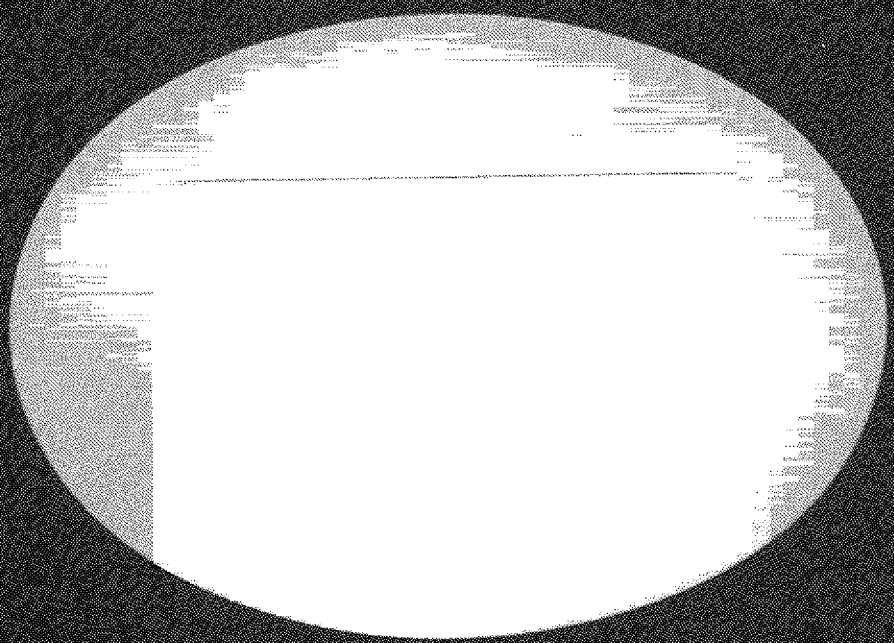

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CHITIN-WHEY AND RELATED SAPPORO PAPERS FROM THE UNIVERSITY OF DELAWARE

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by

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MICROCRYSTALLINE CHITIN: ITS PREPARATION AND PROPERTIES

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ABSTRACT

Microcrystalline chitin, a partially degraded low molecular weight chitin, has apparent potential in both medical and nutrition applications. Procedures for making microcrystalline chitin were described by Dunn and Farr (1974) and have been improved by Austin and Brine (1981). In the latter procedure, better control of the molecular weight of the product was achieved. However, some residual phosphorus from the hydrolysis procedure was present in the final product and seemingly affected its properties. In a further modification of the Austin and Brine procedure, described in this paper, a phosphate-free product has been obtained, and this material has improved solubility vs. other microcrystalline chitins. The crystal structure was disrupted by the presence of phosphorus, as indicated by x-ray diffraction. The phosphorus appears to be present as a phosphate salt of the free amine groups in the chitin and can be removed by washing with dilute sodium hydroxide. Scanning electron microscopy of microcrystalline chitin showed evidence of a subunit structure.

* * * * * * * * * * *

Microcrystalline chitin was first described by Dunn, et al. (1974) in the form of a thixotropic suspension. In general, microcrystalline polymers are prepared by a combination of chemical degradation and high-speed shearing (Battista, 1975). For example, microcrystalline cellulose is prepared by digestion with hydrochloric acid followed by high-speed shearing. The original process for making microcrystalline chitin (Dunn et al., 1974) is derived from the microcrystalline cellulose technology as it uses hydrochloric acid (or other mineral acids) to accomplish the chemical degradation. When this process is applied to chitin there is an apparent large decrease in molecular weight in the first five minutes of acid treatment, indicating extensive molecular degradation through rapid hydrolysis (Austin and Brine, 1981).

A more effective procedure for preparing microcrystalline chitin uses a solution of phosphoric acid in 2-propanol for the hydrolysis (Austin and Brine, 1981). In this way better control of the hydrolytic degradation can be achieved. The product from the Austin and Brine procedure consistently has a levo (-) optical rotation as does native chitin. Additionally, this procedure produces a friable, easily dispersable powder. The procedure of Austin and Brine is as follows:

A slurry of chitin (150 g KY-10 chitin, Hercules, Inc.) in phosphoric acid (85%; 475 ml) and 2-propanol (1 ℓ) was gradually heated to boiling in a 4-liter beaker on a hot plate while mechanically stirring for a period of two hours. It was then quenched with tap-water (2.5 ℓ) and left at room temperature for 15 minutes. The slurry was then centrifuged at 2000 rpm using an International centrifuge, model V-2. On removal of the supernatant liquid, the chitin was washed twice with hot water followed by one wash with acetone. After each wash (15 minutes) the slurry was again centrifuged and the supernatant liquid removed.

This product was filtered under suction and sheared in water using a Waring "blendor" at 20,500 rpm. The emulsion thus obtained was evenly spread on trays and freeze-dried. The dry microcrystalline chitin was then subjected to a dry shearing process using a Waring "blendor" at high speed (20,500 rpm) and finally ground to a 40-mesh white powder using a Wiley laboratory mill. The yield of microcrystalline chitin was 125-130 g.

Although this procedure offers greater control of molecular weight (75,000-5,600) it is not without its limitations. The microcrystalline chitin prepared in this manner typically contains 0.4-0.8% phosphorus and has limited solubility in the N,N-dimethylacetamide-LiCl solvent system used for chitin (Austin, 1977).

Effects of phosphorus on microcrystalline chitin

In an attempt to improve the solubility of the Austin and Brine microcrystalline chitin the nature of the associated phosphorus was studied. Infra red spectroscopy indicated trace amounts of phosphorus present in some type of P-O linkage. The phosphorus content was found to be unaffected by treatment with lithium borohydride, a strong reducing agent, and could only be partially removed by extraction with aqueous hydrochloric acid. However, dilute aqueous sodium hydroxide removed all of the phosphorus rapidly. This observation, coupled with the previous information, indicated the presence of a phosphate salt of the free amine groups in the microcrystalline chitin.

X-ray diffraction of microcrystalline chitin containing various amounts of phosphorus showed changes in the crystal structure that became more pronounced at higher concentrations of phosphorus (Table 1). Note the shifts in the d spacing relative to KY-10 chitin, the starting material for all three microcrystalline chitins.

Table 1. The effects of phosphorus on the crystal structure of microcrystalline chitin.

d Spacing (Å)			
Microcrystalline Chitin			
KY-10 0.00% P	2.98% P	3.49% P	3.88% P
4.77	4.71	4.70	4.57
4.45	4.04	3.96	3.90
4.02	3.58	3.52	3.40
3.55	3.34	3.34	3.22
-	3.26	3.26	3.11
3.30	3.17	3.17	3.05
2.96	2.67	2.66	2.66

Some broadening of the diffraction bands was observed in the microcrystalline chitins and may be due to the presence of phosphate salts.

Not only is the crystal structure of the microcrystalline chitin affected, but also its properties. As mentioned earlier, the phosphorus containing microcrystalline chitin is less soluble in N,N-dimethylacetamide-LiCl than is the parent chitin. Phosphorus-free microcrystalline chitins, prepared by washing microcrystalline chitin with dilute aqueous sodium hydroxide, have an increased solubility over the phosphorus-containing product having a solubility equal to the parent chitin.

The presence of phosphorus, or phosphate salts, also has an effect on the dispersibility of the microcrystalline chitin a product containing about 2.5% P forms stable thixotropic dispersions at concentrations of 22% down to 10%. Phosphate-free product forms a paste at a concentration of 22% and does not form a creamy dispersion. At a concentration of 14% the phosphate-free product begins to separate from the water. Addition of a dispersing agent, such as "Tween 20," to the phosphate-free product will produce a creamy, stable dispersion. It appears that the phosphate salts act as self-dispersing agents.

Preparation of phosphate free microcrystalline chitin.

Information gained in the study of phosphorus in microcrystalline chitin was used to modify the procedure of Austin and Brine to produce a phosphate-free product. The following procedure was evolved:

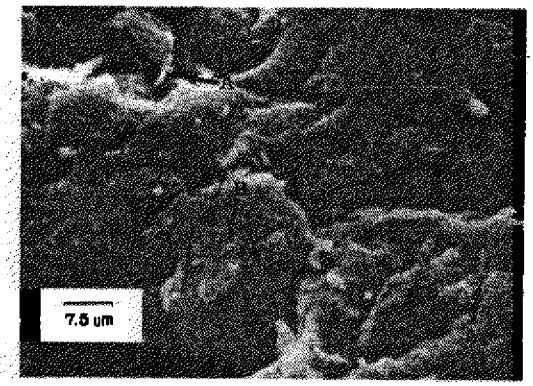
Chitin (150 g Madera chitin, Madera Products Inc.) was suspended in a solution of 1250 ml 2-propanol and 473 ml 85% phosphoric acid. This mixture was heated to its boiling point (about 80°C) with constant stirring and maintained at that temperature for 1.5 h. The mixture was then quenched with 1 liter of tap water and the supernatant liquid removed by centrifugation. The solids were then washed with 1% sodium hydroxide until a neutral or alkaline pH was reached (3 washes were usually sufficient). Then washed twice with tap water and once with deionized water. (The supernatant liquid was removed by centrifugation between washes.) The solids were transferred to a Waring "blendor" with approximately 2.5 volumes of deionized water, a minimum quantity needed for satisfactory shearing to produce a mayonnaise-like product. The sheared product was then placed in a tray and freeze-dried.

Subunit structure.

When a microcrystalline chitin dispersion was viewed through a phase contrast microscope it was observed that a large portion of the particles were 8-12 microns long with some as small as 4 microns (Figure 1) plus a small population of large (greater than 100 microns) particles. Closer examination of the surface of these large particles by scanning electron microscopy (SEM) revealed what may be a regular subunit structures (Figure 2), which give rise to the microcrystals seen in Figure 1. Note the partially unhinged crystals (A) in Figure 2 and the agglomerations of crystals (B). Gardner and Blackwell (1971) describe ribbons with widths of 200-300 angstroms in chitinous diatom spines. Combinations of these units, or similar structures, could be responsible for the apparent subunits observed in microcrystalline chitin. The uniformity

Figure 1. Aqueous dispersion of microcrystalline chitin viewed under phase contrast.

Figure 2. Surface feature of a large microcrystalline chitin particle as seen in SEM



of the particles in Figure 1 along with the regular crystalline features seen in Figure 2 suggest that the size of the particles in the final product may be controlled by a subunit structure in the chitin.

SUMMARY

1) Microcrystalline chitin prepared by the method of Austin and Brine contains phosphorus. Evidence indicates this phosphorus is present as a phosphate salts of the free amine groups. These salts deform the crystal structure and reduce the solubility in N,N-dimethylacetamide-LiCl (DMAc-LiCl).

2) A modified procedure, using dilute sodium hydroxide washes, has been developed to produce a phosphate-free microcrystalline chitin. This phosphate-free product has an increased solubility in DMAc-LiCl over microcrystalline chitins containing phosphorus.

3) The phosphate salts present in the Austin and Brine microcrystalline chitin act as dispersing agents and improve the stability of the dispersions.

4) Particle size in microcrystalline chitin dispersions is generally 8-12 microns.

5) Particle size in microcrystalline chitin appears to be determined by a subunit structure within the chitin.

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CHEMISTRY OF CHITIN ISOLATES

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ABSTRACT

Chitins vary in chemical and physical properties as a function of source and method of isolation, and are appropiately termed chitin isolates. Representative commercial and laboratory chitin samples were found to contain substantial and differing amounts of amino acid residues.

Chemical reactions of the alkyl N-acetylglucosamine (GlcNAc) glycosides, as prototypes of the chitin isolates, provide insight as to the behavior of these biopolymers. For example, tertiary amide-LiCl solvent systems for the chitin isolates appear to function by exchange of the complexed salt from solvent to polymer, as in the case of the alkyl GlcNAc glycosides. New tertiary amide-LiCl solvents have been found, but offer little advantage over known systems.

Other monomer-polymer parallels are indicated in the effects of C-2 substituents on hydrolysis and anomerization. Stabilization of the glycoside linkage by a free C-2 amine group apparently accounts for the unusual resistance of chitosan to acid hydrolysis. Anomerization of portions of the β-glycosidic bonds and consequent disorder of polymer chains may contribute to the variation in properties of chitinous products.

INTRODUCTION

In the period since the first chitin/chitosan conference in 1978 there has been much clarification of many of the subtleties of the chemistry of chitin isolates. Both our own research and the copious flow of publications worldwide have contributed to this understanding. In particular, variation in chitin properties has encouraged the reporting of chitin source and history in many investigations.

The chemical reactions and physical properties of the monomeric alkly N-acetyl-D-glucosamine (GlcNAc) glycosides have been found quite comparable and predictive of the behavior of the more complex chitin isolates and chitosan. It is this theme that will be elaborated here, especially with respect to the prevalance of amino acid residues in chitin isolates, factors affecting the dissolution of chitin in tertiary amide-LiCl systems and the effect of C-2 substituents on the chemical behavior of the glycosides, including chitin and chitosan.

EXPERIMENTAL

Materials and Methods. Commercial chitin was obtained from several sources: Dungeness crab (Cancer magister), pink shrimp (Pendalis borealis) and king crab (Paralithodes camtschatica) from the former Food, Chem. & Res. Lab., Seattle, WA; Japanese red crab (Chionectes opilio) from Eastman Kodak Co.; Tanner (snow) crab (Chinoecetes bairdi) from Madera Products, Inc., Albany, OR; and brown shrimp (Panaeus aztecus) from Hercules, Inc. (no longer suppliers). Horseshoe crab (Limulus polyphemus) (local, uncalcified) chitin was isolated by treatment with 5% NaOH at room temperature. Chitin from the blue crab (Callinectes sapidus) was prepared by treatment of ground local shell material with 1 N HCl solution at room temperature, followed by 1 N NaOH solution at 50°C for 6 hr, washed with deionized water, then acetone and air dried. Ethyl GlcNAc glycoside from Pfanstiehl Laboratories, Inc., Waukegan, IL, was recrystallized from a 2-propanol-ethyl acetate (1:2) mixture (5). Other chemicals were obtained from commercial suppliers and purified as required. Amino acid analyses were carried out on a Durrum D-500 amino acid analyser following procedure detailed previously (10). Data given in Table 1 are the average of two determinations.

Effect of LiCl on Optical Rotation of Ethyl GlcNAc Glycoside. To compare the effect of LiCl in chitin solvent systems with the simpler monomeric glycoside, the effect of LiCl concentration on the optical rotation of ethyl GlcNAc glycoside in ethanol was determined. Anomer ratio of test glycoside sample was 83:17 (α:β) (in water). Experiments were carried out in absolute ethanol at 45°C; change in rotation occurred in about 1 minute. Specific rotation, $[\alpha]_D$ was calculated from the observed angle of rotation, ℓ = 2 dec., c = conc. of glycoside, 0.1M, equal to 0.0249 g/mℓ; $[\alpha]_D$ of α-glycoside anomer is +134°, $[\alpha]_D$ of β-anomer is -44.3 (water) (26). Results are detailed in Table 2.

Chiroptical Behavior of Ethyl GlcNAc Glycoside in Acid Solution. To obtain a picture of the competetive rates of hydrolysis and anomerization of a simple glycoside, an experiment was designed to follow the change in optical rotation of ethyl GlcNAc glycoside in aqueous acid

solution. Concentrations of 1.0M, 0.1M and 0.01M HCl were tried both at room temperature and at 53°C, starting with an ethyl GlcNAc sample with an α:β anomer ratio of 83:17. At room temperature no change occurred in 30 days. Best results were obtained at 53°C in 1M HCl with significant change of rotation in a reasonable time (Table 4). In this experiment the concentration of the glycoside was 0.1M, equal to 0.0249 g/mℓ. Cell length was 2 dec. $[\alpha]_D$ of α-anomer is +134°; $[\alpha]_D$ of GlcNAc, α-anomer is 55.6.

RESULTS AND DISCUSSION

Residual Amino Acids in Chitin Isolates. There is a growing realization that chitin varies considerably in physical and chemical properties because of both species source and method of preparation, as reviewed previously (4, 9, 10, 15). Hence it appears appropriate to call most chitins "isolates". In our prior work, chitin isolates were prepared by consistent techniques to help establish the species variation and one point stands out: each isolate retained some portion of covalently-bound residual amino acids from the parent mucopolysaccharide, despite rigorous alkaline hydrolysis (1N NaOH, 100°C, 48 hr). Accordingly, several commercial and laboratory chitin samples were analyzed for their amino acid content to determine whether such residues were a common feature of these products. As indicated in Table 1, amino acids were indeed present in all of the representative chitins. Proteinaceous material amounted to approximately 0.4 - 5.6 percent in the samples tested (based on 118 as the average mol. wt. of the amino acids).

Table 1. Chitin Isolates: Amino Acid Content

Species	Amino acids μ mole/g
Dungeness crab	72.3
Brown shrimp	33.5
Japanese red crab	330.3
Tanner (snow) crab	139.5
Horseshoe crab	152.5
Blue crab	477.8
King crab	235.4

Atropic (tertiary) Amide Solvent Systems. The amides that provide effective chitin solvent systems in conjunction with lithium chloride in every case are tertiary amides and to date only lithium chloride has proven to be a co-activator. N,N-dimethylpropionamide (17) and N,N'-dimethyl cyclic ethylene urea (1,3-dimethyl-2-imidazolidinone) are new. However, they offer neither lower viscosities nor more concentrated chitin solutions.

Turning to the chemistry of the chitin solutions themselves, with N,N-dimethylacetamide (DMAc) and LiCl it has been shown (18) that the lithium ion is associated with the carbonyl oxygen to yield a complex carrying a positive charge. It was also indicated that the N-H groups in a polymer chain are associated with chloride ions, which with the positive lithium complex, gives an essentially neutral entity, soluble in DMAc.

In another investigation (20) caprolactam, a secondary amide (like chitin) was found by IR spectra to form a complex with LiCl containing 4 moles of caprolactam to one of Li. The complex melted at 98.5°C and in the crystal the Li^+ ion was coordinated to the carbonyl oxygen of each of the 4 lactam molecules. The chloride ions were said to be hydrogen bonded to the N-H hyrdogens. Both of these formulations are similar and in support of the amide-LiCl complex structure, is the observation that chitosan, which has few acetamide groupings to function in this type of complex, is insoluble in DMAc-LiCl.

After finding the DMAc-LiCl solvent system for chitin we studied briefly its effect on ethyl GlcNAc glycoside. In both DMAc and absolute ethanol, LiCl caused an immediate and significant drop in specific rotation, apparently because of the formation of a complex having different optical rotation properties.

Because of our experience with anomerization of this glycoside in alcohol solution, a study was made of the effect of various concentrations of LiCl in absolute ethanol on optical rotation. As may be seen from Table 2, about 2 moles of LiCl to 0.1 mole of ethyl GlcNAc glycoside was required for maximum change. No specific ratio of LiCl to glycoside in a complex was indicated, but rather a mass action effect to favor complex information in the equilibrium ethanol system. Lithium acetate dihydrate was also effective in reducing the value for specific rotation, indicating that lithium ion is the active complexing agent.

These data imply also that the slow optical inversion (renaturing) of chitin (2, 22) in DMAc-LiCl solutions (change of $[\alpha]_D$ form (+) to (-) or an increase in (-)

character) may result at least in part merely from the effect of the solvent itself. The slow change observed with chitin may result from slow penetration and complexing. The relatively low molar concentration of LiCl in DMAc (5% LiCl = 1.2M) may also account for the better chitin solvency of 1-methyl-2-pyrrolidinone (9% LiCl or 2.1M) solution. An ethanol-LiCl complex is probably in competition with the glycoside-LiCl system.

A concomitant action of LiCl in these systems may be to reduce the crystallinity of the chitin, presumably by disruption of hydrogen bonds. Such an effect of lithium salts has been observed with other polyamides, and with polycaprolactam and the dilithium salt of perfluoroglutaric acid (1).

Table 2. Effect of LiCl Concentration on Optical Rotation of Ethyl GlcNAc Glycoside (0.1M) in Ethanol.

LiCl conc., M	Rotation α^a	$[\alpha]_D$
0.0	6.2°	124.5°
0.2	6.0	120.5
0.6	5.6	112.4
1.2	5.3	106.4
1.6	5.2	104.4
2.0	5.1	102.4
3.0	4.8	96.4
3.5	5.1	102.4

[a] Theta (θ) is observed rotation

These several findings taken together are directly pertinent to the solubility of chitin in these systems: 1. Only fully-substituted amides are effective solvents, 2. An increased concentration of LiCl in the solvent favors complex formation and greater solvent power, 3. If lithium in these systems has a coordination number of 4, up to 4 chitin molecules may be associated in solution, which could account in part for the high viscosity of these systems.

A very significant consequence of chitin complex formation is that many of our physical measurements are only comparative, rather than absolute. Degree of polymerization, solubility parameter and specific rotation all depend upon a known chemical species to define molecular weight and density, which with chitin/LiCl is uncertain at best and probably variable.

Conformation of Terminal Aldohexose Groups and Anomerization. The terminal aldehyde group of chitin isolates, although a relatively small proportion of the polymer molecule, nevertheless plays an important role in determining polymer properties such as denaturing by cross-linking with free amine groups, mutarotation of the α and β anomers and one of the likely points of attachment for the protein portion of the mucopolysaccharide.

The conformation and mutarotation of the aldohexose sugars are determined by steric and electrostatic effects, which in turn are markedly influenced by the nature of the C-2 substituents. In the simple case of D-glucose, steric effects favor the boat form of the β-anomer in which all of the side groups occupy an equatorial position. Electrostatic effects, however, favor the α-glucose form in which the dipoles of the C-1 OH group and the C-5 ring oxygen are opposed (anomer effect) (14, 24); the equilibrium ratio of α:β is 37:63. However, also favoring the alpha anomer structure are the acetamide group at C-2 (23, 25) and relative acidity of the system (16). The cumulative result is a increased tendency toward formation of alpha anomers; with GlcNAc, the equilibrium ratio is 68:32, α:β. Mo and Jensen, (13) indicate that the monohydrate of N,N'-diacetylchitobiose, $(GlcNAc)_2$, crystallized by slow evaporation of aqueous 2-methyl-2,4-pentanediol, has a 90:10 ratio of its α:β anomers. Barker et al., (7) have reported the influence of terminal aldohexose groups on infrared absorption in a series of GlcNAc oligomers. They found that alpha-anomers exhibited mutarotation for the di-and tri-saccharides, but the effect decreased as the oligomer chain lengthened. These equilibrium anomer ratios are indicative of the tendency toward the alpha-anomer conformation as affected by C-2 substitution. Such factors become of increased significance when applied to the chitinous polymers, as the total effect is a strong propensity for anomerization to the alpha conformation under favorable environmental conditions.

C-2 Substituent Effects on Glycoside Hydrolysis and Anomermization. Perhaps the most important structural feature in chitin/chitosan that determines its chemical behavior is also the C-2 substituent: hydroxyl, acetamido, amino or, occasionally, a simple 2-deoxy (methylene) group in the glycoside structure itself. The principal competitive reactions encountered with the glycosides are hydrolysis and optical inversion, promoted in acidic systems by protonation of the O-1 oxygen (hydrolysis) or of the O-5 oxygen (anomerization) (Fig. 1). The integrity of the polymer chain, anomerization and helical conformation of the molecules are all dependent upon these factors.

The hydrolysis of the glycosidic linkage in chitin is well known (8, 23). The optical inversion (anomerization) of the glycoside from the natural beta form to an alpha-glycoside is seldom considered. Yet in the simple alkyl GlcNAc glycosides acid-catalysed conversion of beta to alpha anomer and base-catalysed inversion of alpha to beta-anomer has been demonstrated (5, 19)(Fig. 1).

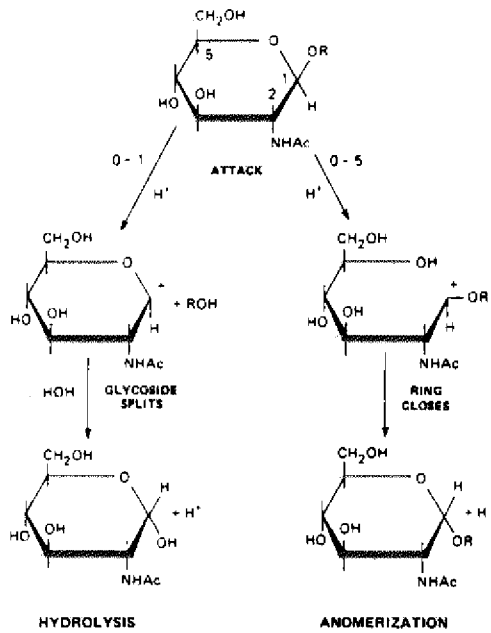

Fig. 1. Protonation at 0-1 and 0-5 Oxygens Leading to Hydrolysis and/or Anomerization of Alkyl GlcNAc Glycosides (and Chitin/Chitosan).

Hydrolysis rates of C-2 substituted methyl glucosides give the clearest picture of the substituent effect (Table 3) (8). These effects are reflected in the comparative behavior of the chitin and chitosan polymers themselves.

The hydrolytic stability of the glycosides (and chitinous products) increases dramatically from C-2 acetamido (alkyl GlcNAc glycosides, chitin), through hydroxyl (glucosides, cellulose) to amino (alkyl GlcN glycosides, chitosan). Thus chitin is much more sensitive to acid attack than cellulose, and chitosan in acetic acid solution has a useful life of well over a month (3). Chitosan was not completely hydrolyzed by boiling 3.3 N HCl in 3 days (7). The surprising stability of such chitosan solutions is of tremendous advantage in the commercial applications of this product. The optical inversion (anomerization) at the C-1 carbon atom in an alkyl GlcNAc glycoside, as well as in chitin or chitosan, is less apparent, but nonetheless real and important. Anomerization occurs because both the 0-1 and 0-5 oxygens are receptive to protonation and results in the formation of a planar C-1 carbonium ion and consequent anomerization (14). Notably with opening the ring at the C-5 site it does not rupture the polymer chain (Fig. 1).

Table 3. Effect of C-2 Substituents on Methyl D-Glycoside Hydrolysis and Specific Rotation.

C-2 Structure		Methyl glycoside hydrolysis rate Half-life, min. [a]	$[\alpha]_D$	Ref.
>CH-NH-Ac	α	18 min	+131.5°	(26)
	β	14	- 44.3	(26)
>CH-OH	α	48	+158	(13)
	β	24	- 33	(13)
>CH-NH_2·HCl	α	540	+100	(12)
	β	540		

[a] Determined in 2.493N HCl at 100°C (8).

Further, the glycoside structure itself increases the anomeric effect and stability of the alpha-conformation (24). In support of this alpha-structure propensity, is the report (8) showing that the hydrolysis rates as half-lives of the methyl glycosides of GlcNAc and glucose (Table 3), are substantially longer for the α-anomers.

In our studies of the preparation and resolution of the mixed optical isomers (anomers) of ethyl GlcNAc glycoside (5, 19) it was found that short reaction times of α-GlcNAc and alcohol favored formation of the beta anomer (with optical inversion). In 135 minutes 40% of beta anomer was obtained, while with a 205 minute reaction only 22% of beta anomer was isolated. Apparently, in the competitive reactions of α-GlcNAc to β-glycoside conversion and the subsequent acid-catalysed anomerization of the β- to α-anomer, the shorter times favored the beta-structure as the first step, while the slower anomerization trailed. Again, these reactions involve similar proton attack of the 0-1 and 0-5 oxygen positions in the GlcNAc structure (Fig. 1).

The alkyl GlcNAc glycoside preparational studies were carried out in ethanol solution, of course, to press the desired synthesis reaction. In view of the concurrent anomerization, however, we studied this factor independently, starting with a high-alpha anomer mixture of ethyl GlcNAc glycoside in a similar acidic

ethanol solution. In 24 hours at 53°C with 0.1M HCl a sample of the glycoside containing 83% α-anomer was completely converted to the α-anomer.

These studies were then extended to aqueous systems, with which we are usually concerned in chitin isolation and reactions. At 53°C in 1M HCl there was little change for several hours, followed, however by an accelerated decrease in optical rotation (Table 4). Since hydrolysis to GlcNAc would decrease the specific rotation (55.6° for α-anomer, 41.3° for equilibrium value (25) and anomerization would increase it (α-ethyl GlcNAc glucoside, = 134°) these results, suggest that the reaction rates of hydrolysis and anomerization are about equal in this system. Hence, early in the experiment, the products of the two reactions balance each other in specific rotation. After anomerization is largely complete, hydrolysis to GlcNAc continues and the specific rotation steadily drops.

Table 4. Changes in Optical Rotation of Ethyl GlcNAc Glycoside in 1.0M HCl at 53°C

Time, hrs.	θ[a]	$[\alpha]_D$
0	4.6°	92.4°
1.0	4.6	92.4
2.5	4.6	92.4
4.0	4.5	90.4
6.0	4.3	86.3
11.5	4.1	82.3
18.0	3.8	76.3
24.0	3.5	70.3
30.0	3.1	62.2
40.0	2.6	52.2

[a] Theta (θ) is observed rotation

Anomerization of Glycoside Linkages in Chitin and Chitosan. The possibility of anomerization of glycoside linkages in chitinous polymers has been suspected since the early work of Falk et al. (11) who studied the structure of "chitan", a completely acetylated chitin from Thalassiosira fluviatilis. By means of polarized infrared absorption spectra they found that initially the anomeric centers had a β-configuration. However, after deuterium exchange in 10 N HCl at 20°C, the characteristic absorption band shifted progressively and in time indicated an α-configuration. They questioned the significance of this finding, but in view of the strong acid conditions and our current thoughts on anomerization, the development of portions of an alpha structure seems quite logical.

Anomerization of glycoside linkages in chitinous products is perhaps not usually observed because of short reaction times involved to avoid hydrolysis, and somewhat comparable rates of hydrolysis and anomerization. However, there are additional strong indications in chitin/chitosan behavior in which anomerization is believed to be involved. Again, recall that ring opening and anomerization can take place without rupture of the polymer chain.

First there is an example of chitosan with surprisingly slow rate of hydrolysis as judged from monomer studies (Table 3). In some of our earlier work (3) one sample of chitosan was found to be completely racemized with 0° rotation. Further, there was no change in rotation on treatment in mildly acid systems (pH 2.0 - 5.5) normally leading to a more negative rotation (3, 6). In the light of the above comments, it now appears likely that in the preparation of this chitosan sample (some details unknown), much anomerization occurred, propensity for helical conformation was destroyed and a completely racemized product was obtained.

A second example is the unexplained variation in behavior of commercial chitin samples in their ability to renature or reverse their initial positive optical rotation in a dimethyl acetamide lithium chloride (DMAc-LiCl) solution (Table 5) (2, 22). It appears that in non-reversing samples, the preparational conditions led to some anomerization, partial racemization and loss of helicity. Some of the samples also contained insoluble fractions, perhaps caused by heat or acid denaturing which conditions are also conducive to anomerization. High initial values of specific rotation could result from α-anomeric linkages, also.

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

Chitin	% Sol. Material	$[\alpha]_D$ Initial	$[\alpha]_D$ In 2 wks.
Horseshoe crab	82	-56°	-56°
Blue crab	58	+33	-52
Red crab	76	+65	-22
Pink shrimp	62	+24	-54
Brown shrimp (KY-10)	92	-36	-36

It is now recognized that a portion of the above reversal from (+) to (-) specific rotation may result from the difference in the solvent system itself as indicated previously. The slow change in rotation observed with chitin in DMAc-LiCl solution may result in part from the relatively low concentration of LiCl (1.2M), the strength of its complex with DMAc and hence the slow shift of LiCl to the chitin molecule. However, the magnitude of the change and the similar reversal behavior of chitosan in certain acidic media still favor the random coil to helix conformation concept postulated (3, 4, 22).

In a recent crystallographic study of N,N'-diacetylchitobiose $(GlcNAc)_2$ (13) it was shown that the O(3')---O(5) bond distance is too great to allow formation of a normal intramolecular hydrogen bond and constrains only mildly the conformational freedom about the glycosidic bridge.

Taking these several indications together and considering the facile anomerization of the simple alkyl GlcNAc glycosides, one can hardly escape the conclusion that chitin and chitosan do anomerize to some extent under certain conditions and that in consequence will vary in such physical properties as solubility, crystallinity and chiroptical behavior because of this induced heterogeneity. In any case the possibility of anomerization should be considered in future studies of the chemical and physical behavior of chitinous products.

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CHITIN-PROTEIN INTERACTIONS

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ABSTRACT

It has been demonstrated recently that chitin is not a singularly defined compound but represents a family of closely related chemical entities whose compositions are a function of both species of origin and method of preparation. Moreover, these species differences appear to be largely a result of subtle variations in the natural chitin-protein complexes; that is, the manner in which protein is chemically bound to chitin.

Analyses of chitin isolates from several species of marine invertebrates, prepared by a series of carefully controlled hydrolytic treatments, indicate that all conventional chitin samples contain at least trace amounts of residually linked amino acids and confirm a marked species variation in their identity and content. Further, specific chitin-protein bond breaking treatments and subsequent structural analyses by IR indicate that amide type bonding of chitin to protein predominates in all species through N-glycosidic linkages to aspartic acid (asparagine).

INTRODUCTION

The nature of the association between protein and chitin in crustacean shells has been the source of speculation for a long time. Although the ratio of exoskeletal protein to chitin is known to vary substantially with species, chitin always appears to occur in association with it (1, 2, 3). Hackman (4) attempted to react cuticular protein, peptides, and amino acids with both N-acetylglucosamine (NAG) and purified chitin but the very weak bonds which did form were suspected to be of a Schiff's base type between the terminal aldehyde groups in the NAG and the amine groups of the amino acids. Based on this result, he concluded that hydrogen bonding must be the principal binding mechanism in the protein-chitin complex. Giles, at al (5), proposed that non-acetylated glucosamine units in the chitin polymer would permit direct cross-linking to tanning quinones and thereby to protein. Lipke and Geoghegan (6) were able to eliminate this possibility by using N-bromosuccinimide to cleave quinone linkages and produce peptidochitodextrins from insect cuticle. Their isolation of fragments of mucoprotein with glycosyl, N-acetylglucosaminyl and peptidyl residues further strengthened the arguments for the existence of stable covalent linkages between chitin and protein.

Hackman (7) reported that exclusively aspartyl and histidyl amino acid residues remained with native chitin from crab (Scylla serrata), squid (Logilo) and insect shells after hot alkali treatment and in each case, were the last to be removed by hot acidic hydrolysis. He proposed N-acylglucosamine covalent cross-links from an amino acid carboxyl group to the free NH_2 group of the occasional glucosamine unit in the chitin polymer, concluding that this type bond would possess the required stability to accommodate the data. His data suggested chemical homogeneity for the chitin-protein complex and apparently, a common covalent bonding pattern with a ratio of 1 strongly bound amino acid residue per 200-300 glucosamine units in chitin. Rudall (3) alternatively suggested the possibility of an amide link between the carboxyl group of an occasional N-acetylmuramic acid (Fig.1) in the chitin polymer chain and the amino group of a terminal alanyl unit in a peptide chain.

Hunt (2) pointed out that the results of previous research would favor aspartic acid as the amino acid directly linked to the polysaccharide (8), although this linkage had been demonstrated in other glycoproteins to be of N-glycosylamine type (Fig.2) between the reducing end group of the polysaccharide and the amide group of asparagine. Hunt reasoned that if this type protein linkage is accepted, then the bound protein must be found only at the extremeties of the chitin chains since these are unbranched structures. Rudall and Kechington (9) have interpreted the repetitive beading along the chitin chain observed in election micrographs together with the extra reflections in the chitin-protein complex x-ray diffraction pattern, which do not belong to purified chitin, as indicating regular repeats of bound protein along the chitin chain at exactly 31 Å intervals. Their apparent removal by proteolytic enzymes seemed to indicate that these proteins are arranged exactly every 6 NAG units and that natural chitin is deacetylated on a repetitive basis with the free amine groups serving as the linking points to the protein chains (10).

Attwood and Zola (11) also cast considerable doubt on the universality of Hackman's results when, in repeating his studies with different species, they were unable to confirm the linkage of aspartyl or histidyl residues to chitin, finding their alkali treated chitin-protein complexes contained a long list of trace amino acid residues, with no predominance of either aspartic acid or histidine. Karlson, et al. (12) found that the horseshoe crab (Limulus polyphemus) shell complex was relatively rich in glycine and alanine even after mild alkalai treatment. Herzog, et al. (13), employing proteolytic enzymes to investigate the chitin-protein complex in crayfish (Astacus fluviatilis), found the strongly bound protein to be rich in glycine and concluded that the chitin is present in a stable complex with the protein, covalently bound through glycine. Recent findings (14) indicate that chitin isolates from several marine invertebrates retain significant quantities of covalently bound amino acids even after mild alkaline extraction and that up to 0.5 MOL% of

amino acids remain following rigorous hydrolysis. Overall, it is evident that while the data accumulated on the various species run contrary to the initial findings of Hackman which indicated a universal chitin-protein complex, there has been little other consensus on the subject. The purpose of this study was to gain further insight into the role that differences in covalent bonding in the chitin-protein complex play in the natural variation of chitin.

EXPERIMENTAL

Fresh samples of the blue crab (Callinectes sapidus), the stone crab (Menippe mercenaria), the red crab (Geryon quinquedons) and the horseshoe crab (Limulus polyphemus) were used as starting material. The shells were cleaned, dried and ground as previously described (14). In order to analyze for the covalent bonding type in the chitin mucopolysaccharide of the samples species, a common starting point was established. For the horseshoe crab the product was defined as that remaining after sequential extraction of the ground shell with water (20°C, 24 hr.), 0.16 M Na_2SO_4 (2°C, 72 hr.), and 7 M urea (2°C, 48 hr.). The mucopolysaccharides from the blue, red and stone crab samples were defined as those remaining after ethylenediaminetetracetic acid (EDTA) decalcification of the shell and 7 M urea extraction of physically associated protein (11, 13, 14, 21).

Specific chemical treatment methods adapted from glycoprotein studies, were selected to aid in the differentiation of the types of covalent bonding occurring in the chitin-protein fractions starting with the covalently bound, chitin mucopolysaccharide from each species (see Fig.4 for summary of treatment scheme). The first treatment, 0.01 N NaOH at 20°C for 5 hours, was used to cleave weak, double covalent bonds such as Schiff's bases to extract the proteinaceous residues bound in this manner (11). Portions of the resultant samples were saved after filtration, washing with deionized water and acetone, and air-drying for amino acid analyses. As a next step, lithium borohydride ($LiBH_4$) treatment was employed as a method of specifically cleaving acetal type bonds, O-glycosidic linkages, such as those which have been documented in other glycoproteins between N-acetylglucosamine and serine (NAG-Ser) and N-acetylglucosamine and threonine (NAG-Thr) (15), while not significantly affecting amide or stronger linkages between protein and carbohydrate. This method involved refluxing pre-weighed portions of sample in 0.3 N $LiBH_4$ in dry tetrahydrafuran for 20 hours. Following the spending of any residual $LiBH_4$ with cold acidic methanol, filtration and acetone washing, the samples were air dried and portions retained for amino acid analyses.

Hydroxylamine treatment has been used extensively in studies of polysaccharide-protein linkages to cleave acetal and amide linkages (16), was employed in this study to break the proposed amide type linkages in the chitin-protein complexes, such as N-glycosyl and N-acylglucosaminyl bonds. The method consisted of stirring pre-weighed portions of sample for 9 hours at 37°C in 4 M $NH_2OH \cdot HCl$ which had been adjusted to a pH of 12.20 with NAOH. Following treatment, the hydroxylamine solution was filtered, the samples were washed with deionized water and acetone, air-dried and portions retained for amino acid analyses. As this hydroxylamine treatment, by itself, does not further differentiate between the N-glycosyl (Fig.2) and N-acylglucosaminyl type (Fig.3) linkages, infrared (IR) spectrometry was employed in conjunction with this treatment and amino acid analysis for this purpose. Another type of linkage which may survive cleavage by the treatments mentioned above and which has been postulated (17) between chitin and protein is an ether type linkage such as those associated with N-acetylmuramic acid (Fig.1). To ascertain the presence of these kinds of linkages evidence was sought for the hydroxylamine treated samples by comparing their amino acid analysis chromogragrams with ones generated for a pure N-acetylmuramic acid standard.

Proteolytic enzyme hydrolysis coupled with subsequent amino acid analysis was also employed to aid in the elucidation of the bonding of those amino acid residues most strongly bound to chitin (15). Beginning with the covalently bound chitin mucopolysaccharide, each sample species was treated separately with papain (Sigma Chemical Co.) following established procedures (13). The papain treatments were carried out for 20 hours at 65°C in a sodium acetate buffer at pH 5.6 with a concentration of 15.0 g of papain (2.6 U activity/mg) per 1 of buffer. After completion of these hydrolyses, the resulting products were prepared for amino acid analysis by filtration, washing with ethanol and ether, and drying.

Amino acid analyses were carried by placing a sample in air-tight hydrolysis tubes to which 12 N HCl and n-leucine standard had been added. Following standard procedures, the samples were hydrolyzed in vavuo at 110°C for 24 hr., evaporated to dryness, and diluted with a pH 2.2, sodium citrate buffer (18). The samples were then centrifuged, a 20 µl of sample of supernatant removed, and analysis of amino acid content performed on a computer equipped Durrum D-500 amino acid analyzer. A standard mixture of 16 common amino acids for machine calibration, containing 0.5 µmol/ml of each amino acid, was analyzed following the same procedures. Similarly prepared standards of pure N-acetylglucosamine and N-acetylmuramic acid (Pfanstiehl Laboratories, Inc.) were analyzed to determine their peak patterns, to allow for quantification of the glucosamine content in the sample species and to aid in the detection of possible muramic acid residues in samples. Samples were all run in duplicate. Instrumental error was assumed to be ±1% (manufacturer's specifications).

Infrared spectrometry was employed in this study to aid in the determination of the presence of free amine groups in the treated chitin-protein fractions. This method was previously employed

with success by Pearson, et al. (19) to observe the deacetylation of chitin (i.e., the production of free amine groups) during chitosan formation. For the purposes of this investigation, IR spectra of the chitin-protein fractions of all species were taken after both the $LiBH_4$ treatments and the hydroxylamine treatments for comparison. Each sample was prepared for analysis by grinding and sieving to less than 100 μ thorough low temperature (60°C) drying in a vacuum desiccator over silica gel, and pressing into a potassium bromide (KBr) pellet using a Wilks Scientific Co. manual pellet die. The pellet mixture in each case consisted of 25 mg sample/150 mg KBr homogeneously mixed. The spectra was then run and recorded on a Perkin-Elmer Model 180 grating IR spectrophotometer. The IR spectra of the enzyme treated samples were taken to ascertain the extent of free amine group formation resulting from these hydrolyses and to compare them with the spectra mentioned above. The IR spectra of well-characterized, commercially obtained chitin and chitosan (Hercules Co.) from a single species source, the brown shrimp (Penaeus aztecus), were also obtained to provide a comparative measure of the effects of deacetylation (increased free amine) on the characteristic spectral bands.

RESULTS AND DISCUSSION

In recent related studies (21), it has been shown that all chitin isolates even those prepared by carefully controlled series of hydrolytic and extractive treatments of the native chitin-protein complexes, contained covalently bound, proteinaecous residues. Moreover, these protein fractionation experiments revealed that the strongly covalently bound protein in the chitins prepared from a range of test species constituted 25.0 - 45.9% of the total shell protein. It has been reported recently as well that after exhaustive alkaline hydrolysis, a process known to be partially degradative to the chitin polymer but useful in attempting to elucidate the core chitin-protein linkages, discernible amounts of residual amino acids remained very strongly bound to the chitins derived from five different invertebrate sources (14). The data derived from that study regarding the amino acid composition of the rigorously extracted chitins indicates that event after prolonged, stringent alkaline hydrolysis, the residual chitins retained 0.05-0.5 MOL % of amino acid and that, importantly, aspartic acid, glycine and serine were present in all. The persistence and predominance of aspartic acid further leads one to postulate that it plays a key role in the covalent, core linkage of chitin to protein.

Arising as well from these earlier studies is considerable evidence indicating that there are probably several types and strengths of covalent bonding that may be present in the chitin-protein mucopolysaccharides. As such, a series of chemical treatments (Fig.4) designed specifically to cleave certain classes of protein-carbohydrate bonds in a sequential fashion was performed on the covalently bound portions of the chitin mucopolysaccharides of the various species. Coupling this fractionation with amino acid and infrared spectrometic analyses of the resultant chitin-protein products, it was possible to differentiate and quantify the kinds of covalent bonding occurring in each sample. Accordingly, the molar percent of the total covalently bound protein for each sample which was associated with each bond type was calculated from the amino acid analyses data assuming that the sum of the fractions listed above comprised 100% of the covalently bound protein in the samples. From the results of these calculations, compiled in Table 1, it can be observed that the amide type bonding is the predominate covalent linkage found in the chitin-protein units of all the samples, although there is variation in the actual percentage among the species.

TABLE 1 - Covalent Bonding Type Fractions of Protein in Chitin-Protein Mucopolysaccharides of Sample Species

Bond Type	Molar Percent of Bound Protein			
	Horseshoe Crab	Blue Crab	Stone Crab	Red Crab
Double Covalent (Schiff's Bases)	5.8	22.6	3.5	18.7
Acetal (C-1, O-Glycosidic)	3.6	16.0	23.2	29.0
Amide (C-1, N-Glycosidic) or (C-2, N-Acylglucosaminyl)	58.3	54.9	51.1	43.5
Other (Residual, Strong)	32.3	6.5	22.2	8.8

Since there appeared to be a substantial amount of strongly bound protein remaining associated with chitin in the residual fractions, ranging from 6.5% in the blue crab to 32.3% in the horseshoe crab, a further attempt was made to classify the chitin-protein linkages. Based on proposals in the literature (3, 17) that this "other", residual protein may be linked by strong ether type bonds such as those between N-acetylmuramic acid and peptide chains in the peptidoglycan of bacterial cell wall material (Fig.1), the amino acid analysis of pure, N-acetylmuramic acid was run to determine its characteristic chromatogram. However, comparison to the chromatograms of the chitin-protein fractions for all species failed to indicate the presence of N-acetylmuramic acid type linkages in any of the samples.

The amide type bonding fraction, determined to be the predominate class of covalent bonding in the chitin-protein complexes of all species, actually may consist of protein portions bound in two distinct manners since the hydroxylamine fractionation technique will not differentiate between N-glycosidic (Fig.2) and N-acylglucosaminyl (Fig.3) type linkages. To aid in the elucidation of these structures, infrared red (IR) spectrometric analysis was employed since hydroxylamine cleavage of N-acylglucosaminyl type bonds would produce free amine ($-NH_2$) groups in the residual chitin-protein product whereas cleavage of N-glycosidic linkages would not. By carefully analyzing the IR spectra, the relative differences in free amine groups present in the chitin-protein fractions before and after the hydroxylamine treatments can be qualitatively determined and thereby, bonding types indicated.

To illustrate the relative differences in free amine groups to be expected the IR spectra of commercially prepared brown shrimp chitin and chitosan were run and are presented in Fig. 5. These spectra features agree well with published data on chitins and chitosans from other species sources (20,22). Since chitosan is formed from chitin by vigorous deacetylation resulting in free amine group production, the absorption bands to note are those associated with the carbonyl and amide interactions of the acetamido group in chitin (95% acetylated). Specifically, progressive weakening and shifting of the spectral bands attributed to the bound C-2 amide group at 3265, 3100 and 1550 cm^{-1} (N-H bending) and those associated with the acetyl carbonyl group at 1625 and 1650 cm^{-1} (C=O stretching vibration) as a consequence of deacetylation in chitin and free amine ($-NH_2$) formation in chitosan are theoretically expected and are observed in comparing these spectra. Additionally, it can be observed that absorption bands associated with the acetamido methyl group at 2970 and 2940 cm^{-1} (CH_3-(C=O) stretching vibrations) in the chitin spectrum are nearly absent from the chitosan spectrum since about 95% of the acetyl groups have been hydrolyzed.

The IR spectra generated from the chitin-protein fractions before $LiBH_4$ treatment and after hydroxylamine treatment for the test species are depicted in Fig. 5. Additionally, the spectra of the enzyme hydrolyzed chitin mucopolysaccharides for each species are presented to provide a cross reference since treatment with papain, a proteolytic enzyme, might also be expected to cleave a portion of the N-acylglycosaminyl (C-2) linkages between chitin and protein, if they exist, and product similar changes in IR absorption bands. From these spectra it is clearly apparent that there are virtually no significant differences in the key spectra absorption bonds associated with the IR spectra of the three blue crab fractions and thus, no evidence to support the presence of N-acylglucosaminyl bonds in the blue crab chitin-protein complex. Similarly, there is little data arising from the comparison of the spectra of the stone crab fractions which would conclusively indicate a rise in free amine content after hydroxylamine treatment, although very slight weakening of a possible bound amine bond at 3100 cm^{-1} appears to be occurring in the spectrum of the hydroxylamine treated fraction. In comparing the data for the horseshoe crab and red crab fractions, there seems to be a slight diminution of the bound amide bands at 3265 and 3100 cm^{-1} from the spectrum of the $LiBH_4$ treated fraction to that of the hydroxylamine treated fraction. There is also some slight weakening in the carbonyl bands at 1625 and 1550 cm^{-1}. The spectrum of the papain treated horseshoe crab and red crab fractions, however, do not mirror these differences and appear most similar to the spectrum of the $LiBH_4$ treated fractions. Thus, the data supporting the presence of N-acylglucosaminyl linkages in the red crab and horseshoe crab chitin-protein complexes are inconclusive.

SUMMARY

1. There are several distinct covalent bonding type fractions in the chitin-protein mucopolysaccharides of all species.
2. Amide type bonding is the predominant covalent linkage between chitin and protein in all the species.
3. There is no evidence that N-acetylmuramic acid, ether type linkages are present in the most strongly covalent bound fraction of any of the species.
4. N-glycosidic linkages appear to be the principle type of amide bonding, and, coupling these results with the data on the core linkage structure, aspartic acid (asparagine) is the probable amino acid involved in this linkage

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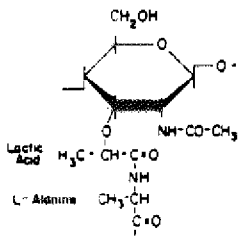

Figure 1 - An N-acetylmuramic acid type ether linkage formed between an *N*-acetylglucosamine unit in the polysaccharide chain and lactic acid. This in turn is amide linked to an amino acid residue (for example L-alanine)in a peptide chain.

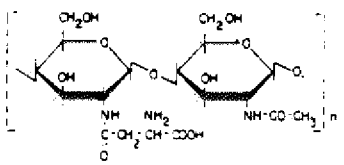

Figure 2 - Linkage at a free (deacetylated) amine group along the chitin chain with aspartic acid. This is an N-acylglucosamine type linkage to the β-carboxyl group of the aspartic acid.

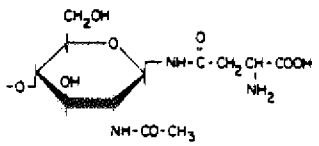

Figure 3 - N-glycosidic linkage between the terminal N-acetylglucosamine of the chitin chain and asparagine.

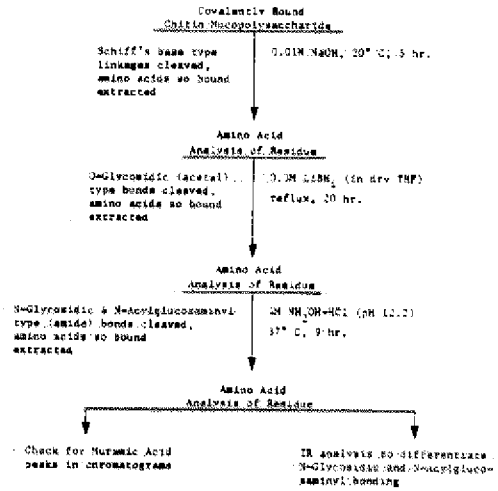

Figure 4 - Sequential chemical treatments of differentiate types of covalent bonding in the chitin mucopolysaccharide.

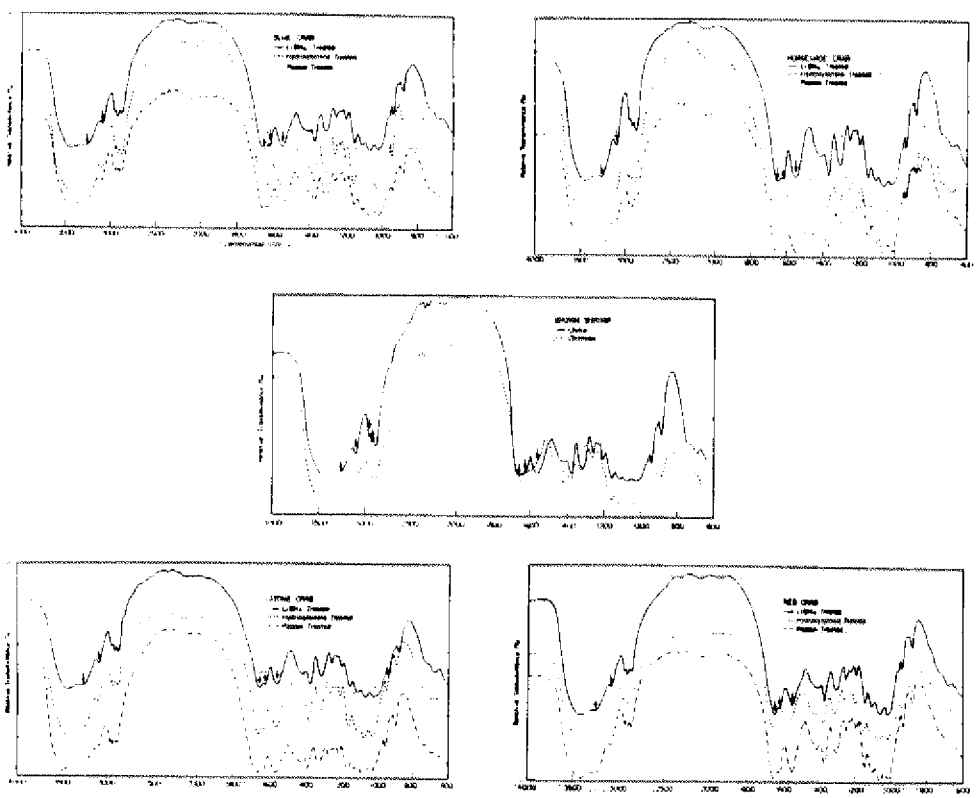

Figure 5 - Infrared absorption spectrum of brown shrimp chitin and chitosan and the covalently bound chitin-protein fractions in the test species.

UTILIZATION OF CHITINOUS PRODUCTS AND WHEY IN ANIMAL NUTRITION

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ABSTRACT

Billions of kilograms of chitin and whey are produced annually in the U.S. as by-products of the shellfish processing and cheese manufacturing industries, respectively. Most of these by-products are disposed of as waste, a practice that creates a serious environmental pollution problem and a loss of valuable nutrients.

Nutritional studies have shown that a combination of chitinous products and whey in isonitrogenous isocaloric diets enabled broiler chickens to utilize whey more efficiently. Chickens fed a diet containing 20% dried whey plus 2% double-sheared chitin for 31 days were significantly ($P < 0.05$) heavier than the control groups. Another 6-week broiler experiment was conducted with four diets [diet 1, consisted of 100% University of Delaware lactose-free diet (UDLFD); diet 2, 94.5% UDLFD + 2% chitin; diet 3, 63.5% UDLFD + 20% whey; and diet 4, 57.5% UDLFD + 20% whey + 2% chitin]. Analysis of variance demonstrated that diet significantly ($P < 0.0005$) affected final body weights. There were no significant differences in weight gain between diets 1, 2, and 4. However, birds fed diet 3 (whey without chitin) were significantly lighter in weight ($P < 0.01$) and had a significantly higher ($P < 0.01$) feed efficiency than those fed diets 1, 2, or 4. The feed efficiency of diets 1, 2, 3, and 4 were 1.83, 1.86, 2.10, and 1.81, respectively. In addition to the low weight gain, control birds on 20% whey without double-sheared chitin or ground chitin developed severe diarrhea. Furthermore, the abdominal fat pads of chickens fed the preferred 3-component diet 4 weighed significantly less ($P < 0.01$) than those of birds fed diet 1.

INTRODUCTION

For the U.S. seafood processor, shell waste presently represents a serious disposal problem since its only real outlet has been as a minor animal feed additive. Although it contains 20-30% protein and 10-30% chitin, it also contains more than 65% calcium salts. This high salt level makes it unsuited for bulk use in poultry feeds in particular. As a consequence, a large portion of the more than 100 million kg of shellfish processing waste (1,2) is released annually into sewage systems, streams, and landfill disposal sites. In view of the nature of this material and the geographic concentration of the seafood processing operations, this practice constitutes a serious, continuing environmental pollution problem for the adjacent coastal zone areas.

Chitin [poly-β-(1 → 4)-N-acetyl-D-glucosamine] obtainable from crab, shrimp, and lobster shell waste (3) continues to show promise as a new marine resource (1,4). Several new uses of chitin have been demonstrated in recent years including medical sutures, film and fiber products, and derivative compounds (5,6,7).

Whey, the serum of milk, is the liquid mixture formed in the process of making cheese. The largest production of whey occurs in the United States where it was nearly 19 billion kg in 1977 (8). Approximately 40% of that amount is being utilized (9) The remaining 10.8 billion kg of unused whey is normally released into the environment. In addition to the economic and nutrient losses, the dumping of whey constitutes the most potent pollutant of all dairy wastes and one of the strongest wastes of any kind (10). It was estimated that 100 kg of liquid whey has the polluting strength equivalent to sewage produced by 45 people in one day (10,11). Furthermore, the pollution problem associated with the disposal of whey worsens each year as the demand for cheese production continues to grow (8). Yet this so-called waste is a valuable nutrient for it retains about 55% of the nutrients in whole milk. Among other things, dried whey contains about 13% protein of high biological value, most of the water-soluble vitamins in milk, 8% minerals (primarily calcium and phosphorus), 1% fat, and about 70% lactose. This very high quantity of lactose in dried whey is the reason for its underutilization as a food source since the prevalence of lactose malabsorption and intolerance ranges from 70-90% in some populations studied in Africa, Asia, Latin America, and the U.S. (12,13,14,15,16,17,18). A similar incidence of intolerance exists in most animal species. The symptoms of lactose intolerance are severe and include diarrhea, gas production, cramps, bloating, dehydration, and death.

The problem of intolerance begins shortly after weaning when consumption of lactose-containing food is reduced. This event is followed by a similar reduction in the synthesis of lactase (also called ß-D-galactosidase, found in the brush border of the intestinal mucosal cells) which hydrolyzes the ß-linkage of lactose. The main problem is then how to increase the ability of an individual to digest larger amounts of lactose in the diet. Since lactase is not an inducible enzyme, attempts are made to supply the enzyme by encouraging the growth of certain desirable lactase-containing bacteria in the gut. Some 30 years ago, it was shown that Bifidobacterium bifidus var. Penn (Lactobacillus bifidus var Penn by the earlier terminology) predominated in the intestine and and feces of breast-fed infants while these were limited or absent from the intestine and feces of infants fed cow's milk (19,20,21). György (19) demonstrated that this strain of Bifidobacterium requires specific factors found in human milk for growth. These factors are virtually absent from cow's milk (19,20). Other studies (22,23) have shown that N-acetyl-D-glucosamine (GlcNAc, the monomer of chitin), bovine milk casein digest, and human milk casein and some of its derivatives also stimulate growth of B. bifidus var. Penn. Isolation studies have shown that the human milk growth factors are either entirely carbohydrates (such as GlcNAc or oligosaccharides) or glycoproteins (23,24,25,26,27). The growth promoters serve as a source of glucosidically bonded GlcNAc residues for bacterial cell wall biosynthesis.

We followed these leads in animal feeding studies using various chitinous materials including propyl-GlcNAc glycoside, ethyl-GlcNAc glycoside, microcrystalline chitin (MCC), double-sheared chitin, and chitin. In earlier nutritional studies employing various GlcNAc glycoside supplements in the diet, we were able to increase the tolerance to lactose in the rat (28,31). We also found MCC to be effective in promoting the digestion of whey in chickens (28,32); however, because GlcNAc and MCC are expensive and unavailable commercially in large quantities, any large scale application of this technology would be economically unfeasible. Nevertheless, the chicken is a better animal model to study this problem for the following reasons: 1) The chicken gut contains chitinolytic activity (29), 2) it is the least efficient utilizer of whey (9,30), and 3) if chickens could utilize whey efficiently, the poultry industry could be one of the largest users of whey. By supplementing the chicken diet with chitin and whey, we would achieve the following. The chitinolytic system in the gut would hydrolyze chitin to its oligomers and monomers. In turn, these saccharides would promote the growth of Bifidobacteria present in the gut and concurrently increase lactolytic activity. The net result would be an increase in the animal's ability to metabolize lactose thereby making whey a desirable feed for animals.

EXPERIMENTAL

The first of two experiments involved a small number of broiler chickens and double-sheared chitin as a chitinous supplement to whey. In this preparation, chitin (purchased from Hercules, Inc., Wilmington, Delaware, no longer a supplier, extracted from Brown shrimp shells) was reduced in particle size in dry form using a high-speed Waring Blendor. Then it was ground in a Wiley mill to pass a 40 mesh seive. This double sheared chitin had a molecular weight of 350,000-450,000. The dried sweet whey was purchased from Kraft, Inc. Distributors, Chicago, IL and had a lactose content of 68%. Each of the four diets shown in Table 1 were fed to a group of 5 male and 5 female one-day old Ross X Arbor Acre broiler chicks. The chicks were purchased from Allen's Hatchery (Seaford, Delaware). They were sexed, debeaked, vaccinated (at hatchery, for Marek's Disease and at 10 days of age for New Castle, Infectious Bronchitis, and Infectious Bursal Disease), identified by numbered wing bands, and randomly assigned to dietary groups. For the first 4 days, they were maintained on a commercial starting lactose-free diet (CLFD, purchased from Southern States Cooperative, Newark, DE) which contained a coccidiostat. Water and feed were provided ad libitum. All diets were formulated to be isonitrogenous (23.5% protein), isocaloric (3160 kcal/kg metabolizable energy), and were fortified with equal amounts of vitamin and trace mineral supplements. To facilitate uniform mixing of the CLFD with the double-sheared chitin and dried whey, the CLFD was passed through a hammer mill (6 mm screen). For the first 3 weeks, chicks were maintained in heat-controlled brooder batteries and thereafter transferred into wire cages. The body weights of the birds were recorded at the start of the experiment and at 7, 14, 21, and 31 days of age. At the end of the experiment (31 days), necropsies were performed on all birds.

The second broiler experiment involved a larger number of birds and ground chitin in a completely randomized block design of pens. Each of the four diets shown in Table 2 were fed to three replicate pens, (numbered 1 to 12; diet 1 included replicates 1-3; diet 2, 4-6; diet 3, 7-9; and diet 4, 10-12) each containing 35 male one-day old Ross X Arbor Acre broilers. All diets were formulated to be

isonitrogenous (23.5% protein), isocaloric (3160 kcal/kg metabolizable energy), and were fortified with equal amounts of vitamin and trace mineral supplements. Chitin used in this experiment was purchased from Madera Products, Inc., Albany, Oregon who extracted it from Tanner (snow) crab shells. It was ground to 60 mesh and had a molecular weight of 2,300,000 and a specific rotation of $[\alpha]_D = -35^o$. The dried whey was acid whey purchased from Gopez Milk Product Co., Cincinnati, Ohio. The lactose content of this by-product was 73%. None of the diets used contained coccidiostat. In order to simulate commercial conditions, the chicks were raised in floor pens. Each pen provided 4.60m^2 of floor area covered with dried peanut hulls and equipped with heat lamps. Cylindrical hanging feeders and automatic waterers were provided for free choice feeding and drinking.

The chicks were purchased from Allen's Hatchery (Seaford, Delaware). They were sexed, debeaked, vaccinated and identified as in the previous experiment. For the first 4 days, chicks were maintained on a University of Delaware lactose-free diet (UDLFD) without coccidiostat. Following this period, birds were fed their randomly assigned experimental diets. Body weights were recorded at the start of the experiment and at 9, 24, and 44 days of age at which time the experiment was terminated. The well-being of the chicks was observed two to three times daily for the duration of the study. Samples of feces from each pen were collected fresh twice per week and examined for the presence of coccidia. This was done by mixing the collected feces with 15 ml phenol-sugar solution and strained through cheese cloth. The mixture was examined by light microscopy at 40X. The amount of feed consumed was recorded for all pens. At the end of the trial, 120 chickens were collected randomly, 10 birds from each pen, and dissected to check the vital organs (e.g., liver, heart, lungs, gall bladder, etc.) for any abnormalities. Also, 70 birds (10 birds each from replicates 1, 3, 4, 5, 7, 11, and 12) were picked randomly, the abdominal fat pads removed, and the fat weights recorded. The body weight of birds were matched with their abdominal fat pad weights and a ratio was determined by dividing body weight by the weight of the abdominal fat pad. Statistical analyses were performed using the Statistical Package for Social Science (33) and the Delibr/Naovmain computer program (34).

RESULTS AND DISCUSSION

The data in Table 3 show the progressive growth of chickens in the first experiment. Analysis of variance of the mean weights at 31 days of age indicated a significant ($P < 0.05$) effect due to diet. The Duncan multiple range test results shown in Table 3 indicate that birds fed the chitin-whey diet 4 weighed significantly more ($P < 0.01$) than those receiving diets 1, 2, or 3. These results demonstrate that growth retardation from dietary whey was overcome by the addition of double-sheared chitin to the diet. Three or four days after placing the birds on their respective diets, chicks in treatments 3 and 4 developed diarrhea. Although the diarrhea of the chickens fed diet 3 worsened with time and became severe within 17-20 days, the condition of chicks in diet 4 improved and by the end of the trial, they were nearly normal or only slightly diarrhetic. In addition, neither whey nor double-sheared chitin had any adverse effect on the appetite of the birds. There was no mortality in any pens for the duration of the experiment. Necropsies at the end of the experiment revealed that all internal organs of birds fed diet 1 were normal. The gall bladders of birds in diet 2 were slightly smaller and their intestines larger than normal. All birds examined from treatment 3 showed intestinal hemorrhage, enlarged intestine, and enlarged ceca. Birds in the chitin-whey diet 4 had larger livers and gall bladders by visual inspection than those in diet 1. The gizzards of birds in diet 4 peeled easily (a desirable characteristic), the skin was firm, and the flesh under the peel was smooth. Subjectively, the abdominal fat pads of birds in diet 4 were smaller, which led us to weigh this tissue in the second experiment. The intestines of chicks fed diet 4 were large and distended, suggesting vigorous ongoing fermentation.

Table 4 lists the results from the second experiment. The growth trends are similar to those observed in the first trial. Analysis of variance of the mean weights at the end of the experiment indicated a significant ($P < 0.0005$) diet effect. The Duncan multiple range test results shown in Table 4 indicate that there were no significant weight gain differences among diets 1, 2, and 4. However, chickens fed diet 3 weighed significantly less ($P < 0.01$) than those fed 1, 2, or 4. Furthermore, birds raised on diet 3 had a significantly higher ($P < 0.01$) feed efficiency than those fed diets 1, 2, or 4 (Table 4). As in the case of the double-sheared chitin and previous studies (28), these results establish that chickens gained the least body weight in the diet containing 20% whey without chitinous supplement (diet 3). In addition to the poor growth, birds in this diet suffered from severe diarrhea, a condition that persisted throughout the duration of the study. Chickens on the chitin-whey diet 4 were initially diarrhetic but their feces

gradually (within 15-20 days) became fairly normal. Weekly checks of the feces showed that birds in all diets were virtually devoid of coccidia. Necropsies at the end of the experiment showed that the condition of internal organs of the birds in the four treatments were similar to those in the first experiment. For the duration of the experiment, there were three mortalities all from diet 3 (two from replicate 7 and one from replicate 9).

Seventy chickens (10 birds each from replicates 1, 3, 4, 5, 7, 11, and 12) were picked randomly, dissected, the abdominal fat pads removed, and the weights recorded. Ratios of individual body weight to fat pad weight were determined. Table 5 shows Duncan multiple range test comparisons of mean ratios of body weight to fat in the abdominal fat pad with the various replicates. These analyses demonstrated that the differences were not statistically significant in replicates 11 versus 12, 1 versus 3, 4 versus 5, and 5 versus 7. However, the differences were highly significant ($P < 0.01$) in comparisons between replicates 1, 3, 4, 5, and 7. Figure 1 shows a visual comparison between the abdominal fat pads from 10 chickens each of replicates 3, 11, and 12 (identified as groups 2, 11, and 12). These results confirm our previous observation that a diet containing 20% whey plus 2% double-sheared chitin or ground chitin produced chickens with significantly less abdominal fat, without a reduction in body weight, than chickens raised on a commercial broiler ration. This is an interesting observation since others have shown that the addition of corn oil to the diet increased body fat (35,36). However, in these studies the level of energy and/or protein were not held constant, therefore, direct comparison is not appropriate. Furthermore, subjective tasting of cooked chickens from diets 1 and 4 suggested that the meat of birds raised on the chitin-whey modified diet 4 were tastier than those grown on diet 1. Sensory evaluation of the meat from birds fed chitin and whey is necessary to determine if these ingredients added product acceptability.

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Table 1. Composition of experimental diets used in experiment 1.

Ingredients	Diet 1	2	3	4
Commerical lactose-free diet[1]	100	94.5	63.5	57.5
Dried whey	0	0	20.0	20.0
Double-sheared chitin	0	2.0	0	2.0
Soybean meal	0	2.5	12.0	15.0
Corn oil	0	1.0	4.5	5.5

[1] Southern States Cooperative, Inc. starting and growing mash with coccidiostat.

Table 2. Composition of experimental diets used in experiment 2.

Ingredients	Diet 1	2	3	4
University of Delaware lactose-free diet[1]	100	94.5	63.5	57.5
Dried whey	0	0	20.0	20.0
Ground chitin	0	2.0	0	2.0
Soybean meal	0	2.5	12.0	15.0
Corn oil	0	1.0	4.5	5.5

[1] This diet contained: corn 53.75%, soybean meal 32.10%, blended fat 5.00%, meat and bone meal 7.00%, dicalcium phosphate 0.30%; iodized salt 0.35%, DL-methionine 0.20%, choline chloride 0.20%, vitamin premix 0.50%, and trace mineral premix 0.50%. This diet was formulated to provide 23.5% protein, 3160 kcal/g, 0.90% calcium, and 0.55% available phosphorus.

Table 3. The effect of double-sheared chitin and whey on the mean ± SD weight of broilers from the first experiment.

Diet	Days 0	7	14	21	31
			grams		
1	81	190	347	546	988^{b} ± 29.2
2	87	182	348	518	956^{b} ± 26.1
3	86	180	298	480	906^{a} ± 25.9
4	80	194	359	609	1105^{c} ± 31.6

^{a,b,c}Means ± SD with different superscripts are significantly different ($P < 0.01$).

Table 4. The effect of ground chitin and whey on the mean ± SD weight and feed efficiency of chicks from the second experiment

Diet	Days 0	9	24	44	Feed Efficiency[a]
			grams		
1	77	221	803	1739^{b} ± 72.3	1.83^{b} ± 0.04
2	75	229	823	1745^{b} ± 67.1	1.86^{b} ± 0.09
3	78	214	788	1669^{b} ± 70.1	2.10^{c} ± 0.10
4	78	246	831	1782^{c} ± 78.6	1.81^{b} ± 0.02

[a]Calculated at 44 days of growth

^{b,c}Means ± SD with different superscripts are significantly different ($P < 0.01$).

Table 5. Duncan multiple range test comparisons of mean ratios of body weight to fat.

Replicate Comparison	Mean Ratio	Level of Significance
12 vs. 1	125 vs. 52	0.01
12 vs. 3	125 vs. 51	0.01
12 vs. 4	125 vs. 66	0.01
12 vs. 5	125 vs. 71	0.01
12 vs. 7	125 vs. 78	0.01
12 vs. 11	125 vs. 107	NS
11 vs. 1	107 vs. 52	0.01
11 vs. 3	107 vs. 51	0.01
11 vs. 4	107 vs. 66	0.01
11 vs. 5	107 vs. 71	0.01
11 vs. 7	107 vs. 78	0.01
1 vs. 3	52 vs. 51	NS
4 vs. 5	66 vs. 71	NS
5 vs. 7	71 vs. 78	NS

NS = not significant

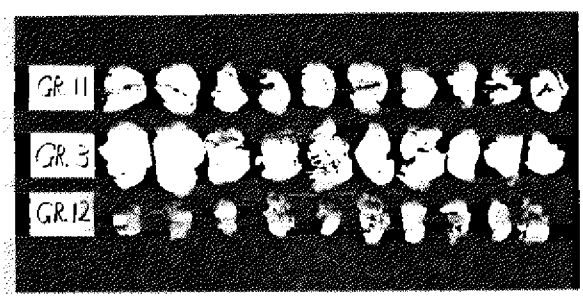

Figure 1. A picture showing visual comparisons of abdominal fat pads from replicates 11 and 12 (from diet 4) and replicate 3 (from diet 1).

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