AND THEIR USE IN SALMONID PIGMENTATION

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ABSTRACT

A process was developed for the recovery of shrimp protein as a by-product of a chitin-recovery operation. The protein was extracted and precipitated from shrimp waste by acid and heat treatment. The coagulum was found to be 80.5% water and 7.6% ash, or 10% calcium and 74% protein on a dry-weight basis. It was low in bacteriological counts, had little odor and compared favorably to reported amino-acid profiles for other shrimp meals. The protein and pigment extract of the meal was fed to rainbow trout. After two weeks, significant amounts of astaxanthin and its isomers were incorporated into the flesh and skin of the trout. The results show that the shrimp coagulum could be used as a pigment and protein source for salmonids raised in aquaculture.

INTRODUCTION

Agricultural and marine food industries are established for primary products such as grain, canned vegetables, shrimp or crab meal and fish fillets. These industries are often established without much thought being given to environmental problems or by-products. In some cases, as in the crustacean meat industries, the waste material can represent an amount greater than 80% of the landing. Disposal operations in the future will have to meet increasingly more stringent ecological standards, as well as attempt to recover valuable protein and other products. These waste products must compete with other products that they might replace, such as protein, lipids, etc., or they must create their own market. The economic stability of a processing plant cannot help but be improved if by-products can be developed from waste. The total utilization of renewable resources certainly is a goal that is worth pursuing and can be reached as new technology is developed.

In terms of dollars, shrimp is an extremely valuable fishery. Current domestic and imported shrimp amount to more than a half billion pounds per year (7, 8). In processing shrimp, the head and hard carapace are removed during semi-mechanized peeling operations. Waste material accounts for approximately 70% of the whole shrimp and may approach 85% in the processing of other crustaceans such as the Atlantic deep-sea red crab.

It has been estimated that the solid waste from the crab industries is now in excess of 10^6 Kg/year in the United States (7). Shrimp and crab meal made from this waste has had limited use as a protein supplement because of the large proportion of exoskeleton material in the meal and, in most cases, the taste of the product. Although the protein level on a dry-weight basis varies from 25-40%, the high calcium level may have a deleterious effect on its overall nutritive value.

Crustacean meals have also been used for their pigment content, especially astaxanthin and astaxanthin ester. Lambertsen and Braekkan (5) determined the astaxanthin content of a number of Norwegian and foreign dried shrimp meals and compared these to vacuum dried meals. The astaxanthin content of industrial meals is generally low, and can even be absent in some commercially available meals. Higher levels were obtained with vacuum dried meals but even these, under storage, lose pigmentation. Other authors (4, 9, 11) have shown that careful drying of crustacean meals allows their use as a pigment source. The use of the whole meals from deep-sea red crab as a pigment source has not been satisfactory, however, because of the difficulty of the biological extraction, and other meals have proved equally unsatisfactory because of inert material and pigment instability under normal storage conditions.

We would like to describe the preparation of a shrimp-protein coagulum as a by-product of chitin processing at Marine Commodities International in Brownsville, Texas, in which the recovered protein was intensely red (3). This red protein was evaluated as a protein and was tested as a pigment source for salmonids.

The shrimp-waste material from mixed shrimp species was obtained from processing plants in Mexico. The mechanical separation of the proteins from shrimp-processing waste was accomplished by an initial grinding to produce a particle size no greater than 10 mm. The resulting slurry was passed through a Baader Fish Bone Separator to remove the shell material. The protein slurry was acidified with acetic acid to the isoelectric point of the protein (pH 4.5), treated with 0.1% TBHQ Tenox (Eastman Chemical) as an anti-oxidant and allowed to coagulate for one hour at 80° C in a stainless-steel tank. The coagulum was dewatered by filtering in cloth filter bags or centrifuged in a Sharples P-600 horizontal bowl centrifuge. The coagulum was packaged in air-tight bags, frozen and shipped by air freight in dry ice to Rhode Island.

The characteristics of the coagulum were determined by several independent laboratories as well as by Marine Commodities International (MCI) and the University of Rhode Island (URI). Amino-acid analysis was performed by Texas A & M University laboratories (personal communication with R. Nickleson) according to the method of Cobb and Hyder (1) for which a fully automatic 120C Spinco amino-acid analyzer was employed. Microbial counts were performed by Texas A & M University laboratories (personal communication with R. Nickleson) using the method of Surkiewicz (14). Moisture, ash and protein were determined by the AOAC methods (1970).

The carotenoids were extracted and identified by the method of Saito and Regier (9) and Kuo et al. (4).

A commercial trout feed manufactured by Zeigler Brothers Feed Mills, Inc., of Pennsylvania was used to feed the control fish. This diet was found to contain 35.3% crude protein, 6.3% crude lipid, 4.9% fiber, 10.4% ash, 10.0% water and 33.1% nitrogen-free extract (4), plus amounts of β-carotene (1 µg). Astaxanthin was not isolated from this diet (4). The fish were fed this mixture at the hatchery prior to our purchase, and the feed was continued as a control.

The desired amount of carotenoids extracted from the frozen shrimp protein was dissolved in a small amount of petroleum ether (PE), and this was added to the control pellets. The PE was removed under vacuum in a flash evaporator at 40° C. The pigmented diet was kept in a dark refrigerator prior to feeding.

The shrimp protein was mixed with other nutrients to constitute 10% of the mixture by dry weight. The diet was used on the Oregon Test Diet (6). The procedure was similar to that used by Kuo et al. (4).

Fish culture

Rainbow trout (Salmo gairdneri) were purchased from American Fish Culture, Rhode Island, and held in 125-gallon fiberglass tanks with flowing water (2 gal/min) and supplemental aeration. The water temperature was maintained at 11° C and the O_2 level was maintained at 8 ppm (6, 4). Sixty fish of 140 g average size were distributed evenly between the four tanks. After 30 days, the fish were fed the diets at the rate of 3% of body weight per day.

Analysis of carotenoids in fish

The fish were sacrificed after the feeding periods. The fish flesh was cut into pieces and ground in a blender in acetone to extract the pigments. The pigments were purified and identified (3, 4).

The protein coagulum was found to have the following analysis: moisture 80.5%; ash, 1.5%; calcium 0.2%; protein 14.4% (2.2% N x 6.25 uncorrected for chitin N). Table 1 shows the amino-acid profile of the protein coagulum compared to casein and to other crustacean meals. The protein coagulum is marginally different from other shrimp meals. The shrimp protein is higher than casein in arginine, aspartic acid, glycine; lower in valine, glutamic acid and proline; and similar in the other amino acids. Tryptophan and cystine were not analyzed by this method. The protein coagulum gave a calculated PER of 2.709. Bacteriological analyses on the coagulum were conducted on similarly prepared products and were found to be low in bacterial contamination.

The carotenoid content of the shrimp meal as received from MCI was determined on the frozen samples and on a tray-dried sample (Table 2). The frozen sample contained trace amounts of the typical plant pigments lutein and zeaxanthin. These pigments were the main pigments in the fish because of the commercial feed that was used. Other minor pigments were isolated which are characteristic of crustacea (10). Astaxanthin and astaxanthin ester together constituted the main pigments.

A rather large amount of astacene was also isolated, presumably as a result of the various handling and processing steps prior to freezing. It can be seen that the tray drying (70° C, 12 hours) of the coagulum resulted in a great loss of pigmentation. While the dried protein was red, it contained only small amounts of astaxanthin and astacene.

Table 1. Amino-Acid Profile (as gAA/16g Nitrogen) of Freeze-Dried Shrimp Coagulum and Other Proteins (3)

Amino Acid	Casein (15)	ISP (15)	Sun-Dried Shrimp Meal (8)	Spray-Dried Shrimp Meal (2)	Freeze-Dried Coagulum
Arginine	4.07	7.8	6.31	8.06	6.31
Histidine	3.02	2.5	1.90	2.97	2.09
Isoleucine	6.55	4.9	3.26	5.17	4.68
Leucine	10.05	7.7	7.57	8.14	8.10
Lysine	8.01	6.1	6.17	8.34	7.36
Methionine	3.08	1.1	2.84	2.60	2.62
Phenylalanine	5.39	5.4	4.56	5.05	4.01
Threonine	4.28	3.7	4.28	3.91	4.65
Tryptophan	1.33	1.4	1.26	6.73	
Valine	7.39	4.8	4.42	5.62	5.02
Tyrosine	5.82	3.7	3.64	3.61	3.31
Alanine	3.35	3.9	5.29	7.14	7.11
Aspartic Acid	7.39	11.9	10.74	8.63	11.13
Cystine	0.38	1.2	1.59		
Glutamic Acid	23.05	20.5	15.46	17.8	14.38
Glycine	1.99	4.0	4.29	7.52	6.45
Proline	11.75	5.3	3.44	4.54	7.05
Serine	6.65	5.5	4.53	4.69	5.20

A pigment extract was made of the frozen shrimp meal, and this was fed together with a commercial ration. Table 3 shows the results of feeding the shrimp-protein extract for two weeks. Astaxanthin, astaxanthin ester and astacene were absorbed in about equal amounts. The higher levels of the feed pigments (lutein and (zeaxanthin) found in the fish were unexpected and may represent an increased absorption of pigments due to higher lipid levels.

Table 3 also shows the results of feeding a 10% shrimp-protein diet for two weeks. This diet contained less than 1/10th the amount of pigment contained in the 20 mg % diet. These results show that the fish also contained somewhat less than 1/10th the amount of pigment. We expected this level to be far lower since some difficulty was experienced in getting the fish to take either the control or the shrimp-meal diet. Initial studies with a diet of corn oil as a lipid source supplemented with ω-3 containing lipids was not eaten by the fish. The results reported here were with a fish diet containing herring oil. The initial mean weight of the fish was 140 g. At the end of the feeding trials, the control fish and the fish fed the two pigmented diets averaged 174-175 g.

Table 2. Composition of Carotenoids in Shrimp Meal before and after Drying (3)

Carotenoids	Before Drying (µg/g dry basis)	After Tray Drying
3,3'-Dihydroxy-β-carotene	1.36	
Echinenone	0.25	
Isocryptoxanthin	0.38	
Canthaxanthin	0.39	
4-Keto-4'-hydroxy-β-carotene	0.74	
Dihydroxypiradixanthin (?)	4.66	
Lutein	trace	
Zeaxanthin	trace	
Astaxanthin ester	66.1	10.3
Astacene	55.4	6.26
Astaxanthin	7.19	trace

Table 3. Composition of Caroteoids in Fish Fed Pigmented Diets for 2 Weeks (3)

Carotenoids/µg/g dry weight	Control	Pigmented	Control	10% Shrimp Meal
α-Cryptoxanthin	0.10	0.18	trace	trace
Canthaxanthin	trace	trace	-	trace
Lutein	1.79	2.04	1.3	1.76
Zeaxanthin	0.75	0.9	0.16	0.21
Astaxanthin ester	-	0.30	-	0.075
Astacene	-	0.28	-	trace
Astaxanthin	-	0.28	-	trace

In related experiments, whole freeze-dried red-crab waste (Geryon quinquedens) was fed to rainbow trout (4). Fish were sacrificed after 15- and 23-week feeding periods (small fish) and 4-week feeding periods (large fish). In the control fish, small amounts of lutein ester, trace amounts of α-cryptoxanthin and a few unidentified carotenoids were found. The experimental fish had a mean weight of 34 g after 15 weeks (2.21 µg total carotenoids per fish) and 51 g after 23 weeks (3.162 µg total carotenoids/per fish) of feeding on the 20% crab meal diet (15.4 µg crude protein/g diet). Astaxanthin ester, free astaxanthin and astacene were isolated from these fish. In addition, the plant pigment lutein ester was also isolated (Table 4). The amount of total carotenoids per gram fish

was almost the same in the fish after 15- and 23-week feeding periods--0.065 and 0.062 µg total carotenoids per gram respectively. Large fish (132 g) were fed the 20% crab-meal diet to assess the effect of size on the uptake of carotenoids from the crab meal. After 4 weeks the control (167 g) and the crab-fed fish (165 g) had increased in size, but the level of astaxanthin was the same as in the smaller fish (Table 4).

Table 4. Carotenoid Content of Fish Fed 20% Crab-Waste Diet (4)

	Concentration of Carotenoids (µg/g)				
Pigments	Control* Diet 15 Weeks	Crabmeal Diet 15 Weeks	Crabmeal Diet 23 Weeks	Control** Diet 4 Weeks	Crabmeal Diet 4 Weeks
Total:					
Astaxanthin	-	0.051	0.052	-	0.056
Zeaxanthin	-	-	-	0.26	0.23
Lutein	0.052	0.014	0.01	0.33	0.26
Canthaxanthin	-	-	-	0.01	trace

*25 g fish at the beginning of the experiment
**132 g fish at the beginning of the experiment

Large fish (85 g and 132 g) were fed commercial trout pellets on which pigment extracts (0.1 and 0.2 mg/g diet) had been adsorbed. After 4 (Table 5) or 7 (Table 6) weeks on this experimental diet, the trout had a strong orange-pink flesh and fins and a vivid red streak on the skin, whereas the control trout flesh remained grayish white in color and only a faint yellow-orange streak was present on the skin. The pigmented-diet-fed fish as well as the controls exhibited similar mortalities over the feeding periods.

Table 5. Carotenids of Rainbow Trout Which Were Fed on Pigmented Diet (0.2 mg carotenoids/g diet) for Seven Weeks (4)

Experiment No.	No. of Fish	Wt. per Fish (g)	Color of Flesh	Main Carotenoids (µg/g fish)*				
				Astaxanthin Ester	Astaxanthin	Astacene	Lutein Ester	Lutein
1	2	91.5	faint pink	0.5	0.3	0.18	1	trace
2	2	115	pink	1.1	0.39	0.20	0.1	0.08
3	1	172	strongly orange-pink	0.61	1.57	0.09	0.56	0.02

*The concentration of carotenoids was calculated in saponified forms.

A second experiment was run with larger fish (132 g with 0.1 mg/g diet) over a 4-week period (Table 6). The final average weight of the 5 fish sets were 167 g for the control and 169 g for the extract-fed fish. The larger fish were found to be rapidly pigmented over the 4-week period with 0.1 µg/g carotenoid content added to the feed.

Table 6. Carotenoids in the Rainbow Trout Fed on 0.1 mg/g diet for 4 weeks (4)

Pigment	Concentration (µg carotenoid/g Fish) Control	0.1 mg/g
Total astaxanthin	-	0.88
Zeaxanthin	0.26	0.21
Lutein	0.33	0.27
Canthaxanthin	0.013	0.0008
α-Cryptoxanthin	trace	trace

The fish in one study were of a nearly uniform size at the start of the feeding experiments. After a 4-week adaptation period on a control diet and a 7-week period on the pigmented diet, the fish went from an average weight of 85 g to a range of 90 g to 172 g. The color of the flesh ranged from a faint pink to a strong orange-pink. The large fish contained the greatest concentration of pigment. The fish clearly obtained astaxanthin from the diet as had been suggested by Steven (13).

DISCUSSION

The total utilization of crustacean waste has been the subject of the reports of numerous investigators. The waste is composed mainly of calcium carbonate, chitin, protein and carotenoids. Variation in proximate analyses will depend on the species, sex, metabolic state and subsequent processing, handling and storage. The shrimp-protein concentrate used in these experiments was prepared by MCI as a by-product of a chitin-chitosan recovery procedure. Normally an alkaline digest is used for the recovery, but experiments were conducted with a mild acid coagulation since alkali is known to convert astaxanthin to astacene. The resulting product was found to be low in calcium and bacterial contamination and high in protein quality and astaxanthin ester/astaxanthin content.

The results of these experiments show that the protein or a pigment extract of the protein would be effective in the pigmentation of salmonids. These results also show that the protein should have properties similar to the protein in shrimp meal.

The red or yellow color of the fins, skin and flesh of salmonid fish is due to the carotenoids. The carotenoids that occur most commonly are astaxanthin and its ester, cantaxanthin, lutein and, to a lesser extent, β-carotene (Figure 1). Trout and salmon reared on most commercial feeds lack the natural red color because these red pigments are not contained in the feeds. A number

of authors have fed salmon various preparations containing these pigments and have established the fact that the diet must contain these pigments if the flesh is to contain the natural coloration (4). At the moment, there is no good, stable source of natural salmon pigments. A number of authors have shown that various crustacea such as the pelagic red crab (11), extracted or whole, shrimp waste (12), crab and shrimp waste (9), and deep-sea red crab (4) can be used. These reports would not appear to be a long-term answer to the needs of aquaculture for pigments. The limitations are as follows:

1) Difficulty of pigment extraction from the meal. This varies from very difficult for the salmonid in the case of deep-sea red crab to less difficult for shrimp meal.

2) Problem of the stability of the pigments. There are, at the moment, serious problems in pigment stability on drying and storage (Table 2).

3) Whole meals contain inert material such as chitin and deleterious elements such as calcium that detract from the nutritional value of the feed.

There is a market for these pigments in the aquaculture industries. For instance, the price of aquaculture-raised products can be more than 25¢ per pound higher for the pigmented fish.

Because of the high water content and perishable nature of the shrimp protein reported here, the fresh protein is not the complete answer. Drying the protein is also not effective because of pigment loss.

What would appear to be practical would be an integrated approach to chitin recovery in which the pigment was either extracted along with the other lipids or precipitated with the protein at a mildly acid pH (lower levels of pH or alkaline conditions would rapidly destroy astaxanthin). The protein could easily be extracted for its pigment content. Thus, the limitation on shrimp or crab meals for animal use would be removed by the separation of the pigment and other lipids and the protein into separate products. The protein would be colorless and odorless and the pigment would be in an oil that could be suitably protected. The chitin would be unaffected by the removal of products for which there are demonstrated markets.

ACKNOWLEDGMENTS

The author wishes to mention that the results reported here are taken from the work published with T. Kamata, H-C. Kuo and Mr. and Mrs. James Collins. Rhode Island Agricultural Experiment Station No. 1740.

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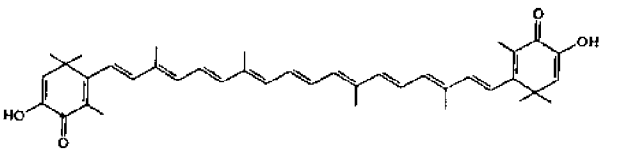

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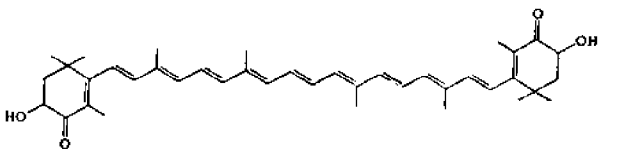

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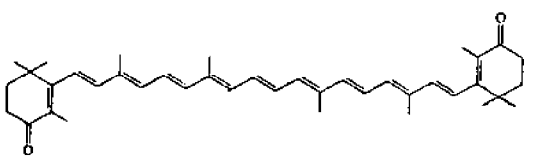

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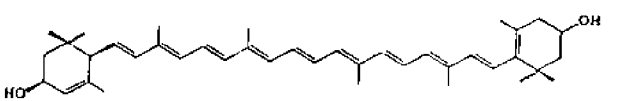
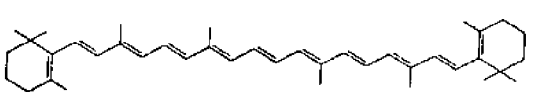


Lutein



β-Carotene

Figure 1. Structure of some carotenoids found in shellfish.

THE CHELATING PROPERTIES OF KYTEX H CHITOSAN*

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ABSTRACT

Kytex H** chitosan has been evaluated as chelating resin by physical and chemical techniques. Porosimetry studies suggest that the flat, angular particles are "gel-like" rather than macroporous with very large (~70 A median diameter) pores. The anticipated rapid absorption of ions was confirmed by direct kinetic measurement.

In contrast to the behavior of commercially available chelating resins, Kytex H chitosan exhibits only an 8% increase in volume upon exposure to base from acid. Columns packed with this chitosan exhibit low back pressures, an effect probably due to the irregular shape of the flake. Chitosan's thermal stability was demonstrated through use of differential thermal analysis and thermogravimetric techniques.

The metal binding capacity of Kytex H chitosan compares favorably with commercially available chelating resins. Chitosan is relatively inert to alkali metals and alkaline earth ions; it is thus superior to the other polymers tested for the recovery of transition metals in alkaline or saline waters. Chitosan's main disadvantage is its solubility in all acid media except sulfuric acid. Although long-term exposure to sulfuric acid does indeed break down the polymer, short-term cycling in dilute acid (< 3%) should have little effect.

INTRODUCTION

Chitosan, a high-molecular-weight linear polymer of anhydro-N-acetyl-D-glucosamine has for many years been recognized as a scavenger for metal ions. This property is derived from the amine function in the C-2 position of the glucose ring. As a weak-base anion-exchange polymer, chitosan, unlike commercial weak-base anionic-exchange resins, may also be classified as a chelating resin. Its complexing ability is a direct consequence of the base strength of the primary amine, and it is most effective for those metals that form complexes with ammonia, e.g., copper, zinc and mercury. Characterization of Kytex H chitosan has revealed a number of advantages for this material over commercially available chelating resins. This work relates the physical and chemical characteristics of Kytex H chitosan and compares them with commercially available chelating polymers, Chelex 100 resin (Bio-Rad Laboratories) and Amberlite XE-318 resin (Rohm and Haas Company).

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*Hercules Research Center Contribution No. 1698

**Hercules trademark for cationic marine polymers.

DISCUSSION OF RESULTS

Physical characterization

Kytex H chitosan polymer exists as flat, angular particles with a ridged structure. A photograph of this material at 1200x magnification is shown in Figure 1. The Kytex H chitosan "flake," sized to pass through a np 8 sieve, is of coarse-particle distribution, 94% greater than 50 mesh. Other pertinent data are given in Table 1. The results of repetitive analysis are shown parenthetically in the table. To obtain a more homogeneous, and thereby more representative, sample of Kytex H polymer, the chitosan was milled through a 30-mesh screen after the addition of dry ice. The chitosan was milled repeatedly and resieved to the desired mesh after the dry ice had evaporated thoroughly. All subsequent experiments were based on the milled material.

Table 1. Kytex II Chitosan, as Manufactured

Shape	Flat, angular particles with ridged or layered structure
Particle distribution	>20 mesh 54.8% 20-50 mesh 40.5% 50-100 mesh 4.4% 100-200 mesh 0.3%
Moisture	9.8% (9.4%)
Ash	1.9%
N	8.2%
NH_2	7.3% (7.71, 7.77%)
% deacetylation	77.9% (82.6%)
Viscosity	2800 centipoises (1% in 1% acetic acid)
Bulk density	0.14 g/cc

Functional group capacity (NH_2)

The total exchange capacity of the resin, 4.8 meq/g, is derived from the amine content of the dry weight of the polymer. The volume capacity, 3.3 meq/ml, was determined by measuring the interstitial column volume of a packed column (1). Interestingly, the fractional column void volume observed, 0.39, compares closely with that for a column of spherical particles, 0.40. Apparently, the irregular shape of the milled and sieved chitosan flake does not permit a tight compaction (the bulk density of the column was 0.28 g/cc).

Thermal stability

A rather surprising observation is the high temperature stability of chitosan, a decomposition temperature of 230° C in air. This value was obtained as the point at which the exotherm (differential thermal analysis, DTA) deviated from the baseline. In a nitrogen atmosphere, no decomposition was observed until ∿260° C. The thermally induced weight loss of chitosan and commercially available chelating resins is shown in Figure 2. The predominant reaction through ∿100° C is that of water loss. The chitosan and Amberlite resins display little change in behavior until about 225° C.

These data do not contradict the result of the differential thermal analysis described above; the weight-loss data indicate production of volatiles, while DTA gives a general indication of chemical or physical transformation.

The low water content of chitosan is a definite advantage when considering thermal stability. Columns packed at ambient temperatures could be operated at, say, 75° C with minor changes in bed characteristics. This would hardly be the case for the commercial resins, which experience approximately 40% weight loss at 70° C. The high temperature limit of chitosan stability (230-256° C, depending upon the atmosphere) may suggest unique uses for this material; chelation is highly unlikely at those temperatures.

Volume expansion

One of the disadvantages of the general class of ion exhchangers is the marked change in bed volume with ion absorption. This swelling or contraction can lead to column breakage or plugging of the bed: a threefold increase in resin volume on passing from the acid to base form is not uncommon. Chitosan, on the other hand, exhibits very little volume change on exposure to acid or base; water:acid:base = 1.0:0.99:1.07. It is difficult to estimate the absolute degree of swelling (volume change) of the chitosan from comparison of its specific volume in organic versus aqueous media. The sponge-like hydrophilic structure of the chitosan permits water to enter the interstices, and therefore the flake appears to have a smaller volume in water than in organic solvents (Table 2). The marked affinity of chitosan for water is reflected in the "weight swelling" value, 0.5 g water/g water-saturated polymer. However, this high water retention does not necessarily lead to a severe increase in volume. The lack of swelling of the polymer was apparent in experiments designed to evaluate the volume capacity; a column was drypacked to a chitosan bulk density of 0.28 g/cc, yet there was negligible resistance to water flow on back-flushing. Quantitative estimates of the pressure drop of chitosan-packed columns also suggest that swelling is minimal (Table 3).

Solubility

One of the prime requirements for an ion-exchange resin is that it remain totally insoluble in the working solution. Although chitosan is reported to be insoluble in sulfuric acid, long-term exposure to sulfuric acid does indeed break down the polymer. After 57 days, 6.7% of the chitosan had dissolved in 10% H_2SO_4, while 1.1% had dissolved in 3% H_2SO_4 (these values are corrected for $CaCO_3$ and are based on a chitosan carbon content of 45%). Since chitosan exhibits varying degrees of solubility in organic and other inorganic acids, sulfuric acid would appear to be the reagent of choice for the elution of bound metal ions. However, these data strongly suggest that the chitosan flake must be modified, i.e., crosslinked, to withstand prolonged severe treatment. Short-term cycling in dilute sulfuric acid should have little effect.

Porosimetry

The porosity of an ion-exchange resin can determine the rates of the ion-exchange reactions and the limiting size of ions that can penetrate a resin

Table 2. Volume Expansion of Kytex H Chitosan (50-100 mesh) by Pycnometry (25°C)

Sample	Medium	Specific Gravity (g/cc)	Specific Vol. (cc/g)
Freeze-dried[a]			
0.4% H_2O	H_2O	1.572	0.636
2.5% H_2O	H_2O	1.604	0.624
2.5% H_2O	Iso-octane	1.490	0.671
Oven-dried[b]			
	H_2O	1.621	0.617
	Iso-octane	1.464	0.683
	Heptane	1.447	0.691
	n-Butanol	1.475	0.678
	Ethylene glycol	1.409	0.710
Water-saturated[c]			
49.4% H_2O	H_2O	1.216 1.564[d]	0.822 (0.639)[d]
	H_2O	1.223 1.592[d]	0.818 (0.628)[d]
Water-saturated; acid/base			
Control	H_2O	1.246	0.803
1 N H_2SO_4	H_2O	1.262	0.792
1 N NaOH	H_2O	1.166	0.858

[a] 16-18 hrs; H_2O determined by Karl Fischer method

[b] 6 hrs, 105-110° C

[c] Water-saturated material aspirated for 30 minutes; water determined by drying at 105-110°C for 6 hrs

[d] Corrected for water content

Table 3. Pressure-Drop Characteristics of Columns Packed with Kytex H Chitosan

Mesh	Column Dimensions	Pressure at Flow[a]
50-100	35.6 cm, 0.4 cm i.d.	None at 10 ml/min[b]
100-170	18.4 cm, 0.4 cm i.d.	160 psi at 10 ml/min

[a] Pressure characteristics maintained even after flow kept at 2 ml/min for 90 minutes

[b] Evidence of channeling

matrix. Ion-exchange matrices are essentially of two types: (A) a single-phase homogeneous gel, e.g., Chelex 100; or (B) a two-phase, heterogeneous structure often termed macroporous, e.g., Amberlite XE-318. Physical methods used to determine the pore volume, size, surface area, etc., on the dry material have questionable significance when solution behavior is to be predicted. This is especially true for homogeneous gel-type particles. Nevertheless, porosimetry can be of value as an indicator of solution behavior in nonswelling systems (organic solutions or nonswelling polymers, e.g., chitosan). Figure 3 details the pore characteristics for the three chelating polymers, chitosan, Chelex 100 and Amberlite XE-318. A quantitative summary of the data, obtained on an Aminco 60,000-psi porosimeter, is presented in Table 4. The similarity between the chitosan and Chelex resin suggest that chitosan, too, has a gel-like structure, i.e., very large pores. Some of the pore structure of chitosan may be due to inter-ridge spacing seen in the micrograph. This view is supported by a severe decrease in porosity on drying the polymer (as opposed to lyophilization), an effect not observed with the more structured macroporous Amberlite resin. As is expected, grinding the chitosan to a smaller mesh leads to increased pore area and thereby a more accessible pore volume (Table 4). The observation of very large pores predicts reasonably rapid ion absorption for the chitosan; the converse should hold for the Amberlite resin. This prediction was confirmed by the direct measurements of the rate of absorption of silver ion.

Surface functionality

Electron spectroscopy for chemical analysis (ESCA) of the three chelating polymers in the silver form (Kytex H, Chelex 100, Amberlite XE-318) was employed to identify the surface atoms and establish the surface coordination dentate number, the number of ligand atoms per silver atom; ESCA detects those atoms within 20-60 Å of the surface. Although not unequivocal, the data presented in Table 5 suggest that chitosan has the most surface nitrogen available for chelation. The other resins appear to have a more carbonaceous outer layer, higher C/O. The Amerlite resin bead seems to have its oxygen and nitrogen functionality "buried internally." The necessity for the presence of N and O ligands is suggested by binding capacity data and pH behavior.

Metal binding capacity of chitosan

Most of the work concerning the chelating properties of chitosan has been done by R.A.A. Muzzarelli (4). In addition to the general literature, M.G. Muzzarelli holds a patent covering the chelating properties of chitosan (3). Masri, Reuter and Friedman (2) have compared the metal-binding capacity of chitosan with poly (p-amino-styrene). The binding capacity of chitosan for various metal ions varies between fractions of a millimole to about 3 mmols depending upon the author. The apparent discrepancy between these values can be resolved by realizing that the total binding capacity is often a function of the total metal ion concentration exposed to the polymer. For the data offered by R.A.A. Muzzarelli, the maximum metal ion the polymer has seen is approximately 0.1 mmol per gram; for Masri et al. the ratio of metal/chitosan is 20 mmol/g. Table 6 compares the metal binding capacity of Kytex H chitosan with the two commercial chelating resins. The proton binding capacity is determined by the number of amino groups, in the case of Kytex H, and by the nominal values on the bottle of resin for the commercial

Table 4. Porosimetry of Chelating Polymers

Sample	Treatment	Mesh	Pore Characteristics: Median [Dia.(μ)]	Total Vol. (cc/g)	Av.Dia.(μ) (4 V/A)*	Total Area (m^2/g)
Kytex H (Chitosan)	As manufactured	>20	48.6	0.41	22.9	0.072
		20-50	69.8	0.89	23.7	0.15
		50-100	73.4	2.61	42.3	0.25
	Dry-ice milled	>50	66.1	0.72	21.2	0.14
		50-100	73.2	1.80	43.3	0.17
	Room temp. milled	50-100	58.8	1.03	12.3	0.33
	Dried-ice milled Lyophilized	50-100	64.6	1.71	1.94	3.52
	Dry-ice milled Dried 6 hrs @105°C	50-100	81.6	1.59	19.2	0.33
Chelex 100	As manufactured Lyophilized	50-100	31.6	1.94	12.6	0.61
	As manufactured Dried 6 hrs @105°C	50-100	64.5	0.73	49.1	0.060
Amberlite XE-318						
	As manufactured Lyophilized	50-100	0.094	0.90	0.096	37.7
	As manufactured Dried 6 hrs @105°C	50-100	0.077	0.53	0.065	32.4

*Assumes cylindrical pores of circular cross-section.

Table 5. Surface Functionality[a] of Chelating Polymers (Ag^+ Form) as Determined by ESCA

Sample[b]	Carbon / Oxygen	Nitrogen / Silver
Kytex H (20-50 mesh)	2.00	1.25
Kytex H	1.92	1.28
Chelex 100	2.82	1.07
Amberlite XE-318	5.92	0.38

[a]Only those atoms listed were considered

[b]Resins are 50-100 mesh unless otherwise indicated

products. The anion exchange capability of chitosan is demonstrated most effectively by the very high binding capacity for sodium dichromate. The variation in copper binding capacity with pH leads to the prediction that the protonation constant of the chitosan polymer is at least $10^{3.8}$. This is estimated from the decade increase in copper-binding capacity between pH 3.4 to 4.8. It is important to bear in mind that the metal/chitosan ratio for this work is 2-3 mmol/g versus the 20 mmol/g used to obtain the data in reference 2. Thus, our binding capacity would naturally be somewhat lower than that observed by those workers.

An outstanding characteristic of the chitosan polymer is the lack of binding capacity for alkali metal and alkaline earth ions. This is shown most dramatically for magnesium sulfate in Table 6. To exploit this phenomenon, the chitosan was exposed to low levels of copper and zinc ions in moderately alkaline, medium salinity water (Table 7). The pH's observed were those measured in the presence of the polymers. Prewashing of the ion-exchange resins did not affect these values. As the data indicate, chitosan has a clear advantage for the binding of transition metal ions that form strong ammonia complexes in the presence of high concentrations of calcium and magnesium. This was also demonstrated by Muzzarelli at microgram levels. A possible objection to the data is that, for the Chelex and Amberlite resins, the metal ions were removed from the solution by hydroxide formation and were thus not available for binding to the resin. However, pre-analysis of the metal ion stock solutions confirmed the desired pH and concentrations. The increasing pH must therefore be due to impurities within the ion exchange resins. If in fact the pH of the solutions in the presence of these commercial ion-exchange resins is $\sim$9-10, then the metal hydroxides would have precipitated on the resins and would have been removed from solution. At that point, it is of little concern as to where the hydroxide is formed, so long as the metal ion is indeed removed from solution.

Kinetics of ion absorption by chelating resins

The microkinetics of ion absorption of chelating resins is influenced not only by diffusion of ions to the resin, but by the second-order reaction of the

Table 6. Comparison of Metal-Binding Capacities of Chelating Polymers

(polymers are 50-100 mesh)

Solution	Salt	pH	mmol/g (Dry Basis) Kytex H	Chelex 100	Amberlite XE-318
	H+		4.8	2.9	5.3
A	$Cr(NO_3)_3$	3.0-3.4	0.011		
		5.0-4.4	0.19		
A	$Na_2Cr_2O_7 \cdot 2H_2O$	2.4-3.4	4.85		
		5.0-5.7	4.45		
A	$CuSO_4$	2.0	0.011		
		2.6		2.23	
		3.0-3.4	0.24		
		4.8-4.5	2.60		
		5.0			2.62
C	$Cu(NH_3)_4^{2+}$ (0.3 M NH_3)	10	2.65	2.70	2.96
A	$MgSO_4$	2.0-2.1	0.0033		
		4.0-6.0	0.012		
		8.5	0.043		
		7.6		1.99	
		9.2			2.20
B	$Zn(NO_3)_2 \cdot 6H_2O$	6.1-6.2	1.34		
		3.1-3.0		2.07	
		6.2-5.8			3.54
B	$Cd(NO_3)_2 \cdot 4H_2O$	7.2-6.5	1.25		
		3.8-3.5		1.81	
		4.0-3.1			2.35
B	$Pb(NO_3)_2$	5.1	1.71		
		4.0-3.1		2.35	
		4.6			3.07

Test Conditions: Solution 15 ml volume
A: 15 ml of 0.2 M salt, 2 ml of 0.16 M Na_2SO_4
B: 15 ml of 0.2 M salt
C: Stock: (34 ml of 0.2 M $CuSO_4$, 5 ml of 0.16 M Na_2SO_4, 2 ml concentrated NH_4OH) to 50.0 ml

Polymer: Kytex H: ~115-01.17 g
Chelex 100, Amberlite XE-318: 0.71 g
Polymers are of equivalent weight on a dry basis.
Equilibrate with stirring for 20 minutes.
Filter (glass disk) wash with 3 20-ml aliquots of distilled water
Analyze ashed polymer for metal content by atomic absorption

Table 7. Comparison of Metal-Binding Capacities of Chelating Polymers in a Synthetic Industrial Cooling Water[a]

Chelating Polymer	Amount Recoverable (10^2 mmol/g)			
	pH	Cu^{2+}	pH	Zn^{2+}
(50-100 mesh)				
Kytex H (Chitosan)	6.9-7.0	2.96	7.4-8.0	2.13
Chelex 100	9.2-10.0	1.02	9.6-10.0	1.00
Amberlite XE-318	10.8-10.9	1.12	10.6-10.8	1.54

Test Conditions: Solution: 25 ml volume

$Cu(NO_3)_2 \cdot 3H_2O$: 1.69×10^{-4} M (10.7 ppm Cu) in cooling water

$Zn(NO_3)_2 \cdot 6H_2O$: 1.76×10^{-4} M (11.5 ppm Zn) in cooling water

Polymer: Kytex H: 0.15-0.17 g
Chelex 100, Amberlite XE-318: 0.71 g
Polymers are of equivalent weight on a dry basis

Equilibrate with stirring for 30 minutes.

Filter (glass disk) wash with 3 25-ml aliquots of distilled water.

Analyze ashed polymer for metal content by atomic absorption.

[a]Composition of synthetic industrial cooling water:

$CaCl_2 \cdot 2H_2O$	442 mg	3.10×10^{-3} M	300 ppm Ca hardness as $CaCO_3$
$MgSO_4$	120	9.97×10^{-4}	100 ppm Mg hardness as $CaCO_3$
NaCl	61	1.04×10^{-3}	400 ppm Cl as NaCl
Na_2SO_4	360	2.53×10^{-3}	500 ppm SO_4 as Na_2SO_4
$NaHCO_3$	17	2.20×10^{-4}	10 ppm alkalinity as $CaCO_3$

in 1.00 liter distilled water, adjusted to pH 6.3 with 1 N H_2SO_4

ligand with the metal ion to form the chelate complex. In some cases, these are responsible for the slow kinetics of ion absorption. The data in Table 8 illustrate the rate of silver ion uptake by chitosan in comparison with the Chelex resin and Amberlite resin. These values are to be considered as relative rather than absolute; the kinetics are strongly influenced by stirring, total volume of the solution and dispersion of the polymers. A conclusion that may be drawn without equivocation is that the Amberlite ion-exchange resin is slower under the comparable conditions. The chitosan may have responded more rapidly had a pre-wetted sample been added to the reaction flask; the commercial resins are essentially 70% water. The 96% reaction time was used instead of the more common 50% reaction time to eliminate the influence of mixing the polymers, which is obvious at short times. The difference between 7-10 minutes for Kytex H, 50/100 mesh, is due to the precision of reading the recorder chart; the difference corresponds to approximately 0.4 millivolt, twice the precision of measurement of the recorded potential.

Table 8. Kinetics of Silver-Ion Absorption of Chelating Polymers

Polymer	Mesh	pH	Capacity (mmol/g)		$t_{96\%}$ (min)
			Tritrimetry	Potentiometry	
Kytex H (Chitosan)	94% > 50	6.0	2.36		15-hr equilibration
	50-100	6.5	2.19	2.42	4
		3.1-6.0	2.23	2.14	7-10
	20-50	2.8	2.19	2.36	7
Chelex 100	50-100	3.0-4.4	3.88	3.74	3
		4.5	3.84	3.88	1
Amberlite XE-318	50-100	2.8	4.64	4.45	13

Test Conditions: Solution: 75 ml of 0.1 M $AgNO_3$ + 0.1 M $NaNO_3$
5 ml of 0.015 M HNO_3 for pH 3 solutions

Polymer: Equivalent of 1.0 g dry weight

Potentiometry: Orion $Ag^+/S^=$ Electrode no. 94-16A
Orion Double Junction Reference no. 90-02-00
Beckman Research pH Meter
Heath Potentiometric Recorder and associated modules

Titrimetry: Polymer filtered, washed with 3 75-ml aliquots of H_2O, excess Ag^+ determined by Volhard procedure.

CONCLUSIONS

The metal-ion binding capacity of Kytex H chitosan has been examined under various conditions and shown to be comparable to that of commercial ion-exchange resins. Chitosan has a number of advantages over the resins: it is nonswelling in either acid or alkaline media, "indifferent" to alkali metal and alkaline earth ions, and thermally stable. In addition, the flake material exhibits a low back pressure in columns due to the irregular shape of the particles. Chitosan's main disadvantage is its tendency to solubilize at moderately acid pH's, except in a sulfuric-acid medium.

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Figure 1. Scanning Electron Micrograph of Kytex H Chitosan, 1200X

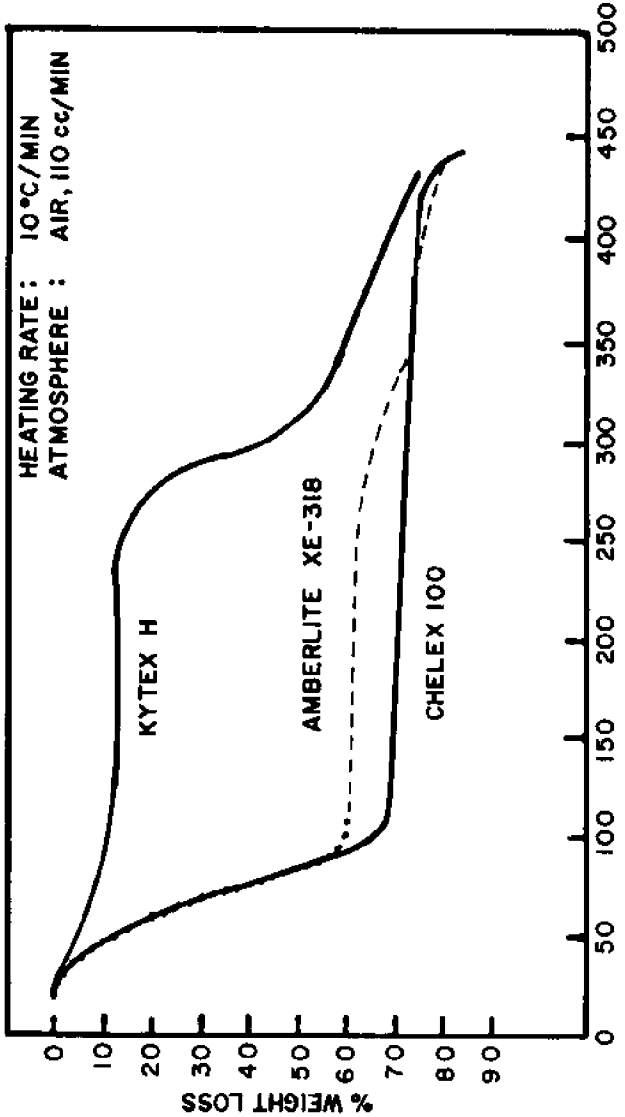

Figure 2. Thermogravimetric Analysis of Chelating Polymers (50-100 mesh). Heating rate: 10°C/min; Atmosphere: Air, 110 cc/min

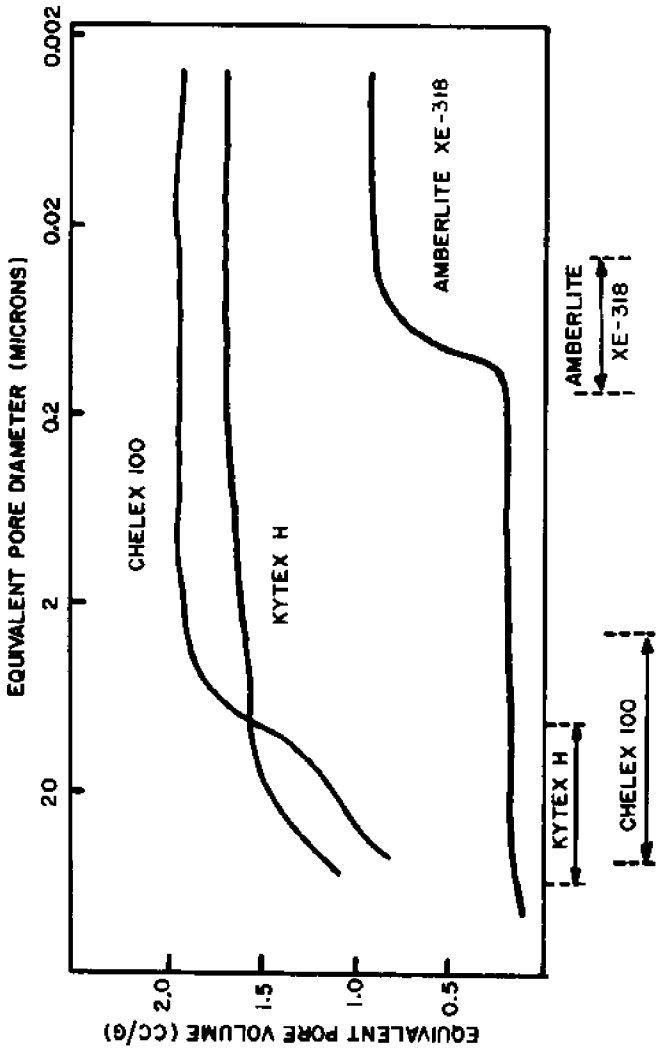

Figure 3. Porosity Profile of Lyophilized Chelating Polymers (50-100 mesh).

CHITOSAN AND CHITOSAN DERIVATIVES FOR REMOVAL OF TOXIC METALLIC IONS FROM MANUFACTURING-PLANT WASTE STREAMS

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ABSTRACT

We showed previously the binding of various metallic ions by chitosan in equilibrium tests with known salt solutions. In this report, we demonstrate, in batch tests and in column-flow operations, the effectiveness of chitosan for the treatment of actual waste streams to remove, or reduce to acceptable levels, the toxic metallic ions. Wastes treated were: (a) from electroplating and metal-finishing operations (with disposal problems mainly of cyanide and salts of chromium, cadmium, zinc, lead, copper, iron and nickel); (b) from a nickel-salt manufacturing plant (disposal of nickel and alkali); (c) from a lead-battery manufacturing plant (disposal of sulfuric acid and lead salts); and (d) from exhausted dyebath for wool fabrics in which dichromate is included in the bath (disposal of chromium). The chitosan was effective for reducing the content of copper, cadmium, iron, zinc, lead and nickel salts and sulfuric acid. Cyanide appears to have been partially removed. Although chitosan as such is useful for treating these wastes, it may be advantageous to use chitosan that has been partially crosslinked with di- or polyfunctional reagents to impart increased resistance to solubilization with effluents of acidic pH. After the chitosan or its partially crosslinked derivatives are used for treating the waste effluents, it is desirable to have procedures for regeneration of the chitosan matrix for reuse. Such procedures are being investigated and appear feasible through the use of appropriate buffers.

INTRODUCTION

In previous studies (1-4) we surveyed the binding of toxic and heavy metallic ions by different types of synthetic resins and biopolymers in search of inexpensive and readily available insoluble supports for treating industrial waste effluents containing these ions. Chitosan (CHT) was among the promising polymers we tested (4). It is particularly attractive: it has a high capacity and a fast binding rate for many ions; it filters well, and effluents can be passed through it in a column at relatively fast flow rates; it has a buffering capacity for hydrogen ions due to its high content of amino groups, and its free amino groups provide means for chemical modification. It is also a renewable natural resource that could potentially be made abundantly and inexpensively available as a waste by-product of fishery operations.

Chitosan has one undesirable property, however, from the standpoint of its use as a filter for scavenging metallic ions; it forms salts with acids, and most of these salts are soluble in water. There are some exceptions, for example the sulfate- and sulfite salts insoluble at ambient temperature (but soluble in boiling water). The insoluble salts may also be converted to soluble products in the presence

of an excess of other acids due to the ionic nature of the salts. In addition, the sulfate or sulfite ions occupy the binding amino sites, and this, in some instances, may not be conducive for binding before these ions are exchanged. Insolubility of the supports at wide pH range and solute composition are essential requirements for their practical application as filters for metallic ions. In our recent studies of polymer derivatives based on CHT for enzyme insolubilization and as finishing agents for shrinkage control of wool fabric (Masri and Randall, these Proceedings), we found that partial crosslinking of CHT overcomes the problem of solubility in acids, and we have taken advantage of this in some of the experiments we report here on metallic-ion binding. Chitosan and its crosslinked products supplement other waste treatment methods in uses that are based on chemical precipitation, oxidation-reduction, adsorption, ion exchange, ultrafiltration, or removal with starch xanthate (5-7).

EXPERIMENTAL

Chitosan (Food Chemical and Research Laboratories, Seattle, Wash.) was ground in a Wiley mill to pass 1mm screen, and a 30-40 mesh (Tyler) fraction was used.

Crosslinked CHT was prepared as follows: to 300 g air-dried CHT was added 150 ml of a 25% aqueous solution of glutaraldehyde (GA), plus 1200 ml water, and the mixture was kept at 20° C for 1 hour with occasional stirring. The mixture was then filtered, and the product (designated CHT-GA-1.4) was washed with water and methanol and air-dried to give 342 g.

In other preparations, the above procedure was repeated except that the ratio of GA to CHT was changed (from the 0.5 ml GA solution/g CHT) to (1) 0.25 ml/g (product designated CHT-GA-0.7); (2) 0.75 ml/g (product CHT-GA-2.0); or (3) 1 ml/g (product CHT-GA-2.6).

Waste liquors

The following waste solutions were obtained for treatment. All were filtered before use.

Solution 1. Nickel waste: this was from a plant which manufactures nickel carbonate; the waste had 7.2 ppm Ni and a pH 8.8 (KOH is used in the manufacture).

Solutions 2 and 3. Lead wastes: these were from a lead-battery manufacturing plant; Solution 2 had 1.8 ppm Pb and 30 ppm Fe, while Solution 3 had 1.8 ppm Pb and 14.5 ppm Fe. Both solutions were acid (pH 1-2, sulfuric acid).

Solutions 4-6. Plating wastes: these were from a supplier providing a waste collection and disposal service. These solutions were from metal plating and finishing operations. They were designated as received from the supplier: copper, cadmium, and lead wastes (4, 5, 6 respectively). The copper and the cadmium wastes contained cyanide, and this was noticeable by smell. Metal-ion analysis by atomic absorption (AA) showed the following concentrations in part per million (ppm):

Solution 4: Cu, 138; Zn, 3.5; Pb, 3.5; an Hg. 0.1.
Solution 5: Cd, 2800.
Solution 6: Pb, 4.4; Zn, 227; Ni, 7.9; Cu, 3.6; Mn, 4.8; As, 1.8; Ca, 65; and K, 55.

Initial pH values of solutions 4-6 were: 9.1, 7.45, and 6.55 respectively. Estimated solids content of the three solutions were 2.1, 6.4, and 0.7% respectively.

Solution 7. Chrome waste from chrome dyebath: dye was exhausted onto wool fabric in the usual way by boiling in a chrome dyebath which contained excess potassium dichromate as mordant. The exhausted bath had 260 ppm chromium; it was not analyzed for Fe, Zn, Ni or Cu, nor were salts of any of those metals added intentionally. Analysis of the CHT, after it was used to treat the bath, by x-ray fluorescence analysis (XFA) showed, however, that these metal ions were present (see Results). Possible origins of these ions were the hot tap water, the bath kettle (made from No. 315 stainless steel), or the dyestuff used (not a refined grade).

Tests for metallic-ion binding

The uptake of metallic ions by CHT or its crosslinked derivatives (CHT-GA-0.7, and -1.4) from known salt solutions was done using 2 g substrate in contact with 100 ml of 0.2 M solution for 1 hr with stirring on a magnetic stirrer. The substrates were then separated, rinsed with water, air-dried, and their metal-ion content determined by XFA. In most tests the filtrates were contacted once more, each with a fresh 2 g batch of substrate for another hour, followed by separation and analysis of the substrates.

With the nickel waste (Solution 1), 500 ml of the waste and 2 g CHT were used for 1 hr. The CHT matrix was then analyzed by XFA and the treated liquor by AA for nickel.

With the lead-battery wastes (Solutions 2 and 3), 50 ml of each solution was contacted for 1 hr with 2 g CHT, and the liquors and substrates were analyzed for lead and iron.

With the plating-waste solutions 4-6, batch tests were done with each solution using 100 ml samples. Each 100 ml sample was contacted successively for 2 hrs with three 2-g batches of CHT; after each contact, the substrate was separated before the next 2-g batch was contacted with the filtrate. In the course of the total contact time of 6 hrs, the liquors were analyzed by AA by withdrawing a small aliquot of 1-3 ml (sampling at 1, 2, 3, 4, and 6 hrs). The three lots of CHT obtained with each waste solution were also analyzed by XFA after they were separated, rinsed and air-dried.

With the dyebath Solution 7, tests similar to those with the plating solutions were done, except that only two 2-g lots of CHT were used (for 2 hrs each) with the (same) 100 ml.

In a few tests, CHT was packed in columns, and the waste solutions were passed through (with lead-battery, nickel and copper wastes). Typically, 3-10 g CHT was packed in glass tubing of 9-12 mm internal diameter, and the influent solutions were passed through in the upflow

direction at a rate 150-450 ml/hr. Effluent fractions from the columns were monitored by AA. At the end of a run, the CHT in the columns was washed with a few bed volumes of water and then extruded in sequential segments for analysis by XFA. Results with these columns will be mentioned briefly below and will be reported in detail elsewhere.

All analyses for metal-ion content were done by AA with liquids and by XFA with the solid CHT substrates (3, 4).

RESULTS

Reaction of chitosan with glutaraldehyde

Although the reaction of native unsolubilized CHT with GA is by its nature a non-homogenous two-phase reaction, it is remarkable that the reaction appears to have proceeded rapidly to completion at ambient temperature; almost all the GA reagent added with the different preparations appears to have been used up, as reflected in weight increases in the products and as corroborated by elemental nitrogen analysis of the products. The number appearing as a suffix after the CHT-GA symbol denotes the number of millimoles GA bound/g original CHT as estimated from the observed weight increases and nitrogen analysis.

Titration of crosslinked chitosan

The CHT-GA products titrated hydrogen ion in amounts less than with CHT itself and depending on the extent of crosslinking. Values are shown in Table 1.

An important difference between CHT and its crosslinked products is thtat titration of CHT, with most acids such as hydrochloric, acetic or formic, yields water-soluble salts at 20° C (insoluble salts with sulfuric and sulfurous acids), while, in contrast, titration with any of the acids of the crosslinked CHT-GA products yields insoluble salts. The crosslinked products are thus useful for removal of hydrogen ion and (as will be shown) metallic ions from water media. Although the least amount of crosslinking that is required to give insoluble acid salts has not been worked out precisely, our tests at present indicate that about half as much crosslinking as in CHT-GA-0.7 would be sufficient (i.e., $\sim$0.4 mmol GA/g CHT).

Table 1. Uptake of Hydrogen Ion by Chitosan (CHT) and CHT that had been Crosslinked to Different Extents with Glutaraldehyde (GA)*

CHT Product	Buffering Capacity (H^+ uptake, meq/g)	pH at Midpoint of Plateau of Titration Curve (when half amount of H^+ was used	pH at End of Buffering Capacity (when indicated amount of H^+ was used)	Weight Increase of Titrated Product (mg/g original substrate)
CHT	4.1	5.7	4.8	290
CHT-GA-0.7	3.7	4.9	4.2	240
CHT-GA-1.4	3.5	4.2	3.4	140
CHT-GA-2.0	3.2	3.8	3.1	150
CHT-GA-2.6	3.0	3.9	3.1	110

*In this experiment, 0.1 M H_2SO_4 was used to titrate 10 g of each CHT product to construct a titration curve.

Binding of metallic ions by crosslinked chitosan

Metallic ion uptakes by CHT-GA-1.4, CHT-GA-0.7, and CHT itself, after 1 hr contact with known salt solutions, are shown in Table 2. For comparison, uptakes in 24 hr by native CHT (from a previous study [4]) are also shown.

Table 2. Metallic Ion Uptake by Chitosan (CHT) and Two Preparations of Crosslinked CHT (CHT-GA-0.7 and CHT-GA-1.4) from Known Salt Solutions (in meq metallic ion/g substrate)[a]

Solution		CHT-GA-1.4 2 lots of 2 g each contacted successively for 1 hr each with the same liquor				CHT-GA-0.7 1 lot of 2 g for 1 hr		CHT[b] Uptake in:	
		Lot 1		Lot 2					
Salt	Initial pH	pH at end of 1 hr	Uptake (1 hr)	pH at end of 1 hr	Uptake (1 hr)	pH at end of 1 hr	Uptake (1 hr)	1 hr	24 hr
$HgCl_2$	3.2	3.1	3.57	3.9	3.61	3.4	3.61	4.76	5.60
$CdCl_2$	5.1	6.5	0.74	6.6	1.16	6.5	1.38	2.20	2.78
$Pb(NO_3)_2$	4.0	5.0	1.07	5.4	1.24	5.0	1.41	1.79	3.97
$ZnCl_2$	5.4	5.5	0.35	5.9	0.36	5.9	1.04	2.04	3.70
$Co(NO_3)_2$	5.5	7.0	0.21	7.1	0.26	6.7	0.31	1.00	2.47
$NiCl_2$	5.1	6.7	0.24	6.7	0.14	6.4	0.29	0.94	3.15
$K_2Cr_2O_7$	3.8	6.1	0.85	6.3	0.65			1.45	
$Cr(NO_3)_3$	2.4	3.3	0.68	3.3	0.64	3.2	0.75	c	0.46[c]
$CuCl_2$	3.6	3.8	0.52	3.8	0.61	3.5	1.38		3.12
$FeCl_3$	1.6	1.5	0.63	1.7	0.62	1.7	0.78	d	d
$KMnO_4$	8.2	9.0	3.83	9.3	1.48			d	
$Mn(NO_3)_2$	1.9	5.7	0.11	7.1	nil	5.7	0.17		
$AgNO_3$	5.3	7.0	2.07	6.2	1.79		2.12	2.46	3.26
$HAuCl_4 \cdot 3H_2O$	2.3	2.1	2.83	2.2	0.93[e]	1.7	3.07		5.84
$H_2PtCl_6 \cdot 6H_2O$	1.8	2.2	2.32	2.3	1.30[e]				4.52

[a]100 ml of 0.2 M solutions plus 2 g CHT substrate were used except with Au and Pt salts. With those: 1 g salt plus 600 mg substrate were used (see note e). When not given, values were not obtained.

[b]The 24 hr uptakes are from a previous study (4).

[c]Much of CHT became solubilized, and the remainder was soft and gelatinous.

[d]CHT became completely solubilized.

[e]Most of the gold and platinum salts were taken up by the first lots of substrate, and all the remaining available salts in filtrate were taken up by the second lots.

The uptakes were based on the weight of the CHT products after the contacts and on the metal ion content of the treated products as determined by XFA. The results show substantial and rapid binding of the salts by the crosslinked products. The partial crosslinking apparently did not seriously interfere with accessibility of the potentially available binding sites that were not utilized (blocked) by the crosslinking. Of course the abolished or blocked amino group sites in CHT-GA-1.4 are substantial (potentially as much as 2.8 meq/g CHT), and a consequent decrease in the uptake profile would be expected; but, as the measurements show, sufficient binding sites remain in the crosslinked products for use with most wastes, which usually would contain the metal ions in the parts-per-million range. In our opinion, the partial loss of binding capacity is a favorable trade-off for the improved solubility property (insolubility in acids). As mentioned earlier, less crosslinking than in CHT-GA-0.7 may also be sufficient to stabilize the CHT to yield products with uptake profiles approaching those with the native CHT. No solubilization, gelation or softening of the CHT-GA products occurred after treatment with any of the salt solutions. In contrast, solubilization or gelation was observed in tests with CHT with some of the acidic salt solutions, especially with concentrated ferric chloride, potassium dichromate, potassium permanganate and chromium nitrate.

Although CHT-GA, by overcoming the solubility problem (at some loss of binding capacity), is more suited in our opinion than CHT itself for treatment of wastes, most of our work with the present wastes had been done with CHT itself before we prepared the crosslinked products. Nevertheless, the results with the wastes using CHT, together with the above results with known salt solution using CHT-GA, point to the effectiveness of the biopolymer and its derivatives for waste treatment.

Results of treatment of waste solutions with chitosan

Nickel waste, Solution 1: treatment of this liquor with CHT (500 ml/2 g for 1 hr) reduced the nickel-ion content from 7.2 to 1.2 ppm. Removal of the indicated amount of nickel from this analysis of the liquor (by AA) was corroborated by direct analysis by XFA of the CHT used, which increased in weight to 2.064 g and had 1445 ppm Ni.

In a column experiment, 4.9 l of the waste was also passed through a bed of 3 g CHT packed to 15 cm in a glass tubing at 150 ml/hr. Analysis of the column effluent indicated no trace of nickel by AA throughout the run. Analysis of the extruded CHT by XFA indicated removal of 93% of the total nickel in the feed by the first half, and 99.8% by the first two-thirds of the adsorbent bed.

Lead-battery waste, Solutions 2 and 3: treatment of these wastes with CHT (50 ml/2 g for 1 hr) reduced the lead concentration in the liquor to less than 0.05 ppm and the iron concentration to 8.7 (Solution 2) and 4.3 (Solution 3) ppm. Both solutions had nearly neutral pH after treatment. The relatively high ratio of CHT to liquor we used with these solutions was to neutralize the high content of sulfuric acid. Neutralization with alkali was not very satisfactory, as it resulted in a precipitate which was difficult to filter; the filtrates after neutralization, however, had less than 0.05 ppm Pb and 0.1 ppm Fe. Thus further treatment of the

filtrates was superfluous. The 2 g lots of CHT used with Solutions 2 and 3 increased in weight to 2.320 g and 2.310 g respectively; most of this weight increase very likely was due to the uptake of sulfuric acid.

Metal-plating wastes, Solutions 4-6: results with these solutions are shown in Tables 3-5. Analyses of both the liquors and the CHT matrices are shown.

Results in Table 3 (copper waste) show a large decrease in copper concentration in the treated solution even though the solution had a high alkaline pH from the start. A high percent of copper was found, especially in the first two lots of CHT (5.6 and 1.0%), after treatment of the waste. In a column experiment, the CHT bed was also very effective in removing the copper from this waste, and the bound copper was readily desorbed with a solution of ammonium hydroxide-ammonium chloride of pH 10. Thus the original pH of the liquor (9.1) probably was not optimal for binding to CHT. (Details of column experiments will be reported elsewhere.)

Results with the cadmium waste are shown in Table 4. These results show the following points:

Table 3. Removal of Copper from Plating Waste (Solution 4) by Chitosan (CHT) in Batch Tests

Analysis of Liquor by AA

at 0-6 hrs after contact with 3 lots of CHT. The lots were contacted successively for 2 hrs. each with the same liquor (100 ml)

After Contact with	Initial	CHT-1		CHT-2		CHT-3
Time (hrs)	t_0	t_1	t_2	t_3	t_4	t_6
pH	9.1	9.1	9.1	9.2	9.2	9.1
Cu, ppm	790	230	135	21	17	14

Analysis of the Contacted 3 Lots of CHT by XFA

	CHT-1	CHT-2	CHT-3
Weight Incr.	384	284	180
mg/lot (2 g)	55766	10182	352
Cu, ppm	(5.6%)	(1.02%)	(0.035%)

Table 4. Removal of Cadmium Ion from Cadmium Plating Waste (Solution 5) by Chitosan (CHT) in Batch Tests

Analysis of Liquor

at 0-6 hrs after successive contact with three 2 g batches of CHT. Each contact was for 2 hrs with the same liquor (100 ml)

Contact with CHT lot		CHT-1		CHT-2		CHT-3
Time (hr)	t_0	t_1	t_2	t_3	t_4	t_6
pH	7.45	7.80	7.80	7.95	8.05	8.20
Cd, ppm	2800	2500	2400	2200	2200	2000

Analysis by XFA of contacted CHT matrices after successive 2-hr contact times with the same liquor

	CHT-1	CHT-2	CHT-3
Weight incr.	280	286	200
mg/lot (2 g)	23849	19149	508
Cd, ppm	(2.4%)	(1.9%)	(.05%)

a) The initial concentration of cadmium in the waste was relatively high at 0.28%; the waste apparently was a concentrate (its dry matter content was 6.4%). The treatment with CHT decreased the cadmium concentration only to 0.2%, but that nevertheless represents the substantial removal of 80 mg cadmium ion/100 ml waste. This was corroborated by direct XFA analysis of the CHT.

b) The XFA analyses of the three lots of CHT showed that only the first and second lots were effective in the treatment, both removing about 50 and 40 mg cadmium respectively. On the other hand, the third lot removed only 1 mg.

c) All three lots showed large weight increases after the treatment (280, 286 and 200 mg), indicating uptake of some material other than cadmium, perhaps cyanide.

d) During the run, the pH of the liquor rose from 7.45 initially to 8.2 at the end (6 hrs total contact time with the three lots of CHT); the pH was already 8.1 when contact with the third lot was started, and this was very likely not optimal for binding.

It appears that the only moderate effectiveness of CHT with the waste in this experiment was due to the high original concentration of cadmium (and other materials) in the waste and to the rise in pH to an unfavorable alkaline level which tends to favor desorption rather than binding of the cadmium ion. The rise in pH was probably due to binding of cyanide complexes by the CHT. We did not acidify the waste because of its content of cyanide; perhaps retesting this liquor and maintaining the pH near neutral during the treatment, or after removing the cyanide from the waste, would prove more effective.

Results with the lead-plating waste (Solution 6) are shown in Table 5. Analysis of the CHT used in the treatment showed the presence of many metal ions in addition to lead. This prompted us to check the liquor for other

ions (see Experimental for analyses of solution). The 3 lots of CHT effectively reduced the content of these ions in the treated solution. Comparison of the metallic-ion profiles of the lots of CHT by XFA suggests that a preferential order of uptake of the various ions took place, which may have been dependent on the particular ion species itself, the relative concentrations of the various ions in the medium which were changing with the successive contacts, and perhaps on the changing pH during the whole contact period (from pH 6.5 initially to 7.7 at the end).

Treatment of the exhausted chrome-dye bath (Solution 7) with CHT (100 ml solution, successively with two 2-g lots, for 1 hr with each lot) decreased the chromium in the liquor from 260 to 29 ppm after the first hour with the first lot; no further decrease was detected by AA after contact with the second lot.

Analyses of the two CHT lots by XFA are shown in Table 6. The high content of bromine detected (not originally expected) is undoubtedly due to inclusion of tetrabromophthalic anhydride in the original dye bath, in connection with another study (with flame retardants for wool).

Table 5. Removal of Lead Ion from Lead-Plating Waste (Solution 6) by Chitosan (CHT) in Batch Tests

Analysis of Liquor

at 0-6 hrs after successive contact with three 2-g batches of CHT, each contact for 2 hrs with the same 100 ml liquor

Contact with	None (Initial)	CHT-1		CHT-2		CHT-3
Time (hr)	t_0	t_1	t_2	t_3	t_4	t_6
pH	6.55	6.8	7.0	7.4	7.5	7.7
Pb, ppm	4.4	0.5	0.5	0.4	0.4	0.4

Analysis of contacted CHT matrices after successive 2 hr contact times[a]

	CHT-1	CHT-2	CHT-3
Weight Incr. mg/lot (2 g)	176	220	140
Pb, ppm	350	53	44
Zn, ppm	8280	1174	123
Ni, ppm	284	18	30
Cu, ppm	99	12	12
As, ppm	151	23	10

[a]No Ca, K or Mn was detected by XFA.

Table 6. Analysis by XFA of Two Lots of Chitosan (CHT) (2 g each) Contacted Successively with the same (100 ml) of Chrome Dye Bath (Solution 7)

Ion detected	Ion Concentration, in ppm	
	First CHT lot	Second CHT lot
Cr	7984	325
Fe	219	158
Ni	20	25
Cu	13	18
Zn	73	31
Br	3691	309

With the above wastes, the calculated amounts of ions removed, based on AA analyses of liquors, are reasonably close to those based on XFA of CHT substrates, considering that the two independent techniques are basically different and that the concentrations involved are relatively low.

DISCUSSION

Our experiments show that CHT and partially crosslinked CHT appear to be promising substrates for the treatment of wastes to remove metallic ions for pollution abatement. Desorption of the ions appears feasible by appropriate pH adjustment, thereby allowing regeneration of the substrates and conservation of the industrial metallic ions by their recovery. Elution is effected with alkaline or acidic buffers; if acid pH is used for elution of the metallic ions, a subsequent step of treatment with alkali, such as potassium hydroxide, may be required to regenerate the free base polymers, since elution with acids results in the uptake of hydrogen ion by the binding amino group sites.

The purpose of crosslinking CHT for this application is to increase its stability, prevent its degradation upon extended and repeated use, and to impart insolubility in acids. This insolubility of the polymer in acids allows for its use to treat acidic wastes and for elution of bound ions by acidic buffers preliminary to regeneration of the adsorbent polymer. The crosslinking of the native CHT (from the solid state) can be easily done by simple methods under mild conditions in water and with relatively inexpensive reagents. This reaction is rapid, even though it is heterogenous in nature. The native physical structure and ultrastructure of CHT remain essentially intact in the reaction. The physical structure is desirable for easy filtration; the native ultrastructure, with its many intricate internal spaces and surfaces, is conducive for penetration of solutes into the polymer and their binding (Masri and Jones, these Proceedings). Initial experiments also indicate that insolubilization of the CHT can be achieved merely by blocking part of the amino groups without crosslinking the polysaccharide (e.g., with formaldehyde). Uptake profiles with such preparations are not completed.

The CHT polymers bind metallic ions and hydrogen ion by competitive interaction and salt formation with the free amino group sites. We have detected, by x-ray photoelectron spectroscopy analysis of treated CHT substrates, the binding also of the associated anions together with the bound

cations. Binding of the anions from a mixture of salts is likely to be preferential and competitive and may be independent of which cations are bound. For example, with potassium dichromate, the chromium, as the dichromate anion, is bound to the substrate, but the potassium cation is not (hydrogen ion from water is likely to be bound instead, resulting in a rise in pH).

This competitive binding of metallic ions and hydrogen ion (and of anions) results in sensitivity of the binding to the pH of the solution. The optimal pH for treatment of a waste depends in part on its composition, and it can be found experimentally (e.g., in batch tests done at various fixed pH's that are maintained throughout the different tests).

ACKNOWLEDGMENTS

We thank Gary M. McDonald and Ray Young, from our own laboratory, for the metal-ion analyses and Drs. David A. Fellers and John W. Finley for helpful review of the manuscript.

Reference to a company and/or product name does not imply approval or recommendation of that product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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THE ROLE OF CHITIN AND OTHER ORGANICS IN THE TRANSPORT OF METALS TO THE WORLD'S OCEANS

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ABSTRACT

Chitin, chitosan and several humic substances have the ability to complex a number of transition elements in the natural aqueous systems. The total production of chitin in the sea is enormous, but because of its rapid biodegradability, the chitin content in marine sediments is very small. Chitosan is also widespread in nature and has chelating ability. Humic and fulvic acids, the main constituents of natural organic substances, have chelating characteristics that are several orders of magnitude greater than those of the inorganic ligands.

While chitosan and humic substances form relatively stable organo-metal complexes, chitin-metal complexes are unstable. This makes chitin an important agent for metal transport, while chitosan and humic substances act as sinks for metals in the natural aqueous systems. For every metal, there is an organism capable of concentrating it relative to the lithosphere, either through its skeleton-building activities or through the requirements of its metabolic functions. Those metal pathways are largely influenced by the nature of the biota and the type of organic matter consituting the biots.

Most natural organic substances contain all three varieties: chitin, chitosan and humic substances. In natural systems, the stability of metals in the water is probably controlled by the stability of polyligand-metal complexes. Thermodynamics tell us that metal complexes with multiple ligands are more stable than single-ligand complexes, but very large complexes are likely to be important only in the sediment phases, as organic coatings on clay minerals.

Many of the natural biots such as seaweeds have a complexing ability that depends on the chemical characteristics (pH, salinity, etc.) of their aqueous environment because these regulate the metal content of the water.

Considering the volume of the biodegradable chitins in the skeletons of arthropods, the amount of metal associated with them must be enormous. When the chitin-metal complexes break down, they release a large amount of these metals into the sea water. The decomposition of these complexes could very well be considered as one of the sources for localized metalliferous sediments in the marine environment.

INTRODUCTION

In natural aqueous systems, metals are distributed in a number of available forms: dissolved ions, dissolved organic complexes, colloids, metal hydroxide coatings, organic coatings, clay exchange sites and clay lattice sites. Most metals are

sensitive to pH and Eh variations in the natural water. However due to the limited solubility of most metal compounds, metals tend to be found in suspended or deposited fraction sites. The ability of a number of organic compounds to chelate metals is very well known (6), but the importance of this ability for natural metal transport mechanisms is not well understood.

Fresh waters contain dissolved organic materials totaling up to 9 parts per million (9); sea water contains much less (about 1 ppm). While the major organic compounds in the dissolved state consist principally of carbohydrates, nitrogenous compounds, such as chitin, chitosan, protein, amino acids, etc., and lipids those in the suspended or sediment fraction consist mainly of a group of compounds collectively known as humic substances. Thorstenson (17) predicts that high molecular weight organics are thermodynamically unstable in natural aqueous systems and that the two most important lighter organics are carbohydrates and alanines. Even though the chelating properties of humic substances with molecular weights ranging from 2000 to 3000 are well recognized (10) in the natural environments, they are likely to be more important as sinks rather than as transport agents, except in the weathering zones. However, humic metal chelates are very stable once they are formed; they can be maintained in the dissolved state, even under an excess partial pressure of oxygen, as has been shown for Fe^{+2} organic complexes (16). Since humic substances comprise a mixture of the three major natural organics described above, the role of nitrogen organics, chitin and chitosan, in metal chelation becomes obvious. Although inorganic ligands such as CO_3^{--}, SO_4^{--}, etc., will compete with organics for complexing metals in solution (14), the structural complexity of organics in natural waters suggests that polyligand-metal complexes are very important in the aqueous geochemistry of metals.

PROPERTIES OF CHITIN AND OTHER ORGANICS

Figure 1 illustrates the biogeochemical cycle of metals. Individual metals may have their own subcycles: for example, heavy metals (Pb, Cu, Zn, etc.) may be cycled through the soil (weathering)→living organisms→sea→sea-bed, while metals such as Na and K may complete the entire cycle. The length of the cycle of individual elements will be determined by the extent to which that metal has an affinity to a given sink. Goldberg (5) states that organisms and plants concentrate metals up to 10,000 times the amount found in the hydrosphere or lithospheres.

Chitin and chitosan are constituents of the natural dissolved and particulate organics and part of the supporting tissues of invertebrate animals, of fungi and of bacteria; they are consequently abundant in the natural environment. Since arthropods have chitin in their exoskeleton, the amount of chitin produced in the marine environment is enormous. To maintain the production of C and N in the sea in balance, the mineralization of chitin is likely to take place rapidly (20). On the other hand, chitosan--the deacetylated chitin--forms relatively stable chelating polymers (11). Table 1 summarizes the chemistry of a number of natural organics. Table 2 summarizes the x-ray diffraction data for a number of chitin and a chitin-Cu chelates prepared in the laboratory. While Duursma (2) speculates that chitin will not break down, at least in surface sea water, Yoshinari and Subramanian (19) have clearly shown that metal-chelated chitin is unstable even in surface waters and is highly biodegradable.

ORGANICS IN METAL MOBILIZATION

Baker (1) extracted very large amounts of a number of metals from industrial ore minerals by chelation with soil organic materials. The humates dissolved individual metals such as Pb, Zn, Cu, Fe, Ni and Mn up to 1000 times the values dictated by their equilibrium solubility products in water. Similarly, it was (3) observed that seaweeds from the coastal environment concentrated a number of heavy metals through metabolic activity and regulated the dissolved metal levels in water as well. The relative solubility products of metal-inorganic ligands and metal-organic ligands (14) suggest that in an actual mixed system, chelation may not be very important as a direct mechanism in metal mobilization. Hence the metal uptake by living organisms is likely to be the influencing factor in the metal's biogeochemistry. Since chitin is one of the important constituents in the chemistry of animals, mechanisms of metal fixation should be briefly discussed.

Table 1. Chemical Analysis of Chitin and Some Other Natural Organics

	%C	% H	% N	% O	References
Chitin	43.11	7.18	7.68	42.03	(15)
Organic acids in fresh water	45.54	3.19-5.1	1.5-4.2	38.8-47.9	(18)
Fulvic acid	48.8	4.0	2.9	44.60	(7)
Organics in Fe-Mn nodules	0.66-1.21	NA	NA	NA	(4)
Organics in recent sediments	3.52-4.18	NA	NA	NA	(8)
Soil humic acid	50-60	6-9	2-6	30-35	(12)
Ocean-dissolved humic acids	50-60	2.8-5.2	5-10	0-35	(2)

NA: not available

MECHANISMS OF METAL MOBILIZATION BY CHITIN

The formation of a metal-organic complex such as Cu-chitin can proceed along the following paths, starting with a Cu-mineral:

1. $CuS \rightleftharpoons Cu^{++} + S^{--}$
2. $Cu^{++} + e^{-} = Cu^{+}$
3. $Cu^{+} + (Chitin)^{-} \rightarrow Cu\text{-}Chitin$

Here, the negative colloids of chitin absorb the cuprous ions from the water and form a colloidal complex. Since the pH is one of the important factors in the stability

Table 2. X-ray Diffraction Data

Authors	Cell Parameters			Examined Material
Lotmar and Picken (1950)	9.40	10.46	19.25	Chitin
Darmon and Runall (1950)	9.30	10.40	19.20	Chitin
Falk et al. (1966)	3.37	4.57	6.80	Chitin
Aspinall (1970)	4.76	10.28	18.84	Chitin
Subramanian et al. (15)	3.36	4.44	8.85	Chitin
Subramanian et al. (15)	4.43	5.53	8.79	Cu-Chitin

Abstracted from Subramanian et al. (15)

of complexes, the decay of carbohydrate (with a resultant pH change) can affect the colloidal nature of chitin and hence the stability of such complexes. For example:

4. $2CH_2O \rightarrow CO_2 + 4H^+$

5. $(Chitin)^- + 4H^+ \rightarrow (H_4chitin)^+$

The reversal of the colloidal charge of chitin will destabilize the Cu-chitin complex and release the metal ions back to the solution. Metal ions can also be released directly into the solution by direct biodegradation of the complex, as shown in equation 6. Thus, while the complex can form in one region of the ocean, say the near shore, it can decompose elsewhere in the ocean supplying those waters with metals. Due to the enormous amount of chitin in animal and fungal exoskeletons, degradation of the metal-chitin complexes will make available a large quantity of metals in solution for possible inorganic precipitation through mechanisms such as:

6. $(Fe\ chitin) \rightarrow Fe^{+2} + CO_2 + NO_3^- + OH^-$

7. $Fe^{+2} + O_2 \rightarrow Fe^{+3} + e^-$

8. $Fe^{+3} + 2OH^- \rightarrow Fe(OH)_2^+$

9. $Fe(OH)_2^+ + OH^- \rightarrow Fe(OH)_3$ Precipitate

It may be noted here that hydrated oxides of Fe constitute an important portion of recent sediments in general and recent metalliferous sediments, in particular.

Plants and organisms require micro-nutrients for a variety of reasons: Fe is required for the synthesis of protoplasm, Zn for the synthesis of growth hormones, Mn for chlorophyll synthesis, and Cu is used up as an enzyme activator. The main source of these metals for the biota is water, though some organisms are filter feeders. Some zooplanktons extract nearly 50% of the total Pb supplied by rivers to the world's oceans; in case of other metals, the organic uptake could be several thousand times that of the water. Not all organisms simply act as extractors; prawn is known to extract Zn^{+2} from water (for use in metabolism) and to release it back in the water as $ANOH^{+}$ or Zn-organic complex (13). Since chitin occurs mostly in exoskeletons, the chitin-metal association in the marine environment probably follows a similar pathway, that is, the metals extracted by the organisms are complexed with the chitin or chitosan present in the exoskeleton and are released either as colloidal complexes or as dissolved organic complexes. Both of these eventually biodegrade to release the complexed metals, through reactions cited above. Though the actual mechanisms of metal incorporation by chitin in an animal's body is not clear, the experimental verification of the easy biodegradability of chitin-metal complexes would suggest that chitin has potential industrial applications, including water treatment for toxic elements, similar to the application of the chelation properties of chitosans reported by Muzzarelli et al. (11).

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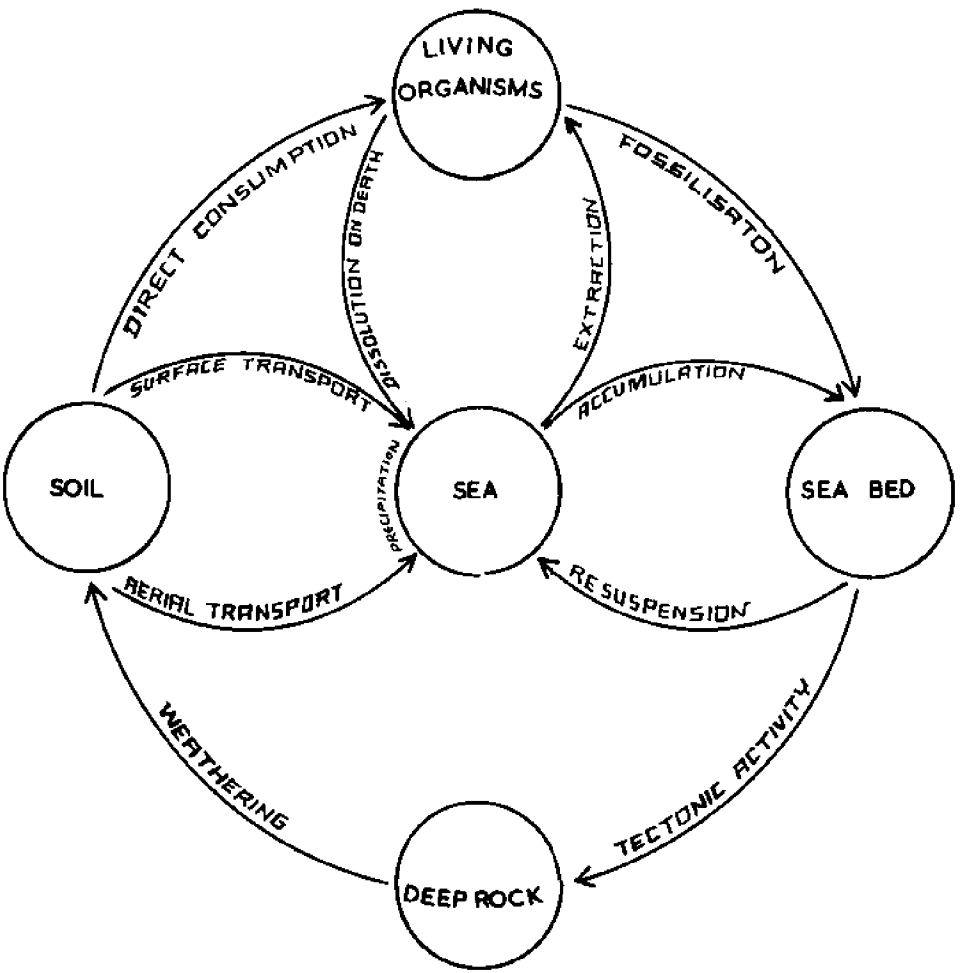

Figure 1. Biogeochemical cycle of elements

VI. SPECIAL APPLICATIONS

APPLICATIONS OF CHITIN AND CHITOSAN IN WOUND-HEALING ACCELERATION

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ABSTRACT

Chitin, chitosan and partially depolymerized chitin were found to be higher in wound-healing accelerator activity than the standard acid-pepsin digested cartilage preparations (Catrix) (14,15,21,28). The degree of acceleration of the wound-healing process was determined in animal tests by measuring the bursting strength of the newly formed tissue of the wound after seven days by the method of Prudden (21). In human clinical tests, the acceleration of healing was confirmed by effecting healing at a normal rate in both slow-healing and non-healing wounds (ulcers).

Shrimp chitin and chitosan showed a wound-healing activity of +30% by the Prudden method, while chitin from lobster and king-crab shells showed activities of up to +75%.

Regenerated chitin fibers, non-woven mats, sponges and films were prepared by the viscose xanthate process and tested by Prudden's wound-healing model (21). All showed activities of +30% or substantially higher.

Standard silk and catgut sutures coated with regenerated chitin or chitosan showed wound-healing activities only slightly lower than the all-chitin fibers.

Surgical cotton gauze coated with regenerated chitin was substantially more active than the uncoated control.

INTRODUCTION

Wounds caused by major or minor injuries, by surgery, by burns, and slow-healing and non-healing wounds such as ulcers represent the most widespread treatable condition encountered by humans and by animals as well. Since most wounds, when not infected, heal spontaneously in endocrinologically and nutritionally normal animals, it was considered axiomatic that the rate of healing represents a biologic maximum and therefore could not be accelerated. This conviction was changed only after overwhelming evidence was presented by several investigators that cartilage is indeed effective in accelerating the healing of wounds (1,11,14-18,21,22,26-28).

The acceleration of wound healing with processed cartilage powders (Catrix) and cartilage extracts (Catrix-S) derived primarily from bovine tracheal cartilage, but also from cartilage from a variety of other sources, has been extensively described in the medical literature. This work was done principally by Prudden and his co-workers (1,11,14-18,21, 22,28). Prudden found that the primary factor in the acceleration of the wound-healing process can be attributed to the presence of hexosamines and, more specifically, to the presence of N-acetyl-D-glucosamine (NADG) (20). Prudden also found that the cartilage effect in wound healing depended not only on the quantitative presence of NADG, but even more on the molecular structure of which it is a component. The

importance of the molecular structure of the active cartilage preparations was demonstrated when we destroyed most of the wound-healing activity by hydrolyzing the cartilage substance, either with acids or with alkalis. It became obvious that NADG is one of the most important factors responsible for the wound-healing activity of the cartilage mucopolysaccharides and glycoproteins.

The relatively low level of wound-healing acceleratory activity of monomeric NADG has been known for some time (9,10,23,24). It is assumed that the observed level of activity was due to the high degree of solubility of NADG and its consequent rapid excretion through the kidneys. When administered intravenously, 30% of the NADG was excreted within the first hour. The solubility of NADG and its rapid absorption by the tissues when it was applied topically to the wounds allowed for too short a residence time in the wounds to enable it to exert a major effect on the healing process.

Since topical NADG did have a wound-healing acceleratory effect, albeit not to a significant degree, it was thought that some polymers of NADG, perhaps even chitin, might equal or at least approach the wound-healing acceleratory capacity of cartilage. But, the generally recognized resistance of chitin to chemical attack, except that of the most drastic nature, made it appear to be a most unpromising candidate for a wound-healing accelerator. Yet, in contrast to its chemical inertness, chitin was known to be readily attacked by chitinase enzymes, notably by lysozyme, which is transported to the wound sites by the inflammatory cells (polymorphonuclear leucocytes). On the premise that the availability of abundant lysozyme at the wound site (19) would gradually break down the chitin to the active NADG dimer and provide for its sustained release, it was decided to explore the use of chitin and its derivatives in our wound-healing studies. At the first application of a finely ground (approximately 70 microns) lobster chitin in a test with 12 pairs of rats (12 chitin-treated rats and 12 untreated), it was found that chitin gave results not only greatly superior to the untreated controls, but also significantly superior to rats treated with processed cartilage (Catrix). Further investigation demonstrated that the wound tissues were indeed able to utilize the chitin due to the presence of lysozyme, which does depolymerize the chitin to its oligomers and eventually to the dimer. This is then responsible for the wound-healing activity observed. Because lysozyme acts slowly on the chitin particles, chitin becomes a continuous source of NADG dimers and other soluble oligomers as long as the wound contains inflammatory cells releasing lysozyme from their lysosomal sacs. The inflammatory reaction, and therefore the release of lysozyme, continues until the wound is finally healed.

MATERIALS AND METHODS

Chitin powders

The chitin powders were prepared from the starting materials (e.g., the carapace of the crustaceans or fungal mycelia) by alternate treatment with dilute NaOH and dilute HCl, followed by washing with de-ionized water until the effluent was free of chlorine. The chitin component of the shells, which were still colored after the above treatment, was extracted with acetone, thereby removing most of the remaining color. We avoided bleaching the chitin in order not to degrade the chitin polymer any more than was made necessary by the purification process. The dry chitin materials were powdered by ball milling in a porcelain jar mill with ceramic balls. Hammer

milling was not used, because it was found that in some cases abrasion of the metal hammers caused sufficient contamination by the alloy components to give erratic results in a number of wound-healing tests. The ground chitin was screened to a particle size of 70 microns with an average of 40 microns.

The fungal chitin required no milling, as it had a sufficiently small particle size for use in topical applications.

The chitin and chitosan obtained from outside sources, as indicated in Table 1, were washed with de-ionized water until all water-soluble residues were removed.

Regenerated chitin materials

The Southern Research Institute of Birmingham, Alabama, was retained by us to prepare various regenerated chitin materials via the chitin viscose process of Thor (29,30), but modified to suit the materials available and the equipment on hand.

A. Preparation of xanthate dope

50 g of chitin is steeped in one liter of 50% NaOH solution under vacuum for 2+1/2 hours at 30°C. The excess NaOH solution is removed by vacuum filtration for 30 minutes. The alkali-chitin cake is then placed in a polyethylene bag and pressed on a hydraulic press at approximately 34 atmospheres (500 psi) until the weight of the cake is reduced to 2.3 times the original weight of the chitin. The pressed cake (116 g) is shredded for about 3 minutes in a Waring blender. Crushed ice (1544 g) stripped of free water is then added to a beaker immersed in an ice/salt bath at 0°C., followed by the shredded alkali-chitin and 11.6 g carbon disulfide, and then rapidly mixed by hand with a Teflon spatula. The mixture is hand stirred intermittently at about 30-minute intervals for 3 hours at 0°C. The beaker is covered with a polyethylene bag between mixing periods. The partially reacted mixture is transferred to a glass container, sealed and stored for 18 hours at 0°C.

After storage, the chitin viscose is filtered at room temperature under approximately 3.4 atmospheres (50 psi) nitrogen pressure, three times through a filter pack of a single filter cloth (Chicopee 6970950) backed with two 40-mesh stainless-steel screens. This is followed by filtration of approximately 34 atmospheres (500 psi) nitrogen pressure through a filter pack of two layers of filter cloth (Chicopee 6721900 and 6936100) backed with two 40-mesh stainless-steel screens. The filtered solution is stored in sealed containers at 0°C., and it is then ready for use. Chitin viscose prepared by this procedure contains about 7% alkali-chitin and has a Brookfield viscosity of between 16,000 and 30,000 cps at 0°C., depending on the chitin source and the care in preparation. The material degrades on storage.

B. Chitin fibers

The chitin viscose dope was forced through rayon-type spinnerets into a coagulating bath of a solution of 8% sulfuric acid, 25% sodium sulfate and 3% zinc sulfate in water at about 40°C. The yarns were collected as skeins.

The fibers prepared by this process were relatively weak, between 1.0 and

Table 1. Wound Healing with Chitin and Chitosan Powders
Topical Application to Sherman-Strain Albino Rats

Material	Animals No. Pairs	Wound (21) Burstg. Str.	Note & Source of Material
Control: no treatment	200	100.0= .0%	Average strength of untreated rats
Cartilage, bovine (Catrix)	200	+29.0%	Average of Catrix-treated rats
D-Glucosamine HCl	24	+ 2.0	Aldrich Chem. Co.
N-acetyl-D-glucosamine	20	+ 4.6	Prudden (20)
Chitin, shrimp [1]	70	+27.8	Prudden (20)
Chitin, king crab [2]	48	+47.0	Lescarden
Chitin, Dungeness crab [3]	12	+21.0	Food, Chem. & Res. Labs.
Chitin, rock lobster [4]	24	+34.0	Robinette Res. Labs.
Chitin, lobster (Maine) [5]	18	+49.0	Lescarden
Chitin, *Penicillium notatum*	12	+45.0	Fungal chitin, Lescarden
Chitin, *Aspergillus niger*	12	+30.0	Fungal chitin, Lescarden
Chitin, *Phycomycetes mucor*	28	+35.6	Fungal chitin, Lescarden
Chitosan, Dungeness crab	24	+30.5	Food, Chem. & Res. Labs.
Chitosan, shrimp	18	+26.0	Deacetylated Chitin, Robinette Res.
Chitosan, shrimp	12	+24.0	Lescarden
Chitosan, lobster	12	+60.0	Lescarden
Chitosan, shrimp	12	+21.0	Robinette Res. Labs.

[1] *Penacus steiferus*
[2] *Paralithodes camschatica*
[3] *Cancer magister*
[4] *Palinuridae*
[5] *Homarus*

0.6 g/d tenacity; with sufficient expenditure of time and effort, however, fibers equal in strength to high tenacity rayons can be developed. The fibers prepared by the above process were utilized in the tests shown on Table 2.

C. Chitin nonwoven fabrics

Nonwoven fabrics were made by the wet-laid process. A number of nonwoven mats were prepared and tested. The mats that appeared to be the most suitable for surgical use in covering large areas of wound surface were prepared with fibers of 0.15 mm in diameter and were cut by hand into lengths of about 30 mm. The fibers were dispersed in water containing Triton X-100, a wetting agent, using mixing equipment that would not cut the fibers. The fiber suspension was poured into a TAPPI standard handsheet mold, where the mat was formed on a paper screen and sandwiched between wet sheets of filter paper. The sandwiches were sealed in polyethylene bags. The wet mats are pliable and are well suited for direct application to wounds.

D. Chitin sponges

Chitin sponges were prepared by the procedure commonly used to make cellulose sponges. Chitin viscose was placed in a beaker to form a layer about 3 cm thick. Sodium sulfate crystals were stirred into the viscose, and a 5% sulfuric acid solution was poured into the beaker to regenerate the chitin. The chitin mass containing the sodium sulfate was washed with de-ionized water until the effluent showed no trace of sulfate ions.

The moist chitin sponges are soft, pliable and suitable for use on or in wounds. The sponges were stored in polyethylene bags.

E. Chitin films

Chitin films ranging in thickness from 1 to 3 mm were prepared by casting films of chitin viscose onto glass plates with a doctor blade, regenerating the chitin by immersing the coated plates in an acidified sodium sulfate bath, removing the chitin films from the plates and air-drying the films in a tenter frame.

F. Chitin-coated catgut sutures

The sutures (000 Ethicon, Johnson & Johnson) were pretreated by immersing them in a 10% solution of hydrochloric acid for 1 min, and then washing them with de-ionized water. The pretreated sutures were coated by placing them in a hypodermic syringe fitted with a No. 18 needle, with one end of the suture threaded through the needle. Chitin viscose was poured into the syringe, and the plunger of the syringe was then inserted. The suture was pulled slowly through the needle while the plunger was pressed downward to extrude the viscose with the suture. The suture coated with the viscose was placed in an acidified sodium-sulfate bath to regenerate the chitin. The chitin-coated sutures were washed with de-ionized water and stored in isopropanol.

WOUND-HEALING STUDIES IN HUMANS

Topical application of chitin powder, Lescarden's Poly-NAG^{R}

Before commencing with tests on humans, the chitin powder to be used in the following human studies was subjected to a complete series of

Table 2. Wound-Healing with Regenerated Shrimp Chitin from Its Xanthate Topical Application to Rats

Material	Animals No. Pairs	Wound (21) Burstg. Str.	Note & Source of Material
Control: no treatment	200	100.0= .0%	Average of untreated rats
Chitin fibers	12	+32.9%	Applied as fibers
Chitin sponge	12	+25.0	Applied as powder
Chitin nonwoven mat	12	+30.0	Applied as powder
Chitin nonwoven mat	12	+120.0	Applied as a mat imbedded in wound
Chitin-coated cotton gauze	12	+20.0	Applied over wound
Chitin-coated catgut sutures	12	+30.0	Used as the suture

toxicological and pharmacological tests that showed it to be safe for its intended use.

Case #217-85-79, F.F., female, age 55; slow healing surgical incision on left ankle following greater and lesser saphenous phlebectomies for stasis varicosities of the left leg on 5/14/73. Poly-NAG powder applied topically to the incision on 6/6/73 and continued on a daily basis until 6/23/73, when the incision was completely healed. Healing in 17 days (14).

Case #224-32-41, S.G., male, age 29; perineal wound following total colectomy with end ileostomy and abdomino-perineal resection on 9/28/73, carried out because of chronic ulcerative colitis with vesico-sigmoid fistula and chronic perineal fistulization. Poly-NAG powder instilled into the perineal wound on 11/1/73 on a daily basis; continued the treatment to 11/20/73, at which time the wound was healed. Healing in 20 days.

Case #221-54-44, M.L., male, age 57; draining sinus tract following partial gastrectomy with bilateral vagectomy and Billroth II anastomosis for bleeding ulcer. Operation on 4/25/73. No spontaneous healing commenced by 5/17/73. Treatment with Poly-NAG powder was begun by instilling it into the sinus tract and continuing it on a daily basis until 5/21/73, when the tract was completely healed. Healing in 4 days.

Case #200-01-21, T.M., female, age 17; non-healing wound following excision of a keratosis from the left temple in May 1973. When seen on 9/27/73, the affected area showed a non-healing "proud flesh" area of 1.5 x 0.5 cm covered by a dirty scab. The scab was removed, the area cleansed, and Poly-NAG powder applied generously with a powder insufflator. The patient was instructed to carry out this procedure twice each day, and when she was seen again on 10/5/73 the wound had healed over by approximately 50%. The daily treatment was continued and by 10/19/73 the wound had completely healed. Healing in 14 days.

Case #200-05-25, M.S., female, age 20; post-total colectomy non-healing ulcers: umbilicus, left labia, and perineal defect with probable sinus tract at the fourchette. Poly-NAG powder was applied to the ulcers on 6/28/73 and the treatment continued on a daily basis. By 7/13/73 there was a remarkable contraction of all wounds, and when last seen on 9/27/73 further excellent progress was noted. The wounds had contracted by about 70% in all areas. The patient has not been seen since 9/27/73, but it is assumed that her progress continued at the same rate, with complete healing probably within the next month.

Case #200-15-25, J.S., female, age 53; non-healing wound of the left leg secondary to trauma, patient was seen in September 1973. The wound required six stitches which were removed two weeks later. When seen on 11/21/73, the wound was still open and was indurated and blue. Poly-NAG powder was applied and covered with Adaptic, a dry sterile dressing, and an Ace Bandage. On 11/26/73 the wound appeared healthy with the healing process commencing. Poly-NAG was reapplied. On 11/29/73 there was further progress noted with only a small ulceration remaining. Poly-NAG was reapplied. On 12/5/73 the wound had essentially healed. Poly-NAG was again applied. On 12/10/73 the wound had completely healed. Healing in 19 days.

Case #213-07-50, S.W., female, age 47; necrotic area on the side of a left below-knee amputation stump. Treatment with Poly-NAG powder began on

5/15/73 and continued on a daily basis. By 6/27/73 the wound had essentially healed, and, when last seen on 7/16/73, it was noted to be completely healed. Healing in less than 2 months.

DISCUSSION

Table 1 demonstrates that applied topically in animal wounds, chitin and chitosan are, in general, at least as effective in accelerating the healing process as is bovine cartilage (Lescarden's Catrix). Chitin from king crab, and lobster and fungal chitins from Penicillium notatum and Phycomycetes mucor, gave significantly greater wound-healing acceleration than cartilage, as indicated by the relative bursting strength of the wounds when tested by Prudden's method (21).

The wound-healing acceleration obtained with chitosan from various sources was in the same range as with cartilage, with one exception--that of lobster chitin--which gave twice the bursting strength of cartilage.

Regenerated chitin materials gave wound-healing results by the Prudden method (21) in the same range as cartilage. A notable exception was the nonwoven mat imbedded in the wound, which gave four times the bursting strength of cartilage. This increase in strength is at least partly attributed to the mechanical support that the fabric gave to the wounds. It is to be noted that the coated catgut sutures gave satisfactory acceleration to the healing, although the thin film of chitin coating represents a much smaller quantity of material than applied in the wounds in all other tests.

Human tests confirm the wound-healing acceleratory effect of chitin.

CONCLUSIONS

Chitin and chitosan are effective wound-healing accelerators (3-7) in both animal and human tests. The source of chitin and chitosan has an important bearing on its wound-healing efficacy, indicating significant differences in the structure of the chitin polymer.

Surgical adjuncts of regenerated chitin are physiologically compatible, bio-absorbable and effective wound-healing accelerators (6).

Chitin, chitosan, oligomers, or lower polymers of N-acetyl-glucosamine, probably both the D and the L form, should find important applications in medicine and surgery wherever healing of wounds is a problem. The physiological compatibility of chitin with living tissues, combined with its ability to form readily sulfate esters which are non-thrombogenic (25), appears to make chitin a most promising candidate for prosthetic structural devices of any desired shape or size. These could serve as replacements for bone or cartilage, for arteries and veins, for musculo-fascial replacements in large hernias and for many other uses. We feel that chitin, chitosan and their derivatives are opening a fertile field for development in some of the most important branches of medicine and surgery.

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USE OF CROSSLINKED CHITOSAN IN THE FINISHING TREATMENT OF WOOL FABRIC FOR LAUNDERING-SHRINKAGE CONTROL

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ABSTRACT

Interfacial deposition of crosslinked chitosan on woven wool fabric is shown to impart laundering-shrinkage control. Additives such as flame retardants could also be included in the crosslinking procedure. The crosslinking of chitosan was accomplished in both a two-step and a one-step method. In the two-step method, the chitosan was solubilized in dilute acids and used to impregnate the fabric. After the fabric was dried, it was treated with a reagent to crosslink the deposited chitosan. The crosslinker could be a difunctional or polyfunctional reagent reactive with chitosan, especially through its abundant amino groups, such as glyoxal, glutaraldehyde, solubilized dialdehyde starch, or dihydroxydimethylol ethylene urea. In the one-step method, the solubilized chitosan and the suitably blocked crosslinking agent were mixed together and applied to the wool fabric. The crosslinking was then effected by unblocking the crosslinker either through curing with heat or by pH adjustment. The fabric so treated had improved resistance to shrinkage.

INTRODUCTION

Interfacial deposition of polymers on wool fabric is an accepted method of imparting laundering-shrinkage resistance. Examples are the deposition of polyamide or polyurea resins by reaction of diacid chlorides or diisocynates with diamines (2-4, 9-12). In these reactions, the wool acts as the physical support on which polymerization occurs, although active participation of the wool (through reaction of lysine residues with the diacid chlorides or diisocynates) may occur with consequent grafting of the polymers (10). In any case, the deposited resin stabilizes the fabric against shrinkage.

Treatment with chitosan (CHT) was also reported to result in shrinkage resistance (6); however, the effect with wool does not endure repeated laundering by machine, as the CHT is apparently not firmly enough anchored to the wool. At best, only ionic interaction of wool and CHT can be visualized (between amino groups of CHT and carboxyl groups of aspartyl and glutamyl residues, for example).

In our work on immobilizing enzymes, we achieved insolubilization of the enzymes by entrapment and by fixing them in polymer gels based on crosslinking dissolved chitosan (Masri and Randall, these Proceedings). This polymerization of CHT solutions to give insoluble polymers suggested the potential application of this method as a finishing treatment to produce a shrinkage-resistant fabric. With this system, the potential of grafting and covalent attachment of the deposited CHT polymer to the wool would depend on the choice of a crosslinker that might react not only with CHT alone but also with functional groups on the wool (e.g., lysine, arginine, histidine or serine residues). The covalent coupling of CHT to wool could

occur if the crosslinker made a two-point attachment--one to the amino group of CHT and the other to the wool--through reaction with ε-amino groups of lysine or hydroxyl of serine, for example.

To test whether CHT polymers would be suitable for use in obtaining shrinkage resistance, we initially carried out the deposition-polymerization in two steps: the CHT and the crosslinker were each applied separately, with drying of the fabric between applications. This two-step application was done because mixing the CHT with the crosslinkers usually resulted in rapid polymerization to a gel that was too thick to apply to the fabric. In later experiments, application of the polymerization mixture in a single step was achieved by chemically blocking the crosslinker before its addition to the CHT solution, thus holding the polymerization until after the application on the fabric. The polymerization was then started by curing the fabric at a suitable temperature. The single-step method, aside from being simpler and more practical, also resulted in more even treatment and allowed for inclusion of additives such as a flame retardant to the polymerization mixture. The glutaraldehyde (GA) and glyoxal (GX) crosslinking reagents were blocked by capping with bisulfite. Polymerization of CHT with dihydroxydimethylol ethylene urea (DHDMEU) was sufficiently slow at 20°C so that a one-step application from a mixture of CHT and DHDMEU was feasible; after application, polymerization was done by curing at 150°C.

Results with both the two-step and the single-step methods show that deposited CHT polymers improve shrinkage resistance.

EXPERIMENTAL

Materials

Chitosan (Food Chemical Company, Seattle, Washington) was ground to pass through a 1 mm screen and then solubilized in dilute hydrochloric or acetic acid using 4 mmol/g CHT to give 1-3% solutions.

Bisulfite-capped GA and GX were prepared according to a described method (8) starting with aqueous 30% GX or 25% GA solutions. Dihydroxydimethylol ethylene urea as a 40% aqueous solution was obtained as Permafresh 183 from Sun Chemical Company.

The fabric used (from J. P. Stevens Company) was undyed plain-weave wool, 6-1/2 oz./sq. yd., with 31 ends/inch (warp) and 27 picks/inch (fill), constructed with 2.8 woolen run yarn. The fabric was washed with trichloroethylene and air dried before use. Circles of fabric 3-1/2 inches in diameter (cut from the fabric with a special die) were made for shrinkage tests using the accelerotor; swatches 6 inches by 11 inches were used for the machine-wash tests (see below). Flame-retardant tetrabromophthalic anhydride (TBPA) was used in some of the treatment tests.

Test methods

Area shrinkage of treated wool fabric was measured either (a) in the accelerotor after a 2-minute wash (7) or (b) after a standard 15-minute wash in a home-model washing machine followed by a 30-minute tumble dry (1). Circles of fabric, 3-1/2 inches in diameter, were used in the accelerotor test; the circles were preconditioned in 1% sodium acetate.

Other conditions were 1780 rpm fixed speed rotor, a 2-minute wash, 200 ml 1% sodium oleate and 38-40°C. The washing machine 15-minute wash was at 40°C with a 4-lb load (ballast) and 60 g of a low-suds detergent; the 30-minute tumble dry was at a medium temperature setting.

Experience had suggested that an area shrinkage of 10% or less in the accelerotor test would represent a satisfactory and adequate degree of shrinkage resistance (1). Flammability tests were done on a 10-inch by 3-inch cloth suspended vertically according to a modified ASTM procedure D1230.

Treatment of fabric (polymerization procedure)

Experiment 1: Two-Step Application. In this exploratory experiment (Table 1), pre-cut fabric circles, 3-1/2 inches in diameter (1.1 g), were used for treatment. The CHT·HCl was applied and dried on the fabric in an oven at 70°C; then the GA was applied and dried. This order of application was also reversed. The dried treated fabrics were then rinsed by hand under warm tap water and air dried before the accelerotor shrinkage test. With some circles, TBPA was also applied before either the CHT or GA, or after one of these reagents had been applied. The CHT·HCl and GA (aqueous) solutions contained 1% isopropanol to wet the wool. The TBPA was dissolved in dimethylformamide (DMF). The reagents were applied in a volume just sufficient to wet the circles. Rough estimates of the amounts of reagents applied per circle were: 50-100 mg CHT, 100-200 mg GA and 50-100 mg TBPA. The TBPA was included in this experiment to check whether it would interfere with the polymerization.

Experiment 2: Two-Step Application. Here swatches of fabric were treated in two steps as in Experiment 1, except that bisulfite-capped GX or GA was used instead of the unblocked aldehydes (Table 2). The method of application was also different: the CHT·HCl solution (3% in water containing 1% isopropanol) was always padded on the fabric first. Excess solution was removed by passing the cloth between two squeeze rolls (40 lb pressure); the cloth was then dried before the next reagent (capped GX or capped GA) was applied. The capped aldehydes were padded on the fabric from 8% solutions, and excess liquid removed by the squeeze rolls. After a partial drying for 10-15 minutes at 70°C, the fabrics were then cured at 150°C for 10-15 minutes. The wet weight pickup with the different solutions varied in this experiment between 80 and 120% of the weight of the wool. A main purpose of this experiment was to check whether curing the treated fabric at 150°C would unblock the capped aldehydes to polymerize the CHT. One test was included in this experiment in which the CHT·HCl was padded on the fabric and the excess solution was removed with the squeeze rolls. Then the fabric, while still wet, was exposed to ammonia vapor to precipitate the free base CHT on it. The fabric was then dried in the oven.

Experiment 3: Single-Step Application. In this experiment (Table 3), the CHT plus the bisulfite-capped GX or GA was applied in a single step as a mixture. The solutions were padded on, and excess liquid removed by the squeeze rolls. The fabric was then dried and cured as in Experiment 2, hand-rinsed under tap water, and air dried before testing for shrinkage in the washing machine or accelerotor. Circles of 3-1/2 inch diameter were cut from the treated fabric for the accelerotor test. Weight add-on, when measured, was based on the weight increase of the treated fabric determined after the hand rinse and air drying, but before the machine wash.

In Experiment 3, each of the following 9 solutions in water was used (separately) to treat fabrics; all solutions contained 0.1% Igepal 610 and 1% Isopropanol to help wet the wool: (1) 2% bisulfite-capped GA (C. GA); (2) as Solution 1, but also containing 3% TBPA; (3) 3% CHT·HOAc plus 2% C. GA; (4) same as Solution 3, plus 3% TBPA; (5) 3% CHT·HCl plus 2% C. GA; (6) same as Solution 5, plus 3% TBPA; (7) 6% DHDMEU plus 0.5% $ZnCl_2$; (8) 3% CHT·HOAc plus 3% DHDMEU plus 0.5% $ZnCl_2$; and (9) same as Solution 8, plus 3% TBPA. The wet weight pickup with solutions containing CHT varied between 78-146% of the weight of the wool and with those not containing CHT between 53-79%. The higher wet pickup with solutions containing CHT is very likely due to its high viscosity.

RESULTS

Results of shrinkage tests with treatments of Experiments 1-3 are shown in Tables 1-3, respectively. The following points can be made:

1. Deposition on the fabric of free-base CHT from its hydrochloric acid salt by neutralization with ammonia, without crosslinking the CHT, resulted in shrinkage resistance (Tables 1-2) in the standard machine wash or the accelerotor. The effect was not stable to a second 2-minute accelerotor wash (Experiment 1, Table 1).

2. When the CHT was crosslinked with GA in Experiment 1 (with the 2-step deposition method), the obtained shrinkage resistance endured a second 2-minute accelerotor wash (Table 1). It appears from this experiment that the shrinkage effect was better when the CHT was applied to the fabric first. This order of application also resulted in much less (but still unacceptable) discoloration of the fabric; discoloration was severe when GA was applied first.

3. The two-step method in Experiment 2, using the bisulfite-capped dialdehydes (with curing at 150°C), instead of the unblocked reagent, to polymerize the CHT, also resulted in shrinkage resistance (Table 2) with only slight discoloration (off-white, yellowish cast). The CHT·HCl was applied before the capped GA or GX with all fabrics in this experiment.

Table 1. Experiment 1: Shrinkage of Wool-Fabric Circles with a Chitosan-Glutaraldehyde (CHT-GA) Polymer Deposited in a Two-Step Application

Treatment (reagents in order applied)	No. of Fabric Circles	Approx. Add-On %	% Area Shrinkage after Accelerotor Wash	
			1st 2-min wash	2nd 2-min wash
None: control	3		46	-[a]
GA only	4	6	42	-
T[b] only	1	8	41	-
CHT·HCL only[c]	1	1	44	-
CHT·HCl + NH_3	2	9	12	31
CHT·HCl + GA	3	14	2	3
GA + CHT·HCl	2	-	24	-
CHT·HCl + T + GA	1	17	0	4
GA + T + CHT·HCl	1	-	31	-
T + CHT·HCl + GA	2	15	14	12

[a]Hyphen = not obtained.

[b]Tetrabromophthalic anhydride.

[c]CHT·HCl was dried on fabric, then fabric was hand rinsed with water and air dried before the accelerotor wash test.

4. When capped GA was used with CHT (hydrochloric or acetic salts) in the single-step application method (with curing at 150°C), shrinkage resistance was obtained with very slight or no discoloration (Table 3).

5. Similarly, a one-step application of a mixture of CHT·HOAc plus DHDMEU, with $ZnCl_2$ as catalyst and with curing at 150°C also gave shrinkage resistance (Table 3). The fabric was essentially not discolored and had good handle (feel to the touch) and mechanical properties. Although shrinkage resistance was not complete with this single-step method, we have not worked out optimal treatment conditions (e.g., ratio of CHT to crosslinker, level of add-on, curing time and temperature, pH of polymerization mixture, or pH adjustment during curing).

6. Inclusion of TBPA in the polymerization mixture did not appear to interfere with polymerization of CHT or the shrinkage-resistance effect. Fabrics whose treatment included TBPA in Experiments 2 and 3 were tested for flammability after the standard machine wash. The flammability tests indicated marginal improvement (the results are not shown), but other work from our laboratory indicates that a higher level of treatment (above 5% add-on to the weight of the wool) is required for improvement regarded as adequate.

Table 2. Experiment 2: Shrinkage of Wool Fabric with Crosslinked Chitosan (CHT) Deposited in a 2-Step Application of, First, Solubilized CHT, Then Bisulfite Capped Glutaraldehyde (GA) or Glyoxal (GX), with curing at 150°C

Treatment (reagents in order applied)	Shrinkage (percent) Machine-Wash Tumble-Dry (swatches) Warp	Fill	Area	Accelerotor Wash Area
None: control[a]	14.8	5.5	20	49
CHT-HCl + NH_3	2	2	4	16
CHT-HCl + GX[b]	2.5	3	5	-[c]
CHT-HCl + TBPA + GX	0	1	1	-[c]
CHT-HCl + GX with suspended TBPA	0	0	0	20
CHT-HCl + GA	0	0	0	1

[a]Average of 3 treated fabrics; see also accelerotor wash data Table 1.

[b]Average of 4 treated fabrics.

[c]Not done.

DISCUSSION

Deposition of CHT polymers on wool fabric appears to be a potentially practical basis for stabilizing the fabric against laundering shrinkage. A one-step application method is possible, using a mixture of dissolved CHT plus a blocked crosslinker (e.g., bisulfite capped di- or polyaldehydes or di- or polyisocynates), or a slow reacting unblocked crosslinker (e.g., DHDMEU). After application of the mixture, polymerization on the wool can be started or enhanced by curing at elevated temperature or by pH adjustment. Optimal treatment conditions with CHT polymers need to be worked out. These include the choice of crosslinker, proportions of CHT and crosslinker, pH of the mixture that would be suitable to prevent polymerization before application to the fabric, but would be compatible for polymerization during curing, optimal curing time, temperature and pH levels that will not yellow or mechanically damage the fabric, and adequate level of treatment (percent of add-on). With the CHT sample that we used, a 2-3% solution is also relatively viscous. It would be desirable to use lower viscosity CHT fractions (lower molecular weight polymer) to facilitate application and to allow a higher level of add-on, if desired.

Table 3: Experiment 3: Laundering Shrinkage of Wool Fabric Treated to Deposit Polymer in One-Step Application of a Mixture of Chitosan (CHT) and Bisulfite Capped Glutaraldehyde (C. GA), or a Mixture of CHT and Dihydroxymethylol Ethylene Urea (DHDMEU), with or without Tetrabromophthalic Anhydride (T)

Experiment 3 Solutions	Treatment (mixture)	No. of Treated Fabrics	Approx. add-on (%)	Shrinkage (percent) Machine-Wash Tumble-Dry (swatches) Warp	Fill	Area	Accelerotor Wash (circles) Area
	None: control[a]	3		14.8	5.5	20	49
1	C. GA only	1	2	7.3	5.5	12	46
2	C. GA + T	1	5	7.7	5.5	13	46
3	CHT·HOAc + C. GA	1	[b]	2.6	1.5	4	21
4	CHT·HOAc + C. GA + T	1	7	2.7	2	5	26
5	CHT·HCl + C. GA	2	4	1.8	1.5	3	35
5	CHT·HCl + C. GA	2	9	0.3	0.4	1	14
6	CHT·HCl + C. GA + T	2	7	1.8	1.5	3	32
7	DHDMEU only	2	2	6.6	5.8	12	46
8	CHT·HOAc + DHDMEU	2	7.5	0.8	0.7	2	7
9	CHT·HOAc + DHDMEU + T	1	9	0	0.25	0.3	7

[a]Data from Table 2.

[b]Not measured.

Inclusion of certain additives (depending on their nature) to the polymerization mixture for other desired properties (e.g., improved flammability and insect resistance) may be compatible in the single-step treatment, without interfering with the polymerization of shrinkage-control efficacy of the deposited CHT polymers.

The shrinkage resistance effect of the deposited CHT polymers was not unexpected; it has analogies with other treatment methods now in use. For example, deposition of polyurea or polyamide polymers by reaction of diisocynates or diacid chlorides with diamines is in principle similar to our present method, in which the polyamine polymer CHT replaces the diamine monomer in the crosslinking reaction and the difunctional crosslinkers GA, GX or DHDMEU replace the difunctional diisocynates or diacid chlorides. In exploratory tests, we in fact obtained polymers by reaction of CHT with an aliphatic diisocynate (DDI, dimer acid diisocynate, General Mills) or with a polyurea prepolymer with pendent isocynate groups (Synthaprett LKF, Bayer), but the reactions were slow.

In the treatment with CHT polymers, the reagents used to crosslink the CHT very likely also react to some extent with the wool itself, through its many kinds of functional groups, thereby covalently anchoring (grafting) the

CHT polymers to the keratin. For example, the reaction of aldehydes with proteins (lysine and arginine residues) is known; reaction of DHDMEU with proteins was reported (1). In our experiments, treatment of wool with GA alone resulted in an intense yellow-brown coloration and a weight increase (Table 1); crosslinking of wool by dialdehyde starch also was reflected in a brownish coloration, weight increase, and in amino acid analysis (5). When fabrics were treated with GA before application of the CHT in the 2-step method, discoloration was also more severe and the shrinkage protection poorer than with treatments in which the CHT was applied before the GA. Apparently, the effectiveness of GA to crosslink (the CHT) is partly diminished by reaction with wool, whose reactive sites for grafting are thus also partly abolished (if the GA reacts with the protein by a 2-point crosslinking reaction). The application of CHT as the first reagent in the treatment appears to have a sparing effect on the lysyl and other reactive residues of wool, the CHT providing a high concentration of amino groups for reaction with GA. As regards the reaction of CHT with wool, very likely only adsorptive ionic interaction can occur (only 1% add-on with CHT, Table 1). Tetrabromophthalic anyhdride appears to enter reactions with both CHT and wool. For example, when wool was heated with TBPA in DMF at 70-80°C in the oven for about an hour and then thoroughly washed with DMF, water and methanol, a distinct weight increase of the wool occurred (Table 1). Similarly, when a clear-solution mixture of solubilized CHT and dissolved TBPA in a water-DMF solvent mixture was heated on the steam bath, a gel polymer formed. Clearly the choice of additives, the concentrations at which they are employed, and the manner of their inclusion may have bearing on the polymerization of CHT and the shrinkage-control effect. The covalent participation of the additives in the polymerization reaction would lead to washfastness from the fabric.

ACKNOWLEDGMENTS

From our laboratory we thank Clay E. Pardo, Lona M. Christopher, Buenafee T. Molyneux and Iva B. Farrell for their help, and Attila E. Pavlath and Walter J. Thorsen for reviewing the manuscript.

Reference to a company or product name does not imply approval or recommendation of that product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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STUDIES ON THE PREPARATION OF CHITIN FIBERS

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ABSTRACT

Chitin was solubilized in different solvents by a freezing procedure and dispersed in 99% formic acid after freezing the mixture repeatedly. A clear solution of chitin viscose was also obtained by applying the freezing method to alkali chitin and carbon disulfide. Chitin was found to be soluble in dichloroacetic acid or methanesulfonic acid at room temperature, though the molecular weight of chitin tended to be reduced rapidly in dichloroacetic acid. Chitin fiber was obtained from a solution of chitin-formic acid containing 8% dichloroacetic acid or chitin viscose, but it had the fatal defect of poor loop tenacity. Chitin film was difficult to prepare from chitin viscose, but it was possible to prepare it from formic acid solution. Chitin viscose and cellulose viscose were blendable in any ratio. Cellulose fiber containing 3% chitin had a ramie-like feeling and a high dyeability; the properties of viscose-rayon fiber were also improved.

So many studies have been made on acetyl cellulose that it is now as well known as acetate fiber. But few studies have been made of acetyl chitin, so the preparation of acetyl chitin was studied in order to examine the properties of acetyl chitin fiber and film. Chitin was acetylated by nearly 30% after treatment of alkaline chitin with acetic anhydride at 0° C overnight. The acetyl chitin acetylated in various components was derived through the treatment of chitin in a mixture of acetic anhydride and methanesulfonic acid at 0° C overnight. The fiber and film of acetyl chitin obtained from the 99% formic acid solution were found to be more flexible with the increment of acetylation than are those of chitin.

Diacetyl chitin was prepared by the following methods: acetylation in excess of acetic anhydride (a) by the saturation of HCl at 0° C for 10 days; (b) by an excess of acetic anhydride in methanesulfonic acid at 0° C overnight; and (c) by an excess of acetic anhydride in the presence of perchloric acid at 0° C for 3 hours.

INTRODUCTION

Among the polysaccharides widely distributed in nature, chitin has a chemical structure most similar to cellulose, but it has thus far not been found useful because there are few suitable solvents for it, owing to the strong resistance of its micelle structure to chemical reagents. When the micelle structure of chitin is broken by some physical or chemical procedure without decomposition of the chitin molecule, the solubility or reactivity for the chemical reagents will be changed and the synthesis of chitin derivatives will be further developed. For this reason, a freezing procedure was used to break the micelle structure, and chitin viscose (2) or chitin-formic acid solution was obtained. Although chitin was also found

to be easily soluble in dichloroacetic acid or methanesulfonic acid, the molecular weight tended to be reduced if it was left standing for too long a time in these solvents.

Chitin fiber obtained from the viscose showed poor loop tenacity, and the chitin film prepared from formic acid solution was slightly brittle. But the fiber and film are not yet useful for practical purposes owing to this brittleness. Chitin viscose and cellulose viscose, however, can be blended in any ratio without phase separation. Viscose-rayon fiber blended with 3% chitin showed excellent qualities of loop tenacity, dyeability and ramie-like texture. Some studies on chitin derivatives such as glycol chitin (5), chitin nitrate (6), carboxymethylated chitin (7) and acetyl chitin (3) have been reported, and many have been done on acetyl cellulose (1) as an acetate fiber. But there are few available on acetyl chitin and almost none on acetyl chitin fiber and film.

Nearly 30% acetylated chitin was prepared by the freezing method (0° C) from acetic anhydride and alkali chitin. Furthermore, variously acetylated chitins were prepared from chitin through treatment in a mixture of acetic anhydride and methanesulfonic acid at 0° C overnight. The 100% acetylated chitin was synthesized by the following methods: (a) chitin was kept for several days at 0° C in an excess of acetic anhydride saturated with hydrogen chloride; (b) chitin was reacted with an excess of acetic anhydride in methanesulfonic acid at 0° C overnight; and (c) chitin was reacted with an excess of acetic anhydride containing perchloric acid at 0° C for 3 hours.

The solubility of acetyl chitin in 99% formic acid increases with the degree of acetylation. The diacetyl chitin, when completely acetylated, was soluble in 85% formic acid. The film formation and spinnability of acetyl chitin were studied. The quality of both the acetyl-chitin fiber and the film improved in loop tenacity, flexibility and brightness with the degree of acetylation.

EXPERIMENTAL

Chitin

Powdered chitin of crab shell was obtained from the Nippon Suisan Co. Ltd. It was found that chitin binds tightly 1/2 mole of water per N-acetylglucosamine unit. Anal. found: C; 44.96%; H; 6.50%; N: 6.57%. Calcd. for $[C_8H_{11}NO_3(OH)_2 \cdot 1/2H_2O]$; C; 45.28%; H; 6.60%; N; 6.60%.

Chitin viscose

One part of the chitin powder was steeped in three parts of 40% aqueous sodium hydroxide for 2 hours at 11-13° C and for 10 hours at 0-5° C to prepare an alkaline chitin. The alkali chitin was pressed to three times the original chitin weight to remove the excess alkali and then crushed to powder with a Waring blender. The powder was put into a separatory funnel and left for 10 hours at -20° C *in vacuo*. Xanthogenation proceeded at 30° C for 15 hours with the addition of carbon disulfide, corresponding to one-half

the weight of the original chitin. Aqueous sodium hydroxide was then mixed with the xanthate at 0° C to make a 5% chitin and a 4.5% alkali concentration. The mixture of swollen xanthate was frozen at -20° C and was then slowly melted over 3 hours to obtain a clear homogeneous chitin viscose.

Cellulose viscose

Cellulose viscose was prepared from pulp according to the usual method via alkaline cellulose.

Aging of chitin viscose and the preparation of the spinning solution

The viscosity of the chitin viscose was reduced to 130 poises by aging for 14-18 hours. As the viscosity was still too high to spin a fiber, however, an equimolar amount of urea per N-acetylglucosamine residue was added. The viscosity dropped to 100 poises, and the solution was filtered through calico and flannel for spinning.

Solution of chitin in a chemical reagent

Solution in formic acid

Five g of chitin was suspended in 100 ml of 99% formic acid for several hours at room temperature and then frozen at 0° C overnight. The frozen mixture was melted gradually at room temperature. This procedure was repeated several times until a clear solution was obtained. As the viscosity of this chitin solution was too high to spin a fiber, the solution was diluted to a concentration of 4% chitin by adding 25 ml of 99% formic acid, followed by 10 ml of dichloroacetic acid to prepare a 100-poise homogeneous chitin solution.

Solution in dicloroacetic acid

Chitin was easily dissolved in dichloroacetic acid at room temperature. The viscosity tended to drop noticeably, however, with a corresponding fall in molecular weight, if the solution stood for too long a time at room temperature.

Solution in methanesulfonic acid

Chitin was dissolved in methanesulfonic acid at a low temperature with a high concentration of acid.

Acetylation of chitin and the solution of acetyl chitin

Acetylation of chitin through alkaline chitin

Alkaline chitin was prepared by the same method as chitin viscose and acetylated by an excess of acetic anhydride. It was assumed from the elemental analysis that 0.3 moles of OH groups per N-acetylglucosamine residue was acetylated. Anal. found: C; 45.93%; H; 6.69%; N; 6.21%. Calcd. for $[C_8H_{11}NO_3(OH)_2 \cdot 1/2\ H_2O]_{0.7}[C_8H_{11}NO_3(OH)(OCOCH_3) \cdot 1/2\ H_2O]_{0.3}$; C; 45.95%; H; 6.50%; N; 6.23%.

(A) Acetylation in methanesulfonic acid

To a mixture of 4 parts of methanesulfonic acid and 6 parts of glacial acetic acid, a calculated amount of acetic anhydride was added at 0° C; one part of chitin powder was then added with stirring at 0° C, and the mixture was left overnight at 0° C. The reaction mixture was poured into ice water to precipitate acetyl chitin. The precipitate was filtered and washed with water. The product was resuspended in distilled water, neutralized with ammonium hydroxide and boiled for 1 hour. Acetyl chitin was collected by filtration and dried in vacuo. Variously acetylated chitins were prepared by this method. The amounts of acetic anhydride and the degree of acetylation are shown in Table 3.

(B) Preparation of diacetyl chitin

a) One part of chitin powder and 5 parts of acetic anhydride were mixed, saturated with hydrogen chloride and kept for 10 days at 0° C. This reaction mixture was added to ice water, and the precipitate was washed with water and ethanol. It was easily soluble in 99% formic acid, but was also soluble in 85% formic acid. Nearly 1.6 moles of OH groups per N-acetylglucosamine residue was assumed to be acetylated from the elemental analysis. Anal. found: C; 48.48%; H; 6.07%; N; 5.18%. Calcd. for $[C_8H_{11}NO_3(OCOCH_3)_2]_{0.6} \cdot [C_8H_{11}NO_3(OH)(OCOCH_3) \cdot 1/2\ H_2O]_{0.4}$; C; 49.09%; H; 6.06%; N; 5.11%.

b) One part of chitin was added to the mixed acids (6 parts of acetic anhydride and 4 parts of methanesulfonic acid) with stirring at 0° C for 5 hours. This reaction mixture became viscous and was kept at 0° C overnight. The product was precipitated by the addition of ice water. The precipitate was treated by the same procedure as described in (A) (acetylation in methanesulfonic acid). Anal. found: C; 49.46%; H; 6.00%; N; 4.72%. Calcd. for $[C_8H_{11}NO_3(OCOCH_3)_2]$; C; 50.17%; H; 5.92%; N; 4.88%. It was easily soluble in 99% formic acid and still soluble in 85% formic acid.

c) To the aqueous perchloric acid (60%), an equimolar amount of acetic anhydride corresponding to the water in perchloric acid was added at 0° C and kept overnight at 0° C to prepare a solution of perchloric acid in glacial acetic acid. One part of chitin powder was added to a mixture of 10 parts of acetic anhydride and 2 parts of the perchloric-acid solution. This reaction mixture was stirred for 3 hours at 0° C, and the sludgy product was poured into ice water to precipitate. The precipitate was collected, resuspended in distilled water and treated by the same procedure as described in (b) above. Anal. found: C; 50.00%; H; 6.14%; N; 4.76%. Calcd. for $[C_8H_{11}NO_3(OCOCH_3)_2]$; C; 50.17%; H; 5.92%; N; 4.88%.

Spinning solution of fibers and spinnings

Chitin fibers

a) The spinning solution of chitin viscose was debubbled at room temperature and spun into a coagulation bath (10% sulfuric acid, 25% sodium sulfate

and 1% zinc sulfate) through a stainless-steel nozzle (50 holes, 0.1 mm ϕ or 30 holes, 0.2mm ϕ). Ethanol was used for the elongation bath at room temperature. The fiber was washed with water and ethanol (Fig. 1-B).

b) Chitin solution in formic acid [4% chitin (w/v), 8% dichloroacetic acid (v/v)] g was spun through a platinum nozzle (50 holes, 0.1 mm ϕ) into isopropyl ether (first coagulation bath) and passed through ethanol-glacial acetic acid-water (5:1:1 v/v) (second coagulation bath). The elongation proceeeded in cold water as shown in Fig. 1-A; the elongation ratio was 1.1.

Acetyl chitin fiber

10 g of acetyl chitin (1.1 moles acetylated) was dissolved in 150 ml of 99% formic acid; 12 ml of dichloroacetic acid was added to this solution making the spinning solution about 100 poises [6.2% acetyl chitin (w/v), 8% dichloroacetic acid (v/v)]. The spinning followed the same procedures as described in (b) above. The elongation ratio was 1.32, and the fiber was washed with boiled water (Fig. 1-A).

Diacetyl chitin fiber

The spinning solution (20 g of diacetyl chitin in 100 ml of 99% formic acid and 50 ml of ethylene chloride of around a 100-poise viscosity) was prepared by the addition of ethylene chloride to the solution of 20% (w/v) diacetyl chitin in formic acid; it was spun by the same procedure as was used for the chitin fiber (b) above. The elongation ratio was 1.45, and the fiber was washed with boiled water (Fig. 1-A).

RESULTS AND DISCUSSION

Chitin-viscose solution

The chitin viscose could not be prepared by the same method as was used for cellulose viscose, but a freezing process was applied successfully to obtain a homogeneous chitin viscose. The effect of aging on the viscosity was also observed in a way identical to that for cellulose viscose. It proved difficult, however, to reduce the viscosity of a 5% chitin viscose to the spinnable 100-poise level. The spinnability was also fatally affected by lowering the chitin concentration. Urea was found effectively to reduce the viscosity; as shown in Fig. 2, the viscosity of chitin viscose was reduced rapidly by increasing the urea concentration. The equivalent mole amount of urea to N-acetylglucosamine residue was enough to achieve a viscosity of 130 to 100 poises. This seems to suggest the presence of an intermolecular hydrogen bond due to the aminoacetyl groups of chitin.

Chitin fiber and cellulose chitin fibers

As shown in Table 1, chitin fibers prepared from chitin viscose at a low temperature seem to be useful, as they have a high Young's modulus and dry tenacity. Poor loop tenacity and poor wet tenacity were observed, however, and properties of chitin fiber seem to be changed seriously by the absorption of water. On the other hand, chitin fiber prepared from the formic-acid

solution seems to have better qualities than the fibers prepared from the viscose. The dyeability of chitin fibers by acidic or direct dyes was much better than for cellulose-rayon fiber; the higher dyeability was observed to increase with the chitin content of the cellulose-rayon fiber. The properties of cellulose-chitin fibers are shown in Table 2. The addition of 3% chitin was found to improve the properties of cellulose-rayon fiber.

Acetyl chitin

Chitin was acetylated by nearly 30% per N-acetylglucosamine residue through the reaction of alkaline chitin with acetic anhydride. Variously acetylated chitin was also obtained in methanesulfonic acid-acetic anhydride mixture by varying the amount of acetic anhydride, as shown in Table 3.

Diacetyl chitin was also prepared with an excess of acetic anhydride in methanesulfonic acid according to the above method. The acetylation of chitin proceeded in an excess of acetic anhydride saturated with hydrogen chloride, according to P. Shoruigin and E. Hait (7), although it was difficult to acetylate completely and took a very long time. When perchloric acid was used as a catalyzer, however, diacetyl chitin of a high molecular weight was prepared in a short time. The higher acetylated chitin was the more soluble in 99% formic acid, and the diacetyl chitin became soluble in 85% formic acid. The spinning of diacetyl chitin was carried out successfully by the use of a formic-acid solution containing ethylene chloride. It was spun into isopropyl ether, treated with ethanol-glacial acetic-acid water (5:1:1, v/v) and elongated in water at room temperature. The fibers showed some fine qualities. The properties of the fibers are shown in Table 4.

Composition of chitin and acetyl chitin

Chitin is assumed to bind water tightly, as shown by thermal analysis, infrared absorption spectrum and elemental analysis. The composition of the chitin molecule is $[C_8H_{11}NO_3(OH)_2 \cdot 1/2\ H_2O]$ after it has been dried several hours at 100° C *in vacuo*. Completely acetylated diacetyl chitin binds no water; the composition is $[C_8H_{11}NO_3(OCOCH_3)_2]$. The composition of the intermediates of acetylation agrees well with the results of the elemental analysis, assuming that the N-acetylglucosamine residue binds a half-mole of water even in monoacetyl-chitin $[C_8H_{11}NO_3(OH)(OCOCH_3)\ 1/2\ H_2O]$. There is no bound water on diacetyl-chitin $[C_8H_{11}NO_3(OCOCH_3)_2]$. The details of these findings will be published elsewhere.

ACKNOWLEDGEMENT

We are very much indebted to H. Shiota for his skillful help with sample preparations and to Y. Kabashima for his improvements of the spinning apparatus. We would like to express our thanks to Mitsubishi Rayon Co. and Nippon Suisan Co. for support in testing the fiber properties and for the supply of chitin powder.

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Table 1. Properties of Chitin Fibers

Sample No.		1	12	13	14
Denier		3.08	8.16	11.30	17.78
Tenacity (g/d)	Dry	1.17	1.52	1.30	0.90
	Wet	0.22	0.15	0.10	0.02
Elongation(%)	Dry	11.2	5.8	5.1	3.9
	Wet	10.9	4.7	6.4	2.2
Knot	Strength (g/d)	0.18	0.10	0.12	0.08
	Elongation (%)		9.3	6.0	7.7
Loop	Strength (g/d)		0.07	0.06	0.07
	Elongation (%)		4.2	4.6	5.2

Table 2. Properties of Cellulose-Chitin Fibers

Chitin Content (%)		0	1.5	3	5	10
Denier		12.9	11.2	23.7	25.8	26.9
Tenacity (g/d)	Dry	2.09	2.08	1.75	1.37	1.20
	Wet	1.06	1.02	0.66	0.53	0.44
Elongation (%)	Dry	18.4	15.6	19.3	15.1	14.4
	Wet	28.4	24.2	26.1	23.3	19.2
Knot	Strength (g/d)	1.42	1.12	1.19	0.99	0.88
	Elongation (%)	17.3	10.5	19.5	14.1	15.6
Loop	Strength (g/d)	1.26	0.96	1.56	1.10	1.05
	Elongation (%)	3.1	2.8	6.7	5.4	4.4

Table 3. Acetylation in Methanesulfonic Acid plus Acetic Anhydride

Molar ratio*	Acetylated group**	Elemental analysis C(%)	H(%)	N(%)
1.6	1.1	47.78	6.41	5.73
2.6	1.4	48.57	6.18	5.39
3.5	1.7	49.16	6.14	5.11

*equivalent mole of acetic anhydride/N-acetylglucosamine residue.
**equivalent mole of acetyl group/N-acetylglucosamine residue.

Table 4. Properties of Chitin and Acetyl-Chitin Fibers

Properties		No:	1	2	3	4	5	6	7	8
		Sample:	Chitin	Chitin	0.3Ac NaOH	0.3Ac NaOH	1.1Ac CH_3SO_3H	1.6Ac CH_3SO_3H	2.0Ac CH_3SO_3H	2.0Ac $HClO_4$
Denier (d)			25.46	3.22	50.06	3.55	3.98	4.28	9.22	7.88
Tenacity (g/d)	Dry(20°C, 65%RH)		1.32	0.68	1.73	0.94	1.89	1.52	1.13	1.48
	Wet(20°C, 100%RH)		0.18	0.23	0.74	0.33	0.75	0.58	0.50	0.56
	Wet(90°C, 100%RH)		0.18	0.23	0.73	0.37	0.75	0.65	0.43	0.39
Elongation (%)	Dry(20°C, 65%RH)		2.7	2.9	3.7	5.7	11.7	5.3	7.5	7.0
	Wet(20°C, 100%RH)		7.8	10.8	15.6	13.7	22.7	12.9	14.1	15.4
	Wet(90°C, 100%RH)		7.1	13.0	15.7	15.2	22.1	14.4	15.9	20.6
Knot (20° C, 65% RH)	Strength (g/d)		0.45	0.45	0.41	0.44	0.64	0.39	0.14	0.31
	Elongation (%)		--	2.2	--	1.6	1.6	1.1	1.9	1.8
Young's Modulus (g/d)	Dry(20°C, 65%RH)		71.48	42.66	70.80	45.91	55.67	75.17	46.69	63.96
	Wet(20°C, 100%RH)		2.74	2.39	5.48	2.51	6.02	6.38	3.40	4.66
	Wet(90°C, 100%RH)		2.39	1.97	5.43	2.03	4.98	5.09	2.52	1.70
Absorption of moisture (%)			12.9	13.0	21.3	13.9	11.9	9.3	8.5	9.5
Density (g/ml)			1.3817	1.3469	1.3883	1.3551	1.3281	1.3147	1.2444	1.3132

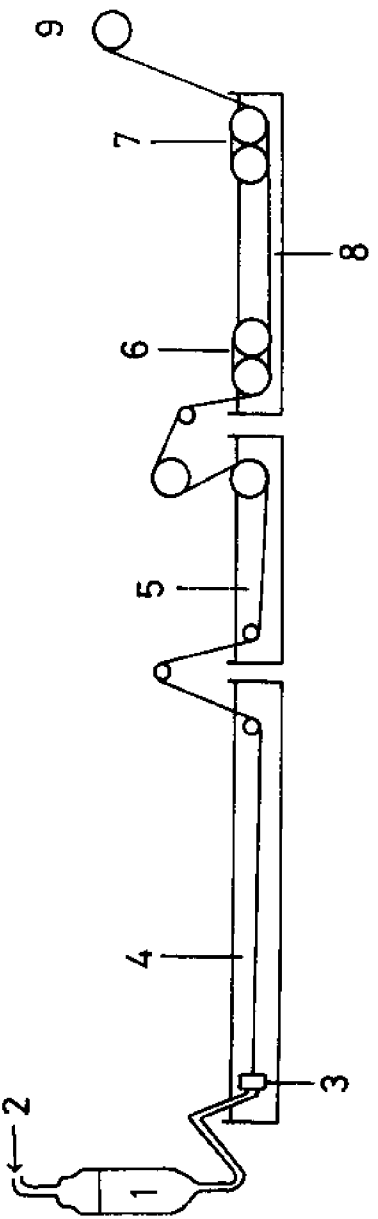

Fig. 1-A. Spinning apparatus for chitin, acetyl-chitin and diacetyl-chitin fibers. 1: Spinning solution. 2: Pressure. 3: Nozzle. 4: First coagulation bath (isopropyl ether). 5: Second coagulation bath(ethanol-glacial acetic acid-water, 5:1:1, v/v). 6: First roller. 7: Second roller. 8: Elongation bath (water). 9: Winder.

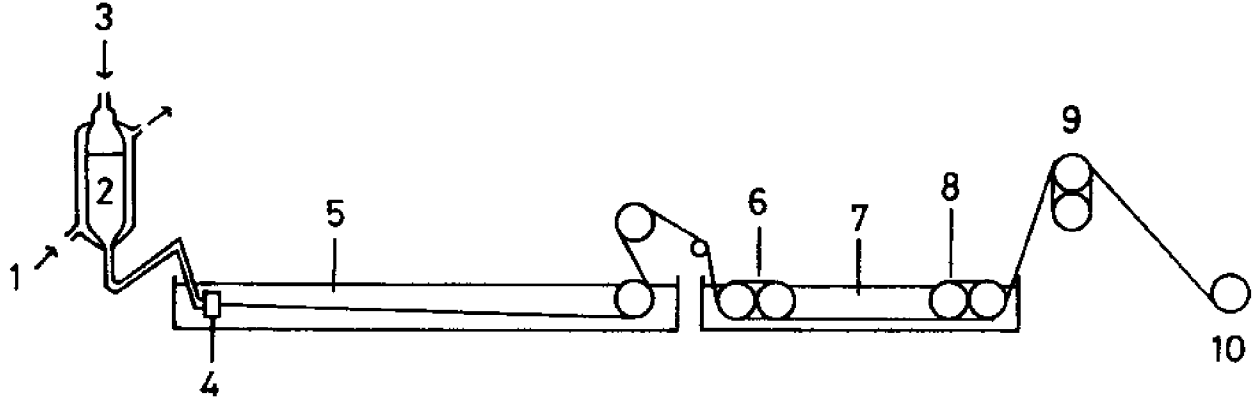

Fig. 1-B. Spinning apparatus for chitin-viscose fibers.
1: Cold water. 2: Spinning solution. 3: Pressure. 4: Nozzle.
5: Coagulation bath (10% sulfuric acid, 25% sodium sulfate and 1% zinc sulfate). 6: First roller. 7: Elongation bath (Ethanol).
8: Second roller. 9: Hot roller(110°C). 10: Winder.

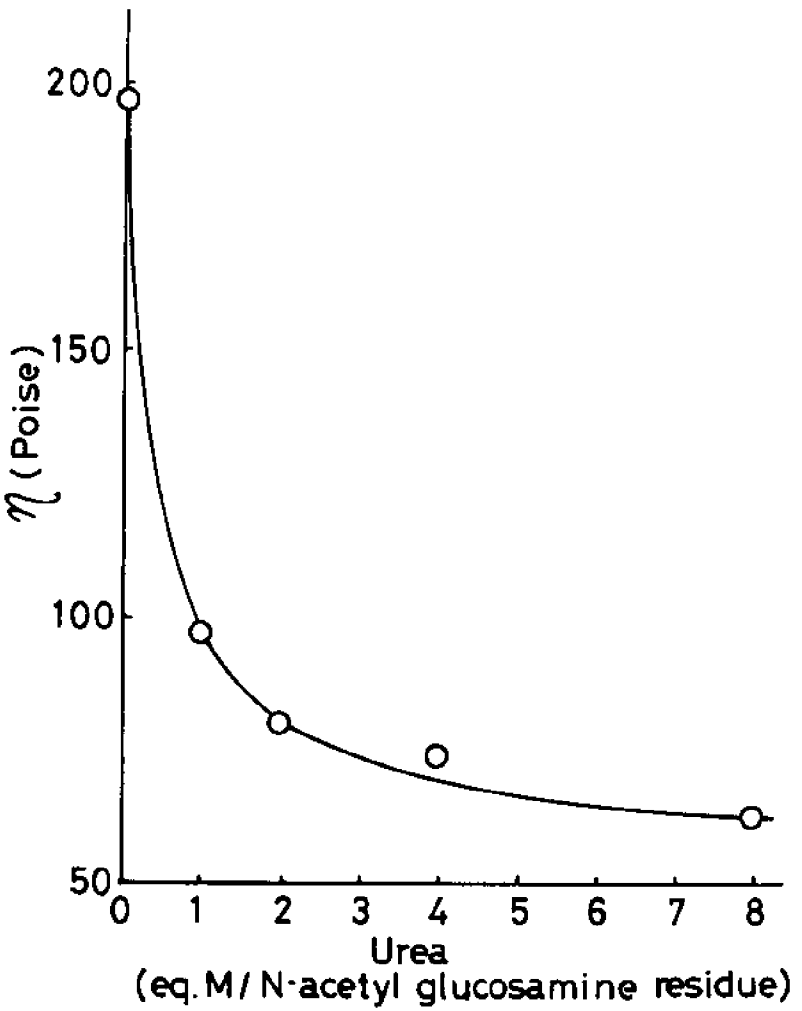

Fig. 2. Effect of urea on the viscosity of chitin viscose.

THE POSSIBLE USE OF CHITIN AND CHITOSAN AS ANIMAL FEED

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ABSTRACT

Shrimp processing is a major industry in Nigeria, and a high proportion (85%) of what is produced is waste. Shrimp waste has a high protein content (44.1%) of good protein value. Its amino-acid profile is similar to that of fishmeal, and it is high in lysine and methionine (6.2 and 2.5 g per 100 g protein). Preliminary experiments show that the protein is easily digestible (78.7%) and has good PER (2.1) when fed to rats at a 10% level. It also contains a high amount of chitin (12.5%) and of ash (27.3%) made up mainly of calcium, and this might affect its value as animal feed. The potential of shrimp waste as animal feed is discussed in detail.

INTRODUCTION

One way of combating human protein malnutrition is by increasing the use of animal protein, but that form of protein is becoming more and more expensive in developing countries, and will soon be out of the reach of the poor. The price of fishmeal has tripled within the last year or two, and soybean and maize are also becoming very expensive. It is therefore imperative to seek locally available cheap sources of nutrition and protein. Cassava has since been substituted for maize; more recently, the question has arisen whether shrimp waste could not be substituted for fishmeal.

The first commercial shrimp fishing in Nigeria started in 1965 when two 70-foot Gulf of Mexico vessels, using a double rig of two 45-foot trawls in conjunction with a try net, began fishing out of Lagos, with an average daily catch that increased from 200 lb of tails in January to over 1,000 lb in June. By 1973 the Nigerian government had banned the importation of stockfish from Norway and Iceland, and this, coupled with the discovery of shrimps in commercial quantities in the Nigerian waters, has led to a rapid growth of the fishing industry. Around this period as many as 1358 metric tons of shrimps were caught, and that amount has since grown even larger. It later became clear that commercial fisheries would have to be developed in order to meet the demand for fish in the country. Loans were given to merchandize fishing boats, purchase nets, and process and market the

fish. According to the federal fisheries Annual Report of 1973 about 23 fishing companies had been registered, some operating on a charter basis, others engaged in coastal fishing for fish and shrimps for freezing and export to foreign markets. At the port, the heads, tails and shells are removed and discarded. These could be dried and made into a meal by any of the usual processing methods, such as steaming, or hot-air or sun drying. The meal could be high in nitrogen, depending on the source, with some proportion contributed by chitin.

Since a high proportion of the shrimp brought to shore (about 85%) is made up of shrimp waste that is usually discarded, an attempt is made in this preliminary report to determine the chemical composition of the waste and evaluate the possibility for using it as animal feed.

EXPERIMENTAL

Shrimp waste was obtained from the factory and brought to the laboratory for drying. The meal was then ground up into a powder and used for various analyses.

Proximate analysis and lipid determinations were carried out by AOAC methods of analysis (1), and chitin by the method of Meyers et al. (7). Amino-acid analysis was done after hydrolysis with 6N HCl for 16 hrs on a Perkin Elmer automatic amino-acid analyzer model KL-3B; available lysine analysis was carried out by Carpenter's method (4). In vitro digestibility was determined using the method of Saunders and Kohler (10) with pepsin followed by pancreatin.

In the rat feeding assays, a modification of the Rippon basal protein-free diet was used (9). The composition of the diet is shown in Table 1.

Table 1. Composition of Basal Protein-Free Diet

Ingredient	Amount
	g
Sucrose	140
Butter (Danish)	200
Vitamin mixture	100
Non-nutritive cellulose	100
Salt mixture	80
Cod-liver oil	20
Corn starch	1360

All the test diets were prepared by incorporating each material separately at the expense of the cornstarch in the basal protein-free diet to give 10% protein (N x 6.25). Both the basal protein-free diet and the test diets were made into a thick paste with a small quantity of water to reduce spillage by the animals. Weanling litter-mate male rats of the Wistar strain from our own colony were used. They were collected at 23-24 days of

age, numbered and housed individually in screen-bottomed cages. They were weaned to the stock diet in the experimental cages for one week. At the commencement of the feeding trial the rats were 30-31 days old and weighed between 50-60 gm.

One group of four rats was fed the protein-free diet for ten days and a second group was fed the shrimp-waste diet. For comparative purposes, casein at the 10% protein level was also fed to a third group of four male rats for ten days. Water and food were given ad libitum. The weights of the animals were recorded every other day. The first three days were regarded as an acclimatization period during which no records were kept of food consumption, and no collection of feces was made.

Collection of feces was made daily for the last 7 days of the feeding experiment. The feces of individual rats were pooled, dried at 105° C for 24 hours and ground into powder for fecal nitrogen estimation.

At the end of the experimental period the animals were sacrificed and the carcass nitrogen determined by Kjeldahl digestion and analysis. The feeding trial was then repeated.

RESULTS

Table 2 gives the chemical composition of the shrimp waste.

Table 2. Proximate Analysis of Shrimp Waste

	%
Moisture	6.3
Total crude protein	49.5
Corrected crude protein	44.1
Ether extract	4.3
Crude fiber	12.1
Ash	27.3
Calcium	11.4
Phosphorus	1.8

It includes a large amount of crude protein (44%), with a high proportion of non-protein material (11%). Although the lipid content is low (0.73%), the iodine number (142) shows that it is highly unsaturated (Table 3). The fat consists of phospholipid (57.9%) and non-phospholipid (42%) fractions in similar amounts. The rest of the lipid is made up of triglycerides (24.3%), sterol ester (14%), diglycerides (8.2%), free fatty acids (12.9%), monoglycerides and sterols. The chitin content is 12.5% which corresponds approximately to the crude-fiber content (12.1%). The true protein N can be obtained by subtracting the percentage of chitin-N (0.86%) from the total N. From this true protein-N (7.06%) the true protein (44.1%) is obtained by multiplying by 6.25. The ash content is very high (27.3%); it consists mainly of calcium (11.4%) in the form of $CaCO_3$, but contains some phosphorus (1.8%) and other minerals.

Table 3. Chemical Composition of Shrimp Waste

Constituent		%
Lipid (Iodine value of lipid:142)		0.73
Sterol ester	of extracted fat	14.0
Triglyceride		24.3
Diglyceride		8.2
Monoglyceride		2.2
Sterol		3.7
Free fatty acids		12.9
Phospholipids		57.9
Non-phospholipids		42.1
Hydrocarbons		36.8
Chitin		12.5

The amino-acid analysis in Table 4 shows that shrimp waste is made up of proteins of good quality.

Table 4. Amino-Acid Analysis of Shrimp Waste (% of dry matter)

Thre	Gly.	Val	Met	Isoleu	Leu	Phen	Lys*	His	Arg
1.9	2.1	1.8	0.8	1.7	2.8	2.1	2.7	1.6	2.8

*Most of the lysine is in the available form, but the lysine digestibility is low (63%).

The PER and digestibility of the proteins are low (1.9 and 71.5% respectively), but if allowance is made for the chitin N then the figures are reasonable (2.1 and 78.7% respectively) as shown in Table 5.

Table 5. Nutritive Value of Shrimp Waste

	Uncorrected			Corrected		
Ration	PER	TD	In Vitro Digesti-bility*	PER	TD	In Vitro Digesti-bility*
Shrimp Waste	1.9	71.5	58	2.1	78.7	63.8
Casein	--	--	--	2.5	86.8	99.5

*The low in vitro digestibility is probably due to the chitin.

DISCUSSION

The high salt (sodium chloride) and calcium contents could limit the use of shrimp waste as animal feed. Salt is usually added to prevent spoilage during the sun-drying process, and the concentration may vary from 0 to 7% in the final product. The ash content is variable, making up from 17 to 30% of the final product, as it is derived mainly from the exoskeleton. The exoskeleton is very rich in $CaCO_3$, and can make up from 5 to 27% of the product (11).

The high mineral content is probably responsible for the low chemical digestibility (71.5) and the low PER (1.9) value. In this preliminary experiment, the shrimp waste was used exclusively as the main source of protein, but in subsequent experiments now in progress, it is being used in combination with leaf-protein concentrate. From the pattern of the amino acids it appears a priori to be a good animal feed. The composition is comparable to the compositions of fishmeal and of bonemeal. It has a high content of lysine and methionine, and most of the lysine is in the available form. In addition, it contains an appreciable amount of carotenoids which makes it valuable as chicken feed. Jarquin et al. (5) found that it reacted in a slower growth rate in chicks when compounded with fishmeal than fishmeal alone did when given at the same protein level. Supplementation with lysine improved the weight gain, but the addition of methionine produced no further response. Addition of phosphoric acid also improved growth. Lovell et al. (6) found that shrimp waste was as good as amino-acid fortified soya as the sole source of protein for rats. Bray et al. (3) found that shrimp meal was superior to tankage as a supplement to maize or to maize and rice polishing, regardless of whether these supplements were fed alone with maize or in combination with cottonseed meal and other protein feeds in growing-finishing swine diets. Perez (8) found that satisfactory results could be obtained when shrimp meal was used as a supplement at 5% level.

The amount of chitin is closely related to the crude-fiber content, probably because the method of estimation is similar. Other workers have also found that crude-fiber content is a good indication of chitin (2). It is very doubtful if chitin is utilized by monogastric animals, though it may possibly be used by ruminants, and this lowers the nutritive value of shrimp waste. The PER and TD obtained could be corrected for chitin, and these would then give a PER of 2.1 and a TD of 78.7%, which are more reasonable and promising for a source of protein.

Another aspect that might pose a problem in the use of shrimp waste as animal feed is the variable composition of the meal. The head, which constitutes about 40-44% of the shrimp, is known to contain the highest amount of protein, and it is the source of a series of enzymes (7). The hulls contain less protein and more chitin and ash, so that the final composition of the meal will depend on the proportions of the various parts that are mixed together. In addition, the method of processing will also have an effect on the composition. In cases where the shrimp heads are separated at sea, a period of time will have elapsed before final processing, and this may result in loss of nutritive

value. A 10% reduction has already been observed in the protein when the heads were allowed to spoil for 24 hrs. The rapid degradation is brought about by the enzymes concentrated in the head. This autolytic and microbiological degradation can be reduced by a treatment with acid for 6-24 hrs, followed by neutralization, which destroys the enzymes.

CONCLUSIONS

With proper handling, shrimp waste can be a new source of protein for animal feed, and it would be economical, both because it is expensive to get rid of this waste due to its high BOD requirement and also because of its nutritive value.

ACKNOWLEDGMENT

This work was supported by a grant from the Nigerian Institute of Oceanographic and Marine Research, Lagos, for which the authors are very grateful.

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VII. CHEMICAL ASPECTS OF CHITIN RESEARCH

MODIFIED CHITOSANS AND THEIR CHROMATOGRAPHIC PERFORMANCES

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ABSTRACT

Chitosan is applied in anion-exchange, chelation, ligand-exchange, affinity and thin-layer chromatography. Chitosan is the most powerful collector of vanadium so far known among polymers and its column capacities are 0.347 g VO^{2+} per gram and 2.327 g VO_4^{3-} per gram. By taking advantage of chitosan's indifference to manganese ions, it can be used to recover cobalt, nickel and copper from marine nodules, after fusion of the manganese nodules in sodium hydrogen sulfate at 215°C. Copper-loaded chitosans are used to collect amino acids with high yields, even under unfavorable pH conditions. Lysozyme is isolated from accompanying proteins on chitosan columns at pH 9.0 and is eluted with a 2 % solution of propylamine. The chicken egg white lysozyme thus recovered keeps 55 % of the original activity. Chitosan thin layers are prepared for the first time and used to perform quality checks on a number of dyes of interest in histology; they also help resolve dye mixtures used in food technology.

INTRODUCTION

Chitosan has been fully described in recent books (15,16,17), and since the first reports on its chelating ability (14,24), several Laboratories have produced data of interest in various branches of chromatography.

Chitosan can be considered a strong base as its primary amino groups, whose pK_a is 6.3, easily form quaternary nitrogen salts at low pH values; thus, in acidic solutions chitosan has high anion capacity. At higher pH values, however, it is a weak base because the primary amino groups are not protonated and therefore do not interact with anions and do not dissociate neutral salts: for instance, they do not retain chloride from sodium chloride neutral solutions. This is a peculiar feature of chitosan, in so far as it would be classified as a strongly basic anion exchanger with no dissociation capacity for neutral salts.

Chitosan is relatively indifferent to alkali metals and alkaline earth ions and is thus superior to other polymers tested for the recovery of transition metals in saline waters. The complexing ability of chitosan is a direct consequence of the base strength of the amino groups and is most effective for those metals which form complexes with ammonia, e.g. copper, zinc and mercury. The amino groups are regularly distributed at C^2 of each anhydroglucose ring and the nitrogen percent is up to 8.4.

Thus, while sharing desirable properties with other biopolymers, such as hydrophilicity, chitosan alone possesses certain features due to its amino groups, namely basicity, high capacity for transition metal ions, fast binding rate, fast flow-rate for solutions percolating through chitosan columns, buffering capacity for hydrogen ions and negligible swelling.

Characterization of chitosan has revealed a number of advantages for this material over man-made commercially available chelating resins such

as Amberlite XE-318 and Dowex A-1, otherwise called Chelex 100 (Srafion is not considered a chelating resin (20)). For instance, chitosan exhibits very little volume change with exposure to acids, bases and brines, while man-made resins undergo volume changes as high as 40 %.

Chitosan can be easily derivatized: for example, the immobilization of enzymes and other substances has been performed with glutaraldehyde, however, in view of the features of the chitosan surface and chemical constitution, adsorption does not necessarily require chemical modification. Cross-linking with suitable reagents would presumably obviate the tendency to solubilization that chitosan shows in certain media.

The purpose of this paper is to offer evidence of the usefulness of chitosan in anion-exchange chromatography, chelation chromatography, ligand-exchange chromatography, adsorption or affinity chromatography and in thin-layer chromatography. Important separations are presented for each of these branches.

As for anion-exchange, particular attention is paid to the possibility of collecting vanadium in large amounts instead of traces, as previously investigated (21,22). In fact, there is interest in collecting vanadium from fly ashes derived from petroleum products (5) and from waste waters.

In the field of chelation chromatography, the separation of valuable metals from manganese nodules is of importance; marine ores, mainly composed of manganese oxide with some iron oxide and containing up to 2.7 % of cobalt, nickel and copper, await exploitation for the industrial production of the latter three elements (3,7,8,13,27).

Ligand-exchange chromatography has found a variety of applications, during the last several years, mainly for isolation or fractionation of amines (9,19,25), amino acids (1,6) and nucleosides (4); the capacity of the chromatographic supports depends on the amounts of metal ions being retained on them and therefore chelating polymers having high capacity for transition metal ions are highly desirable. In addition, the metal ions should be retained under the operation conditions and no leakage should occur. Chitosan combines a number of features which qualify it as a good support for ligand-exchange.

By taking advantage of chitosan's surface characteristics (10,18,26), several enzymes have been immobilized on it, among others α-chymotrypsin, acid phosphatase, proteinase, trypsin and subtilisin. It is known that the biochemistry of lysozyme has been studied with the aid of oligochitosaccharides. Chitosan is not hydrolyzed by lysozyme (2) and therefore is an attractive chromatographic support for this enzyme. Thus, the isolation of lysozyme from natural products would extend the choice of techniques for the purification of this important enzyme.

The preparation of thin layers of chitosan, here reported for the first time, has been hindered so far by the inability to produce chitosan powder thin enough for homogeneous layers. In the past, thin layers were produced with chitosan-coated cellulose; the chitosan often being in the formate or acetate forms as a very minor component of the layer, the results were not representative of chitosan itself. Chromatographic checks on some cationic dyes are often necessary to ensure reproducible staining of histological preparations (11,12). In another group of dyes currently used in the food industry, one is presently suspected of having adverse effects and

foods containing it are being withdrawn from the market in Italy and other Countries. A sudden need exists for rapid identification of the red dyes to produce evidence of illegal products.

EXPERIMENTAL

Instrumentation

A Perkin-Elmer 305 atomic absorption spectrometer equipped with both flame atomizer and PE-70 graphite atomizer, deuterium background compensator and strip chart recorder was used for metal determinations. A Beckman model 25 double beam spectrophotometer equipped with a printer unit, an automatic strip chart recorder and a thermostat was used to determine amino acids and enzyme activities. Effluent fractions from a reference column with no enzymes were normally used as blanks. The selected wavelengths were 280 nm for proteins and 600 nm for Remazol Brilliant Blue R, to determine the immobilization yields and the activity percentages, respectively. Amino acids were determined at 540 nm. A Perkin-Elmer DSC-2 differential scanning calorimeter was used to obtain physical evidence of chemical modifications introduced into chitosan: it was operated in the temperature interval -173 ÷ +100°C with cooling and heating rates of 80°K×min^{-1}, range 20 mcal×sec^{-1}, chart speed 10 mm×min^{-1} and helium pressure 5 atm.

Chitosan

Chitosan was supplied by Food, Chemical and Research Laboratories, Inc., (Kypro Company), 4900 9th street N.W., Seattle, WA 98101 (batch number 3-73 Dungeness); it was milled, sieved and used in powder form. The jacketed columns were as follows: for vanadium 30×10 mm, 20 ml×min^{-1} (0.5 g preconditioned chitosan); for manganese 60×5 mm, 3 ml×min^{-1} (0.2 g preconditioned chitosan); for lysozyme 80×9 mm, 5 ml×min^{-1} (1.5 g chitosan) and for amino acids 85×4 mm, 0.7 ml×min^{-1} (0.5 g chitosan loaded with 0.150 g copper from copper sulfate solution). The columns were connected to a Gilson HP-4 peristaltic pump. For the preparation of thin layers, chitosan powder was first dissolved in 2 % acetic acid and then precipitated with ammonia; after washing on a large Buchner funnel, the gel was introduced into a dialysis tube and kept against distilled water for several days. The gel was then liophilized at -30°C, then it was disrupted in a mill at 20,000 rpm for a couple of minutes. A suspension of the resulting powder in water+ethanol (9:1) was used to prepare 0.25 mm thin layers with the Desaga apparatus. Scheicher & Schuell DEAE-cellulose and Carlo Erba Stratocrom Al-backed silica gel thin layers (0.25 mm) were also used. The latter were developed according to previous publications (12).

Chitosan derivatives

A number of chitosan derivatives have been prepared by suspending the chitosan powder in the proper solution and stirring according to the following descriptions. The solubility assay was done in 5 % acetic acid at 20°C and at boiling. All the derivatives obtained were free-flowing powders.

TU-GLA-chitosan: chitosan (2 g) was reacted with 0.2 M thiourea (40 ml) and 25 % glutaraldehyde (1 ml). Insoluble.

U-GLA-chitosan: chitosan (2 g) was reacted with 0.2 M urea (40 ml) and 25 % glutaraldehyde (1 ml). Insoluble.

U-GLY-chitosan: chitosan (2 g) was reacted with 0.2 M urea (40 ml) and glyoxal (0.5 g). Insoluble.

AMSA-chitosan: chitosan was reacted with S-acetylmercaptosuccinic anhydride (molar ratio 1:10) in little water at boiling for a few minutes. The violet product was washed with cold water until white. Insoluble.

EPIC-chitosan: chitin (50 g) in form of flakes was stirred in 40 % sodium hydroxide (500 g) at 0°C for 48 hr. During this time, the temperature was allowed to rise to 15°C three times. Water (2 l) at 15°C was added and the flakes were pressed in a nylon filter. After addition of epichlorohydrin (50 g) the reaction was allowed to proceed for 48 hr at 5°C. The amino groups were thus protected (except for about 15 % of them, naturally occurring in the free form); the obtained epichlorohydrin chitin was deacetylated according to Broussignac to yield epichlorohydrin chitosan (17). Insoluble.

DIAZ-chitosan: chitosan (2 g) was reacted with excess nitrous acid according to the current diazotization procedure. Evolution of nitrogen was evident even at low temperature.

O-Hydroxyethyl chitin covalently labelled with Remazol Brillinat Blue R was prepared and used for the determination of lysozyme activity.

Copper-loaded chitosan was prepared by stirring chitosan (50 g) with copper sulfate pentahydrate (37.5 g) in water.

Reagents

Metal ion solutions were obtained by dissolving sulfates, except for mercuric chloride, trisodium orthovanadate and sodium hydrogen phosphate. The solubilization of the manganese nodules has been carried out in sodium hydrogen sulfate or in potassium hydrogen sulfate. The temperature required for fusion was around 215°C; this is important because the process hereby proposed requires very little energy, in accordance with well-known restrictions related to the locations of the future processing plants. The resulting mass is soluble in water and yields a clear solution of manganous ions; the sulfate anion favors the subsequent chromatographic separation. The amino acid solutions were 0.3 mM. The eluate (2 ml) was added to 1 % ninhydrin reagent (2 ml) kept in a boiling water bath for 10 min, cooled and diluted with 4 ml of 50 % ethanol. The dyes were all supplied by Merck AG, Darmstadt, and were applied as water+ethanol solutions.

RESULTS AND DISCUSSION

Anion-exchange chromatography

The data in Table 1 show that chitosan and all of its derivatives collect the total amount of vanadate brought to their presence in aqueous solution at pH 2.5. The lowest results are obtained with DIAZ-chitosan. It was observed that the chitosan powder, which is white, first became orange and then green upon collection of the yellow vanadate. As vanadate is reduced to vanadyl by fairly mild reducing agents, this observation can be explained by admitting that part of the vanadate is progressively reduced to vanadyl when left in contact with chitosan by the combined action of some carbonyl groups and light; therefore vanadium would be retained in

part as vanadate (ion-exchange) and in part as vanadyl complex (chelation). Several measurements on the collection of vanadyl from aqueous solutions have been carried out on chitosan; the breakthrough curves have been studied as functions of the pH of the vanadyl solution, the most favorable one being at pH 6.0. The column capacity, in this case, has been calculated to be 265 mg of vanadium per gram of chitosan, from the equation:

$$\text{column capacity} = \frac{\frac{1}{2}\,(V_1 + V_2)\times C}{\text{ml}_{\text{chitosan}}}$$

where C is the metal ion concentration in $\text{mmol}\times\text{l}^{-1}$; V is the effluent volume in liters at points 1 and 2 (initial and final points of the breakthrough curves). Thus, both vanadate and vanadyl ions can be easily recovered on chitosan.

Vanadate can also be recovered from brines; several measurements of the collection yields on several derivatives from 3 % NaCl solutions have been included in Table 1. As the most interesting derivatives in this respect appear to be U-GLY-chitosan, the breakthrough curves for vanadate from chitosan and U-GLY-chitosan have been studied as a function of pH: the highest column capacity for chitosan was found at pH 4.0, while for U-GLY-chitosan is at pH 3.0. The breakthrough curves for vanadate from these columns under the best experimental conditions are in Fig. 1. It is clear

Table 1. Collection Percentages of Metal Ions from Aqueous Solutions at pH 2.5, by Chitosan and Chitosan Derivatives (200 mg) after 24 hr at 20°C under Stirring

Polymer	VO_4^{3-}		Mn^{2+}		Co^{2+}		Ni^{2+}		Cu^{2+}	
	2.5	brine	2.5	5.5	2.5	5.5	2.5	5.5	2.5	5.5
Chitosan	100	100	0	0	10	10	46	54	99	100
TU-GLA-chitosan	99	67	0	0	0	0	0	11	11	17
U-GLA-chitosan	99	34	0	0	5	5	5	23	38	91
U-GLI-chitosan	100	90	0	0	4	9	18	35	64	85
AMSA-chitosan	99	43	0	0	8	16	25	58	94	96
EPIC-chitosan	99	69	0	0	4	11	10	23	86	100
DIAZ-chitosan	60	33	0	0	0	0	0	0	0	0

For symbols, see under Experimental.

that the chitosan collects more vanadate from 3 % NaCl brines than from water. The column capacities are: 196 and 320 mg of vanadium per gram of U-GLY-chitosan and 718 and 1032 mg of vanadium per gram of chitosan. These values are given as minimum values obtained by extrapolating the breakthrough curves; actual values would possibly be higher, because the effluent concentrations are always slightly lower than the concentrations of the solutions fed into the columns. They take into account the high specific weight of U-GLY-chitosan (0.8 g instead of 0.5 g for similar columns of chitosan).

The chitosan treated with nitrous acid (DIAZ-chitosan), as a consequence of the destruction of the amino groups, completely loses its chelating ability as shown in Table 1; in fact, DIAZ-chitosan is a deeply modified substance as revealed in Fig. 2: the calorimetric profiles and areas relevant to chitosan, chitosan orthovanadate and DIAZ-chitosan testify of the altered identities.

The chelation ability is not lost with other derivatives: EPIC-chitosan, a cross-linked chitosan, keeps most of its chelating power. This means that chitosan, when suitably cross-linked to impart insolubility, is still apt for chelation chromatography. An illustration of this point is given in Fig. 3, where the breakthrough curves for mercuric ion on chitosan, EPIC-chitosan and AMSA-chitosan are plotted.

While the results for chitosan and AMSA-chitosan are equivalent, there is a little drop of column capacity with EPIC-chitosan. The column capacities are: chitosan, 565; AMSA-chitosan, 526 and EPIC-chitosan, 173 mg of mercury per gram.

Chitosan and U-GLY-chitosan columns conditioned at pH 5.5 with sulfuric acid, were used to study the collection of phosphate from 2 ppm solution of phosphorus as sodium dihydrogen orthophosphate. Under these conditions, the breakthrough points were at 640 ml and 80 ml for chitosan and for U-GLY-chitosan, respectively. Evidence of the high capacity of chitosan for orthophosphate is presented in Fig. 4. Chitosan columns (1 g) were conditioned at various pH values with the mixture sulfuric acid + ammonium sulfate (0.1 M) and with ammonia. The phosphate concentration was plotted against the volume of effluent. The highest capacity was found at pH 6.0.

Chelation chromatography

It appears from the data presented above that cobalt, nickel and copper are retained on chitosan, while manganese is not. It is also known that iron in the ferrous state is not retained.

The breakthrough curves for the mixture of the five metals in the ratios Mn 52.6 %, Fe 31.6 %, Co 5.2 %, Ni 5.2 % and Cu 5.2 % are presented in Fig. 5. Ascorbic acid was added to keep iron in its lower oxidation state. However, most of the marine nodules contain very little iron and its collection on chitosan would not appreciably depress the collection of Co, Ni and Cu, thus the reducing agent is just optional. It should be noted that these results refer to a very short column, which was selected to obtain the five curves in reasonable interval for analytical purposes, but columns with a more favorable length to diameter ratios would give more distinct intervals between the Mn and the Co curves.

Chitosan columns have been recycled 20 times according to the following sequence: a) metal ion solution (1 ml) at pH 6.0 with added ascorbic acid in the ratio ascorbic acid/iron = 100 and sodium sulfate (4 $g \times l^{-1}$), final volume 50 ml; b) water (20 ml) to wash out excess disodium sulfate; c) 0.1 M sulfuric acid + ammonium sulfate mixture to perform elution; d) water (20 ml); e) 1 M ammonia (10 ml) and finally, f) water, to reach pH 6.0. The columns did not lose their chelating ability as the results of the 20th cycle are similar to those of the first (23).

Ligand-exchange chromatography

The copper-loaded chitosan (100 mg) was stirred with amino acid solution (3 ml) for 1 hr at room temperature. The same was done with the copper-loaded chitosan in the ammonia form. The results are presented in Table 2 and indicate that all the amino acids tested can be collected on the chitosans loaded with copper, which have high capacities for a few of them. Results for the series relevant to Cu-chitosan are higher than for the series of Cu-NH_3-chitosan.

Table 2. Collection Percentages of Amino Acids (3 ml of 0.4 mM solutions) on Cu-Chitosan and on Cu-NH_3-Chitosan (100 mg) after 1 hr Shaking

Amino acid	initial pH	Cu-chitosan	Cu-NH3-chitosan	chitosan
L-tryptophan	6.6	100	65	0
glycine	6.8	100	37	0
L(+) histidine HCl	5.2	83	58	0
L(+) aspartic a.	4.3	100	87	0
L(+) isoleucine	6.6	69	19	0
DL serine	7.4	88	59	0
L(-) histidine	6.5	62	59	0
L(+) glutamic a.	3.9	100	87	0
L(+) cysteine	5.5	94	0	0

The final pH values were: on Cu-chitosan 5.5; on Cu-NH3-chitosan 6.5 ÷ 7.0 and on chitosan 8.0 ÷ 8.4.

When 0.3 mM amino acid solutions are percolated through the columns, L(+)-cysteine shows a black band in the upper part of the column, which can be eluted with ammonia. The breakthrough curve for this amino acid under the said conditions is rather flat; some breakthrough curves are in Fig. 6.

Affinity or adsorption chromatography

The immobilization yields of lysozyme are shown in Fig. 7. Aqueous solutions of lysozyme at the desired pH (3 ml at 1 $mg \times ml^{-1}$) were introduced into chitosan columns and kept in contact 30 min at 30°C. The columns were then washed with water (60 ml) at the same pH, and water fractions (5 ml) were collected for spectrophotometric determinations. The optimum pH for lysozyme retention is 9.0. When the solution is percolated through the column at the flow-rate of 5 $ml \times min^{-1}$, without allowing for a 30 min standing, the retention is 75 % at pH 9.0.

Lysozyme can be easily and quantitatively eluted from chitosan columns by using 2 % propylamine solution at pH 11.5. Good elution yields can be also reached with carbonate buffers.

Chicken egg white (85 ml) was treated with sulfuric acid in the volume ratio 1:3 and shaken for 1 hr at pH 5.5 after adjustement with 1 M sulfuric acid. After 12 hr at 0 ÷ 5°C and centrifugation at 20,000 rpm for 2 hr, the pH was adjusted to 9.0 and the supernatant was passed through a chitosan column. Various aliquots, for instance 0.5, 1.0, 2.5 and 5.0 were diluted to 20 ml and submitted to chromatography.

Most of the high molecular weight proteins are eliminated during the sulfuric acid treatment; other proteins quickly saturate the chitosan column and pass through during the adsorption step while lysozyme is preferentially adsorbed. The column was then washed with buffer at pH 9.0 (60 ml) and eluted with propylamine. Washing had the effect of removing selectively most of the proteins other than lysozyme; propylamine had the effect of eluting lysozyme in the interval 5 ÷ 15 ml. Table 3 shows that the protein recovered in the process of elution (lysozyme) is proportional to the volume of egg white treated; the same can be said for the enzymatic activity found on the elution fractions. The enzyme activity was found to be about 55 % of the activity of the untreated enzyme.

Table 3. Recovery of Lysozyme from Chicken Egg White, after Treatment with Sulfuric Acid and Chromatography on Chitosan Columns

Treated egg white, ml	Optical density at 280 nm of the eluate fraction	Enzymatic activity of the eluate, percent of original
0.5	0.205	50
1.0	0.430	52
2.5	1.010	58
5.0	2.150	56

Thin-layer chromatography

The separations obtained on thin layers of chitosan for a number of dyes commonly used in histology are illustrated in Table 4; they were achieved in less than 1 hr, by developing with water + methanol + 2 % diethylamine (8:1:1). To show how different the behavior is of the dyes on DEAE-cellulose, data for DEAE-cellulose thin layers developed in the same tank are included.

As a general trend, the dyes on DEAE-cellulose do not move or produce long tails; only Methyl Red is resolved. On chitosan thin layers, the separations of several components are visible; particularly remarkable are those relevant to Phenol Red, Eosin Yellowish, Leishman's Eosin Methylene Blue and Methyl Red. With more diethylamine in the developing mixture (water + methanol + 2 % diethylamine, 1:1:8), Toluidine Blue is resolved into 3 fluorescent components at 00, 74 and 88 and Neutral Red

Table 4. $R_f \times 100$ of several dyes used in histology resolved into components on chitosan thin layers developed with water + methanol + 2 % diethylamine (8:1:1) over 1 hr at room temperature. Data on DEAE-cellulose are included for comparison.

TB	E	PR	O	EY	NR	CV	AO	EM	BCB	AB	MR	MV
Chitosan												
84 f 79 f	96 br 80 p t 38 v t 10 v t	92 r 87 y	50 o	96 f 93 f 89 f 82 f 70 p 64 p 55 p 51 p	04 y	11 v	06 y	33 f 28 p f 00 b	08 lb	06 lb	48 f 40 y	23 v
DEAE-Cellulose												
00 f	16 br t	05 v	00 o	00 r	06 y	75 v t	47 y t	30 lb t	40 lb t 12 o	00 lb	33 f 20 y 00 f	33 v t

TB = Toluidine Blue, E = Ematoxilin, PR = Phenol Red, O = Orange II, EY = Eosin Yellowish, NR = Neutral Red, CV = Crystal Violet, AO = Acrydine Orange, EMB = Leishman's Eosin Methylene Blue, BCB = Brilliant Cresyl Blue, AB = Amido Black 10 B, MR = Methyl Red, MV = Methyl Violet.

b = blue, br = brown, g = grey, lb = light blue, p = pink, r = red, v = violet, y = yellow; f = fluorescent under UV light at 254 nm; t = tailing, o = orange.

gives 3 spots at 05 (yellow) and 12 and 23 (fluorescent).

About the dyes which belong to this group and to the one previously studied (11), the following considerations can be made: while silica gel is superior in resolving Crystal Violet and Acrydine Orange, chitosan is superior in resolving Neutral Red because this dye could not be resolved on silica gel thin layers.

As for the dyes currently used in the food industry, their R_f on chitosan thin layers are as shown in Table 5: the E-123 dye is sharply separated and distinguished from the other dyes because its red spot has a relatively low R_f value.

Table 5. $R_f \times 100$ of Dyes Used as Food Additives, on Chitosan Thin Layers Developed with Water + Methanol + 2 % Diethylamine (8:1:1) over 1 hr at Room Temperature. Color Codes as under Table 4

E-102	E-103	E-104	E-110	E-123
93 y	75 y 00 y	78 y 64 y	71 o	61 r
E-124	**E-126**	**E-131**	**E-151**	**E-152**
78 r	94 f 87 r	95 b	44 v	28 g

CONCLUSIONS

Chitosan is suitable for applications in various branches of chromatography. It possesses impressively high capacity for vanadate, the ratio vanadate/chitosan being 2.3/1 by weight in the product obtained. Phosphate is another anion that can be retained on chitosan with very high yields: chitosan forms insoluble salts whose anions can undergo exchange.

Chitosan can be easily modified to obtain a range of chitosans insoluble in acetic acid, for special chromatographic applications. Insolubilization can be easily confered with simple reactions with relatively inexpensive reagents in aqueous media; the reactions are rapid even though heterogeneous in nature. Percolation of solutions through the modified chitosans is as facile as on chitosan itself due to the native ultrastructure which is conserved. Therefore, the solubility of chitosan in certain acids should not be considered a drawback: on the contrary it is an advantage because one can improve the surface characteristics of chitosan by simply dissolving and reprecipitating it, for the purposes of gel chromatography and of thin-layer chromatography.

The selectivity of chitosan in chelation chromatography has been put to use in recovering valuable metals from manganese nodules: the proposed

method requires very little energy, thus taking into account the location of future treating plants in energy-deficient regions such as Hawaii: this is a remarkable advantage over the proposed methods, one of which includes melting a salt mixture and keeping it at 1,000°C for 5 hr to carry out halidation and vaporization of the halides (7). Another method reacts nodules with air and sulfur dioxide to form a leach solution which after filtration and pH adjustement to 2.0, is extracted with hydroxyquinoline or similar soluble chelating substances; the valuable metals are then recovered after a number of steps which make the overall process a very expensive and complicated one. The chitosan process, on the contrary, requires commonly available chemicals of low price, is a one-step separation and yields the metals as a dilute sulfuric acid solution that lends itself to further treatments.

In addition to the use of chitosan in the chromatography of inorganic substances, the use of chitosan in connection with amino acids, proteins and enzymes and their isolation or recovery is another expanding field. Lysozyme is an important addition to the list of enzymes so far treated on chitin and chitosan. Lysozyme can be isolated on chitosan and eluted with 55 % of its original activity. The proposed method is an advance over the current procedures for the preparation of lysozyme, which involve many chromatographic and salting-out steps followed by desalting and dialysis.

Evidence is produced of the peculiar characteristics of chitosan thin layers: they are definitely different from cellulosic thin layers, as can be seen from the data presented in this paper and in a previous one (12). They have been used to resolve several dyes into their components; Neutral Red is better resolved on chitosan than on any other support so far tested. The detection of the E-123 dye in mixtures of dyes used in food technology is only possible on chitosan; no sufficient resolution was obtained on cellulosic and silica gel supports.

Chitosan finds a distinguished place among the various chromatographic supports, because it combines in itself several desirable characteristics which make it unique and versatile.

ACKNOWLEDGMENTS

This work was performed under the auspices of the North Atlantic Treaty Organization (NATO Contract No. 810). Thanks are due to M. G. Muzzarelli, R. Rocchetti, G. F. Scarpini and F. Tanfani for assistance in producing the experimental data.

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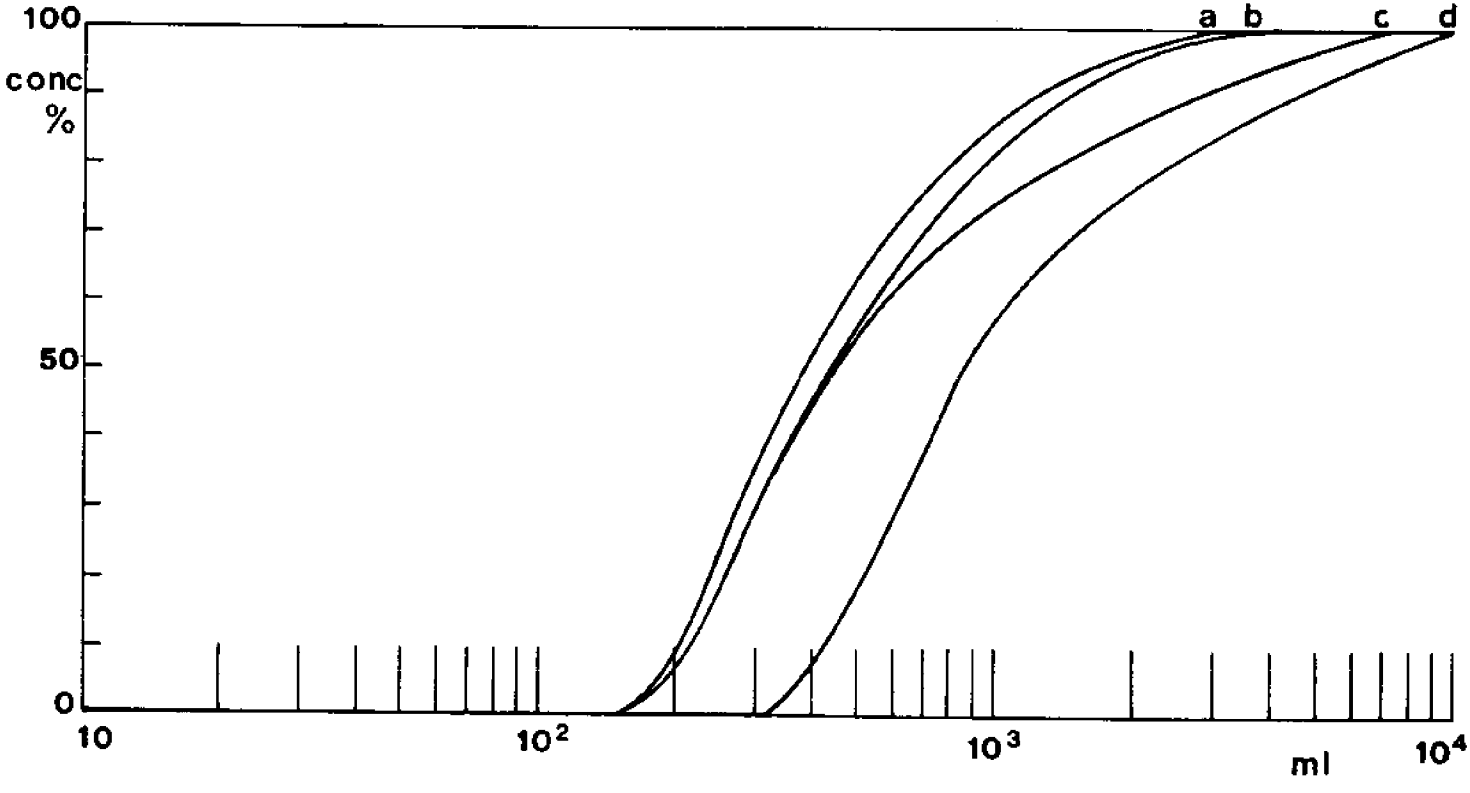

Figure 1. Breakthrough curves for orthovanadate from chitosan and U-GLY-chitosan columns. The orthovanadate solution contained 100 ppm vanadium at pH 4.0 for chitosan and at pH 3.0 for U-GLY-chitosan. The columns were 3 × 1 cm and contained 0.5 g chitosan and 0.8 g U-GLY-chitosan. a = U-GLY-chitosan; b = U-GLY-chitosan in 3 % NaCl brine containing 100 ppm vanadium; c = chitosan; d = chitosan in 3 % NaCl brine containing 100 ppm vanadium. Flow-rate 20 $ml \times min^{-1}$.

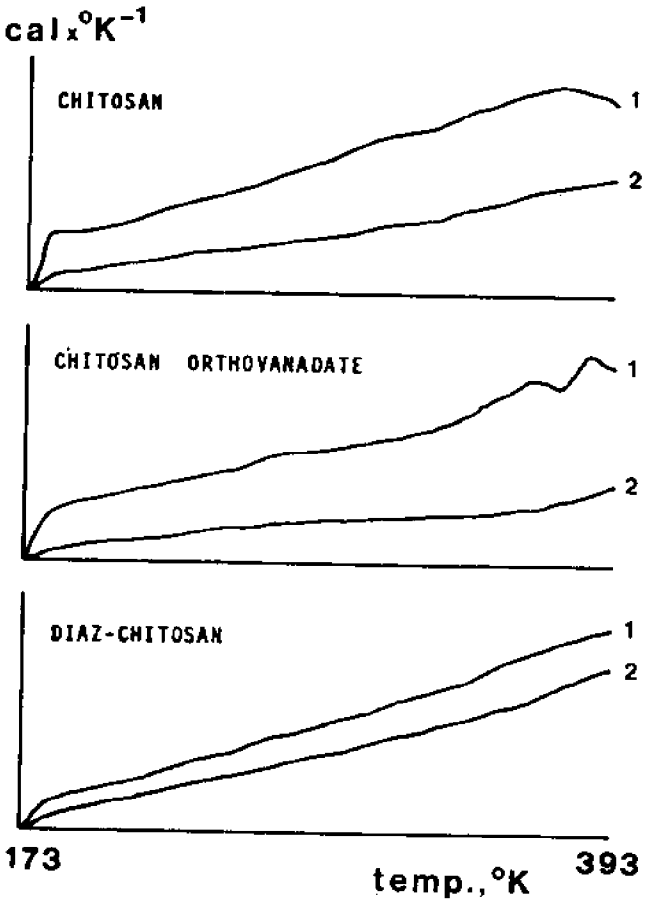

Figure 2. Differential scanning calorimetry readings for chitosan, chitosan orthovanadate and DIAZ-chitosan, in the temperature range -100 ÷ +120°C. Freeze-dried samples. Numbers refer to cycles.

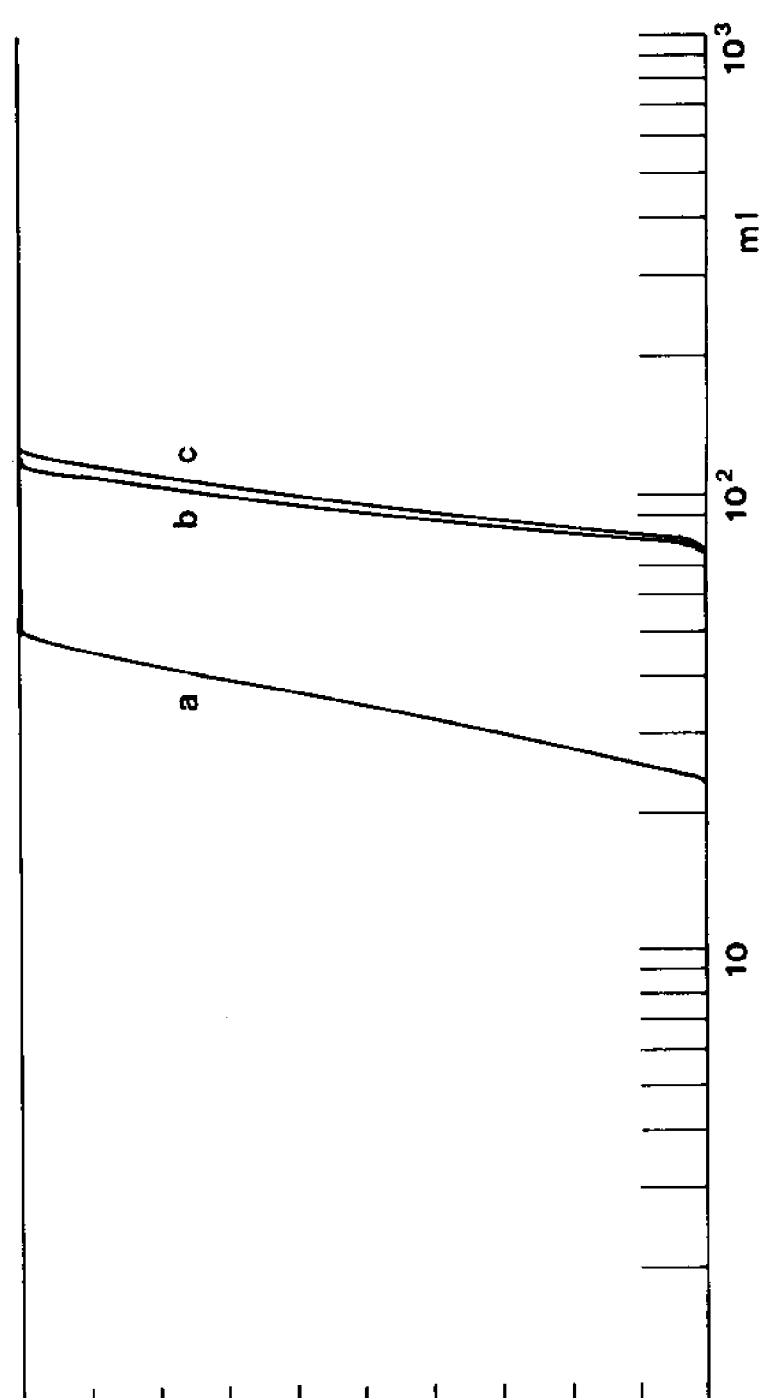

Figure 3. Breakthrough curves for mercuric ion from: a = AMSA-chitosan; b = EPIC-chitosan, and c = chitosan. The mercuric chloride solution was 0.5 mM at pH 4.3. The columns were ≈9 × 0.5 cm and contained 200 mg powder. Flow-rate 6 ml×min^{-1}.

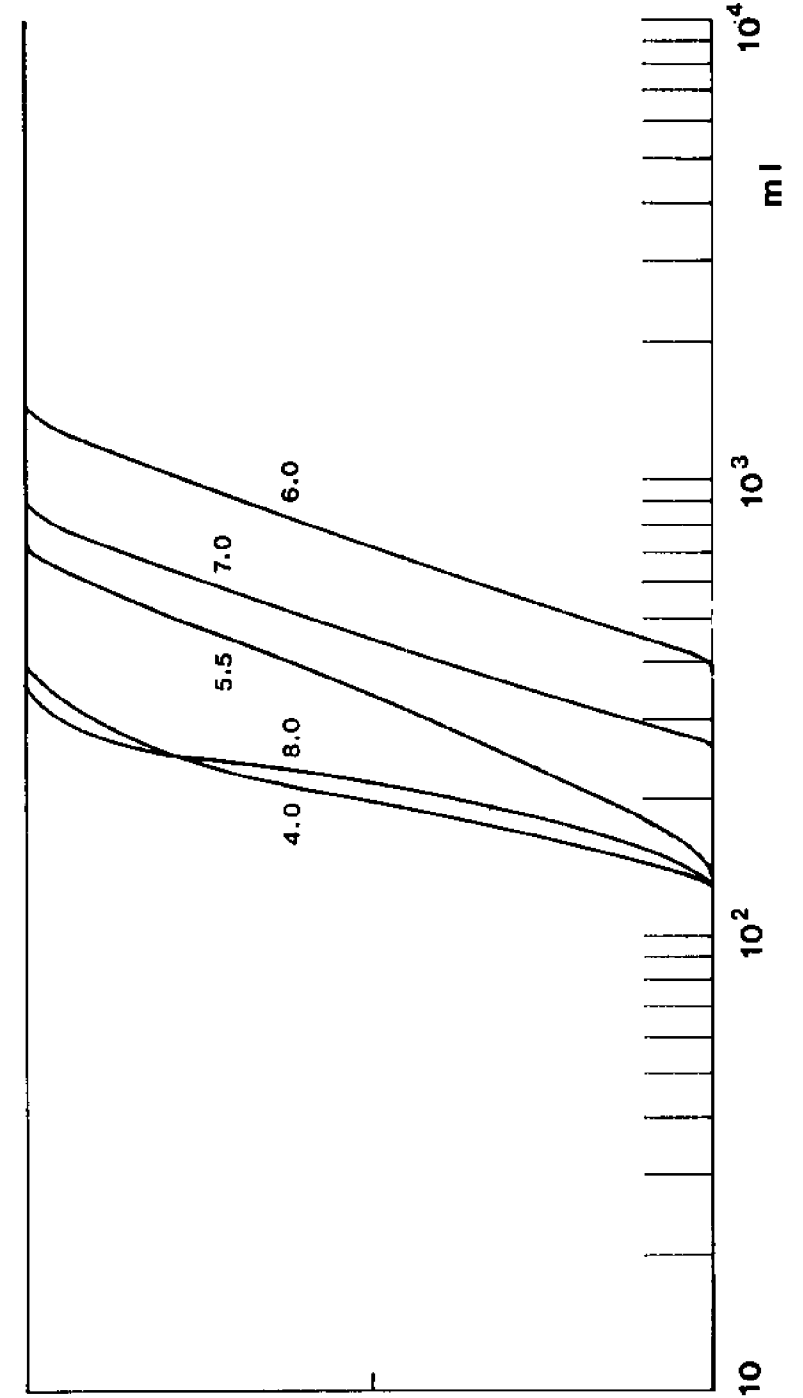

Figure 4. Breakthrough curves for orthophosphate from chitosan columns. The orthophosphate solution contained 2 ppm of phosphorus at various pH values. The columns were 3 × 1 cm and contained 0.5 g of chitosan pre-conditioned with 0.1 M ammonium solfate + sulfuric acid solution. Flow-rate 6 ml×min^{-1}.

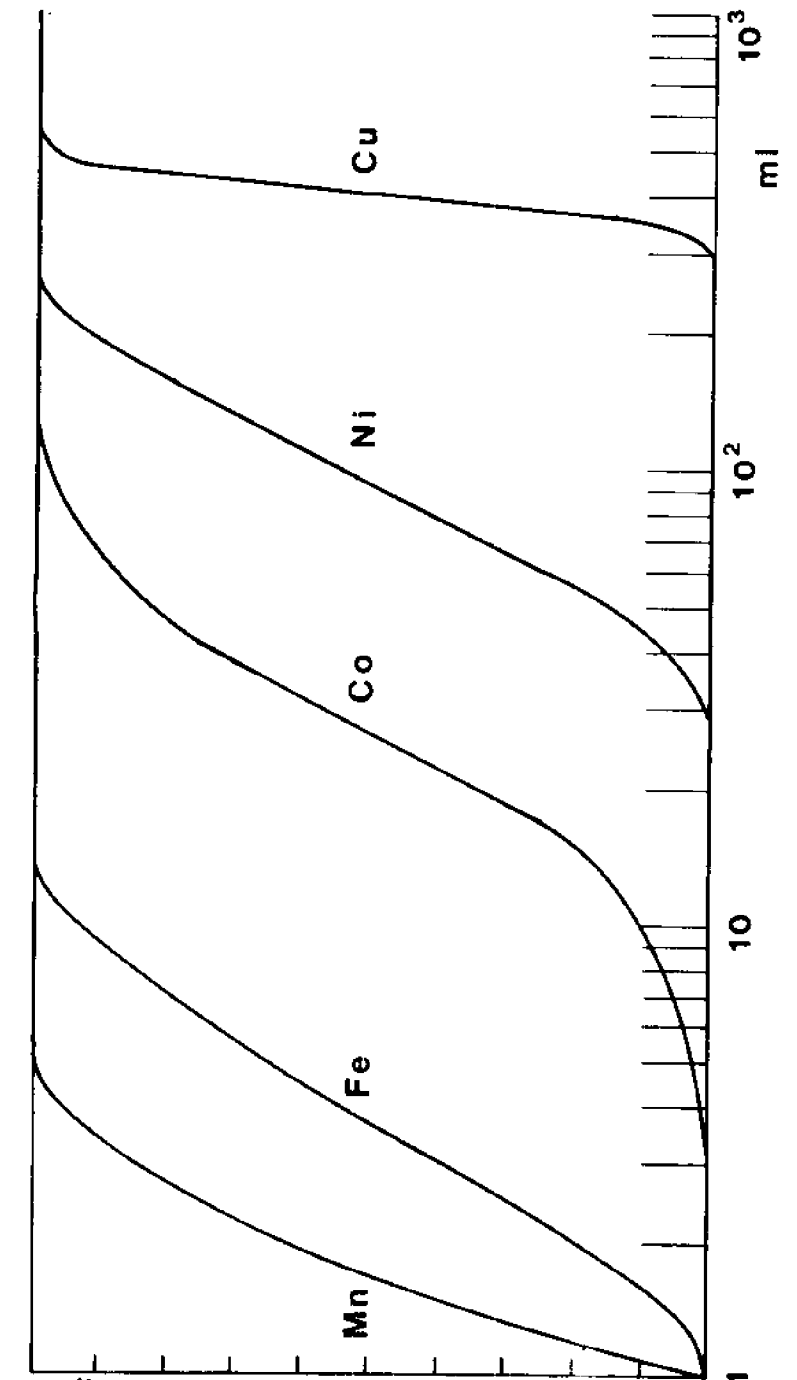

Figure 5. Breakthrough curves for a 200 mg chitosan column on which a mixture of metal ions of interest in marine nodule technology was passed (diam. 5 mm, pH 6.0, molar ratio ascorbic acid/iron = 100, flow-rate 3 ml·min^{-1}).

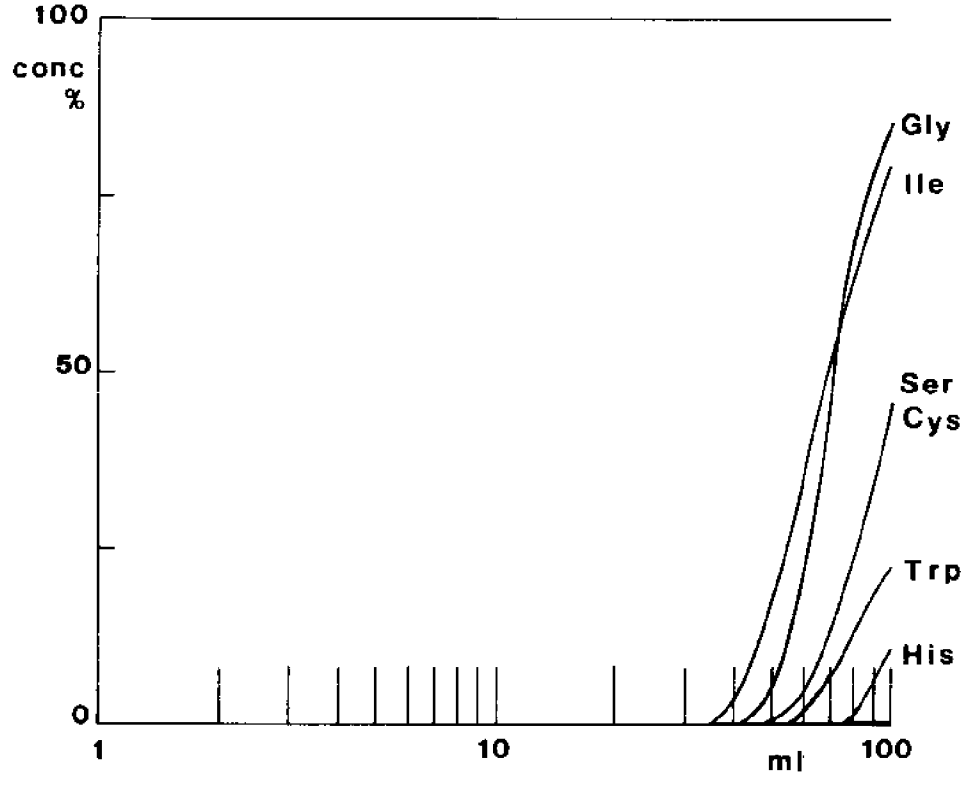

Figure 6. Breakthrough curves of amino acids from a chitosan column loaded with copper in the amino complex form (500 mg chitosan loaded with 376 mg copper sulfate pentahydrate and excess ammonia; diam. 4 mm, flow-rate 0.7 ml×min^{-1}).

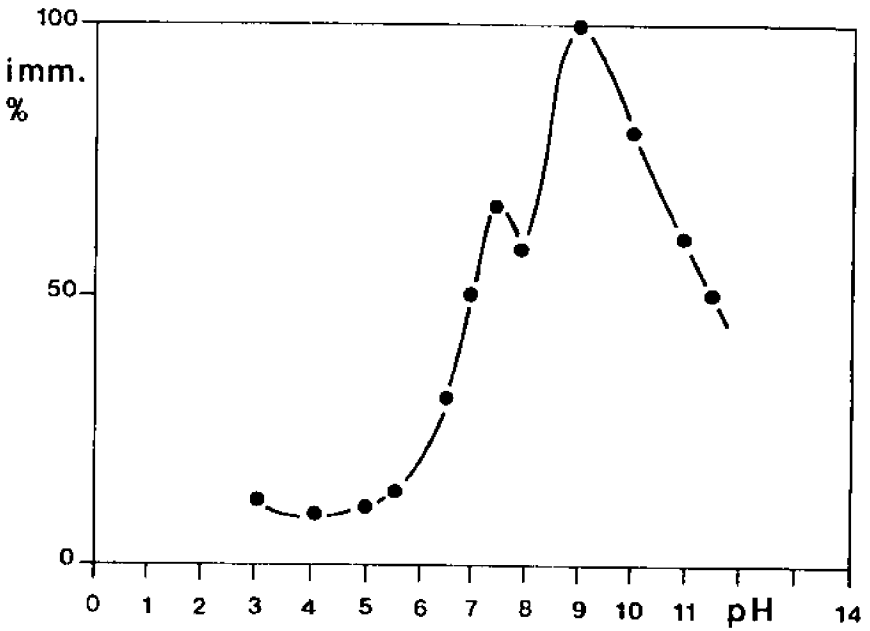

Figure 7. Immobilization percent of lysozyme on chitosan. Lysozyme solution (3 ml, containing 3 mg) was left in contact within a chitosan column (1.5 g) for 30 min.

USE OF CHITIN POWDER AS ADSORBENT IN THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

The applicability of powdered chitin, isolated from king-crab shell, was examined by thin-layer chromatography. The ability of the chitin layer to separate mixtures of either phenols, amino acids, nucleic acid derivatives, or inorganic ions (Cu-group) was almost equal or superior to that of crystalline cellulose, silica gel and polyamide layers. The developing time was generally much shorter when operating with the chitin layer than with other layers.

EXPERIMENTAL RESULTS

Chitin, isolated from the shell of king crab (2), was sifted after powdering by ball-mill. The chitin layer was a 250 $\sim$ 300 mesh powder.

Separation of some phenols

Figure 1 shows a comparison of the thin-layer chromatograms of some phenols on polyamide, silica gel, and chitin layers (4). With the chitin layer, a more satisfactory separation and clearer spots were obtained than with other layers. Water, as a developing solvent, as well as 20% aqueous acetone, gave good results on the chitin layer.

Separation of amino acids

Table 1 shows a comparison of Rf-values of some amino acids on silica gel (1) and on chitin layers (4). On the chitin layer, the basic amino acids traveled faster, while the acidic amino acids were slower, than on a silica-gel layer. This difference might be due to the basicity of the chitin powder, the acetamido groups of which were partially deacetylated during the process of isolation.

The two-dimensional thin-layer chromatogram of the hydrolysate of casein on the chitin layer is presented in Figure 2.

Separation of nucleic-acid derivatives

Figures 3 and 4 show a comparison of thin-layer chromatograms of nucleic-acid bases and nucleosides on chitin and crystalline cellulose layers (5). On the chitin layer, nucleic-acid bases, nucleosides and nucleotides generally traveled faster than on the cellulose layer.

Table 1. A Comparison of Rf'-values of Amino Acids in 1-Propanol/ Water (64:36 w/w) on Silica Gel and Chitin Layers

Amino acid	Rf'-value[a] x 100		ΔRf'[c]
	Silica gel[b]	Chitin	
Gly	58	68	10
Ala	67	76	9
Val	82	94	12
Pro	47	76	29
Met	93	95	2
Cys	58	36	-22
Asp	60	21	-39
Glu	64	33	-31
Arg	4	60	56
Lys	4	64	60
His	36	73	37
Phe	105	100	-5
Tyr	104	88	-16
Ser	64	71	7
Leu	100	100	-

[a] Rf'-value relative to the Rf'-value of leucine.

[b] Calculated value based on the results by Brenner et al. (1).

[c] Difference between Rf'-value on chitin and on silica-gel layer.

Separation of some inorganic ions (Cu-group)

Figure 5 shows a comparison of thin-layer chromatograms of the mixture of Cu^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+} and Bi^{3+} on chitin and crystalline cellulose layers. On the chitin layer, only Cu^{2+} turned violet; the remainder turned yellow or brown with potassium-iodide solution.

Figure 6 shows the thin-layer chromatograms of the Cu-group fractionated from the hydrolysates of oyster and viscera of cuttlefish. On the chitin layer, Cd^{2+}, Cu^{2+} and Pb^{2+} (Hg^{2+} ?) in oyster and Cd^{2+} and Cu^{2+} (Hg^{2+} ?) in cuttlefish viscera were detected, while the cellulose layer had no separating ability for these fractions.

Highly deacetylated chitin powder as coating material

The slurry of a highly deacetylated chitin powder, which was treated with a hot sodium-hydroxide solution, produced less uniform layers than other coating materials and the thin layers produced cracked during drying and activation. As a result, our attempts to obtain a thin layer and to chromatograph on the highly deacetylated chitin powder failed.

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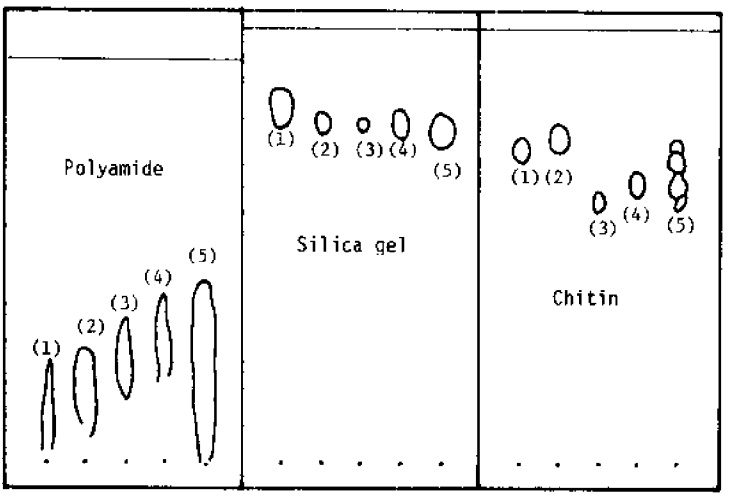

Figure 1. Comparison of thin-layer chromatograms of phenols on polyamide, silica gel, and chitin layers, eluting with 20% aqueous acetone, made visible with diazotized benzidine solution. 1 = Resorcinol, 2 = hydroquinone, 3 = pyrogallol, 4 = phloroglucinol, and 5 = mixture.

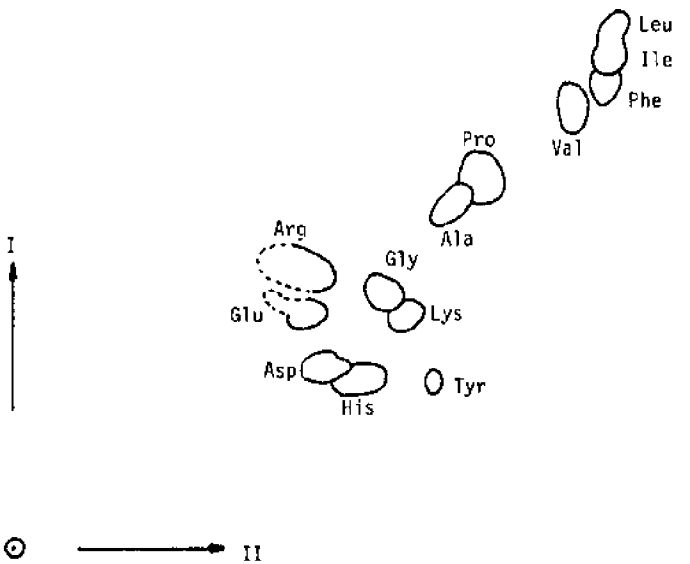

Figure 2. Two-dimensional thin-layer chromatogram of casein hydrolysate on chitin layer, eluting with solvent-I of 1-butanol/acetic acid/water = 3:1:1 and solvent-II of 1-propanol/34% aqueous ammonia = 67:33. Casein was hydrolyzed with concentrated hydrochloric acid for 19 hours at 120° C in a sealed tube. Made visible with ninhydrin-copper nitrate solution.

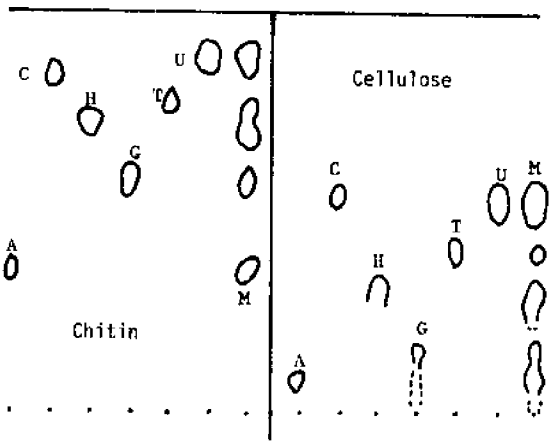

Figure 3. A comparison of thin-layer chromatograms of nucleic-acid bases on chitin and cellulose layers, eluting with solvent of saturated aqueous ammonium sulfate solution/7M sodium acetate/ 2-propanol = 40:9:1. Made visible by UV light. Abbreviations of nucleic acid bases: A = adenine, G = guanine, H = hypoxanthine, T = thymine, C = cytosine, U = uracil, and M = mixture.

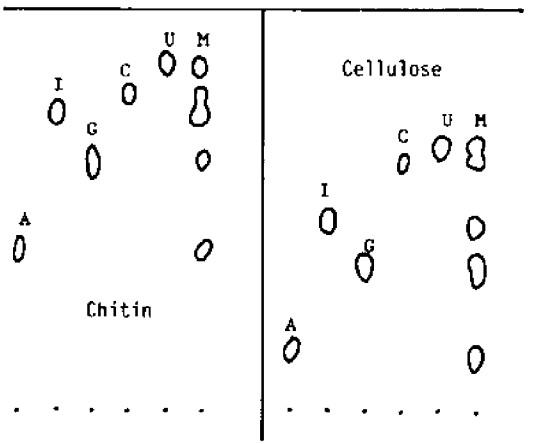

Figure 4. A comparison of thin-layer chromatograms of nucleosides on chitin and cellulose layers, eluting with the same solvent as in Figure 3. Made visible in UV light. Abbreviations of nucleosides: A = adenosine, G = guanosine, I = inosine, C = cytidine, U = uridine, and M = mixture.

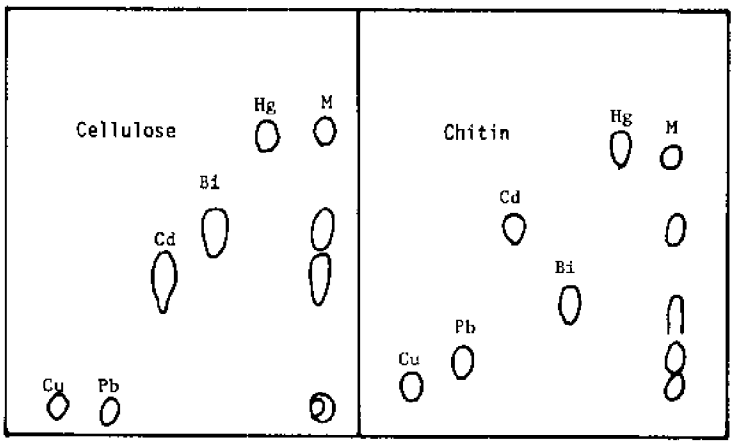

Figure 5. A comparison of thin-layer chromatograms of some inorganic ions (Cu-group in analytical chemistry) on chitin and crystalline cellulose layers, eluting with solvent of 1-butanol/1.5M hydrochloric acid/acetylacetone = 100:20:5. Made visible with 2% potassium-iodide solution, ammonia gas, and hydrogen-sulfide gas (3).

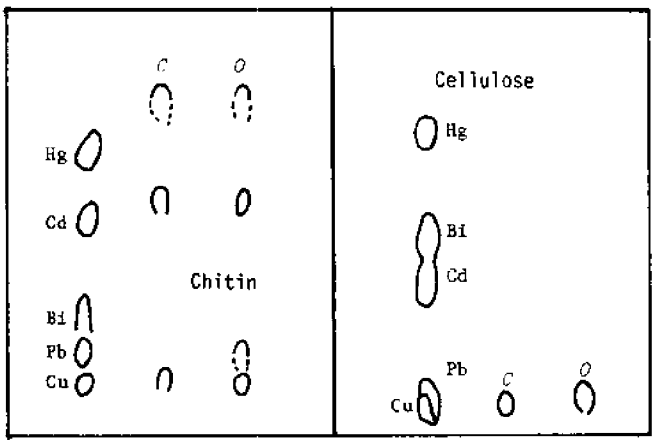

Figure 6. A comparison of thin-layer chromatograms of the Cu-group in hydrolysates of oyster and viscera of cuttlefish. Samples were hydrolyzed with the mixture of sulfuric, nitric, and perchloric acids, followed by fractionation with thioacetamide (3). Elutant and detection are identical to those in Figure 5. Abbreviation of hydrolysates: O = oyster and C = cuttlefish viscera.

INSOLUBILIZING ENZYMES WITH CHITOSAN AND CHITOSAN-DERIVED POLYMERS

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ABSTRACT

The fixing of enzymic activity on chitosan matrices by insolubilization of enzymes such as lactase and invertase was achieved through several strategies. In one procedure, chitosan that has been solubilized by neutralization with dilute acids to about pH 4-5 is mixed with an enzyme dissolved in water or a suitable buffer. This is followed by back titration with alkali, in an amount equivalent to the acid used for solubilizing the chitosan. The resulting precipitated chitosan product retains enzymic activity. In this procedure, inorganic sulfate can be used instead of alkali to reprecipitate the chitosan product, since the sulfate salt of chitosan is also insoluble in water at ambient temperature (though soluble in boiling water). Alternatively, the chitosan sulfate is first prepared (from acid-solubilized chitosan and inorganic sulfate or by direct titration of chitosan flakes with sulfuric acid) without the dissolved enzyme and then used to fix the enzyme.

In another strategy the acid solubilized chitosan (pH 4-5) is mixed with the enzyme solution; then, a difunctional or polyfunctional crosslinking agent such as glyoxal, glutaraldehyde or solubilized dialdehyde starch is added. This results in conversion of the mixture to a gel within minutes; the gel then hardens within an hour and can be broken into granular form with retained enzymic activity. The Schiff base polymer produced in this way can be reduced with sodium borohydride or sodium cyanoborohydride with retention of activity. The advantage of reducing the gel is to enhance its conversion to a granular form and to stabilize the Schiff base formed. The products obtained are very hydrophilic, retaining about 85-90% water after being sucked dry on a filter connected to a water aspirator. The ε-amino groups of lysyl residues of the enzyme probably participate in the Schiff base formation, and thus the enzyme becomes covalently bound to the crosslinked polymer. The enzyme can also be fixed in the polymer, due to physical entrapment or interfacial deposition. A variation of the above procedure is to fix the enzyme by crosslinking with dialdehydes or polyaldehydes on chitosan flakes suspended in water rather than on solubilized chitosan. Filtration through the products formed in this way is much easier, and the products retain much less water.

INTRODUCTION

The current interest in preparing insolubilized forms of enzymes by immobilizing them on insoluble physical supports and the potential industrial application of such enzyme preparations in food processing or treatment of food-processing wastes prompted us to explore the potential use of chitosan for insolubilizing enzymes.

Various techniques have been used for insolubilizing enzymes. In one procedure, for example, the enzyme is fixed to an insoluble support by entrapment such as in the formation (polymerization) of polyacrylamide. In another, adsorption or ionic interaction of enzymes with a suitable

insoluble matrix such as ion-exchange resins or alumina is used. A third procedure involves covalent coupling to a reactive group on an insoluble polymer, such as coupling enzymes to diazonium salts of polymers or a Schiff base formation between lysyl residues of enzymes and polyamino polymers (1). Although considerable technological knowledge exists and advances have been made in this field, industrial application of the available information has been lagging, perhaps because of costs, and, to some extent, because of the complexity of some reactions and steps involved in preparation of the products. The finding of an inexpensive and abundantly available suitable support material and of a simple method of preparation may stimulate industrial applications.

Chitosan is an attractive substance from this point of view: it could be considered a waste by-product of fishery operations; it is a renewable natural resource; and presumably it could be made available inexpensively if sufficient demand and a large enough market could be generated for its use. Its high content of functional (free amino) groups offers a versatile chemical handle for chemical modification, such as in covalent binding to enzymes or ionic salt formation (1). Its content of free amino groups can be varied by the extent of deacetylation of its precursor, chitin, with concomitant change in the resistance to solubilization in acid media of the partially de-acetylated products. Another way in which resistance of chitosan to solubility in acids can be increased is to effect partial crosslinking of the chitosan through some of its amino groups. We have used such partially crosslinked chitosan in enzyme work and toxic metallic ion removal in experiments operated at acid pH.

The chemistry of enzyme insolubilization has been reviewed (2,3). Chitin had been used for insolubilizing enzymes with glutaraldehyde (4). In this report, we describe some of our experiments exploring the use of chitosan for enzyme insolubilization.

EXPERIMENTAL

The chitosan used was from Food Chemical and Research Laboratories (Seattle, WA). The sample was ground in a Wiley mill to pass through 1 mm screen and further sieved to various mesh sizes. The major fraction, 30-40 mesh size, was used in the experiments described here. The product had about 9% moisture and titrated about 4.5 milliequivalents of hydrogen ion/g to a pH of about 4.2.

Lactose is a crude acid-tolerant β-galactosidase from Aspergillus niger; it was obtained from Wallerstein Company (Morton Grove, Illinois 60053) as lactase LP. Two lots were used: lot 1 hydrolyzed 10 μmol lactose/min/mg crude enzyme at 40°C in potassium acetate buffer pH 4; lot 2 hydrolyzed 7 μmol/min/mg.

Invertase is a standardized liquid Sucrovert preparation derived from yeast. It was obtained from SuCrest Corporation (New York, N.Y.). The solution hydrolyzed 6.7 mmole sucrose/min/ml at 40°C at pH 5.

Lactase activity was measured on a shaker water bath at 40°C, with a 0.4M lactose solution in 0.1M potassium acetate buffer (pH 4.0) as substrate. Usually about 0.2-1.0 g of the chitosan products were used in 25 ml of substrate solution; filtered 1 ml aliquots were withdrawn at different time intervals to determine the rate of glucose production during incubation. Glucose was measured by the glucose oxidase-chromogen procedure. It was

supplied by Worthington Biochemical Corporation (Freehold, New Jersey) as Glucostat. Clinistix reagent strips (Miles Laboratory, Elkhart, Indiana) were also used for semi-quantitative measurement of glucose in exploratory tests.

Invertase activity of products was measured from the rate of glucose formation in the batch incubation mixture, in a way similar to lactase at 40°C, with 0.1M sucrose in 0.1M phosphate buffer pH 5.0 as substrate.

Preparation of insolubilized enzymes

Essentially, the following three strategies were tried for insolubilizing lactase and invertase on chitosan:

1. Entrapment and adsorption by the precipitation of solubilized chitosan: In this method, chitosan is first solubilized with acetic acid or hydrochloric acid (~4-5 mmol/g) to about pH 4.5. The enzyme dissolved in a small volume of water is then mixed with the chitosan solution; this is followed by back titration of the acid used with alkali, which causes the chitosan to precipitate, entrapping the enzyme. The ionic and adsorptive binding of the enzyme to the chitosan matrix may also take place. Alternatively, instead of back titration with alkali, an equivalent amount of inorganic sulfate (2-2.5 mmol/g chitosan) could be used to effect precipitation of the chitosan as the sulfate salt. Since this salt is insoluble in cold water and only with difficulty soluble in boiling water, it starts precipitating as a gel upon cooling to about 50°C.

A variation of this method using sodium sulfate for precipitation is to prepare the chitosan sulfate first in the absence of the enzyme; dissolve the sulfate salt in boiling water; cool it to about 50°C; add the enzyme to the cooling chitosan sulfate, which is starting to precipitate; and then chill the mixture rapidly to avoid heat denaturation of the enzyme.

Another variation of the sulfate-salt method is to prepare the chitosan sulfate by direct titration of chitosan flakes with the requisite amount of dilute sulfuric acid (about 2 mmol/g chitosan) to about pH 4.2, in the presence of the enzyme. Alternatively, the chitosan sulfate flakes can be prepared in the absence of the enzyme, washed with water, and then used to fix the enzyme by contacting the flakes with the enzyme dissolved in a suitable buffer, as in the following example:

> Example 1. To 10 g of air-dried chitosan flakes (30-40 mesh) was added 250 ml of 0.1M H_2SO_4 for an hour with stirring. The treated flakes (chitosan sulfate) were filtered, washed with water, and air dried to give 12.9 g of product. Then 2.0 g of this chitosan sulfate product was contacted with a mixture of 10 ml of water plus 0.2 ml of the stock Sucrovert solution for an hour. The mixture was then filtered, rinsed thoroughly with distilled water (only), and air dried.

2. Fixing enzymes by crosslinking chitosan solutions: A second strategy of fixing lactase and invertase to chitosan involves dissolving chitosan in acetic or hydrochloric acid to pH 4.2 to give about 2% solution of chitosan. The enzyme dissolved in a small volume of water is then mixed, and this is followed by the addition of a crosslinking agent such as glyoxal dialdehyde starch (DAS) or glutaraldehyde (GA). A gel forms, which has retained activity and could be used in this form or, after reduction

with sodium borohydride (reduction of the Schiff base polymer produced by the reaction of these aldehydic reagents with the amino groups of chitosan), the gel gives a granular polymer with improved filterability and retained enzymic activity. The following examples are illustrative of this method.

Example 2. Chitosan (7 g) was suspended in water (220 ml); 1N HCl (30 ml) was then added. To the dissolved chitosan hydrochloride solution, lactase (0.5 g of lot 1 dissolved in 50 ml of water) was added. The mixture (20°C) was stirred on a magnetic stirrer for 5 minutes. Then 7 g of DAS that had been solubilized in 100 ml of water plus 2.5 mmol sodium carbonate was added to the chitosan-enzyme mixture. The DAS solution was at pH 7. After 10-15 minutes, the mixture became a thick gel; after about 30 minutes it was broken into a granular product in a Waring blender. A small sample was removed to test for activity (qualitatively, with Clinistix reagent strips) after thorough washing with saturated KCl, phosphate buffers (pH 7 and 4), and distilled water. The sample, which was active, was then returned to the blender. The mixture in the blender was cooled to 10°C, and 2 g of sodium borohydride dissolved in a small volume of water was added while the mixture was being blended. Treatment with the borohydride caused rapid and noticeable shrinkage of the gel and the release of the liquid from the gel, which became an easily filtered and very granular resin. The polymer was washed with water, saturated KCl and phosphate buffers (with soaking) and finally washed with distilled water. It was then filtered with suction on a coarse-sintered glass funnel connected to a water aspirator. The filtered moist product had about 90% water and was stored in this condition.

Example 3. A similar experiment to that described in Example 2 was performed, except that glyoxal was used in place of DAS for crosslinking. Specifically, the differences were that 30 ml of a 30% glyoxal (in H_2O), 1 g crude lactase (lot 1), and 5 g sodium borohydride were used. The filtered moist product also contained about 85-90% water.

Example 4. Lactase was insolubilized as in Examples 2 and 3, but glutaraldehyde was used for crosslinking. In a typical experiment, 2.4 g of chitosan was dissolved in 5 ml of 2M acetic acid plus 120 ml of 0.1M sodium acetate buffer of pH 5.4 (the final pH of the solubilized chitosan was also 5.4). Lactase (120 mg, lot 1) in 10 ml H_2O was added and mixed, followed by the addition of 275 mg glutaraldehyde in 150 ml of water. Sodium borohydride (100 mg) was used to reduce the Schiff base polymer. The washed moist polymer had about the same water content as the DAS and glyoxal products.

Example 5. The procedure of Example 4 was followed exactly, except that yeast invertase (1 ml Sucrovert solution) was used. Again, about the same water content was obtained in the filtered moist product.

3. Insolubilizing enzymes by crosslinking insolubilized chitosan: In this preferred method, chitosan flakes are first partially crosslinked (for example with GA) without dissolving them in acid. To a suspension of

chitosan flakes in water, GA is merely added for an hour; the flakes are then washed and are ready for use to insolubilize enzymes. The reaction of GA with the chitosan flakes is rapid and complete (as reflected in gravimetric measurements, nitrogen analysis and change of solubility properties of the treated flakes). The amount of GA can be selected to crosslink any fraction of the potential free amino group content of the chitosan (about 5 meg/g). In the present experiments, the product used was prepared by adding 1.4 mmol GA/g chitosan. The product, unlike chitosan itself, was insoluble in acidic water. Preparation of the product and its use for insolubilizing lactase and invertase are described in the following examples.

Example 6. To 300 g of air-dried chitosan (30-40 mesh) was added a mixture of 150 ml of a 25% GA (in water) and 1200 ml of water at 20°C for 1 hour with occasional stirring. The mixture was then filtered; washed with water, methanol and ethyl ether; and air dried to give 342 g product (designated CHT-GA). It contained 6.56% N (on about 9% moisture-content basis) compared to a corresponding value of 7.37% N for the starting chitosan (also 9% moisture).

To 5 g of the CHT-GA suspended in 100 ml of 0.2M sodium acetate buffer (pH 5.0) at 20°C was added 1 ml of the stock invertase solution (Sucrovert). The mixture was stirred for a few minutes on a magnetic stirrer before 1.9 ml of a 25% GA solution was added, drop by drop, while stirring. After 1 hour the flakes were filtered; washed thoroughly with water, saturated KCl and 0.2M acetate buffer (pH 5), then with distilled water; and air dried to give 6.2 g of product.

Example 7. This experiment was similar to that of Example 6, except that only 0.38 ml of the 25% GA solution was used to fix the invertase (1 ml Sucrovert) to 5 g of CHT-GA. The yield was 5.5 g of air-dried product.

Example 8. Here, experiment of Example 7 was repeated, except that only 0.08 ml of 25% GA was used (diluted with a small volume of water). The air-dried product weighed 5.27 g.

Example 9. To test whether the addition of the small amount of GA was necessary at all to fix the enzyme to the CHT-GA product, we repeated the experiment of Example 8, except that we omitted the addition of 0.08 ml GA. The product of this experiment weighed 5.13 g.

Examples 10-13. These represent four parallel experiments analogous to those of Examples 6-9, respectively, except that in each example, 0.5 g lactase (lot 2) was used instead of invertase. The air-dried products weighed respectively: 6.02 g, 5.69 g, 5.27 g and 5.44 g.

RESULTS

The chitosan sulfate-invertase product of Example 1 hydrolyzed sucrose at an initial rate of 98 μmol/min/g (retention of 15% of added activity). The invertase activity, however, was not firmly bound, and the enzyme leaked into the incubation medium (see Discussion). The initial rate was

determined by measuring the rate of glucose production from measurements taken after short incubation periods (3 and 5 minutes).

The hydrolytic activities of the enzyme-chitosan products that were prepared by crosslinking solutions of chitosan in the presence of lactase or invertase, using DAS, glyoxal or GA, are shown in Table 1 (Examples 2-5).

Table 1. Lactase (or Invertase) Activities of Polymers from Crosslinking Chitosan Solutions with Aldehydic Reagents in the Presence of Enzymes (measured by the rate of glucose formation from lactose or sucrose substrate)

Example	Enzyme	Crosslinker	Activity	
			µmol glucose/min/g Moist Polymer*	Insolubilized (% of added)
2	Lactase	DAS	21.3	59
3	Lactase	Glyoxal	43.0	76
4	Lactase	GA	31.0	70
5	Invertase	GA	48.0	14

*Moist products contained 85-90% water. Activities were measured at 40°C at pH 4 for lactase and pH 5.0 for invertase. 1 ml aliquots were withdrawn (filtered) at measured intervals and glucose content determined.

Invertase or lactase activities of products that were made from insolubilized chitosan flakes treated with GA before and after they were contacted with the enzymes (Examples 6-9 with invertase and 10-13 with lactase) are shown in Table 2.

Table 2. Invertase (I) and Lactase (L) Activities by Insolubilizing Enzymes with Different Amounts of GA on Chitosan Flakes that were also Pretreated with GA

Example and Enzyme	Amount of GA Used after Contacting Enzyme with 5 g Pretreated Chitosan (ml of 25% GA)	Activity	
		µmol glucose/min/g Air-Dried Polymer*	% of Loading
6 (I)	1.9	39	3.6
7 (I)	0.38	276	23.2
8 (I)	0.08	206	16.0
9 (I)	0.0	183	14.2
10 (L)	1.9	17	2.8
11 (L)	0.38	27	4.4
12 (L)	0.08	190	29.0
13 (L)	0.0	262	40.0

*0.2 to 0.4 g air-dried products incubated at 40°C with 25 ml of 0.1M sucrose in phosphate buffer (pH 5.0) or 0.4M lactose in 0.1M potassium acetate buffer (pH 4.0).

DISCUSSION

In our experiments, we tried three approaches to insolubilize enzymes on chitosan. These were (a) entrapment and ionic adsorption by precipitation of solubilized chitosan in the presence of dissolved enzyme by alkali or inorganic sulfate; (b) crosslinking solutions of chitosan containing dissolved enzymes; and (c) partial crosslinking of native (unsolubilized) chitosan flakes with GA before and after contacting the flakes with dissolved enzyme. Of these three approaches, the first was the least attractive, since the products obtained by precipitation had very poor filterability and appeared gelatinous. It would be difficult to wash unbound or unencapsulated enzyme from such preparations, but qualitative tests indicated retention of enzyme activity. The products were not further characterized.

The potential use with enzymes of the sulfate salt of chitosan, as prepared from the native flakes by direct titration with dilute sulfuric acid rather than from chitosan solutions plus inorganic sulfate, however, still appeared plausible. First, filterability would not be a problem; second, the intricate ultrastructure of native chitosan flakes and the large internal surfaces and spaces of this microarchitecture appeared potentially conducive to sequestering or binding the enzyme (see Masri and Jones, these

Proceedings); and, third, the bound or entrapped enzyme could conceivably become stabilized and locked in the chitosan matrix by the potential ionic crosslinking of adjacent polysaccharide chains (by the divalent sulfate ion through salt formation with amino groups of the chains). Some of our exploratory experiments also suggested adsorptive or ionic affinity of the proteins to the polyamino polysaccharide. Results with the chitosan sulfate-invertase product of Example 1 showed that an appreciable amount of invertase was bound to the matrix, based on the initial rate of sucrose hydrolysis measured at short intervals after the start of incubation (presumably to minimize the effect of leakage). The initial rate was 98 μmol sucrose/min/g, representing 15% of the added activity. The enzyme leaked into the medium, however: with longer periods of incubation, the rate of glucose production appeared to rise. When portions of the aliquots that were withdrawn for the rate measurements were not boiled immediately after withdrawal (to inactivate any enzyme that might have leaked), but were left at 20°C for 1-3 hours before their glucose contents were determined, very much higher glucose values were obtained,compared to values with corresponding portions that were boiled. In fact, almost all the available substrate was hydrolyzed with some of the unboiled samples. Initial trials indicate that the adsorbed enzyme could be fixed to the chitosan-sulfate matrix with GA to prevent leakage. Furthermore, the chitosan-sulfate flakes without GA may be useful for enzyme isolation and purification.

The second approach we used of insolubilizing enzymes by crosslinking chitosan solutions yielded satisfactory products which filtered well and had relatively high activities, despite the high water content (Table 1), and the activity was firmly bound. It is likely that the enzyme in these preparations is bound not only by entrapment and adsorption in the gel but also by covalent coupling. The free ε-amino groups of lysyl residues of the enzyme probably participate in the crosslinking through Schiff base formation with the aldehyde reagents. This participation leading to covalent binding of the enzyme to the Schiff base polymer is diagrammatically depicted in Figure 1.

There are two main disadvantages, however, to this procedure. First, the high water content of the polymers may not be desirable; second, the preparation of the products requires solubilizing the chitosan to give acidic solutions of about pH 4.2 to which the dissolved enzyme must be exposed. This acidic pH is not compatible with many enzymes and may cause their inactivation.

The third procedure of fixing enzymes on unsolubilized chitosan flakes with GA is the procedure we prefer. Relatively high specific activities can be achieved by appropriate control of the amount of GA used after contacting the dissolved enzyme with the partially pre-crosslinked chitosan flakes (Table 2). Coupling of the enzyme to the matrix can be carried out at a pH to which the enzyme is stable. The products are easy to handle, have excellent filtering properties, and can be air dried. In addition, the native physical structure and microarchitecture of the chitosan referred to earlier are essentially preserved in the products.

The sensitivity of the enzyme activities in the products to the GA added in the procedure is striking; it may be different for different enzymes. Evidently the high reactivity of GA with the protein and the large differences in molecular weights of the reagent and the protein are responsible

for this sensitivity. Very likely a greater extent of crosslinking by GA, though favoring firmer attachment of the protein to the matrix, may also cause excessive crosslinking of the enzyme to itself and to the matrix, thereby masking and limiting accessibility of the active sites and movement of the substrate and products to and from the sites. The optimal extent of crosslinking (namely, the greatest possible for maximal fixing without serious loss of activity), however, can easily be determined experimentally for specific cases.

Experiments of examples 9 and 13 show that the partially crosslinked CHT is itself suitable for coupling enzymes without further addition of GA, merely by contacting the enzymes with the CHT-GA product. This is not surprising, since the CHT-GA product would be expected to contain free aldehyde groups produced by some of the GA originally added. The fact that the precrosslinking of CHT was carried out in a two-phase manner (solid CHT and dissolved GA) enhances the probability of the one-point attachment. The content of free aldehyde groups in CHT-GA was apparently sufficient to be effective for coupling lactase (Example 13) and, to a lesser extent, invertase (Example 9).

In all our experiments, we intentionally used relatively high enzyme loading (i.e., initial dissolved enzyme added/g chitosan) to obtain products with high specific activities, and the percent of added activity retained on the polymers should be viewed in this light. For potential practical application, a high specific activity is desirable if not a prerequisite. The following example illustrates this point. The experiments of Examples 8 and 10 were repeated, but this time using double the amount of enzymes. The resulting products had specific activities comparable to those of the parallel examples (183 μmol sucrose/min/g, and 16 μmol lactose/min/g). We have not determined the minimum ratio of enzyme to matrix loading beyond which no improvement of specific activity could be realized. Another illustrative point is that in some tests the mother liquors from enzyme-coupling reactions retained appreciable activities that could be fixed on fresh batches of chitosan matrices.

Although in the main we used lactase and invertase as models in the experiments reported here, we have also tried a few experiments of fixing other enzymes such as glucose oxidase and horseradish peroxidase using GA pretreated chitosan flakes, and the products were active.

In conclusion, our studies of immobilizing enzyme on chitosan matrices point to two promising procedures. They employ relatively inexpensive and readily available reagents and materials, mild conditions and simple methods of preparation. The insolubilized enzyme-chitosan filter easily, and a relatively high specific activity can be attained in the products. The procedures are applicable to insolubilizing enzymes in general at wide pH ranges. It is hoped that the simplicity and versatility of the methods and the low potential cost and satisfactory properties of the immobilized enzyme products will stimulate interest for industrial application. The present experiments warrant further study of the products of the two procedures to evaluate their behavior under actual-use conditions and in continuous operation with media of various pH, ionic strength and composition.

In one procedure, a solution of chitosan plus an enzyme is treated with a crosslinking agent to give a gel polymer with retained enzyme activity,

which is very likely bound covalently to the polymer. With this procedure, acidic pH is required (which may not be suitable for some enzymes) and the hydrogel products have high water uptake, although this does not appear to interfere with their use.

In a second procedure, chitosan flakes are first treated to effect partial crosslinking of the chitosan in the solid state without dissolving the flakes. The treated flakes are then used to couple enzymes at suitable pH's merely by contacting them with solutions of the enzymes followed by a further addition of a small optimal amount of the crosslinker, thereby fixing the enzymes to the flakes. Throughout this procedure the flakes remain undissolved even if coupling is done in acid media, since the preliminary crosslinking renders them insoluble even in acid. Thus a wide pH range best suited for a particular enzyme can be used. Rendering the flakes insoluble in this way also preserves the desirable original native physical structure and ultrastructure of the chitosan. The enzyme-chitosan products obtainable with this procedure have high specific activities, excellent filtering properties, low water uptake and can be air dried for ease of handling and storage.

The resistance to solubility in acids of the partially crosslinked chitosan flakes suggests their use in other applications, including the removal of toxic metallic ions from acidic waste effluents (see Masri and Randall, these Proceedings).

Finally, ionic binding of enzymes to chitosan sulfate suggests its use for enzyme isolation and purification.

ACKNOWLEDGMENTS

We thank Alfred C. Olson and Glenn G. Watters of our laboratory for constructive review of the manuscript.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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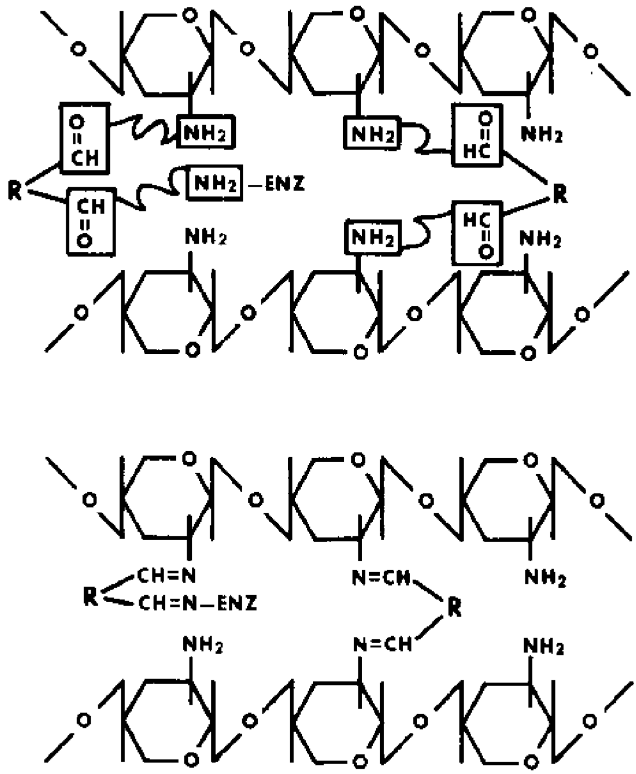

Figure 1. Crosslinking chitosan by polyaldehydes in presence of lactase.

INTERACTION OF WHEAT-GERM AGGLUTININ WITH CHITIN OLIGOMERS AND MICROBIAL CELL-WALL POLYMERS

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ABSTRACT

Wheat-germ agglutinin (WGA) is a protein capable of agglutinating erythrocytes and other types of cells, especially tumor cells. The agglutination can be inhibited by N-acetylglucosamine (GlcNAc) and by chitin oligomers [$\beta(1\rightarrow4)$-linked $(GlcNAc)_{2,3,4...}$], which exhibit even higher inhibitory activity. The ability of WGA to bind GlcNAc and chitin was used for the purification of the agglutinin by affinity chromatography on columns of a Sepharose-bound GlcNAc derivative or on ground chitin.

The purified WGA, when excited at 280 nm, presents a typical tryptophan fluoresence-emission spectrum centered at 348 nm. The addition of GlcNAc or chitin oligomers enhances the protein fluorescence intensity, and this alteration in fluorescence was used to measure the binding affinity of the chitin oligomers to WGA by spectrofluorimetric titrations. The binding constant was found to increase with oligomer length up to the tetramer.

The ability of WGA to interact with polymers that contain GlcNAc residues was studied by employing microbial cell-wall components. WGA binds and precipitates with soluble bacterial cell-wall peptidoglycan and teichoic acids. It also binds to and agglutinates bacteria that carry GlcNAc-containing polymers on their surface. Studies on the binding of a fluorescent WGA derivative (FITC-WGA) to fungi demonstrate that the agglutinin binds almost exclusively to hyphal tips and septa of chitin-containing fungi (e.g., Trichoderma viride). Studies with radioactive chitin-precursors indicate that WGA binds to the sites where chitin is intensively synthesized. The pattern of binding of FITC-WGA indicates that chitin is exposed in the tips and septa. Binding of WGA inhibits the incorporation of precursors into chitin, and, as a result, the extension of hyphae and germination of spores are inhibited.

Binding of FITC-WGA to hyphal tips and septa of isolated mycobionts (Lichen fungi) suggests that they may contain chitin in their cell walls and that it is exposed at the same sites as in free living chitin-containing fungi.

INTRODUCTION

Lectins are proteins or glycoproteins that bind mono- and/or oligosaccharides with great specificity. The use of lectins for investigating structural and organizational aspects of carbohydrate-containing cell surface-membrane components of animal cells is very well documented (28,31).

Many lectins agglutinate bacteria and precipitate with bacterial polysaccharides. Sumner et al. (32) observed agglutination of Mycobacteria by concanavalin A (ConA). This agglutination was a result of the interaction

of ConA with an arabinogalactan present on the mycobacterial walls (13). ConA also precipitated with the polyglycosyl glycerol phosphate teichoic acid from Bacillus subtilis (10). A lectin from snail (Helix pomatia) precipitated lipopolysaccharides of rough mutants of Salmonella typhimurium (17), streptococcal group-C polysaccharide, and the teichoic acid of Staphylococcus aureus 3528 (15,16). Phytohemmagglutinin (14) and soybean agglutinin (7) have been shown to bind specifically to those Rhizobia that participate in symbiotic relationship with the plants from which these lectins were purified. These lectins interact with isolated O-antigen-containing lipopolysaccharides of their symbiont Rhizobia (33).

Very few studies on the interaction of lectins with fungi have been reported. Spores of Fusarium roseum and F. solani were agglutinated differentially by several lectins (20). Spores of Ceratocystis fimbriata were agglutinated by various host-plant extracts in a specific pattern, which suggested the possible involvement of the spore agglutinating factors (assumed to be lectins) in establishing host specificity (21).

We have investigated the interactions of wheat-germ agglutinin, a lectin specific for N-acetylglucosamine residues (1,8), with chitin oligomers, bacterial cell-wall polysaccharides and their constituents, as well as with intact bacteria and fungi (22-26,30). Here, we present the results of our investigations as well as of subsequent recent studies. All these observations point out the specificity of the interaction of WGA with GlcNAc-containing polymers in solution or on intact cells and suggest the use of this lectin for localization of such polymers, especially chitin on microbial cell walls.

Sugar-binding properties of wheat-germ agglutinin

N-acetylglucosamine and its oligosaccharides have been shown to inhibit agglutination of cells by WGA (1,8). It was concluded that WGA is capable of binding these saccharides. We have used this property for the purification of WGA by affinity chromatography on an immobilized GlcNAc-derivative: the 2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-glucopyranosylamine-Sepharose (22,23). Since WGA binds chitin oligomers, it was possible to purify it on a column of ground chitin (6).

Chitin oligomers were much more potent inhibitors of agglutination by WGA than the monosaccharide GlcNAc. Allen et al. (1) proposed that the saccharide binding site in the lectin may be composed of several subsites, similar to what has been found in lysozyme. The binding of chitin oligomers to lysozyme induced changes in the protein fluorescence, and a method was developed to measure association constants by spectrofluorimetric titrations (9). We attempted to apply a similar procedure, because of the similarity to lysozyme, in order to investigate the interaction of WGA with chitin oligomers.

WGA contains 3 tryptophan residues per subunit, and, upon excitation at 280 nm, the agglutinin presents a typical tryptophan fluorescence centered at 348 nm. As can be seen in Figure 1, the addition of GlcNAc increases the fluorescence. Chitotriose $(GlcNAc)_3$ induced a higher enhancement of fluorescence and a shift of 7 nm to a lower wave length. These results are probably due to changes in hydrophobicity near one or more of the tryptophans or to conformational changes induced by saccharide binding. Measurement of changes in WGA fluorescence induced by increasing quantities of various chitin

oligomers allowed calculation of the corresponding association constants presented in Table 1. The binding affinity increases with the chain length up to the tetramer, supporting the suggestion that the sugar-binding site in WGA is extended and comprised of 3 to 4 subsites (1). Equilibrium studies using chitotetraose reduced by sodium [^{3}H]borohydride demonstrated the presence of 2 saccharide-binding sites per subunit (29). Since WGA is composed of two subunits (27), the native molecule possesses 4 binding sites. Loss of saccharide-binding and cell-agglutinating activities by WGA, in which tryptophan residues were oxidized with N-bromo-succinimide, indicates that in each binding site there is one tryptophan residue directly involved in sugar binding (30).

Table 1. Association Constants and Free Energy of Association of Chitin Oligomers with Wheat-Germ Agglutinin Determined by Fluorimetric Titrations

Saccharide	10^{-4} x Ka (liter/mole)	- ΔFa* (K cal/mole)
$(GlcNAc)_2$	1.3	5.6
$(GlcNAc)_3$	2.2	5.9
$(GlcNAc)_4$	3.6	6.2
$(GlcNAc)_5$	3.2	6.1

*Calculated from the relationship $\Delta F^{\circ}_{a} = - RT \ln Ka$

Interaction of wheat-germ agglutinin with isolated bacterial cell-wall polymers

Cell walls of both gram-negative and gram-positive bacteria contain a common constituent, peptidoglycan, which consists of linear polysaccharide strands composed of repeating units of a disaccharide N-acetylglucosamine and N-acetyl muramic acid (MurNAc). The latter residue is usually substituted with peptide units through which the polysaccharide strands are crosslinked. Gram positive bacteria also contain one or more heteropolysaccharides, which are usually attached by covalent linkages to the glycan strands of the peptidoglycan. Most prominent among these are the teichoic acids, which are polymers of ribitol or glycerol phosphate linked by phosphodiester bonds. In these polymers the hydroxyl groups of the alcohol are often substituted by one or more glycosidically linked monosaccharides (e.g., glucose or GlcNAc).

We have isolated a soluble form of peptidoglycan from a medium of cultures of Micrococcus luteus grown in the presence of penicillin G. Polyribitol phosphate teichoic acid, either substituted with GlcNAc residues or non-glycosylated, was isolated from cell walls of S. aureus H and S. aureus 52A2,

respectively. The interaction of WGA with these polymers was studied directly, by testing the ability of the lectin to precipitate the polymers, and indirectly, by measuring the ability of the polymers and their lower molecular weight constituents to inhibit the hemagglutinating activity of WGA. We found that WGA precipitated only polymers that contain GlcNAc residues, such as the soluble peptidoglycan from M. luteus and the GlcNAc-containing teichoic acid of S. aureus H, whereas the teichoic acid of S. aureus 52A2, which is devoid of GlcNAc residues, was not precipitated (25). Hapten inhibition of polymer precipitation and of hemagglutination by WGA indicates that both activities are mediated via saccharide binding, since they can be inhibited by GlcNAc, chitin oligomers and by GlcNAc MurNAc and its dimer (Table 2). These experiments also demonstrated that, based on their molar content of GlcNAc, the soluble bacterial cell-wall polymers are much better inhibitors than $(GlcNAc)_3$ or $(GlcNAc\ MurNAc)_2$.

Interaction of wheat-germ agglutinin with intact bacterial cells

Agglutination of animal cells by lectins is a result of the binding of the lectins to cell-surface glycoproteins. Since WGA was found to bind certain bacterial cell-wall polymers, its ability to agglutinate bacteria was studied. As shown in Table 3, WGA agglutinates bacteria that are "coated" by polymers containing GlcNAc residues, but does not agglutinate cells of S. aureus 52A2, on which the teichoic acid is devoid of GlcNAc residues and apparently hinders access of WGA to the underlying GlcNAc-containing peptidoglycan. Cells of E. coli PAT 84, which are rapidly agglutinated by WGA, were chosen to demonstrate the binding of fluorescein isothiocyanate-labeled WGA (FITC-WGA), since the cells, when grown at 42°C, do not divide, but instead form long filaments which are large enough to be observed clearly in the fluorescence microscope (Figure 2). The binding to and agglutination of the E. coli cells by WGA strongly suggest that WGA can interact with cell-wall lipopolysaccharides, which are known to contain GlcNAc residues (18). To test the effect of WGA on the growth of the agglutinable bacteria, they were grown in the presence of WGA (0.5 mg/ml). No inhibition of the bacteria could be observed.

Interaction of wheat-germ agglutinin with fungi

Since WGA binds chitin oligomers as well as chitin (1,6,8), the binding of the lectin to fungi having chitin in their cell walls was investigated. The Deuteromycetes Trichoderma viride and F. solani that belong to fungal group V (3), all of which have chitin-glucan cell walls, were used. FITC-WGA was found to bind to hyphal tips and to septa, whereas very little binding at other parts of the hyphae occurred (Figure 3). The binding was specific and could be inhibited by $(GlcNAc)_3$. There was no binding to hyphae of Phytophthora citrophthora, which does not contain chitin.

In fungi with chitin-glucan hyphal walls, hyphal extension and septa formation involve the synthesis of chitin (11). Short pulse labeling of T. viride colonies with [^{3}H]acetate followed by autoradiography revealed that the radioactivity was localized in the hyphal tips and septa (Figure 4 A) as originally reported by Galun (11). Since these sites of chitin synthesis are exactly those where FITC-WGA was found to bind, we concluded that the chitin in those regions is not covered by glucan and therefore is accessible to WGA. Binding of WGA prior to labeling with [^{3}H]acetate markedly inhibited incorporation of the precursor into the hyphal tips (Figure 4 B) and prevented extension of the hyphae (26).

Table 2. Inhibitory Effect of Various Saccharides and Cell-Wall Polymers on the Hemagglutinating and Precipitating Activities of WGA

Precipitation is that of the teichoic acid of S. aureus H, and agglutination is that of trypsinized rabbit erythrocytes. For comparison, the concentration of inhibitors was normalized to their molar content of GlcNAc. For relative inhibitory activity the hemagglutination inhibitory activity of GlcNAc was taken as 1. Apparent Ka was calculated from the relative inhibitory activity using the Ka for $(GlcNAc)_3$ ($Ka = 2.2 \times 10^4 M^{-1}$), which was determined by spectrofluorimetric titration.

Compound	Concentration (mM) Required for 50% Inhibition of: Precipitation	Agglutination	Relative Inhibitory Activity	10^{-4} x Ka (liter/mole)
Ribitol	(0% at 300 mM)	(0% at 300 mM)		
MurNAc	(0% at 300 mM)	(0% at 300 mM)		
GlcNAc	(20% at 300 mM)	75	1	0.006
GlcNAcMurNAc	9	1.5	50	0.3
$(GlcNAcMurNAc)_2$	1.2	0.32	235	1.4
$(GlcNAc)_3$	0.22	0.21	360	2.2
Linear peptidoglycan	n.t.	0.012	6,250	38
Teichoic acid (S. aureus H)	-	0.0035	21,400	130

Table 3. Interaction of Wheat-Germ Agglutinin with Bacterial Cells

Bacterial Strain	Polymer in Cell Wall	WGA Concentration (μg/ml)[a] Required for 50% Agglutination
S. aureus H	GlcNAc-containing teichoic acid peptidoglycan	48
S. aureus 52A5	peptidoglycan	4
S. aureus 52A2	GlcNAc-deficient teichoic acid peptidoglycan	Nonagglutinable at 512
M. luteus NCTC 2665	peptidoglycan	32
E. coli PAT 84	lipopolysaccharide	24

[a]Bacterial suspensions were incubated with varying concentrations of WGA, and the agglutination was determined from the decrease in the absorbance at 580 nm. The values represent the concentration of WGA that caused 50% of the maximal reduction in absorbance.

The germination of spores involves emergence of a hyphal tube and intensive chitin synthesis in the tip of that tube (4). Incubation with WGA inhibited germination of T. viride spores (26). The effect is probably due to binding of WGA to the tip of the emerging hyphal tube rather than to the spore coat, since no binding of FITC-WGA or agglutination of the spores was observed. Kleinschuster et al. (20) also reported that there were no receptors for WGA on spores of F. solani, but trypsinization exposed such receptors.

Recently, Galun et al. (12) and Barkai-Golan et al. (2) extended our studies to other fungi and other lectins. Penicillia and Aspergilli contain chitin in their cell walls. FITC-WGA was bound to young hyphal walls of such fungi, especially to the hyphal tips and septa. WGA inhibited incorporation of [^{3}H]acetate into young hyphae of Aspergillus ochraceus, and germination of the spores was also inhibited (2). Binding of FITC-WGA to hyphal tips and septa of mycobionts isolated from the lichens Xanthoria parietina, Tornabenia intricata and Sarcogyne sp., as well as incorporation of [^{3}H]GlcNAc into these regions during short-pulse labeling, suggested that chitin may be a constituent of the mycobionts' hyphal wall (12).

A different approach to localization of receptors for WGA was reported by Horisberger et al. (19). They prepared gold granules labeled with WGA and used scanning electron microscopy to detect binding of WGA to Saccharomyces cerevisiae. No binding was observed prior to treatment of the yeast with α-mannanase. After such treatment, gold granules were observed exclusively on bud scars, on the mother cell-bud junction and on the bud, all of which contain chitin (5).

CONCLUSION

We have demonstrated that binding of WGA to microbial cells and cell-wall polymers is specific and mediated via its affinity for GlcNAc; it is even more so for polymers containing numerous GlcNAc residues in either hetero- or homopolysaccharides. Thus the binding of WGA to microbial cells may indicate the presence of GlcNAc residues exposed on their cell walls.

Binding to fungi, where GlcNAc is predominantly found in chitin, may demonstrate the presence of chitin. The specific binding of WGA or other lectins to microbial cell walls may provide preliminary information on the nature of saccharides on cell walls even prior to the more time-consuming chemical analyses which require larger amounts of cell-wall material.

Since plants and bacteria do not contain chitin, FITC-WGA may be used for specific identification of chitin-containing fungi in biological material, e.g., in cases of plant infection by phytopathogenic fungi that contain chitin on their cell walls.

ACKNOWLEDGMENTS

These studies were carried out while the author was in the Department of Biophysics, the Weizmann Institute of Science, Rehovoth, Israel. Thanks are extended to Drs. N. Sharon, D. Mirelman and E. Galun for their collaboration and for communication of recent observations.

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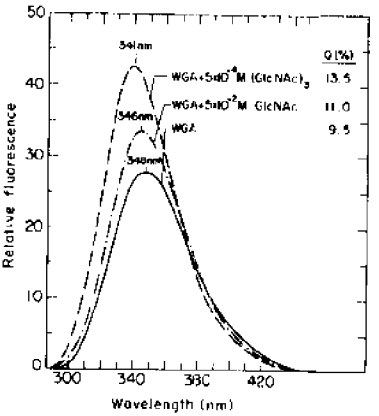

Figure 1. Fluorescence emission spectra of WGA (60 μg/ml) and of its complexes with saccharides. The measurement was at pH 6.4 in 0.1 M sodium phosphate buffer. Excitation wavelength was 280 nm.

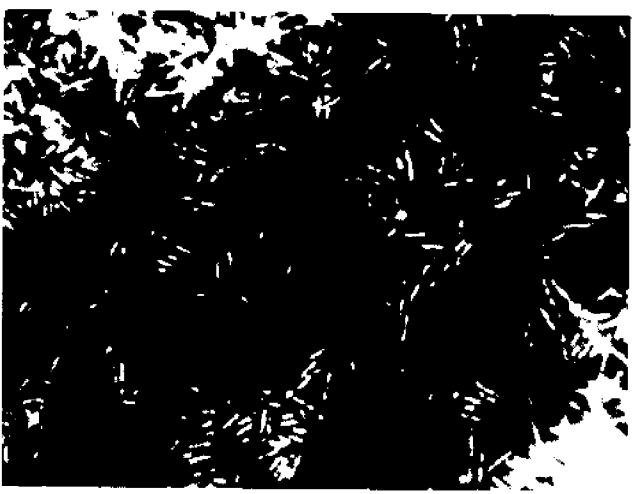

Figure 2. Microscopic appearance of the binding of FITC-WGA to cells of *E. coli* PAT 84.

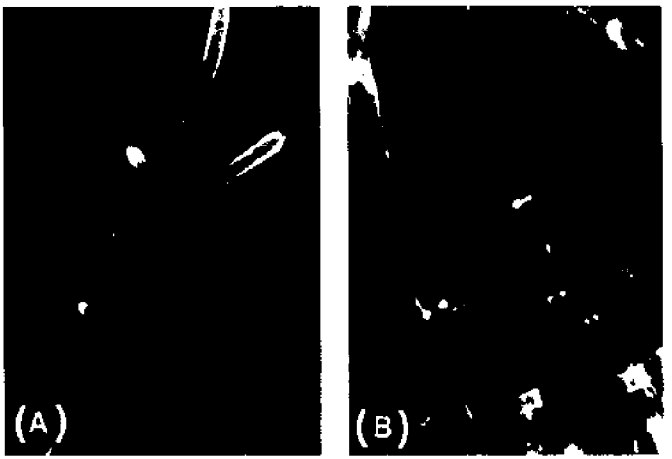

Figure 3. Microscopic appearance of *T. viride* hyphae treated with FITC-WGA. Binding to hyphal tips (A), to septa (B).

Figure 4. Microautoradiographs of *T. viride* hyphae. (A)– Hyphae from a colonial front after incubation for 10 min with [^{3}H]acetate. Note heavy labeling of tips and septa; (B)--Hyphae from a colony which was preincubated with 250 μg WGA and then labeled as in A. Note inhibition of labeling at hyphal tips.

CHITOSAN-METAL COMPLEXES AND THEIR FUNCTION

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ABSTRACT

Chitosan has the characteristics selectively to combine particular metal ions to form metal complexes. These tendencies are most predominant in Cu (II), Hg (II), Fe (II), Ag (I), Cd (II), Ni (II); they are weakest in Mn (II), Co (II) and Cr (III). The presence of alkali earth metals, Sr (II), Mg (II), Ca (II) and Ba (II) with, e.g., Cu (II) ion in an aqueous solution did not influence the Cu (II) chitosan-complex formation. Chitosan film also made complexing easy; the elimination of metal ions from the complex was performed at approximately pH 2. The recovery of complex-formation ability by the chitosan film was then about 100%. A water-soluble glucosamine oligomer Cu (II) complex was prepared to use as a model of a chitosan Cu-(II) complex. Spectrophotometry confirmed that one mole of cupric ion coordinated with four moles glucosamine. The chitosan-Cu (II) complex displayed oxidation-reduction catalytic activity in ordinary temperatures and atmosphere. The decomposition-reaction rate of hydrogen peroxide was accelerated ten times by chitosan-Cu (II) complex; this reaction was recognized to proceed according to the same mechanism as an enzymatic reaction. It was found that the dehydrogenative polymerization of coniferyl alcohol, using chitosan-Cu (II) complex as a catalyst, led to the formation of lignin-like polymeric substances.

INTRODUCTION

Chitin is an abundant natural polymer. Chitin's estimated yield per year is supposed to amount to 100 billion tons, approximately the same as cellulose production. The estimated amount of chitin produced by plankton in the Antarctic Ocean is over several billions tons every year. When this plankton becomes widely used as a protein source, the utilization of such chitin will be indispensable. Fundamental and practical studies of chitin as a worldwide source of protein are vital.

Earlier we prepared a chitin film (5) and studied its characteristics. At the same time with Muzzarelli (4), we also found that the chitin film, chitosan film and chitosan powder combined metal ions selectively (2) and obtained significant information with respect to the catalytic activity of the chitosan-metal complex. In this paper, the summary of these latter results will be reported.

EXPERIMENTAL

Preparation of chitosan

Chitin prepared from shells of crab and plankton was used as the experimental material. Chitosan was then prepared by the modified Hackman method from purified chitin. Chitin (160 g) in a platinum beaker was heated with 40% NaOH solution (1.6 kg) under a nitrogen stream. The temperature was maintained at 100° C for 2 hours and then at 150° C for 30 minutes. After cooling to room temperature, the alkaline solution was poured into ice-cold dilute aqueous acetic-acid solution. The still alkaline solution was neutralized with acetic acid to pH 7.8. The precipitated chitosan was collected by successive centrifugation. The precipitate was suspended again into water and then filtered, washed with water until the washings were neutral, then washed with ethanol and ether. The yield was 119.7 g.

Preparation of chitosan-metal complex

To the chitosan (500 mg), an aqueous solution (10 ml) of metal salt (4 millimole) was added and shaken overnight at room temperature. As metal salts, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $CdSO_4 \cdot 8H_2O$, $Pb(CH_3CO_2)_2 \cdot 3H_2O$, $AgNO_3$, $HgCl_2$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $K_2Cr_2(SO_4)_4 \cdot 24H_2O$ and $MnSO_4 4 \cdot 6H_2O$ were used. After completion of the reaction, the chitosan-metal complex was filtered and washed with water until no metal ions were detected against sodium sulfide or sodium chloride. Quantitative determination of metal ions contained in the chitosan was conducted by using a Varian Techtron Model 1000 Atomic Absorption Spectrophotometer, after decomposing the metal-chelating chitosan with nitric acid (see Table 1).

Measurement of the saturated binding amount of metal ions (see Fig. 3) was performed according to the procedure described in reference 2.

Preparation of glucosamine oligosaccharide by partial degradation of chitosan (6)

To a solution of chitosan (30 g) in 2.25 l of 0.1N-HCl, 100 ml of 2.5% $NaNO_2$ solution was added and the mixture was stirred for 15 hours at room temperature. The degradate was subjected to fractional precipitation, using aqueous ethanol solution of various concentrations containing a small amount of hydrochloric acid. Three fractions were obtained (F-1, F-2, and F-3) and purified by gel filtration on a Sephadex G-15 column. The F-1 fraction containing 17 glucosamine units was used for preparation of the water-soluble Cu (II) complex.

Preparation of water-soluble glucosamine oligosaccharide Cu (II) complex and measurement of the absorption spectra

An aqueous solution of F-1 hydrochloride containing 0.2 mole of glucosamine units per liter was prepared. An aliquot of 2 ml was then placed in test tubes to which 0.2 mole $CuSO_4$ solution was added until

the [Cu (II)]/[Cu (II)] + [glucosamine unit] ratios came to the following values: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0. The same volumes of 1N-NaOH were then added to each tube. The tubes were shaken vigorously, and the excess Cu (II) ion precipitated as $Cu(OH)_2$ was removed by centrifugation. The absorption spectra of Cu (II) complexes were measured by using a Hitachi Recording Spectrophotometer Model EPS-2.

Measurement of catalytic activity of chitosan-Cu (II) complex on decomposition reaction of hydrogen peroxide

The reaction mixture containing 70 ml of hydrogen peroxide solution, 10 ml of buffer and the chitosan-Cu (II) complex (including 5×10^{-5} g-atom of cupric ion), was stirred at 20° C. When a degradation ratio was measured at various pH's, the initial concentration of hydrogen peroxide was controlled to 7.5 m mole/l. The rate of decomposition reaction of the hydrogen peroxide was measured by titration with potassium permanganate.

Dehydrogenative polymerization of coniferyl alcohol using chitosan-Cu (II) complex as catalyst

Coniferyl alcohol was synthesized from vanillin via acetyl ferulate. M.p. 71.5 - 73° C.

Chitosan-Cu (II) complex containing 1.6 millimole Cu (II) per gram complex was used as catalyst in the polymerization reaction. A solution of coniferyl alcohol (200 mg) in acetone (4 ml) was added to a mixture consisting of distilled water (8 ml), buffer (4 ml) and chitosan-Cu (II) complex (5-100 mg). To the resultant solution of a ph 5.6 were added two equivalents (2.2 ml) of hydrogen peroxide (0.5 N). The reaction mixture was shaken at 30° C at atmospheric pressure for 16-140 hours. The mixture was then dialyzed against running water for 48 hours and concentrated in reduced pressure. The resulting precipitate was separated, dissolved in dioxane and freeze-dried in order to remove chitosan Cu (II) catalyst (Fr. A). The filtrate was directly concentrated and freeze-dried (Fr. B). Coniferyl alcohol (200 mg) and chitosan-Cu (II) complex (5-100 mg) were placed in test tubes to which 4 ml of acetone were added to dissolve the coniferyl alcohol. Eight ml of water and 4 ml of buffer (pH 4.0-7.0) were added to each tube and the contents were frozen. Then 0.5 N-H_2O_2 (2.2 ml) was added to the frozen mixture and it was frozen again. Air in each tube was replaced with N_2, and then the tube was sealed with a flame *in vacuo*. Polymerization was carried out at 30° C for 16 hours. The resultant polymerisate was treated in a manner similar to that described above.

The average molecular weight of the polyconiferyl alcohol was determined on the dioxane solution, using a vapor-pressure osmometer. The NMR spectra were measured on the acetylated polymers in $CDCl_3$. Determination of phenolic OH was carried out by the $\Delta\varepsilon_i$ method.

RESULTS AND DISCUSSION

Chitosan-metal complex

We have previously reported (5) on the preparation of chitin film by the solid-phase acetylation of chitosan film. When either chitosan film or partially acetylated chitosan film was immersed in separate solutions containing Cu (II), Co (II) and Ni (II) ions, the films turned blue, red and green, respectively. The chitin film did not change color. Independently, Muzzarelli et al. (4) reported on the formation of metal complexes of chitin and chitosan with transition metals or post-transition metals, and elsewhere we have reported (2) on the amounts of metal ions complexed with chitosan and the selectivity of chitosan for these ions. The amounts of metal ions complexed with powdered chitosan are presented in Table 1.

Table 1. Metal Contents in Chitosan-Metal Complexes

Metal Ion	Metal Content, m mole/g
Ag (I)	0.88
Hg (II)	2.55
Cu (II)	2.29
Cd (II)	0.78
Cr (III)	0.0026
Mn (II)	0.191
Fe (II)	1.28
Co (II)	0.033
Ni (II)	1.18
Pb (II)	0.088

Metal ions of Ag (I), Hg (II), Cu (II), Cd (II), Fe (II), and Ni (II) took up more than 0.5 millimoles per gram of chitosan. The affinities for Hg (II), Cu (II), Fe (II) and Ni (II) ions were especially predominant in chitosan. IR absorption spectra of chitosan-metal complexes were investigated. By combining with metal ion, some of the IR absorption bands of chitosan shifted to longer wave lengths. The following shifts were observed in IR absorption bands of O=C-NHR (1650 cm^{-1}), $-NH_2$(1590 cm^{-1}) and $-NH_3$ (1560 cm^{-1}), respectively: 1650 → 1620, 1590 → 1575 and 1560 → 1510 cm^{-1}. Further, 1650 → 1640 and 1560 → 1530 cm^{-1} in Fe (II)-complex were also noticed. These results indicate that the chelation actually occurs between the metal and chitosan.

The amounts of Cu (II) ion combined with chitosan depended largely upon the pH of the aqueous solution of Cu (II) salt--that is, the combined

amount of Cu (II) increases as the pH approaches 6 from the acidic side (Fig. 1). At a lower pH range, Cu (II)-chitosan chelation is decomposed and the desorption of Cu (II) ion takes place. The experiment of removal of Cu (II) ion from chitosan-Cu (II) complex was conducted by using phthalate buffer of pH 2.3. The results are shown in Fig. 2. About 100% Cu (II) ion was removed after 5 hours. The binding capacity of Cu (II) ion of the chitosan film that had been regenerated from its Cu (II)-complex was the same as that of the original one.

Fig. 3 shows the amount of Cu (II) ion combined to chitosan powder in the presence of alkali earth metals; it indicates that the binding capacity of Cu (II) ion was not influenced by the co-existence of alkali earth metals in solution.

Preparation of glucosamine oligosaccharide and its Cu (II) complex

Chitosan-Cu (II) complex was insoluble in water, and consequently it was too difficult to deal with the structure quantitatively. A water-soluble glucosamine oligosaccharide-Cu (II) complex was therefore prepared as a model of the chitosan-Cu (II) complex. The preparation method for glucosamine oligosaccharide previously reported seemed to be unsuited to obtaining the desired oligomers on a large scale. As 2-amino-2-deoxy-D-glucosyl linkage in chitosan is very resistant to acid hydrolysis, the preparation of glucosamine oligosaccharide by partial hydrolysis with acid is difficult. Chitosan in 0.1N-HCl was therefore treated with a specific amount of sodium nitrite, causing a partial deamination and cleavage of the chain on the chitosan molecule.

The rate of the deamination reaction was determined by measuring the amount of 2, 5-anhydromannose formed by deamination of aminosugar with nitrous acid (Fig. 4). In this case, a 1/5 equivalent of nitrous acid was used for each glucosamine unit, about 10% glucosamine in chitosan was deaminated to 2, 5-anhydromannose, suggesting that a decasaccharide may be formed as a main degradation product. From these preliminary experiments, a 1/5 equivalent of nitrous acid was used in order to obtain the decasaccharide of glucosamine. Preparation and fractionation of of oligosaccharides were carried out as shown in Fig. 5.

Three kinds of oligosaccharide, viz., F-1 hydrochloride (Pn: 18), F-2 sulfate (Pn: 16) and F-3 sulfate (Pn: 10), were obtained as a result. Paper chromatography and gel-filtration chromatography were conducted for confirmation of the homogeneity of these kinds of glucosamine oligosaccharides. In a paper chromatogram, F-1 hydrochloride was not contaminated with lower molecular weight oligosaccharides. The elution curves of gel filtration using Sephadex G-15 column also indicated no contaminants present in F-1 hydrochloride.

Water-soluble glucosamine oligosaccharide-Cu (II) complex was prepared from F-1 hydrochloride and $CuSO_4$ solution. In general, when a chelate compound is formed in solution, the color intensity of the solution is enhanced; a colorimetric determination can then be used to estimate the composition of the chelate compounds. This can be carried

out, using the method of continuous variation, introduced by Job (1). The solution of F-1 hydrochloride was mixed with aqueous solution of $CuSO_4$. At pH 5.6, the spectrumof the complex was identical with that of $CuSO_4$ and had a maximum at 764 nm. The formation of the complex was seen from the fact that the absorbance at 764 nm increased with the increasing amount of the added F-1 solution to a definite volume of $CuSO_4$. Glucosamine oligosaccharide-Cu (II) complex was then prepared in alkaline solution, where excess cupric sulfate would precipitate as cupric hydroxide. Only the spectrum of the water-soluble complex resulting from the formation of the coordinate compound was measured. The complexes obtained in alkaline solution gave spectra having λ_{max} at 510 nm and 620 nm (Fig. 6). As these spectra were based upon the complex alone, the molar ratio of Cu (II) and the glucosamine unit in the complexwere determined by applying the method of continuous variation (1). The solutions having [Cu (II)]/[Cu (II)] + [glucosamine unit] ratios between 0.0 and 1.0 were prepared, and their spectra were observed. The molecular extinction coefficient (ε) and λ_{max} (510 and 620 nm)were plotted against the [Cu (II)]/[Cu (II)] + [glucosamine unit] ratio, and the obtained curves showed the maximum values at 0.2 molar ratio. From the experimental results described here, it became obvious that the complex consisting of one Cu (II) ion and four moles of glucosamine units was formed, all of the glucosamine molecules coordinating to the cupric ion as shown in Fig. 8.

Catalytic effect of chitosan-Cu (II) complex on decomposition reaction of hydrogen peroxide

It is generally known that some metal ions such as Fe (III) and Cu (II) have the catalytic activity of an oxidation-reduction reaction. Recently, the catalytic activities of the polymer metal complex have been studied, and it has become obvious that the activity depends on the degree of polymerization. We studied the catalytic activities of chitosan-Cu (II), chitosan-Fe (II) and chitosan-Ni (II) complexes on the decomposition reaction of hydrogen peroxide. Only chitosan-Cu (II) complex showed a considerable activity on decomposition of hydrogen peroxide at neutral pH. The decomposition rate of hydrogen peroxide was measured at pH 4.5-8.0 using a chitosan-Cu (II) complex as a catalyst. At pH 4.5, hydrogen peroxide was not decomposed by the chitosan-Cu (II) complex, but about 80% of the hydrogen peroxide had decomposed in the course of the catalytic reaction of chitosan-Cu (II) at pH 6.9 after 7 hours (Fig. 9). When only Cu (II) ion or chitosan was used as catalyst under the same conditions, the decomposition of hydrogen peroxide did not occur at all. The catalytic activity of chitosan-Cu (II) complex was enhanced at pH 8, but Cu (II) ion alone also showed the catalytic activity. The relationship of the initial velocity of decomposition of hydrogen peroxide to the concentration of hydrogen peroxide (substrate) indicated that as the substrate concentration was increased, the initial velocity of decomposition of the hydrogen peroxide became constant (Fig. 10). This result indicates that the reaction proceeds through a catalyst-substrate complex, that is, according to the Michaelis-Menten equation:

$$\underset{\text{enzyme}}{E} + \underset{\text{substrate}}{S} \rightleftharpoons \underset{\text{complex}}{ES} \longrightarrow \underset{\text{enzyme}}{E} + \underset{\text{product}}{P}$$

From the values of Fig. 10, the Michaelis constant (Km) was calculated to be 0.59. The value of Km was larger than an ordinary enzymic reaction. The larger value of the Km constant means that the affinity of chitosan-Cu (II) complex with hydrogen peroxide was smaller than catalase. A higher concentration of the substrate is therefore necessary to elevate the velocity of the reaction catalyzed with the Cu (II) complex.

Catalytic activity of chitosan-Cu (II) in the dehydrogenative polymerization of coniferyl alcohol

Coniferyl alcohol, a precursor of lignin, polymerizes to give the lignin in plant tissue by the dehydrogenase system. On the other hand, coniferyl alcohol is dehydrogenated by peroxidase in the presence of hydrogen peroxide as shown in Fig. 11; the formed radicals then polymerize by a coupling reaction to gi e synthetic lignin (DHP). Because the chitosan-Cu (II) complex showed catalytic activity in the decomposition reaction of hydrogen peroxide, the catalytic activity of the chitosan-Cu (II) complex in dehydrogenative polymerization of coniferyl alcohol was studied. The polymerization of coniferyl alcohol using the chitosan-Cu (II) complex was carried out under the same conditions as the enzymic dehydrogenative polymerization using peroxidase reported by Lai Yuan-Zong et al. (3). The polymer was obtained in a 37% yield at pH 5.6 and 30° C after 20 hours. When the polymerization was conducted without a catalyst, the polymer yield was only 5% (Table 2). The polymerization of coniferyl alcohol using chitosan-Cu (II) as a catalyst was separately performed in an ampule in vacuo. The polymer yields and analytical data are shown in Table 3. The polymer with a higher molecular weight was obtained at pH 5.6 in about a 30% yield. NMR spectra of the polymers obtained by both methods are shown in Fig. 12. In the case of polymerization conducted at atmospheric pressure, the structure of the resultant polymer was similar to native MWL. The optical rotations of these polyconiferyl alcohols were measured in dioxane. The polyconiferyl alcohol obtained at atmospheric pressure was optically inactive, but the polyconiferyl alcohol obtained in vacuo showed positive optical rotation. The optical activity is due to the polyconiferyl alcohol itself, since chitosan-Cu (II) complex used as a catalyst is insoluble in dioxane. If a slight amount of the chitosan were dissolved in dioxane, it might show a negative optical rotation in water. From these results it was assumed that the polyconiferyl alcohol synthesized in vacuo had a more regular structure than that of native MW. That is, it was recognized that the chitosan-Cu (II) complex not only had a catalytic activity in the polymerization of coniferyl alcohol, but also functions as a matrix, which gives a regular structure to a polymer.

Table 2. Polymer Yields of Polyconiferyl Alcohol Obtained at Atmospheric Pressure

Catalyst	Chitosan-Cu (II)					None
Polymerization time, hr.	20	44	68	92	140	140
Yield, %	37	23	18	22	19	5

pH: 5.6, at 30° C

Table 3. Analysis of Polyconiferyl Alcohol Obtained in Vacuo

Catalyst		Chitosan-Cu (II)					$CuSO_4$
pH		4.5	5.0	5.6	6.0	7.0	5.6
Yield, %	Fr. A	18.1	5.2	29.1	13.0	13.1	8.0
	Fr. B		8.8	7.0	5.6	8.6	2.5
Mn		600	1510	2200	900	720	590
λ_{max}, nm		274		279	280	273	279
$E^{1\%}_{1\ cm}$		290		268	241	282	221
Phenolic OH, %		5.5		6.0	5.7		4.4
$[\alpha]^{20}_{D}$		+53.4		+22.2		+112.5	

Cu (II) ion: 0.032 m mole at 30° C for 16 hours.

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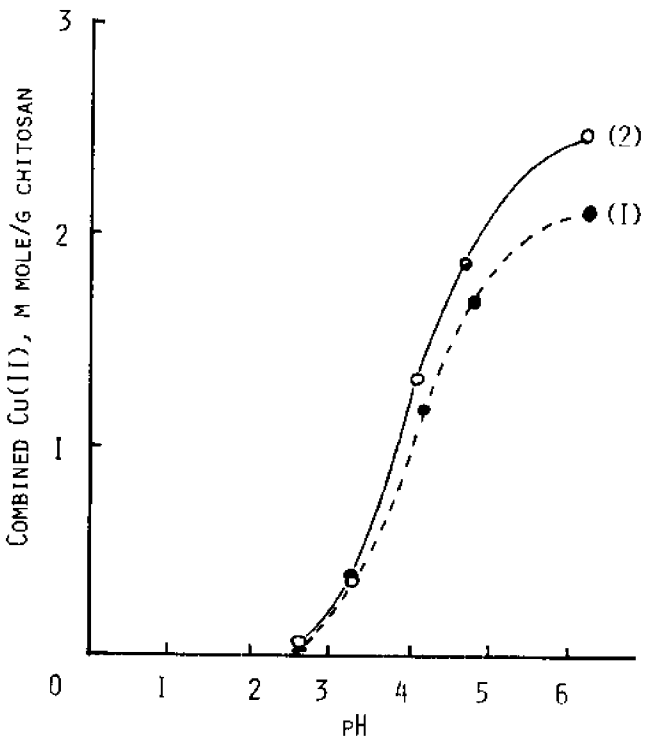

Fig. 1. PH dependence of the amount of Cu(II) ion combined with chitosan. Initial concentration of Cu(II) ion: (1) 5×10^{-3}, (2) 8×10^{-3} mol/l.

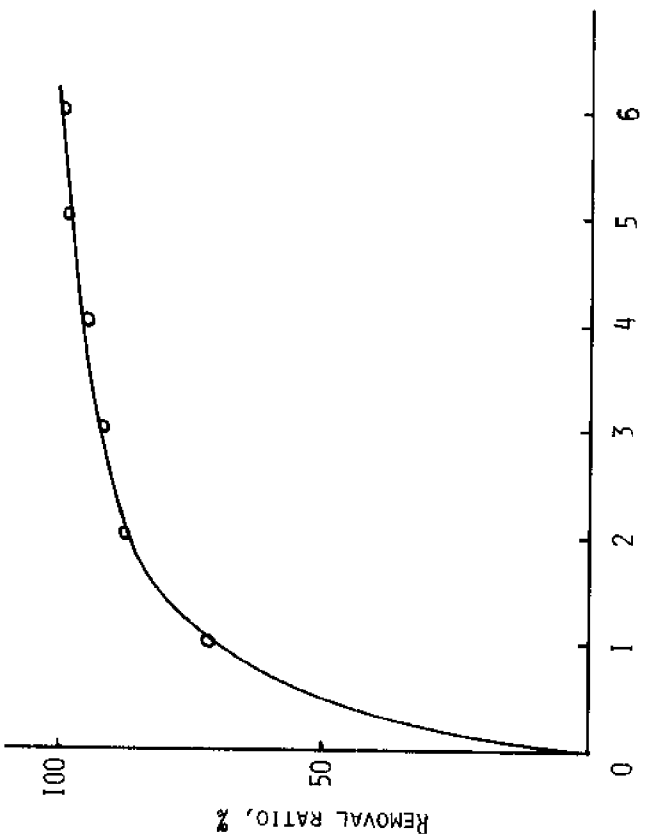

Fig. 2. Removal of Cu(II) ion in phthalate buffer(pH 2.3) from chitosan film-Cu(II) complex

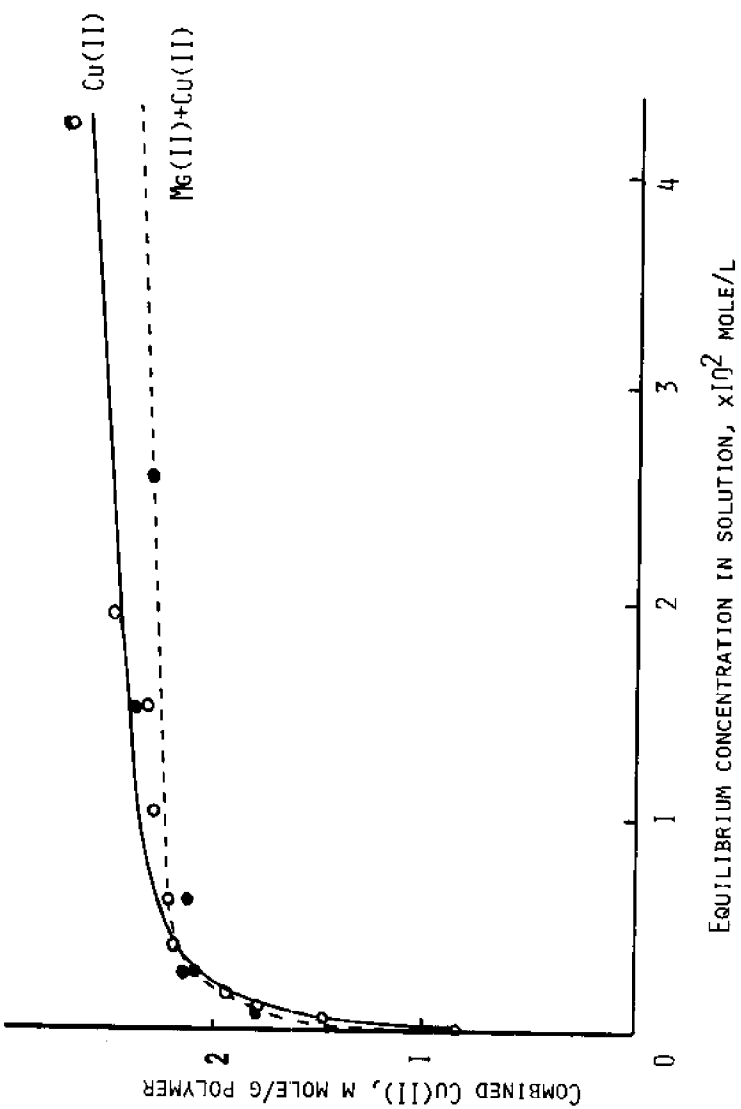

Fig. 3. Equilibrium curves for Cu(II)-chitosan at 30°C.

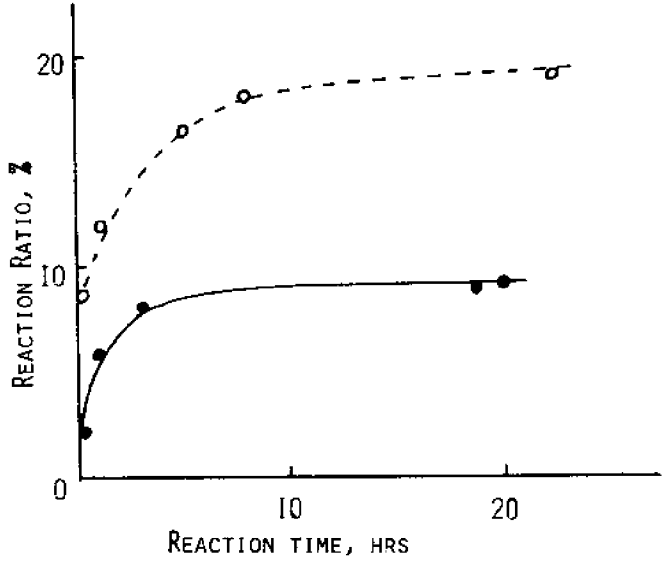

Fig. 4. Determination of degradation ratio of chitosan with nitrous acid. Chitosan: 1.67 milimole.
●——● $NaNO_2$: 0.36 milimole.
○- - -○ $NaNO_2$: 0.72 milimole.

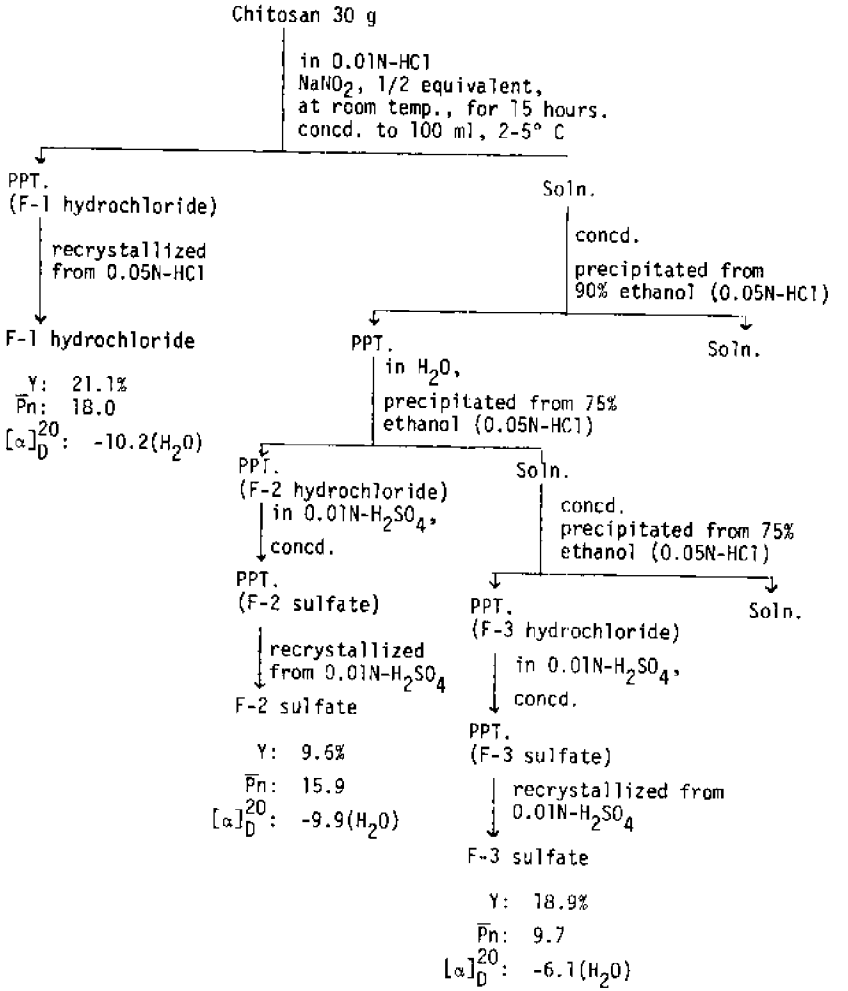

Fig. 5. Preparation of glucosamine oligosaccharide

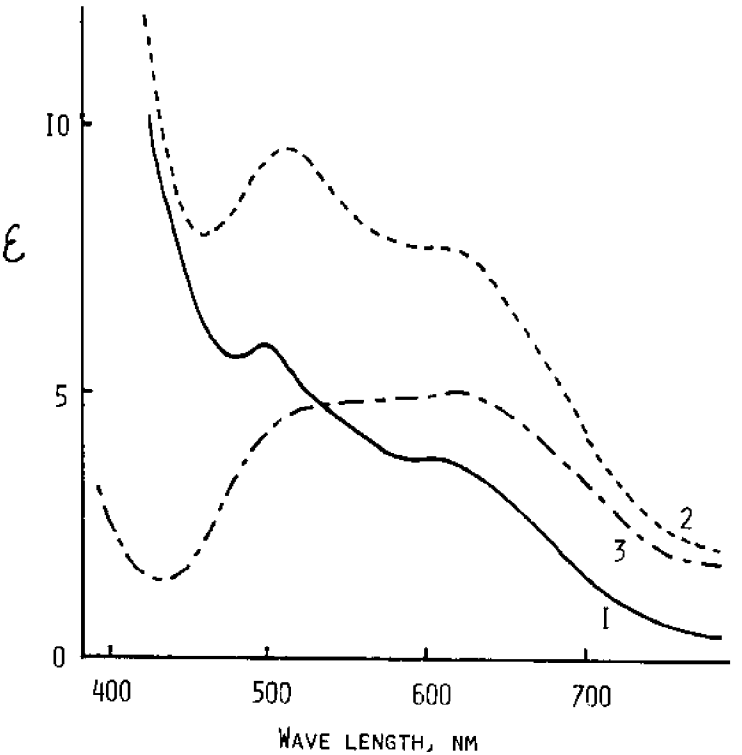

Fig. 6. Absorption spectra of glucosamine oligosaccharide Cu(II) complex.
1, [Cu(II)]/[Cu(II)]+[glucosamine unit]: 0.1.
2, [Cu(II)]/[Cu(II)]+[glucosamine unit]: 0.2.
3, [Cu(II)]/[Cu(II)]+[glucosamine unit]: 0.5.

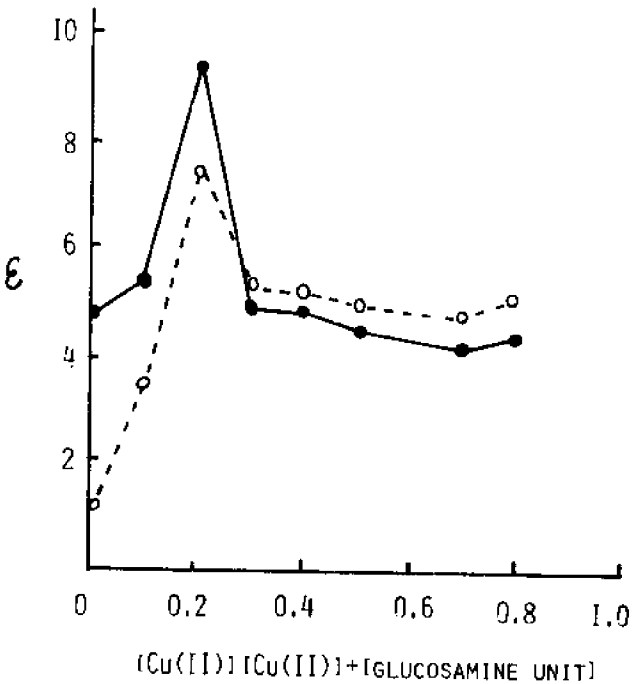

Fig.7. Determination of molar ratio in glucosamine oligosaccharide-Cu(II) complex.
o - o 620 nm. ●——● 510 nm.

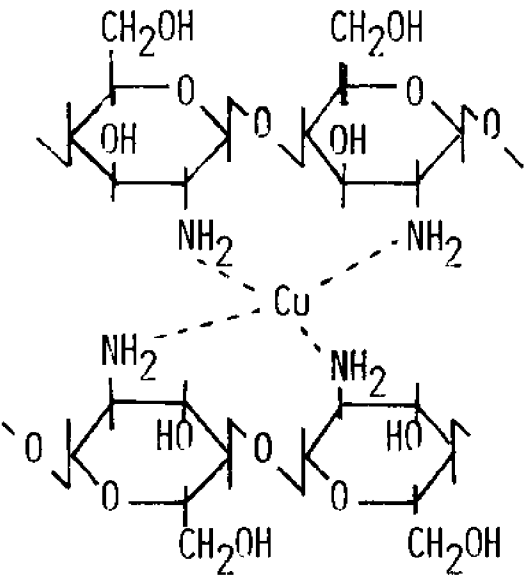

Figure 6. Screw dislocation formed by a chain end in a crystalline polymer.

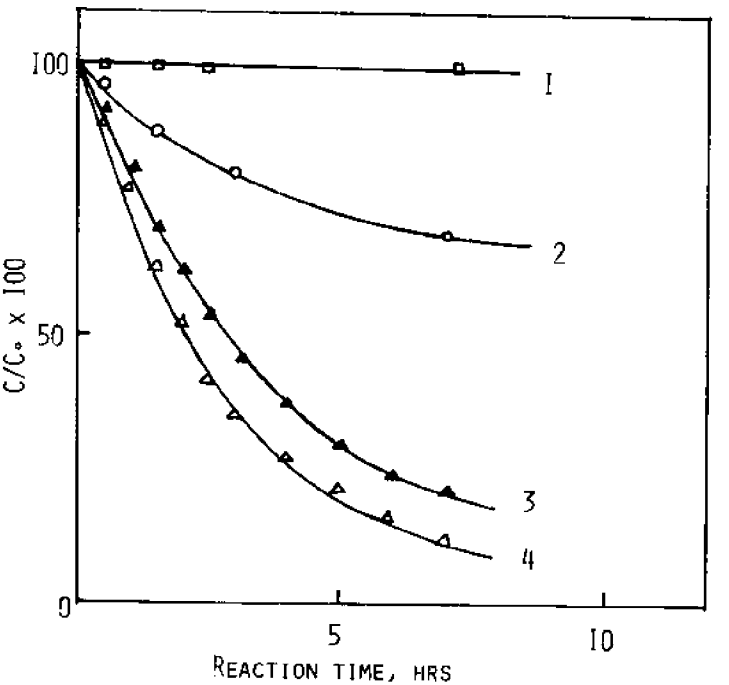

Fig. 9. Decomposition reaction of hydrogen peroxide.
1, Cu(II), pH 6.9. 2, Cu(II), pH 8.0.
3, Chitosan-Cu(II), pH 6.9. 4. Chitosan-Cu(II), pH 8.0.

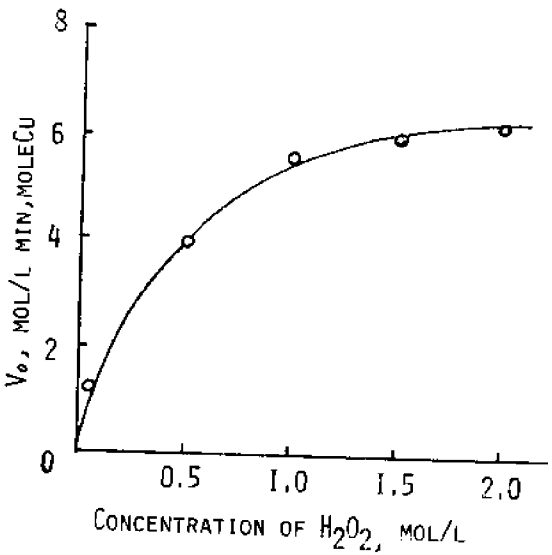

Fig. 10. Relationship of concentration of H_2O_2 with initial velocity of decomposition reaction of H_2O_2 using chitosan-Cu(II) catalyst.

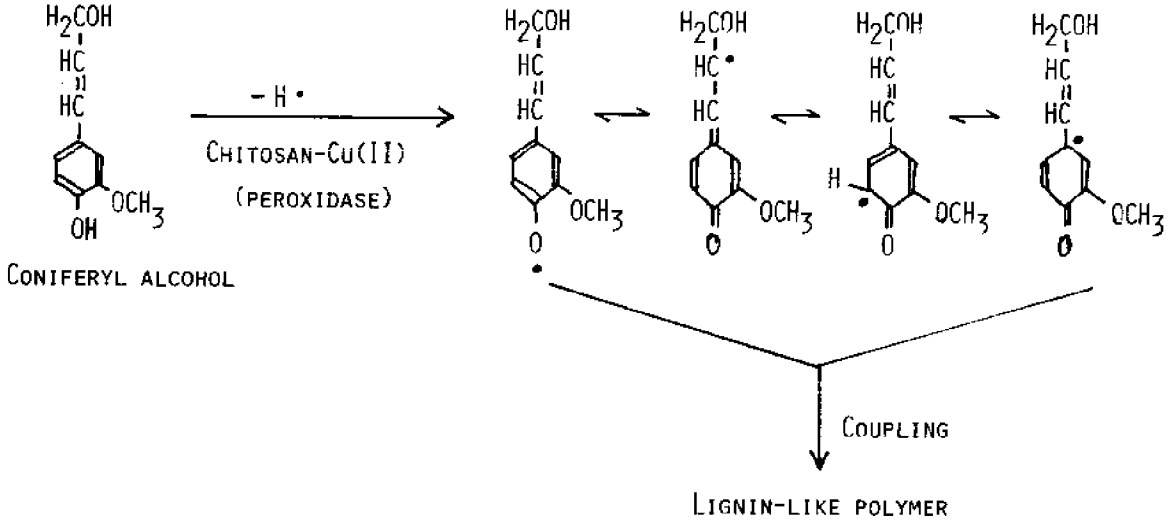

Fig. 11. Dehydrogenative polymerization of coniferyl alcohol

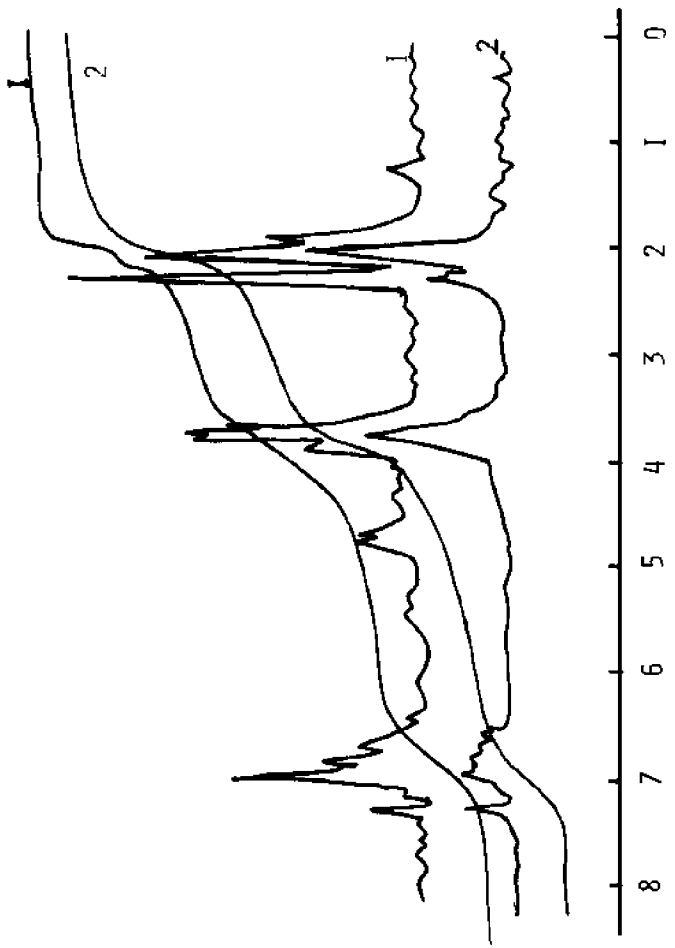

Fig. 12. Nmr spectra of polyconiferyl alcohol.
1: Polymer was obtained in vacuo.
2: Polymer was obtained in atmosphere.

CHARACTERIZATION OF CHITOSAN. II: THE DETERMINATION OF THE DEGREE OF ACETYLATION OF CHITOSAN AND CHITIN

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ABSTRACT

Several methods for the rapid determination of the degree of acetylation of chitosan have been evaluated. The most promising are the titration of chitosan hydrochloride and the evaluation of the mass spectra of chitosan and related polymers.

Chitosan hydrochloride was prepared by adding an excess of concentrated hydrochloric acid or sodium chloride to an acetic-acid solution of chitosan. The hydrochloride, which is soluble in water, exhibits the properties of a weak polyelectrolyte. The degree of acetylation can be determined by titration of the ammonium cation ($-\overset{+}{N}H_3$) in silver nitrate with a base or chloride ion. The measurement of the specific conductivity of aqueous solutions of chitosan hydrochloride is another method for determining the ionic concentration of the hydrochloride.

Preliminary studies of the mass spectra of chitosan hydrochloride indicate that the degradation temperature and the fragmentation patterns are characteristic of the polymer and that it will be possible to determine the degree of acetylation of chitosan or deacetylation of chitin from the mass spectra. The mass spectra of three commercial samples of chitosan were found to be distinctly different, indicating that the mass spectrometer may be a convenient tool for monitoring production procedures and the properties of the polymer.

INTRODUCTION

The properties of chitosan are related to the average molecular weight and molecular-weight distribution of the polymer, i.e., to the extent of deacetylation and chain degradation that occur during the alkaline hydrolysis of chitin and these, in turn, depend upon the hydrolysis conditions: concentration of alkali, reaction temperature, reaction time, and the presence or absence of oxygen. If oxygen is present, degradation and oxidation proceed by a free radical mechanism similar to that reported for cellulose (1).

Direct evidence for the variable composition of chitosan is the variation in color and solubility of chitosan from batch to batch. The viscosity of aqueous acetic-acid solutions of chitosan (4) and the coagulative powers of chitosan solutions (5; Wu and Bough, these Proceedings) have been reported to vary with composition and hydrolysis conditions.

A unique characteristic of chitosan is that it is insoluble in water and common organic solvents, but soluble in acid solutions. Chitosan dissolves slowly with degradation in warm solutions of hydrochloric, perchloric and nitric acids. It forms precipitates with dilute solutions of polyanionic acids such as sulfuric and phosphoric acids and also with concentrated hydrochloric acid (2). Chitosan is soluble in the aqueous solutions of a number of organic acids. Muzzarelli (3) has reported that chitosan is soluble in aqueous solutions of formic, acetic, citric, pyruvic and lactic acids, as well as in glycolic, maleic, malic, malonic, tartaric and many other acids. We have also reported that chitosan dissolves in oxalic and dichloroacetic acid solutions with the formation of thermoreversible gels (these Proceedings).

The variations in the extent of deacetylation and degradation that accompany the hydrolysis of chitin, combined with the solubility restriction, have made characterization of the polymer difficult and have hampered the study of the properties and reactions of chitosan. The objective of this study was to develop simple and rapid methods for determining the degree of acetylation of chitosan. Chitosan hydrochloride was selected as a suitable intermediate, as it is easy to purify, is soluble in water and was expected to exhibit the properties of a weak polyelectrolyte. The mass spectra of several polymers were taken and examined to determine if the fragmentation patterns would aid in the characterization of chitosan and related polymers.

In this paper, we report the determination of free amino groups by potentiometric titration and argentimetric titration of chitosan hydrochloride, the conductance of chitosan hydrochloride solutions and the results of preliminary mass spectra obtained from the degradation of chitin, chitosan and chitosan hydrochloride.

EXPERIMENTAL

Materials

Commercial chitin and chitosan were purchased from the Kypro Company of Seattle which has incorporated the Food, Chemicals and Research Laboratory. Chitosan was prepared in the laboratory from chitin purchased from the Kypro Company by refluxing 50g samples of chitin in 2.4 l of 40% sodium hydroxide for six hours.

Chitosan hydrochloride

Chitosan (approximately 2.5g) was dissolved in 10% acetic acid (85 ml) by stirring at high speeds for about 15 minutes. Undissolved

particles were removed by suction filtration using polyester cloth as the filter. The filtration time varied between 5 and 10 minutes, depending on the viscosity of the solution. Concentrated hydrochloric acid (~18 ml) was added slowly to the chitosan with rapid stirring until no further precipitation of chitosan hydrochloride was observed. The temperature rose 6° C during the addition of the acid. The precipitate was filtered through a very coarse sintered glass frit. The solid was made into a slurry with 100 ml of methanol and refiltered. This operation was repeated until the washings were free of chloride ion. A total of 5 or 6 washings was required to remove the excess hydrochloric acid. Filtration of the methanolic slurry required 5-10 minutes, much less than the 45 minutes needed to filter the viscous acetic acid slurry.

The off-white chitosan hydrochloride was dried in an oven at 50° C overnight and then placed in a constant-humidity chamber at room temperature to ensure that all the chitosan hydrochloride would attain the same water content before samples were weighed for titration, a precaution made necessary by the hygroscopic nature of the hydrochloride.

The dry chitosan hydrochloride is light brown in color, how light depending on the particular chitosan used and the temperature at which it is dried. The hydrochloride decomposes when air dried at 125° C.

Chitosan hydrochloride may also be made by adding a saturated solution of sodium chloride to the chitosan acetic-acid solution until no further precipitation is observed. Chitosan forms the hydrobromide, or hydroiodide, when sodium bromide, or sodium iodide, is added to acetic-acid solutions of chitosan. Both salts are water soluble, and chitosan can be reprecipitated by addition of sodium hydroxide. Both salts turn dark brown on standing.

Titration of chitosan hydrochloride

(a) Titration with sodium hydroxide

About 1.200 g of the dried chitosan hydrochloride was dissolved in water by shaking for about 0.5 hour and the solution was diluted to 100 ml. Two 25 ml aliquots of this solution were titrated against 0.1000 M sodium hydroxide, with the use of a Corning Model 5 pH meter. A third 25 ml aliquot was titrated with the same base using phenolphthalein as an indicator. A solution of glucosamine hydrochloride was titrated in a similar manner.

(b) Titration with silver nitrate

In order to compare the concentrations of $-\overset{+}{N}H_3$ and Cl^- ions, 4.1136 g of chitosan hydrochloride were dissolved in 250 ml of water. Three 25 ml aliquots of solution were titrated with 0.1000 M potassium hydroxide and three with 0.1000 M silver nitrate, using phenolphthelein and 2,7-dichlorofluorescein, respectively, as indicators.

Conductivity of chitosan hydrochloride solutions

The conductivity of chitosan hydrochlorides of seven different concentrations was measured at 25° C in a conductance cell constructed with a Beckman conductivity bridge ($K = 0.5516\ cm^{-1}$).

Mass spectra of chitin, chitosan and chitosan hydrochloride

A DuPont (model 21-491) double-focusing mass spectrometer was used to obtain the mass spectra of chitin, chitosan and chitosan hydrochloride at several temperatures and to measure the degradation temperature of each polymer. The samples were placed in a direct inlet probe and heated to a predetermined temperature. An ionizing voltage of 82V was used to ionize the vapors.

RESULTS

The aqueous solutions of chitosan hydrochloride behaved as expected and exhibited properties characteristic of quaternary ammonium-salt solutions. Titration curves for chitosan hydrochloride and glucosamine hydrochloride are shown in Figure 1.

The concentration of $-\overset{+}{N}H_3Cl^-$ groups per gram chitosan hydrochloride are given in Table 1.

Table 1. Moles NH_3^+ per Gram Chitosan Hydrochloride (x10-3)

Sample	Potentiometric	Indicator
K-1[a]	3.833	3.816
K-2	3.816	3.800
K-3	3.860	-----[c]
L-1[b]	4.026	3.983
L-2	4.133	4.050
L-3	3.866	3.833

[a] K samples were purchased from Kypro Co., Seattle.

[b] L samples were prepared in the laboratory.

[c] Poor end point because of high viscosity of solution.

The agreement between the chitosan concentrations, expressed as moles $-\overset{+}{N}H_3$ determined by potentiometric and indicator titrations is satisfactory. Chitosan hydrochloride solutions of low viscosity must be prepared in the indicator method to ensure sharp end points. The extent of deacetylation of chitin (or the acetyl groups remaining in chitosan) can be estimated from the moles $-\overset{+}{N}H_3/g$, by assuming a value for the "equivalent weight" of the repeat unit in the chitosan polymer.

The argentimetric titration of chitosan hydrochloride was evaluated as another simple procedure for determining $-\overset{+}{N}H_3$ and Cl^-. The results of the titration of 25 ml aliquots containing 0.4114 g chitosan hydrochloride were:

a) KOH titration with phenolpthalein indicator
4.067×10^{-3} mole $-\overset{+}{N}H_3/g$ chitosan.HCl

b) $AgNO_3$ titration with 2,7-dichlorofluorescein indicator
4.033×10^{-3} mole Cl^-/g chitosan.HCl.

The agreement between these titration values indicates that the acidic properties observed are entirely due to the $-\overset{+}{N}H_3$ functional groups present in the chitosan hydrochloride. Potassium dichromate is not a suitable indicator for this titration, as the chromate anion forms an insoluble complex with chitosan and gives an indefinite end point.

The specific conductivities of seven chitosan hydrochloride solutions are given in Table 2.

Table 2. Specific Conductivities of Chitosan Hydrochloride

Solutions	
Chitosan Hydrochloride (g/l)	Specific Conductivity (k)
6.8770	3570
3.4385	1902
1.7193	1022
0.4298	327.3
0.2149	193.5
0.1075	116.4
0.0537	67.0

A plot of concentration of chitosan hydrochloride expressed as g/l vs. specific conductivity, is linear over a wide range of concentrations, while a plot of k/c vs. $\sqrt{c}$ (where c is concentration in g/l) gives a curve, a result typical of a weak electrolyte. Such plots may be useful in monitoring chitosan hydrochloride concentrations.

The mass spectra of chitin, chitosan and chitosan hydrochloride have been determined over a range of temperatures. Interpretation of these preliminary mass spectra of commercial chitosan samples is hampered by the lack of detailed information concerning the hydrolysis condition under which the chitosan samples were prepared. However, a cursory analysis of these spectra indicate that such spectra will provide information on polymer degradation temperature, polymer identification, the degree of deacetylation of chitin (or the degree of acetylation of chitosan) and, perhaps, the ratio of free amine groups to N-acetylamine groups in chitosan.

The degradation temperatures of the three polymers are listed in Table 3.

Table 3. Degradation Temperature of Chitin, Chitosan and Chitosan Hydrochloride

Polymer	Degradation Temperature(°C)
Chitin	~300
Chitosan, K-1	270-280
K-2	288-292
K-3	280
Chitosan. HCl	210-220

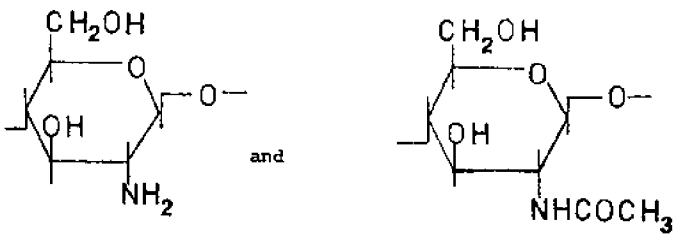
The thermal stabilities of the three polymers are significantly different, with chitin being the most stable. Chitosan hydrochloride is the least stable, presumably because the hydrogen chloride liberated in the probe at elevated temperatures attacks the β-glycosidic links between the monomer units, causing rapid depolymerization. This observation confirms the earlier observation that chitosan hydrochloride darkens rapidly when heated in an oven at 125°.

Mass spectra for three samples of commercial chitosan, commercial chitin and a sample of chitosan hydrochloride are depicted as bar graphs in Figures 2-6. In these spectra, all the peaks below mass 25, as well as 18 (H_2O), 28 (N_2), 32 (O_2), and 44 (CO_2), have been omitted, even though some of them may provide useful information. For example, the 28/32 ratios in most spectra were greater than 4, suggesting the presence of CO from the acetyl group in the 28 peak.

The mass spectra of each polymer are distinctive. Chitin (Figure 2) has a low volatility and exhibits a very simple fragmentation pattern until the degradation temperature is approached. When chitin degrades, approximately 200 mass ions appear. Chitosan hydrochloride also exhibits a simple fragmentation pattern (Figure 3) and, as noted earlier, the hydrogen chloride liberated promotes rapid depolymerization.

The mass spectra of the chitosan samples are much more complex than those of chitin. The chitosans gave relatively complex fragmentation patterns, even at relatively low temperatures. Although all three were marketed as chitosan and purchased from Kypro Company over a period of several years, the mass spectra of each are distinctive. Of particular interest is the high volatility of K-2 (Figure 6) compared to the other samples (Figures 4 and 5). Sample K-3 is the least volatile of the three, as attested by the simple spectrum at 135°C (Figure 5). The fragmentation pattern for K-2 is much more complex at 175°C than the spectra for K-1 and K-3 at temperatures above 200° C. In fact, the spectra for K-2 extended to masses above 200. Without knowing the history of these samples, one can only speculate as to the reason for the high volatility of K-2 compared to the other two. Variable amounts of a mass ion at 36, attributed to HCl, were found in these samples.

The fragments observed are derived from the monomer units



Clusters of ions can be observed in the mass spectra. Fragments to which some of these ions can be attributed are listed in Table 4. The peaks attributed to $-COCH_3$ (mass 43) and $-NHCOCH_3$ (mass 60) can be used to estimate the degree of acetylation, and it may be possible to determine the $-NH_2/-NHCOCH_3$ ratio. The ability to determine the degree of acetylation and the $-NH_2/-NHCOCH_3$ ratio could be used to monitor the effect of hydrolysis conditions and could be indicators of the chemical properties of chitosan.

Table 4. Mass Ions and Associated Ion Fragments

Peak	Fragment
29	$>CHNH_2$
31	$-CH_2OH$
36	HCl
43	$-COCH_3$
55	$>CHCHCH$ $\quad\quad NH_2$
59	$-CHOHCHNH_2$
60	$-NHCOCH_3$
71	$>CHNHCOCH_3$
72	$-CHOHCHNH_2CH$
84	$>CHCHNHCOCH_3$

CONCLUSIONS

Chitosan hydrochloride exhibits the properties expected of a weak polyelectrolyte. The number of moles of the $-NH_2$ functional groups per unit weight can be determined by titration of $-\overset{+}{N}H_3$ with a standard base or Cl^- in standard silver nitrate. This data can be related to the extent of acetylation by assuming an "equivalent weight" for the monomer repeat unit. The measurement of specific conductivity is another method that can be used for the rapid determination of chitosan hydrochloride.

Mass spectra provide information on degradation temperatures and polymer identification and, with further work, will permit the determination of the degree of acetylation in a polymer and possibly the $-NH_2/-NHCOCH_3$ ratio. The mass spectra of three commercial chitosans exhibit significant differences. Mass fragmentation patterns of chitosan and chitin may prove to be a convenient and precise method for the investigation of the influence of hydrolysis conditions on solubility, viscosity and coagulative properties.

SUGGESTIONS FOR FUTURE WORK

The ability to determine the degree of acetylation and the -NH2/ $-NHCOCH_3$ ratio in chitosan would provide other useful tools for the study of the preparation, the properties and the reactions of this interesting polymer. Accordingly, we plan to continue development of rapid and simple procedures for the determination of these parameters either from mass spectra alone or from some combination of mass spectra with other methods, such as hydrolysis of the hydrochloride and determination of total nitrogen.

ACKNOWLEDGMENTS

We wish to thank the Fisheries and Marine Services of Environment Canada for an operating grant, Dr. Clive Elson for assistance with the mass spectra studies and Mr. Ross Kean and Mr. Jairajh Mattai for assistance with the experimental work.

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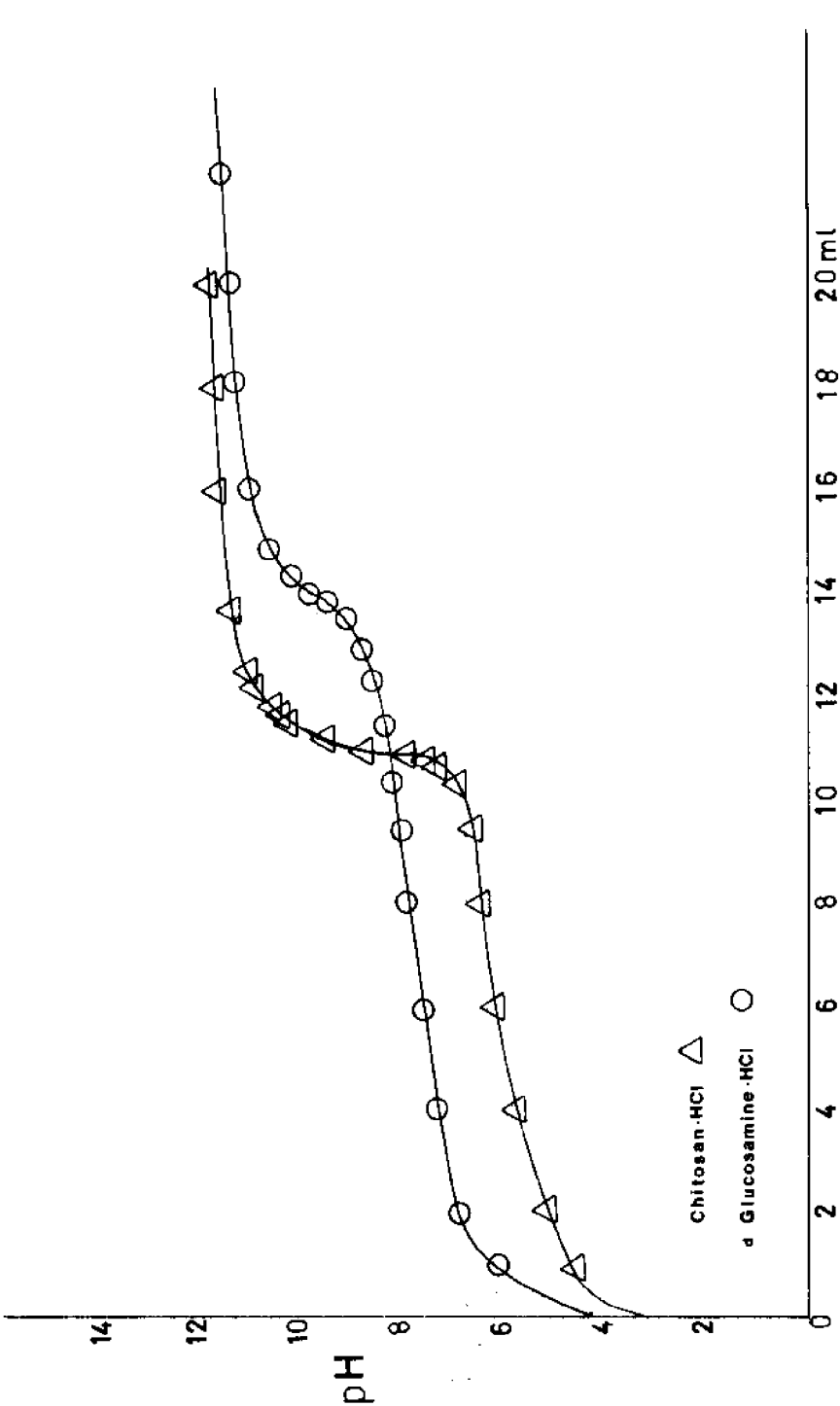

Fig.1 Titration Curve for Chitosan·HCl and d-Glucosamine·HCl

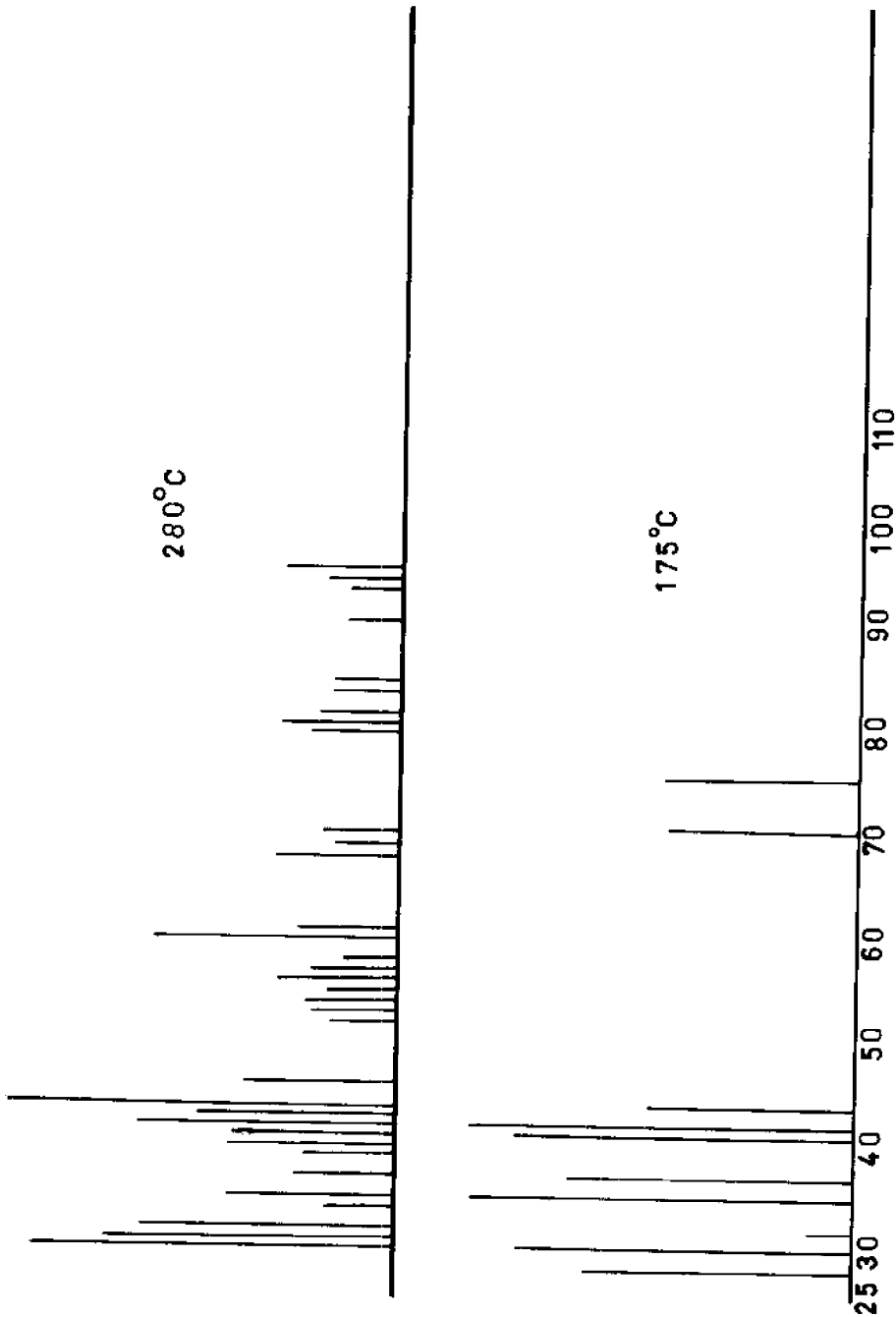

Fig.2 Mass Spectrum of Chitin

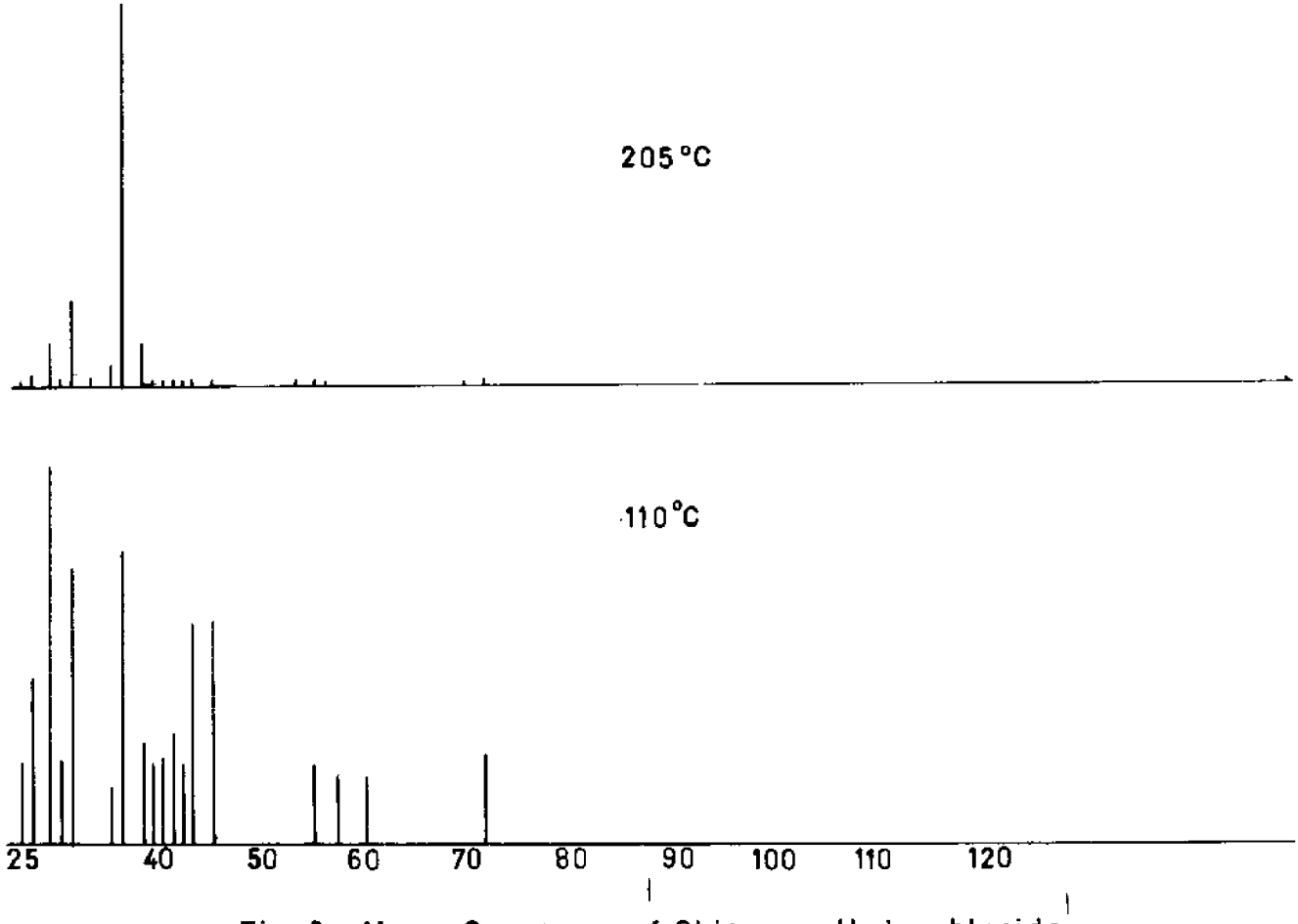

Fig. 3 Mass Spectrum of Chitosan Hydrochloride

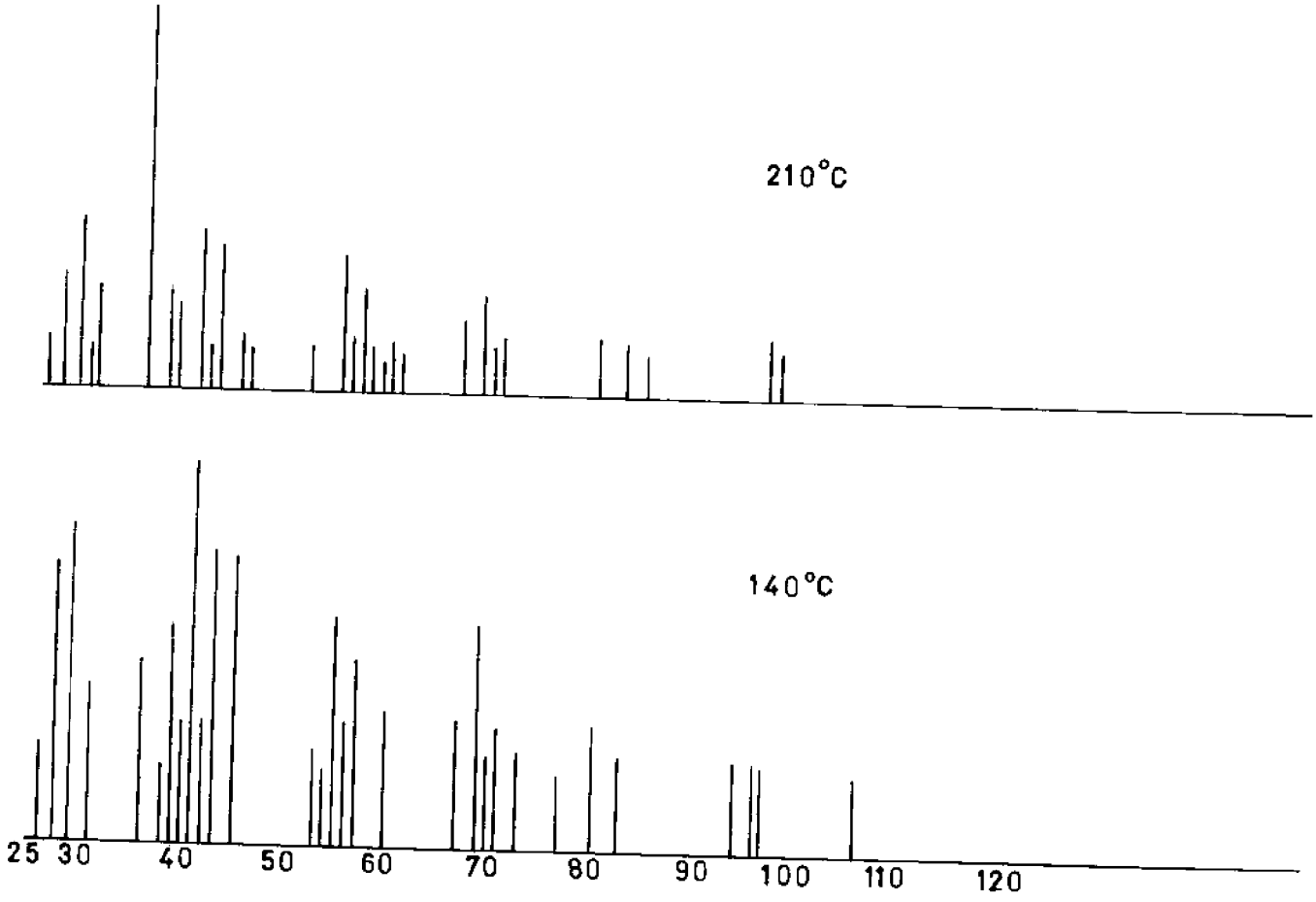

Fig. 4 Mass Spectrum of Chitosan, K-1

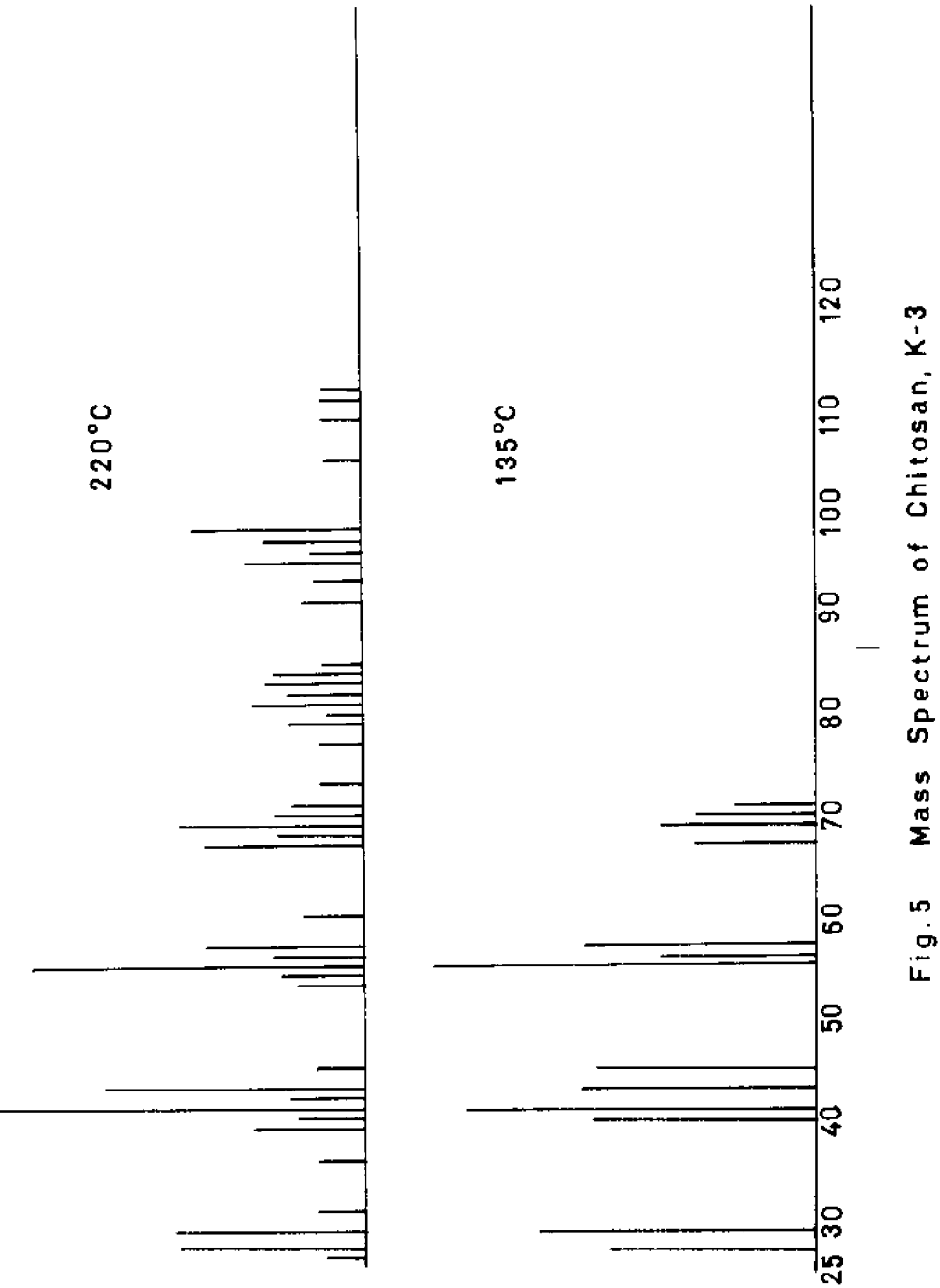

Fig.5 Mass Spectrum of Chitosan, K-3

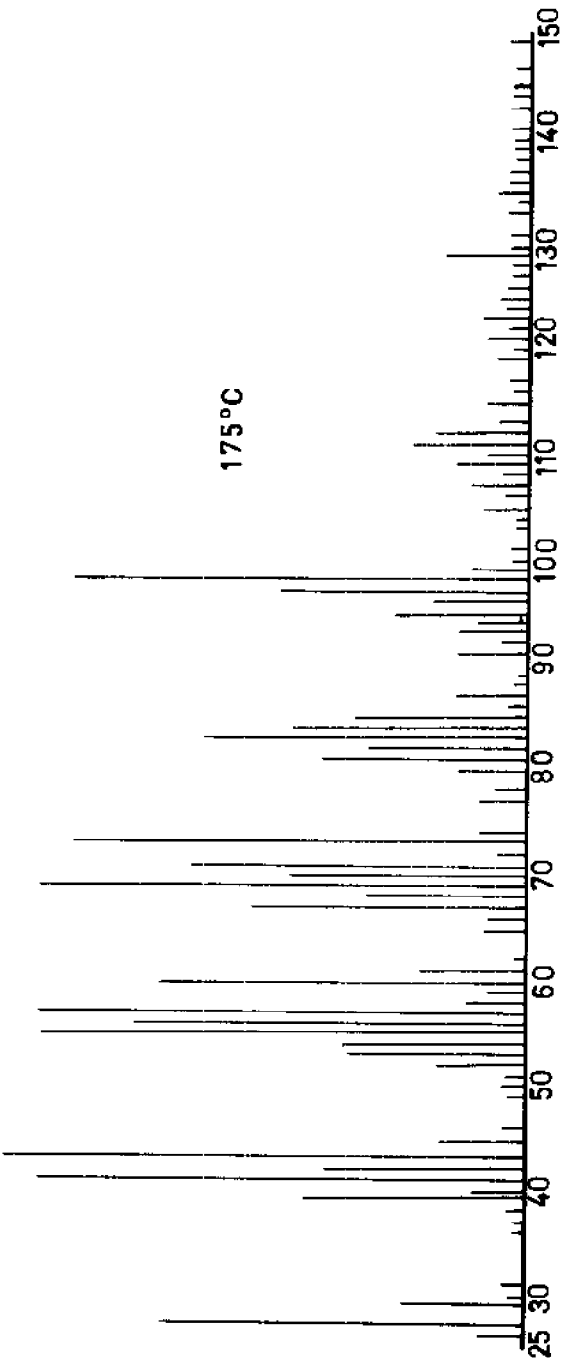

Fig.6 Mass Spectrum of Chitosan, K-2

STUDIES ON THE ACETYLATION OF CHITOSAN

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ABSTRACT

A study of the acetylation of chitosan film has been carried out using IR spectroscopy to follow the reaction. Acetylation techniques similar to those used for cellulose proved ineffective, but a facile technique for selective N-acylation has been developed, involving treatment with carboxylic-acid anyhydride/methanol solutions at room temperature. A wide range of other solvents has been examined, but methanol has been found to be by far the most effective.

The N-acyl chitosans so far produced have been O-acetylated in both acidic and basic media, and the rates of O-acetylation were found to be dependent upon the size of the N-acyl group. The N-acetyl and the N-propionyl chitosans undergo O-acetylation extremely slowly, while N-butyryl and N-hexanoyl chitosans react at greatly increased rates. This strongly supports the suggestion that the difficulty in acetylating chitosan and chitin is due to the inaccessibility of the polymer chains.

A new method for measuring the extent of deacetylation of chitosan samples is reported.

INTRODUCTION

The acetylation of chitin and chitosan is extremely difficult, despite their similarity to cellulose. Several attempts have been reported in the literature using extreme conditions such as acetic anyhdride at elevated temperatures (9) or acetic anhydride with dry hydrochloric acid as catalyst (10). Selective N-acylation of chitosan under mild conditions in solution has recently been reported (3-7), using acid anhydride/acetic acid (10%)/methanol mixtures. In the present work, the acetylation of chitosan has been studied in an attempt to develop a simple procedure for the preparation of fully acylated derivatives. Chitosan in film form has been used throughout because of the ease of handling and of following the extent of reaction by IR spectroscopy.

EXPERIMENTAL

The chitosan used in the initial studies was supplied by the U.S. National Oceanic and Atmospheric Administration and that used in the later work was supplied by Hercules Incorporated. The extent of deacetylation of the samples was determined in two ways:

(1) by treatment with excess aqueous hydrochloric acid and titrimetric determination of the excess acid (2);

(2) by treatment with a solution of sodium periodate to cleave the α-aminoalcohol units and determination of the unconsumed periodate by titration with sodium arsenite.

The average values for the extent of deacetylation were 72% by method 1 and 77% by method 2.

The films were prepared by dissolving chitosan flakes in aqueous acetic acid (5%) and casting onto a glass plate. After drying, the films were removed from the plate, steeped overnight in methanolic sodium hydroxide (5%) to convert the amine salt to the free amine, rinsed in methanol and air dried. The IR spectrum of each sample was recorded prior to any subsequent treatment.

The acylation reactions were carried out in stoppered flasks at room temperature, except for the studies on the rates of acylation which were carried out at 25°C, 35°C and 45°C, using a thermostated water bath. The reagents used were General Purpose Reagent grade, with the exception of methanol which was Analar grade. The IR spectra were recorded on a Perkin Elmer 157 recording spectrophotometer.

RESULTS AND DISCUSSION

Initial attempts to acetylate the chitosan films involved treatment with acetic anhydride/glacial acetic acid mixtures, together with perchloric acid (82%) as a catalyst. This is a recognized method for the acetylation of cellulose (11), and it was thought that chitosan might react in a similar manner. The extent of reaction was very limited, however, even when the reaction time was extended beyond 120 hours. Two possible explanations for this lack of reactivity are:

(1) limited accessibility of the functional groups, due to the close packing of the chains, restricts the extent of reaction;

(2) protonation of the amine groups, due to the acidic nature of the reaction medium, causes a reduction in the extent of their acetylation and in the extent of O-acetylation, through destabilization of the transition state structures required for the acetylation of the adjacent C(3) and C(6') hydroxyl groups. The influence of the C(2) amine salt on the adjacent hydroxyl groups has been shown by Horton et al. (8), who reported specific oxidation of the primary hydroxyl groups of chitosan when the counter-ion of the protonated amine groups was the perchlorate ion.

Attempts to swell the film using various water/methanol mixtures were impracticable, as the film became unworkable. Treatment with acetic anhydride at room temperature for 120 hours followed by refluxing in acetic anhydride for 2 hours gave good N-acetylation together with some O-acetylation. Extending the reaction times gave no noticeable increase in the extent of acetylation, nor did the use of triethylamine as an acid scavenger. From other studies on chitosan, to be reported later, it became clear that the use of methanol as the reaction medium gave an increase in the reactivity of chitosan. Initial experiments using methanol/acetic anhydride mixtures showed that complete N-acetylation could be obtained in less than 12 hours at room temperature. Some O-acetylation also occurred with increased reaction times, but it was not complete even after prolonged treatment.

A range of organic solvents was then examined to determine their

influence on the N-acetylation of chitosan. The solvents studied included alcohols up to hexanol, glacial acetic acid, dimethylformamide, formamide, pyridine, dimethylsulfoxide, tetrahydrofuran, dioxan and chlorinated hydrocarbons. The range of the solubility parameter values covered by the solvents examined was 7.4-19.2 Hildebrands (7.4-19.2 cal$^{\frac{1}{2}}$ cm$^{-\frac{3}{2}}$). It was found that only methanol and formamide gave any appreciable N-acetylation, even after 24 hours reaction time. A series of binary mixtures of ethanol/methanol and of methanol/formamide (δ = 19.2) was prepared, and the extent of reaction determined after 30- and 60-minute treatment. The extent of acetylation was calculated by measuring the increase in the absorbance of the amide I band at 1660 cm^{-1}, using the absorbance of the hydroxyl band at 3450 cm^{-1} as an internal standard to take into account variations in film thickness.

Figure 1 is a plot of the extent of N-acetylation after 30- and 60-minute reaction times, expressed as the percentage of amine groups acetylated, against the solubility parameter values of the reaction media. The latter values have been calculated to take into account the contribution of the acetic anhydride present. The figure shows that there is a very considerable increase in the rate of N-acetylation in the reaction media that have solubility parameter values in the range $12.75<\delta<14.75$, and that it reaches a maximum at $13.1<\delta<13.5$.

Apart from changes in their intensity, the majority of the absorption bands in the IR spectrum of chitosan are unaffected by the increasing extent of N-acetylation. A major exception is the amide II band at 1595 cm^{-1}, which shifts to 1550 cm^{-1} as N-acetylation proceeds. The presence of hydrogen bonding in simple secondary amides has been found (1) to increase the absorption frequency of the amide II band, and this shift to lower frequencies as N-acetylation proceeds indicates gradual disruption of hydrogen bonding involving the amide group that was present in the original chitosan film.

N-acylation of chitosan

A comparison of the rates of N-acylation of chitosan by several carboxylic-acid anhydrides has been made, using methanol as the reaction medium. The acid anhydrides used were acetic, propionic, butyric, hexanoic and benzoic. The reactions were followed by monitoring the change with time of the absorbance of the amide I band until acylation was complete. The absorbance of the 3450 cm^{-1} band was again used as an internal standard. In all cases, a shift in the amide II band to the lower frequencies on N-acylation was observed.

The results for the reaction of butyric anhydride are shown in Figure 2. The extent of N-butyrylation is expressed as A_t-A_o, where A_t and A_o are the absorbances of the amide I band at time t and at time t = 0 respectively, both values being corrected for film thickness. Figure 2 shows evidence of an induction period for the reaction, which is particularly noticeable in the curve obtained at 25°C. In general, the induction period increased with an increase in the size of the acid-anhydride molecule and decreased with an increase in the temperature. The induction period could be completely eliminated by pretreatment of the film sample in methanol for several hours at the temperature at which acylation is to be carried out, which shows that the induction period is due to the time required for the methanol to swell the film before diffusion of the anhydride into the chitosan can take place. All the aliphatic-acid anhydrides

required approximately similar reaction times for complete N-acylation, on the order of 10-12 hours, but benzoic anhydride was considerably slower, and reaction times in excess of 72 hours were required for full N-benzoylation at 25°C.

Approximate values for the Arrhenius energies of activation have been calculated from the measured rates at 25°C, 35°C and 45°C. These are in the order of 95-90 $kJmol^{-1}$ for acetic, propionic and butyric anhydrides, and about 75 $kJmol^{-1}$ for hexanoic anhydride. The value for benzoylation was not determined. Although the values for acetic, propionic and butyric are close together, they do form, together with that for hexanoic anhydride, a series in which the energy of activation decreases with an increase in size of the anhydride molecule.

O-acetylation of N-acyl chitosans

A series of films of N-acyl chitosans were prepared at room temperature using methanolic solutions of acetic, propanoic, butyric and hexanoic anhydrides. In addition, N-benzoyl chitosan was prepared by refluxing in a methanolic solution of benzoic anhydride. After the N-acylation was complete, the films were steeped overnight in alcoholic potassium hydroxide (5%) to remove any O-acyl groups (3-7). The films were then acetylated for 1 hour at room temperature in either acetic anhydride/glacial acetic acid/perchloric acid, or acetic anhydride/pyridine. The extent of O-acetylation was measured using the absorbance of the carbonyl band at 1740 cm^{-1} and the 3450 cm^{-1} band as an internal standard.

The values of A_{1740} (corrected for film thickness) are plotted against the calculated lengths of the respective N-acyl groups in Figure 3. This shows that in both acetylation media the extent of reaction of N-acetyl chitosan is very small and that there is only a slight increase in the extent of O-acetylation when the substrate is changed to N-propionyl chitosan. However, there is a 12- to 15-fold increase on changing the substrate to N-butyryl chitosan, followed by a further small increase with N-hexanoyl chitosan. These results show that the spacing between the polymer chains is the controlling factor in the O-acetylation of chitosan and that there is a critical separation between chains below which O-acetylation does not occur to any appreciable extent. Further separation of the chains above this critical distance has only a small effect upon the ease of acetylation. Although the N-benzoyl group is intermediate in length between the N-butyryl and the N-hexanoyl groups, its influence on the ease of O-acetylation is less than that of either of these, but greater than that of the N-propionyl group. This may be due to the planar nature of the benzene ring, which means that the N-benzoyl group will only separate the polymer chains in one dimension, while the aliphatic acyl groups will cause disruption in two dimensions.

CONCLUSIONS

The work reported here demonstrates that the major obstacle to the ready acetylation of chitosan and chitin is one of restricted accessibility of the polymer chains. Although protonation of the amine groups of chitosan would be expected to reduce the extent of N-acetylation, it would appear to have little effect on the extent of O-acetylation, as evidenced by the similar difficulty in O-acetylating chitosan film both before and after N-acetylation.

Facile, selective N-acylation of chitosan films can be carried out using carboxylic-acid anhydride/methanol mixtures. When the reaction is carried out on untreated chitosan films, there is an induction period, but this can be removed by presteeping the films in methanol. The experimental activation energies for N-acylation decrease with an increase in the size of the anhydride molecule.

The ease of O-acetylation of N-acyl chitosans increases with an increase in the size of the N-acyl group, with a dramatic increase in going from the N-propionyl to the N-butyryl chitosan. Further increase in the size of the N-acyl group has only a small effect on the ease of O-acetylation.

The results of this work are being used to produce a variety of organosoluble derivatives of chitosan for characterization and study.

ACKNOWLEDGMENTS

The authors thank the U.S. National Oceanic and Atmospheric Administration and Hercules Incorporated for gifts of chitosan used in this study, and Mrs. F. A. Wood for assistance in recording the IR spectra. G. K. M. thanks the Science Research Council for financial support.

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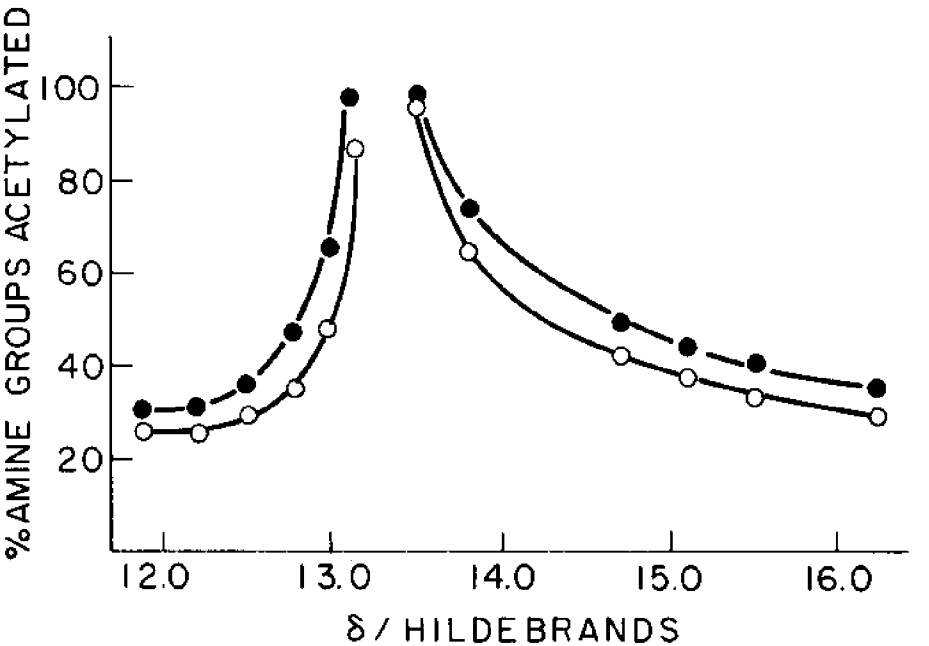

Figure 1. Plot of the percentage of the amine groups acetylated versus the solubility parameter value of the reaction medium; O 30 minutes reaction time; ● 60 minutes reaction time.

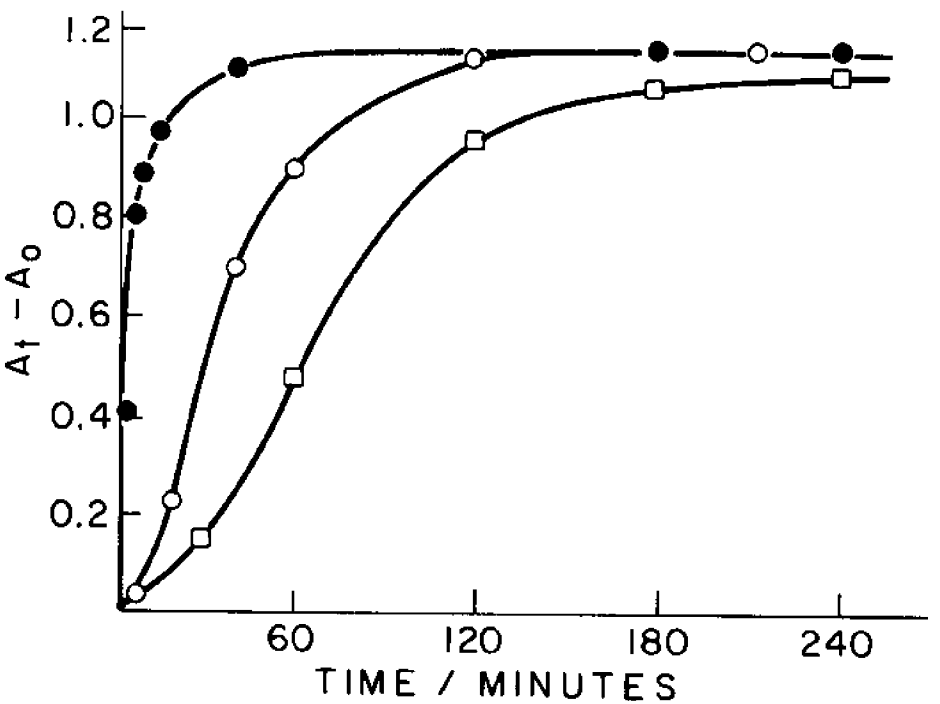

Figure 2. Plot of $A_t - A$ versus time for N-butyrylation of chitosan film; □ 25°C; O 35°C; ● 45°C.

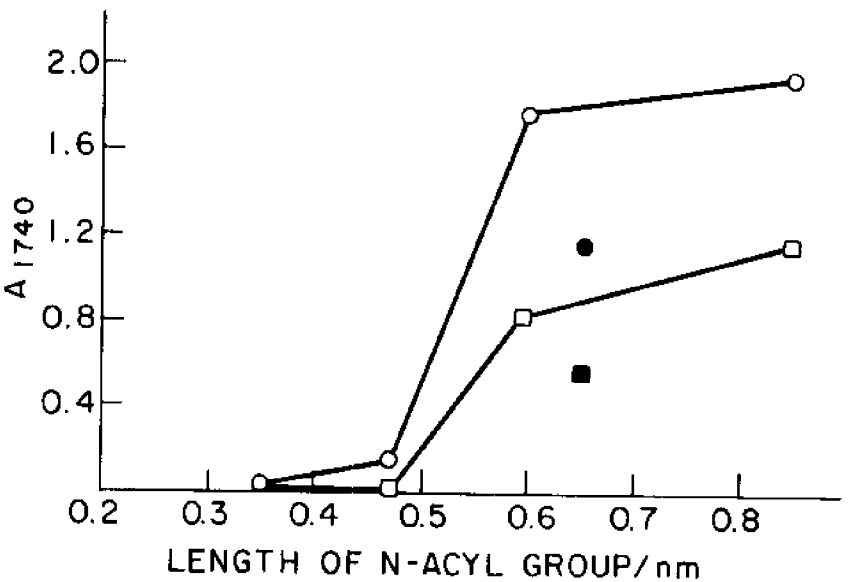

Figure 3. Plot of the extent of O-acetylation after 1 hour reaction time versus calculated chain length of the N-acyl group for various N-acyl chitosans; ○ acetic anhydride/glacial acetic acid/perchloric acid; □ acetic anhydride/pyridine; ● N-benzoyl chitosan in acetic anhydride/glacial acetic acid/perchloric acid; ■ N-benzoyl chitosan in acetic anhydride/pyridine.

CHITIN FROM MARINE ORGANISMS AND ITS USE AS AN ADHESIVE

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ABSTRACT

Chitin from the polyzoan Scrupocellaria bertholetti gave a greenish brown color reaction with the typical chitosan test, and when the alkali treatment was prolonged for an hour, a violet color reaction. Sugar chromatography disclosed that the chitin contained, in addition to glucosamine, fucose, which seemed to be responsible for the atypical color reaction.

Purified chitin from the coelenterates Tubularia sp., Physalia sp., Velella sp., Porpita sp. and Millipora sp. contained glucose, galactose and mannose in association with glucosamine. The coenocium of the pterobranch Rhabdopleura sp. yielded identical results as that of S. bertholetti.

Chitin from these marine organisms could be used to prepare good-quality adhesive. The alkali-purified chitin was completely soluble in 70% sulfuric acid at room temperature to give a brown solution. After neutralizing with 17% alkali, a xanthate was prepared by stirring the solution with carbon disulfide. The xanthate was a golden brown viscous fluid which served as a strong adhesive for decorative and commercial plywood, hard board and furniture joints. The concentrated solution could be spread to prepare films or sheets of various thicknesses, or it could be drawn into fine fibers. These became brownish, hard and vitreous upon drying. The paste that formed when highly concentrated could be used as a molding compound.

INTRODUCTION

The chitin from the cuticle of Polyzoa is of special interest. While Richards and Cutkomp (6) reported that it gave a greenish brown color reaction to the chitosan test, Hyman (3) reported that it gave a typical violet color reaction. This difference is still to be explained. No x-ray diffraction study has yet been done on the chitin from this aberrant group.

In Coelenterata, chitin occurs in the periderm of hydrozoans, in the pneumatophore of Siphonophora and the coenosteum of Millipora. Rudall (8) observed that none of the coelenterate specimens studied by him gave the highly crystalline x-ray diffraction pattern that could be obtained from purified arthropod chitin. He suggested that coelenterate chitin may have a second principal sugar component, but little is known of this secondary constituent.

The present investigation attempts to throw some light on these problems. Incidentially, it was found that an excellent quality of adhesive can be prepared from the chitin of these marine organisms.

EXPERIMENTAL

The marine organisms used in this study are listed in Table 1. Materials fixed in 5% formalin (in sea water) were decalcified with aqueous EDTA at pH 3 (2). The decalcified material was boiled in 5% KOH solution for 24 hrs, followed by prolonged washing in cold water (9). The resultant material was used for all further studies.

The chitosan test and its variants were performed according to Krishnan and Sundara Rajulu (4). Nitrogen was estimated by the microkjeldahl method of Steyermark (9). Hydrolysate of the material was prepared in sealed vessels with 6N HCl at 100° C for 12 hrs, unless otherwise stated. After drying the hydrolysate over P_2O_5 and KOH, the residue was taken up in water and run on paper-partition chromatograms, using 8 different solvent mixtures (Table 2). The chromatograms were sprayed with aniline hydrogen phthalate, silver nitrate, or Elson and Morgan reagents (7). The relationships of glucosamine with the other sugars was studied, using Dowex 50-X8- H+ columns (1).

The x-ray photographs were taken in a cylindrical camera after drying the material *in vacuo* over phosphorous pentoxide.

RESULTS

Chitosan test

For a typical chitosan test involving treatment with saturated alkali for 30 min at 160° C, the chitin from *S. bertholetti* gave a greenish brown color reaction; but when the alkali treatment was prolonged for more than 50 min, it turned to violet. Similar reactions were obtained by the coenocium of *Rhabdopleura* sp. and by the materials from all the five species of coelenterates studied.

Nitrogen content

The nitrogen content of the chitin from the materials studied is given in Table 1. The values range from 4.2% to 5.9%.

Table 1. Nitrogen Content of the Chitin from Some Different Species of Marine Organisms

Sl. No.	Name of Species			Nitrogen %
1	*Scrupocellaria bertholetti*	(Polyzoa)	Whole colony	5.9
2	*Rhabdopleura* sp.	(Pterobranchia)	Coenocium	5.7
3	*Tubularia* sp.	(Coelenterata)	Whole colony	5.3
4	*Physalia* sp.	(Coelenterata)	Pneumatophore	4.4
5	*Velella* sp.	(Coelenterata)	Pneumatophore	4.2
6	*Porpita* sp.	(Coelenterata)	Pneumatophore	4.6
7	*Millipora* sp.	(Coelenterata)	Coenosteum	4.3

Sugar analyses

Chromatographic analyses of the hydrolysate of the chitin from all the species studied revealed the presence of one or more neutral sugars in addition to glucosamine (Table 2). The neutral sugars are glucose, galactose, mannose and fucose.

Studies on cation-exchange columns

It was noted that the neutral sugars passed through the Dowex 50- H+ columns, while glucosamine was retained by the resin, when the material was hydrolyzed for 12 hrs. When the hydrolysis was only partial, after only 6 to 7 hrs, the neutral sugars were also retained by the cation-exchange resin and eluted along with the glucosamine in all cases.

X-ray diffraction studies

The x-ray diagrams obtained for the purified chitin from the cuticle of the polyzoan and the coenocium of the pterobranch are given in Figure 1a and 1b. It was seen that, in both instances, there are resemblances to that yielded by the β-chitin from the Loligo pen.

Chitosan sulfate crystals from α- and β-chitins

It is well known that chitosan prepared from chitin by treatment with hot saturated alkali is soluble in acetic acid and that a precipitate of chitosan sulfate is formed on the addition of dilute sulfuric acid. In the present study, these chitosan spherite crystals were examined under an ordinary light microscope. It was found that the chitosan sulfate crystals from the α- and β-chitins show distinct differences in their contour, as shown in Fig. 2.

PREPARATION OF ADHESIVE

An interesting observation from the commercial point of view is that the chitin from all these marine organisms can be used to prepare good-quality adhesive. The procedure is as follows:

The alkali-purified and washed chitin was completely soluble in 70% sulfuric acid at room temperature, giving a slightly brown liquid. After neutralizing with 17% sodium-hydroxide solution at room temperature, a xanthate was obtained by stirring the neutralized solution in CS_2. The xanthate was golden brown in color and gave a smooth solution.

The solution, in dilute condition, can serve as a strong adhesive for decorative and commercial plywood, hardboard and furniture joints. The concentrated solution can be spread to prepare films or sheets of various thicknesses, or it can be drawn into fine fibers; upon drying, these become brownish in color, hard and vitreous in character and almost impossible to grind. The paste that forms when highly concentrated can be used as a molding compound.

Table 2. Sugars Present in the Chitin from Some Marine Organisms

Sl. No.	Species	Rf values								Identity of Sugars
		1	2	3	4	5	6	7	8	
1	S. bertholetti	0.166	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.52	0.64	0.54	0.39	0.74	0.52	0.54	0.23	Fucose
2	Rhabdopleura	0.16	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.52	0.64	0.54	0.39	0.74	0.52	0.54	0.23	Fucose
3	Tubularia	0.16	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.38	0.53	0.46	0.31	0.66	0.46	0.39	0.16	Glucose
4	Physalia	0.16	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.38	0.53	0.46	0.31	0.66	0.46	0.39	0.16	Glucose
		0.35	0.49	0.43	0.22	0.62	0.44	0.34	0.14	Galactose
5	Velella	0.16	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.38	0.53	0.46	0.31	0.66	0.46	0.39	0.16	Glucose
		0.35	0.49	0.43	0.22	0.62	0.44	0.34	0.14	Galactose
6	Porpita	0.16	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.38	0.53	0.46	0.31	0.66	0.46	0.39	0.16	Glucose
		0.35	0.49	0.43	0.22	0.62	0.44	0.34	0.14	Galactose
7	Millipora	0.16	0.27	0.25	0.10	0.38	0.25	0.32	0.50	Glucosamine
		0.38	0.53	0.46	0.31	0.66	0.46	0.39	0.16	Glucose
		0.44	0.62	0.48	0.27	0.69	0.49	0.46	0.18	Mannose

Solvent mixtures:
1. Butanol, acetic-acid, water (4:1:5)
2. Butanol, acetone, water (2:7:1)
3. Butanol, pyridine, water (5:4:3)
4. Butanol, ethanol, water (4:1:1)
5. Isopropanal, pyridine, acetic-acid, water (8:8:4:1)
6. Phenol, water (8:2)
7. S-collidine (water-saturated)
8. Isobutyric acid

DISCUSSION

The results reported in the foregoing study reveal that the greenish brown chitosan color reaction is not an anomaly, but a definite phenomenon, for which the presence of sugars other than glucosamine seems to be responsible. This could be inferred from the observation that in all the species of the marine organisms studied a greenish brown chitosan reaction was observed and that in one of them neutral sugars, such as galactose, glucose, fucose and mannose, were found along with the glucosamine.

The presence of neutral sugars introduces the question of the nature of the macromolecules or molecules to which these sugars are bound. The obvious possibilities are (1) that these neutral sugars may be components of one or more polysaccharides other than chitin, or (2) that they may be integral parts of the chitin itself.

Had a polysaccharide composed solely of these neutral sugars been present, the neutral sugars should have passed through the resin irrespective of the extent of hydrolysis. The retention of the neutral sugars by the cation-exchange resin and their elution with glucosamine seem to indicate that they may be integral parts of the chitin moiety.

The results of the nitrogen analyses of the materials in question substantiate the above inference. For arthropod chitin, which has only glucosamine, the nitrogen content is 6.4% (6). In contrast, the amounts of nitrogen present in the chitin of the marine organisms studied are lower. In the four coelenterate species, i.e., Physalia, Velella, Porpita and Millipora, the nitrogen content ranged from 4.2% to 4.6% and the values of nitrogen for the other three species studied are 5.3% to 5.9%. These two ranges seem to be related to the number of neutral sugars present. In the four species, there are two neutral sugars: the chitin from the coenosteum of the Millipora has galactose and mannose, while the other three species have glucose and fucose. In the other three species, in contrast, there is only one neutral sugar, either glucose or fucose.

The explanation for these results can be that when there are more neutral sugars in the chitin molecules, the nitrogen values are lower. Conversely, in the presence of only one neutral sugar nitrogen content increases.

These observations lend support to Rudall (8) who suggested, based on x-ray diffraction studies, that the coelenterate chitin may have additional sugar components. It might be added here that the chitin of the "bone" of cephalopods also has two neutral sugars (9).

CONCLUSION

Chitin has hitherto been considered as N-acetyl D-glucosamine, and it is thought that this aminopolysaccharide has the same chemical composition in all groups (5). The results recorded here, as well as those reported by other workers (9, 11), suggest that chitin from different sources may not be chemically identical. The differences may relate to the constituent sugars and nitrogen content. Kandaswamy (personal communication) records differences in the amounts of acetyl groups in α- and β-chitins.

The α- and β- chitins, which previously could only be distinguished on x-ray diffraction studies, can now be differentiated on the basis of variations in the type of the chitosan spherite crystals yielded by them.

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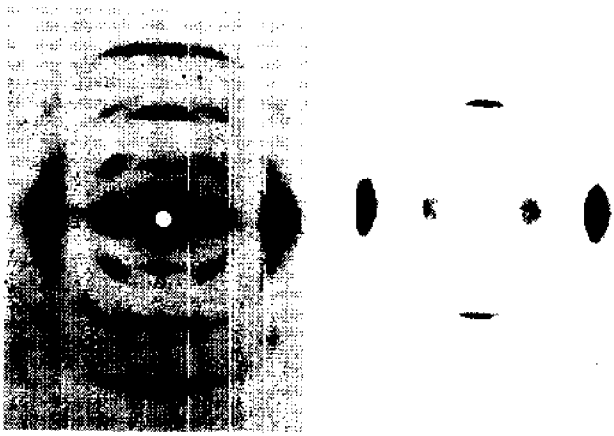

a b

Fig. 1. X-ray diffraction diagram of the alkali purified chitin from S.bertholetti (a), and that by the coenocium of Rhabdopleura.

a b

Fig. 2. A chitosan sulphate crystal yielded by the chitin from S.bertholetti (a) and that by the chitin from Periplaneta americana.

A METHOD FOR OBTAINING PURE CHITIN OF COMMERCIAL VALUE

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ABSTRACT

Chitin is an important material found in the skeletal structure of arthropods and in other invertebrate groups. In all cases, protein is bound by covalent bonds to the chitin, forming stable complexes, with the result that numerous workers have reported the impossibility of preparing an uncontaminated sample of chitin.

The present work reports a possible method for obtaining the purest possible sample of chitin. When all proteinaceous impurity was removed, chitin also showed several properties that suggested possibilities for commercial exploitation.

The materials used were the common marine crabs, Neptunes sanguinolentus and Uca pugilator, in different stages of the molt cycle: soft crab (Stage A), paper-shell crab (stage B), hard crab (stage C), and pillans (stage D3 and D4). Nitrogen estimations of the alkali-purified material showed that the values were from 6.50% to 6.54% in A, B and C, and in the pillans stage, from 6.90% to 6.92%, very near the theoretical value of 6.89%. This result indicates that the chitin from the cuticle in the pillans stage could be maximally purified.

This finding was substantiated by other observations. The ninhydrin reaction was only faintly positive for the purified material in the pillans stage. The chromatograms of the hydrolysate of the material disclosed only one unidentifiable feeble spot with ninhydrin in this pillans stage. In stage C, three amino acids were present, and in stages A and B two amino acids were found. These observations suggest that chitin in the cuticle of the crustaceans studied can be purified to the maximum possible extent in the D3 and D4 stages. The commercial advantages of the chitin obtainable in the pillans stage over that in the other stages are discussed.

INTRODUCTION

Chitin is an important structural material found in the cuticle of arthropods and in the skeletal structures of other groups of invertebrates (5). Numerous workers have reported that protein has been bound by covalent bonds to chitin, forming stable complexes, and that it is consequently impossible to prepare samples of chitin that are not contaminated (2, 7, 8). The present investigation reports a possible method for obtaining the purest possible samples of chitin. The purity was tested by analyses of protein and amino acids, nitrogen estimations and x-ray diffraction studies. In the absence of any proteinaceous impurity, the chitin produced a chitosan sample which in solution had a much higher viscosity than the controls did.

EXPERIMENTAL

The materials used were the marine crabs Neptunes sanguinolentus and Uca pugilator, which are commonly available along the Ernakulam coast of India. Every natural collection contains these crustaceans in various stages of the molt cycle: soft crab (stage A), paper-shell crab (stage B), hard crab (stage C) and pillans (stages D3 and D4), according to the criteria proposed by Travis (10).

In all these cases, the cuticle was freshly obtained and freed from the underlying tissues by manual cleaning in sea water. The material was later purified by boiling in 5% NaOH for 24 hrs (6). The residue was collected, washed in tap water several times, in ethanol 3 times and in ether 3 times, and finally dried *in vacuo* over phosphorous pentoxide. The dried material was used for all further studies.

Nitrogen estimations were made by the standard microkjeldahl method of Steyermark (9). The hydrolysate of the material was prepared in 6N hydrochloric acid in sealed vessels at 100° C for 12 hrs. After drying the hydrolysate over KOH and phosphorous pentoxide, the residue was taken up in water and analyzed by 2-dimensional paper-partition chromatography. The solvent for the first run was 2-butanol and 3% ammonia (5:2, V/V) and, for the second run, 2-butanol, acetic acid and water (15:3:2, V/V/V). The amino acids were identified by specific spot tests (1) as well as by comparison with standard amino acids run simultaneously under conditions identical to the controls.

The x-ray photographs were taken in a cylindrical camera, after the material was dried *in vacuo* over phosphorous pentoxide.

A chitosan solution was prepared according to the procedure outlined by Moorjani et al. (4), and the viscosity was determined by the Ostwald technique.

$$\text{Relative viscosity} = \frac{d_1 t_1}{t_2}$$

where: d_1 = density of chitosan solution

t_1 = time of flow for chitosan solution

t_2 = time of flow for the same volume of water

Absolute viscosity = relative viscosity x 0.00895 poises, where 0.00895 is the absolute viscosity of water at 25° C.

RESULTS

Nitrogen estimation and ninhydrin reaction

Nitrogen estimations of the purified chitin show that the values are 6.50% to 6.54% in stages A, B and C, and 6.90% to 6.92% (Table 1) in the pillans stage.

The ninhydrin reaction is only very faintly positive for the purified materials in the pillans stage which is the lowest amount of all the stages tested (Table 1).

Chromatographic analyses of the amino acids

The chromatogram of the hydrolysate of the materials discloses only one unidentifiable feeble spot with ninhydrin in the pillans stage. In stages A and B, histidine and aspartic acid are present, while in stage C three amino acids (histidine, aspartic acid and glutamic acid) are found.

Table 1. The Nitrogen Content and the Ninhydrin Reaction Shown by the Chitin of the Cuticle of Crabs in Various Stages of Molting

Molting Stages	Nitrogen Content %*	Ninhydrin Reaction
N. sanguinolentus		
Soft crab	6.54	+
Paper-shell crab	6.51	+ +
Hard crab	6.50	+ + +
Pillans crab	6.90	±
U. pugilator		
Soft crab	6.52	+
Paper-shell crab	6.53	+ +
Hard crab	6.51	+ + +
Pillans crab	6.92	±

*Average of 5 estimates

Key for ninhydrin reactions: ± faintly positive
+ positive
++ strongly positive
+++ very strongly positive

X-ray diffraction studies

The X-ray diffraction patterns are sharp and well defined, without any extra reflections typical of α-chitin in the pillans stage (Fig. 5). The patterns are diffuse in the other stages studied (Fig. 6).

The commercial advantage of chitin in the pillans stage

Chitosan was prepared from the chitin of the cuticle of two species of crabs in different stages of the molt cycle by deacetylation with saturated sodium hydroxide at 100° C for one hr. The thoroughly washed chitosan (1 g) was dissolved in 100 ml of 2% acetic acid. The viscosity of the solution was then studied.

From the data presented in Table 2, it can be seen that the chitosan solution prepared from the chitin of the cuticle in the pillans stage is more viscous in both the species than those from the other stages. The relative viscosities

of the chitosan solutions from the pillans stage are 367.5 and 346.1; the absolute viscosities from this stage are 3.165 and 3.078 poises. The highest viscosity from the other stages is less than half of these values.

Table 2. Viscosity of the Chitosan Solution Prepared from the Chitin of Crabs in the Various Molting Stages

Molting Stages	Relative Viscosity	Absolute * Viscosity
N. sanguinolentus		
Soft crab	176.7	1.4321
Paper-shell crab	118.2	0.6514
Hard crab	74.3	0.5229
Pillans crab	367.5	3.1650
U. pugilator		
Soft crab	142.2	1.1530
Paper-shell crab	111.9	0.6256
Hard crab	56.4	0.3641
Pillans crab	346.1	3.0780

*Expressed in poises of 1% chitosan in 2% (W/V) acetic acid

DISCUSSION AND CONCLUSIONS

It is known that the theoretical value of nitrogen in chitin is 6.89% (5). This value is never obtained in nature, however, because of the protein contaminant found in the chitin which forms stable complexes with it (7). In all prior studies, the chitin from the cuticle of arthropods in the intermolt stages was studied for this purpose (2). But since in the intermolt stages the cuticle is fully hardened and polymerization of the various components is completed (5), it should theoretically be possible to obtain a less contaminated sample of chitin in the earlier stages of the molt cycle.

This has been achieved in the present investigation. The nitrogen values of the chitin obtained from the two species of crabs studied are 6.90% and 6.92% from the pillans stage, which is very near the theoretical value. In the other stages, the values are much lower, from 6.50% to 6.54%; it was therefore inferred that the purest possible chitin sample could be obtained from the pillans stage. In this stage, the chitosan solution is more viscous than in the contaminated state. The viscosity of the chitosan solution could be of great value to textile and paper industries for use as a sizing and adhesive material (4).

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Fig. 1. Two dimensional chromatogram of the hydrolysate of the purified chitin from the cuticle of N.sanquinolentus (a), and U.pugilator (b) in 'pillans' stage

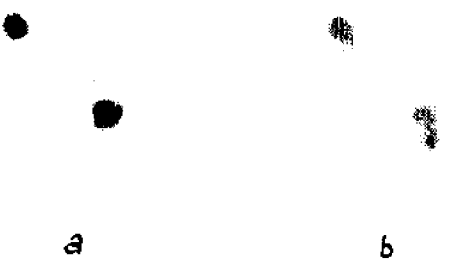

Fig. 2. Two dimensional chromatogram of the hydrolysate of the purified chitin from the cuticle of N.sanquinolentus (a), and U.pugilator (b) in soft crab stage

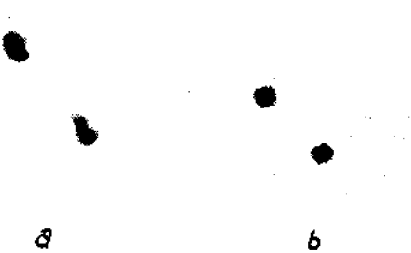

Fig. 3. Two dimensional chromatogram of the hydrolysate of the purified chitin from the cuticle of N.sanquinolentus (a), and U.pugilator (b) in paper shell stage

Fig. 4. Two dimensional chromatogram of the hydrolysate of the purified chitin from the cuticle of N.sanquinolentus (a), and U.pugilator (b) in hard crab stage

Fig. 5. X-ray diffraction figure given by the purified chitin from the cuticle of N.sanquinolentus in 'pillans' stage

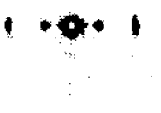

fig. 6. X-ray diffraction figure given by the purified chitin from the cuticle of N.sanquinolentus in 'hard crab' stage

METAL-BINDING PROPERTY OF CHITOSAN FROM PRAWN WASTE

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ABSTRACT

Three samples of chitosan were prepared from a single species of prawn waste, comprised of head and shell. Three different temperatures (100 ± 2°C, 90 ± 2°C and room temperature, 28-30°C) were employed at the stage of deacetylation. Assessments were made of the samples for their differences in metal-binding capacity (Cu^{++} from aqueous solution of copper sulfate), if any, as influenced by the temperature of deacetylation and its probable relationship with viscosity. The adsorption of Cu^{++} by chitosan is found to be almost independent of the temperature of deacetylation, while significant variations occur in viscosity--it increases as temperature decreases. The adsorption of copper from copper-sulfate solution on chitosan was rapid at the initial stages, with a maximum rate of removal occurring during the first few minutes. After one hour of treatment, the reaction slowed down and became nearly complete in three hours. The capacity of chitosan for binding different metal ions (Cr^{+++}, Ni^{++}, Zn^{++}, Fe^{+++} and Mn^{++}) from their salt solutions was also studied. The rate of adsorption of these metals was in agreement with the observation for copper. Viscosity and adsorption capacity of chitosan are, therefore, qualities independent of each other.

INTRODUCTION

The prawn-processing industry of India turns out over 40,000 tons of waste, mostly of head and shell. Prawn shell contains chitin, the cellulose-like substance that is its main structural component. The deacetylated derivative of chitin called chitosan is known to have potential application in a number of industries, such as textiles, paper, cosmetics and food. The utilization of prawn waste for the preparation of chitosan can, in addition to solving the problem of the disposal of huge quantities of waste, promote another small-scale industry. This possibility has created so much interest that a number of research programs on different aspects of the production and application of chitosan are being carried on in India. Earlier works reported on chitosan (3,4,6,7) mainly describe the effects of various processing parameters (such as the temperature at which deacetylation takes place and the concentration of acid used for demineralization) on the quality of the chitosan as determined by its viscosity (1% solution in 1% acetic acid). The capacity of chitosan conditioned in ammonium sulfate and/or sulfuric acid for collection of metal ions has been discussed by Muzzarelli et al. (5). Reports on the assessment of the quality of chitosan based on any property other than viscosity measurements or on any other possible quality control parameter that can be reliably applied in industrial production have been scarce. A number of known and potential uses of chitin and chitosan and explanations of research and development programs and operations of pilot plants have been published (2). The film-forming capacity of chitosan and chelation of heavy metal ions from their solutions have also been studied (1). Because chitosan samples prepared in our experiments showed great variations

in their viscosities, depending upon changes in the processing conditions, it was felt necessary to study the properties of chitosan, other than viscosity, that could be used as a reliable index for quality. The capacity of chitosan to adsorb metals from their salt solutions as influenced by the temperature of deacetylation and the rate of adsorption as influenced by viscosity are both discussed in this paper.

MATERIALS AND METHODS

Three different samples of chitosan were prepared from a single lot of prawn-shell waste derived from a single species of prawn and collected from a prawn-processing factory at Cochin. Except for the temperature at which deacetylation was carried out, all conditions of preparation of chitosan were kept constant. Deacetylation was effected at 100 ± 2°C for sample no. 1; 90 ± 2°C for sample no. 2; and at room temperature (28-30°C) for sample no. 3. The samples had viscosities of 155.3, 430.4 and 902 cp. respectively, in a 1% solution in 1% acetic acid.

For determining the adsorption capacity, 1 g of coarsely powdered chitosan (sample no. 1) was added to 100 ml of dilute salt solution with a known concentration of Cu^{++}, Cr^{+++}, Ni^{++}, Zn^{++}, Fe^{+++} and Mn^{++} and sampled periodically. For determining the rate of adsorption compared to viscosity, 1 g each of all the three samples of chitosan was added to 0.1 M copper-sulfate solution, samples were drawn every 10 minutes, and residual copper in the solution was estimated. Viscosities of the respective samples were determined on a 1% solution in 1% glacial acetic acid at room temperature, using a Redwood Viscometer no. 1250.

RESULTS AND DISCUSSION

Figure 1 indicates the quantity of copper sulfate adsorbed from the solution against the time of treatment for all the three samples studied. The rate of removal of copper sulfate from the solution is generally at a maximum during the first 60 minutes; thereafter it proceeds slowly and becomes more or less complete after three hours. Though there is a slight increase in the rate of adsorption in the initial stages with sample no. 3 and less so with sample no. 2, both become slower and the maximum adsorption is lower than that of the other two samples. There is not much difference in the adsorption properties of samples no. 1 and no. 2, with the maximum adsorption of sample no. 2 slightly less than that of sample no. 1.

Table 1. Adsorption of Metals by Chitosan from Salt*Solutions

Time in Minutes	Quantity Adsorbed (mg/g)					
	Cu++	Cr+++	Ni++	Zn++	Fe+++	Mn++
30	747	1253	80	1203	777	nil
60	1049	1353	131	1259	1165	-
90	1245	1514	152	1546	1261	-
120	1240	1514	312	1649	1359	-
	1526	1514	327	1837	1456	-

*Salts used and the initial concentration of the metal ion in solution (g/100 ml): copper sulfate, 0.6356; chromium aluminum sulfate, 0.1617; nickel sulfate, 0.1469; zinc sulfate, 0.4634; ferric sulfate, 0.3794; manganese sulfate, 0.1989.

As can be seen from the Table, there is a great variation in the affinity of chitosan for different metals. In agreement with the observations represented in Figure 1, the rate of adsorption of other metals by chitosan shows a peak value in the initial stages, which falls in the subsequent phases. The affinity observed among the metals tested varied significantly; this observation may be of great importance in its industrial applications for selective removal of metals.

We observed (3) that the temperature of deacetylation had a great influence on viscosity, and we considered viscosity as a suitable index of quality. The present investigations show that viscosity had no significant effect on the metal-binding property of chitosan: the maximum adsorption for the three samples studied was almost the same. The progressive increase in viscosity exhibited by samples with a decrease in temperature of deacetylation is therefore probably due to the greater chain length of the polymer, since the extent of degradation of the polymer chain might have been less at lower temperatures. With an increase in temperature, there is a greater possibility of degradation of the chain length; this accounts, perhaps, for the low viscosity of the sample prepared at high temperature.

From the foregoing, it appears that viscosity and affinity for metals are properties independent of each other. They cannot be used as a general index of the quality of chitosan for a process-control measurement. Process controls during production of chitosan should therefore be selected to suit the application envisaged for the product.

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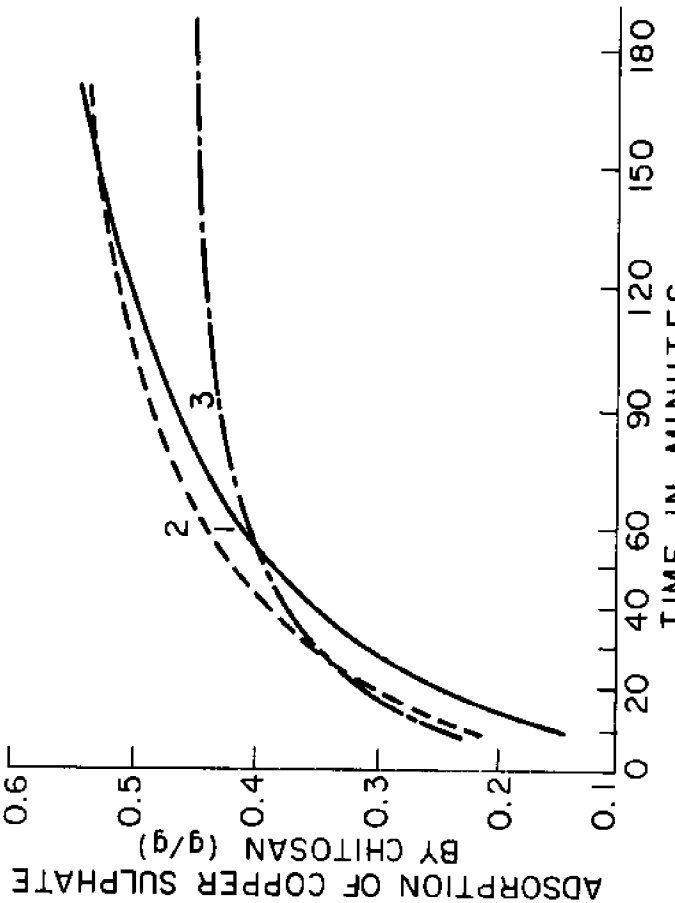

Figure 1. Rate of adsorption of copper sulphate by different samples of chitosan

VIII. BIOCHEMICAL AND MICROBIOLOGICAL RESEARCH

SYNTHESIS OF CHITIN MICROFIBRILS IN VITRO BY CHITIN SYNTHETASE PARTICLES, CHITOSOMES, ISOLATED FROM MUCOR ROUXII

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ABSTRACT

Chitin synthetase activity in cell-free extracts of the yeast cells of Mucor rouxii can be separated as particles with an average sedimentation coefficient of 105 S. These spheroidal particles, called "chitosomes," measure 40-70 nm in diameter. Their appearance varies after negative staining; some exhibit a well-defined shell 6-12 nm thick and have granular structures inside; others show no internal structure, but have a pronounced depression of the external surface. In thin section, isolated chitosomes appear as microvesicles with a tripartite shell.

Almost all the isolated chitosomal chitin synthetase occurs in a latent, "zymogenic" state that requires treatment with proteases for activation. The acid proteases from Mucorales are most effective. The proteolytic activation of the zymogen is blocked by pepstatin. These and other properties of purified chitosomal chitin synthetase are similar to those manifested by crude fractions of the cell-free extract: kinetics, requirement for divalent metals (Mg^{2+} or Mn^{2+}), stimulation by free N-acetyl-D-glucosamine, inhibition by the antibiotic polyoxin or by uridine diphosphate. One notable difference between crude and purified preparations, however, is the greater stability of the purified chitosomal chitin synthetase zymogen against spontaneous activation and destruction.

Chitosomes can be dissociated by treatment with digitonin. The chitin synthetase thus solubilized has a sedimentation coefficient of 16 S and retains most of the enzymatic properties of the chitosomal enzyme.

Upon addition of substrate (UDP-GlcNAc) and activators, chitosomes undergo a seemingly irreversible sequence of transformations. The internal structure changes, and a coiled chitin microfibril appears inside the chitosome; frequently the shell is opened or shed, and a long extended microfibril can be seen arising from the coiled microfibril.

Chitosomes have been isolated from several genera representing the major groups of fungi: Neurospora crassa (Euascomycetes), Saccharomyces cerevisiae (Hemiascomycetes), Mucor rouxii yeast and mycelial forms (Zygomycetes), Allomyces macrogynus (Chytridiomycetes), and Agaricus bisporus (Homobasidiomycetes). We regard the chitosome as a cytoplasmic

conveyor of chitin synthetase zymogen to sites of wall synthesis on the surface of fungal cells.

INTRODUCTION

The biosynthetic steps involved in chitin synthesis, from the conversion of glucose into N-acetyl-D-glucosamine to its polymerization into a long chain of sugar residues joined by β-1,4 links, are fairly well known (5), except perhaps for the lingering possibility that a lipid intermediate may enter into the final step(s) of the pathway (17). In all systems studied so far, fungal and animal, uridine diphosphate N-acetyl-D-glucosamine (UDP-GlcNAc) is the universal glycosyl donor (Fig. 1).

As important as this knowledge is, it leaves unanswered some fundamental questions of chitin biogenesis. Chitin is not a soluble product but a highly ordered microfibrillar structure. Hence, we need to know how the polysaccharide chains become assembled in an orderly manner into a microfibril and how these microfibrils are integrated into the structure of cell walls. The spatial and temporal components and controls of chitin microfibril synthesis in fungal cells must be understood. Specifically, we need answers to the following questions: (1) where in the cell is the chitin chain synthesized; (2) how and where are these chains assembled into a microfibril; (3) where is chitin synthetase made and how is it transported to its site of operation; and (4) how is chitin synthesis regulated and coordinated with other concomitant aspects of wall growth? In the last decade, the search for answers to these questions has been initiated. Progress has been made in the following areas:

1. With respect to spatial controls, a major piece of the machinery of localized wall synthesis in fungal cells was identified by the discovery that the actively growing regions of fungal cells, conspicuously the hyphal tips, have a large accumulation of cytoplasmic vesicles of different size and stainability in the vicinity of the expanding walls (9,11,12). These secretory vesicles are the likely candidates for explaining the specific discharge of enzymes and/or precursors for wall growth at precise sites on the surface of the fungal cell where wall expansion takes place. Several lines of evidence indicate that cell-wall microfibrils are not preformed in the cytoplasm and then exported to the wall (1,2). It is more likely that microfibril assembly takes place *in situ*. Accordingly, the process of delivering chitin synthetase to its final site of operation is a pivotal component of the mechanism of chitin fibrillogenesis in fungi.

2. With respect to temporal controls, Cabib and co-workers (6,7) discovered an important, perhaps the principal, mechanism for timing the operation of chitin synthetase. They found that the chitin synthetase in *Saccharomyces* spp. can exist in a latent zymogenic form. Upon treatment with protease B, the zymogen is activated. The system has an additional controlling factor--a soluble protein which serves as a specific inhibitor of protease B. In their model, the zymogen is visualized as being located in the plasma membrane; its activation occurs with the arrival of protease-carrying vesicles.

3. Before 1974, the evidence for chitin biosynthesis in fungi rested exclusively on radiotracer studies showing the incorporation of radioactivity from UDP-GlcNAc (labeled in the glycosyl moiety) into an

insoluble product (8,10,13-15,18,19). The sources of chitin synthetase were various crude particulate fractions obtained by centrifugation of cell-free extracts. This precluded any observation of either the enzyme or the product. The product was identified by its solubility properties and digestibility with chitinase. In 1974, studies from the yeast form of Mucor rouxii (20,21) revealed that the chitin synthetase of crude membrane preparations (e.g., mixed membrane fraction, or MMF) was highly zymogenic and unusually stable. A procedure was discovered to separate chitin synthetase from other components of the crude MMF (20): the MMF was exposed to chitin synthetase substrate (UDP-GlcNAc) in the cold and centrifuged at 81,000 g for 30 min. A transparent "solution" of chitin synthetase was thus obtained which, upon incubation at 20-25°, produced a copious precipitate of microfibrils indistinguishable by electron microscopy (shadow-casting) (Fig. 2) or x-ray diffraction (powder camera) from microfibrils formed in vivo (20).

4. Electron microscopy of shadow-cast preparations of "solubilized" chitin synthetase, incubated with substrate and activators, indicated that chitin microfibrils were formed from particles containing chitin synthetase that appeared as "granules" measuring 35-100 nm (23). Some of these granules could be seen attached to the ends of microfibrils. The particles containing chitin synthetase could also be recovered directly from a high-speed supernatant of the cell-free extract. Subsequent detailed examinations of these particles by negative staining revealed a more complex structure than that seen by shadow-casting; furthermore, the particles underwent an extensive transformation during microfibril synthesis (4). These structures containing chitin synthetase were named "chitosomes" (4).

The remainder of this article is a summary of our observations on chitosomes.

CHITOSOMES

Isolation and morphological characterization

Part of the chitin synthetase activity of cell-free extracts of Mucor rouxii remains in the supernatant after walls and membranes are removed by sedimentation at 1,000 g and 54,000 g respectively (22). All of the chitin synthetase activity in this supernatant is in the form of chitosomes.

Chitosomes can be separated and identified by the procedure depicted in Fig. 3. The final step in this procedure is a sucrose density gradient centrifugation (Fig. 4). Electron microscopic examination of gradient fractions (4) shows a predominance of spheroidal particles, most of them measuring 40-70 nm in diameter in the peak fractions of chitin synthetase activity (Fig. 5). These particles, or chitosomes, have a variable morphology. Two major types have been recognized. Some chitosomes (cycloid type) exhibit a well-defined shell 6-12 nm thick and have an internal granular appearance (Fig. 7). Others show no internal structure but have a pronounced depression of the external surface (proctoid type) (Fig. 6).

Thin sections of pelleted chitosomes reveal structures in the same size range as those seen by negative staining (Fig. 8). The structures are bounded by a shell measuring only 6.5-7 nm thick in thin section. The shell has a tripartite appearance similar to that of a biological membrane.

In thin section the chitosomes have the overall appearance of microvesicles (4).

General properties of chitosomal chitin synthetase (22)

In sucrose gradients, chitosomes sediment as a single peak (Fig. 4) with an average sedimentation coefficient of 105 S. Chitosomes are excluded from Sepharose 6B or Biogel A-5m gel beds, but are included in Sepharose 4B or 2B (22). This behavior is consistent with particles of the size found by electron microscopy. The median size of chitosomes in the peak fraction is 60-70 nm.

The chitin synthetase of purified chitosomes (22) has essentially the same enzymatic behavior observed in crude fractions of cell-free extracts of M. rouxii (3,17,18,21). The pH optimum is 6.5; Mg^{++} or Mn^{++} at 5-10 mM is required for maximum activity. Mn^{++} is slightly more effective, but it becomes highly inhibitory at higher concentrations. Free N-acetyl-D-glucosamine (GlcNAc) is a strong stimulator, particularly at low substrate concentration. GlcNAc is not incorporated into the chitin chain; its stimulatory effect is allosteric (17,19). GlcNAc seems to mimic the effect of UDP-GlcNAc, which is the allosteric effector in vivo (17,22). Chitosomal chitin synthetase is inhibited by the antibiotic polyoxin D with the same K_i (0.65 µM) obtained for crude chitin synthetase fractions from mycelium of M. rouxii (3).

Zymogenicity

Nearly all the isolated chitosomal chitin synthetase of M. rouxii is in a latent or "zymogenic" form that requires treatment with a protease for its activation (22,23). The acid proteases of Mucorales are the most effective activators. The proteolytic activation of the zymogen can be blocked by pepstatin, an inhibitor of acid proteases (unpublished data).

Solubilization of chitosomal chitin synthetase

Chitin synthetase can be solubilized from chitosomes of M. rouxii with 0.5% digitonin. The solubilized enzyme has a sedimentation value of 16 S determined by centrifugation in sucrose-density gradients. The enzyme thus solubilized maintains its initial zymogenic state and can be activated by acid proteases. Activation is prevented by pepstatin. The product synthesized by the solubilized enzyme is microfibrillar and has been characterized as α-chitin by x-ray diffractometry (unpublished results).

Electron microscopy of microfibril formation (4)

Within 30 seconds after substrate and activators are added to a chitosome suspension, clusters of chitin microfibrils can be discerned. In short incubations (< 15 min), the clusters of microfibrils are associated with large numbers of rounded particles in the size range of chitosomes (Fig. 9). But these particles do not have the proctoid or cycloid appearance of unincubated chitosomes. Instead, they consist of one or more chitin fibrils coiled into a spheroidal clew or "fibroid" (Fig. 10). A long extended microfibril is frequently seen arising from such fibroids (Figs. 10, 12, 13). Some of the fibroids retain a small or major portion of the original chitosome shell (Figs. 11, 12). This is a clear indication that the chitosomes produced these fibroid coils and the associated

extended microfibrils (4).

The fibroid coils are probably the result of the chitin assembly being confined within the chitosome shell. The role or significance of the fibroid coil is not entirely clear; possibly fibroids are not formed *in vivo* but are the result of fibril assembly in chitosomes that had not opened up sufficiently during the initial stages of chitin synthesis in the *in vitro* experiments.

In prolonged incubations of chitosomes with substrate and activation, the proportion of fibroids to extended microfibrils is drastically reduced (Fig. 13). The average thickness of straight microfibrils increases with time of incubation (4). Thin microfibrils can be seen merging into thicker ones (Fig. 13).

Occurrence in other fungi

Chitosomes have been isolated by the procedure shown in Fig. 3 from several fungi representing the major groups of fungi (unpublished findings):

Group	Organism
Chytridiomycetes	*Allomyces macrogynus*
Zygomycetes	*Mucor rouxii*
Hemiascomycetes	*Saccharomyces cerevisiae*
Euascomycetes	*Neurospora crassa*
Basidiomycetes	*Agaricus bisporus*

Upon incubation with substrate plus activators, the chitosomes of these fungi produce extended microfibrils and coiled fibroid structures similar to those described for *Mucor rouxii*.

Occurrence in vivo

There is no doubt that the cell-free extracts of *Mucor rouxii* and other fungi have at least part of their chitin synthetase in the form of chitosomes. However, unequivocal proof for the existence of chitosomes in the living cell has not yet been obtained. Although a remote possibility remains that chitosomes are artifacts produced by fragmentation of larger cellular structures during the severe cell-disruption procedure employed (high-speed homogenization with glass beads), we believe that this was not the case. (A) Experiments with cells labeled with ^{14}C-choline or inorganic ^{32}P-phosphate indicate that only a minute portion (< 1%) of the protein and phospholipid of the cell is associated with the total chitosomal mass (22). Hence it seems unlikely that the chitosome fraction represents mere fragments of major membranous organelles. (B) A chitosome population can be isolated from cells broken by milder procedures such as low shear homogenization of hyphae of *M. rouxii* with a razor-blade chopper (unpublished data). (C) Morphological correlates of isolated chitosomes can be found in thin sections of unbroken cells of *M. rouxii* (D). These are microvesicles, about 35-50 nm in diameter, found either suspended in the cytoplasm among the ribosomes or located inside a larger multivesicular body.

CONCLUSION

Although the experimental evidence is still limited, we can offer the following hypothesis to explain the process of chitin fibril formation by a living fungal cell: chitin synthetase is probably produced as a zymogen in the endoplasmic reticulum and packaged in the form of chitosomes. The chitosomes act as cytoplasmic conveyors of chitin synthetase and migrate to the cell surface areas where wall synthesis takes place. Upon reaching the plasma membrane, a chitosome must interact with it in some undetermined manner, perhaps by fusion or extrusion. Chitosomal chitin synthetase probably ends up somewhere in the region of the plasma membrane-wall interface. Whether it remains in the "periplasmic space" or becomes primarily affiliated with the plasma membrane or the wall is not yet known. The chitin synthetase zymogen becomes activated by cell-surface protease(s), and the many chitin synthetase subunits derived from, or contained in, a single chitosome synthesize a corresponding number of individual chitin chains. These chains assemble collectively into a long ribbon by the intrinsic forces of crystallization of the chitin molecules. A simultaneous or parallel migration of chitosomes along with the larger secretory vesicles associated with growing walls could be a major device to coordinate microfibril synthesis with other aspects of cell-wall metabolism (1) that are necessary to make a complete cell wall.

ACKNOWLEDGMENTS

The investigations described herein were supported in part by grants from the National Institutes of Health (AI-05540), National Science Foundation Latin American Program (INT 75-08378), Consejo Nacional de Ciencia y Tecnología, México (847 and PNCB 03011104), and a Brown-Hazen Grant from Research Corporation. Journal Paper No. 6740 of the Purdue University Agricultural Experiment Station. Portions of this work were performed with the technical assistance of Eleanor Lippman, Catalina Montecillo and Emma Reyes.

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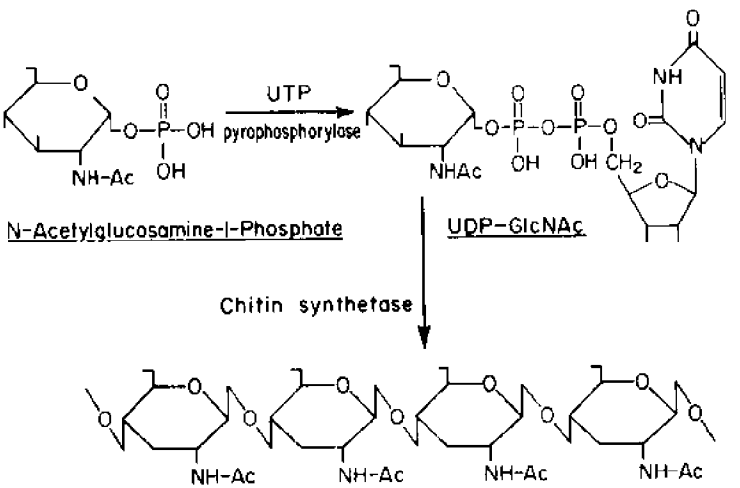


Fig. 1. Chitin biosynthesis

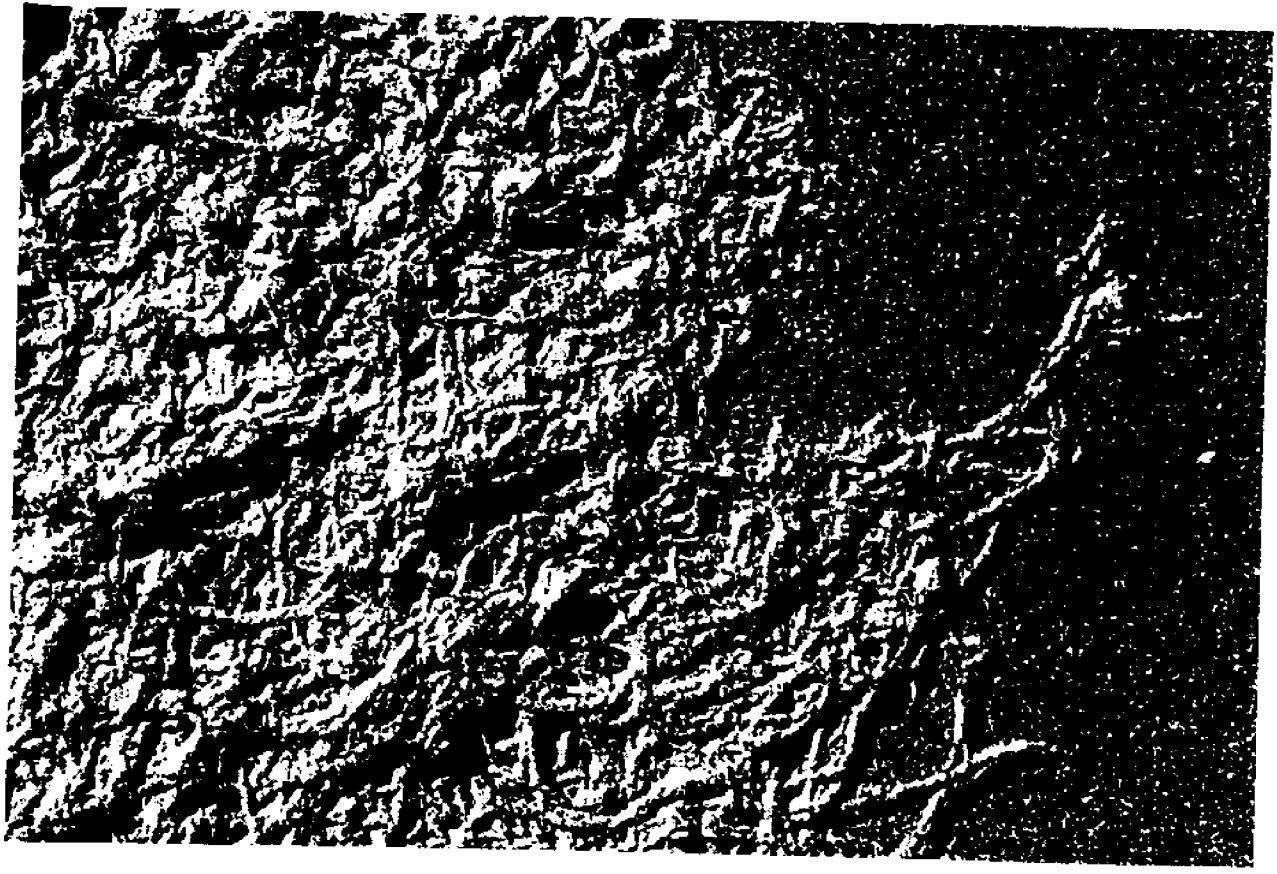


Fig. 2. Chitin microfibrils synthesized in vitro by substrate-solubilized chitin synthetase. Magnification 64,000 X (20).

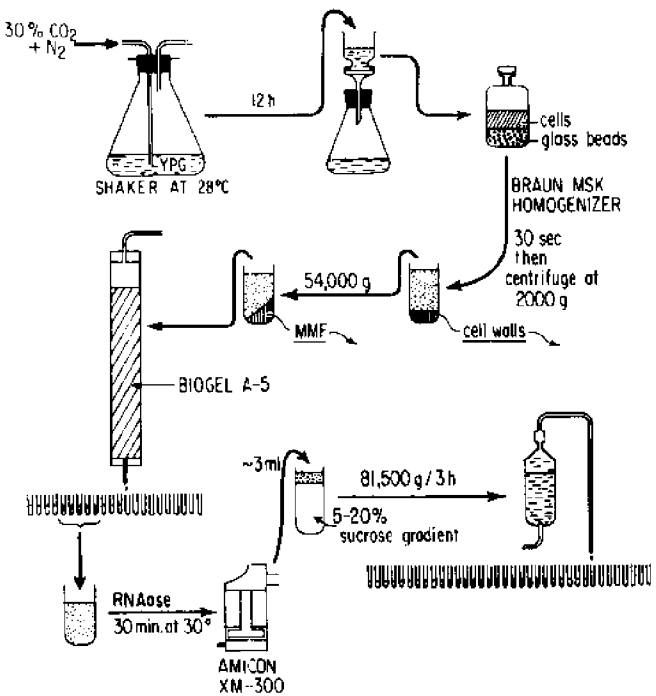


Fig. 3. Procedure for the isolation of chitosomes from yeast cells of Mucor rouxii. Details in references 2, 4 and 23. After the RNAse treatment the samples were centrifuged at 10,000 g for 20 min (not illustrated) to remove precipitate before being applied to Amicon filter. Samples were removed from the fractionated sucrose gradient and assayed for chitin synthetase activity (see Fig. 4).

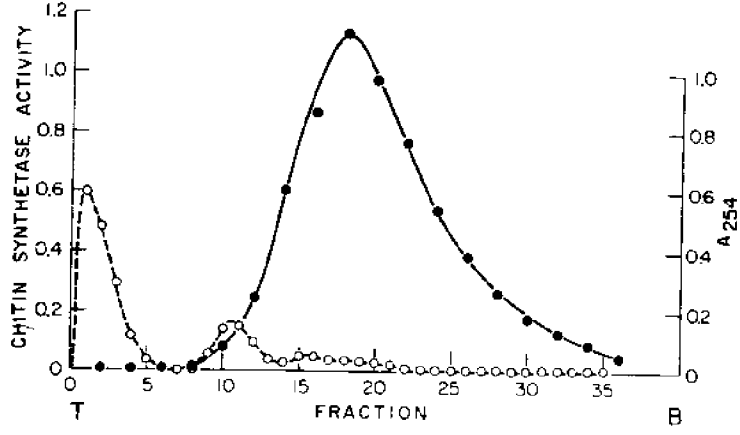
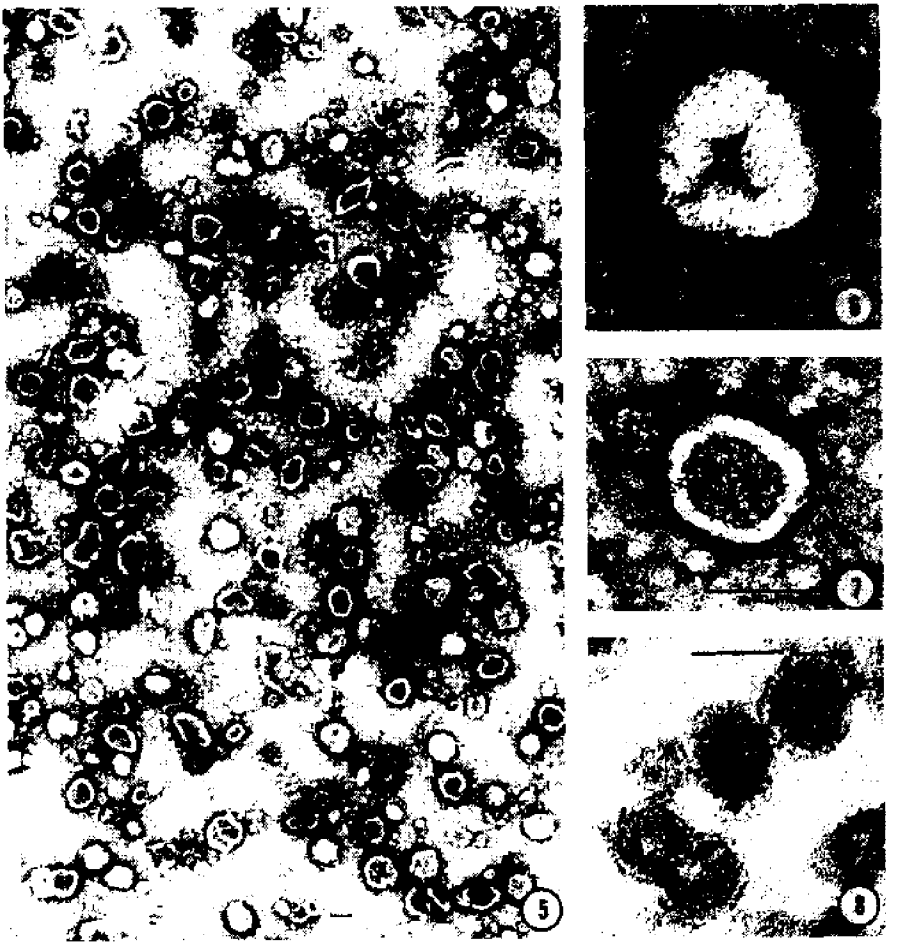
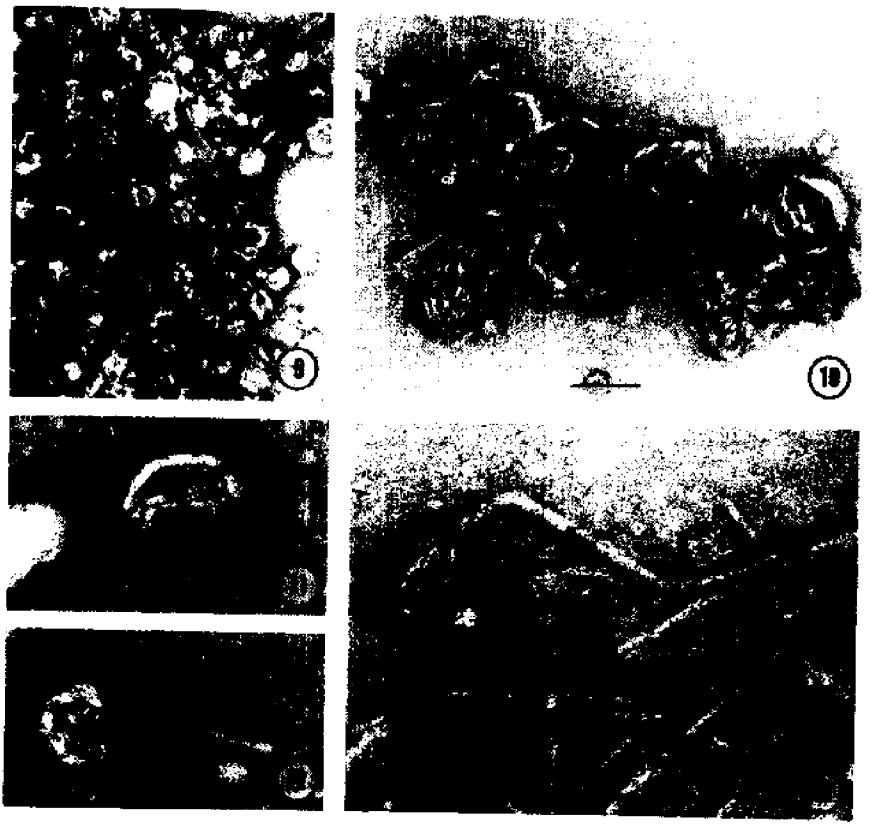


Fig. 4. Sedimentation of chitosomal chitin synthetase in a linear 5-20% sucrose gradient. Gradient was fractionated into 1 ml fractions. Absorbance at 254 nm (O) was continuously recorded. Chitin synthetase activity (●) was assayed in 100 µl samples and expressed as total nmoles of GlcNAc incorporated into chitin per min per fraction (22).



Figs. 5-8. Chitosomes from Mucor rouxii (4). Fig. 5, negatively stained sample from peak fraction of chitin synthetase (see Fig. 4) showing a predominance of chitosomes of proctoid (P) and cycloid (C) appearance. Fig. 6, detail of a proctoid chitosome. Fig. 7, detail of cycloid chitosome. Fig. 8, thin section view of isolated chitosomes. Magnification Bar = 50 nm.



Figs. 9-13. Synthesis of chitin microfibrils by purified chitosomes (4). Fig. 9, cluster of microfibrils with fibroid coils produced after 5 min incubation. Fig. 10, detail of the fibroid coils. Fig. 11, chitosome with a fibroid coil inside and part of the original shell. Fig. 12, chitosome with a long extended microfibril. Note the fibroid coil inside the chitosome shell. Fig. 13, cluster of long microfibrils with few fibroid coils produced after 30 min incubation. Magnification Bar = 50 nm.

IN VITRO CHITIN BIOSYNTHESIS BY CUTICULAR DISKS OF MANDUCA SEXTA LARVAE IN THE V INSTAR

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ABSTRACT

A simple yet efficient method of short-term culturing is described for the quantitative analysis of chitin biosynthesis by epidermal tissue of Manduca sexta larvae in the V instar. After 1 hour of incubation at 21° C in a modified Robb's phosphate-buffered saline solution (1969) at pH 7.6 containing ^{14}C-N-acetylglucosamine, cuticular disks showed substantial production of chitin. The in vitro rate of synthesis was substantial compared to the calculated in vivo rate of 0.18µg chitin/mm^2/hr. The use of such a system may have applications in studying other biochemical pathways or inhibitors and represents the first step toward cell-fractionation procedures.

INTRODUCTION

The complete pathway of chitin biosynthesis has been known for quite a while (1, 4, 8). Numerous fungal studies have reported substantial in vitro production of chitin from uridine-5'-diphospho-N-acetylglucosamine (UDPAGm) using microsomal fractions (12, 16, 17). Similar attempts to duplicate high incorporation rates using animal microsomal fractions, however, have not exceeded 4% of the total label added (2, 6, 8, 11, 14). Despite the large amount of chitin produced by many animals in short, well-defined periods, higher rates of label incorporation have not been obtained in the few published studies. In addition, in vitro studies have not calculated the actual in vivo rate of chitin biosynthesis for the chosen animals. Evaluation of the production of chitin in vitro with the calculated in vivo rate would replace the relative measure of synthesis, such as the percent incorporation of total label, with an absolute one, such as µg chitin/unit area/hour. In addition, a system that is simple and would allow demonstration of rapid chitin biosynthesis has not been reported for animal tissues.

EXPERIMENTAL

Calculation of the in-vivo rate of chitin biosynthesis

Larvae of the tobacco hornworm Manduca sexta (Lepidoptera: Sphingidae) were grown from eggs on an artificial diet (Bell, personal communication) and kept under 12D:12L light conditions at 25°. Animals were cultured individually in plastic containers so that their precise age after ecdysis to the fifth instar would be known. At 12-hour intervals, larvae were eviscerated and the cuticle scraped to remove all soft tissues. A template was used to obtain 578.5 mm^2 sections of cuticle. Protein was removed using a modified method of Hornung and Stevenson (7). Cuticle was placed in hot 10% NaOH for 30 minutes, rinsed and then placed in 89% formic acid for 1 hour at room temperature. Rinsed sections were oven dried and weighed. Any weight differences between successive intervals would be due to additional synthesis of chitin. Thus the amount of chitin produced per unit area per hour was calculated. Figure 1 illustrates the relationship of chitin production and larval age as well as the calculated rate of 0.18µg chitin/mm^2/hr for the interval from 0 to 120 hours post-ecdysis to the fifth instar.

Preparation of cuticular disks for in-vitro biosynthesis

Larvae 24-60 hours post-ecdysis to the V instar were eviscerated. The cuticle was cleared of fat and muscle by gentle scraping. Cuticular disks of known area were made reproducibly, using a #7 cork borer. Disks with intact epidermis were immediately placed in test tubes containing 1 ml of chilled saline solution, to prevent cell degeneration. When all the samples were prepared, the tubes were warmed to 21° C for 10 minutes prior to the addition of label.

Culture medium and conditions

Cuticular disks were cultured in 0.5 or 1 ml of Robb's phosphate-buffered saline solution (15) which lacked $CaCl_2$, since preliminary results indicated that $CaCl_2$ reduced the production of chitin (Table 5). N-acetylglucosamine (AGm) was added to the medium to a concentration of 10 mM; pH at 7.6 and incubation at 21° C were found to be optimal for chitin biosynthesis (Tables 3 and 4). ^{14}C-acetyl-1-glucosamine (^{14}C-AGm) was added to the medium which was then stirred and incubated for 1-3 hours.

Synthesis was stopped by the addition of about 5 ml of 10% NaOH. However, tests indicated that newly synthesized chitin was being solubilized, and therefore rinsing and continued incubation of the disks for a total of 3 hours were performed (cold phase) to obtain more accurate rates of biosynthesis.

Calculation of the in vitro chitin biosynthetic rate

Following incubation, the disks were deproteinized as described for the calculation of the in vivo rate of synthesis. Each disk was placed in a scintillation vial and then cut into fine pieces with scissors. Ten ml of a toluene-POPOP-PPO scintillation cocktail were added, and the samples were counted for 10 minutes in a Mark II scintillation counter. ^{14}C counting efficiency was 60%. For each concentration of AGm used in the medium, the theoretical incorporation of ^{14}C-AGm into chitin was calculated. In other words, a known amount of label should have been incorporated to equal the calculated in vivo rate. The actual incorporation of label was then compared to the in vivo rate and presented on a percent basis as in Tables 1 and 2.

RESULTS

Verification of chitin biosynthesis

The conclusion that ^{14}C-AGm was incorporated into chitin is based on the following facts. First, chitin is one of the few macromolecules able to withstand the harsh chemical deproteinization procedures. Next, enzymatic hydrolysis of labeled deproteinized cuticle by chitinase and β-glucosidase (presumably contaminated with chitobiase [Jeuniaux, personal communication]) produced a purple spot with the Elson-Morgan test (20). Descending chromatography of the hydrolysate using the organic phase of butanol (4V): acetic acid (1V): water (5V) (13) or 95% EtOH (7V): HOAc (3V) (9) produced a single purple spot which co-chromatographed with AGm. Direct scintillation counting of 15 x 20 mm paper sections in toluene-POPOP-PPO showed that the radioactivity present was correlated with the purple regions shown by the Elson-Morgan test. In addition, micro-Kjeldahl analysis (5) of deproteinized

cuticle yielded a mean nitrogen percent of 7, compared to the theoretical value of 6.9% (10). Finally, TH-6040 (Dimilin), a known inhibitor of chitin synthesis (3, 18, 19) appreciably reduced the incorporation of ^{14}C-AGm into disks at concentrations as low as 6.4 x 10^{-16} M.

Effect of AGm concentration on the incorporation of ^{14}C-AGm into chitin

Table 1 illustrates current typical efficiencies of incorporation compared to the in vivo rate. Incubation was at 21° for 3 hours at a pH of 7.6. Disks from larvae 24-48 hours post-ecdysis to the V instar were made using a #7 cork borer. 8.8 x 10^5 DPM of ^{14}C-AGm were added to 1 ml of Robb's saline minus $CaCl_2$ ("n" indicates the number of disks used).

Table 1. Effect of AGm Concentration on the Incorporation of ^{14}C-AGm into Chitin

AGm conc	n	Mean DPM	Mean Efficiency	Range of Efficiency
5mM	4	17,597	47.7%	34-60%
10mM	4	8,252	44.3%	40-48%
15mM	4	5,160	44.6%	20-62%
20mM	4	3,930	42.2%	36-55%
40mM	4	1,383	29.6%	15-48%

Effect of glucose concentration on the incorporation of ^{14}C-AGm into chitin

Table 2 illustrates earlier work in which the effect of glucose concentration on the incorporation of ^{14}C-AGm was examined. Conditions were essentially the same as for Table 1 except that AGm concentration was at 5mM. Efficiencies are based on the in vivo rate of synthesis ("n" indicates the number of disks used).

Table 2. Effect of Glucose Concentration on the Incorporation of ^{14}C-AGm into Chitin

Robb's Medium Minus $CaCl_2$	n	Mean DPM	Range of DPM	Mean Efficiency
No glucose	4	9,040	6,424-13,633	24.5%
.5mM glucose	4	10,632	3,592-14,385	28.8%
1mM glucose	4	8,500	4,321-13,806	23.0%
5mM glucose	3	10,050	9,628-10,300	27.2%
10mM glucose	3	7,817	7,078-8,341	22.2%
20mM glucose	3	10,568	7,398-12,294	28.7%

Effect of pH on the incorporation of ^{14}C-AGm into chitin

Table 3 compares the incorporation rates vs pH for various Robb's phosphate-buffered saline solutions. Results for the pH's tested are shown as the percent incorporation at pH 7.6, where the rate of incorporation was maximal. Note the secondary peak at pH 6.8.

Table 3. Effect of pH on the Incorporation of ^{14}C-AGm into Chitin

pH	% of Maximal Rate
6.4	5-25
6.6	30-40
6.8	75-85
7.0	30-40
7.2	15-25
7.4	30-45
7.5	40-60
7.6	100
7.7	40-60
7.8	30-45
8.0	15-25
8.2	30-40
8.4	30-40
8.6	30-45

Effect of temperature on the incorporation of ^{14}C-AGm into chitin

Table 4 illustrates the effect of temperature on the incorporation of ^{14}C-AGm into chitin. Culture conditions were essentially those described under "Culture medium and conditions." Data are listed for each temperature as the percent of incorporation at 21° C where the incorporation rate was maximal. Note the increase in incorporation at 33° C.

Table 4. Effect of Temperature on the Incorporation of ^{14}C-AGm into Chitin

Temperature	% of Maximal Rate
19	75-85
21	100
23	75-85
25	35-50
29	50-60
33	80-90

Effect of media modification on the incorporation of ^{14}C-AGm into chitin

Table 5 shows the effects of changes in the composition of the Robb's phosphate-buffered saline solution on the incorporation of AGm into chitin. Tests were performed at 21° C at a pH of 7.6. Section A lists results as the percent of incorporation compared to Robb's complete medium. Section B presents the results as the percent of incorporation compared to Robb's saline minus $CaCl_2$.

Table 5. Effect of Media Modification on the Incorporation of ^{14}C-AGm into Chitin

	Solution	% of Maximal Rate
A.	Robb's complete	100
	Robb's minus $CaCl_2$	230
	Robb's minus $MgSO_4$ minus $MgCl_2$	8
B.	Robb's minus $CaCl_2$	100
	Robb's minus $CaCl_2$ plus 4μM ATP	64
	Grace's modified medium (contains $CaCl_2$)	17

DISCUSSION

This is the first report of results in which *in vitro* data are compared to a calculated *in vivo* rate of chitin biosynthesis for animal tissues. One test, which did not utilize the addition of unlabeled AGm, indicated that 37% of the total label was converted to chitin by the cuticular disks, yet represented only 1.5% of calculated *in vivo* rate. This suggested that relative values such as the percent of incorporation of label might be misleading and may only detect the residual activity of certain enzyme systems, making evaluation of previous work on chitin biosynthesis, in animal systems in particular, very difficult. This is why an approximation of the *in vivo* rate of synthesis would be useful in assessing results and discussing implications.

That the amount of chitin per unit area remains constant while the total chitin per larvae is decreasing after approximately 144 hours was noted, and is at present under investigation.

The system examined the complete pathway of chitin biosynthesis, rather than an isolated step. The particular optima for one enzyme may not reflect the optima for the system as a whole. The bimodal curve of the effect of pH on the incorporation of ^{14}C-AGm (Table 3) may illustrate that point. The increase in activity at 33° C was expected as a typical response in enzymatic activity. But the elevated temperature may not allow prolonged stability and may also accentuate the variability shown by the present system. Due to the relative crudeness of this system, 3-5 samples are required for each data point. It was not possible uniformly to control the condition of the epidermis following removal of the other tissues. Epidermis such as that lining the carapace of the crayfish or lobster, which has minimal muscle attachments, may be ideal for such procedures.

The rapid and substantial incorporation of AGm into chitin suggests that an active AGm kinase enzyme is present in the larvae. This is especially likely since the AGm was labeled in the acetyl moiety and since changes in the glucose concentration failed to alter the incorporation of ^{14}C-AGm.

Current work involves the continued modification of media conditions and procedures to increase the absolute rate of incorporation. However, as Table 1 shows, the conditions used allowed substantial rate of synthesis to proceed. Isolated samples have equaled or exceeded the in vivo rate of synthesis, but conditions have not been found which reproducibly provide such results. The use of ^{14}C-UDPAGm as a substrate may provide even higher rates of incorporation, since AGm must pass through additional steps prior to incorporation and these may be operating under less than optimal conditions. This would be especially true if the present conditions are closest to those required by the enzyme chitin synthetase.

This work is a first step toward cell-fractionation procedures that will examine chitin biosynthesis and chitin synthetase from animal epidermis. The present methodology allowed an evaluation of progress from in vivo to cell fractionation and proved useful for the study of inhibitors, as seen by the extremely low levels of TH-6040 needed to depress chitin biosynthesis.

CONCLUSIONS

The ability of cuticular disks of Manduca sexta larvae in the fifth instar to produce chitin from ^{14}-AGm has been demonstrated. At present, the optimal conditions involve the use of Robb's phosphate-buffered saline solution minus $CaCl_2$ at pH 7.6 at 21° C. One hour of incubation was sufficient to demonstrate chitin biosynthesis. The value of reporting incorporation based on in vivo rates is discussed. The simplicity and relatively good efficiency of the described system merits further consideration. Perhaps additional pathways could initially be studied using such a system, avoiding the burdensome and expensive aspects of more elaborate culturing procedures. The effects of certain insecticides or inhibitors can readily be examined using this system.

ACKNOWLEDGMENTS

Funds for this work were provided by NIH grant no. ES00650. The U.S. Agricultural Dept. at Fargo, North Dakota, supplied the eggs for this study. We would like to thank Dr. William Petri for his assistance.

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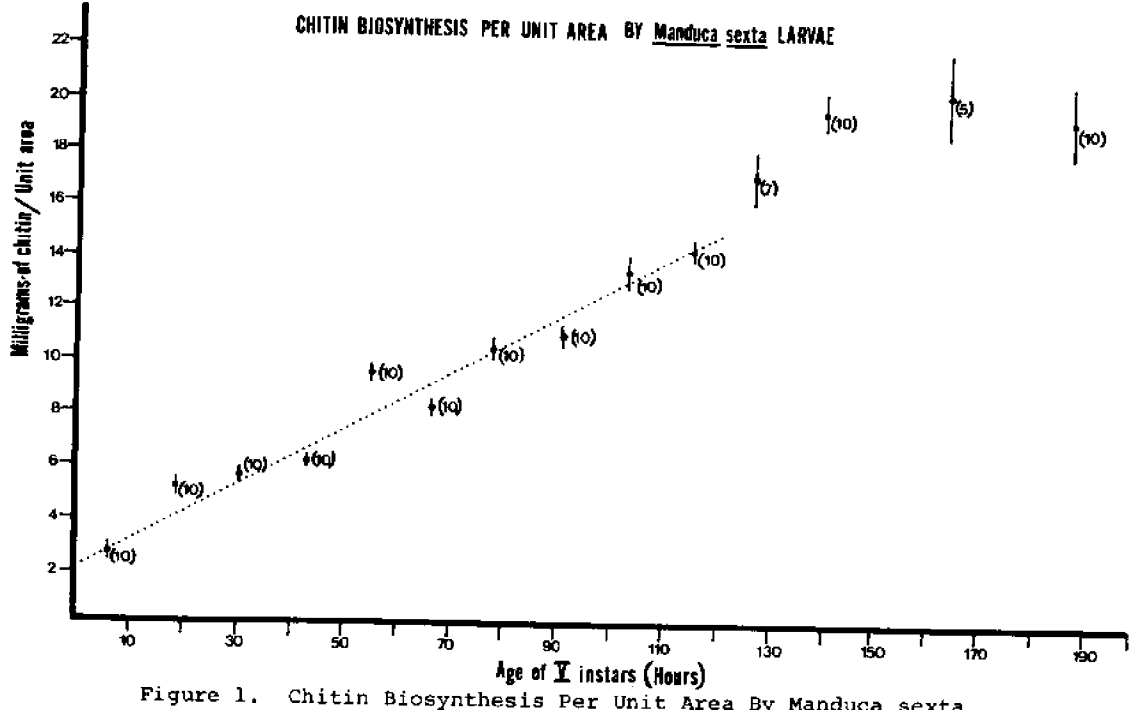

Figure 1. Chitin Biosynthesis Per Unit Area By Manduca sexta Larvae At 12 hour intervals larvae were eviscerated and the chitin content of an area 578.5 mm^2 of cuticle was calculated. Each data point represents the mean for each interval. The standard errors pass through each mean. The dotted line indicates the mean rate of synthesis from 0-120 hours post-ecdysis to the V instar. Values beyond 120 hours show means and standard errors for larvae placed in vermiculite prior to pupation. The rate of synthesis was calculated as 0.18µg of chitin/mm^2/hr. The number of animals used for each data point are in parentheses.

ENZYMATIC BREAKDOWN OF THE CHITIN COMPONENT IN INSECT CUTICLE DURING THE MOLT

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ABSTRACT

Insects degrade and resorb products of the old cuticle extensively during the molt. Using a modified chitinase assay giving reproducibly high chitinase activity, we have demonstrated that molting-fluid chitinase has an apparent requirement for calcium ions, although it is inhibited by high levels of calcium. It has a pH optimum near neutrality when attached to chitin substrate. The high endogenous specific chitinase activity previously reported as localized in old cuticle during the molt, is shown to have its origin in molting fluid. Cuticle chitin is not accessible to chitinase prior to exposure to molting fluid, and evidence is presented suggesting that trypsin-like proteases are involved in unmasking cuticle chitin so that molting fluid chitinase can attach to it and degrade it to soluble products. Molting-fluid chitinase activity can be separated into one fraction that adheres tightly and specifically to chitin substrate and one that does not. Molting-fluid chitinase activity adsorbed to chitin substrate separates into two narrow adjacent protein bands during disc gel electrophoresis; when eluted and separated after reaction with SDS, these give four protein bands. The existence of several enzymes degrading cuticle chitin serially and combined into one or more chitinase complexes is proposed.

INTRODUCTION

Insects, like other arthropods, are invested with an exoskeleton composed of chitin and protein which must be molted to permit changes in size or, in the case of holometabolous insects, in shape. With respect to the cuticle, the molting sequence may be said to begin with apolysis, i.e., the old cuticle is detached from the underlying epidermal cells which previously secreted it (7). Following apolysis, the epidermal cells lay down most of the outer layers of the new cuticle; this is followed by extensive degradation of the old cuticle and resorption and reutilization of the breakdown products (6). The sequence ends with ecdysis when the remnant of the old cuticle is shed. During the molting sequence, the space between old and new cuticle is filled with molting fluid. Passonneau and Williams (17) showed the presence of both chitinase and protease activity in molting fluid from Hyalophora cecropia (Lepidoptera: Saturniidae); Jeuniaux and Amanieu (11) investigated chitinase in molting fluid from Bombyx mori (Lepidoptera: Bombicidae), which was followed further in Jeuniaux's monograph (9). Bade reported the localization in molting cuticle of very active chitinase showing high activity in the alkaline region (1); this activity rose to considerable height following apolysis and declined abruptly prior to ecdysis (2). We are at present reinvestigating the problem of cuticle breakdown during the molt in an effort to discover more about the properties of the enzymes involved in it; using these enzymes, we hope ultimately to elucidate details of the cuticle structure of arthropods that are not readily accessible to purely chemical investigation.

Chitinase assay

Whenever "chitinase" activity is to be investigated, one is confronted with a decision as to the substrate to be used and the product to be measured. This is not the place to review and compare the many methods utilized by various investigators; suffice it to say that chitins of different origins do not give the same activity with the same enzyme, even if rate of evolution of the same product is measured (M. Bade, unpublished) and that commercially available "chitin" tends to yield substrate giving different and usually rather low rates of activity with the same enzyme when substrates prepared in successive runs of chemical preparation of chitinase substrate are compared. Substrate prepared from crustacean cuticles may be inhibiting (Table 1, Exper. 4.) and tends to be so inactive that assay times of 3 to 6 hours for chitinase activity are not uncommon (R. Keller, personal communication; 14, 15, 16, Okutani, these Proceedings).

Table 1. Chitinase Activity Under Various Conditions

Enzyme Source	Additions	Nanomoles N-Acetylglucosamine $\times$ min^{-1} $\times$ mg protein^{-1}	Residual Activity %
Manduca Molting Fluid	None*	33.6	100
	EDTA (0.05M)	3.5	10
	EDTA (0.05M) + Ca^{++}(0.05M)	38.3	114
	Ca^{++} (0.1M)	4.0	12
Streptomyces (Sigma Chemical Company)	None*	11.4	100
	EDTA (0.1M)	1.3	11
Manduca Molting Fluid	None*	10.8	100
	Ca^{++} (1×10^{-4}M)	30.8	281
Manduca (molting) Old Cuticle	None*	12.6	100
	1.2 mg colloidal chitin (from K&K "chitin")	0.4	3

*Assayed in absence of added Ca^{++}

We now report that colloidal chitin prepared from larval cuticle of insects consistently gives very high activity with chitinase preparations from both insects and Streptomyces; its use, together with other modifications to be mentioned below, permits us to assay chitinase activity in 10 minutes. Our assay system contains in a total volume of 1.0 ml: 500 mM phosphate-acetate buffer pM 7.0, 0.5 M $CaCl_2$, and 10 mg colloidal chitin substrate. Reaction is initiated with 0.01-0.1 ml of enzyme; the mixture is incubated at 37° with stirring for 15 minutes, and N-acetylglucosamine determined (18) in 0.1 ml samples removed at 5 and 15 minutes. Our enzyme unit is defined as

the amount of enzyme giving 1 nanomole of N-acetylglucosamine per minute. The presence of ample chitobiase activity should be ascertained with this assay; this can be done by measuring the rate of evolution in the presence of excess nitrophenyl N-acetylglucosaminide of material absorbing at 510 nm with suitably diluted enzyme for 10 minutes, or alternatively chitobiase may be added if this enhances the rate of production of N-acetyl glucosamine in the assay (10).

Several details deserve comment. A ten-fold higher substrate concentration than recommended in the literature (10) and customarily used (e.g., 15) was necessitated by the finding that concentrations of chitin substrate of 10 mg/ml or higher are required to give maximal chitinase activity (Figure 1). Both insect and Streptomyces chitinase appear to require calcium ions for maximal activity (Table 1); however, added calcium must be kept low since insect chitinase is inhibited by calcium above about 50 mM (Table 1) and molting fluid usually contains some calcium (Bade, unpublished). As will be presently shown, a part of molting-fluid chitinase is tightly absorbed to chitin substrate; the absorbed chitinase activity has a pH optimum near neutrality (Figure 2), i.e., much closer to the pH of molting fluid (M. Bade, unpublished; A. Jungreis, unpublished, 12) than the acid pH optimum previously reported. All activities here reported were, therefore, measured at pH 7.0.

Origin of cuticle chitinase

Localization of very high chitinase activity in Manduca old cuticle has previously been reported (1); this activity has shown to be held tenaciously and to rise abruptly following apolysis and to fall precipitously prior to ecdysis (2). With the use of highly active chitin substrate it was possible to demonstrate a low grade chitinase activity in new cuticle as well (Table 2); however, chitinase activity in cuticle prior to apolysis is directed entirely against exogenously added substrate. "Endogenous," i.e., tightly held chitinase activity which needs no added exogenous substrate

Table 2. Chitinase Activity in Cuticle Prior to Initiation of the Molt Sequence

	mg N-acetylglucosamine per hour per mg protein*	
	With Added Substrate	Without Added Substrate
Chitinase in Premolt Cuticle	7.3 ± 1.3	0.18 ± 0.18

*24 experiments ± std. deviation; all assayed without adding Ca^{++}

for its demonstration, can be elicited in cuticle harvested from Manduca larvae 40 hours prior to apolysis for the larval-to-pupal molt (Figure 3, curve B); chitinase activity was measured by incubating cuticle fragments for the indicated time with molting fluid, then washing them free of molting fluid and determining rate of N-acetylglucosamine production from chitin endogenous to the cuticle fragments. The phenomenon of development of endogenous activity at a linear rate upon incubation with molting fluid, together with much additional

evidence seemingly consistent with it, led to the proposal that chitinase is laid down as the proenzyme in cuticle at the time of cuticle synthesis and that one of the functions of molting fluid is the activation of cuticle prochitinase at the time of molting (3,5). However, the demonstration that similar "endogenous" activity develops more rapidly to even higher specific activities when cuticle is deproteinized prior to incubation with molting fluid (Figure 3, Curve A) put an end to such speculation.

The gradual development of "endogenous" chitinase activity as intact cuticle by incubating with molting fluid is, therefore, in part apparently due to gradual penetration of molting-fluid chitinase activity into the fairly dense layers of cuticle chitin. It must, however, also be due in part to a process which renders the chitin of intact cuticle competent to serve as substrate for chitinase. It has already been mentioned that endogenous cuticle chitin is not degraded by the low chitinase activity present in larval cuticle prior to onset of the molt; as the molt progesses, cuticle chitin becomes able, at first gradually and then rapidly, both to attach molting-fluid chitinase and to serve as substrate for it.

Cuticle transformation during the molt

One can advance several possible mechanisms for rendering cuticle chitin initially unreactive, and explanations for later rendering it competent as substrate would vary accordingly. One of these mechanisms, the activation of a prochitinase, has been ruled out decisively. Another one, masking of cuticle chitin by protein which is removed by a protease contained in molting fluid, is made likely by the finding that the development of endogenous chitinase activity in intact cuticle through exposure to molting fluid is inhibited by trypsin inhibitors. This is shown in Table 3. It can also be seen that chitinase reaction with colloidal cuticle chitin is not affected by the presence of the same inhibitors; it therefore appears probable that the effect of trypsin inhibitors is on some reaction associated with the state of chitin substrate in intact cuticle rather than on chitinase itself.

The nature of molting chitinase

Following Jeuniaux (10) the chitinolytic system is commonly stated to consist of a chitinase (EC 3.2.1.14), which breaks down the substrate macromolecule and smaller polymers, and a chitobiase (EC 3.2.1.29), which produces N-acetylglucosamine from chitobiose. The presence of a considerable level of chitobiase activity in Bombyx molting fluid has been confirmed by Kimura (14, 15), and we have demonstrated the presence of chitobiase in molting fluid as well as in blood of the molting and non-molting Manduca larvae. However, persuasive evidence exists suggesting that not all residues in animal chitin are acetylated (19) and that animal chitin is a glycoprotein, i.e., is covalently attached to (cuticle) protein (20). If one were to accept hydrolysis of such chitin by a chitinolytic system with just one chitinase, one would have to postulate an enzyme with so little specificity that it is equally ready to break bonds in the vicinity of a nitrogen atom that bears a bulky acetyl group and one that carries a full positive charge. Further, no provision exists in this scheme for hydrolyzing the bonds that may anchor the chitin to protein. We therefore propose that several enzymes act serially and together to degrade animal cuticular chitin, different enzymes splitting the acetal bond involving the anomeric carbon adjacent to free and acetylated nitrogens, respectively, and separate ones splitting whatever bonds bind chitin to protein.

Table 3. Chitinase Activity in Whole Cuticle vs. Chitinase Activity with Colloidal Chitin: Effect of Trypsin Inhibitors (n = number of trials)

Addition	n	% Residual Endogenous Chitinase Activity Following Incubation with Molting Fluid: Whole Cuticle	n	Insect Colloidal Chitin
None	8	100 ± 20	2	100
O-phenanthroline*	2	89	-	n.d.
TPCK (chymotrypsin inhibitor)	2	117 ± 17	-	n.d.
p-methylsulfonyl fluoride	3	42 ± 6	2	97
TLCK (trypsin inhibitor)	3	17 ± 2	-	n.d.
Soybean trypsin inhibitor*	3	23 ± 1	2	100
Ovomucoid trypsin inhibitor*	2	45 ± 4	2	99
DFP	2	17 ± 1	-	n.d.

*Assayed in the presence of 8-hydroxyquinoline, which tends to enhance chitinase activity.
n.d.: not determined.

Are a variety of chitin-splitting enzymes present in molting fluid? The literature provides hints, and our own most recent work indicates that the molting-fluid chitinolytic system may indeed be heterogeneous. In 1975, Winicur and Mitchell (21) published a paper showing with each larval molt in Drosophila a rise of enzymatic activity that splits chitosan. The authors called it chitinase activity, but, as just stated, identity of chitinase and chitosanase seems somewhat unlikely. Kimura in 1976 showed that molting-fluid chitinase and chitobiase activity can be separated into several fractions by ammonium sulfate precipitation and by salt gradient elution from DEAE-cellulose (15). Using our sensitive chitinase assay, we have shown that some of the chitinase activity present in Manduca molting fluid adsorbs preferentially to chitin; this is shown on Table 4. Preferential adsorption of chitinase to chitin has been known for some time, and indeed was used by Jeuniaux in purifying Streptomyces chitinase (8). The interest in our new data lies in the following observation: when molting fluid is treated once with chitin, 10% of protein present (and 10% of protease) is adsorbed to chitin, while over 50% of chitinase is adsorbed. A much smaller fraction of the chitinase left in solution is adsorbed to chitin, however, when the supernatant from the first adsorption is cycled past chitin a second time; in fact, nearly the full amount that would be present if the second chitinase adsorption were non-specific is recovered in the supernatant from the second adsorption. Thus, molting-fluid chitinase can be divided into a fraction that specifically and nearly irreversibly adsorbs to chitin, and one that does not.

Table 4. Adsorption of Chitinase to Chitin during Successive Treatments of Supernatant with Chitin

	Chitinase Initially Present	Chitinase Precipitating with Chitin		Chitinase Remaining in Solution	
	Enzyme Units	Enzyme Units	%	Enzyme Units	%
1st adsorption Found	117	66	56	51	44
2nd adsorption Found	31	6	20	26	84
Expected with specific adsorption		17	56		
Expected with non-specific adsorption				28	90

We have subjected the fraction that specifically adsorbs to chitin to electrophoresis on polyacrylamide gels. At neutral or alkaline pH, 8 bands can be visualized by staining with Coomassie Brilliant Blue. Chitinase activity can be shown to be present in two narrow bands running close together in about the middle of the column. When these two bands are eluted and subjected to SDS-electrophoresis, 4 SDS-protein bands are obtained. We suggest that the "chitinase" activity strongly adsorbing to chitin may be a complex of several enzymes and that both the complex and the chitinase fraction which does not strongly and specifically adsorb to chitin may play a role in normal cuticle degradation during the molt.

ACKNOWLEDGMENTS

This work was supported by Grant ES00650 from the National Institutes of Health. Manduca eggs were furnished by the Department of Agriculture Field Station at Fargo, North Dakota. We are grateful for the dedicated and skillful assistance of Alfred Stinson.

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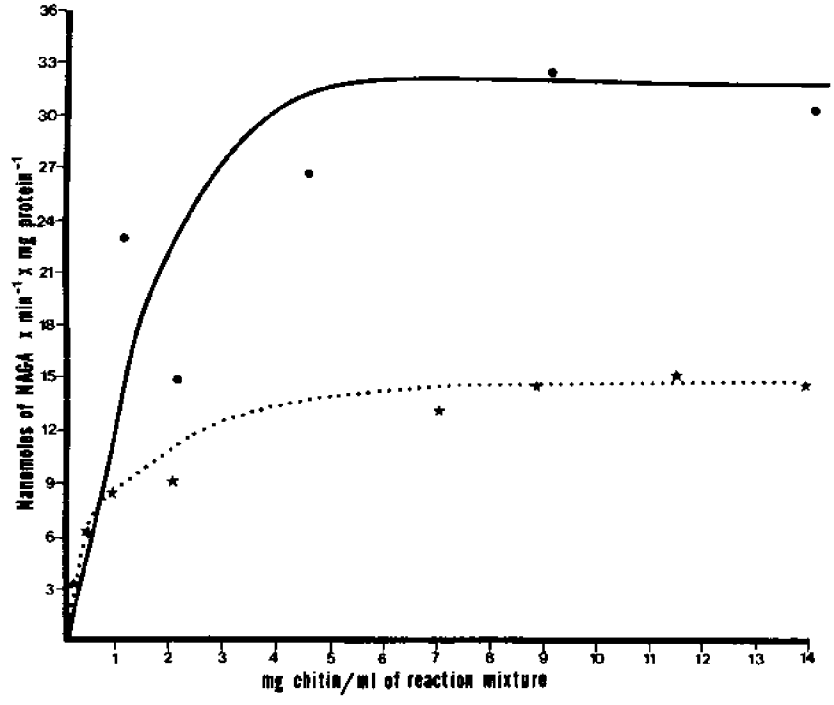

Figure 1. Initial Rate of N-acetylglucosamine Production by Chitinases Incubated With Varying Amounts of Insect Colloidal Chitin.
• Molting Fluid Chitinase
⋆ Streptomyces Chitinase
NAGA: N-acetylglucosamine

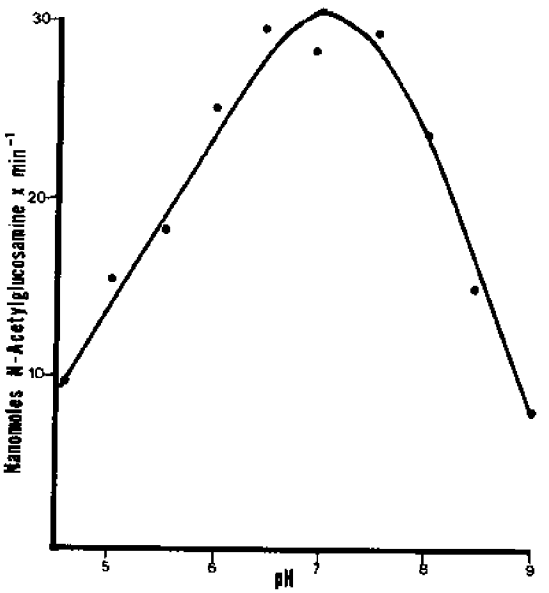

Figure 2. pH-Activity Curve for Molting Fluid Chitinase Adsorbed to Insect Colloidal Chitin.

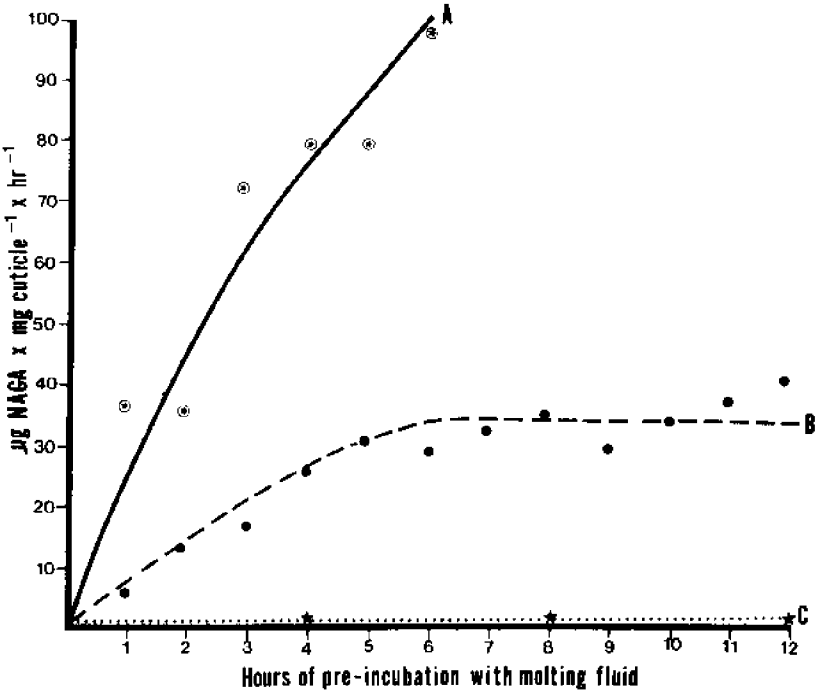

Figure 3. "Endogenous" Chitinase Activity Elicited In Cuticle Through Incubation With Molting Fluid
⊛ Deproteinized Cuticle
• Intact Cuticle
★ Boiled Molting Fluid

ULTRASTRUCTURE OF CRAB CHITIN AND CHITOSAN REVEALED WITH THE SCANNING ELECTRON MICROSCOPE

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ABSTRACT

In the search for inexpensive supports for enzyme insolubilization and adsorbents for scavenging toxic metallic ions from industrial wastes, we found several natural substances and by-products such as bark, waste wool and other keratins and crab-shell chitin and chitosan to be suitable. The use of these biopolymers involves processes of interfacial non-homogenous interactions when they are employed as insoluble matrices in contact with solutes. For this reason, the physical structure and ultrastructure of the solid polymers may have a bearing on the nature and course of the interactions. To gain insight into the role that the physical ultrastructure and the matrix might play, we examined crab shell and related materials with the scanning electron microscope. Ultrastructural features not yet reported were revealed. Two main features are: fine laminated layers of microfibrils in the plane of the layers and a columnar microarchitecture produced by inter-layer microfibrils with mineral grains laid down among them.

INTRODUCTION

In our work on enzyme insolubilization and on removal of toxic and heavy metals from industrial wastes, we examined a number of biopolymers and waste by-products in search of inexpensive physical supports that would be suitable for those purposes (4,5). Chitin and chitosan were among the promising substances examined. From these experiments we propose the use of chitosan flakes in their native state as prepared from chitin with alkali, without solubilizing the chitosan. In some of this work, the chitosan chips before use were first partially crosslinked under mild conditions that left their native structure essentially intact (Masri and Randall, these Proceedings). The crosslinking was done to impart insolubility to the treated chips in acidic media. The binding of metal ions and the fixing of enzymes to the chitosan or crosslinked chitosan chips (as well as the crosslinking reaction itself) proceeded rapidly and extensively, and equilibria were quickly established, despite the fact that these interactions with reagents or solutes occurred by two-phase heterogeneous processes. This result prompted us to examine chitosan chips and related chitinous materials with the scanning electron microscope to check whether ultrastructural features were present that could explain these fast interactions. The microarchitectural features observed contribute to the understanding of the rapid penetration of solutes into the native chitosan matrix and subsequent interactions with it. The main observed features will be shown and described. Pertinent references on the morphology of the crustacean exoskeleton appear in the literature (1-3,6,7). Our findings correlate with, and supplement, these studies.

EXPERIMENTAL

Observations were made on demineralized chitin and chitosan chips (presumably derived mainly from king and some Dungeness crab shells) obtained from Food Chemical Company, Seattle, Washington. A limited number of shell fragments from king-crab legs and whole Dungeness crab purchased from a local market were also examined, together with the shell of a red crab caught in the Bolinas lagoon in northern California. The crab-shell fragments were examined as received, or after decalcification with dilute hydrochloric acid, or after deacetylation of demineralized fragments with alkali in an autoclave.

A Jeolco scanning electron microscope was used, operated at 25 kV. Specimens were mounted on metal holders with double-coated sticky cellophane tape and silver cement. Objects were gold coated. Methods described by Wise for molluscan shell examination were followed (8).

RESULTS AND DISCUSSION

From many fascinating photographs of different views taken, we have selected nine (Figures 1-9) to show the main ultrastructural observations. All photographs are scanning-electron micrographs except Figure 1, which was taken with the light microscope. The chitosan flakes referred to in the figures are from the commercial sample (see above, Experimental) which was presumably mainly from king crab. The intricate microarchitecture newly revealed in the photographs includes the characteristics described below.

Laminated layers: These were parallel to the outer surface of the shell. Although the fineness varied even with specimens from different parts of the same shell, in general, the king crab showed the finest laminations of 12,000 layers/cm, the Dungeness crab had intermediate fineness of 4,000-6,000 layers/cm and the red crab had the coarsest lamination of 1,200 layers/cm. The fineness also varied in the fragments of commercial chitosan from 7,000 to 12,000 layers/cm, but as many as 55,000 layers/cm were observed in highly iridescent chips. As would be expected, these finely laminated chips give multiple film interference colors. These observed differences in fineness of lamination are likely due not only to differences in species but also to the particular location on the exoskeleton, age and stage of development, individual variations, and sampling. Figures 1-4 and 7 show this layering.

Planar and interlaminar fibrils: The layers themselves appear to be made up of fibrils whose orientation within a layer could not be clearly seen, although the arrangement appears to be ordered rather than random as suggested in Figures 5 and 6. In addition to the inner-layer fibrils, transverse fibrils bridging the layers were also present (Figures 2,4,8-9). Holes and short "spikes" in the layers probably represent points where the transverse cross-fibrils have been sheared or torn out of adjacent layers. Alternatively, the holes and spikes may represent a native structure rather than an artifact; in this case, the holes provide channels for the inter-layer communication, and the spikes constitute either rigid or flexible supports that help to maintain layer separation (Figures 5 and 6).

Columnar microarchitecture: The inter-layer spacings appear to consist of interlaminar (transverse) fibrils with the mineral grains laid down

between them; the arrangement gives a columnar appearance that could readily be seen in mounted fragments that fortuitously broke off obliquely (Figures 7-9). When the mineral was removed with dilute hydrochloric acid, the columnar structure disappeared and the transverse fibrils appeared collapsed on the edges of the main layers; the surface then appeared to be covered with a gelatinous film.

These observations contribute to the understanding of the rapid penetration and interaction of solutes.

ACKNOWLEDGMENTS

We thank Drs. Daniel H. Gould and Wilfred H. Ward from our laboratory for critical review of the manuscript, Robert David Wong for art work in mounting micrographs, and Dawn M. Thorne for help in preparing the manuscript.

Reference to a company and/or product name does not imply approval or recommendation of that product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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Fig. 1. Cross-section of a chitosan flake in water. Upper section: 11,000 layers/cm; lower: 7,000 layers/cm (light microscope).

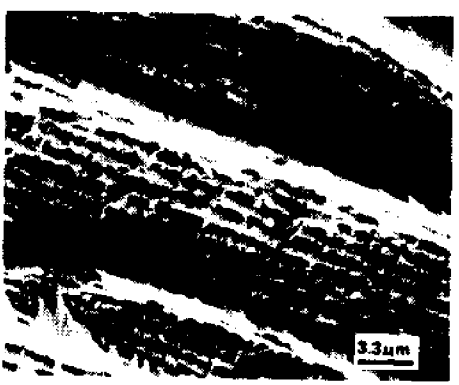

Fig. 2. Chitosan cross-section: 9,000 layers/cm showing interlaminar (transverse) fibrils holding layers together.

Fig. 3. Cross-section of raw shell of red-crab carapace. Finer lamination nearer inner surface (lower part of fig.).

Fig. 4. Red-crab carapace showing layers and interlaminar (interlayer) fibrils. Tip on right was briefly dipped in dilute hydrochloric acid then quickly rinsed to remove surface calcium carbonate; mineral grains are still present on left.

Fig. 5. Peeled layers of crab chitosan, obtained by tearing dry flake, showing planar (intra-layer) fibrils, holes in layers and spikes protruding from layers. Holes and spikes are probably produced by peeling, causing transverse fibrils to be pulled out and broken. One end (spike) remains attached to one layer and pulled out from the adjacent layer, leaving a hole.

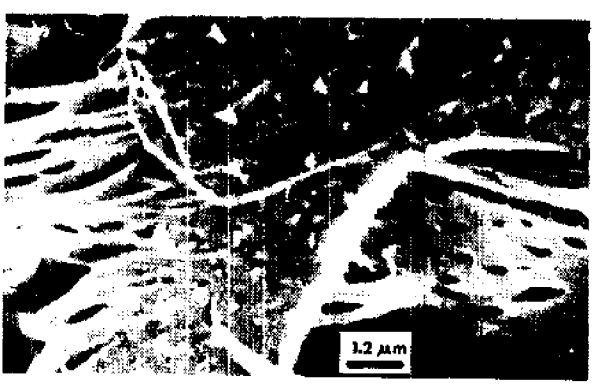

Fig. 6. Photographically enlarged part of Fig. 5 showing holes and spikes in layer.

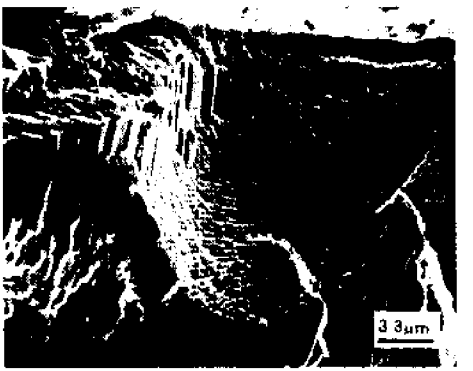

Fig. 7. Cross-section of Dungeness-crab carapace. Outer part (top) broken obliquely, showing both layers (middle center) and columnar structure (toward upper left); calcium carbonate is present (chip not demineralized).

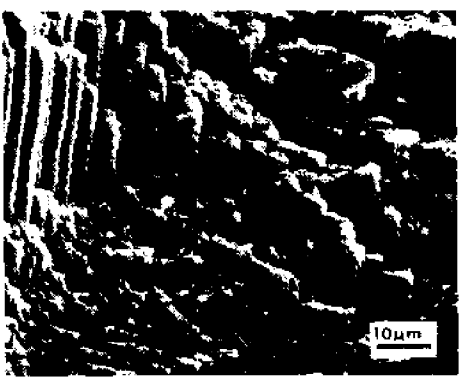

Fig. 8. Close-up part of Fig. 7 (by electron microscope).

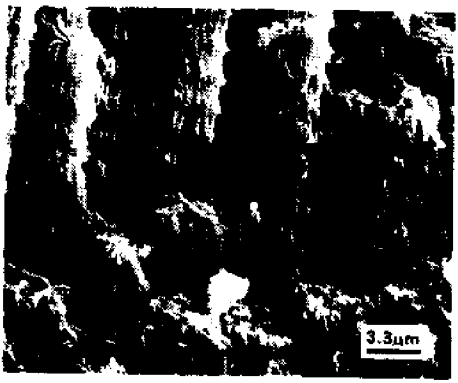

Fig. 9. Enlarged view from Fig. 8 (by electron microscope) showing bundles of transverse (interlaminar) fibrils in the substructure of the columns containing the calcium carbonate.

CHITIN FIBER ARRANGEMENT IN ARACHNID AND MYRIAPOD CUTICLE

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ABSTRACT

The chitin fiber arrangement in arachnid and myriapod cuticle was studied, employing the common Indian forms, viz., the black and brown scorpions Palamnaeus bengalensis and Buthus tamulus gangeticus, the whip-scorpion Thelyphonus giganticus, the giant spider Poecilotheria regalis and the millipede Thyroglutus malayus. Light microscopy and x-ray diffraction after gentle extraction of the cuticle were used.

The chitin fiber arrangement provides several laminae to the cuticle as discrete and separable sheets of varying lengths. The laminae are composed of "lamellae," i.e., patterns appearing in oblique sections due to a helicoid arrangement, perhaps of continuous fibers, and anastomosing into a mat in the laminae. These become separable or torn as sheets along the horizontal plane but never along the vertical planes, the fibers being discontinuous in the interlaminar zone.

X-ray diffraction patterns of the extracted cuticle, i.e., chitin, revealed a marked heterogeneity along the axis normal to the body, indicating that the fibers were oriented equally in all directions among the various horizontal planes of the cuticle. Crystallinity appeared along both the axis parallel to the surface and transverse to the body and the axis parallel to the surface and longitudinal to the body, indicating a longer i.e., higher, pitch of the helices.

INTRODUCTION

The fine structure of the arthropod cuticle has attracted the attention of several workers in the recent past. Drach (8, 9) found that the laminae of the crab cuticle were separable, though connected by fibers continuing from one lamina to the next as parabolic arcs. This view was supported by Dennell (4) and Locke (11). Locke (12) described how the endocuticle of the insect cuticle was composed of numerous lamellae, which were shown in an electron microscope as patterns of microfibers arranged in sheets and curving out at right angles between the sheets. They had, however, the same orientation in the adjacent sheets.

Bouligand (1, 2, 3), studying the cuticle of a crab Carcinus maenas (L), reported that it consisted of fine fibers arranged in a helical manner which gave rise to a laminated appearance in vertical and oblique sections and to a plumose appearance in the interlaminar region in oblique sections. The parabolic arcs seen crossing between the laminae were not due to continuous fibers but were patterns resulting from the differing angles of sections of the fibers in successive layers. Neville and Berg (14), Neville and Luke (15, 16), and Neville, Thomas and Zelazny (17) have supported the view of Bouligand.

Dennell (5) examined the cuticle of Carcinus maenas (L) by light microscopy and suggested that the laminae were discrete and separable plates which could be pulled off from one another. Laminae were, however, connected by interlaminar

fibers which were branched and frayed in appearance. Between these fibers were long unoriented macrofibers which did not stain with anilin blue. The interlaminar fibers and the macrofibers accounted for the plumose appearance of the interlaminar zone. Ejike (10) also found discrete lamellae, as described by Dennell (5), in the cuticle of another crab, Callinectes gladiator. He, however, described large, sinuous, unbranched macrofibers in the main calcified zone, which often traversed parabolic paths and passed from one interlaminar zone to the next. In the overlying pigmented zone, macrofibers were branched and densely packed In addition, there were finer fibers between the macrofibers which were straight and parallel. The macrofibers were found to be hollow.

Dennell (6) also found that in the cuticle of Cancer pagurus L. and Carcinus maenas (L) the fine fibers passed obliquely through the laminae in planes parallel to the four faces of a gently sloping pyramid. The parabolic arcs (interlaminar fibers) were found to lie in a plane tilted at a steeper angle and oblique to the planes of the fine fibers. The laminae were reported to be discrete, as they preserved their identity around the angle formed by two verticle faces meeting at right angles as Drach (9) found. Thus the view of Dennell (6) did not support the model proposed by Bouligand (1, 2, 3).

Mutvei (13) studied the special arrangement of the chitin-protein fibers in the cuticle of two decapod crustaceans, Homarus gammarus L and Carcinus meanas (L), with the scanning electron microscope and described numerous horizontal lamellae in the cuticle, which gave rise to numerous vertical lamellae and were connected with them. Vertical lamellae emerged from both sides of each horizontal lamella, and hence they were not artifacts. Both the horizontal and the vertical lamellae were pierced by pore canals. Each pore canal had a wall of its own composed of vertical fibers. Dennell (7) also described the laminae in the scorpion Pandinus imperator (Koch) as being composed of horizontally arranged fibers associated with a laminar membrane, and he reported that curved continuous sheets of fibers passed from one lamina to the next through the interlaminar region.

In view of these recent findings about the ultrastructure of insect and crustacean cuticles, it was decided to verify and extend the studies to other arthropods, viz., the arachnids and the myriapods.

MATERIALS AND METHOD

The common Indian arachnids and myriapods, e.g., the black and the brown scorpion, Palamnaeus bengalensis and Buthus tamulus gangeticus, the whip scorpion, Thelyphonus giganticus, the giant spider, Poecilotheris regalis and the diplopod Thyroglutus malayus were used in this study. Pieces of cuticle were taken from regions where it was thickest, e.g., the cephalothorax of Palamnaeus and Buthus, the pedipalpi of Thelyphonus and Poecilotheria and the dorsal tergites of Thyroglutus. Cuticle pieces fixed in 5% neutral formalin were extracted with 5% solution of KOH for a period of one month and then washed thoroughly in water. The millipede cuticle was decalcified before extraction with 30% aqueous solution of sodium hexametaphosphate (20). Sections (4-6μ in thickness) were cut on a cold microtome (Cryo-cut) and were stained with Mallory's triple stain. The preparations were examined under the phase-contrast microscope. A piece of cuticle was cut obliquely from its two adjacent sides, and the corner between these two sides was examined under phase-contrast microscope.

For x-ray diffraction studies, rectangular cuticle pieces cut lengthwise along the body axis of the scorpion Palamnaeus bengalensis, as described above, were gently KOH extracted, washed and piled together to give approximately a 2 mm sheet. Pieces from this sheet were variously cut and mounted over a narrow slide hole so as to provide orientations in three directions: axis normal to the body, axis parallel to surface and transverse to body, and axis parallel to surface and longitudinal to body. X-ray diffraction photographs of these samples were taken using Kα radiations. The x-ray tube was operated on 30kV, 10mA. A flat film Laue camera was used, the distance between the sample and the x-ray film being 5 cm and exposure varying from 4 to 6 hours.

OBSERVATIONS

The arrangement of chitin fibers is described in the different arachnids and myriapods examined, as follows:

Palamnaeus bengalensis: Typical plumose lamellae (Fig. 1) numbering 12-16 were seen in oblique sections of cuticle in regions where it was thick. The interlamellar distance increased with increasing obliquity of sections. Parabolic emerged at acute angles from lamellae.

Four to five horizontal sheets, i.e., laminae, became separable in the alkali-extracted cuticle. Mechanical separation of laminae was also seen to have taken place in transverse sections cut on cold microtome.

The chitin fiber arrangement was present in the surface view of all the sections cut horizontally, a polygonal pattern of dense lines (Fig. 2) enclosing shining dots representing the cut ends of the chitin fibers. In regions of these sections where the cuticle begins to tilt, these patterns and dots also become progressively elongated and parallel (Fig. 3) and finally merge into a plumose pattern (Fig. 4).

Helical pore canals, as distinct from dermal gland-duct openings, could be identified under changing focus in regions where some of the pore canals ar especially large, e.g., on tubercles in pedipalpi.

Buthus tamulus gangeticus: Chitin fiber arrangement was the same as in Palamnaeus. Three to four laminae and nine to ten lamellae were seen in obliq sections. The dermal gland duct's oblique cut ends were also seen along with the lamellae (Fig. 5). These showed helical patterns above the exocuticle in the surface view (Fig. 6). Pore canals distinct from lamellae were seen in transverse sections in regions of tubercles (Fig. 7), but these were more clearly distinguishable in a surface view of the cuticle, because they ended peculiar funnel-shaped dilations in Buthus (Fig. 8).

Thelyphonus giganticus: Eight to ten laminae were seen mechanically separated in transverse sections (Fig. 9). Parabolic emerged at acute angles, but became straight in interlamellar regions (Fig. 10). Patterns of fibers enclosing polygonal areas seen in the surface view were present in the separated laminae (Fig. 11).

Thyroglutus malayus: Four to five laminae were separable in the inner endocuticle region. Laminae could also be seen at angles of obliquely cut

cuticle pieces (Fig. 12). Eight to ten lamellae were present only in the inner endocuticle with typical parabolic in the interlaminar regions (Fig. 13); lamellae were absent from the outer endocuticle.

Poecilotheria regalis: Four to five laminae and 12-14 lamellae (Fig. 14) were distinguishable. Helical pore canals distinct from lamellae were clearly seen in transverse sections (Fig. 15). However, polygonal patterns were not seen in surface view of all horizontal sections below the exocuticle. These were present only in the exocuticle which was separable as a lamina (Fig. 16).

X-ray diffraction patterns: In the beam normal to the body, continuous rings were obtained (Fig. 17), indicating a marked heterogeneity. On the other hand in both the beam parallel to surface and transverse to body (Fig. 18) and the beam parallel to the surface and longitudinal to the body (Fig. 19) crystallinity appeared, but it was not sufficient to characterize the type of chitin present.

DISCUSSION

These observations show that the cuticle of arachnids and myriapods, as exemplified by the cuticle of the scorpions Palamnaeus bengalensis and Buthus tamulus gangeticus, the whip scorpion, Thelyphonus giganticus, the giant spider, Poecilotheria regalis, and the millipede, Thyroglutus malayus is formed of helicoid chitin fibers as reported by Bouligand (1, 2, 3). These fibers produce typical plumose and lamellate patterns with parabolie in the interlaminar regions seen in oblique sections, the interlamellar distance increasing toward the inner side of the cuticle, except in Thelyphonus where they decrease. Chitin fibers are periodically discontinuous; hence several laminae became separated in the extracted cuticle or were mechanically detached, as in the transverse sections cut on a cold microtome. Furthermore, in the spider cuticle the exocuticular lamina bearing polygonal patterns visible in surface view and distinct from the rest of laminae became separated in the extracted cuticle, suggesting that laminae are realities, while lamellae are simply patterns. Dennell (5) reported that in crab cuticle the upper surface of the lamina showed a distinct bounding membrane and that interlaminar fibers arose from the lower surface of this bounding membane and were continuous from one lamina to the next. But in the arachnid and myriapod cuticle neither the bounding membranes nor the interlaminar fibers are present.

In addition to the microfibers, macrofibers were reported by Dennell (5) as present in the interlaminar zone where they pursued an oblique course across a number of interlaminar zones, crossing each other as they did so. Ejike (10) reported two types of macrofibers in two different zones of the crab cuticle. In the pigmented zone the macrofibers were branched, while in the underlying main calcified zone the macrofibers were unbranched, and they traversed sinuous and often parabolic paths. They lay in the interlaminar zone and traversed the laminae, passing from one interlaminar zone to the next. Macrofibers could not be detected in the present study. Helical pore canals, as reported by Shrivastava (10), pierced the cuticle of the scorpions Palamnaeus and Buthus from below up to the inner boundary of the epicuticle and, in the Buthus described, ended in peculiar cup-shaped dilations beneath the epicuticle. These have been confused with the chitin-fiber cut ends in the surface view of the cuticle of the scorpion Pandinus imperator (Koch) (7).

The fine structure of the arachnid and myriapod cuticle thus seems to conform, on the one hand, with the basic insect pattern suggested by Bouligand (1, 2, 3) viz., constituted of helicoid fibers responsible for plumose and lamellate patterns in oblique sections. On the other hand, it resembled the crustacean cuticle in possessing separable laminae (5), perhaps because of periodic discontinuity of the chitin fibers. Further modifications, such as the presence of bounding membrane in laminae and of interlaminar fibers emerging from the lower surface of bounding membranes, as reported for the crab cuticle, were not present in the arachnid and myriapod cuticle. Nevertheless, a more detailed and extensive survey convering these groups of arthropods is warranted.

X-ray diffraction patterns of the extracted cuticle chitin revealed a marked heterogeneity along the axis normal to the body, indicating the fibers were oriented equally in all directions among the various horizontal planes of the cuticle. Crystallinity appeared along both the axis parallel to the surface and transverse to the body and the axis parallel to the surface and longitudinal to the body indicating a longer, i.e., higher, pitch of the helices of chitin fibers. Some reflections corresponding to those of chitin as given by Rudall (18), viz., (a) depicting separation of chitin chains in planes parallel to the sugar ring and (b) depicting separation of chitin in the plane perpendicular to the sugar ring, and some other reflections of the three main layer lines were obtained. The first row line drawn by Rudall corresponded in this specimen to a β-chitin pattern, although this seems highly unlikely. Since the reflections were not sufficient to characterize the type of chitin present, another sample with better aligned fibers should therefore be sought.

ACKNOWLEDGMENT

The award of a University Grants Commission (India) Junior Research Fellowship to Archana Dutt is gratefully acknowledged.

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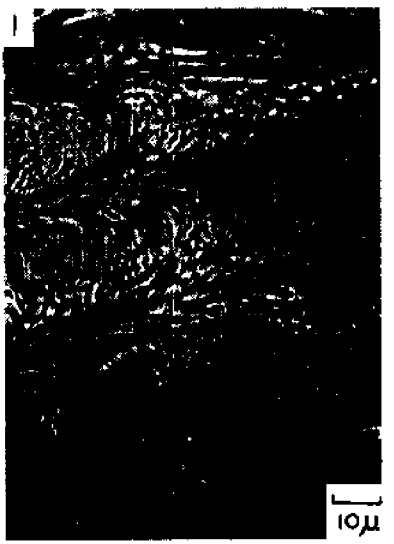

Fig. 1. Photomicrograph of oblique T.S. through KOH treated pedipalp cuticle of Palamnaeus bengalensis.

Fig. 2. Photomicrograph showing polygonal patterns of dense lines and shining dots seen in surface view of the cuticle of Palamnaeus bengalensis.

Fig. 3. Photomicrograph showing polygonal patterns becoming progressively elongated and parallel seen in surface view of the cuticle of Palamnaeus bengalensis.

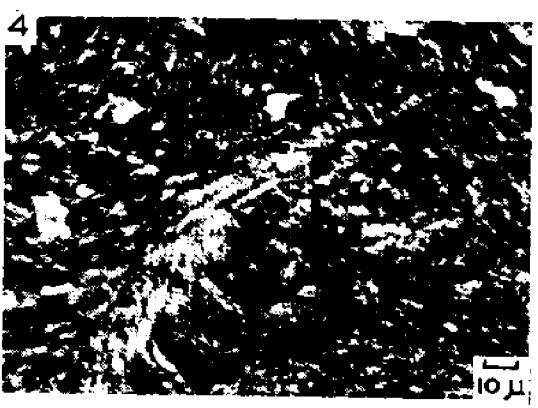

Fig. 4. Photomicrograph showing polygonal patterns emerging into a plumose pattern as seen in surface of the cuticle of Palamnaeus bengalensis.

Fig. 5. Photomicrograph of oblique T.S. through KOH treated cuticle of Buthus tamulus gangeticus.

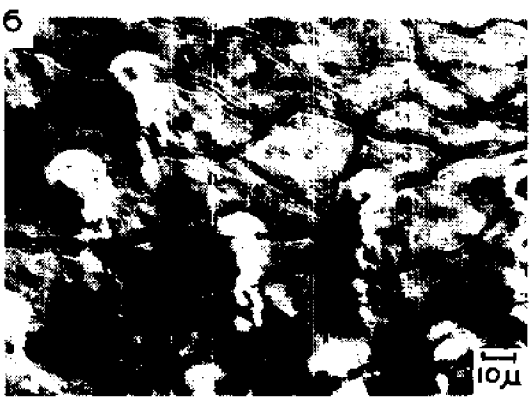

Fig. 6. Photomicrograph showing helical patterns of dermal gland duct seen obliquely in surface view of the cuticle of Buthus tamulus gangeticus.

Fig. 7. Photomicrograph showing pore canals in oblique T.S. of KOH treated tergite cuticle of Buthus tamulus gangeticus.

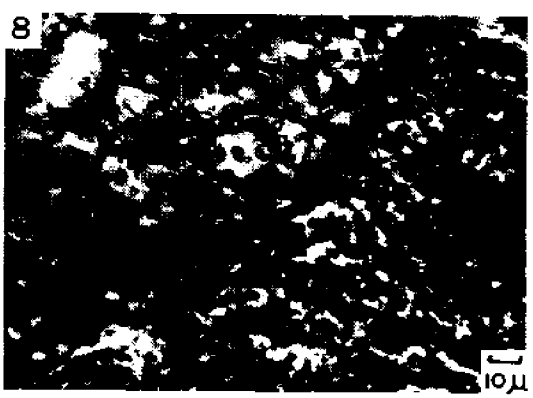

Fig. 8. Photomicrograph showing cup shaped dilations of pore canals as seen in surface view of the cuticle of Buthus tamulus gangeticus.

Fig. 9. Photomicrograph of T.S. through KOH treated pedipalp cuticle of Thelyphonus giganticus showing a few of the laminae separated from the cuticle.

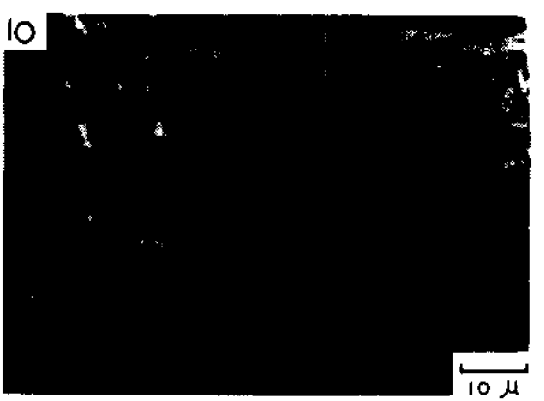

Fig.10. Photomicrograph showing microfibres in oblique T.S. of KOH treated pedipalp cuticle of Thelyphonus giganticus.

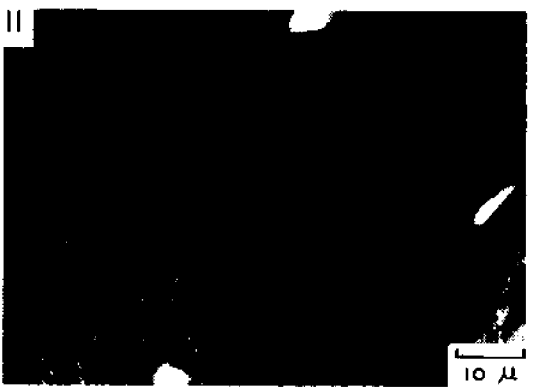

Fig.11. Photomicrograph showing pattern of microfibres in surface view of a laminae of KOH treated pedipalp cuticle of Thelyphonus giganticus.

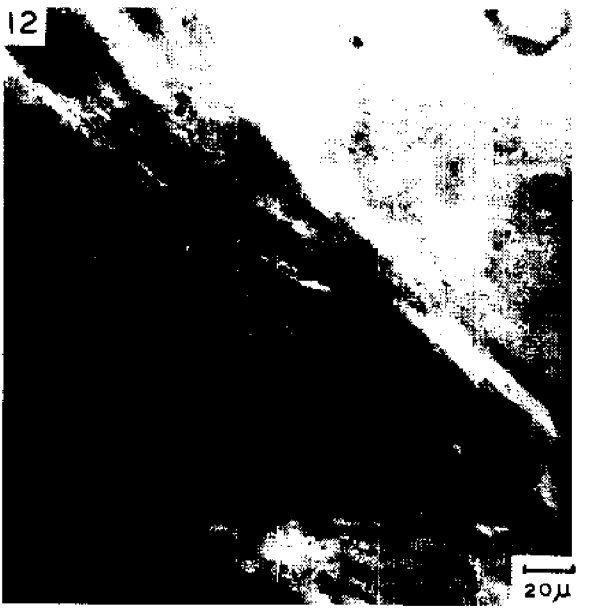

Fig.12. Photomicrograph showing the laminae in KOH treated cuticle of Thyroglutus malayus being continuous from one face of the obliquely cut corner to the other.

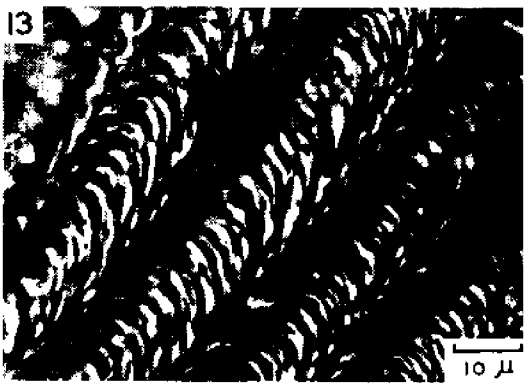

Fig. 13. Photomicrograph showing microfibres in oblique T.S. of KOH treated tergite cuticle of Thyroglutus malayus.

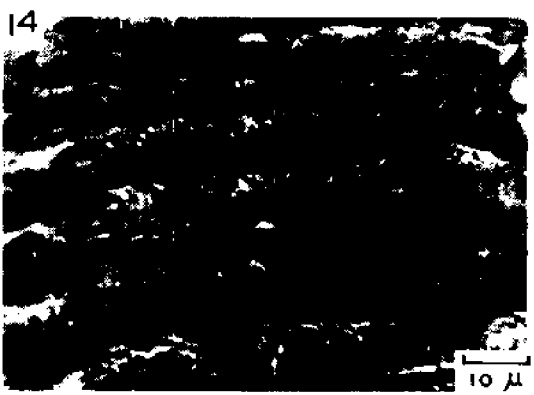

Fig.14. Photomicrograph showing microfibres in oblique T.S. of KOH treated pedipalp cuticle of Poecilotheria regalis.

Fig. 15. Photomicrograph of oblique T.S. through KOH treated pedipalp cuticle of Poecilotheria regalis.

Fig. 16. Photomicrograph showing lamina of exocuticle bearing polygonal patterns as seen in surface view along with the underlying lamina of the cuticle of Poecilotheria regalis.

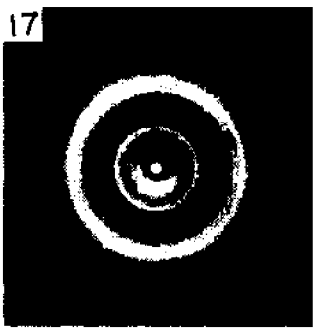

Fig. 17. X-ray diffraction photograph of extracted cuticle of Palamnaeus bengalensis with beam normal to the body.

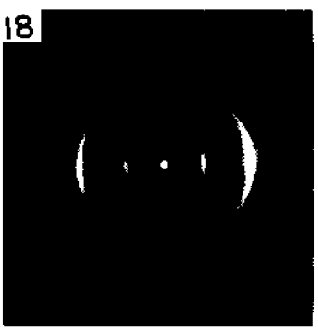

Fig. 18. X-ray diffraction photograph of extracted cuticle of Palamnaeus bengalensis with beam parallel to surface and transverse to body.

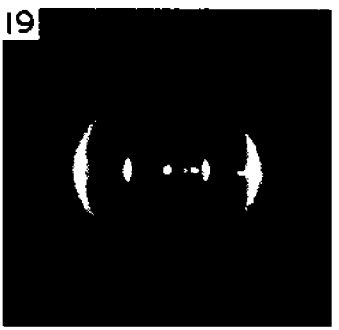

Fig. 19. X-ray diffraction photograph of extracted cuticle of Palamnaeus bengalensis with beam parallel to surface and longitudinal to body.

CHITIN CONTENT AND VARIATION WITH MOLT STAGE AND CARAPACE LOCATION IN THE BLUE CRAB, CALLINECTES SAPIDUS

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ABSTRACT

Employing a modified chitin-specific spectrophotometric method of analysis, the chitin content and its variation as a function of molt stage and carapace location was determined for the blue crab, Callinectes sapidus Rathbun.

It was found that chitin content, on a dry-weight basis, over all samples tested averaged 14.9% for the blue-crab exoskeleton. The chitin content for all locations on the main dorsal carapace averaged 15.8% while the claw sections averaged 11.6% chitin. Molt-stage variation in chitin was significantly greater, ranging from 12.2% to 24.4%, exclusive of the freshly exuviated carapace, which had an 8.5% chitin content.

The variations in chitin content with molt stage and carapace locations in the blue crab no doubt are biologically significant and have links to the physiological functions and life cycle of the crab.

INTRODUCTION

This study was undertaken as part of an investigation into the potential supply of chitin available from blue-crab (Callinectes sapidus Rathbun) processing waste and to provide some basic data on variations in chitin production during the blue-crab molting cycle. It also appeared opportune to determine chitin content in relation to carapace locations. It should thus be possible to interpret the results along basic physiological and biological lines with relationship to the life cycle and morphological structure of the blue crab.

Since no chitin-specific, direct method of quantitative analytical determination was available from the literature, an attempt was made to modify and optimize the spectrophotometric technique of Strickland and Parsons (12) for particulate chitin to determine chitin in crustacean exoskeletons. Previous studies (6, 8) in which chitin has been determined in the decapod, Hemigrapsus nudus, and the crayfish, Orconectes obscurus, have relied on the traditional digested residue dry weight as the quantitative measure of chitin content. Even when tissue grinding is thorough and the method for digestion of all proteinaceous and carbonate material ostensibly is complete, subtle variations in the cuticle matrix structures and chitin losses during the rigorous digestion process may result in significant, non-systematic errors.

Accordingly, the chitin determinations were made by the improved spectrophotometric technique which is described in this paper.

MATERIALS AND METHODS

Blue-crab chitin source and isolation

For the purpose of analytical chitin determination, 19 live blue crabs, Callinectes sapidus Rathbun, were sampled from the tributaries of the Delaware Bay by conventional trapping. The molt stages were determined using standard biological criteria (3, 4, 10). The blue crabs were preserved in isopropanol and kept under refrigeration at 4° C until removal for chitin determination.

Each blue-crab exoskeleton analyzed was surgically cut with a laminectomy trephine to obtain 9 sections. Figure 1 illustrates the dorsal carapace of the blue crab, indicating the locations of these sections. Each section was then dried at 100° C, for 10 minutes, precision weighed, and decalcified in 1N HCl, at 25° C for 12 hours, followed by 3 deionized water washings and deproteinization in 5% NaOH, at 25° C for 12 hours. The chitin content of these sections was then determined spectrophotometrically on a Beckman DB spectrophotometer.

Analytical methods

The spectrophotometric method employed was a modification of the technique delineated in Strickland and Parsons (12) that takes into account critical observations made by Boas (1) and by this author. It is based on the Elson-Morgan method for glucosamine determination (5). The method spectrophotometrically measures the intensity of red color that develops when glucosamine hydrochloride is boiled in basic solutions with acetyl acetone, quick cooled, and then the pyrrole thus formed, 3-acetyl-2-methyl-5-tetrahydroxybutyl pyrrole, is condensed by Ehrlich's reagent, p-dimethylaminobenzaldehyde, in 95% ethanol (5). The chitin to be determined was hydrolyzed in excess of 6N HCl into the glucosamine hydrochloride. Modifications of the method include optimization of the hydrolysis period, control of acetylation and condensation reaction times, mild preparative hydrolysis of samples to decalcify crab exoskeletons and reduce interfering chromogens, and use of pure chitin as calibration standards.

Boas (1) observed that the chitin hydrolysis conditions always represent a balance between glucosamine liberated and destroyed, since in greater than 2N HCl at 100° C, some significant percentage of glucosamine is destroyed within 15 hours. Therefore, optimal hydrolysis conditions must be set for each type of sample depending upon its response to the hydrolysis conditions. This also implies, however, that in 2N HCl (or weaker) no loss of glucosamine occurs. Since it has also been shown that 1N HCl deacetylates chitin very minimally (1), 1N HCl was employed in this study to reduce interfering substances and to decalcify the samples.

The first major modification to the analytical procedure delineated by Strickland and Parsons (12) was in the make-up of the standard solution used in calibrating the spectrophotometer. For this study it was felt that a much truer calibration could be achieved by hydrolyzing pure chitin in known concentrations in the same manner actual samples would be treated and then determining their optical absorbance at 5300A° rather than relating glucosamine

hydrochloride absorbance readings to true chitin by the suggested formula factor. In other words, the real condition measured at any time t in the chitin determination is the balance between the glucosamine liberated to that time and that which has been destroyed:

$$\Sigma \text{ chitin} = \Sigma \text{ glucosamine} \cdot HCl = \int_0^t \frac{dGLU_{lib}}{dt} - \frac{dGLU_{deg}}{dt}$$

This argument points to the second major modification. The contention was made above that there is an optimum time of hydrolysis unique for each type of sample, i.e., a distinct moment when the greatest optical density is recorded for the balance between glucosamine liberated and destroyed. The results of an experiment conducted on pure chitin samples showed very distinctly a 4.5-hour hydrolysis produced optimum results, while in the case of the blue-crab exoskeletons, a six-hour hydrolysis period seemed optimum.

Implementing these modifications, the standard calibration curve in Figure 2 was generated. From linear regression analysis, it had a correlation coefficient greater than 0.998 and the standard error was found to be ±.002 absorbance units. Running a six-hour hydrolysis determination for four samples at each of five precisely known concentrations of chitin (along the range of determination) and statistically analyzing the data, the percent standard deviations were found to vary from 6.6% at the low end of the determination range to 3.0% at the high end.

RESULTS AND DISCUSSION

The results from the determination of chitin-content variation with carapace location are summarized in Table 1 below. These chitin-content values are the average at each location for the 19 crab samples.

Table 1. Chitin-Content Variation with Carapace Location in the Blue Crab

Carapace Location*	Average % Chitin Content (Dry Wt.)
1	15.5 ± 4.9
2	15.0 ± 6.8
3	16.2 ± 6.7
4	17.7 ± 6.5
5	13.7 ± 6.0
6	10.8 ± 5.1
7	12.3 ± 4.8
8	15.2 ± 4.7
9	16.7 ± 5.0

*See Figure 1

The chitin-content values for the main dorsal carapace sections 1-5, 8, 9 fall within a narrow range, averaging 15.8%. The claw sections 6, 7 average 11.6% chitin. This difference undoubtedly has a biological significance. It can be explained by the physiological fact that the claws are the defensive instruments of the blue crab and, as such, have a relatively higher deposition of calcium salts in the cuticle for strengthening and protective purposes. Furthermore, the relative trends in chitin content with carapace location seemed to follow a consistent pattern in the individual crab samples.

The results from the spectrophotometric determination of the variation of chitin content with molt stage in the 19 blue-crab exoskeletons are listed in Table 2 below and summarized by molt stage in Table 3.

Table 2. Chitin Content of Blue Crab, Dorsal Carapace

Sample No.	Sex	Carapace Width (mm)	Molt Stage	Descriptive Stage	Chitin Content (% dry wt)*
1	F	91.2	A_1	Earliest paper	24.43 ± 4.88
2	F	67.0	A_2	Early paper	21.02 ± 4.68
3	F	107.6	A_2	Early paper	21.34 ± 5.78
4	M	113.6	B_1	Paper	17.47 ± 10.28
5	F	77.5	B_2	Paper	13.63 ± 3.56
6	M	56.1	C_1	Late paper	12.84 ± 1.08
7	M	64.1	C_2	Early Hard	12.52 ± 3.29
8	F	82.9	C_3	Hard	13.25 ± 5.24
9	M	91.9	C_3	Hard	12.17 ± 4.83
10	M	102.7	C_3	Hard	12.43 ± 2.63
11	M	86.3	D_1	Earliest peeler	14.33 ± 6.18
12	F	86.5	D_2	Early peeler	15.60 ± 6.13
13	M	74.4	D_2	Early peeler	15.33 ± 4.77
14	M	108.5	D_2	Early peeler	15.18 ± 2.25
15	M	81.2	D_3	Peeler	13.29 ± 3.62
16	F	81.8	D_3	Peeler	13.44 ± 3.54
17	F	91.0	D_3	Peeler	13.45 ± 3.15
18	M	80.9	D_4	Late peeler	12.87 ± 3.51
19	F	91.2	E	Exuviated carapace	8.54 ± 4.86

*Values reported are the average for nine locations on carapace. Shown in Figure 1.

As a function of molt stage, the chitin-content values range from 8.5% to 4.2% chitin by dry weight. However, except for the exuviated carapace (Stage E) all values fall in a closer 12.2% to 24.4% range. The highest chitin content is

observed for the early post-molt stages, A_1 and A_2. As the blue crab proceeds through the life cycle, it is observed that percent chitin content in the exoskeleton decreases steadily to a relative low point during the hardening stages (C_1, C_2, C_3). This is due to the increase of calcium salt deposition for the physiological purpose of strengthening the exoskeleton (3, 4, 9). The chitin content then relatively increases as the crab begins to prepare for molting stages, D_1 and D_2, by reabsorbing calcium salts (10, 3, 4). It falls off rapidly in stages D_3 and D_4 to exuviation. This is due to removal of chitin by either reabsorption by the crab for reutilization (6, 8) or by some external process such as rapid microbial degradation upon exuviation (11, 7).

Table 3. Chitin Content Variation with Molt Stage in the Blue Crab

Molt Stage	% Chitin Content (Dry Weight)*
A_1	24.4 ± 4.8
A_2	21.1 ± 5.2
B_1	17.4 ± 10.2
B_2	13.6 ± 3.5
C_1	12.8 ± 1.0
C_2	12.5 ± 3.2
C_3	12.6 ± 4.2
D_1	14.3 ± 6.1
D_2	15.3 ± 4.3
D_3	13.3 ± 3.4
D_4	12.8 ± 3.5
E (Exuvia)	8.5 ± 4.8

The soft-shell crabs, stages A_1 and A_2 averaging 22.3% (±5.1%), are the only group with chitin content significantly higher than the 14.9% (±4.3%) average over all molt stages for the 19 crabs tested. The most likely physiological cause for this occurrence is that the carapace calcification process, hardening, has not proceeded sufficiently to affect significant calcium deposition. This does not, however, specifically indicate the stages of the molt cycle during which chitin deposition is occurring. The variations of chitin values shown in Table 3 do emphasize real changes which are occurring in the blue-crab exoskeleton and do have a definite relationship to the stage in the molt cycle.

SUMMARY AND CONCLUSIONS

The chitin content for all locations on the main dorsal carapace of the blue crab fell within a narrow range, 13.7% to 16.7%, averaging 15.8%, while that for the claw sections averaged less, 11.6%.

The chitin content variation with molt stage showed a much greater range, 12.2% to 24.4%, exclusive of the freshly exuviated carapace. This exuvia had a chitin content of 8.5%, indicating either rapid microbial degradation or significant amounts of chitin reabsorption by the crab.

It can be concluded that the variations in chitin content that depend upon molt stage and carapace location are of biological significance and have links to the physiological functions and life cycle of the blue crab.

An improved, spectrophotometric method of chitin determination has been developed.

ACKNOWLEDGMENTS

This investigation was sponsored in part by NOAA, Office of Sea Grant, Department of Commerce, under Grant No. 04-3-158-30. The contributions of Drs. Paul R. Austin, John Born and Karl-Heinz Szekielda to the guidance and conduct of this research and review of this manuscript are greatly appreciated.

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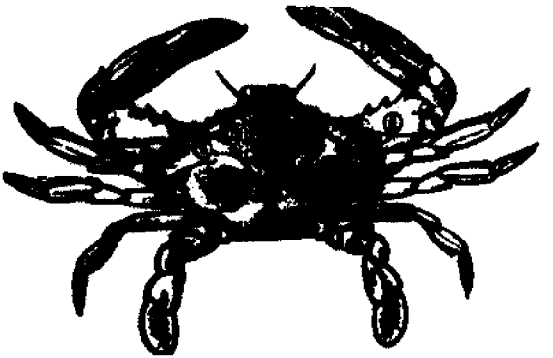

Figure 1. Blue crab dorsal carapace indicating the locations used in the chitin content determinations

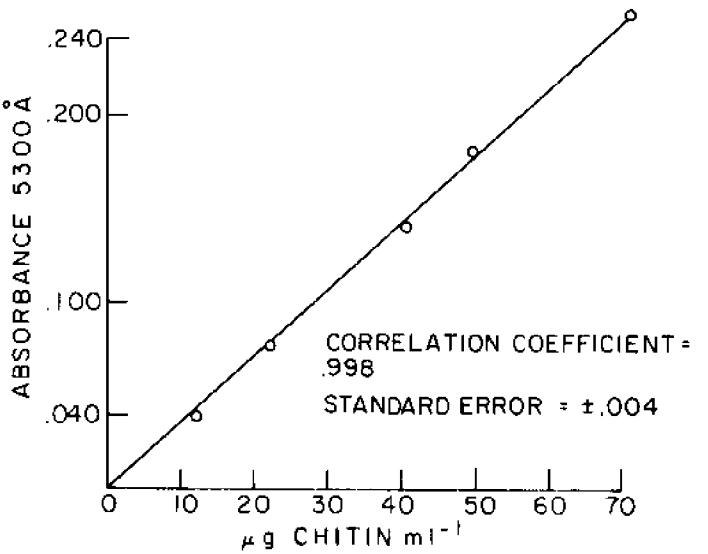

Figure 2. Calibration curve for chitin determination (6 hr. hydrolysis period)

CHEMICAL DIFFERENCES IN THE α- AND β-CHITINS AND THEIR SIGNIFICANCE IN THE QUALITY OF THE BY-PRODUCTS OF CHITIN

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ABSTRACT

Chemical differences exist between α- and β- chitin in addition to different crystalline forms, which expalin the conversion of the α form into the β form upon treatment with concentrated nitric and hydrochloric acids.

The materials used were the pen of Loligo indica and the chaetae from the polychaete annelid Nereis diversicolor. The acetyl content of previously alkali-purified and washed materials was estimated: L. indica had a 9.62% and N. diversicolor had a 9.57% acetyl content.

After treating with fuming nitric acid or 6N hydrochloric acid, the acetyl content of these chitins was reduced to between 7.4 and 7.6%. The nitrogen content of the experimental and the control pieces, however, remained the same.

The significance of these differences in acetyl content is discussed in relation to the x-ray diffraction patterns yielded by the α- and β-chitins. The relation of those chemical differences to the quality of the chitosan solution is also discussed.

INTRODUCTION

It is well known that the x-ray diffraction patterns yielded by the chitin occurring in arthropods and some other invertebrates differs from those given by the chitin found in some parts of certain invertebrates (the skeletal pen of the squid and the chaetae of annelids [3,5]). These are designated α- and β-chitins, respectively. It has been reported that the α-chitin yields the β-chitin pattern after treatment with 45% fuming nitric acid or 6N hydrochloric acid (5,2). In view of this conversion, it was thought that these two kinds of chitins were merely two crystalline forms of polyacetylglucosamine and that there were no chemical differences between them. The results reported in the present investigation reveal some definite differences in the chemistry of the two kinds of chitin.

EXPERIMENTAL

Preparation of the materials

The materials used were the pen from Loligo indica Pfeffer and the chaetae from the annelids Nereis diversicolor. These animals are available in great abundance in Cochin. Chitin from these structures was purified by extracting the material with 1N aqueous NaOH at 100° C for 16 to 18 hrs. For the chaetae, the residue was collected by centrifugation and washed in running tap water for several days. The material was then dialysed against distilled water for 24 hrs (changing the distilled water several times during this period), collected by centrifugation, washed three times with ethanol and three times in ether, and finally dried in vacuo over phosphorous pentoxide. The yield was 42% to 46%. For the purification of chitin from the pen of Loligo the procedure was the same except that centrifugation was omitted, as the amount of material was very large.

Estimation of acetyl groups

1.5g of finely ground material was dissolved in 25 ml of 80% sulfuric acid. The solution was allowed to stand for 3 hrs at 0° C in a refrigerator and then diluted to 500 ml with double distilled water. The solution was autoclaved for 1 hr and distilled in steam; 5 liters of distillate was collected. The distillate was titrated equivalent to 31 cc of 0.105 N sodium hydroxide. The volatile acid was characterized as acetic by the Virtanen-Duclaux method. The residue from the distillation of the volatile acid was extracted in a continuous extractor with isopropyl ether for the non-volatile acid. The extract, when taken up with water contained only a trace of acid.

Estimation of nitrogen

The nitrogen content of the chitin samples was estimated by the standard microkjeldahl method of Steyermark (6).

X-ray photographs

The x-ray photographs were taken in a cylindrical camera after drying the material in vacuo over phosophorous pentoxide.

Viscosity of the chitosan solution

Chitosan solutions from the two kinds of chitin were prepared according to the procedure of Moorjani et al. (4), and the viscosity was determined by the Ostwald technique.

$$\text{Relative viscosity} = \frac{d_1 t_1}{t_2}$$

where: d_1 = density of chitosan solution

t_1 = time of flow for chitosan solution

t_2 = time of flow for the same volume of water.

Absolute viscosity is determined by multiplying by 0.00895 poises (the absolute viscosity of water at 25° C).

RESULTS

Three samples of purified pen of Loligo were selected, one of which was kept as a control. Of the remaining two, one was treated with fuming nitric acid and the other with 6N hydrochloric acid at room temperature for 4 hrs. These were then washed thoroughly in running distilled water which removed the acid present. The x-ray pictures taken of these two experimental pieces yielded an α-chitin pattern (Fig. 1), while the control piece gave a β-chitin pattern (Fig. 2).

The quantitative estimates of the acetyl groups yielded for the control piece was 9.62%, and for the experimental pieces 7.4% to 7.6%. These estimates were repeated 3 times and the values were consistently within a narrow range (Table 1). The nitrogen content was similar, however, for the experimental and the control pieces.

The purified chitin from the chaetae of Nereis diversicolor had an average of 9.57% acetyl content (Table 2). This value was reduced to an average of 7.5% after treatment with fuming nitric acid. A still lower value of 7.4% was yielded by the chitin after treatment with 6N hydrochloric acid.

The purified chitin from the shell of a crab Neptunes sanguinolentus which is well known as an α-chitin, has an acetyl value of from 7.4% to 7.6%. This value does not decrease after treatment with acids (Table 3).

Table 1. Estimation of Acetyl Content from the Purified Chitin of the Pen of Loligo

Sl no.	Material	Acetyl Content %	Nitrogen Content %
A.	Control piece:		
1	Trial 1	9.47	6.42
2	Trial 2	9.68	6.49
3	Trial 3	9.71	6.58
	Average	9.62	6.49
B.	Experimental piece after treating with fuming nitric acid:		
1	Trial 1	7.49	6.44
2	Trial 2	7.29	6.53
3	Trial 3	7.42	6.39
	Average	7.40	6.45
C.	Experimental piece after treatment with 6N hydrochloric acid:		
1	Trial 1	7.75	6.60
2	Trial 2	7.67	6.38
3	Trial 3	7.38	6.57
	Average	7.60	6.52

Table 2. Estimation of the Acetyl Content from the Purified Chitin of the Chaetae of Nereis

Sl no	Material	Acetyl Content %	Nitrogen Content %
A.	Control pieces:		
1	Trial 1	9.51	6.38
2	Trial 2	9.56	6.55
3	Trial 3	9.64	6.46
	Average	9.57	6.46
B.	Experimental piece after treatment with fuming nitric acid:		
1	Trial 1	7.52	6.51
2	Trial 2	7.49	6.49
3	Trial 3	7.63	6.42
	Average	7.54	6.47
C.	Experimental piece after treatment with 6N HCl:		
1	Trial 1	7.37	6.54
2	Trial 2	7.41	6.48
3	Trial 3	7.46	6.49
	Average	7.41	6.50

Table 3. Estimation of the Acetyl Content from the Purified Chitin from the Shell of the Crab N. sanguinolentus

Sl no	Material	Acetyl Content %	Nitrogen Content %
1	Control piece	7.53 ± 0.12	6.42 ± 0.19
2	After treatment with fuming nitric acid	7.21 ± 0.25	6.32 ± 0.14
3	After treatment with 6N hydrochloric acid	7.34 ± 0.28	6.41 ± 0.16

Differences in the quality of the chitosan solution in the two types of chitins

Chitosan was prepared from the purified chitin of the pen of Loligo, chaetae of Nereis and the shell of N. sanguinolentus by treatment with saturated NaOH solution at 100° C for one hr. The thoroughly washed chitosan (1 gm) was dissolved in 100 ml of acetic acid. The viscosity of the solution was then studied; the results are presented in Table 4: the chitosan from

Table 4. Viscosity of the Chitosan Solution Prepared from the Purified Chitin of the Pen of Loligo, Chaetae of Nereis and the Shell of N. sanguinolentus

Sl no.	Material	Relative Viscosity	Absolute* Viscosity
A.	Pen of Loligo:		
1	Control piece	264.3	1.5720
2	After treatment with fuming nitric acid	96.2	0.5845
3	After treatment with 6N hydrochloric acid	112.8	0.6772
B.	Chaetae of Nereis:		
1	Control material	176.5	1.3941
2	After treatment with fuming nitric acid	87.1	0.5788
3	After treatment with 6N hydrochloric acid	94.9	0.6305
C.	Shell of N. sanguinolentus:		
1	Control piece	74.6	0.5332
2	After treatment with fuming nitric acid	69.4	0.4893
3	After treatment with 6N hydrochloric acid	71.8	0.5127

*Expressed in poises of the 1% chitosan in 2% (W/V) acetic acid.

the pen of the cephalopod and that from the chaetae of the polychaete were 264.3 and 176.5 respectively. Treatment of the original material with fuming nitric acid and with 6N hydrochloric acid considerably reduced both the relative and the absolute viscosity of the chitosan solution.

The chitosan solution prepared from chitin of the control piece of N. Sanguinolentus and from the samples of chitin after treatment with fuming nitric acid or with 6N hydrochloric acid have similar viscosities.

DISCUSSION AND CONCLUSIONS

Clark and Smith (1) reported that treatment with hydrochloric acid reduces the acetyl content of chitin. The results reported in the present study substantiate this observation: the β-chitin has a 9.57 to 9.62% acetyl content. These values are reduced to 7.40% to 7.54% after treatment with fuming nitric acid and to 7.41% to 7.60% following treatment in 6N hydrochloric acid.

The materials that were originally giving a β-chitin x-ray pattern yielded an α-chitin pattern after treatment with these acids. In view of the observations recorded, it is likely that the partial deacetylation of the β-chitin may result in the conversion to α-chitin.

Estimations of the acetyl content of the original α-chitin from the shell of crab lend support to this inference. It contains from 7.40% to 7.60% acetyl content. We suggest that such differences in acetyl content can be employed as a criterion to distinguish the α- and β- chitins.

Treatment with either fuming nitric acid or 6N hydrochloric acid does not affect the acetyl content of the α-chitin of the crab shell. This may mean that these acids cannot remove the acetyl groups under these conditions.

The number of the acetyl groups seems to have a bearing on the viscosity of the chitosan solution. The acetyl content is directly proportional to the viscosity of the chitosan solution.

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Fig. 1. X-ray diffraction pattern yielded by the pen of Loligo after treatment with fuming nitric acid

Fig. 2. X-ray diffraction pattern yielded by the pen of *Loligo*, control piece

ENZYMATIC HYDROLYSIS OF CHITOSAN*

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ABSTRACT

One of the components of the cell wall of the Mucorales is chitosan. Fungi were examined for their production of chitosan; it was found to occur only in the Mucorales, but was present in each of the six families examined. Its degree of acetylation is low (10%) and decreases further with the age of the culture.

In our study of the lysis of fungal cell walls, chitosanase, a new class of enzyme, was found to be essential in the degradation of the Mucorales to form protoplasts. Thus we studied the microbial degradation of chitosan and we found that a wide variety of microorganisms from soil and water were capable of degrading it. Some microbes degraded only chitosan, while others could degrade both chitin and chitosan. Purified chitosanases from both groups of organisms were found to hydrolyze chitosan, but not chitin. The enzymes degrading chitosan can be subdivided into two major classes according to their specificity--those that hydrolyze only chitosan and those that hydrolyze chitosan and carboxymethyl cellulose. Chitosanases from individual organisms show different hydrolytic-action patterns, and the degradative action of chitosanases is dependent on the degree of acetylation of the substrate. Penicillium islandicum chitosanase shows high specificity toward cleavage at the C-1 N-acetyl glucosamine linkage in the polymer. The practical implications of chitosanases are considered.

INTRODUCTION

We initially became interested in chitosan while studying the cell walls of mucoraceous fungi. Certain of these fungi, Mucor and Rhizopus, are capable of invading burn wounds of debilitated patients (6). Effective treatment of such fungal infections is difficult, and amputation of a limb or an extremity can be the only effective cure (6). One potential alternative approach to the control of such fungi is the selective enzymatic attack of the cell wall of the mold. Thus we screened for microorganisms that could enzymatically degrade the cell wall of the Mucorales. We found a wide range of fungi, bacteria and actinomycetes with this ability. Some of the more effective lytic enzymes that we obtained from them were later found to degrade chitosan. Since these enzymes had not previously been described, we decided to characterize them more fully and explore their possible uses. This paper reviews our approach to the enzymatic hydrolysis of chitosan.

THE NATURAL OCCURRENCE OF CHITOSAN

Chitosan was first discovered in nature by Kreger in 1954. By the use of x-ray analysis and chemical determinations, he characterized it in the cell walls and sporangiophores of Phycomyces blakesleeanus (19). Bartnicki-Garcia

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and Nickerson subsequently reported it in the cell walls of Mucor rouxii (3). On the basis of these two reports, Bartnicki-Garcia had the perspicacity to use chitosan as a chemotaxonomic marker for the Zygomycetes in his classification of fungi based on cell-wall composition (2). We have since confirmed this concept by characterizing chitosan in the cell walls of one member of each of the Mucorales families tested (Table 1) and showing its absence in the other fungal groups (9, 14, 22). There are isolated reports of chitosan occurring in the cell walls of the green alga Chlorella (21), in the spermatophore stalk of Podura aquatica (27), and in the spore walls of the basidiomycete Puccinia graminis (14). Apart from these studies, we have been unable to find other reports of naturally occurring chitosan. However, chitin that is 20% and 40% deacetylated occurs respectively in cuttlefish and in the pen of Loligo (10).

The degree of acetylation of fungal chitosans is low as measured by PMR spectroscopy. Chitosan from a young culture of Cunninghamella echinulata was shown to be 9% acetylated; the proportion decreased to around 1% as the culture aged from two to eleven days (Fig. 1 [14]).

MICROORGANISMS THAT DEGRADE CHITOSAN

During the investigations on the lysis of fungal cell walls, we (23) and Ruiz-Herrera (26) independently discovered the presence of enzymes that degrade chitosan. We now realize that chitosan-degrading microorganisms are extremely common in soil (10^5 bacterial colony forming units per gram of soil in garden, forest, salt marsh and agricultural samples). The presence of chitosan degraders is probably a reflection of the fact that Mucorales are extremely common soil fungi, and, following their death, a microbial population rapidly develops to utilize their dead hyphae. One result that surprised us was that salt-marsh muds similarly contained high numbers of chitosan-degrading bacteria. As these muds are essentially anoxic, most Mucorales do not thrive there. Why, then, should there be such high numbers of chitosan-degrading microbes present? Possibilities include:

a) The lytic bacteria are facultative and retain their ability to colonize aerated soils containing larger Mucoralean populations than the salt-marsh muds. Thus they retain their ability to degrade chitosan.

b) Chitin could be deacetylated to chitosan, resulting in a secondary population developing to utilize this newly formed substrate. Deacetylation and deamination of chitin have been suggested as potential routes for the utilization of chitin (8, 32). Chitin is common in the salt-marsh environment, due to the abundance of molted arthropod skeletons. Deacetylation would thus result in large amounts of chitosan in the salt-marsh muds. Perhaps analogously, it has been proposed that chitosan is synthesized in the Mucorales by deacetylation of preformed chitin (1).

c) Some other readily available source of chitosan is present in the soil but has not yet been described; perhaps an animal source.

d) The chitosan-degrading enzymes of the lytic bacteria are not specific toward chitosan but may perhaps attack chitin and/or cellulose.

Table 1. Occurrence of Chitosan in Mucorales*

Family**	Genus	Protoplast Formation by Penicillium islandicum *** Chitosanase
Mucoraceae	Absidia	N.T.
	Mucor (3 species)	+ -
	Phycomyces	+
	Rhizopus	+
	Zygorhynchus	+ (with chitinase)
Pilobolaceae	Pilaira	N.T.
Thamnidiaceae	Helicostylum	+
Piptocephalidaceae	Syncephalastrum	+
Kickxellaceae	Kickxella	N.T.
Cunninghamellaceae	Cunninghamella	N.T.
	Mortierella	+

*A composite table based on extraction of chitosan in 0.4N HCl at 100° C of 20 min. and subsequent hydrolysis by acid or purified chitosanase with characterization of the glucosamine and oligosaccharide products (5, 9, 14, 22).

**Based on Webster (31); members of Choanephoraceae and Endogonaceae were not available for testing.

*** + = protoplasts readily formed; + - = protoplasts formed with difficulty; N.T. = Not Tested. Addition of chitinase considerably aids protoplast formation (see Zygorhynchus).

To explore this last possibility, an agar "plate clearing assay" was employed to examine bacterial cultures known to degrade chitosan to see if they also had the ability to degrade chitin and cellulose. Chitosan degraders were replicated onto plates containing chitin or cellulose and screened for the production of clearing zones around the colony. The results showed that chitosan degraders could be subdivided into four groups. Group I was capable of attacking only chitosan. Group II could attack both chitosan and cellulose. Group III could attack chitosan and chitin. Group IV was capable of degrading all three substrates. This showed that there was a group of organisms that contained enzymes specific for chitosan degradation, and that these enzymes could not degrade chitin or cellulose. In the case of the organisms that could degrade more than one substrate, however, it does not resolve the question of broad specificity.

SPECIFICITY OF CHITOSANASES

The question of specificity of these lytic enzymes has been resolved by enzyme purification. The properties of these chitosanases are reviewed in Table 2. Two preparations, one from P. islandicum and one from Streptomyces No. 6 are specific for chitosan. Bacterial preparations from Myxobacter Al-1 and Bacteria K-1 and No. 8 can also degrade both chitosan and carboxymethylcellulose. The question of whether these latter preparations should be termed chitosanases or cellulases remains unresolved. Another preparation from Bacillus R-4 attacks chitosan but not chitin. This characteristic appears to be common to all chitosan-degrading enzymes purified to date. In general, enzymes attacking chitosan fall into two groups: those specific for chitosan, and those that attack both carboxymethylcellulose and chitosan. None hydrolyze chitin to any marked degree. The P. islandicum chitosanase will slowly hydrolyze the pentamer of N-acetyl glucosamine, but not smaller oligomers.

MODE OF ACTION OF P. ISLANDICUM CHITOSANASE

The P. islandicum chitosanase was purified 38-fold to homogeneity from a crude culture broth with a 42% recovery (9). This enzyme attacks chitosan in an endo-splitting manner, as measured by the rapid decrease in viscosity of chitosan and the formation of mainly oligomeric end products. It appears to be similar in action to the other chitosanase enzymes studied to date (Table 2). The action of P. islandicum enzyme toward chitosan depends on the degree of acetylation of the substrate. Greatest activity is shown toward the "30-60%" acetylated chitosan, with less activity toward polymers of lower degrees of acetylation and no activity toward chitin per se (Fig. 2). In comparison, a chitinase from Serratia marcescens (24) is active only toward highly acetylated polymers (Fig. 2).

The mode of action of the enzyme was investigated by analysis of the degradation products from 30% and 60% acetylated chitosan substrates. Long-term incubation with the 30% acetylated substrate yields oligomers plus a trisaccharide and some N-acetylglucosamine (Fig. 3). The 60% acetylated substrate is degraded to oligomers plus a disaccharide and some N-acetylglucosamine. The reducing terminus of the oligomeric products is either glucosamine or N-acetylglucosamine or a mixture of both. These terminal reducing sugars can be identified through reductive tritiation, followed by selective nitrous-acid degradation. In the nitrous-acid degradation of amino sugars the following conversions occur (Fig. 4 [13]):

a) glucosamine to 2,5 anhydromannose as a monomer or within a polymer;

b) glucosaminitol to 2-deoxyglucose;

c) N-acetylglucosamine and N-acetyl glucosaminitol are unchanged.

In order to determine the new reducing-sugar terminus of the reaction products from a 30% acetylated chitosan, the reducing ends were labeled, using sodium borotritide, and the oligomeric and trimer products were purified by repeated gel filtration (Biogel P2). The trimer and the oligomers were then individually treated with nitrous acid and the products analyzed by paper chromatography. The only radioactive product from the trimer is N-acetyl glucosaminitol (Fig. 5), indicating that N-acetylglucosamine is the terminal reducing sugar of the trisaccharide. Furthermore, this N-acetylglucosamine must be linked to

Table 2. Microbial Chitosanases

	Penicillium islandicum (9,22,23)	*Streptomyces* No. 6 (15,23,25,26)	Bacterium No. 8 (7)	Bacterium K-1 (13)	*Myxobacter* A1-1 (11)	*Bacillus* R4 (28)
Specificity						
Chitosan	+	+	+	+	+	+
Chitin	-	-	-	-	-	-
CMC [a]	-	-	+	+	+	N.T. [b]
Action Pattern						
Endo-/exo-splitting	Endo	Endo	Endo	Endo	Endo	N.T.
Constitutive (C) or Inducible (I)	I	I	I	I	C	C
Molecular Weight (daltons)	30,000	30,000	N.T.	50,000	28,900 [c] 31,000 [d]	N.T.
Stability at 37° C	Stable +[Ca^{++}]10^{-3}M]	Stable	Stable	Stable	+	Stable
Optimum Temp - Chitosan	45	32	N.T.	60	70	N.T.
CMC	* [e]	*		N.T.	38	N.T.
Optimum pH Chitosan	4.5-6.0	7.5	4.8-7.0	5.5-6.9	5.0 & 6.8	"7.6"
CMC	*	*	N.T.	5.0	5.0	N.T.
Attacks Zygomycete cell walls	+	+	+	+	+	+
Forms protoplasts from Zygomycetes Crude enzyme	+	+	+	+	+	+
Pure enzyme	+	-	N.T.			

[a] Carboxymethylcellulose

[b] N.T. = Not Tested

[c] From amino-acid composition

[d] From gel filtration

[e] Not appropriate

glucosamine since no radioactive dimer products are formed. Similarly, the labeled oligomers, when treated with nitrous acid, yield only N-acetylglucosaminitol (Fig. 6). No 2-deoxyglucose, the degradation product from glucosaminitol, was observed. These results suggest that P. islandicum chitosanase shows high specificity toward cleavage at the C-1 N-acetylglucosamine linkage in the polymer, and yet also has a requirement for binding of glucosamine. In the idealized illustration of the degradation of a 30% chitosan (Fig. 7), only reaction sequence I produces terminal N-acetylglucosamine residues.

USES OF MICROBIAL CHITOSANS AND CHITOSANASES

1) Food and Drug Administration approval for the use of chitosan films in food packaging requires evidence that chitosan is nontoxic to humans. Mucorales have been used worldwide for centuries to supply the active factor in microbial fermentations of food products (Table 3). We have demonstrated that these fungi contain chitosan in their cell walls, and thus the chitosan in fungal hyphae appears to be non-toxic in long-term consumption. Direct feeding trials with rats, however, show that high levels of chitosan salts (15%) do appear toxic (20).

2) Wound-healing accelerators: This role has been discussed by Balassa and Prudden (these Proceedings). Immobilized chitosanases allow the ready production of a range of large chitosan oligomers that could be used in this intriguing application.

3) The wide occurrence of organisms capable of degrading chitosan alerts us to the dangers of indiscriminate use of chitosan in situations where microbial growth can prevail. More positively, since chitosan is readily degradable, it could be used in situations where degradation is propitious, e.g., in medical sutures. Chitosan is non-allergenic. Sutures of chitosan could be adsorbed, and would have the additional advantage of acting as wound-healing accelerators.

4) Chitosanases are prime tools for converting gummy chitosans into small oligosaccharide units, without the use of harsh reaction conditions. The products are thus amenable to PMR analysis to determine the degree of acetylation (Fig. 1).

5) Chitosanases have been used for the preparation of protoplasts from Mucorales, which can then be used in genetics for somatic hybridization (4, 5) and for studying membrane and cell-wall synthesis and membrane-bound enzymes (29).

ACKNOWLEDGEMENTS

We wish to acknowledge our discussions with Elwyn Reese while developing the concept of chitosanases. We also wish to thank Dr. C. W. Hesseltine, U.S.D.A., Peoria, Ill., for several cultures of Mucorales, and Dr. J. J. Elliott, Exxon Research Laboratories, Linden, N.J., for the PMR spectra. The work was supported by U.S. Army Grant No. DA-ARO-D-31-124-73-G154, the National Oceanographic and Aeronautics Administration, and the Rutgers University Biomedical Sciences and Research Committees.

Table 3. Use of Mucorales in the Preparation of Foods*

Name(s)	Organism(s)	Substrate	Description of Food	Geographic Location
Atsumandie	Mucor sp.	Farinaceous materials	Soft cheese	India
Hon-Fan	Mucor sp.	Soybean	Red cheese	China
Kaffir Beer	Aspergillus flavus Mucor rouxii	Sorghum or starch grains	Alcoholic beverage (riboflavin)	Malawi
Koji	Aspergillus sojae A. oryzae Rhizopus sp.	Rice, soybeans, cereals	Inoculum for propagation of mold for sake, shoyu production	Orient
Lao-Chao	Hansenula sp. Chlamydomucor sp. Mucor sp.	Rice	Foodstuffs	Orient
Levain of Sikkim	Mucor sp.	Rice	Chinese yeast alcohol	Orient
Meitauzaw	Mucor meitauza (Actinomucor elegans)	Soybean cake residues	Foodstuffs	China
Minchin	Paecilomyces sp. Aspergillus sp. Trichothecium sp. Syncephalastrum sp. Penicillium sp. Fusarium sp.	Wheat gluten	Foodstuffs	Orient
Murcha	Mucor sp., Hansenula anomala var. scheggi	Rice	Rice beer	India, Himalayas
Ragi	Chlamydomucor sp. Rhizopus, Hansenula sp.	Rice	Cheese and starter for other fermentation	Orient
Sufu	Actinomucor elegans Mucor sp.	Soybeans	Cheese-like	China, Taiwan
Tane-Koji	Aspergillus sp. Rhizopus sp. Mucor sp.	Rice, soybeans, wheat or cereals	koji inoculum	Orient
Tape Ketan	Rhizopus sp. Aspergillus sp.	Rice and prepared ragi	Alcoholic sweet paste	Indonesia
Tempeh	Rhizopus oligosporus	Soybeans or coconut meal	Food	East Indies
Thamnidum	Thamnidium sp.	Beef	Aged Beef	U.S.A.

*Based on a review of all available references to Mucorales; includes fermentations in which they may be a minor component (17).

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PROTON MAGNETIC RESONANCE SPECTRUM OF CHITOSAN FROM A TWO DAY OLD CULTURE OF CUNNINGHAMELLA ECHINULATA NRRL 1382

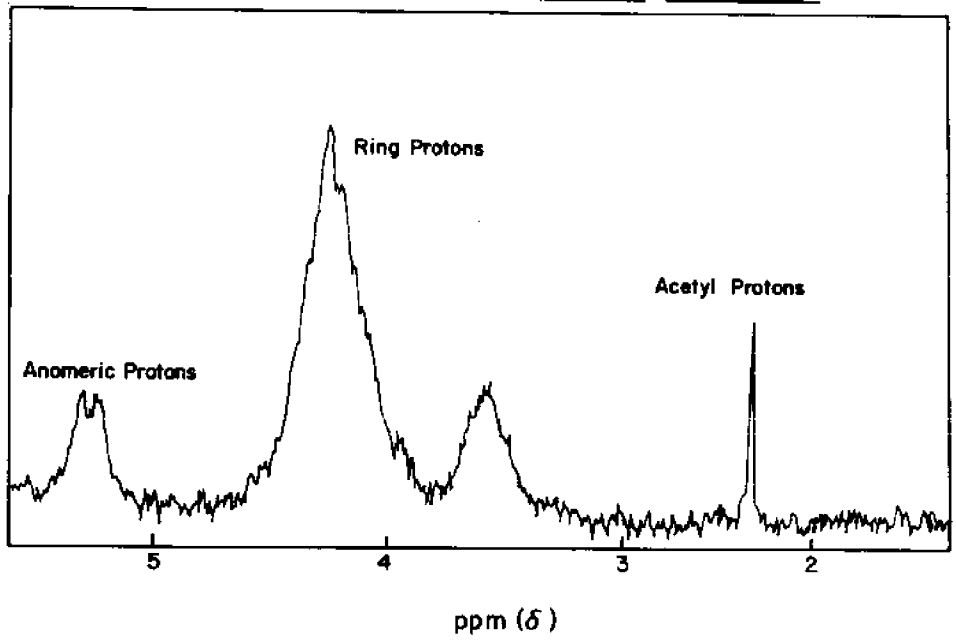


Figure 1. P.m.r. spectrum of chitosan from Cunninghamella echinulata. Chitosan was extracted from dried mycelium in 0.4N HCl, 100°C for 20 min, and purified by acid/alkaline recycling. The final solvent was 1N deuterated trifluoroacetic acid (14). The degree of acetylation can be determined by comparison of the ratio of the acetyl protons to ring protons (3/7 for N-acetylglucosamine). The initial objective was to separate "chitosan" from chitin. The extraction conditions did not promote marked cleavage of the polysaccharide chain, as there was no increase in reducing sugar content. However, some deacetylation could have occurred under these conditions.

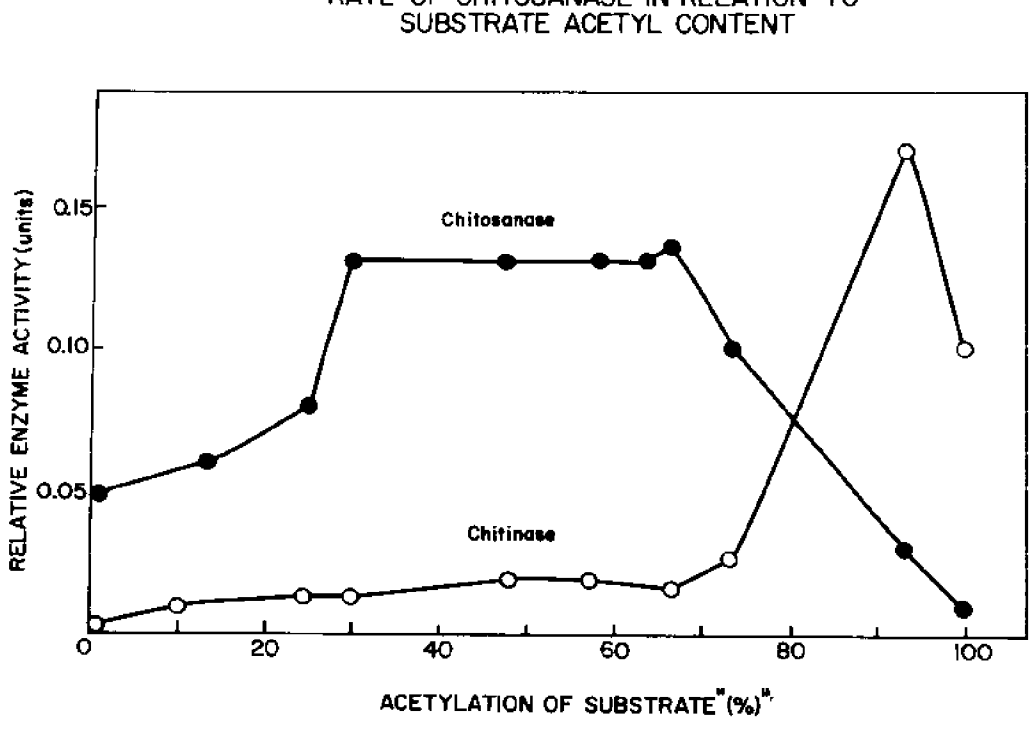


Figure 2. Effect of substrate acetyl content on chitosanase activity. Different percent acetylated chitosans were chemically prepared (12) and their susceptibility to hydrolysis measured in a standard reducing sugar assay (22), containing either 0.1 units of Penicillium islandicum chitosanase or Serratia marcescens chitinase. Percent acetylation is based on the relative fluorescence using fluorescamine (9,30) with glucosamine as a Chitosanase; 0 - 0 Chitinase.

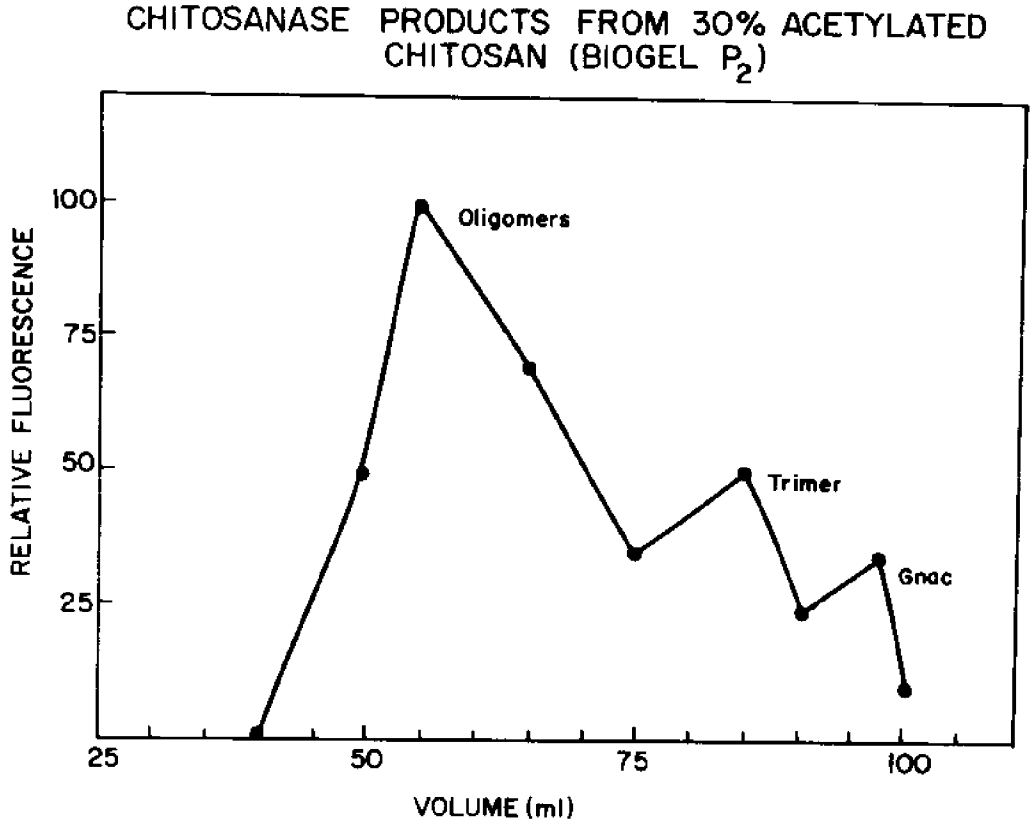
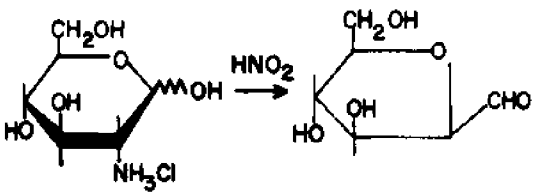
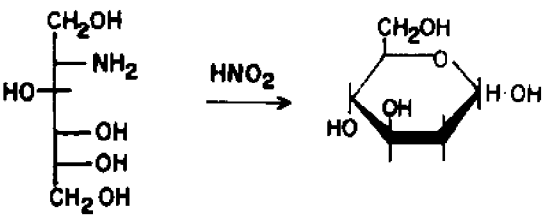


Figure 3. Enzymatic hydrolysis of chitosan (30%). Reaction products of Penicillim islandicum chitosanase analyzed by gel filtration on a Biogel 2 column. Fluorescamine was used as a detection reagent (30).

A.



GLUCOSAMINE 2,5-anhydro D-MANNOSE



GLUCOSAMIN-ATOL 2 DEOXY-GLUCOSE

B.

GN · GN · GNAc · GN · GN (CHITOSAN)

HNO_2 ↓

M + M + (GNAc · M) + M + $[HCl]_n$

C.

GN · GN · GNAc

↓ HNO_2

M + M + GNAc + $[HCl]_n$

Figure 4. Products of nitrous acid degradation of glucosamine glucosaminitol and of chitin/chitosan oligomers (13).

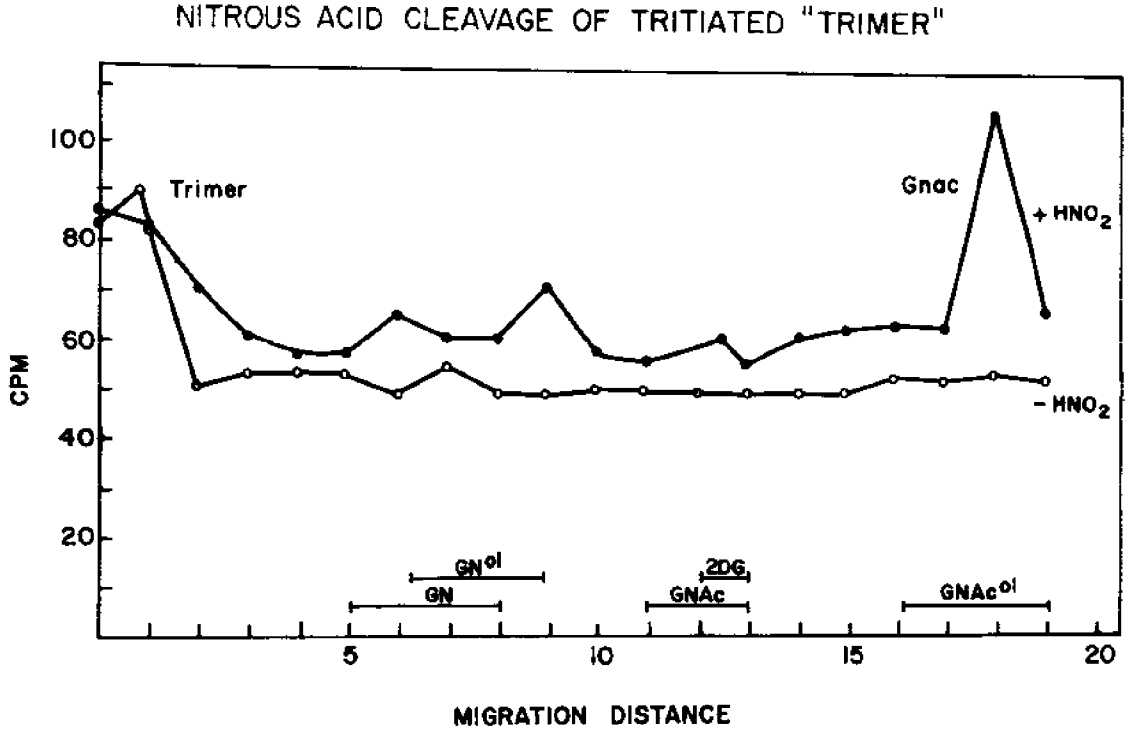


Figure 5. Nitrous acid degradation of purified "trimer" product from 30% acetylated chitosan. The "trimer" produced by Penicillium islandicum chitosanase from 30% acetylated chitosan was tritiated with sodium borotritide, purified by gel chromatography, and subjected to nitrous acid degradation. Samples were extracted, and the radioactive materials located by liquid scintillation (9).

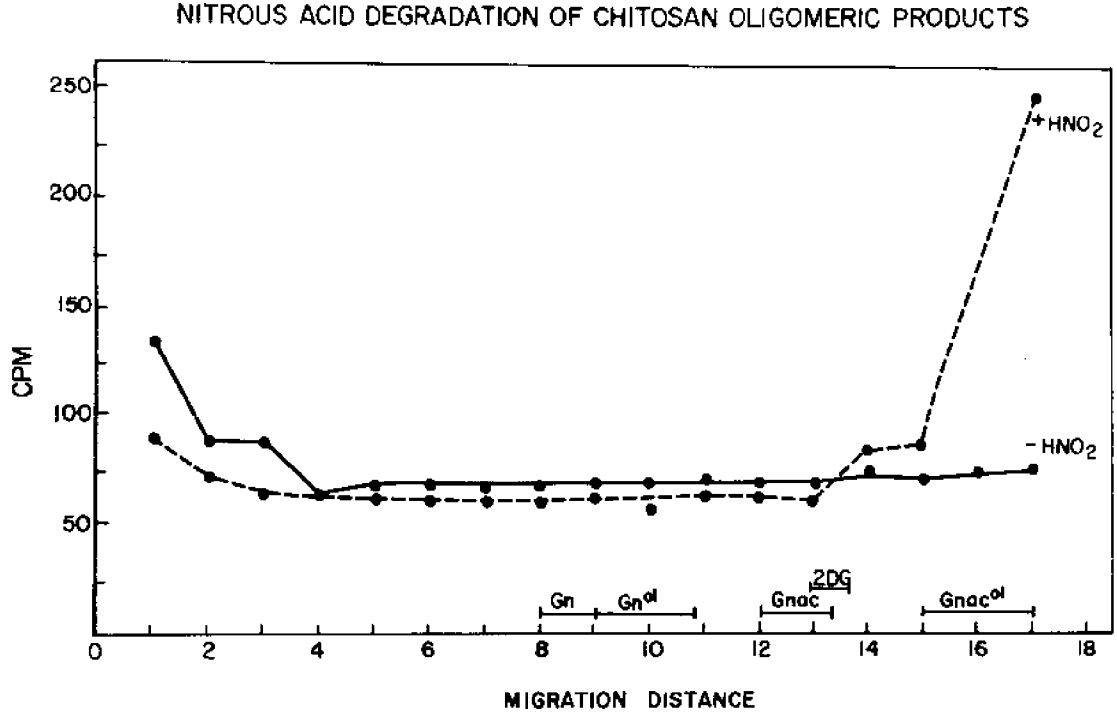


Figure 6. Nitrous acid degradation of purified "oligosaccharide" product from 30% acetylated chitosan. The "oligosaccharide" produced by Penicillium islandicum chitosanase from 30% acetylated chitosan was tritiated with sodium borotritide, purified by gel chromatography, and subjected to nitrous acid degradation. The products were analyzed by paper chromatography. Samples were extracted, and the radioactive materials located by liquid scintillation (9).

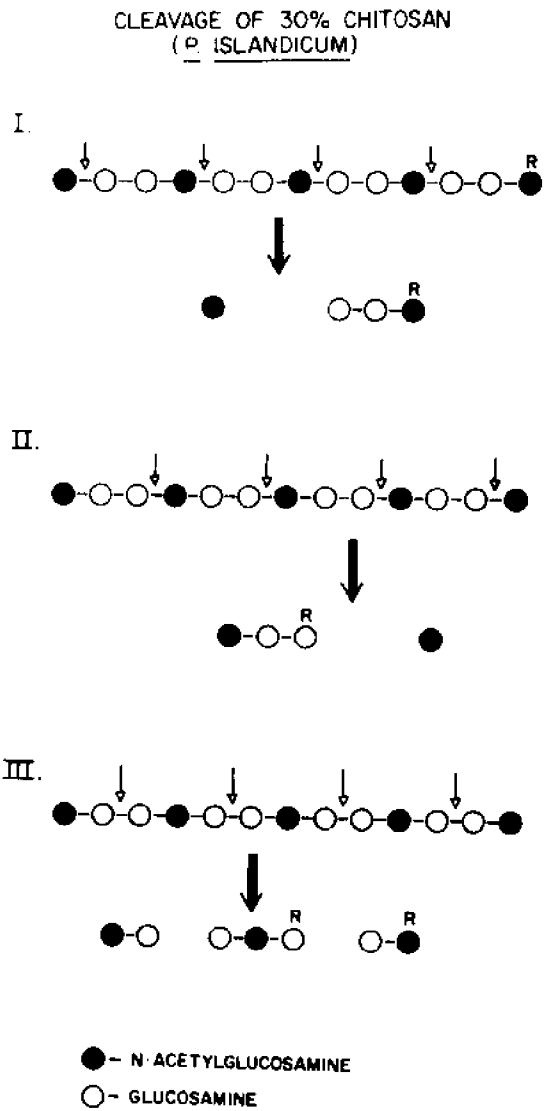


Figure 7. Three potential specific idealized schemes for the enzymatic degradation of 30% acetylated chitosan. Only scheme I yields N-acetylglucosamine as the reducing terminus.

DISTRIBUTION AND ACTIVITY OF CHITINOLYTIC ENZYMES IN THE DIGESTIVE TRACT OF BIRDS AND MAMMALS

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ABSTRACT

The extraction and purification of chitinolytic enzymes devoid of any lysozymic activity, taken from the gastric mucosa of mammals, indicated that these enzymes are true chitinases, rather than lysozymes with chitinolytic activity.

A close relationship was found between chitinase secretions in the digestive tract and the nature of the normal diet of the species considered. Changing the diet of an animal (mouse, rat, hamster, guinea pig) for a few weeks did not modify its ability or inability to secrete chitinase.

In the digestive tract of birds, chitinase is secreted only by the gastric mucosa. All the more or less insectivorous birds so far studied, including chickens, do secrete gastric chitinases. No chitinase secretion was detected in the pigeon or the parrot.

In mammals, gastric chitinases were found in omnivorous and insectivorous species belonging to the orders Insectivora, Chiroptera, Carnivora, Rodenta and primates. In the pig, chitinases are secreted by the gastric mucosa and by the pancreas.

The digestibility of chitin in both a purified and a natural form was estimated in feeding experiments with mice, Japanese nightingales and chickens. From 19% to 58% chitin was digested by mice and chickens that were fed a diet containing pure chitin, and by Japanese nightingales that were fed mealworm larvae.

INTRODUCTION

Since the discovery by Jeuniaux (12) of a secretion of chitinases in the digestive tract of some vertebrates, these enzymes, whose glandular origin was clearly demonstrated by Dandrifosse et al. (7), have been sought in a wide series of species (9, 13, 14, 21, 22).

In order to explain the erratic occurrence of chitinase in the vertebrate species so far studied, it was suggested that the secretion of chitinase by the gastric mucosa or by the pancreas was corrected with the feeding habits of the species, an adaptative correlation that was the result of a regressive evolution (14, 16). This statement was confirmed by the study of a wide series of fish, amphibians and reptiles (21). A correlation between chitinase secretion and diet was also pointed out in the case of mammals belonging to the order Carnivora (4).

Owing to the wide distribution of lysozymes (E.C. 3.2.1.17) (11, 17) and to the fact that these muramidases are able to hydrolyze chitin and some of its derivatives (2, 3, 18, 20), the question arose as to what extent the "chitinases" so far identified in the digestive tract of vertebrates were truly specific for chitin hydrolysis or were lysozymes with chitinolytic activity. It was observed that some pancreatic or gastric extracts of vertebrates with high chitinolytic activity were devoid of any significant lysozymic activity (6, 8). Moreover, Cornelius et al. (5) were able to purify a chitinase devoid of any lysozymic activity from the gastric mucosa extracts of a primate (Perodicticus potto). This enzyme showed both chitinolytic and lysozymic activities. It concluded that the chitinolytic enzymes observed in the digestive tract of vertebrates are "true" chitinases (E.C.3.2.1.14).

It is likely that the secretion of chitinases by some birds and mammals allows the digestion of chitin in the diet to some extent. The ability to digest chitin in vivo, however, has never been indicated. This ability will depend mainly on the chitinase concentration in the gut, on the optimum pH of these enzymes, on the physical and chemical state of the chitin provided with the diet, on the duration of the intestinal transit, and perhaps on the presence or absence of chitobiase (E.C.3.2.1. 29). The aim of this paper is to sum up the quantitative data concerning the distribution and activity of chitinases and chitobiases in the gut of birds and mammals, and to bring out some preliminary experimental results dealing with chitin digestibility.

METHODS

The organs used were dissected, washed, dried on filter paper, weighed ("fresh tissue"), and then homogenized in a mortar with sand and distilled water. The suspension was allowed to stand overnight at 4° C, then centrifuged. The supernatant ("enzyme extract") was kept at -20° C until an enzyme assay could be made.

Owing to the low chitobiase concentration in most enzyme extracts of vertebrates, the chitinase activity was measured according to the method of Jeuniaux (14, 15). This method uses a "native" chitin suspension prepared from cuttlefish bones as a substrate. A 1 ml chitin suspension (5 mg/ml) was incubated in a 1 ml of buffer at pH 5.2 and 37° C with 1 ml of the enzyme extract and 1 ml of chitobiase solution (lobster serum diluted 10 x). The N-acetylglucosamine concentration was measured (23) and the chitinase activity expressed in µg of N-acetylglucosamine (N-AG) liberated x hr^{-1} x g^{-1} of fresh tissue.

The chitobiase activity was also estimated by the above method, using as a substrate a preparation of chitobiose obtained by hydrolysis of chitin with a purified chitinase (14, 15).

The chitin digestibility experiments were performed on mice (Mus musculus, "wild" strain C 57Br, 2-4 months old), chickens (Gallus gallus, 15 days old) and Japanese nightingales (Liothrix lutea, adults). The experimental

animals were fed at regular intervals and reared in individual cages (arranged to collect all the excrement). The mice were fed (for 3 days) a mixture of starch (56%), casein (28%), corn oil (16%), 16.5 g% of purified ground-shrimp chitin, and a gelatin solution as an excipient. The chickens were fed (for 5 days) a commercial food for poultry, with the addition of 4 g% of purified ground shrimp chitin. The Japanese nightingale was fed dead mealworm larvae (Tenebrio molitor) and milk (for 2 days). The excrement was collected during the nutrition experiments for 3 to 5 days, and then for 4 more days. It was then ground, treated with NaOH 2N at 100° C for 3 hours, washed and centrifuged. The residue was hydrolyzed by HCl 11N at 40°C for 30 hours (24). After neutralization, the glucosamine and N-acetylglucosamine concentrations were measured respectively by the methods of Levvy et al. (19) and Reissig et al. (23), in order to calculate the amount of chitin by using a correction factor.

RESULTS

The location and activity of chitinase in 10 species of birds are given in Table 1.

The enzyme extracts of the liver, duodenum and intestinal mucosa of birds showed at most only very low and questionable chitinolytic activities. Chitobiase activity was absent or very low in every case (less than 200 µg N-AG x h^{-1} x g^{-1} fresh tissue), except in the caecal contents of the chickens (1160 µg N-AG x h^{-1} x ml^{-1}).

The chitinase activity in the gastric mucosa and pancreas of some mammals is given in Table 2. Only positive results are given, except for the interesting negative data concerning man. In addition, no chitinase was found in the stomach or in the pancreas of the following mammal species: sheep (Ovis aries), rabbit (Oryctolagus cuniculus), guinea pig (Cavia porcellus), cat (Felis domesticus), stoat (Mustela erminea), ferret (Mustela furo), marten (Martes foina) and sloth (Choloepus hofmanni). The chitobiase activity was very low for the mouse, except in the caecal contents (1160-1364 µg N-AG x h^{-1} x ml^{-1}).

The values of chitin digestibility in vivo are shown in Table 3.

DISCUSSION AND CONCLUSIONS

In birds as in mammals, the distribution of chitinase secretion is not a matter of systematics, but is related to the nature of the usual diet of the species. Gastric chitinases were at least occasionally found in 8 bird species that are more or less insectivorous. No chitinase was found in two strictly grain-eating birds, the pigeon and the parrot.

The same was also true for mammals. Insectivorous and omnivorous species secrete chitinases in the digestive tract, while more specialized species, both carnivorous and herbivorous, do not. However, it has been demonstrated that in rodents the secretion of chitinase was not modified by the addition of chitin to the diet for 1 to 3 months (10).

Table 1. Chitinase in the Digestive Tract of Birds

Species	Activity: μg N-AG x h^{-1} x g^{-1} Fresh Tissue		
	Mucosa of Glandular Stomach	Pancreas	Reference
Passer domesticus (Sparrow)	8470-13360	0	13 and original
Erithacus rubecula (Robin)	4136	0	original
Liothrix lutea (Japanese nightingale)	5620-13280	0	13 and original
Sturnus vulgaris (Starling)	61560	209	original
Turdus merula (Blackbird)	3020	11	13
Corvus corone (Carrion Crow)	3680	0	original
Gallus gallus chicken	1350-4040*	0	original
adult	1780	0	13
Tyto alba (Barn Owl)	9820	0	original
Columba palumbus (Pigeon)	0	0	13
Psittacus erithacus (Parrot)	0	0	original

*Not completely free from muscular tissues

Table 2. Chitinase Activity in Gastric Mucosa and Pancreas of Some Mammals

Species	Activity: μg N-AG x h^{-1} x g^{-1} Fresh Tissues		
	Gastric Mucosa (fundus and pylorus)	Pancreas	Reference
Chiroptera			
Rhinolophus ferrum equinum	5180	0	13
Insectivora			
Talpa europaea	132-178	238-435	13 and original
Erinaceus europaeus	3350-7560	10	13
Suidae			
Sus domesticus	540-832	700-1200	13
Rodents			
Mus musculus			
young	2330 ± 139	0	10
adults	4155 ± 151	0	10
Rattus norvegicus	2254	0	10
Cricetus frumentarius	133	0	10
Carnivora			
Canis domesticus	252	0	4
Vulpes vulpes	1239	0	4
Primates			
Perodicticus potto	1245-5500	0	1
Cebus capucinus	4950	0	original
Homo sapiens *	0	0	original

*Three series of assays, with samples from 3 different individuals.

In birds, the only site of chitinase secretion seems to be the glandular stomach. The chitinase activity in the enzyme extracts of the gastric mucosa is often very high, especially in the starling. In Gallus gallus, the young chickens are as well equipped with gastric chitinases as adults are (Table 2). Chitin was actually digested by the young chickens (15 days old), when purified shrimp chitin was added to the normal food. The digestibility coefficient was 23.5-31.7% (Table 3). The chitin of dead mealworm larvae, without any previous treatment, was more easily digested by a Japanese nightingale (digestibility coefficient: 56.8%).

Chitinases are secreted by the gastric mucosa of different mammals and by the pancreas of the mole and pig. The chitinase activity in the gastric mucosa extracts was generally lower in mammals than in birds. The purified ground-shrimp chitin was digested to some extent by two mice (19% and 58.6% of the ingested chitin) in the experiment reported in Table 3, but was not digested at all by two other mice in a second experiment.

Table 3. Chitin Digestibility in Mice and in Two Species of Birds

Species	Specimen no.	Chitin Ingested (mg)	Chitin in Excrement (mg)	Chitin Digested %
Mus musculus (mouse)	1	457.5	189.4	58.6
	2	353.0	285.8	19.04
Gallus gallus (chicken)	1	2000.0	1530.0	23.5
	2	2000.0	1365.5	31.7
Liothrix lutea (Japanese nightingale)	1	656.15	283.34	56.8

It must be emphasized that no chitinase was found in man, neither in the fundic and pyloric mucosa nor in the pancreas. Gastric chitinases were found, however, in two species of primates.

Owing to the very low chitobiase concentration in the various parts of the digestive tract in all the species studied (with the exception of the caecal content in the chicken and in the mouse), the metabolic utilization of the hydrolytic products of chitin can be questioned. A study of the hydrolysis and absorption of chitobiose and chitotriose in the digestive tract of vertebrates is in progress in our laboratory.

ACKNOWLEDGMENT

This work was supported by grant no. 2.4567.75 of the Fonds de la Recherche Fondamentale Collective.

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METHOD OF EXTRACTING CHITINASE FROM GASTRIC JUICES AND INTESTINAL CHYME OF CHICKEN

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ABSTRACT

We were the first to use the enzyme chitinase to hydrolyze the chitinous layer of the peritrophic membrane covering the gut wall of certain lepidopterous larvae, which facilitates penetration of spores of the entomopathogenic Bacillus thuringiensis from the gut into the hemolymph of larvae. As a result, B. thuringiensis now serves as a basis for several biological insecticides used in many countries because of their pollution-free properties and safety. The addition of chitinase to B. thuringiensis preparations increases their efficacy as protection against the spruce budworm, a serious defoliator of spruce and fir forests in eastern Canada and the United States. At present, chitinase is obtained from the fermentation of microorganisms, and it costs more than $200 per gram. The method proposed here consists of extracting chitinase from the gastric juices of freshly killed chickens at very low cost. A technique is also proposed for the semi-industrial extraction, concentration and purification of chitinase.

INTRODUCTION

The activity of the enzyme chitinase sold by chemical companies is usually in the order of 1200 nephelometric units; it can be kept for a few months at 4° C. It is difficult to obtain quantities of chitinase exceeding 100 mg., however, since most manufacturers do not stock this product because of its high cost ($200 per gram).

Recently, it was determined that the addition of traces of chitinase to B. thuringiensis formulations (10,000 nephelometric units/ha) accelerated the action of the bacillus on larvae of the spruce budworm, a serious defoliator of fir and spruce forests of eastern Canada and the United States. Addition of chitinase favors the pathogenic action of B. thuringiensis even at low environmental temperatures(1).

In order to apply this treatment on a large scale, it is essential that large quantities of chitinase be made available at low cost by finding sources of production other than the fermentation of microorganisms. The process now used by manufacturers, namely fermentation of certain micro-organisms such as the bacteria Streptomyces antibioticus and the fungi Beauveria sp. and Cordyceps sp. is not only expensive but also long and risky.

SOURCES OF CHITINASE

During an evaluation of the different sources of chitinase in organisms, we found that gastropods (the snail Helix pomatia L.), crustacea (Homarus vulgaris M. Edw.), scorpions (Androctonus sp. L.), bactrachians (the frog, Rana temporaria L.), and sparrows (the nightingale, Liothrix lutea seopoli) show great chitinological activity in the intestine (guts) and hepatopancreas. But our attention was centered on the chicken (Gallus gallus L.) because it has high chitinolytic activity and especially because chicken gizzards and intestine (guts) are readily available as waste material from poultry processing.

Many extractions of chitinase and chitobiase from the gastric and intestinal content of chickens were performed in the laboratory, and costs were also determined. Since the tests gave positive results, we developed a method of extraction of chitinase from the chicken gastric and intestinal chyme. 0.5 gm of chitinase having an activity of 890 nephelometric units was obtained from 300 chicken gizzards and intestines.

Method of extraction

1. Raw material: gizzards and intestines from freshly killed chickens were dipped immediately in a solution buffered at pH 5.2 and containing 0.02 M citric acid, 0.02 M disodium hydrogen phosphate (Na_2HPO_4) and 10 mg of thymol per liter of solution (antibiotic).
2. Other substances required: colloidal chitin from technical chitin, concentrated hydrochloric acid, hydrochloric acid diluted 1/10, sodium hydroxide 1/10, ammonium sulfate 55%.
3. Equipment: large centrifuges, filters, tanks.
4. Process for the semi-industrial extraction of chitinase:

The figures given below are for obtaining 100 grams of enzyme with an activity of 850 to 900 nephelometric units, which is sufficient in a treatment with B. thuringiensis.

a) 60,000 gizzards and intestines of freshly killed chickens are placed in 4,000 liters of buffer solution, pH 5.2, as described above.
b) Filtration of the solution through glasswool; centrifugation at 4,500 r.p.m. for 10 minutes. The supernatant is collected and placed in a second container.
c) Addition of 5 kg of colloidal chitin shaken for 10 minutes. The solution is centrifuged at 4,500 r.p.m. for 15 minutes. The deposit is collected.
d) The deposit is washed in 100 liters of buffer solution pH 5.2 then centrifuged at 4,500 r.p.m. for 15 minutes. The precipitate is collected.
e) The precipitate is added to 100 liters of buffer solution, pH 5.2, and maintained at 37° C with light stirring until hydrolysis of the chitin is nearly complete.
f) This solution is centrifuged for 10 minutes at 4,500 r.p.m. The supernatant is treated with 55 kg of ammonium sulfate (saturation at 55%).

g) The saturated solution is centrifuted at 4,500 r.p.m. for 10 minutes. The deposit obtained is chitinase with an activity of 100 to 150 nephelometric units.

h) Purification of the enzyme:

The enzyme is returned in 20 liters of buffer pH 5.2 with 1.2 kg of colloidal chitin. Hydrolysis at 37° C with weak stirring until total reduction of turbidity is obtained.

The solution is centrifuged 15 minutes at 4,500 r.p.m.; the supernatant is saturated in 11 kg of $(NH_4)_2SO_4$, then stirred and centrifuged 10 minutes at 4,500 r.p.m. The deposit is collected.

i) This last operation can be repeated if the activity does not reach 850-900 nephelometric units.

5. Cost of materials to manufacture 100 g of chitinase:

Citric acid	20 kg (maximum)	$68.22
Sodium phosphate dibasic	15 kg	50.75
Thymol	60 g	2.40
Ammonium sulfate	80 kg (maximum)	97.23
Chitin (technical)	10 kg	172.80

6. Preparation of the colloidal chitin:

a) Material: hydrochloric acid (concentrate) 15 gallons $37.44
hydrochloric acid diluted 1/10
sodium hydroxide 1/10

b) Method:

The technical chitin is treated 3 times as follows: 24 hours in 15 gallons of hydrochloric acid 1/10, filtration, and 24 hours in 15 gallons of sodium hydroxide 1/10.

After filtration and drying, the chitin is slightly moistened in acetone and then dissolved in 15 gallons of concentrated hydrochloric acid. This highly viscous suspension is filtrated in fritted glass funnels in a flask containing distilled water (75 gallons, according to the above-mentioned calculation).

The chitin is precipitated in a colloidal suspension, filtered, collected in distilled water and filtered a few times at pH 6.4.

The colloidal chitin is then vacuum dried.

7. The activity of the chitin obtained after purification is determined and checked by nephelometric analyses. It should not be below 850 nephelometric units.

Assessment of chitinase activity

The nephelometric analyses consist in measuring the variation of turbidity by nephelometry of a colloidal suspension of chitin before and after the enzymatic lysis. Lysis is obtained after 2 hours of incubation at 37.5° C, pH 5.3 (buffer solution: citric acid 0.1 M and disodium hydrogen phosphate 0.2. M) Ten nephelometric units measured the activity of 1 mg of chitinase which provoked a 50% decrease of the turbidity of a suspension of colloidal chitin containing 0.3 mg per ml of suspension.

DISCUSSION

The method can be readily used on an industrial scale. The only alteration required is the installation of a collecting device for chicken gastric juices in chicken-processing plants. These juices can easily be concentrated and stored aseptically for subsequent chitinase production. The low-priced chitinase thus obtained can be very useful as an additive in biological insecticides for increasing their efficacy, serving as a base for contact or stomach chitinolytic chemical insecticides. It also has other potential industrial uses.

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CHITIN DIGESTION IN THE DIGESTIVE TRACT OF FISH

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ABSTRACT

Since many Crustacea are consumed as bait by fish, it can be assumed that a chitinolytic enzyme must be present in fish digestive organs.

The present study is concerned primarily with the study of the chitinolytic enzyme secreted from the digestive organs of the fish, Lateolabrax japonicus, and secondarily with the chitin-decomposing enzyme of bacterial origin in its digestive tract.

The chitinolytic activities were investigated by measuring the amount of N-acetylaminosugar liberated, and the decrease of the substrate in the reaction mixture, using precipitated chitin and native chitin as the substrate.

The chitinolytic enzyme of non-bacterial origin was found to be highly active in the stomach, but little or none was discerned in the intestine.

Nearly all the bacteria found in the fish digestive tracts have chitin-decomposing abilities. The populations of chitin-decomposing bacteria were on the order of 10^4 - 10^9 per gram of the contents of the fish digestive tract. These bacteria were classified into the genus Aeromonas, Vibrio and Alginomonas.

Conspicuous differences were observed in the pH stability curve and in the optimum temperature between the properties of the chitinolytic enzyme of non-bacterial and of bacterial origins.

The results obtained show that chitin seems to be decomposed simultaneously by both bacterial and non-bacterial actions in the fish digestive tracts.

INTRODUCTION

For a long time it was believed that the higher animals did not digest chitin, although the possibility of symbiotic bacteria aiding animals in the digestion of chitin was discussed by many investigators.

Chitin-decomposing bacteria were isolated from the intestinal contents of several common marine animals. For instance, from a hundred to more than a thousand chitin-decomposing bacteria per cubic centimeter were found in the stomach contents of squid and other cephalopods which ingest chitinous food (16), suggesting that many bacteria may play an important role as symbionts aiding animals in the digestion of chitin.

Recently, it has been shown that the distribution of chitinolytic enzymes among invertebrates is much more extensive (4, 6, 7, 9, 15). The presence of chitinolytic enzymes among Vertebrata have been found in fish, some lizards, birds and mammals (8-10).

In the digestive tracts of some animals, the decomposition of chitin seems to be carried out simultaneously by both bacterial and non-bacterial actions. The investigation reported here was undertaken to obtain some information on this point.

Japanese sea bass (Lateolabrax japonicus) was used to investigate the chitinolytic enzyme of non-bacterial origin and the chitin-decomposing bacteria in the digestive tract of this fish. A supernatant solution from the homogenates of fish digestive organs was used as the enzyme preparation, and the precipitated chitin and native chitin were used as the enzyme substrate. A chitinolytic enzyme produced by isolated bacteria was prepared by salting out with ammonium sulfate (0.8 saturation) from the culture filtrate. The chitinolytic activities were investigated by measuring the amount of N-acetylamino sugar liberated (13) and the decrease of the substrate in the reaction mixture.

EXPERIMENTAL RESULTS AND DISCUSSION

Non-bacterial origin

Table 1 shows the distribution of chitinolytic activity in the digestive organs of a few kinds of aquatic animals. It indicates that chitinolytic activity was found in the stomachs, livers, and spleens of all fish tested, while, with the cephalopod tested, it was found in the stomach, liver and buccal mass.

Although no attempts at purification of the enzyme were made, several properties of the enzyme reaction were investigated with the crude enzyme preparation from the stomach of Lateolabrax japonicus.

The time courses for the activity of the chitinolytic enzyme are shown in Fig. 1. The increase of oligosaccharides was rapid up to 6 hours, but little monosaccharide was formed in this period of incubation. The optimum pH value was approximately 4. This pH value is similar to that of molds (12) and more acidic regions than to that of Basidiomycetes (1) and Streptomyces (5). The enzyme was stable at pH 3-8 but unstable at pH 9. The optimum temperature was observed at 55-60° C, and the enzyme was stable at 20-60° C but lost almost all activity at 70° C.

Enzymatic hydrolysates of chitin

A portion of a filtrate of the reaction mixture was subjected to thin-layer chromatography (TLC) to determine the products of the enzyme reaction. TLC was carried out on silica gel G, using n-buthanol, acetic acid and water (10:3:7) as the developing solvent.

TLC showed the presence of three spots which were stained with anilin phthalate, anilin oxalate and Elson-Morgan reagents. Two spots have the same Rf values and colors as authentic N-acetylglucosamine and glucosamine respectively.

Table 1. The Distribution of Chitinolytic Activity in the Digestive Organs of a Few Kinds of Aquatic Animals

Species	Organs	Activity
Lateolabrax japonicus	Stomach	+++
	Liver	++
	Gall bladder	+
	Spleen	++
Seriola quinqueradiata	Stomach	+++
	Liver	+
	Gall bladder	-
	Spleen	+
	Pancreas	-
Hippoglossoides dubius	Stomach	++
	Liver	+
	Gall bladder	++
	Spleen	+
Stichaeus grigorjewi	Stomach	+++
	Liver	+
	Gall bladder	+
	Spleen	+
Gadus macrocephalus	Stomach	+++
	Liver	++
	Gall bladder	++
	Spleen	++
Mustelus manazo	Stomach	+++
	Liver	++
	Spleen	++
	Pancreas	-
Ommastrephes sloani pacificus	Stomach	+++
	Liver	+++
	Buccal mass	++
Polypus dofleini	Stomach	+++
	Liver	+++
	Buccal mass	++

-: No activity
+: Slight activity
++: Considerable activity
+++: High activity

Another portion of the reaction mixture was passed through a column of Dowex 50-X8 to adsorb hexosamine and hexosamine oligosaccharides (2). The elution pattern of column chromatography of Dowex 50-X8 showed two fractions. This chromatographic analysis showed the presence of N-acetylglucosamine and its oligosaccharide in the reaction mixture.

In the present study the digestion of chitin by the enzyme obtained from the fish stomach yielded a mixture of N-acetylglucosamine, its oligosaccharide and a little amount of glucosamine. However, it was found the enzyme preparation from the fish stomach apparently is left unchanged if incubated with N-acetylglucosamine, probably because of deacetylation of the chitin substrate (precipitated chitin) during its preparation.

It has been observed by many investigators that the end product of the action of the crude chitinolytic enzyme on native chitin was N-acetylglucosamine (3, 11, 14).

On the basis of the substrate decrease and monomer and oligomer increase, it is assumed that a higher oligosaccharide that can not be depolymerized with glucosidase was formed in the enzyme reaction (Table 2).

Table 2. Correlation of the Decrease of Substrate and the Formation of N-Acetylamino Sugar

Enzyme (ml)	The Decrease of Substrate (mg)	The Formation of N-Acetyl-glucosamine (μg)	
		As monomer	As oligomer
2.0	6.81	32	1168
1.5	6.81	24	1056
1.0	6.67	18	944
0.5	4.58	0	640
0.1	1.97	0	168

Bacterial origin

The bacterial population was estimated by a ZoBell 2216E agar plate medium supplemented with precipitated chitin. As shown in Table 3, it was observed that a considerable amount of chitin-decomposing bacteria was present in the fish digestive tracts, where as many as 10^9 cells per gram of the digestive contents were found. It is therefore necessary to consider the possibility that such bacteria may play an important role as symbionts that aid animals in the digestion of chitin.

The dominant chitin-decomposing bacteria were isolated and determined taxonomically. These bacteria showed the characteristics of genus Aeromonas, Vibrio or Alginomonas.

Table 3. The Population of Chitin-Decomposing Bacteria in the Fish Digestive Tracts

Exp.	Digestive Tracts	Bacterial populations	
		Total*	Chitin**
1	Stomach	5.5×10^9	5.2×10^9
	Pyloric caeca	4.0×10^6	3.9×10^6
	Intestine	3.0×10^7	3.0×10^7
2	Stomach	2.4×10^4	2.4×10^4
	Pyloric caeca	3.5×10^6	3.5×10^6
	Intestine	3.5×10^5	3.5×10^5
3	Pyloric caeca	5.0×10^7	5.0×10^7
	Intestine	3.5×10^8	3.5×10^8
4	Pyloric caeca	1.4×10^8	1.4×10^8
	Intestine	3.3×10^9	3.3×10^9

*Total: Total heterotrophic bacteria
**Chitin: Chitin-decomposing bacteria

From the strains isolated, Aeromonas sp. 9 and Vibrio sp. 15 were used in the enzyme study. The former was isolated in the stomach and pyloric caeca and the latter in the pyloric caeca and intestine.

The optimum pH value for the chitinolytic activity of Aeromonas sp. 9 was found to be 5.5-6.0. This pH range was similar to that of Basidiomycetes (1) and Streptomyces and a more alkaline region than that of molds (12) and the fish stomach. However, the optimum pH value was found to be 7.0 in the case of Vibrio sp. 15.

Between pH 5.0 and 9.0, the enzyme of Aeromonas sp. 9 was stable. It became unstable when the pH value was reduced to less than 4.5. Between pH 5.5 and 9.0, the enzyme of Vibrio sp. 15 was stable; it became unstable at less than 5.0. These pH-stability curves differed considerably from those of the fish stomach.

The optimum temperatures of the chitinolytic activity of two bacteria were observed at about 40° C, a temperature lower than that observed in the stomach (12).

As shown in Table 4, the decrease of the substrate was slightly more than the increase of monosaccharide, and a small amount of oligosaccharide was found in the reaction mixture. On the basis of the data in Table 4, it was assumed that the higher oligosaccharide, which cannot be depolymerized with glucosidase, is present in only very small amounts.

Table 4. Correlation of the Decrease of Substrate and the Formation of N-Acetylamino Sugar

Reaction Temp. (°C)	Decrease of Substrate (mg)	Formation of N-Acetyl-Glucosamine (μg)	
		As Monomer	As Oligomer
25	0.513	394	76
35	0.655	420	124
45	0.981	504	120
55	0.458	336	40

The TLC of the digested products by the bacterial enzyme gave two spots. The rapid spot had the same Rf and color developing as an authentic N-acetylglucosamine. The HCl-hydrolysates of the digested products gave a single spot with the same Rf and color developing as an authentic glucosamine. Accordingly, the digestion of chitin by a bacterial enzyme yielded the mixture of N-acetylglucosamine and its oligosaccharide.

CONCLUSIONS

The chitinolytic enzyme was widely distributed in the fish digestive organs. It was found that the chitinolytic enzyme of non-bacterial origin was highly active in the stomach of fish, but absent or relatively inactive in the pyloric caeca and intestine.

The main substances formed by the action of the enzyme are N-acetylglucosamine and its oligosaccharide, when precipitated chitin is used as the enzyme substrate.

The greater part of the total heterotrophic bacteria present in the digestive tracts of fish was a chitin decomposer. These chitin-decomposing bacteria were classified into genus Aeromonas, Vibrio or Alginomonas.

The main substance formed by the action of the extracellular chitinolytic enzyme may be N-acetylglucosamine. The conspicuous differences were observed in pH stability curves and optimum temperatures between the properties of the chitinolytic enzyme of non-bacterial origin and that of bacterial origin.

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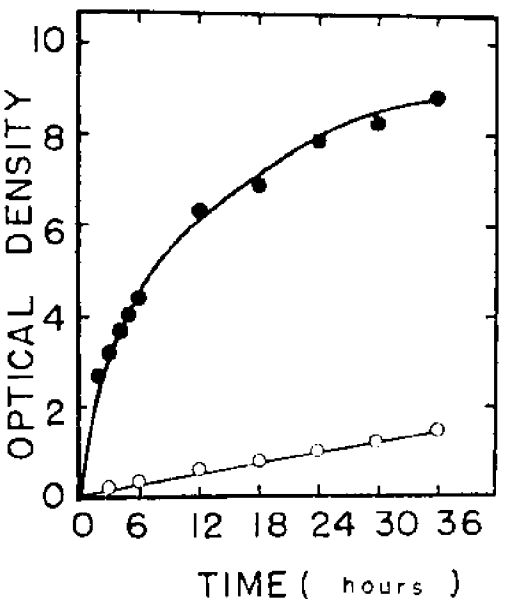

Figure 1. Time courses of the enzyme activity.

—●— Total N-acetylamino sugar (monomer + oligomer)

—○— N-acetylamino sugar (monomer)

To determine water soluble oligomer, the reaction mixture was treated with β-glucosidase (Calif. Corp. for Biochem. Res.).

CHITIN DEGRADATION IN ESTUARINE ENVIRONMENTS AND IMPLICATIONS IN CRUSTACEAN BIOLOGY

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ABSTRACT

Chitin-biodegradation investigations have included analyses of bacterial chitinoclastic activities, studies of chitinase enzymes in the white shrimp (Penaeus setiferus) and associated bacterial biomass, and evaluation of in situ and in vitro degradation rates. Significant rates of chitin decomposition are noted in salt-marsh shrimp-nursery grounds along the southeastern Louisiana coast. Productivity of such estuarine regions is reflected in the noteworthy chitin biodegradative rates reported. In situ activities are temperature related, with maximal chitin mineralization at water-sediment interfaces.

Maximal laboratory degradation rates of 42 mg/day/10^{10} bacterial cells contrast markedly with in situ rates as great as 440 mg chitin decomposed/day. Other factors affecting in situ degradation include available organics, colonization or affinity of bacteria to the substrate, and type, particle size and initial concentration of chitin. Of several types of chitin examined, viz., acid-treated, acid and alkali-treated, freshly molted, and untreated, the last was most readily metabolized. In view of the trace amount of protein associated with natural exoskeleton chitin, processes of co-metabolism may be involved in its decomposition in the natural environment. Correlations are noted between chitin turnover rates and ratio of chitinoclasts to total bacteria.

Chitin transformation in crustacean metabolism is discussed along with use and assimilation of chitinous substrates in diets developed for economically valuable shrimp and prawns in aquacultural systems.

INTRODUCTION

While the abundance of chitin in marine and estuarine environments is well established (5), data on direct in situ rates of substrate decomposition are noticeably lacking. In contrast, rates of chitin degradation under laboratory conditions in closed systems (3,14,15,16) have been reported. Using experimental in vitro rates, Seki (14) calculated that chitin particles <.003 cm in diameter, typical of planktonic crustacea, were decomposed in the coastal waters of Japan at a rate of 27 mg chitin/day. Elsewhere, Liston et al. (11), using a simulated seabed model, determined degradation rates in Puget Sound sediments of 18.9 mg/day

Lack of information on in situ rates warranted studies designed to evaluate previously documented in vitro chitin-degradation reports together

with analyses of chitin transformation in a shallow salt-marsh lake in southeast Louisiana. This site is of considerable interest in view of its high productivity and penaeid shrimp activity, processes contributing to significant exoskeleton deposition and mineralization. Our previous and concurrent investigations (6) on analyses of microbiological and chitinase activities relative to the white shrimp, Penaeus setiferus and its chitinoclastic endosymbionts (8,9,10) have demonstrated the relevance of total chitinolytic processes in penaeid biology. Clearly, chitin transformation information on all aspects of crustacean biology and estuarine energy budgets is needed to understand trophic level interrelationships and extrapolation of data to applied systems such as shrimp and prawn aquaculture.

MATERIALS AND METHODS

All in situ measurements, sediments, and isolated bacteria were from a small semi-enclosed locality (Airplane Lake), within the Barataria Bay salt-marsh estuary. The procedure for the determination of chitin degradation approximates that described by Chan (3) and Okutani (12). Twenty grams dry weight of purified chitin (Calbiochem), ball-milled for 72 hours at 4°C, were dissolved in 300 ml concentrated HCl. The acid-chitin solution was poured into 3 liters of distilled water and the precipitant repeatedly washed by filtration to remove residual acid. The solution was adjusted to a concentration of 5 percent chitin and sonificated until the larger aggregates were disrupted, resulting in a homogeneous chitin-based mixture. The pH was adjusted to maintain a value of 7.6 after sterilization. One milliliter of the evenly suspended sterilized chitin was added to sterile flasks containing 100 ml of a basal broth of 0.5% yeast extract (Difco) and sea water, pH 7.6. The flasks were inoculated with one milliliter of a cell suspension of selected chitinoclastic bacteria of the genus Beneckea (2), designated S/1, S/4, W/4 and S/3. Isolate S/3 was from the white shrimp Penaeus setiferus, while S/1 and S/4 were from sediments and W/4 from water, all taken from the Barataria Bay region.

Each bacterial isolate was grown in yeast extract (0.05%), peptone (1%), and sea-water broth, pH 7.6, for 24 hours at 22°C, centrifuged, washed in sterile sea water, and resuspended in 100 ml sterile sea water. Following inoculation, flasks with the chitin media were incubated at 22°C on a gyratory shaker (30 rpm). Control vessels with chitin, basal broth, and toluene were also incubated. At 24-hour intervals, cell biomass was monitored, using the standard plate-count method and Marine Agar 2216. At selected intervals, flasks were removed, heated to boiling point for 10 minutes, and washed with 2% HCl and 12% KOH. After washing to neutrality, the chitin was collected by filtration, dried at 105°C for 12 hours, and the dry weight determined gravimetrically. The remaining chitin subtracted from the initial concentration represented the amount of substrate decomposed per bacterial cell mass.

Methods used for determination of chitin degradation in vitro have been described by Seki and Taga (16). The top 2 cm of submerged sediments from sites in Airplane Lake were collected aseptically using a Ponar Grab. Sea water at a depth of 6 inches was also collected aseptically. Into sterile flasks containing 100 ml of the collected sea water were added 0.1 g dry weight chitin particles (Calbiochem) of an average size of 1 cm x 0.5 cm with a thickness of approximately 0.2 cm. In other flasks, one gram of sediment, 99 ml of sterile aged sea water and chitin were added. All vessels were incubated at 22°C for 30 days, after which particles were acid- and alkaline-washed and dried at 105°C for 12 hours. The amount of chitin

decomposed was determined gravimetrically.

For in situ studies, native chitin from P. setiferus was removed and washed in tap water. The dorsal portion of the exoskeleton was broken into particles, 1-2 cm^2, with an approximate thickness of 1-0.5 mm. The chitin was dried at 105°C for 12 hours, weighed and placed in acetate bags with a pore size of 60μ x 10μ. The bags were inserted into nylon containers, mesh size 0.25 cm^2, attached to a rod, and placed within a protective metal trap.

The chitin was seeded during the months of September through April, and temperature was monitored during this time. The monitoring station is described by Adams (1). After appropriate time intervals, the bags were retrieved and returned to the laboratory in an ice chest within 24 hours for analysis.

Each bag was submerged in boiling water for 10 seconds and the contents emptied into a U.S. standard metal sieve (No. 100). Tap water was sprayed over the particles to remove accumulated organic matter, and particles were collected and dried at 105°C for 12 hours and weighed. The amount of decomposed chitin was expressed as substrate loss per unit time.

The in situ rates were determined according to the chitin substrate type, particle size, initial substrate concentration, depth of water column, and temperature. The following types of chitin were used: (1) untreated native white-shrimp cuticle composed of approximately 68% chitin, 10% protein and 22% salts as reported by Richards (13); (2) molted P. setiferus exoskeleton containing a relatively higher percent of chitin than native chitin; (3) dilute acid-treated (2% HCl) white-shrimp chitin, also containing a relatively higher percent of chitin than native chitin; (4) acid, alkaline, ethanol-treated chitin as described by Campbell and Williams (4), containing chitin of high purity, and (5) a commercial chitin (Calbiochem), also a highly purified form of chitin. Particle size of the chitin was adjusted by use of various size screens.

The total bacterial biomass was monitored on the chitin substrate using the standard plate-count method. Chitinoclastic bacteria were enumerated as described by Hood and Meyers (7).

RESULTS

In vitro chitin-degradation rates for the predominant chitinoclastic bacteria from the estuarine environment varied from 25-42 mg/day/10^{10} cells at 22°C, with an average rate of 33.8 mg/day/10^{10} cells. The latter value is comparable to that reported by Seki (14) for strains isolated in Aburatsubo Inlet, Japan (30 mg/day/10^{10} cells at 25°C), and by Chan (3) for strains from Puget Sound, Washington (19.2-31.2 mg/day/10^{10} cells at 22°C).

The laboratory degradation rates of chitin in sediments (47 mg/month) and waters (22 mg/month) from Barataria Bay approximate those found in material from Aburatsubo Inlet (14), suggesting that little variation in in vitro chitin-degradation potential exists within these estuary types. Rates in laboratory closed systems do not, however, truly reflect values of chitin degradation found in the actual environment. With this in mind, attempts were made to determine the magnitude of chitin solubilization or

decomposition rates *in situ*.

The *in situ* studies revealed factors that influenced the rate of chitin decomposition; among them are temperature, available organics, colonization or affinity of bacteria to the substrate, and type, particle size and initial concentration of chitin. A strong correlation was noted between decomposition and environmental water temperatures (correlation coefficient, R= 0.95). Maximal degradation was observed in late summer when temperatures averaged 30°C, while lowest rates were in mid-winter when temperatures averaged 8°C.

The depth of the water column has a decided influence on chitin decomposition, with maximum rates (440 mg chitin decomposed/24 hr) at the water-sediment interface or slightly above, compared with values obtained at 2 feet above the interface. Based on the overall greater microbial activity at this interface, these data are not unexpected, but they do demonstrate the need to document the exact depth of the sample test for accurate comparison of data.

The initial concentration of chitin seeded directly affected the rate of substrate degradation, while particle size inversely affected chitin decomposition. Furthermore, decomposition increased as particle size decreased. The type of chitin also affected the decomposition rate (Table 1). Untreated chitin offered the best substrate for microbial degradation, while pure chitin was degraded more slowly.

The purified substrate supported fewer bacteria than untreated chitin, indicating the significance of microbial colonization. In this regard, the protein content of the untreated chitin may provide an attractant, allowing the substrate to be more rapidly colonized and degraded. *In situ* decomposition of untreated chitin was determined over a 23-day period (Table 2). Initial colonization by total bacteria and chitinoclastic types was rapid, as demonstrated by the large cell concentrations at 4 days.

After 8 days, bacterial populations increased. Particularly noteworthy were the relative concentrations of chitinoclasts which increased from 8.9 to 15.6% with concurrent maximum degradation. By the 18th day, 97% of the original substrate was solubilized, a leveling off of the degradation rate and relative concentration of chitinoclasts. The latter appears to be a valid indicator of decomposition rates and may prove to be a useful tool in this regard.

DISCUSSION

While our data on chitin degradation in pure culture and *in vitro* do not differ significantly from those of other workers, *in situ* analyses are noteworthy. Maximal laboratory rates of 42 mg/day/10^{10} cells (axenic culture) contrast markedly with those *in situ* as great as 440 mg chitin decomposed/day. This tenfold difference suggests that earlier reports of *in vitro* tests do not reflect the full ramifications of chitinoclastic processes in the environment. Certainly, *in situ* determinations support observations on the rapid mineralization of chitin and provide additional evidence for the significance of this process in substrate transformation in estuarine ecosystems.

The data from our laboratories have indicated the need to evaluate the

Table 1. *In Situ* Rates of Chitin Degradation on Various Chitin Substrates[1]

Type of Chitin	Rate of Degradation[2] (mg chitin decomposed/24 hr)		
Native chitin	148.4	151.4	146.3
Acid-treated chitin	91.7	92.5	95.8
Molted chitin	127.6	131.3	124.5
Pure chitin	22.2	32.4	28.4

[1]At water-sediment interface in Airplane Lake.
[2]In triplicate.

Table 2. *In Situ* Chitin Degradation Rate and Bacterial Colonization of Native Chitin

Time (days)	Bacterial Population			Native Chitin		
	Total Bacterial Biomass (cells/g)	Chitinoclastic Biomass (cells/g)	Percent Chitinoclastic Bacteria	Amount Degraded (g)	Percent Degraded	Degradation Rate (mg/day)
4	2.7×10^9	2.4×10^8	8.9	0.8995	18.0	250.0
8	4.3×10^9	6.7×10^8	15.6	3.445	68.9	437.5
18	3.9×10^8	5.5×10^7	14.1	4.850	97.0	253.6
23	5.0×10^9	7.0×10^7	14.0	4.942	98.8	220.0

total chitin/chitinoclastic processes, including molting, associated with P. setiferus, for a more definitive understanding of the physiological determinants of penaeid ecology. Shrimp nutritional studies suggest that end products of chitin hydrolysis are important in shrimp growth and maturation. In pond culture of shrimp and prawns, cast exoskeletons must be considered in evaluation of food-conversion rates and overall energy-efficiency calculations. In dietary formulations, levels of shrimp meal as high as 30 percent are not uncommon, with indications that such meals, or specific components therein, may be needed to effect optimal rates of food ingestion and animal growth. Evidence generated to date on chitin degradation within the P. setiferus digestive tract further suggests that bacterial and crustacean chitinase mechanisms play an important physiological role in growth and development of the animal.

As noted, total biomass and species diversity of chitinoclastic bacteria may serve as usable indicators of chitin turnover and substrate transformation in the penaeid shrimp. Substrate specificity tests, revealing maximal enzyme activity on chitin from molts of P. setiferus, indicate an active role for various marsh chitinoclastic bacteria in recycling of this polymer in the natural environment.

ACKNOWLEDGMENTS

This report is the result of research sponsored by the Louisiana Sea Grant Program, a part of the National Sea Grant Program maintained by the National Oceanic and Atmospheric Administration. Additional support was received from the Louisiana State University Agricultural Experiment Station.

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CHITINOLYTIC AND CHITOSANOLYTIC MICROORGANISMS AND THE POTENTIAL BIODETERIORATION PROBLEM IN THE COMMERCIAL APPLICATION OF CHITIN AND ITS DERIVATIVES

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ABSTRACT

Chitinolytic, chitinoclastic and chitosanolytic microorganisms may be of interest as sources of enzyme for the modification of chitinous materials for commercial purposes, but they will then certainly be of concern as potential spoilage organisms, for microbes able to degrade these polymers are ubiquitous. This contribution will concentrate on possible solutions to this problem.

The chitinase system is widespread in both bacteria and fungi. Less is known of the distribution of chitosanase, but several fungi and actinomycetes and a few bacteria are known to produce this enzyme.

Theoretical considerations certainly cannot lead to reliable predictions regarding which member of which of these classes is likely to be dominant in a situation where the biodeterioration of chitinous materials is occurring. Still, examination of available data on spoilage organisms acting in similar or identical situations to those where the use of chitin or its derivatives is envisaged suggests that similar microfloras will be involved. Experience in other fields has shown that simulation experiments can lead to more accurate predictions that may enable us to find solutions before the problems emerge in large-scale commercial use. Some of these possible preventive measures are then outlined in general terms.

INTRODUCTION

Chitin was named about a century and a half ago (13) and chitosan about eighty years ago (9), but both these polymers were discovered even a few years earlier. It is therefore all the more striking that no extensive commercial use of these materials has yet been made, particularly since very large quantities of chitin and chitosan are synthesized annually. An estimated several times 10^9 tons of chitin are produced each year by one group of planktonic arthropod, the copepods, alone (17). Taking into account the chitinous material synthesized by other animals and microorganisms, it seems likely that the total amount formed each year will approach very closely the 10^{11} tons of cellulose which it is estimated are generated annually (17) and which "is a substance of prime importance to man as a raw material for the construction of his artifacts" (18).

These Proceedings, however, represent an effort to end the neglect of chitinous materials, now treated solely as a nuisance, and to encourage their widespread commercial use. It therefore seems appropriate to examine one possible problem area and see to what extent difficulties may be anticipated and solved.

From the microbiological point of view the problems lie in two obvious areas: the use of microorganisms as sources of enzymes for the controlled degradation and modification of the naturally occurring polymers, and the biodeterioration or spoilage of the chitinous product. The first of these is dealt with by Ohtakara et al. in these Proceedings. In this contribution an attempt will be made to assess the potential biodeterioration problem and to outline possible solutions.

MICROBIOLOGICAL BACKGROUND

In one sense our problems would be solved if we could simply expect that deterioration of chitinous materials would not occur; in another, our problems would only have begun, for there would then be an unfortunate accumulation in some environments of discarded wastes such as polyethylene and polyvinylchloride. Indeed, ignoring for a moment the rule of thumb that biosynthesized materials are biodegradable, it might until recently have been expected that chitosan would in fact be resistant to decay since removal of the acetyl residues from the 2-acetamido group of chitin is known to confer upon the polymer resistance to chitinase activity. Since 1973, however, enzymes produced by several microorganisms which specifically degrade chitosan (see Table 1 and Fenton et al., these Proceedings) have been reported. In contrast the first report of a chitin-decomposing bacterium was published in 1905 (14), and chitinolysis is known to be the property of some members of a great many microbial groups (Table 2).

Given the spectra of known chitosanolytic and chitinolytic microorganisms (Tables 1 and 2) and their occurence in just about every conceivable environment, it seems certain that with the establishment of widespread use of chitinous polymers, biodeterioration problems will emerge.

PREDICTION

From the above survey and from what is known of the enzymology of chitin and chitosan degradation, there is no reason to suppose that bacteria or fungi, even though the latter often have chitinous elements in their structure, will overall be more or less dominant in the breakdown of these materials than they are in attacks on other polysaccharides. Nevertheless, it is obviously of interest to know if, in particular instances where the use of chitinous material is envisaged, the primary biodeteriogenic organisms can be predicted.

A first approach to this can be made by examining the microflora of products in which chitinous materials may be used and comparing this with the spectra of known chitosanolytic and chitinolytic types (Tables 1 and 2). This was done in Table 3 for three types of product. A substantial overlap can be seen between biodeteriogenic microorganisms in these situations and types known to degrade chitin and chitosan. At the generic level, then, the biodeterioration microflora of products containing chitin is likely to be similar to that already found in equivalent situations.

It would be unwise to pursue this theoretical analysis by analogy too far, because biodeterioration is an ecological problem: environmental factors

in a particular habitat usually specify particular strains rather than species, let alone genera.

More accurate predictions may be achieved by simulation experiments, such as those used in other fields (e.g., fuel systems of supersonic aircraft [7]). Experience suggests that, given adequate supplies of the particular material liable to biodeterioration and a proper knowledge of the conditions under which it is to be used, indications of the organism(s) likely to be involved can be obtained that will allow preventative measures to be devised.

PREVENTION

The biodeterioration microflora of chitinous products is likely to be similar to that attacking comparable materials in comparable situations. Experience with this microflora will form a valuable basis for devising methods to prevent attack on products containing chitin or its derivatives, provided that some factor is not overlooked (1), underestimated, or was not anticipated. Reference is made to the three types of product considered in the previous section as well as some others. Preventive measures fall into two categories:

1) Rendering the environment inimical to the microorganism

The best way of achieving this is to keep the material dry. This would be most appropriate for chitinous paper products, since it is a method in any case required for optimal transport, handling and storage conditions. If maintaining dry conditions is not possible, lowering of the water activity (a_w) by the addition of solutes may be effective and other physical factors, notably temperature, can be adjusted to control deterioration (1).

Manipulation of the environment by addition of biocides is another approach which falls within this category, and it is one widely used, although there are often contraindicating economic and environmental considerations. Sterilization of materials used in wound treatment is obviously appropriate.

2) Rendering the material inaccessible to the microorganism or to its enzymes

Exclusion of the microorganism may be achieved by suitable hygienic measures. This is an approach that would be applicable to at least some pharmaceutical products (5) containing chitinous materials.

In some cases, the physical or chemical form of the carbohydrate can be designed in its manufactured form so as to hinder or prevent attack; such material might be appropriate in fiber-modification applications, for the formation of chitinous films and for packing material for chromatography columns.

Table 1. Microbial Genera Known to Contain Representatives Producing Chitosanase*

Bacteria	Fungi
Arthrobacter	Aspergillus
Bacillus	Cokeromyces
Sporocytophaga	Gliocladium
Streptomyces	Penicillium
	Rhizopus
	Stachybotrys
	Trichurus
	Trichoderma
	Zygorrhyncus

*Data from References 11, 14, 16, 19.

Table 2. Microbial Genera Known to Contain Representatives Producing Chitinase*

Bacteria	Fungi
Achromobacter	Absidia
Actinomyces	Aspergillus
Aeromonas	Bovista
Agarbacterium	Beauvaria
Alginomonas	Chytriomyces
Arthrobacter	Coprinus
Bacillus	Cordyceps
Benekea	Fistulina
Chromobacterium	Fusarium
Corynebacterium	Gliocladium
Clostridium	Irpex
Cytophaga	Lycoperdon
Erwinia	Metarrhizium
Flavobacterium	Mortierella
Klebsiella	Mucor
Micrococcus	Mycogone
Micromonospora	Paecilomyces
Myxococcus	Penicillium
Nocardia	Phycomyces
Photobacterium	Rhopalophlyctis
Sarcina	Thamnidium
Serratia	Trichoderma
Sporocytophaga	
Streptomyces	
Vibrio	

*Data from References 3, 4, 8, 20, and S. Shimizu and K. Aizawa, personal communication.

Table 3. Microorganisms Involved in the Biodeterioration of Some Products in Which Chitinous Materials Might Be Used

Some Applications for Chitinous Materials		Some Genera Containing Microorganisms Involved in Biodeterioration in Equivalent Applications*	
Industry	Specific Application		
Pharmaceutical	Emulsions; Cosmetics	Acetobacter Arthrobacter* Bacillus* Lactobacillus Proteus Pseudomonas Serratia* Staphylococcus	Aspergillus* Cladosporium* Trichoderma*
Paper	Strength Improvement	Aerobacter Achromobacter* Bacillus* Clostridium* Desulfovibrio Escherichia Flavobacterium* Pseudomonas Serratia*	Alternaria Aspergillus* Chaetomium Cladosporium Penicillium* Polyporus Stachybotrys* Stemphilium Trichoderma*
Textile	Fiber-Property Improvement	Bacillus* Cellulomonas Cellvibrio Clostridium* Cytophaga* Sporocytophaga* Streptomyces*	Alternaria Aspergillus* Chaetomium* Cladosporium* Fusarium* Penicillium* Stachybotrys* Stemphilium* Trichoderma*

*Those genera known to contain representatives producing chitinase and/or chitosanase are marked with an asterisk.

Naturally occurring forms of chitin provide examples of the importance of physical arrangement and/or of the chemical combination in the reduction of deterioration. Purified chitin of the relatively disordered type made from cuttlefish "bone" was more readily attacked by a Bacillus-species chitinase system than the more ordered α-type from shrimp and lobster (unpublished work; see also 12).

Similarly, strips cut from the pro-ostracum of Sepia officinalis, which contains about 49% chitin (6), of which 85% is digestible by chitinase (10), are extensively colonized (Figures 1 and 2) and rapidly degraded (16). Under the same conditions, however, no visible attack occurs on comparable pieces of crab chitin, which, although it contains 75% chitin, has 53% of this masked from the chitinase degradation by glycoprotein and other complexes (10).

CONCLUSIONS

Theoretical approaches to biodeterioration problems cannot be firmly relied on because some factor may be overlooked or underestimated, but evidence suggests that the microflora associated with chitinous material is likely to be similar to that involved with the deterioration of similar or analogous polysaccharides in comparable situations. Simulation experiments should enable a more accurate prediction of the specific types likely to be encountered in a particular environment. The methods for prevention of chitinous biodeterioration are likely to be those applicable to other industrially important polysaccharides.

It is hoped that this contribution will focus attention on this potential problem and encourage the seeking of solutions before the difficulties emerge.

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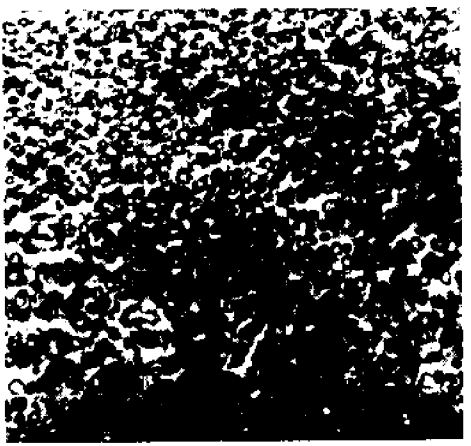

Figure 1. Surface view of an extensively colonized chitin strip cut from the pro-ostracum of Sepia officinalis, incubated at 20°C under anaerobic conditions in a mineral salts medium inoculated with chitin-enriched esturine mud. (Timmis and Berkeley, unpublished.)

Figure 2. Preparation of the same material as in Figure 1 showing morphology of colonizing bacteria. (Timmis and Berkeley, unpublished.)

CHITIN DECOMPOSITION BY MICROORGANISMS IN SOIL

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ABSTRACT

Microorganisms isolated from soil and causing the dissolution of fine particles of Sepia chitin are Bacillus spp (2 strains), Streptomyces spp (4 strains), Arthrobacter sp, Trichoderma sp, Penicillium sp and Aspergillus sp. Observations of the rate of CO_2 release from the chitin-amended soil indicate that most of the purified chitin added to the soil disappeared in about 3 weeks.

INTRODUCTION

Chitin is an aminopolysaccharide made up of N-acetyl-D-glucosamine moities. It is chemically similar to cellulose, differing from it only in the presence of nitrogen in chitin. Among the Protista, chitin has been found in algae, protozoa and fungi. In animals, it has been demonstrated in annelids, arthropods, molluscs, coelenterates and nematodes, often forming a substantial proportion of these organisms, usually in the skeletal structures. The wall of the fungus Apodachyla contains 18% chitin, less than 10% cellulose and about 6.0% protein; insect cuticle contains about 30% chitin (4,5). Because many organisms among the Protista occur in soil, the microbial decomposition of their exoskeletons is of interest. So far as this author knows, pure chitin is found in nature only in the Loligo squid (13), but purified chitin may be obtained from three other sources in a variety of ways (8).

Chitin has numerous uses, including the control of certain plant diseases (6, 12), the acceleration of wound-healing, thin-layer chromatography, and as an adhesive. It will therefore increasingly find its way in quantity into the soil. It is for this reason that it becomes of interest to study its microbial breakdown in soil.

EXPERIMENTAL

Isolation of chitin

Chitin was isolated from the "shells" of Sepia officinalis by treatment with acid to remove the $CaCO_3$. The resulting chitin-protein complex was deproteinized with NaOH, and any chitosan resulting during this treatment was removed with dilute acid. The chitin was shown to be pure by hydrolysis chromatography and infra-red analyses. X-ray diffraction analysis showed it to be of the β-type (8).

Isolation of chitinolytic microorganisms from soil

Two methods for isolation were used. In the first method, strips of chitin (roughly 1 x 5 cm) were buried in a sandy soil obtained at Nsukka and moistened to 60% of its moisture-holding capacity. From time to time, the chitin strips were recovered from the soil when this was possible. The soil particles were picked off, and the strips were sliced into smaller pieces, which were then placed on chitin agar (composition: KH_2PO_4, $MgSO_4 \cdot 7H_2O$ 5 g, NaCl 0.5 g, finely ground chitin 10 g, pH 7). The organisms that developed from them and caused a dissolution of the chitin particles in the chitin agar were purified and studied further. This method mainly yielded fungi.

The second method consisted of making plates from mixtures of soil suspensions and molten chitin agar using the composition given above.

Chitinolytic action of the isolates

The isolates obtained from the chitin strips or from the chitin-soil plates were purified by repeated streaking on glucose-yeast extract agar (7). Thereafter, they were streaked onto chitin agar and incubated at 30° C for up to 10 days. Organisms that showed a clear zone around them were regarded as chitinolytic (10).

Quantification of chitin decomposition

The extent of the breakdown of chitin was followed over a 100-day period by monitoring the release of CO_2 from soil containing chitin as compared with the control, according to the procedure (see Fig. 1) described in reference 9.

RESULT

The bacteria which were shown to "clear" chitin agar after 5-10 days incubation at 30° C were Bacillus spp (2 strains), Streptomyces spp (4 strains), Arthrobacter sp (one strain) and, among the fungi, Trichoderm sp, Penicillium sp and Aspergillus sp.

The percentage of chitin added to the soil and lost as carbon in CO_2 was greatest between the 5th and 20th day (see Fig. 1).

DISCUSSION

The microbiology of chitin decomposition in soil has been studied by a number of workers (2, 3, 11, 14). These studies indicate that chitin is decomposed in soil by a wide range of microorganisms, and the present study confirms this. The ease with which chitin decomposed in soil was shown by the observation that most of the carbon added to soil in the form of chitin had been released in the form of CO_2 in the first three weeks or so. The calculations did not take into account the CO_2 released by priming (1), i.e., by the extra decomposition of organic matter indigenous to the soil resulting from the addition of chitin to soil. The relatively easy decomposability of chitin is important, as chitin has often been confused with chitin-containing structures such as insect cuticles.

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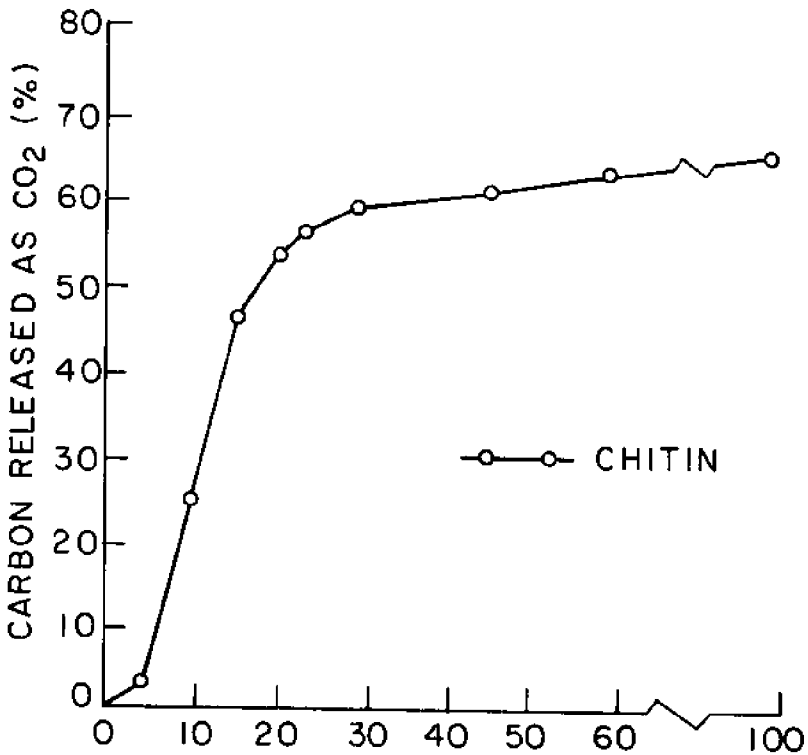

Fig. 1: Cumulative percentage of carbon in chitin released as carbon dioxide.

CHITIN- AND N-ACETYLGLUCOSAMINE-DECOMPOSING BACTERIA IN THE SEA

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ABSTRACT

The present study investigates the range and general characteristics of the standing crop of chitin- and N-acetylglucosamine-decomposing bacteria and the mineralization value of amino sugar in the sea.

The data obtained show that at most 10% of the heterotrophic bacteria were chitin decomposers in the bottom mud, while N-acetylglucosamine decomposers reached a high of 100%, i.e., the population of chitin decomposers was scarce as compared to that of starch decomposers.

In sea water, 1% of the total heterotrophic bacteria were chitin decomposers, while, in contrast, up to 100% of the total heterotrophic bacteria were N-acetylglucosamine decomposers. The populations of glucosamine- and N-acetylglucosamine-decomposing bacteria were the same as that of the glucose decomposers both in sea water and bottom mud.

Mineralization and uptake activities of glucosamine by micro-organisms in sea water were calculated as $V = 0.140 \times 10^{-4}$ mg/l/hr and $V = 0.109 \times 10^{-4}$ mg/l/hr respectively. These V values were considerably lower than those of glucose.

INTRODUCTION

Large quantities of chitin are produced in the aquatic environment of the world each year. From data given by Johnson (2) on the abundance of copepods and their chitin content, it is estimated that this subclass of planktonic Crustacea, some of which form as many as 10-12 chitinous casts through their development stages, produces several billion tons of chitin annually.

Zobell and Rittenberg (10) reported that most chitin is probably utilized by biological agents, since little of it accumulates in marine sediments; its decomposition is probably largely due to microbial action. Bacteria are probably responsible since many kinds of chitin-decomposing bacteria have been isolated from marine environments.

The present study has been directed to an investigation of the range and general characteristics of the standing crop of chitin- and amino-sugar-decomposing bacteria, and the mineralization value in the sea.

Water samples were collected aseptically by using a sterile bacteriological ORIT-type water sampler (9). At the shallow regions, mud samples were collected by using a KK-type mud sampler (3), and in the open sea by using a gravity corer.

The counts of viable heterotrophic bacteria were carried out by using the most-probable-number method, membrane-filter-count method or the agar-plate-count method. PPES-II (9) or Zobell 2216E media were used.

The locations of the sampling stations are shown in Fig. 1. Station 1 has received a considerable amount of attention in recent years for its extreme pollution which has resulted from large amounts of organic material regularly discharged into the sea from industrial and agricultural sources along with human waste (4, 5). Stations 2, 3, 4 were coastal environments and fish-culturing areas. Station 5 and 6 were in the open sea.

EXPERIMENTAL RESULTS AND DISCUSSION

As shown in Table 1, 10^4 -10^6 cells of total heterotrophic bacteria per gram of wet base and 10^2-10^3 cells of chitin-decomposing bacteria were detected in the surface layer of the bottom mud at Station 1. These data mean that at most 1% of the heterotrophic bacteria were chitin-decomposing bacteria; in contrast, N-acetylglucosamine decomposers were present in up to 100% of the total heterotrophic bacteria. Table 1 also shows that populations of chitin-decomposing bacteria were scarce as compared with starch decomposers.

As shown in Table 2, the high densities of glucosamine- and N-acetylglucosamine-decomposing bacteria were observed in marine environments. The populations of glucosamine and N-acetylglucosamine decomposers were on the same order as those of glucose decomposers.

At Station 4, 10^2 cells of chitin decomposer per milliliter of sea water and 10^3 cells per gram of bottom mud were observed. This shows that 1% of the heterotrophic bacteria were chitin decomposers, both in sea water and in bottom mud.

At Station 2, 10^2-10^3 cells of the N-acetylglucosamine-decomposing bacteria per milliliter of sea water and 10^5-10^6 cells of it per gram of bottom mud were observed. These data show that the N-acetylglucosamine-decomposing bacteria were distributed abundantly both in sea water and bottom mud.

At Station 3, 10^2-10^4 cells of chitin-decomposing bacteria and 10^5 cells of heterotrophic bacteria per gram of mud were detected in the bottom mud.

The vertical distribution of the heterotrophic and N-acetylglucosamine decomposing bacteria shows that 0-10^3 cells of the N-acetylglucosamine-decomposing bacteria per 100 milliliter were distributed in sea water and 10^3-10^4 cells per gram were found in bottom mud at Stations 5 and 6.

Several experiments on the chitin-decomposing activities of the marine bacteria were made by Seki (6, 7). He showed that the decomposition of chitin in the mud for 30 days at 20° C was 40-80 mg in a polluted inlet. Seki and Taga (8) showed that marine sediments of this inlet had 10^8 cells of the heterotrophic bacteria per gram of mud and 10^6 cells of chitin-decomposing bacteria.

The mineralization and uptake activities of glucosamine were estimated by using ^{14}C-glucosamine as the substrate (5). These activities were determined kinetically by the Michaelis-Menten formation according to Brezonik (1).

Mineralization and uptake activities of glucosamine by microorganisms in sea water were calculated as $V = 0.140 \times 10^{-4}$ mg/l/hr and $V = 0.109 \times 10^{-4}$ mg/l/hr respectively. These V values were considerably lower than those of glucose.

Chitin is sooner or later decomposed and mineralized through the metabolism of various organisms in the sea. Microorganisms are known to play an especially important role. The decomposition processes of chitin in which microorganisms participate are divided from the chemical point of view into (a) hydrolytic breakdown of chitin into N-acetyl-glucosamine or glucosamine and (b) non-hydrolytic breakdown of the N-acetylglucosamine or glucosamine (mineralization).

Decomposition processes release mineralized nutrients for primary production. The activities of chitin- and amino-sugar–decomposing bacteria have, therefore, both a direct and an indirect influence on the productivity of organisms of various trophic levels in the sea.

CONCLUSIONS

A considerable amount of chitin-decomposing bacteria was observed in the sea in the present investigation, as it has been by earlier investigators.

A chitinase in marine bacteria may be a hydrolytic system (10), and this system acts on chitin-yielding materials, principally N-acetylglucosamine or glucosamine. In the present investigation, the populations of N-acetyl-glucosamine- and glucosamine-decomposing bacteria were distributed widely and abundantly both in sea water and bottom mud. However, the mineralization and uptake activities of glucosamine by microorganisms in sea water were low compared with those of glucose. The author plans to study the decomposition of glucosamine more precisely by using radioisotopic techniques.

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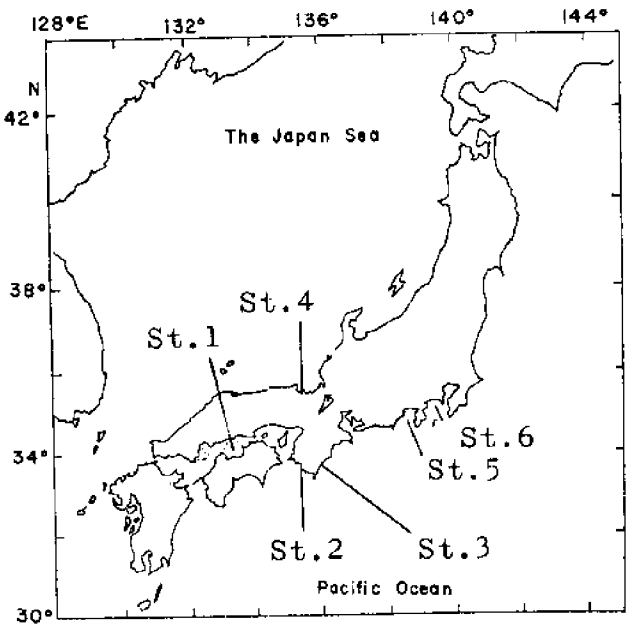

Figure 1. Map of the sampling stations.

CHITINASE SYSTEMS IN MICROORGANISMS AND THE COMMERCIAL USE OF CHITIN

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ABSTRACT

In the screening of microorganisms with potent chitinase activity, an unidentified Gram-negative bacterium was isolated from seashore mud. This strain produced an inducible chitinase system. The amount of chitinase produced was remarkably affected by the levels of glucose and yeast extract in the medium.

In the cultural filtrate of this strain, a chitinase system consisting of chitinase and chitobiase was found. These enzymes were separated from each other using column chromatography on DEAE-Sephadex A-25. It was found that the chitinase fraction probably decomposed chitin to chitin-oligosaccharides, and finally to N,N-diacetylchitobiose. This was further hydrolyzed to N-acetylglucosamine by chitobiase which also decomposed N,N,N-triacetylchitotriose and N,N,N,N-tetraacetylchitotetraose.

Comparison of this chitinase system with those from other microbial sources was made from the standpoint of enzyme production, purification, assay procedure and the mode of action.

INTRODUCTION

In searching for possible uses to which chitin can be put, one area on which attention has been focused has been utilization of the degradation products of this material. For this purpose, active and stable chitinase preparations that are commercially viable are required.

It is well known that chitinase is widely distributed among various kinds of microorganisms (4). However, purified chitinase preparations have been obtained to date mainly from the microorganisms belonging to the genus Streptomyces (2, 6, 26, 29); only a few have been reported from the purification of bacterial chitinases (9, 10, 27). One of the present authors has purified the chitinase system of Aspergillus niger (13, 15) and Streptomyces griseus (19). During further research in our laboratory aimed at obtaining an even more active chitinase preparation, we have isolated two strains of unidentified bacteria from seashore muds.

Using one of these strains, the conditions for chitinase production, the separation of chitinase from chitobiase and the mode of action of the two enzymes separated in the degradation of chitin have been investigated. As part of this study, the biochemical aspect of microbial chitinases will briefly be described.

EXPERIMENTAL

Isolation of bacteria

Seashore muds used as the microbial sources were collected along the coast of the Ariake Sea in the Kyûshû district of Japan. Two loops of mud suspension were inoculated in a test tube containing 10 ml of a medium composed of 1.0% meat extract, 1.0% peptone, 0.5% glucose and 0.5% NaCl, which was adjusted to pH 7.0. After 18 hours of culture at 35° C, 0.1 ml of the culture broth was inoculated on a screening plate with 0.5% colloidal chitin, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.03% $CaCl_2$ and 1.5% agar. Bacteria-decomposing chitins were recognized by the clear zones around their colonies on chitin agar plates and were isolated on yeast-extract peptone (0.5% yeast extract, 1.0% peptone and 0.5% NaCl) slants.

Culture for the testing of chitinase production

The conditions for chitinase production were tested in media containing varying amounts of colloidal chitin, glucose, yeast extract, peptone and NaCl, as shown in Fig. 2. Conical flasks (300 ml) containing 80 ml of the culture medium were inoculated with 1 ml of a 24-hour culture grown in a test tube containing 10 ml of the yeast-extract peptone medium and were incubated at 30° C.

Substrates

Chitin was isolated from crust shell of crab by the usual method (Finely powdered chitin was kindly supplied by Dr. M. Takeda, Shimonoseki University of Fisheries.). Colloidal chitin was prepared by dispersing chitin in concentrated sulfuric acid below 10° C and precipitating it by pouring it into distilled water, according to the procedure of Jeuniaux (5). Glycol chitin was prepared by the method of Senju and Okimasu (25) (carboxymethyl chitin was kindly supplied by Dr. S. Okimasu, Hiroshima Women's University). Colloidal chitosan was prepared from chitosan provided by Seikagaku Kôgyo Co. Ltd.), using the method of Price and Stork (23) (glycol chitosan was the product of Wakô Junyaku Kôgyo Co. Ltd.).

Chitin-oligosaccharides, N,N-diacetylchitobiose (chitobiose), N,N,N-triacetylchitotriose (chitotriose), and N,N,N,N-tetraacetylchitotetraose (chitotetraose) were prepared by hydrolysis of chitin with controlled concentrated hydrochloric acid and separation of the products on a Sephadex G-25 column, using the procedure of Berkeley et al. (3).

Chitinase activity

Chitinase activity was determined by measuring the amount of N-acetyl-glucosamine produced in a reaction mixture containing 1 ml of 0.3% colloidal chitin, 2 ml of 0.05 M phosphate buffer (pH 7.6) and 1 ml of enzyme solution which was incubated at 37° C for 30 minutes. One unit of chitinase activity is defined as the amount of enzyme which produces one μ mole of N-acetyl-glucosamine in one minute.

In the research on the effects of cultural conditions on chitinase production, activity was expressed as the amount (μg/ml) of N-acetylglucosamine produced in the same reaction mixture.

Chitinase activity was also determined by measuring the amount (μg/ml) of reducing sugar, using the method of Schales (1) with N-acetylglucosamine being used as a reference compound, as shown in the chromatographic separation of Figs. 3 and 4.

Chitobiase activity

Chitobiase activity was determined by measuring the hydrolysis of N,N-diacetylchitobiose in reaction mixtures containing 0.3 ml of 3 mM chitobiose in 0.05 M phosphate buffer (pH 7.6) and 0.3 ml of enzyme solution at 37° C for 20 minutes. One unit of chitobiase is defined as the amount of enzyme which hydrolyzes one μ mole of chitobiose in one minute.

N-Acetylglucosamine was determined by the method of Reissig et al. (24).

Column chromatography

A dialyzed enzyme solution corresponding to 147 mg of 0.7 saturation ammonium sulfate precipitate was applied to a DEAE-Sephadex A-25 column (24 x 450 mm) equilibrated with 0.02 M phosphate buffer of pH 7.2. Elution was carried out with 0.02 M phosphate buffer of pH 7.2 and with a linear gradient of NaCl in the same buffer. Flow rate was 60 ml per hour, and 10 ml fractions were collected.

Protein was determined by the method of Lowry et al. (8), bovine albumin being used as a standard.

RESULTS

Isolation of chitin-decomposing bacteria

Of the 11 strains isolated from 19 mud samples, 2 formed strong clear zones on the chitin plates. The time course of the decomposition of colloidal chitin by the 2 strains isolated is shown in Fig. 1. Strain 12 rapidly decreased the turbidity of the colloidal chitin solution in the early stage of incubation, as compared with strain 5. These results indicate that strain 12 is capable of rapidly decomposing chitin to N-acetylglucosamine. This strain is used in all the following experiments; it probably belongs to the genus *Aeromonas*, and will be described elsewhere.

Chitinase production and culture conditions

The effect of the amount of colloidal chitin on chitinase production is shown in Fig. 2. Chitinase activity increased as the amount of colloidal chitin in the medium increased to 0.2%, but it decreased at a concentration of 0.3%. A maximum of enzyme production was attained after 2 days of cultivation.

Using a medium containing 0.2% colloidal chitin, the effect of varying the amounts of other nutrients was examined. The glucose concentration for maximum chitinase production was found to be 0.2%; the addition of glucose higher than 0.3% completely prevented enzyme production. The peptone concentration giving maximum chitinase production was 0.5%. Levels of yeast extract in excess of 0.2% were required to produce any chitinase. Maximum enzyme production was found at concentrations of 0.5%. Although 0.5% NaCl was also added to the medium, the amount of NaCl had no effect on chitinase production.

Table 1. Effect of Chitin and Chitin Derivatives on Chitinase Production

Compound (0.2%)	Chitinase Activity (μg/ml)	
	48 hours	96 hours
Chitin	0.0	3.5
Chitin (Fine powder 1)*	1.8	6.4
Chitin (" " 2)*	2.2	10.6
Chitin (" " 3)*	7.5	16.5
Colloidal chitin	49.2	61.3
Glycol chitin	0.0	0.4
Carboxymethyl chitin	1.8	0.2
Colloidal chitosan	1.7	1.2
Glycol chitosan	0.0	0.0

The composition of media was the same as in Fig. 2.

*The size of the chitin particles is listed in order of increasing fineness from 1 to 3.

Of a variety of compounds tested, colloidal chitin was found to be the most useful inducer of chitinase production (Table 1). The addition of chitin powder resulted in low enzyme production only, although the activity increased as the size of the particles decreased. Very little or no enzyme was produced on compounds such as water-soluble chitin derivatives and deaceylated chitin.

Purification of the chitinase system

As a result of the experiments described above, cultures for the production of enzyme for purification were set up in twenty 300 ml conical flasks containing 50 ml of medium with the composition: 0.2% colloidal chitin, 0.2% glucose, 0.5% peptone and 0.5% yeast extract. The flasks were incubated for 48 hours at 30° C.

The culture broth was brought to 0.7 saturation by adding solid ammonium sulfate; the precipitate produced dissolved in 80 ml of 0.02 M phosphate buffer (pH 7.2) and dialyzed against the same buffer for 48 hours. The dialysis residue contained 81% and 73%, respectively, of chitinase and chitobiase activities of the original culture broth. Ammonium sulfate precipitation followed by dialysis achieved about a seven-fold increase in specific activity.

The separation of the two enzymes was attempted, using column chromatography on DEAE-Sephadex A-25. As illustrated in Fig. 3, three peaks of chitobiase activity appeared in the effluents eluted with both 0.02 M of phosphate buffer and the same buffer containing less than 0.1 M NaCl. Chitinase activity was eluted with around 0.1-0.2 M NaCl, but only about 8% of the original activity was recovered after chromatographic separation. When the activity was assayed by determination of reducing sugar, however, chitinase was found over a wide range of fractions eluted at NaCl concentration higher than 0.1 M. The activities of the two chitobiase fractions, CB_1 and CB_2, and chitinase fraction C, indicated in Fig. 3, are summarized in Table 2. In CB_1, chitobiase activity completely free from chitinase occurs, but in the high specific activity chitobiase fraction, CB_2, a small amount of chitinase was detected.

Table 2. Activities of the Fraction Separated by DEAE-Sephadex A-25 Column Chromatography

Fraction	Volume	Protein	Chitinase			Chitobiase		
			T.A.[1]	S.A.[2]	Yield	T.A.[1]	S.A.[2]	Yield
	(ml)	(mg)	(mU)	(mU/mgP)	(%)	(mU)	(mU/mgP)	(%)
Original	80	147	31,800	216	100	19,900	135	100
CB_1	230	12	0	0	0	6,760	563	34
CB_2	100	1	140	140	0.4	4,930	4,930	25
C	380	40	2,670	67	8	990	25	5

[1] T.A.: total activity

[2] S.A.: specific activity

The chitinase fraction C, concentrated with DIAFLO membrane UM 10, was rechromatographed on DEAE-Sephadex A-25 column, using the same procedure as before (Fig. 4). Although the chitobiase activity was not completely separated, the further lowering of the overall chitinase activity was observed as the chitobiase levels were reduced. The overall chitinase activity could be enhanced by the addition of separated CB_1 material, and the levels of the enhanced overall chitinase activity attained those of the activity determined by measuring reducing sugar. The fraction (No. 113 indicated in Fig. 4) freed from chitobiase activity was used as purified chitinase material in the next experiment.

Enzymatic properties of the chitinase system

The optimum pH for chitinase was found to be between 7 and 8; that for chitobiase, 7.6. Chitinase was more stable than chitobiase when the stability was tested by heating at various temperatures (40-70° C) for 15 minutes. In spite of the complete inactivation of chitobiase with heating at 50° C, about 75% of chitinase activity survived under the same condition. However, chitinase was also completely destroyed at temperatures above 70° C.

The mode of action of chitinase and chitobiase upon chitin and chitin-oligosaccharides was investigated. As shown in Table 3, N-acetylglucosamine was not produced from colloical chitin by the action of either chitinase or

chitobiase alone, but together the two enzymes produced substantial amounts of this sugar. Chitinase decomposed chitotriose and chitotetraose to N-acetylglucosamine slowly, and chitobiase did so somewhat faster. When chitinase and chitobiase acted singly, the formation of N-acetylglucosamine was more rapid in the hydrolysis of chitotriose than in that of chitotetraose, but this was reversed when they acted together. In the hydrolysis of chitotetraose, the amount of N-acetylglucosamine formed by joint action was found to be about three times that calculated from the results of single-enzyme digestion, although the observed values were nearly equal to the calculated ones for the hydrolysis of chitobiose and chitotriose.

Table 3. Decomposition of Various Substrates by Chitinase, Chitobiase and Chitinase plus Chitobiase

Substrate	N-Acetylglucosamine formed (μg)			
	Chitinase	Chitobiase	Chitinase + Chitobiase	
			Observed	Calculated
Chitobiose	0	262	143	131
Chitotriose	38	105	74	72
Chitotetraose	11	79	138	45
Colloidal chitin	0	0	92	0

For the decomposition of chitin oligosaccharides, the reaction mixture containing 0.5 ml (500 μg) of substrates in 0.05 M phosphate buffer (pH 7.6) and 0.5 ml of enzyme solution was incubated at 37° C for 30 minutes. For the decomposition of chitin, the reaction mixture containing 0.5 ml (1,500 μg) of colloidal chitin, 1 ml of 0.05 M phosphate buffer (pH 7.6) and 0.5 ml of enzyme solution was incubated at 37° C for 30 minutes. In the single-enzyme digests, CB_1, which contained 20 mU of chitobiase activity, was used as chitobiase, and fraction 113, which was freed from the chitobiase activity (see Fig. 4), was used as chitinase. In the two-enzyme digests, one-half the amount of each of the enzymes was used.

DISCUSSION

Distribution in microorganisms and chitinase production

Chitinase is distributed among a wide range of microorganisms, as shown in Table 4 (4). Chitin-decomposing bacteria have been isolated from soil and marine mud and from the digestive tracts of animals, and most of the bacteria isolated have produced chitinase as an inducible enzyme. In fungi, chitinase of Aspergillus niger has been produced constitutively in a medium containing glucose and peptone as a carbon and nitrogen source (21), or on a wheat-bran culture (13). Microbial chitinases have been partially purified or found in a homogeneous state (indicated in Table 4 as * and ** respectively) from several kinds of microorganisms.

Table 4. Distribution of Chitinase in Microorganisms

Classification	Genus
Schizomycetes	
Pseudomonadales	Pseudomonas, Vibrio, Aeromonas, Photobacterium
Eubacteriales	Chromobacterium, Achromobacter, Flavobacterium, Klebsiella, Serratia,* Erwinia, Micrococcus, Corynebacterium, Arthrobacter, (partially purified) Bacillus, Clostridium
Actinomycetales	Actinomyces, Streptomyces -(homogeneous preparations)
Myxomycetes	Cytophaga (partially purified)
Phycomycetes	Chytriomyces, Phycomyces
Ascomycetes	Aspergillus - (homogeneous preparations)
Basidiomycetes	Lycoperdon, Irpex, Trametes, Coprinus, Beauveria
Fungi imperfecti	Trichoderma

The strain isolated (no. 12) produced an active chitinase system which was induced effectively only by colloidal chitin and chitin. The addition of up to 0.2% glucose increases chitinase production, probably stimulating the growth of the organism, but glucose in excess of this brings about a rapid decrease of pH at the initial stage of culture and results in complete prevention of enzyme production. Lowering of pH in the medium was also found with yeast-extract concentrations of less than 0.2%. The amounts of yeast extract and peptone required for maximum chitinase production by this strain were substantial, suggesting the possible utilization of yeast extract by this strain as a nutrient rather than as a growth factor. Further work is now in progress to optimize medium composition and to find the best inducer.

Purification and properties

Purified and partially purified chitinase preparations are summarized in Table 5. Homogeneous chitinases have been obtained from the microorganisms belonging to the genus Streptomyces (6, 26, 29) and Aspergillus niger (13). In these studies, selective adsorption on colloidal chitin (6) and column chromatography on DEAE-cellulose (19, 26), hydroxyapatite (13, 26) and SP-Sephadex (29) were found to be effective for the purification of chitinase. Skujiņš et al. (26) have succeeded in crystallizing chitinase from a strain of Streptomyces sp. However, the presence of several enzyme components has been demonstrated during the purification of chitinases of Streptomyces sp. (2, 6, 19, 29).

Partial purification of bacterial chitinase has previously been performed by fractionation of culture broths with ammonium sulfate followed by chromatography on the DEAE-cellulose (10, 27) and DEAE-Sephadex (9) column.

Table 5. Purified and Partially Purified Chitinase Preparations Obtained from Microorganisms

Source	Substrate	Optimum pH	Inactivation Extent and Temperature
Streptomyces griseus (2)	Colloidal chitin	6.3	C1 66%, C2 3% (65°C, 10 min.)
Streptomyces antibioticus* (6)	Colloidal chitin	5.2	
Streptomyces sp.* (26)	Colloidal chitin	4.2-4.8	45% (65°C, 10 min.)
Streptomyces griseus (19)	Colloidal chitin Glycol chitin	3.4-3.8	13% 43% (70°C, 10 min.)
Streptomyces orientalis* (29)	Colloidal chitin	5.5-6.5	I. 52%, II. 60% (65°C, 15 min.)
Aspergillus niger* (13)	Glycol chitin	3.6	21% (70°C, 10 min.)
Serratia marcescens (9)	Chitin Colloidal chitin	6.4	above 50% (50°C, 60 min.)
Cytophaga johnsonii (27)	Colloidal chitin	6.3-6.5	
Arthrobacter sp. (10)	Colloidal chitin	4.9	88% (70°C, 60 min.)
Unidentified bacterium (this study)	Colloidal chitin	7.0-8.0	100% (70°C, 15 min.)

*These enzymes were obtained as homogeneous preparations

Monreal and Reese (9) have reported that the application of a DEAE-Sephadex column for the purification of the chitinase of Serratia marcescens results in very poor recovery of the enzyme. In the present investigation, we have also used a DEAE-Sephadex column for the purification of the enzyme system and have separated chitinase from chitobiase on it. The use of DEAE-cellulose column for purification was unsuccessful, however, because chitinase was bound to the column and was not eluted even with high concentrations of NaCl.

The optimum pH of purified chitinases has been found in a wide acidic region between pH 3.4 and 6.5 (Table 5). As chitinase and chitobiase of this strain have their optimum pH at around 7-8 and the final pH of the medium used in these

studies lies within this range, the enzyme system may be used without separation for the decomposition of chitin to N-acetylglucosamine at neutral pH. It seems, however, that this chitinase is considerably heat-labile as compared with chitinases from other microbial sources (Table 5). For this reason, difficulties remain in using this enzyme system for commercial purposes. We are now seeking a method in our laboratory for obtaining homogeneous chitinase and chitobiase preparation from this bacterium.

Substrate and assay procedures

Chitin and its derivatives are commonly used for the assay of chitinase activity, as shown in Table 6. Since chitin is insoluble in water and does not disperse uniformly in the reaction mixture, chitinase activity has been determined by the turbidimetric method (2, 6) and the viscometric method (12, 28, 30), using colloidal chitin and water-soluble chitin derivatives, respectively, as the substrate. In this investigation, the determination of chitinase activity was performed by measurement of N-acetylglucosamine released from colloidal chitin. This procedure has also been adopted in the purification of chitinase from Streptomyces sp. (26), Streptomyces orientalis (29) and Arthrobacter sp. (10). However, the determination of chitinase activity by this procedure resulted in the decrease of recovery and specific activity on the chitinase fraction obtained from chromatographic separation, as shown in Fig. 3 and Table 3. From this result, the procedure of measuring initial change of the substrates, such as the turbidimetric and the viscometric methods is more suited to the determination of this chitinase activity.

Table 6. Substrate and Assay Procedure of Chitinase Activity

Substrate	Assay Procedure
Chitin	Weight of undegraded chitin Amount of reducing sugar Amount of N-acetylglucosamine
Colloidal chitin	Turbidity* Amount of reducing sugar* Amount of N-acetylglucosamine* Measurement of a diameter of clear zone on the plate
Glycol chitin	Viscosity* Amount of reducing sugar Amount of N-acetylglucosamine*
6-O-Hydroxypropyl-chitin	Viscosity
Chitosan acetate	Viscosity

*These assay procedures were used for the determination of chitinase activity during purification.

Ohtakara (12) has reported a viscometric method for the determination of chitinase activity, using glycol chitin as the substrate, that has the advantage of the solution's viscosity not being affected by the addition of salts or changes of pH, as is the case with chitosan acetate (30). It has been pointed out by Nord and Wadström (11) that the viscometric assay using glycol chitin is more sensitive than the turbidimetric method and gives quite reproducible results with two different batches of substrate. However, no parallel relationship has been found, in the strains of Actinomycetes tested, between the chitinase activity measured by turbidimetric assay and that measured by viscometric assay. Several strains have shown strong turbidimetric activity, regardless of their low activity in viscometric measurement (18). It appears necessary, therefore, to assay chitinase with colloidal chitin, even though glycol chitin may be a more convenient substrate.

Mode of action of the chitinase system

It has been reported by many workers (2, 7, 17, 19) that chitinase randomly cleaves chitin and that the main product in the degradation of chitin is chitobiose. N-acetylglucosamine has also been formed as a direct product of the action of chitinase upon chitin and colloidal chitin, but the quantity was very small as compared with the amount of chitobiose (7). Acting upon chitin oligosaccharides, chitinase has produced chitobiose and N-acetyl glucosamine from chitotriose (2, 19) and chitobiose only (19), or chitobiose with the accompanying formation of chitotriose and N-acetylglucosamine (2), from chitotetraose. Chitinase did not attack either chitobiose or methyl and phenyl N-acetylglucosaminide (2, 15, 19).

In this study, purified chitinase hydrolyzed chitotriose and chitotetraose, but it did not split chitobiose and did not produce N-acetylglucosamine from colloidal chitin. This result probably indicates that chitobiose is the end product in the decomposition of chitin with chitinase alone and that chitobiase is essential for the formation of N-acetylglucosamine. It is clear from a comparison between the observed values and the calculated ones for the hydrolysis of chitin oligosaccharides that chitobiose is a possible product in the decomposition of chitotetraose by purified chitinase (Table 3).

Chitobiase hydrolyzed not only chitobiose but also chitotriose and chitotetraose at a decreasing rate, as the molecular weight of chitin oligosaccharides increased. This enzyme differs from the chitobiase of Streptomyces griseus (2) in that it can digest chitotetraose. It was found that the chitobiase obtained was free from chitinase.

During purification of the chitinase system from other microbial sources, several chitobiases have been separated from chitinases (2, 16, 19). Comparison of the hydrolysis rate of chitobiose and alkyl- or aryl-N-acetylglucosaminide with these chitobiase preparations is shown in Table 7. There are a number of instances where chitobiase activity is dominant, and the chitobiases of Aspergillus niger (16), Aspergillus sp. (20) and Lycoperdon pyriforme hydrolyze chitobiose at a very rapid rate, as compared with the hydrolysis of alkyl- or aryl-N-acetylglucosaminide. In view of this evidence, we have used the name "chitobiase" for one enzyme of the chitinase system throughout this study, although chitobiase (formerly E.C. 3.2.1.29) is now

included with exo-β-N-acetylglucosaminidase (E.C. 3.2.1.30) in the 1972 edition of Enzyme Nomenclature. However, this chitobiase will be further studied from the standpoint of ascertaining the identity of chitobiase with exo-β-N-acetylglucosaminidase.

Table 7. Comparison of Hydrolysis Rate of N,N-Diacetyl-chitobiose and Alkyl- or Aryl-N-Acetylglucosaminide with Chitobiase Preparations

Source	N,N-Diacetyl chitobiose	Alkyl-m or Aryl-m N-Acetylglucosaminide	
Streptomyces griseus (2)	2.19	Ph	1
Streptomyces griseus (19)	6.88	Me	1
Aspergillus niger (16)	1.0	Me	0.012
Aspergillus sp. (20)	112.1	Ph	1.732
Lycoperdon perlatum (22)	15.4*	Ph	13.8*
Lycoperdon pyriforme (4)	4.0**	pNP	5.4×10^{-3}**

The * values are the relative rate of hydrolysis of both substrates except V_{max} (* = μmoles/hr/mg protein, ** = μmoles/min/ml extracted fluid). Me: Methyl-; Ph: Phenyl; pNP: p-Nitrophenyl-.

CONCLUSIONS

An unidentified Gram-negative bacterium isolated from seashore mud produced an inducible chitinase system. This consisted of a chitinase and a chitobiase, which could be separated from each other with a DEAE-Sephadex column. It was confirmed that chitin was decomposed to N-acetylglucosamine with the cooperative action of the two enzymes. Comparison of this chitinase system with those from other microbial sources was made.

ACKNOWLEDGEMENT

We are very grateful to Dr. R. C. W. Berkeley, Bristol University, England, for a critical reading of the manuscript and for his valuable advice.

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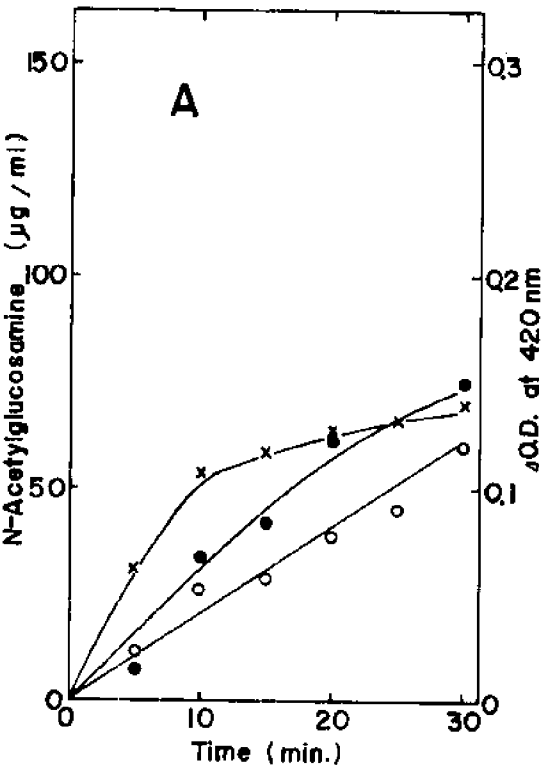

Figure 1 Strain No. 5 (A)

Time course of decomposition of colloidal chitin with the two strains No. 5 (A) and No. 12 (B). The decomposition was followed by measuring the decrease of turbidity at 420 nm (×—×), the increase of reducing sugar (●—●) with the method of Somogyi (1) and the formation of N-acetylglucosamine (○—○) with that of Reissig et al (24), using the same reaction mixture as in the determination of chitinase activity.

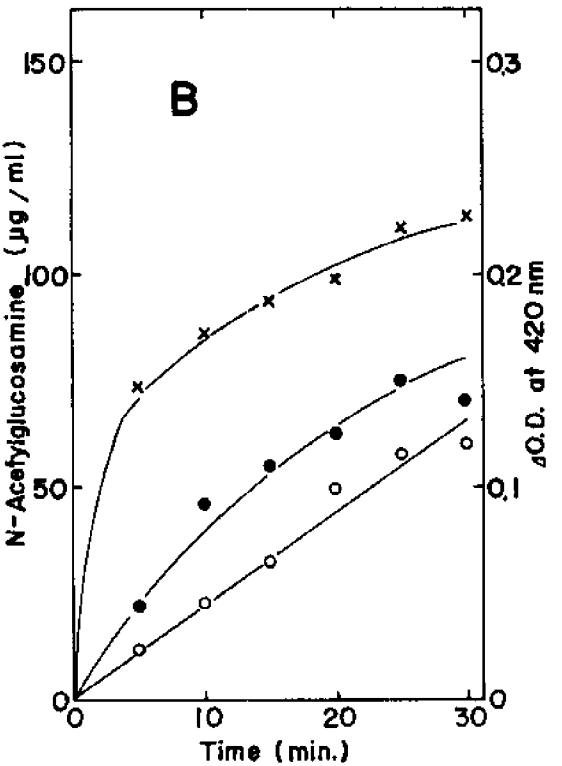

Figure 1 Strain No. 12 (B)

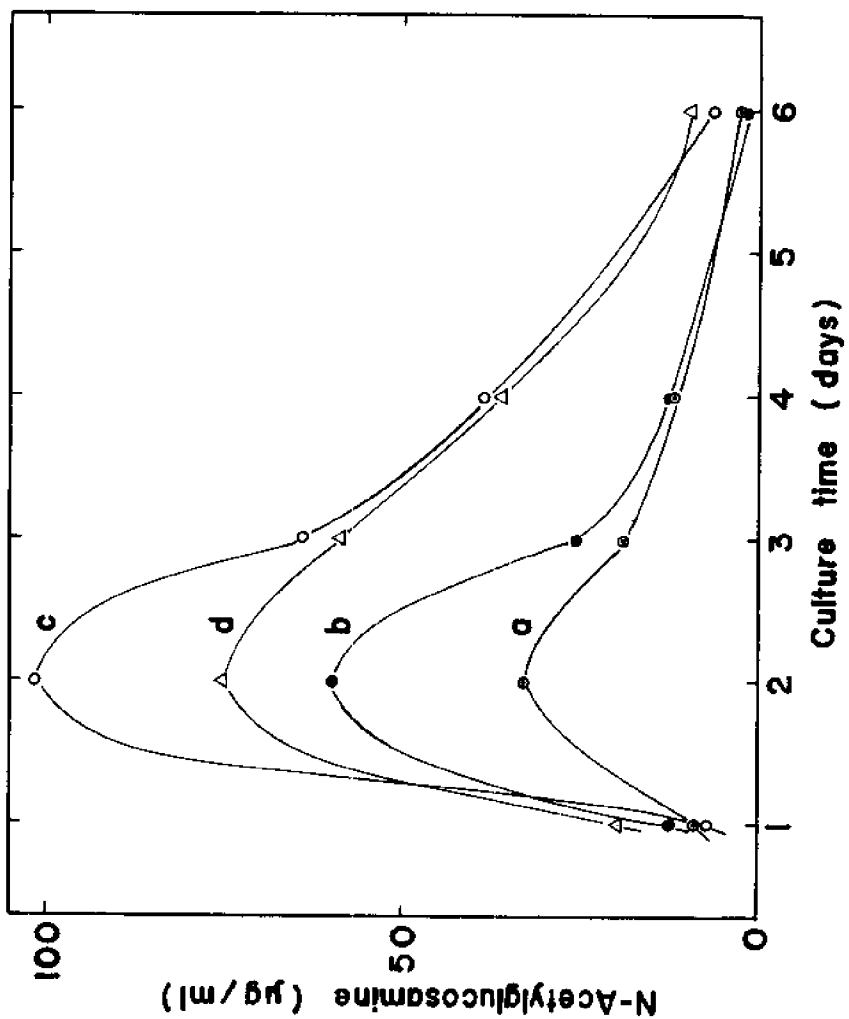

Fig. 2. Effect of colloidal chitin concentration on chitinase production. The medium was composed of 0.2 % glucose, 1.0 % pepione, 0.5 % yeast extract, 0.5 % NaCl and varying concentration (a; 0.05 %, b; 0.1 %, c; 0.2 % and d; 0.4 %) of colloidal chitin.

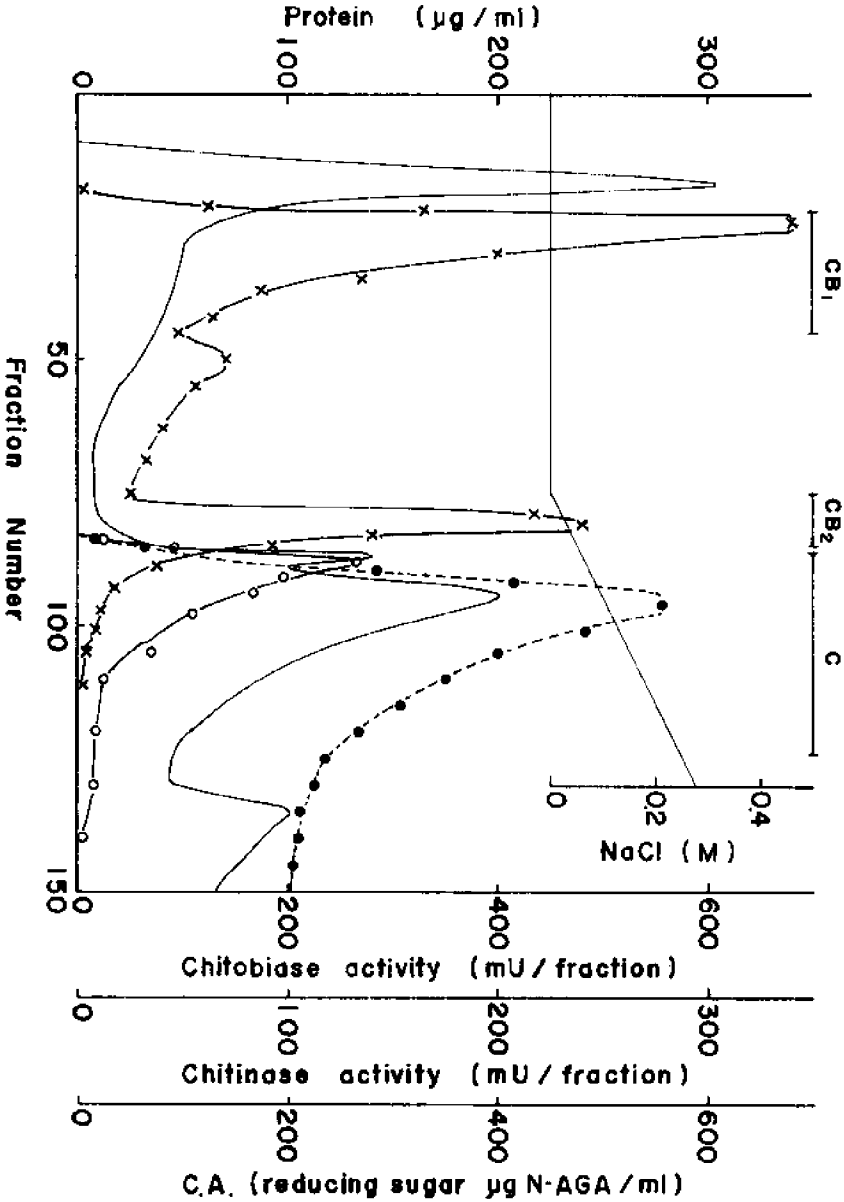

Fig. 3. Column chromatography of the chitinase system on DEAE-Sephadex A-25. C.A. indicates the chitinase activity determined by measuring the amount of reducing sugar (●···●). Chitinase activity (o—o), Chitobiase activity (×—×), Protein (——)

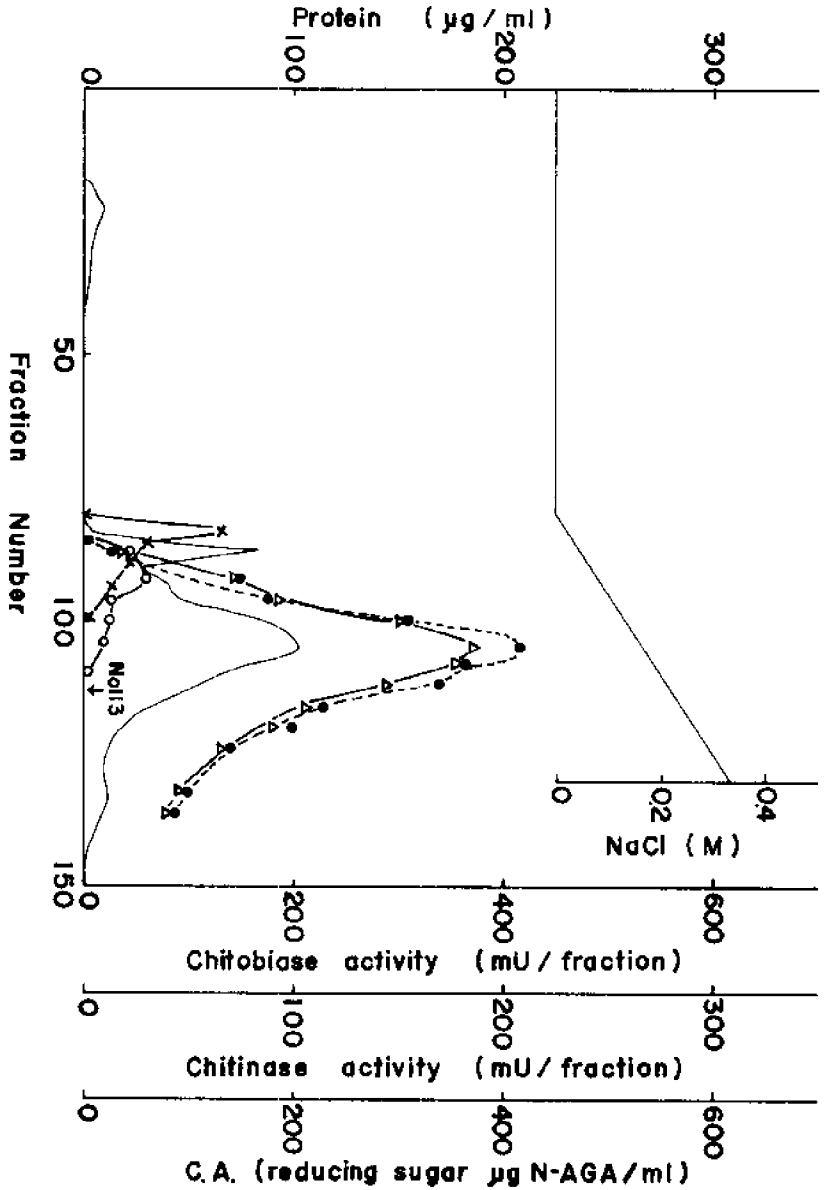

Fig. 4. Rechromatography of C in Fig. 3 on DEAE-Sephadex A-25. Chitinase activity in the presence of chitobiase (△—△) was determined by measuring the amount of N-acetyl-glucosamine formed in the reaction mixture after incubation for one hour at 37°C by the addition of 0.5 ml chitobiase (CB_1 25 mU) to 1.5 ml supernatant of chitinase digest. Symbols were the same as in Fig. 3.

K.M. RUDALL: AN APPRECIATION

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Kenneth Maclaurin Rudall has been the leading figure in research into all aspects of chitin structure for the past thirty years, but his interests during a lifetime dedicated to scientific discovery have been much broader. His fields of active research include animal covering and external secretions, and even that work conceals an extraordinary range of expert knowledge and an even vaster purview of peripheral awareness in the general field of molecular biology.

His career has been almost entirely spent at Leeds University, a vital center of molecular biological research largely inspired by the long-time presence there of W. T. Astbury and several distinguished colleagues, of whom Rudall soon became one. In its early days, that embryonic subject developed rapidly, thanks to the mutual stimulation of these researchers, who combined the classical methods of physics and biology with the single important innovation of x-ray diffraction to form their research techniques.

Rudall was born in New Zealand of an English father and a Scottish mother (his uncle, the mathematician Maclaurin, was president of M.I.T. from 1909 to 1920). He took a degree in 1932 in physics and zoology and then an M.Sc. in zoology at the University of Auckland, where he had his "first conscious contact with chitin, in the canine-type teeth of a carnivorous worm-eating snail. It was dramatic to stew the massive odontophore in dilute alkali, when all disappeared except the radula membrane with its thousands of teeth." Before leaving New Zealand he wrote two papers for the Massey Agricultural College on problems connected with the growth of sheep's wool.

Positions in science, then as now, were in short supply so he moved to England to take a Clothworkers Company Fellowship. Thus the connection with Astbury in 1934 owed something to chance and, as it appeared at the time, something to ill-fortune. At Leeds his research for the Ph.D. centered on "X-Ray and Related Studies on the Form and Constitution of the Biological Cells in Wool and Other Keratin Structures" (1936). His next important paper on the "Physical and Chemical Properties of the Insect Cuticle" (1940), was the result of a fruitful collaboration with G. Fraenkel of the Department of Zoology at Imperial College, London. The subjects they discussed there remained among his major interests.

While continuing this collaboration, Rudall was also developing another field, which came to rank close to chitin among his major achievements; this was the study of the mammalian epidermis. Through his original epidermal preparations, and eventually his isolation of the protein which he named epidermin, he was able to add a great deal to our understanding of the structural changes on extension and contraction of the k-e-m-f group of proteins, a topic we now relate to helix-random coil-pleated sheet transformation. Rudall's contribution was to prove the existence of a

cross-ß structure-- a considerable advance over prevailing ideas. This discovery was then thought to be of great potential significance for our understanding of muscle contraction, although that hypothesis has since had to be discarded.

Several years later, while following another line of inquiry, Rudall more or less accidentally discovered nature's own cross-ß structure occurring in the egg stalk of the lacewing fly, Chrysopa. Although this finding played only a small part in the growth of modern ideas about protein structure, it was significant in confirming beyond doubt the existence of the cross-ß structure (the earlier diffraction patterns had tended to convince only a few experts), and was the first clear demonstration of protein chain folding, proposed even before these general ideas were widely applied to synthetic polymer structures. Now amply confirmed and publicized by the structure determination of crystalline enzymes, the cross-ß structure and the protein fold (the third most common protein conformation) were first set down in Rudall's work on Chrysopa.

One of Rudall's greatest assets was his unusual ability to master new techniques and estimate both their potential scope and their practical application to his own problems. In 1943 Astbury acquired one of the first four R.C.A. electron microscopes provided to Britain under the Lend-Lease arrangement from the United States. Rudall was soon assisting R. Reed in applying this new technique. Results were obtained, but because of competition for the use of the new instrument and because the techniques then in use were not yet capable of providing reliable data for chitin, keratin and epidermis, he did not at that time utilize electron microscopy extensively. It remained a tool well suited to his peculiar kind of experimental ability, however, and eventually it became one of his principle skills and the main subject of his teaching at the university.

A little later, infra-red spectroscopy appeared as a useful tool for structure analysis, and Rudall, in collaboration with S. E. Darmon, soon produced two papers of major importance by applying that new technique to studies of the α-ß transformation (as exemplified by epidermin, the protein over which he had so much structural control) and to the composition and structure of α-chitin and chitosan. Just as Astbury failed to discover the structures that he had brought to the world's attention, so Rudall also failed narrowly to describe the molecular structure of α-chitin. All the necessary information was present in his collaboration with Darmon, but the honor passed to D. Carlstrom in 1957.

The late 1950s found Rudall surveying tissues, membranes and fibers from a wide range of animal sources and applying x-ray diffraction, infra-red and electron microscopy to classify them in the light of the newly discovered structures. General themes link these studies on materials which, at first sight, seem quite randomly selected. One was Rudall's interest in tanning and hardening mechanisms, generated by his earlier work with Fraenkel on the insect cuticle. This led him to examine the collateral glands and their products in many insects. Fascinating discoveries were made, and important work was published on the oötheca of mantids and cockroaches, the egg cases of Aspidomorpha, and the cocoons of Hydrophilus, along with the work on the egg stalks of Chrysopa already mentioned. These findings led

him to make generalizations on 'silks," which he now regarded as being external secretions of insects not necessarily closely related to the commonly known silk from Bombyx mori. He wrote in 1962 of "silks which are collagen, silks which are chitin." The importance of this work lay partly in the wide range of structures found which was thought to owe less to evolutionary pressures operating on extracellular materials than to intracellular biomolecules. This work brought him into collaboration with J. O. Warwicker, F. Lucas and J. T. B. Shaw, all of the Shirley Institute.

Rudall also surveyed the occurrence of α- and β-chitin in the animal kingdom in pursuit of a theory that there might be a relation between the use of collagen by some animals and of the chitins by others. Whatever the outcome of the hypothesis, the classification which he arrived at built a solid foundation for understanding the relation between the α and β forms of chitin originally proposed by Lotmar and Picken in 1950. To these Rudall added a third form, γ, which still awaits general recognition. The most important of these findings was the β-chitin in the pogonophore tubes, a finding made still more significant by the later discovery of the remarkable β-chitin spines on diatoms made by Stacey and his co-workers. These two materials allowed structural determinations (by Blackwell and by Dweltz) a much greater degree of precision than had hitherto been possible.

In the early 1960s, Rudall returned to his interest in wool growth and the hair follicle, an interest renewed by contact with a fellow New Zealander, Dry. This reversal of interests was not so far afield as appears at first glance when one remembers that the general problems of keratinization and the hardening of cuticle may not be dissimilar. In the remaining years before retirement from his university post he was again working on the protein/chitin complex, gaining a deeper and deeper understanding of the relation between protein and chitin, obtaining fresh information from x-ray diffraction, and at last being able to relate the larger details of structure to visible effects seen in the vastly improved electron microscope pictures that were by then possible. He was beginning to form new ideas of the synthesizing and hardening enzymes linked to chitin rods when he retired from active research in 1975.

His published work represents a distinguished record, although, considering the amount and quality of his research, it is not voluminous; of a quiet and reserved disposition and without ambition he was never one to rush into print. Nor was he enthusiastic about large conferences, although he was persuaded to attend a fair number. His most important contributions (especially later on) tended to appear in review articles, into which he was in the habit of inserting his latest results and ideas. As a working scientist his style will always be remembered by those who know him: a slightly built man, seemingly tireless in the long hours spent each day at the bench or studying the literature, he showed no outward sign of the scientific long-distance runner he was.

Rudall preferred to do his own experimental work whenever possible. He had a penchant for the "string and sealing wax" tradition, and, as a result, many of his contrivances had their decidedly humorous aspect; but,

he possessed the scientist's equivalent of the gardener's green thumb, and his specimen preparations, often using ingenious and elaborate procedures, were invariably exactly right for their purpose. What visitors remember are his living colonies of exotic insects, mantids, aspidomorpha, sawflies, lacewings and others, many of them maintained in the laboratory for years when research required it. He had an uncanny ability to provide just the right conditions to maintain a healthy stock. When his interst turned to wool growth, he kept his sheep on a nearby university green, and was apt to be found with a colony of bees loose in his car or a sheep occupying its back seat.

His attitude to students was a little ambivalent. Kindly by nature, he was willing to recognize their existence, but he did not welcome undergraduate teaching and did not go out of his way to attract research students. His interest always lay in the next problem, and he was impatient of the unproductive period that preceded a student's becoming helpful and independent. Although wary of committing his own research to untried hands, he was extremely helpful and generous in giving time, ideas, and advice to any who cared to ask. His criticism was always useful, and problems of any kind, even outside his own wide range of expertise, would always elicit his interest and valuable comments. When, in the 1960s, a number of postgraduate students came to him, he accepted that responsibility, and many were the fortunate beneficiaries of his supervision; a number of them have since had notable research careers. Widely known as a supreme experimentalist, to those students he was also known as an "idea man."

In 1952 he was appointed Reader in Professor Astbury's Department of Biomolecular Structure, an ideal position for him, giving him, as it did, complete freedom for his research and few administrative responsibilities. After Astbury's death, Rudall assumed greater responsibility, and he even surprised many people by his effectiveness as head of a section in the new Astbury Department of Biophysics at Leeds. Administration and committee work did not suit his style, however, which still maintained a colonial's "irreverent disapproval of all formalism and stiffness." After a few years he withdrew from this position and retreated to his research.

Rudall's dedication to research is complete, but he also possesses a dry sense of humor and an independent mind, and is ready always to discuss any topic of the day, serious or trivial. "When science interferes with private life, then something is wrong," he once said to an astonished young research assistant. His private life never seemed to interfere with research, either, however; for his pastimes, he gardened and kept bees. The garden immediately supplied specimens of sawflies, lacewings, food for insects, and weeds, when needed, and the bees made a silk with a remarkably good (and different) α-protein pattern as well as honey. Colleagues benefited from both. When he took holidays or traveled to conferences or to revisit his birthplace, insects and fibers returned in his luggage.

Rudall has retired to an estate with a large garden and outbuildings where, it is rumoured, research still goes on. For any young scientist looking for a problem to try his hand at a visit would be well worth his while.

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Subject Index

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors

Index of Authors