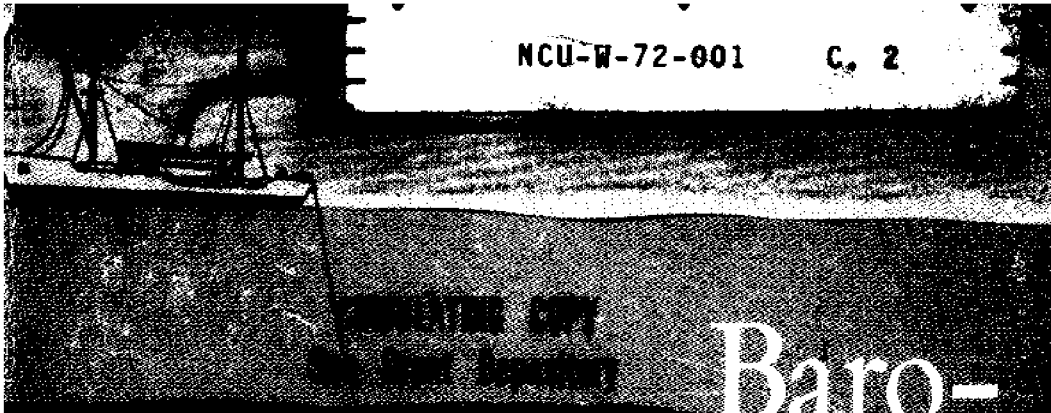

Baro-biology and the Experimental Biology of the Deep Sea

Brauer, Ed.

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ORGANIZING COMMITTEE

Robert R. Hessler
Scripps Institution of Oceanography
La Jolla, California

Heinz A. Lowenstam
California Institute of Technology
Pasadena, California

Robert J. Menzies
Florida State University
Department of Oceanography
Tallahassee, Florida

Richard Y. Morita
Oregon State University
Departments of Microbiology and Oceanography
Corvallis, Oregon

Ralph W. Brauer, Chairman
Wrightsville Marine Bio-Medical Laboratory
Wilmington, North Carolina

BAROBIOLOGY AND THE EXPERIMENTAL BIOLOGY OF THE DEEP SEA

PROCEEDINGS OF THE FIRST SYMPOSIUM ON HIGH PRESSURE AQUARIUM SYSTEMS AS TOOLS FOR THE STUDY OF THE BIOLOGY OF DEEP OCEAN FAUNA AND ASSOCIATED BIOLOGICAL PROBLEMS.

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DEDICATION:

To our students of the past and to those yet to come.

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LIST OF PARTICIPANTS

Numbers in parentheses indicate the pages on which the author's and panelists' contributions begin.

Lawrence J. Albright, Simon Fraser University, Burnaby, British Columbia

Robert M. Avent, Department of Oceanography, Florida State University, Tallahassee, Florida

Robert R. Becker, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon

L. R. Berger, University of Hawaii, Honolulu, Hawaii

Ralph W. Brauer, Wrightsville Marine Bio-Medical Laboratory, Wilmington, North Carolina

R. A. Carr, Naval Research Laboratory, Washington, D. C.

R. A. Courant, Woods Hole Oceanographic Institution, Woods Hole, Mass.

B. G. D'aoust, Virginia Mason Research Center, Seattle, Washington

R. C. DeHart, Southwest Research Institute, San Antonio, Texas

A. Disteche, Institute Van Beneden, Universite de Liege, Liege, Belgium

W. O. Doggett, North Carolina State University, Raleigh, North Carolina

H. Flugel, Institut fur Meereskunde, University of Kiel, Kiel, Germany

Robert Y. George, Department of Oceeanography, Florida State University, Tallahassee, Florida

M. Gilmartin, Hopkins Marine Station, Stanford Research Institute, Pacific Grove, California

M. S. Gordon, University of California at Los Angeles, Los Angeles, California

K. R. Gundersen, University of Hawaii, Honolulu, Hawaii

Robert R. Hessler, Scripps Institution of Oceanography, La Jolla, California

R. A. Horne, J.E.F. Scientific Corporation, Burlington, Mass.

John Kanwisher, Woods Hole Oceanographic Institution, Woods Hole, Mass.

Fred Kalber, Aquatic Sciences, Inc., Boca Raton, Florida

R. C. Kettling, Department of Biology, Georgetown University, Washington, D. C.

B. Kimmel, Office of Naval Research, Washington, D. C.

W. W. Kirk, International Nickel Co., Inc., Wilmington, North Carolina

J. V. Landau, Department of Biology, Rensselaer Polytechnic Institute, Troy, New York

H. A. Lowenstam, California Institute of Technology, Pasadena, California

A. G. Macdonald, University of Aberdeen, Aberdeen, Scotland

Robert J. Menzies, Department of Oceanography, Florida State University, Tallahassee, Florida

R. Y. Morita, Department of Microbiology and Oceanography, Oregon State University, Corvallis, Oregon

Mario M. Pamatmat, Department of Oceanography, University of Washington, Seattle, Washington
Donald Phillips, Department of Oceanography, Florida State University, Tallahassee, Florida
D. Reyss, Centre Oceanologique de Bretagne, Brest, France
John Rowlands, Southwest Research Institute, San Antonio, Texas
W. Y. Saunders, Lockheed Missiles & Space Corp., Sunnyvale, California
R. S. Scheltema, Woods Hole Oceanographic Institution, Woods Hole, Mass.
K. Smith, Woods Hole Oceanographic Institution, Woods Hole, Mass.
John Teal, Woods Hole Oceanographic Institution, Woods Hole, Mass.
H. Theede, Institut fur Meereskunde, University of Kiel, Kiel, Germany
V. Vidaver, Simon Fraser University, Burnaby, British Columbia
J. Morgan Wells, Wrightsville Marine Bio-Medical Laboratory, Wilmington, North Carolina
Charles Woodhouse, Office of Naval Research, Washington, D. C.
Robert Wicklund, Perry Oceanographic Foundation, Riviera Beach, Florida
A. A. Yayanos, Scripps Institution of Oceanography, La Jolla, California
A. M. Zimmerman, University of Toronto, Department of Zoology, Toronto, Canada
Claude ZoBell, Scripps Institution of Oceanography, La Jolla, California

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PRESSURE CONVERSION TABLE

ATMOSPHERES	PSIG	DECIBARS	KG per CM^2
.0680	**1.00**	.690	.0703
.0987	1.45	**1.00**	.102
.136	**2.00**	1.38	.140
.197	2.90	**2.00**	.204
.204	**3.00**	2.07	.211
.272	**4.00**	2.76	.281
.296	4.35	**3.00**	.306
.340	**5.00**	3.45	.351
.394	5.80	**4.00**	.408
.408	**6.00**	4.14	.422
.476	**7.00**	4.83	.492
.493	7.25	**5.00**	.510
.544	**8.00**	5.52	.562
.592	8.70	**6.00**	.612
.612	**9.00**	6.20	.633
.691	10.2	**7.00**	.714
.790	11.6	**8.00**	.816
.888	13.0	**9.00**	.918
.968	14.2	9.80	**1.00**
1.00	14.7	10.1	1.03
1.94	28.4	19.6	**2.00**
2.00	29.4	20.3	2.07
2.90	42.7	29.4	**3.00**
3.00	44.1	30.4	3.10
3.87	56.9	39.2	**4.00**
4.00	58.8	40.5	4.13
4.84	71.1	49.0	**5.00**
5.00	73.5	50.7	5.17
5.81	85.3	58.8	**6.00**
6.00	88.2	60.8	6.20
6.78	99.5	68.6	**7.00**
7.00	103	70.9	7.23
7.74	114	78.4	**8.00**
8.00	118	81.1	8.26
8.71	128	88.2	**9.00**
9.00	132	91.2	9.30

Depth-Pressure Conversion: 1010 decibars is equivalent to approximately 1000 meters' depth in the sea. Note, however, that the exact depth at which a pressure of 1010 db exists depends on local values for gravity and for the density of sea water.

Opening Statement

PARAMETERS CONTROLLING EXPERIMENTAL STUDIES OF DEEP SEA BIOLOGY [1]

by
RALPH W. BRAUER
Wrightsville Marine Bio-Medical Laboratory
7205 Wrightsville Avenue
Wilmington, North Carolina

The decision to invite a conference to discuss various aspects of deep ocean simulators for biological use grew out of an assessment of the state of knowledge in two somewhat distinct areas of inquiry: On the one hand, those concerned with the study of physiology and biochemistry of high pressures could look back on an extended series of investigations which by now have pretty well established that, like temperature or pH, hydrostatic pressure is a parameter the manipulation of which over sufficiently wide ranges is capable of influencing profoundly a wide variety of key biological functions. By the same token, hydrostatic pressure provides a powerful tool for the exploration of many such fundamental problems as control in biological processes and the interplay between structure and function in biological systems. The range of pressures found effective in these investigations falls well within the range of pressures encountered as one passes from the surface of the world oceans to the abyssal and hadal regions. It is therefore a corollary of this work of the experimental biologists that exploration of the experimental biology of deep sea fauna presumes an ability to carry out the requisite manipulations in high-pressure environments.

On the other side of the table, the development of oceanography over recent decades has made intensive exploration of the enormous water masses of the deep ocean appear increasingly important. Problems of evolutionary biology in relation to the emerging history of the ocean bed, problems of the ecology of the benthic community of deep waters and of the role of this fauna in the overall energy flux of the ocean, problems of physiological adaptation and of vertical mobility of species—all of these questions are calling for attention if the biology of these important regions of the deep ocean is to contribute its full share to our understanding of the marine biosphere. At the same time, the view is gaining ground that in order to obtain useful insight into the dynamics of marine biota and of the environment of which they form a part there is called for greatly increased

1. Research supported by Office of Naval Research under contract No. N00014-69-C-0371.

emphasis on experimental as opposed to the more traditional observational methods of approach.

Together, these considerations once again lead to a requirement for systems in which deep sea fauna could be observed and maintained, and experimented with, under conditions which resemble as closely as possible those in their native habitats. Even without the convincing evidence provided by the work of experimental biologists, this requirement would impose a priori need for systems in which one can control at will not only temperature, light, and chemical composition of the aqueous medium surrounding the animals, but also—and especially—hydrostatic pressure, the most uniquely characteristic factor distinguishing the deep sea environment from all others.

While this is not the place to elaborate upon the oceanographic considerations leading to these conclusions—indeed these will undoubtedly be dealt with far more effectively than I could hope to by subsequent contributions to this volume—it seems to me appropriate to discuss somewhat further the significance of hydrostatic pressure as a main limiting factor in the design of systems to meet our needs. Over the years, recognition of the significance of hydrostatic pressure as a factor capable of profoundly influencing living processes has tended time after time to meet with a certain air of incredulity. Except among a small group of the faithful whose interests lay in this direction, there has been an inclination on the part of many investigators to seek to explain such phenomena, when described by others, in terms of almost any factor other than hydrostatic pressure itself. The history of investigation in this field has been one of brief bursts of energy and development, followed by prolonged periods of relative somnolence. There may be some danger that this cycle might be repeated today as oceanographers find occasional species of deep sea fauna that can be transported artificially over wide pressure ranges without actually dying on the spot.

It seems to me important, therefore, to remind you today that the existence of hydrostatic pressure effects upon a wide variety of biological processes is no longer a matter of conjecture. It is a well established fact, and the knowledge of these effects has behind it over 100 years of work, beginning with efforts of French investigators during the second half of the 19th century. These workers demonstrated a wide variety of changes in behavior of many forms of life subjected to hydrostatic pressures of the order of several hundred atmospheres, and they learned early that some of these changes led to the death of the test organisms. After a period of comparative inactivity in this field, work was resumed intensively under the aegis of American investigators such as Marsland and Catell. Using more incisive techniques, these investigators demonstrated the effects of hydrostatic pressures of the order of 100-500 atm. upon a wide variety of specific

vital processes, such as protoplasmic contractility, cell division and the related karyokinetic movements, or the behavior of isolated muscle preparations. Indeed, the work of these investigators, although it, too, slowed down somewhat after a number of years, has never quite subsided, and the recent surge of activity in the molecular biology of hydrostatic pressure effects is in no small measure due to the direct successors of these early workers. Several colleagues from this group have honored this conference with their presence. Meanwhile, two other important lines of investigations were being carried on elsewhere. One of these was the work of the physical chemists who followed upon the footsteps of earlier investigators, many of them students of Professor Bridgman, who turned their attention to the thermodynamic and kinetic effects of hydrostatic pressures upon solutions of electrolytes, solutions of macromolecules, and enzymatic reactions. The theoretical apparatus for the analysis of reaction kinetics under these circumstances was developed in large measure by investigators from the school of Professor Eyring who applied the theory of absolute reaction rates and the concept of the transition complex to the prediction and description of the complex interplay between temperature and pressure. Experimental data on which to test these theories began to emerge presently from more detailed studies of enzyme-catalyzed reactions and from the studies of protein denaturation in high-pressure environments. Another highly important line of inquiry that grew out of this period was based on the discovery by ZoBell and his associates of barophilic bacteria in the deep ocean sediments, and the consequent development of a long series of distinguished investigations concerned with the metabolic processes of micro-organisms in their relation to hydrostatic pressure and temperature.

In every instance and in all of these investigations, it was made abundantly clear that profound biological effects must be expected, and indeed were found, when hydrostatic pressures were varied over a range of values well within that attained by passage from the surface to the deep regions of the world oceans. From the point of view of this conference all of this work can be summed up aptly by paraphrasing a remark of one of the participants in this conference who pointed out in the course of discussion that what needed explaining was not why there should be effects of changes in hydrostatic pressures upon living systems, but rather perhaps why these changes were so relatively mild for a given pressure excursion.

Against this background, it is amusing to contemplate that, as little as five years ago, investigators concerned with the physiology of diving vertebrates had no inkling that hydrostatic pressure effects might presently become a key parameter limiting the penetration of man or other air- or surface-dwelling vertebrates into the ocean depths. When in 1966 we first reported our observations of profound neurological changes in monkeys subjected to simulated dives to pressures of the order of 50 atm., and

suggested that these effects seemed to us likely to be attributable to the influence of the high hydrostatic pressures encountered, the announcement was greeted with wide-spread skepticism. For the next several years, a number of our colleagues insisted that these phenomena reflected the influence of any of a large number of alternative factors rather than hydrostatic pressure. It is perhaps fair to say that today, as a result of painstaking efforts in several laboratories to eliminate such alternatives as temperature changes, oxygen tension problems, difficulties of respiratory exchange, and consequent carbon dioxide accumulation, our original surmise appears to be borne out: Compression of primates to pressures of the order of 50 to 60 atm. gives rise to a complex syndrome which we have called the high pressure neurological syndrome, and which cannot be

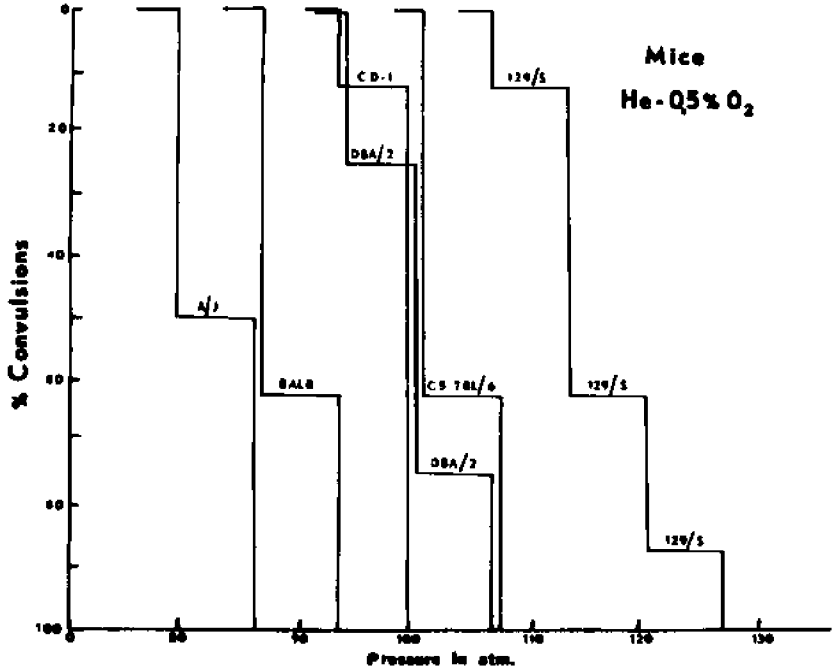

Figure 1. Strain differences in susceptibility and the convulsion phase of the high pressure neurological syndrome in inbred mice. The step curves represent cumulative proportion of animals showing convulsive seizures. Strains A/J, BALB, DBA2, C57 B1/6 and 129/S from Jackson Memorial Laboratory. CD-1 is a less highly inbred strain from Charles River Breeding Laboratories. (Brauer, R. W. and C. Hogue, unpublished data)

attributed to anything other than the effects of hydrostatic pressure (either absolutely or as result of rate of change) upon some aspects of the processes of synaptic conduction in multi-synaptic networks.

I would like to present three sets of data from this body of material because I think these may be helpful in defining numerically some of the problems pertinent to our joint deliberations. Figure 1 shows the distribution of pressures of onset of the convulsive seizure phase of the high pressure hyperexcitability syndrome in six discrete mouse strains, five of them closely inbred, the sixth a stable, but not selectively bred, laboratory mouse strain. The figure illustrates the fact that the mean convulsion thresholds in these strains may vary from 78 atm. for the least susceptible strain to 118 for the most susceptible one. This is one of a number of sets of data which indicate that susceptibility to this syndrome is genetically determined to a large extent, and therefore could well enter into natural selection processes among marine species.

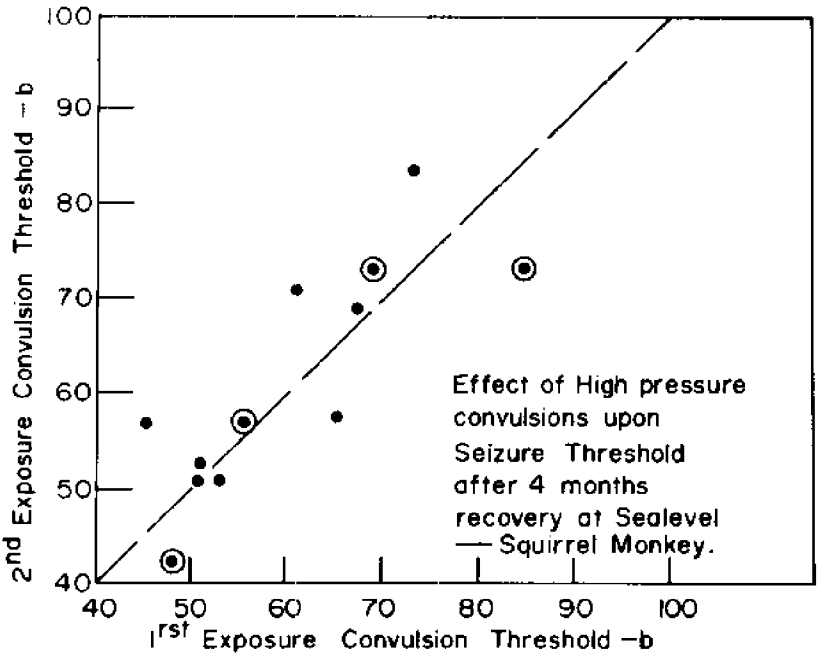

Figure 2. Reproducibility of seizure thresholds in squirrel monkeys compressed in helium/oxygen (...) or in hydrogen/oxygen (...) atmospheres (PO_2 0.5 atm., $dp/_{dt}$ 24 atm/hr.) Pressures recorded in bar. (Brauer, R. W., M. Jordan, R. O. Way - to be published).

The second illustration (Fig. 2) shows the relation between the convulsion thresholds for each of a group of squirrel monkeys who were tested twice, with an interval for recovery of at least four months between first and second exposures. These data are intended to illustrate two points: In the first place, they show that within a given species, susceptibility to the high pressure neurological syndrome may vary quite widely. In the second place they reveal that, despite such variation, for any individual animal the convulsion threshold is quite stable, varying on the average by less than 5 atm. between first and second seizure. Since this figure was prepared, we have had an opportunity to examine some of these animals a third time after an interval of well over a year, and we were impressed by the observation that the three animals in that series showed convulsion thresholds within 2.5, 4.0 and 3.5 atm. of the threshold seen on the second exposure. Both the range of variability within a species and the stability of the threshold are features of the syndrome worth bearing in mind when studies are performed with marine species.

Figure 3, finally, provides data concerning the comparative physiology of the high pressure neurological syndrome as seen in vertebrates. As observed experimentally in the monkeys and mice we have studied most closely, we can distinguish three phases of the high pressure neurological syndrome, coming on at progressively greater pressures: the initial phase is characterized by tremor and motor disturbances, usually first in evidence in connection with movements of head and fore-limbs, but gradually extending to other parts of the anatomy. So far as we can tell this phase does not have any recognizable representation in the electroencephalogram. At somewhat

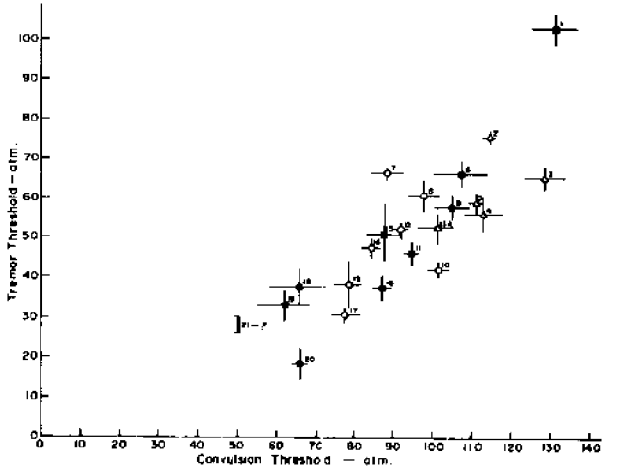

Figure 3. Correlation of threshold pressures for two phases of the high pressure neurological syndrome in 20 species of vertebrates.

(Brauer, R. W., S. Goldman, R. Beaver, M. Jordan, and R. O. Way - to be published)

KEY TO SPECIES

CLASS	ORDER	SPECIES	NO. in figure
Pisces		*Achirus fasciatus*	1
		Paralichthys dentatus	6
		Anguilla rostrata	9
		Symphurus palguisa	13
Reptilia		*Thamnoptulis sistalis*	3
		Anolis carolinensis	4
Aves		*Serinus canarius*	2
		Ectopistes carolinensis	5
		Gallus domesticus	13A
Mammalia	Insectivora	*Erinaceus europaeus*	23*
	Marsuplialia	*Didelphys virginiana*	15
	Edentata	*Dasypus novemcinctus*	8
	Rodentia	*Epimys rattus* S.D.	7
		Mus musculus CE-1	12
		Mus musculus AJ	14
		Cavia porcellus	17
	Lagomorpha	*Oryctolagus cuniculus*	10
	Carnivora	*Procyon lotor*	11
	Primates	*Tupaia tupaia*	20
		Saimiri sciureus	19
		Macaca mulatta	16
		Papio papio	18
		Homo sapiens	21

*Number 23 - location overlaps Number 13-A

higher pressures, electroencephalographic changes are evidenced by isolated spikes and occasional brief localized bursts of high voltage/high frequency discharges. Eventually, these changes are superseded by generalized paroxysmal activity of the electroencephalogram associated with violent motor seizures of different kinds, i.e. by frank convulsive seizures resembling any of several types of epilepsy. The pressure at which the first such seizure occurs is the one we term the convulsion threshold. It is possible to abort this convulsion phase pharmacologically. When this is done, animals can be carried to considerably higher pressures, but finally succumb to a complex of effects eventually associated with circulatory failure. This third phase is only now beginning to be explored by us, and will not concern us at this moment, although I suspect that it bears close relation to the zone in which invertebrate physiologists observe mortality

among their animals. There is some reason for suspecting that the first and second phases of this syndrome reflect the effects of high hydrostatic pressures upon two different sites, either within the central nervous system or, possibly in the case of the tremors, even peripherally. Thus, the relation of the pressures eliciting phase one and phase two, respectively, should tell us something about the pattern of development of the high pressure neurological syndrome, and it is this comparison which we chose as a basis for representing and comparing data on a wide range of vertebrate species. Figure 3 illustrates these relations, in the form of a plot of the mean threshold pressure for tremors against the mean threshold pressure for convulsions in a series of 20 species. These two values are found to be closely correlated ($\rho > 0.85$) for the entire series of species tested, suggesting that indeed the syndrome we are observing is much the same across the entire range of taxa tested. In the case of air-breathing vertebrates, this range extends from reptiles through the higher primates. Of particular interest to the present conference is the fact that four species of fish (Nos. 1, 6, 9, 13 of Fig. 3) subjected to compression at the same rate as the air-breathing species, but tested in the high pressure aquarium system to be described later on in this volume, show relations between the onset of mild motor disturbances and profound generalized convulsive seizures which fit exceedingly well into the pattern set by air-breathing species.

Taken together, these data suggest that at the rate of compression chosen for the series of experiments underlying Figure 3 (24 atm/hr) the upper limits of pressure attainable by surface-reared vertebrates in full possession of their powers are of the order of 100 atm., or, if you prefer, that the greatest depths to which these animals can be expected to penetrate rapidly are of the order of 1,000 m. In other experiments, we have explored the question as to whether this range could be extended by slowing the compression rate. To date, we have explored the range from 50 atm/hr to 0.5 atm/hr. Over this range, the onset of mild motor disturbances seems to vary relatively little, at least in the monkeys and mice for which we have the greatest amount of data. By contrast, the convulsion thresholds are raised somewhat: For both species tested, the 100-fold reduction in compression rate gives rise to approximately 20% increase in the convulsion threshold. While this result is of considerable theoretical interest and of some application in relation to human diving practice, in the present context it does not markedly affect our conclusions. Perhaps these could be re-phrased to suggest that instead of 1,000-m vertical range, surface dwelling species may enjoy a range which potentially might reach to 1200 to 1400 m. This is still a relatively small fraction of the total water column with which we are concerned in the ocean. It is interesting to note that experimental work with a variety of shallow water invertebrate planktonic species suggests that the range accessible to these is rather similar to that outlined above for vertebrate species.

From a biological point of view these observations raise the question whether this kind of effect may not underlie in considerable part the vertical distribution of species in the world ocean at any one point, which had suggested the existence of critical limits to the vertical range of excursion open to individual animals. Such ranges often seem to fall within the 1,000-m bounds suggested above for sufrace dwelling species. Such a figure would be quite compatible, for instance, with the known diurnal excursions of certain planktonic species which rise to the surface some time during the 24-hour cycle, only to return to deeper waters before the day is over. In general these species seem to keep themselves well within the 1,000 to 1,200-m limits here suggested. The same general figure is quite compatible with data presented by Russian observers concerning vertical distribution of most trench fauna.

Thus, workers who have been studying fauna from less than 1,200-m depth stand an excellent chance of having brought up speciments of unimpaired vitality, or specimens whose vitality would be limited solely by considerations such as water temperature, and perhaps by some chemical factors unrelated to pressure (swim bladder fish and the barotrauma to which they are subject form an obvious exception to this statement). However, if one attempts to deal with the fauna of deeper waters, the data available to date strongly suggest that such species are likely to undergo severe stress due to pressure changes in transit to the surface from depths exceeding 1,500 or 1,800m, so that it seems safe to assume a priori that their physiological responses will have been profoundly altered and their survival endangered when they reach the surface. If this view is correct, then it leads immediately to the conclusion advanced at the beginning of this presentation, viz. that experimental work with deep sea fauna presumes the ability to control and to manipulate hydrostatic pressure over the full range of values encountered in the world ocean.

It is perhaps convenient in this context to visualize a spectrum of pressures in the biosphere which might be sketched out as follows:

The range between 0.1 and 1 atm. tends to be dominated by the effects of hypoxia, and toward the low pressure end by problems imposed by water vapor tensions.

The range from 1 to 10 atm. characterizes the uppermost reaches of the oceans, readily accessible to man by present diving techniques, and readily accessible to diving mammals and of course to a wide variety of other surface dwelling marine species. Problems encountered in this zone include those of barotrauma, in swim bladder fish as well as in air-breathing species, and toward the lower end of this zone there are problems of inert gas narcosis in the air-breathing species. Among marine species, there is a suggestion that sensing devices exist which are capable of perceiving pressures in this range, and of guiding the orientation of

many species in this upper water column with great nicety.

The next portion of the spectrum, between 10 and 100 atm. is characterized by the onset and development of the high pressure neurological syndrome in vertebrates, and of its counterpart in many invertebrates.

Finally, the limits of the biosphere are reached in the next segment of the pressure spectrum, between 100 and 1,000 atm. In this zone there are extensive changes in the structure of sub-cellular elements, as well as changes in many fundamental biochemical and cytogenetic reactions in species adapted to surface or to shallow water habitats.

Beyond this realm the pressure spectrum extends to pressures of geophysical import, and changes of biochemical interest include only changes in structure of even relatively modest sized macromolecules over the first few thousand atmospheres of pressure.

Viewed against the background of such a spectrum, the deliberations of this conference, then, will have to deal with the feasibility, and with the degree of desirability, of conducting biological research in the bottom segment of the pressure spectrum of the biosphere, i.e. the segment between 100 and 1,000 atm. If this conference concurs that it is indeed incumbent upon us to further the study of the biology of abyssal and hadal fauna, then it seems to me that the biological data already available do not allow us to evade the task of devising methods for carrying out a large proportion of the operations of the biochemist, the physiologist, the histologist, and perhaps of the student of animal behavior in environments, the most unique single characteristic of which will have to be maintenance of hydrostatic pressures between 100 and 1,000 atmospheres. Whether these values are to be achieved by building hard envelopes to enclose our experimental systems, or whether they could possibly be achieved by operating in the ocean itself using the abyss as our pressure vessel, is one of the considerations with which we shall presently be concerned in the course of our deliberations.

I venture to call your attention, however, to one characteristic of the observations already before us, and this is the relative ease with which incisive physiological data can be obtained when one turns from microscopically small animals to animals that measure at least a few centimeters in length and that thus displace at least 10 or 20 cc of water. Whether it is in the matter of anatomical localization of a particular manipulation, or the ability to make injections or to collect samples, or the feasibility of obtaining electrophysiological measurements—in each case, size is an important factor. To allow the size of test organisms to fall below a modest minimum is certain to involve the investigator in a host of nearly insuperable technical and interpretive difficulties. It is my personal conclusion that this characteristic of our subject will make it mandatory to provide, in whatever systems one will develop eventually, such dimensions in all essential parts

that they can accommodate animals equal to or greater than that minimum size which is compatible with effective experimentation. The devices and results reported in other contributions to this volume suggest that the basic problems of collecting and of manipulating specimens of microscopic size in the deep ocean environment, and of preserving and manipulating them under pressures close to those of the environment in which they were caught, is either solved already, or is amenable to solution by means well within react of present technology. The situation is quite otherwise for macrofauna where problems of capture, retrieval, transfer, and manipulation under ambient pressure all remain to be solved. I submit that, if we agree that the experimental biology of abyssal and hadal fauna is to be developed, then the matter of developing the requisite systems with a view to handling macrofauna will be one of our early and pressing tasks.

In the preceding paragraphs I have emphasized the role of pressure because I think that of the parameters with which we have to deal, this is the only one which imposes severe and novel restrictions upon otherwise conventional methods of operation. From the point of view of the biologist, it is of course evident that pressure is merely one among many parameters that need to be controlled. Systems suitable for the purpose we are envisioning here clearly must be capable of assuring reproducible and reliable controlled environmental conditions, and the factors to be controlled must include besides hydrostatic pressure, the temperature, salinity, oxygen tension, pH, and activity of specific ions, to name merely a few of the more prominent ones.

If we accept the need for high pressure systems, we should realize that measurement of many of these parameters will no longer be trivial or well resolved problems. I am sure that others at this conference will have much to say about solution structure, electrolyte behavior, the properties of electrodes, and the activities of dissolved non-electrolytes, such as oxygen, in systems where pressures begin to approach 1,000 atm. On the experimental side, any systems one may develop will have to accommodate the urgent need for data on cell replication and the related cytokinetic changes, and for studies of growth and differentiation in deep sea forms at various pressures. They will have to accommodate whatever manipulations will be required to provide materials for the study of the histology and cytology of these animals by modern dynamic methods; they will have to provide means for determining metabolic balances, rates of metabolism, and rates of exchange of various body pools with the surrounding media. They will have to provide for a variety of physiological measurements either in intact specimens or in isolated tissues; and, finally, sooner or later they will have to provide for manipulations of the environment as a whole in connection with studies of animal behavior under controlled conditions. If it is realized that all of these manipulations need to be carried out inside heavy

and dense pressure-retaining envelopes, and that the alternative is to carry on similar manipulations several kilometers below the ocean surface—then even the brief and incomplete list of requirements given above should serve to warn us of the complexity of the task we take upon ourselves if we once make the determination that a serious study of the experimental biology of deep sea fauna is to be undertaken.

This very briefly is the conceptual background against which your Organizing Committee developed the workshop conference which gave rise to the monograph before you. We recognized that the transition from observation to experimentation imposes upon any such undertaking the need for multidisciplinary deliberations and eventual action. Once the principles enunciated above are accepted, it follows immediately that successful pursuit of this kind of research presumes a close partnership between all of the biological specialists, and students of such basic scientific areas as, especially, physical chemistry. Beyond this there is a pressing need to include the engineering community in our working partnership. Furthermore, it is evident that no amount of experimental insight, and no amount of brilliant engineering, would serve our purpose without guidance by those who have made operations on the ocean their life work. Thus, at least three major scientific communities will have to collaborate if the venture we are contemplating here is to be brought to a successful conclusion.

Just as it appeared to us evident that the judgments that needed to be made, and the scientific community that would need to be called upon to put them into effect, would have to have an interdisciplinary character, so it appeared clear to us that, if progress were to be made in meeting the scientific needs anticipated, the techniques that would need to be developed would call for a systems approach rather than for the development of discrete pieces of equipment. Capture, retrieval, maintenance, and animal husbandry compartments, experimental assemblies, feedback manipulators, and sensor systems, will beyond doubt have to be integrated in the end to provide an assembly at once capable of performing the tasks we began to recognize, and possessed of the flexibility to respond readily to the whims of the experimenter as he may probe the complex questions characteristic of this field.

The conference upon which the present volume is based was assembled and structured to provide as far as possible an assessment of needs and opportunities, and to review the present state of the art insofar as it concerns deep ocean simulators for biological use. To describe the overall field, we have adopted a term suggested by Dr. ZoBell—Barobiology—and it is at his suggestion that this term appears at the head of this volume. In planning for the deliberations of this conference, every attempt was made to avoid arguing from a preconceived position. Thus, we invited speakers to discuss the pros and cons which might determine the relative priorities to be given to

field exploration, to laboratory simulation, and to the field of inquiry as a whole within the framework of the general structure of biological and oceanographical inquiry. We made arrangements to provide for discussion of alternative methods of approaching the problems that we might define so that one might be able to weigh the merits of the high pressure aquarium systems against experiments in situ conducted from submersibles or by other means. We invited discussion of the problem of gathering valid chemical information in the high pressure environments we anticipated having to cope with. It is only at this point that we anticipated that these deliberations might lead us indeed to seek to develop simulator systems for which the name high pressure aquaria might be in order. Accordingly, we next invited review of two topics dealing specifically with the technology of these devices.

In the first place, we invited distinguished engineers to review for us problems of structure, selection of materials, safety considerations, and problems of corrosion that we might expect to encounter. That part of the conference was designed to give investigators likely to become involved in such an effort access not only to the thinking of some accomplished designers and engineers, but also to the rather specialized and oddly distributed body of scientific, trade, and government literature in which pertinent information of this type tends to be laid down. Finally, we invited those whom we knew to be active in the design and utilization of high pressure aquarium systems or related devices to present to us their experiences—and perhaps to let us in on their thinking and their plans for further development. Throughout, discussions of the conference were recorded faithfully, and they form a part of this volume. They have deliberately been edited lightly because on many occasions the tenor of the discussion may prove quite as pertinent as a means of conveying the attitudes of the speakers as the specific knowledge contained in a particular comment.

An attempt was made to invite to this conference as far as possible all those actively engaged in research on the experimental biology of the deep sea or in fields closely related thereto. Some omissions are due to inability of certain colleagues to accept our invitation. These we regret. Some are due to oversight or ignorance on our part. For these unintentional omissions we apologize. The editor has taken upon himself the task of at least partially rectifying some such deficiences by inviting comment from several workers who did not participate in the actual sessions last spring but whose work seemed directly pertinent to the argument before us.

It is the hope of the Organizing Committee that this conference and the present monograph resulting from it may serve as a report on the State of the Art in an important and exciting field of research, and that it may facilitate the work of those already engaged in this field of ocean and biological science and perhaps entice others to join us in a challenging quest.

INTRODUCTORY REMARKS

By
Claude E. ZoBell
Scripps Institution of Oceanography

Fellow barobiologists and friends. It is a great thrill and pleasure to be here to participate in these exciting sessions. I am looking forward to listening and learning. The pleasure is not from standing here behind this lectern but rather in being with so many friends and big names in barobiology. This, coupled with the cordial reception at the Inn last night, makes me feel like it is a homecoming.

The conference holds high promise of yielding big dividends. These will come from the exchange of ideas and an examination of pertinent problems, particularly problems of high pressure methodology and instrumentation, a field in which our host excells.

The recapitulation of information during the week we are together will be important, but more importantly we should be placing in proper perspective the "state of the art" and unsolved problems of high pressure aquarium systems. What are the significant parameters, what are the techniques, what kinds of hardware are available or required to solve fundamental and basic problems concerning life at high pressure in either aquarium systems or the deep sea? It is our responsibility to assume the leadership in solving these problems and in interesting others in barobiology.

It is quite appropriate that the first paper on the program should be concerned with biochemical aspects of barobiology, with particular reference to microbial cells. Whether dealing with single cells or the largest multicellular organisms, the crucial effects of high pressure appear to be primarily on either cells or their enzymes. This generalization also applies to organisms having gas cavities, assuming that the latter can withstand compression or can be filled with fluids containing essential dissolved gases. Whatever happens to cells or their enzymes can be expected to affect the well-being of the whole organism whether it be a bacterial cell or a diving mammal as big as a whale.

Whether an organism remains metabolically active at increased pressure depends upon the continuance of biochemical reactions. If essential biochemical or physiological reactions are irreversibly stopped by pressure, or by any other kind of adverse condition, the organism ceases to be alive. Also important is the effect of pressure on the rates of biochemical or physiological reactions. Temperature, pH, Eh or redox potential, osmotic pressure, hydrodynamic pressure, and chemical composition of the medium are among the many environmental factors which work synergistically or antagonistically with hydrostatic pressure in affecting physiological or biochemical reaction rates as well as the survival of organisms.

So important is temperature on pressure effects, I hope that throughout these meetings the speakers will stipulate the temperature at which the observations were made. In your otherwise excellent presentation this morning, Dr. Brauer, I was somewhat at a loss to interpret some of your data on the effects of high pressure, because it was not clear to me whether the phenomena occurred at 3°C, the approximate temperature of most high-pressure environments in the sea, at 37°C, the body temperature of many mammals, or at some other temperature. At such sessions as these, as well as in published papers, all too often the temperature remains a secret.

Also important in pressure studies are such other environmental parameters as osmotic pressure, ionic strength, partial pressure of gases, pH, rate of compression or decompression, duration of experiment, and other conditions. Most of these may be understood.

I was interested in your remark, Dr. Brauer, that most of us understand English. In the field of barobiology, I fear, we are still in the Babylonian stage where certain words mean many things to many people. To most barobiologists, "pressure" means more than 1 atmosphere, but some of us have been concerned with the survival of organisms at very low pressures, say 10^{-7} torr. More ambiguous is the term "high pressure." At the reception last night, I talked with diving physiologists who kept saying "high pressure" when they meant 10 or 20 atmospheres. On the *Galathea* Deep-Sea Expedition, "high pressure" was synonymous with hadal, that is 700 atm. or more, the hydrostatic pressure at 7,000 m or more. Many enzymologists concerned with pressure effects differentiate between the effects of "moderate pressure," that is up to 1000 or 2000 atm., and "high pressure," 2000 to 10,000 atm. To the geophysicist "high pressure" may mean millions of bars, the pressure at the center of the earth being 3.6×10^6 bars.

The multiplicity of units used by various workers to express hydrostatic pressure is almost as confusing as the multiplicity of connotations of the adjectives, moderate, high, deep-sea, and so forth. In the slides which we have seen this morning, there appeared both bars and atmospheres. Seeing German and French friends in the audience, I predict that we might be seeing and hearing kilograms per square centimeter (kg/cm^2). As you know, the Metric Convention, to which 33 countries now subscribe, adopted newtons per square meter as the standard unit for expressing hydrostatic pressure.

Conversion tables for 15 other pressure units, as well as other units adopted by the Metric Convention, may be found, for instance, in

"Documentia Geigy, International Tables," (published by Geigy Chemical Corporation, Ardsley, N.Y., 1962 edition, 778 pages). Thus:

$$1.0\ N/m^2 = 1 \times 10^{-5} \text{ bars}$$
$$\text{or} \quad = 1.01972 \times 10^{-5} \text{ kg/cm}^2$$
$$\text{or} \quad = 0.986923 \times 10^{-5} \text{ atm}$$
$$\text{or} \quad = 1.450377 \times 10^{-4} \text{ psi}$$
$$\text{or} \quad = 7.500617 \times 10^{-3} \text{ mm Hg}$$
$$\text{or} \quad = 3.345619 \times 10^{-4} \text{ ft } H_2O$$

Expressing pressure in terms of newtons per square meter seems odd to most of us who are not accustomed to seeing or thinking N m^{-2}. Having been brought up on atmospheres myself, I have to consult conversion tables. I consider the use of atmospheres as a justifiable pressure unit for barobiologists and ecologists, because 1.000 atm. is defined as the absolute pressure at sea level for dry air at latitude 45° at 15° Celsius. Moreover, hydrostatic pressure conveniently increases with water depth by very nearly 1.00 atm. per 10 m, there being some deviation with depth (owing to the compressibility of water), temperature, salinity, and latitude. I dislike pounds per square inch, because this unit is neither metric nor round. Furthermore, most biologists fail to specify whether they mean psig (gauge) or psia (absolute), although they may report the pressure to the first or second decimal place.

I hope there will be some opportunity to discuss units of measurements during this conference. We as leaders in the field of barobiology should set a good example in the use of internationally accepted units.

By way of reiterating some of the "ground rules" outlined by Brauer in his opening remarks, permit me to express the hope that all of you during this session and subsequent sessions will feel free to enter into the discussion. Although, we have named discussants for all of the papers, others in the audience are urged to ask questions or to present information on the subject under consideration.

CHAPTER 1

BIOGEOCHEMISTRY OF HARD TISSUES, THEIR DEPTH AND POSSIBLE PRESSURE RELATIONSHIPS

Heinz A. Lowenstam*

Species of 9 groups of animals have been recovered from the deepest oceanic trenches (1). Therefore, hydrostatic pressures of 1000 to nearly 1100 atm. are no hindrance in nature to the existence of cellular to tissue-grade animals. Yet we know from experimental studies that high ambient pressure affects biochemical processes in various ways. The experimental pressure studies have been concerned so far with cell chemistry, cell divisions, and metabolic processes (2).

Studies of the mineral phases of skeletal carbonates from marine organisms have shown that the aragonite-calcite ratio in species of 5 invertebrate classes are affected by environmental temperatures and in one case by salinity changes also (3). It has been shown further that microelemental concentration, in particular the Sr and Mg contents of skeletal carbonates are, aside from the biochemistry of the species and the crystal chemical effects of the carbonate polymorphs, variously affected by temperature, salinity and the water chemistry (4). The O^{18} contents of skeletal carbonates laid down in isotopic equilibrium with the O^{18} contents of the water are determined by the O^{18} contents of the water and the temperature at which the carbonate was precipitated (5). Nearly all the skeletal carbonates studied to date are based on shallow water species. Hence, the question whether hydrostatic pressure may also affect the mineral phases or the trace element chemistry of hard tissue carbonates did not arise. The depth distribution of benthic animal groups in the oceans indicates that 15 classes of invertebrates which precipitate carbonate in shallower waters range to depths between 6000 to 7000 m and of these, 2 classes are represented at depths greater than 10,000 m (6). Therefore, there is the possibility that the mineral phases and the chemistry of hard tissue carbonates may provide information on pressure effects on the biochemistry of abyssal and hadal species. In the present exploratory phase of the inquiry into this possibility, one would like to know if there are any systematic changes in the biogeochemistry of skeletal carbonates with increase in depth which are distinct from changes induced by other ecologic factors, in particular by temperature.

Non-carbonate minerals are also reported from hard tissue precipitates of marine organisms. This is particularly true for silica, which in the form of opal ($SiO_2.nH_2O$) has been recognized as a hard tissue precipitate in several

* Contribution No. 2010 from the Division of Geological and Planetary Sciences, California Institute of Technology

algal classes and, among marine animals, in the Protozoa, Porifera, and Mollusca (7). Recent efforts to systematically identify hard tissue minerals with the aid of modern techniques have confirmed previously poorly founded suggestions of other non-carbonate minerals and have added a number of others, previously thought to be of solely inorganic origin (8). Figure 4 shows the different minerals which are known at present from hard tissues and the phyla relations of the species in which they have been found. Published identifications of the non-carbonate minerals are based, as in the carbonates, largely on samples of shoal water species. Of the 13 non-carbonate minerals which have been identified from hard tissues of littoral to bathyal species, 9 minerals are found in groups of invertebrates which range to depths greater than 6000 meters. Therefore, non-carbonate minerals may be equally important source areas of information in the search for possible pressure effects on mineralized tissues.

We are in the process of investigating the minerals precipitated by deep water species. It has been shown earlier (Figure 4) that of the 16 minerals currently known from hard tissues of marine organisms, 9 minerals have been found in species of 2 to as many as 16 phyla. Where this is the case,

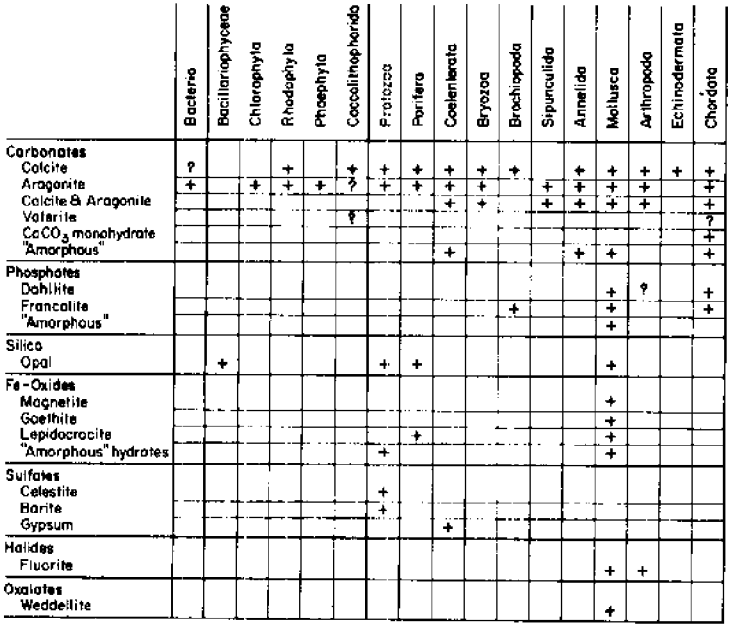

Figure 4. Distribution of skeletal mineral species according to phylum.

we have concentrated our efforts on investigating the mineralogy of the hard tissues of that taxonomic group which has the greatest known depth distribution, to determine whether with increase in depth the mineral precipitates are the same or whether they undergo changes. The maximum depths of the different minerals so far determined are: 6200 m for aragonite (Aplacophora, Monoplacophora, Gastropoda, Bivalvia), 8900 m for calcite (Holothuroidea), 6200 m for opal (Hyalospongia) and 3500 m for dahllite (Pisces), amorphous phosphate minerals (Gastropoda), magnetite (Polyplacophora), goethite (Gastropoda), fluorite (Gastropoda), and weddellite (Gastropoda). According to Dr. Tendal (personal communication), barite (Xenophyophora) has been traced by him to a depth of 6700 m, and Dr. Levi has informed me that opal (siliceous Porifera) has been identified in samples to a depth of 8840 m. Lepidocrocite, (Porifera, Polyplacophora), celestite (Protozoa), and gypsum (Schyphozoa) have been located so far only in species from depths of less than 1000 m.

Figure 5 shows by a solid line the maximum depths which have been substantiated for most of the known minerals. The accompanying broken lines indicate the known depth distributions of the taxonomic groups in which these minerals have been located in part of or in the entire

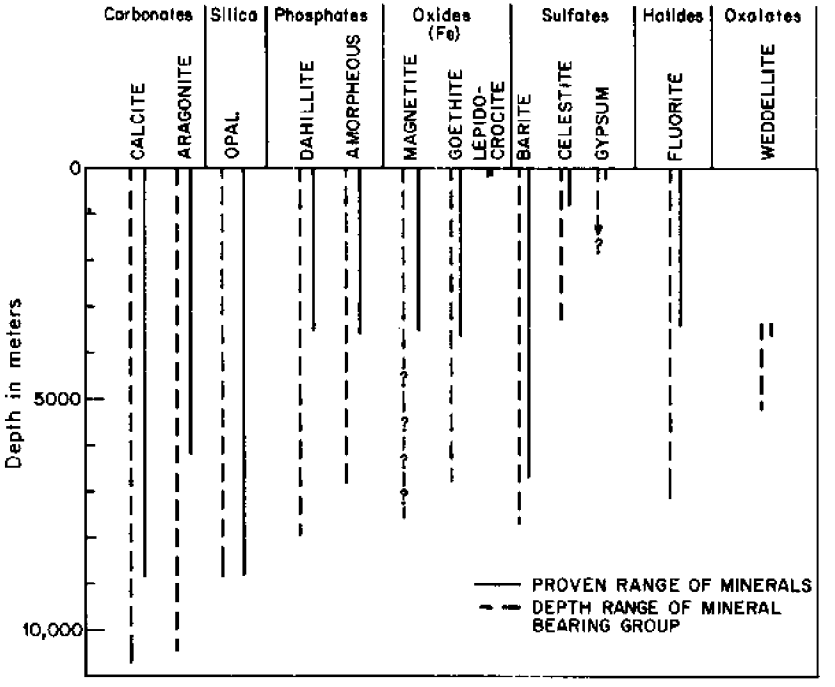

Figure 5. Depth distribution of proven mineral species and depth ranges of mineral bearing groups.

bathymetric range. The graph shows that for almost all eurybathic groups which precipitate a particular mineral in the shallower part of their depth range, data on the mineralogy for deeper water species are lacking. Opal is the only exception, indicating that in this case the mineralogy does not change throughout the known depth range of silica precipitating sponges. In the case of the other minerals, there is at least fair documentation for the greater part of the depth range of calcite precipitation by Holothuroids and for barite by the Xenophyophorans, again indicating no change in hard part mineralogy with increase in depth occupation. However, for most of the minerals which are precipitated by eurybathic groups of animals with a maximum depth range of 6800 m to as much as about 10,600 m, the mineralogy of samples from more than 3500 m remains to be determrmined.

Considering the limited data on the depth coverage of all identified minerals, there is with one possible exception no indication of a change in hard part mineralogy in eurybathic animals. An indication of a possible change in mineralogy with increase in depth might be inferred from species of an opistobranch gastropod genus, which precipitate fluorite and an amorphous phosphate mineral at depths of less than 1000 m and weddellite plus the same amorphous phosphate mineral at depths greater than 3000 m (9). Accepting current taxonomic assignment of the species examined so far, a mineralogic change with increase in depth is indicated. However, anatomical studies of the samples by the author raise questions concerning their congeneric relationship and hence of the inferred mineralogic changes.

The need for additional data is clearly indicated to determine whether depth-related changes in hard part minerals exist, and if so, whether these can be related to a pressure effect.

There is still much discussion on where to place the upper depth limit of the hadal or ultra-abyssal zone in biologic terms (10). If one limits the analyses to deep-sea animals with hard tissues, it appears that the greatest reduction in inferred diversity of mineral precipitates occurs between 6800 m and 8000 m (Figure 5). If substantiated by data on the minerals at the maximum depth for the groups involved, this criterion may be useful for defining the upper depth boundary of the ultra-abyssal bio-zone.

Let us consider next the relationships of the $SrCO_3$ and $MgCO_3$ contents of skeletal carbonates from deeper-water invertebrates as compared to those of related species from shallow waters. It seemed desirable to select groups of organisms which always precipitate the same mineral phase under varying environmental conditions in order to cancel the effect of the crystal chemistry on the Sr and Mg uptake in the samples studied. Also, to avoid possible differences in the concentration levels of the two microelements due to differences in the concentration levels of the two microelements due to differences in biochemical controls, we attempted to select wherever possible, samples of conspecific individuals; and where this was not

possible, at least of congeneric species. We have obtained some data on samples with these qualifications from Polyplacophorans, articulate brachiopods and holothurians. Individuals of 4 subspecies of the eurybathial holothurian species, *Elpidia glacialis*, recovered by the *Ingolf* and *Galathea* expeditions, encompass the greatest depth range of conspecific samples which we have obtained to date. These samples range from 610 m to between 8780 and 8830 m.

In the following discussion, we wish to consider principally the results of our study on the $MgCO_3$ and $SrCO_3$ contents of this eurybathial species and to compare them with those of 3 shallow water species of the holothurian genus *Psolus*. Brief reference will be made further to the data obtained on the $MgCO_3$ contents of the deeper water holothurian species *Scotoplanes globosa* from *Galathea* stations recovered between 2640 and 6620 to 6730 m and of *Oneirophonta mutabilis* from 3570 to between 5850 and 5900 m. The hard tissue minerals in the spicules of all these species were determined by X-ray diffraction patterns to consist of the polymorph calcite.

Temperature is the major ecologic variable which affects the $SrCO_3$ and in particular the $MgCO_3$ contents of the polymorph calcite in hard tissue precipitates. Therefore, to assess the meaning of the $SrCO_3$ and $MgCO_3$ contents of the samples, these data are assessed in terms of their temperature and depth relationships.

Table 1 shows the $SrCO_3$ and $MgCO_3$ contents of the shallow water samples from species of *Psolus*, the data on the localities from which they were obtained, and the mean annual temperatures of their environments. Their $SrCO_3$ and $MgCO_3$ contents are shown to have a positive correlation with temperature. Within the narrow range of depths between 28 to 34 and 60 to 100 m, there is no indication of a correlation of the $SrCO_3$ and $MgCO_3$ contents in relation to depth.

Table 1

Species	Location	Depth (meters)	T°C (mean)	$MgCO_3$ mol %	$SrCO_3$ mol %
Psolus fabricii	off Point Barrow, Alaska	34-40	0°	9.2 ± .2 10.0 ± .1	.23 ± .01
Psolus chitinoides	off Brown Isl. San Juan Archip. Washington	40	9.1°	10.8 ± .2 11.3 ± .4 11.6 ± .1 11.6 ± .2	.27 ± .01 .26 ± .01
Psolus sp.	off Catalina Isl, Calif.	60-100	10-12°	12.0 ± .1 12.4 ± .1 12.6 ± .1	.30 ± .01

Figure 6 shows the $SrCO_3$ contents for samples of the subspecies of *Elpidia glacialis* plotted against temperature. When the experimental points for all subspecies are considered jointly there is an indication of a poorly defined negative correlation with temperature. The indicated relationship between $SrCO_3$ contents and temperature hinges on the experimental points for the Pacific subspecies, *theeli, sundensis, kermadecensis* and *solomonensis*, as defined by Hansen (11). The data for these samples, when considered by themselves, indicate a well-defined negative temperature correlation. However, the experimental points of the Atlantic subspecies, *glacialis*, show a large scatter with a vague suggestion of a positive temperature relationship, which though poorly defined, is in principle similar to that for the shallow water species of *Psolus*. This finding suggests that the $SrCO_3$ contents of the Atlantic subspecies *glacialis* show a response to temperature different from that of the subspecies from the Pacific. In Figure 7, the $SrCO_3$

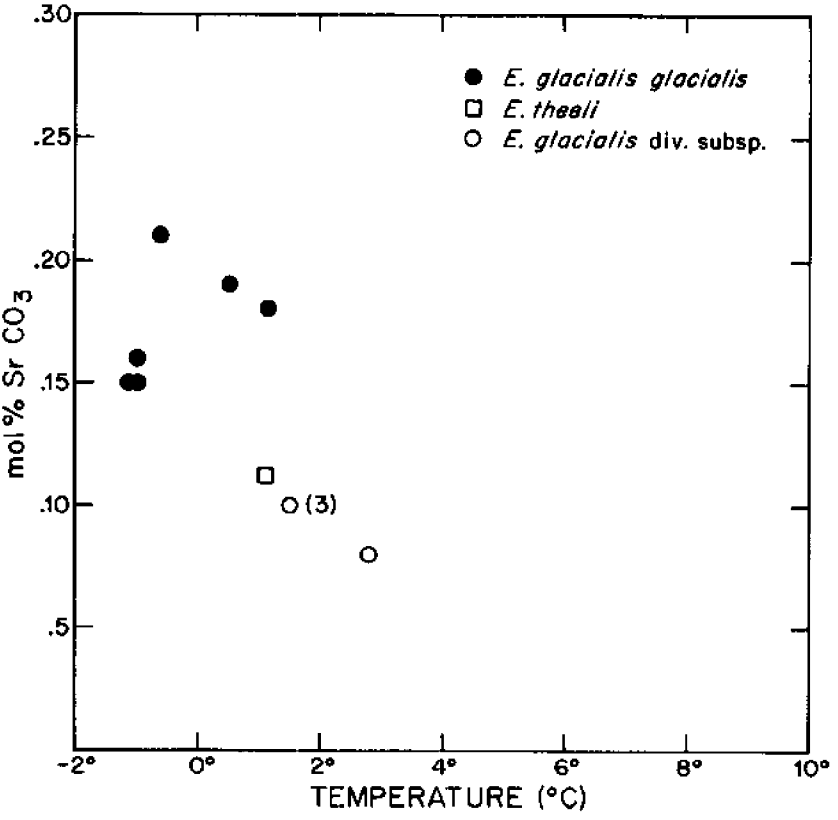

Figure 6. Relation of $SrCO_3$ contents to temperature in *Elpidia glacialis* spicules.

contents of the samples of all subspecies of *Elpidia glacialis* are plotted against the depths from which they were recovered. There is a fairly well-defined trend of decrease in $SrCO_3$ contents with increase in depth. This is due to the fact that the experimental points for the subspecies *glacialis* conform with the trend indicated for those of the other subspecies.

Figure 8 shows the relationship between the $MgCO_3$ samples and environmental temperatures for the same samples of all subspecies of *Elpidia glacialis*. When the experimental points for the samples of all subspecies are integrated, there is no correlation with temperature. However, if one considers the magnesian contents of the samples from the

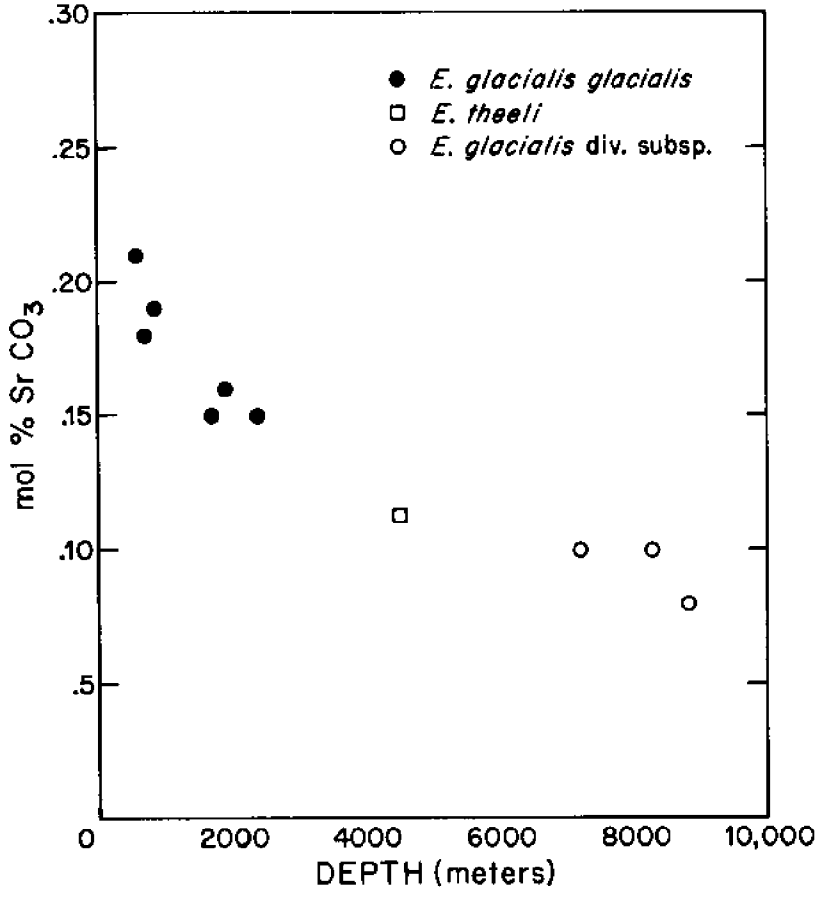

Figure 7. Relations of $SrCO_3$ contents to depth in *Elpidia glacialis* spicules.

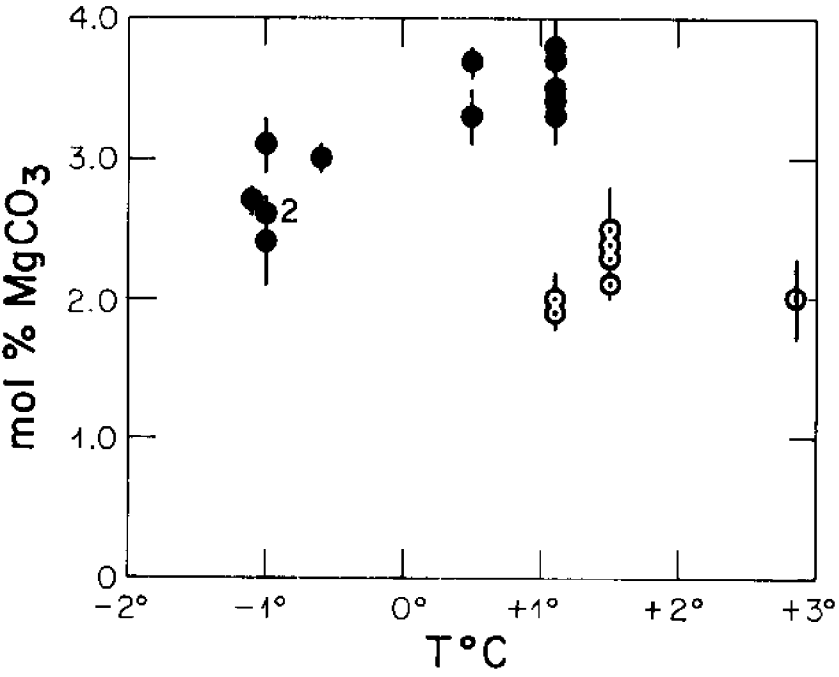

Figure 8. Relation of $MgCO_3$ contents to depth in *Elpidia glacialis* spicules.

Atlantic subspecies *glacialis* (solid circles) separately, one finds that there is a positive temperature correlation, similar to that for the shallow water samples of the *Psolus* species (Table 1), and for that matter, for most calcite-precipitating invertebrates (12). The experimental points for the Pacific subspecies of *Elpidia glacialis* (open circles) show for the same temperature a lower $MgCO_3$ content than for the subspecies *glacialis*. Moreover, there is no temperature relationship between the magnesian contents of the Pacific subspecies samples. Figure 9 shows a plot of the $MgCO_3$ contents of all *glacialis* subspecies against the depths of sample derivation. A negative correlation between $MgCO_3$ contents and increase in depth is indicated. The trend is less sharply defined as compared to the similar correlation between depth and $SrCO_3$ contents. Considered in detail, the negative correlation between $MgCO_3$ contents and depth for the samples of the subspecies *glacialis* can be explained by decrease in temperature with increase in depth. Comparison of the experimental points of the deeper water samples of the subspecies *solomonensis*, *kermadecensis*, and *sundensis* from the Pacific show on the other hand a negative correlation with depth as with temperature (Figure 8).

The experimental point for the Pacific subspecies *theeli* (2 mol % $MgCO_3$) from 4500 m seems to deviate noticeably from the relationship

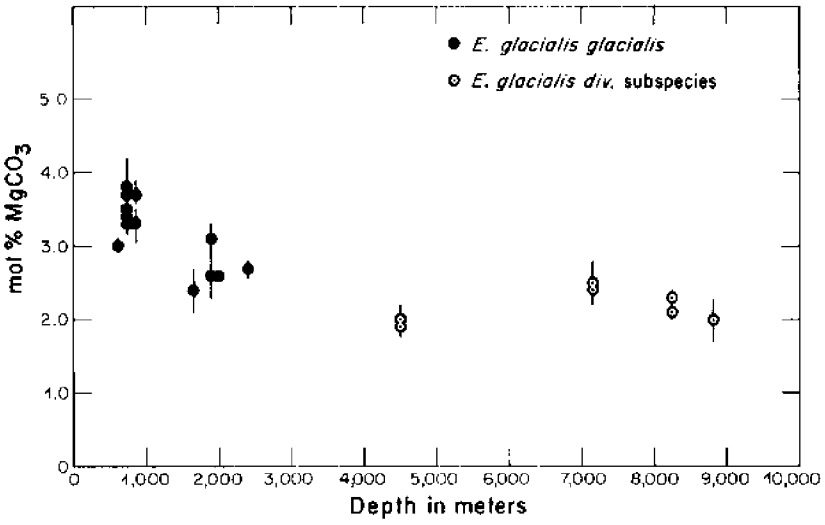

Figure 9. Relation of $MgCO_3$ contents to depth in *Elpidia glacialis* spicules.

between the $MgCO_3$ contents and temperature as well as depth indicated by the subspecies *glacialis* on the one hand and the interrelationships of the 3 other Pacific subspecies with reference to depth. B. Hansen (personal communication) has informed the writer that *theeli* may be a separate species instead of representing a subspecies of *glacialis*.

The present limited number of trace element data for the samples of *Elpidia glacialis* indicate 1.) that the ultra-abyssal subspecies, *solomonensis*, *kermadecensis* and *sundensis* are related biochemically more closely to each other than to the bathyal subspecies *glacialis* from the Atlantic and that *theeli* may be a distinct species; 2.) within the depth range covered by the samples of the subspecies *glacialis*, the $SrCO_3$ contents are more strongly controlled by a depth-related factor than by temperature, whereas their $MgCO_3$ content is primarily dependent on environmental temperatures; 3.) as to the 3 Pacific subspecies which come from 7160 to 8800 m., their $MgCO_3$ contents appear to be more clearly affected by an undefined depth factor than by temperature, whereas in their $SrCO_3$ contents the reverse, if not temperature control alone, seems to be in evidence.

Considering other deep water species of Holothuroidea, the 3 samples of Oneirophonta mutabilis indicate in the range between 3570 and 4500 m a temperature effect, whereas between 4500 and 5850 m a depth-related factor seems to control the $MgCO_3$ uptake of the samples. On the other hand, 4 samples of the $MgCO_3$ contents of the Scotoplanus globosa from 2600 m to between 6620 and 6730 m show neither a correlation with depth or

temperature. The possibility exists that the effects of these two factors may cancel each other. Details are presented elsewhere (13).

Our data on the trace element chemistry of non-carbonate minerals from hard tissues of deep water invertebrates are as yet too limited to indicate any possible existing controls by any ecologic factors.

The foregoing presentation shows that there is a questionable case of a depth-related change in non-carbonate minerals in the hard tissues of one group of deep water organisms. There are some indications of depth-related effects on the $SrCO_3$ and $MgCO_3$ contents of skeletal carbonates at depths in excess of 3000 to 4000 m, but the magnitude of the depth-related effects differs among species. It seems apparent that we need considerably more data on the different kinds of minerals and the trace constituents of the minerals of hard tissues of deep water animals, in particular of eurybathial species. These data should determine which groups of organisms show depth-related changes in the biogeochemistry of their mineralized tissues, how common they are, and the kinds of changes which occur in nature. The information derived from these studies will allow us to intelligently select the species which we wish to recover at ambient pressures for experimental growth studies at varying high pressures in the laboratory. Given this opportunity and keeping all variables constant except for pressure, it should be possible to determine whether depth-related changes in micro-elemental uptake, noted in samples from nature, are partially or entirely pressure-induced.

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Discussion Following Lowenstam Paper

BRAUER: Are there any anatomical differences in the hard tissues of congeneric species found at different depths?
LOWENSTAM: The amount of skeleton is about equal. We have some data on that, where the surface is rough. What we do find, and what still is something I do not understand, is that with one such sub-species which has now been considered a separate species, that is in the case of Elipidium theeli, the spicules stick out in the water. This also bothers me, because at that depth the calcium carbonate ought to be dissolved, which means that it has at least some kind of peculiar mechanism, maybe a monomolecular layer of some organic material.
(Question): Well, you know that they secrete this stuff through their skeletal spicules inside the cell, not toward the outside.
LOWENSTAM: Right, but these spicules go through the cell wall out into the surrounding water in this particular case.
(Question): The cell wall or the body wall?
LOWENSTAM: Body wall. The state of the specimens hardly warrants more detailed statements.
HESSLER: You've done most of the work here on holothurians, and I think it's important to emphasize the fact that holothurians share with most of the deep water echinoderms some very vexing problems which you have alluded to. I'd like to emphasize them. Amongst all the deep-sea creatures, they show the greatest tendencies toward being eurybathial, toward being cosmopolitan, toward exhibiting no diversity within any one community. I don't know what the reason is for this, but they are in a sense very different from most of the other typical deep sea forms in these three characteristics. Now it could be that the systematists really don't have decent specimens to work with, as some of your data might indicate. It may suggest that there's something very basically different about these echinoderms that prefer the deep sea. But from your point of view, I think it would be very interesting to look at some of the other phyla, such as the mollusks, for some of the characters that you're considering. Here you have species that have relatively limited distributions, and which have relatively limited depth distributions, and very high diversities within individual communities, and you may get far more specific answers---generalities more easy to deal with, with some of these other phyla.
LOWENSTAM: The problem is we have already difficulty between the species, even if you take congeneric species. "Depth" would have to be the

major factor to override the scatter which would result from the differences of the biochemical systems of the different individuals. To answer that we have done some biochemical studies in several tissues. For instance, in tritons, we have very good data on lactate and pyruvate down to 4,000 or 5,000 m and we do find that there is a difference. In this case, there are very distinct differences in the slope of the curve, with no overlap, between that for the deep and the surface water species. We have even included some forms from the Mediterraneran, and we have surface-dwelling forms there too. Also we have some evidence in brachiopods.

HESSLER: The reason why I mentioned some of these other groups is that they're very easy to get hold of and they show these other characteristics. You're working with tritons, well, this is one of the groups unusual in the deep sea. You don't find tritons every day—I've found one in my entire sampling career! Some of these other things you can get by the hundreds and fill every demand.

KALBER: Is there any possibility that the change in partial pressures of oxygen we mentioned earlier as associated with hydrostatic pressure will require any recalibration of the method of isotopic ratios for temperature?

LOWENSTAM: No, we have taken samples from depths to 8,000 m, determined the isotopic composition, and corrected for the deflation of the water and come out within 0.5°C of the temperature recorded at that depth. Within the pressure range we are dealing with, there couldn't be any effect, no fractionation unless it is biological. The echinoderms, for instance, do fractionate, in contrast to the mollusks and the brachiopods, where we have very carefully tested them. Is there really fractionation in the sense of a vital effect? I think the vital test probably is to go into the body fluids, and the biochemistry of the organic matrices, and see whether these have fractionated the isotopes. The carbonate then is simply a reflection, so to speak, of a different isotopic composition, and the mineral is laid down in isotopic equilibrium with that of the biochemical, but no that of the oceanic environment.

BRAUER: How about the microconditions under which these things are either preserved or laid down? I'm thinking specifically of a collection of bivalve shell, small bivalves that Dr. Filatova showed me, which, quite obviously, as you look at them, are quite intact around their margin, and quite as obviously have been badly chewed up around their umbo. Dr. Filatova interprets this by saying, "Well, these animals always sit, so to speak, with their face in the sand and their opposite side exposed, and that gets chewed", if I am paraphrasing her properly . This sounds a little bit optimistic to me.

LOWENSTAM: Do you know whether or not an organic shell is present?

BRAUER: No, this is what I'm asking you. Have we got any other data on these, and how would this be affected after death? How quickly does the organic cover that might protect these in fact disappear?

LOWENSTAM: Well, as to the first question, as far as I know, the umbo is frequently injured. So you have initially a chemically exposed situation. As to the second question, I would think it would depend on the kinds of bacteria infesting the area and the population sizes which use organic matrices as a substrate to determine the weight of the exposure. Now it appears that in some areas where you have pretty good carbonate saturation which extends much further in that you can keep the bottom water aerobic and saturated. I suppose you could keep organisms for a much longer period of time or indefinitely, as we are now finding out that sedimentation can take place at various depths. There is much coring going on right now of deep sea sediments, and they go back now as far back as Permian, I think. All you hear of are that foraminifera and such, and in post-Jurassic deposits, diatoms, remain. Now what happens to the megafauna? The population density in terms of food storage should be small, but the probability is that after so many cores we should have been provided with some tissue grade remnants at least.

HESSLER: In partial answer to that question, all I can say from my own observation in working at about 6,000 meters in the Central Pacific is that where you find a normal fauna of calcium carbonate-deposition macrofauna, you never find their dead remains. As soon as they die, they disappear. This observation also includes foraminifera. You find a normal assemblage of calcium carbonate-secreting forams at this depth, but they're never dead.

LOWENSTAM: I got interested in fluorite in micrococcoliths in Mesozoic sediments. I found that two individual specimens had been described, one complete from the Triassic, and a fragmentary one from the limestone of late Jurassic. I was rather amazed, and so I went to my friends who supplied our forams, and asked them for their unknowns. And within two days, I went from Pleistocene to Oligocene, and clear back to the middle Eocene, and they were definitely present. So maybe we have been looking for the wrong thing.

MENZIES: Well, it should be fairly easy to identify a clam!

LOWENSTAM: These are fragmentary!

(Unident): Well, in the Mediterranean cores, at least those we have at Woods Hole, and also those at Lamont, there are a considerable number of clams in those cores. These are piston cores.

EURYBATHIAL BENTHIC ORGANISMS*

By
Robert J. Menzies**
and
Robert Y. George**

Species of marine fauna having a wide range in depth distribution are classified as eurybathial. Most recorded instances of eurybathial species among the isopod Crustacea have been found to be incorrect leading to the suspicion that the previous records of other species in the animal kingdom likewise require correction or at least re-evaluation before one can fully accept the concept of eurybathial species.

A eurybathial species must at the same time be eurybaric, that is, capable of withstanding a wide hydrostatic pressure range as well as a wide range in depth.

Today it may be safely stated that the majority of known instances of eurybathality are restricted to regions of the ocean where temperature conditions are more or less isothermal from the uppermost limit of depth of a species to its lowermost limit. Thus eurybathials are most common in the Arctic, the Antarctic, and the Mediterranean, and on the ocean floor below the permanent thermocline.

Eurybathial Species of the Arctic

Following is a list of 19 species of Arctic invertebrates which have been claimed to show eurybathiality (Gorbunov, 1946). It should be emphasized that each species now requires re-examination taxonomically before these ranges can be verified.

	Depth Range m
Antinoella sarsi	22-3800
Apomatus globifer	20-1900
Eurycope hanseni	460-2750
Ilyarachna hirticeps	12-2600
Haploniscus bicuspis	610-2600
Macrostylis subinermis	1000-2000
Gnathia stygia	20-2550
Arrhis phyllonyx	23-1180
Thenea muricota	300-2363
Colossendeis proboscidea	41-425
Cirroteuthis mulleri	550-2340
Pecten frigidus	1000-2800

*Contribution No. 275 from the Department of Oceanography, Florida State University.
**Department of Oceanography, Florida State University, Tallahassee, Florida,32306, U.S.A.

Tylaster willei	590-2400
Solaster squamatus	90-1600
Elpidia glacialis	100-2800
Ophiopleura borealis	51-1542
Ophioscolex glacialis	300-2500
Raja hyperborea	200-2800
Rhodrichthys regina	1100-2340

Eurybathial Species of the Antarctic

The isothermal (-1.8°C) Antarctic continental margin located south of the convergence similarly shows nine reputed eurybathial species.

	Depth range m
Glyptonotus antarcticus	2-1000
Antarcturus adareanus	11-600
Declopoda australis	shelf-1000
Echinocumis hispida	50-1400
Phomachocrinus kerguelinsis	10-1080
Serolis neaera	280-4000
Serolis meridionalis	1266-2725
Pycnogonum rhinoceros	154-1115
Pycnogonum gaini	shelf-2495
Bathycrinus australis	2514-4636

Eurybathial Species of the Sea Bed Below the Thermocline

At least 14 isopod species have been reported to have a wide bathymetric range along the walls of trenches where the temperature conditions are isothermal. These trench isopod species with wide bathymetric range are listed below:

	Depth range m
Storthyngura benti	5340-7000
Storthyngura chelata	5345-6860
Mesosignum multidens	3470-6330
Haploniscus belyaevi	3102-5495
Ischnomesus andriashevi	4000-6560
Bathycopea ivanovi	2867-4070
Ilyarachna kermadecensis	4540-7000
Ilyarachna kussakini	5461-7230
Bathyopsurus nybelini	4400-7900
Stylomesus inermis	2450-6079
Haploniscus belyaevi	2960-5090
Haplomesus gigas	6156-8430

Eurycope acuticoxalis	4942-7587
Munneurycope murrayi	530-7800

Eurybathial Species of the Mediterranean

At least during the winter the Mediterranean is isothermal being near 13°C both at the surface and the bottom.

Two species of isopod and the holothurian Mesothuria intestinalis are suspected eurybathials. Others could be added.

	Depth range m
Desmosoma chelatum	600-2769
Ianirella bonnieri	1237-2769
Mesothuria intestinalis	20-2000

Eurybathial genera

While the number of known eurybathial benthic species is small, the number of eurybathial genera is higher. In the case of genera, their shallowest occurrence is near the poles but their shallowest occurrence near the tropics is quite deep. These are eurybathial, but it is obvious that they may be most easily collected for study in polar waters. We have called this phenomenon polar emergence of abyssal genera (Menzies, George and Rowe, 1968). The genera typify the abyss and over most of the ocean are found only at abyssal depths (Table I).

Table 1

Least Depth of Occurrence of Several Isopod Genera in Antarctica (75°S) and near the Equator (15°S)

Genus	Least depth m 75°S	15°S
Antarcturus	20	750
Munnopsis	20	1000
Ilyarachna	20	1200
Desmosoma	20	1400
Eurycope	20	2000
Ianirella	20	2500
Storthyngura	500	4000
Microarcturus	1000	4000
Stylomesus	1500	4500

Summary

Eurybathial benthic species are few in number and constitute only a small per cent of the total deep sea fauna (less than 1%). They are found most abundantly in regions of isothermal conditions from the surface to the sea bed. Their taxonomy is poorly known, and they may consist of two or more species with more restricted depth ranges.

The phenomenon of eurybathality has led some workers such as Bruun (1957) to deny any influence of hydrostatic pressure in deep-sea fauna. In view of the rare and inconclusive data on eurybathality this statement appears unjustified, but more definitive data are required both with respect to eurybathial distribution and hydrostatic pressure effects.

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RESPONSES OF SELECTED AQUATIC ORGANISMS TO INCREASED HYDROSTATIC PRESSURE: PRELIMINARY RESULTS*

Robert J. Menzies**
Robert Y. George**
Robert Avent**

Introduction

Observations on the effects of hydrostatic pressure on aquatic organisms reported here are part of a continuing research program at Florida State University. These studies have had the support of the Office of Naval Research, Oceanic Biology (NONR-00014-67-A-0235-0002) and most have been conducted at the Edward Ball Marine Laboratory, Turkey Point, Florida, in the northeastern part of the Gulf of Mexico. Here the marine fauna, in general, is eurythermal and encounters seasonal temperature ranges between 9.0°C to 30°C. Near shore the fauna is moderately euryhaline, responding to seasonal changes in salinity between 20‰ and 30‰. Unless otherwise noted the specimens reported herein come from this nearshore environment.

The apparatus used was that described by Avent, Menzies, and Phillips (1971) and constructed by Mr. Donald Phillips. It is a large volume chamber of 900-cc capacity with view ports permitting observations on animals up to 920 atm. (14,000 psi) or very close to the pressure on the bottom at the greatest depths of the ocean. Hence it reproduces the extreme environmental pressures reported by Flugel and Schlieper (1970).

Our observation that lethal pressures in excess of 1,000 atm. were required to determine even the LD50 for certain groups of animals necessitated the use of a low-volume chamber without viewports capable of 25,000 psi. Prior to the construction of this apparatus the Zobell and Oppenheimer (1950) chamber was used up to a maximum of 18,000 psi.

In our investigation we have used two different methods of pressure application. One of these was used by Avent (1970) in the preparation of his master's thesis. Avent's method consisted of increases of 100 psi with a 3-min. exposure to each increment of 100 psi. The second method involved a continuous increase in pressure at a rate of 250 psi/sec until each response was achieved. With both methods the release of pressure was rapid being not much over 20 seconds in duration. The results obtained differ in the pressure required to achieve a given response. The response itself was reasonably consistent and here each is described.

*Contribution No. 274 from the Department of Oceanography, Florida State University.
**Department of Oceanography, Florida State University, Tallahassee, Florida, 32306.

The specimens were all collected from or near Turkey Point, Florida. All specimens were immersed in artificial sea water (Instant Ocean) of 30‰ and in each case controls were maintained at 1 atm in dishes of volume comparable to the experimental chamber. The animals were of mixed lots as far as size or sex was concerned although few were over 10 cm in greatest length or width.

Our interest in high pressure effects was at first restricted to pressures no greater than 4,000 psi owing to the restrictions of our first observational chambers, which were of low volume and limited and low pressure capability. These limitations kept us from achieving much data until recently on inactivation and on LD_{50}, both of which occur generally at pressures in excess of 5,000 psi.

The limitations noted above have allowed us to pursue observations on behavioral responses that have been earlier of little interest to persons working with the effects of hydrostatic pressure on aquatic organisms. The ecologic significance of these is obvious beacause any significant departure from natural behavior will affect the survival of a species adversely. Most earlier studies on whole animals (vide Fluegel and Schlieper, 1970) have either been concerned with very low hydrostatic pressures or those high enough to induce tetany or death.

We have found that certain behavioral responses are consistently produced by a species at a given hydrostatic pressure. It has been known for many years that animals respond to increasing hydrostatic pressure first by an increase in activity (excitation), second by tetany, third by paralysis (inactivation), and finally by death.

It has generally been conceded that rapid compression and decompression have deleterious effects on cells, but we have found for example that rapid compression and decompression of the isopod Sphaeroma has no observable deleterious effects with pressures up to 20,000 psi, or with a depth equivalent 3,000 m greater than the greatest known depth of the ocean.

Perhaps the most significant result of our preliminary experiments is the discovery that the duration of a given pressure, when once achieved, has the consistent effect of depressing the pressure required to achieve a given response. This effect has the singular meaning that each experiment must be conducted with the same protocol if results are to be meaningful and directly comparable from one set of experiments to another.

In the following detailed presentations of experimental results, N indicates the number of individual animals experimented with and S the standard deviation in pressure.

Species Investigated

Phylum: Ctenophora
Class: Tentaculata

Order: Lobata

1) Species: Mnemiopsis sp. Ecology: Marine, Planktonic, surface

No. investigated: 4

Observations:

R^1: appeared to be an increase in the frequence of the beat of the ciliary comb without loss of synchrony.

R^2: a reduction in the frequency of the beat of the ciliary comb with loss of synchrony.

T=I: this is a general cessation of comb plate beat over much of the body and especially on the tentacular row with persistance of beat (irregular) in parts of the subtentacular row. Here perhaps tetany and inactivity are equal. This was the only point that was repeatedly observed.

Responses

Response	N	mean psi	S psi	psi range	mean kg/cm^2	mean atm
R^1	1	1000	—		70	68
R^2	1	3800	—		266	258
R^3	1	6400	—		448	435
T=I	3	9030	—	8000-9000	632	614

Survival

psi	N	%
9030	3	100

Phylum: Mollusca

Class: Gastropoda

Order: Mesogastropoda

2) Species: Littorina irrorata Ecology: Marine, supralittoral

No. investigated: 90

Observations:

R^1: twisting or rotation of shell.

T^t: retraction of foot and loss of foot-hold with retraction of

I: operculum into shell.

same as T^1.

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R[1]	7	2920	602±	1800-400	2045	196
T[1] & I	9	6620	803±	3800-7800	463	450

Survival

psi	N	%	LD_{50}atm
6,620	10	100	
11,000	10	100	
14,000	10	100	
18,000	10	100	
20,000	20	50	1361
22,000	20	40	

Class: Pelecypoda (Bivalvia)
Order: Eulamellibranchiata
3) Species: Donax variabilis — Ecology: Marine intertidal
No. investigated: 50
Observations:
R[1]: foot extruded from shell and greatly extended and highly active.*
T[1]: retraction of foot and closure of shell.
I: gaping of shell (not observed).

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R[1]	10	3450	354±	2400-4100	242	235
T[1]	10	4700	354±	4400-5200	329	319
I=T[1]						

Survival

psi	N	%	LD_{50}atm
4,700	10	100	
6,190	10	100	
10,690	10	100	
13,250	0	50**	902
16,000	10	0	

*Other responses were seen but never with any consistency.
**LD_{50} interpolated=13,250 psi.

Phylum: Annelida
Class: Polychaeta
Order:
4) Species: Nereis occidentalis (?) Ecology: Marine, intertidal
No. investigated: 20
Observations:
R[1]: a consistently observed violent, writhing activity often to the point of swimming. Earlier inconsistent responses were observed.
T[1]: consisted of tight coiling with convulsive responses to each increases in pressure.
I: cessation of responses; animal tightly coiled.

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R[1]	8	2760	424+	2300-3200	193	188
T[1]	8	5730	1740+	4800-8500	402	390
I	8	9025	985+	7000-10,400	532	613

Survival

psi	N	%
9,025	10	100
16,000	10	100

Phylum: Arthropoda
Class: Crustacea
Order: Amphipoda
5) Species: Telorchestia sp. Ecology: Marine, supralittoral
No. Investigated: 50
Observations:
R[1]: intense swimming activity.
T[1]=I: onset of tetany, loss of swimming capability, inactivation, no movement.

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R[1]	10	1880	254±	1400-2300	132	128
T=I	10	5030	276±	4600-5400	352	342

Survival

psi	N	%	LD_{50} atms
5,030	10	100	
11,000	10	100	
14,000	10	60	
14,700	0	50*	1001
18000	10	0	

*Interpolated.

Order: Isopoda

60 Species: Sphaeroma quadredentatum Ecology: Marine, intertidal

No. investigated: 70

Observations:

R[1]: intense swimming activity

T[1]=I: enrollment and inactivity.

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R[1]	8	1510	218±	1300-1800	106	103
T[1]	9	3220	163±	3000-3400	225	219

Survival

psi	N	%	LD_{50} atm
3,220	10	100	
11,000	10	100	
14,000	10	100	
18,000	10	100	
20,000	10	50*	1440
21,200	0	0	
22,000	10		

*Interpolated.

Order: Decapoda
Suborder: Macrura
7) Species: Tozeuma carolinense — Ecology: Marine, subtidal
No. investigated: 18
Observations:
R^1: vigorous leg movements
R^2: intense swimming activity
T^1 & I: cessation of swimming; inactive

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R^1	8	1150	238 ±	800-1500	80.5	78.2
R^2	8	2360	316 ±	1900-3200	165	160
T^1	8	4000	278 ±	3400-4600	280	272
I	8	5370	230 ±	5200-6000	376	365

Survival

psi	N	%	LD_{50} atm
3400	10	100	
3650	0	50*	245
4000	8	0	

*Interpolated.

Order: Decapoda
Suborder: Anomura
8) Species: Petrolisthes armatus
No. investigated: 50
Observations:
R^1: intense activity.
T^1: retraction of dactyls
I: no movement, often with autonomy of one or both chela (70% of the time).

Responses

Responses	N	mean psi	S psi	range psi	mean kg/cm^2	mean atm
R^1	10	1560	190±	1200-1800	109	106
T^1	10	2750	233±	2400-3100	192	187
I	10	4350	539±	3800-5450	304	296

Survival

psi	N	%	LD_{50} atm
2750	10	100	4
3800	10	100	
4200	0	50*	285
4350	10	40	
5830	10	0	

*Interpolated.

Order: Decapoda
Suborder: Brachyura
9) Species: Eurypanopeus depressus — Ecology: Marine, intertidal
No. investigated: 70
Observations:
R^1: repeated lifting of body.
R_2: intense activity.
R^1=I: retraction of dactyls and inactivity.

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atms
R^1	7	1000	217±	700-1300	70	68
R^2	8	1900	260±	1600-2400	133	129
T^1+I	10	3250	150±	3000-34000	227	221

Survival

psi	N	%	LD_{50} atm
3250	10	100	
4750	10	100	
6230	10	80	
6750	0	50*	459
7750	10	0	
9250	10	0	
11000	10	0	

*Interpolated.

Order: Decapoda
Suborder: Brachyura
10) Species: Uca pugilator — Ecology: Marine, intertidal
No. investigated: 53
Observations:
R[1]: increased activity.
T[1]: tetany.
I: inactivation.

Responses

Responses	N	mean psi	S psi	range psi	mean kg/cm^2	mean atms
R^1	10	1800	50±	1600-2100	126	122
T^1	10	3290	90±	3200-3400	231	207
I	10	5800	708±	5200-7200	406	394

Survival

psi	N	%	LD_{50} atm
3500	3	100	
5800	10	100	
8750	10	80	
10250	10	50	696
11750	10	10	

Order: Decapoda
Suborder: Brachyura
11) Species: Sesarma reticulatum — Ecology: Marine, intertidal
No. investigated: 75
Observations:
R[1]: increased activity
T[1]: tetany
I: inactivation

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atms
R^1	20	2030	113±	1800-2300	142	141
T^1	20	3250	127±	3100-3500	227	221
I	20	4100	147±	3900-5000	287	279

Survival

psi	N	%	LD_{50} atm
4100	20	100	
5740	5	100	
7250	10	100	
10250	10	100	
11500	0	50*	883
11750	10	40	

*Interpolated.

Phylum: Echinodermata
Class: Ophiuroidea
Order:
12)Species: Ophiopholis (?) Ecology: Marine, subtidal
No. investigated: 100
Observations:
R^1: increased activity
R^2: swimming in water
T^1=I: tight coiling of certain rays (4)

Responses

Response	N	mean psi	S psi	range psi	mean kg/cm^2	mean atms
R^1	10	950	233±	600-1100	66.5	64.5
R^2	10	1460	376±	1100-2000	102	99.3
T^1=I	10	3250	298±	2600-3400	228	221

Survival

psi	N	%	LD_{50} atm
3250	10	100	
4700	10	100	
6200	10	100	
7700	10	100	
9200	10	100	
11000	10	100	
14000	10	60	
15400	0	50*	1048
16000	10	20	
22000	10	0	

*Interpolated.

Phylum: Chordata
Subphylum: Vertebrata
Class: Teleostomi
Order: Syngnathiformes
13) Species: Hippocampus zosterae (?) Ecology: Marine, subtidal
No. investigated: 3
Observations:
R[1]: increased activity
T[1]=I: arching of body and inactivity

Responses

Responses	N	mean psi	S psi	range psi	mean kg/cm^2	mean atms
R[1]	3	933		800-1000	65.4	53.5
T[1]+I	3	3160		2900-3400	221	215

Survival

psi	N	%	LD_{50} atm
3160	3	100	
7000	0	50*	476[1]
11000	3	0	

*Interpolated.
[1]Probably much too high.

Results

1. RESPONSE OF INCREASED ACTIVITY(R^1)

One of the most consistent responses by most organisms is an increase in activity. The variation in pressure of this response may or may not be great. We have recorded this variation in mean, in range of psi, and one standard deviation (S) in the following list.

	Species	R^1 Mean	PSI Range	S ±
1.	Littorina irrorata	2920	1800-4000	602 ±
2.	Donax variabilis	3450	2400-4100	354 ±
3.	Nereis occidentalis	2760	2300-3200	452 ±
4.	Talorchestia sp.	1880	1400-2300	254 ±
5.	Sphaeroma quadridentatum	1510	1300-1800	218
6.	Tozeuma carolinense	2360	1900-3200	316
7.	Petrolisthes armatus	1560	1200-1800	190
8.	Eurypanopeus depressus	1000	700-1300	217
9.	Uca pugilator	1800	1600-2100	50
10.	Sesarma reticulatum	2030	1800-2300	113
11.	Ophiuroid	950	600-1100	233
12.	Hippocampus	933	800-1000	—
13.	Ctenophora	1000	—	—

2. RESPONSE OF TETANY (T)

The contraction of (voluntary) muscles in response to pressure is defined as the tetany response. It is expressed as a prolonged retraction of the body (when body is contractile), to a bending or tight flexion of the body (when it is capable of bending), or a flection or an inactivation of the appendages or parts of appendages.

The great similarity between brachyurans in the response to tetany is no coincidence, 3250-3290 psi. Because there is a small range of psi of tetany and a slight standard deviation for each species with means nearly equal. Hence a group effect seems a stong liklihood for brachyura. The response of 12 species follow :

	Species		T^1 Mean	Psi Range	S±
1.	Littorina irrorata		6620	3800-7800	803
2.	Donax variabilis		4700	4400-5200	354
3.	Nereis occidentalis		5730	4800-8500	40
4.	Talorchestia sp.		5030	4600-5400	276
5.	Sphaeroma quadridentatum		3220	3000-3400	163
6.	Tozeuma carolinense		4000	3400-4600	278
7.	Petrolisthes	Brachyura	2750	2400-3100	233
8.	Eurypanopeus	Brachyura	3250	3000-3400	150
9.	Uca	Brachyura	3290	3200-3400	90

10.	Sesarma	3250	3100-3500	127
11.	Ophiuroid	1460	1100-2000	376
12.	Hippocampus sp.	3160	2900-3400	—

3. THE DIRECT RELATIONSHIP BETWEEN INCREASED ACTIVITY AND TETANY

On Figure 10 we have plotted the relationship between the R^1, T (tetany), and the LD_{50}, using a minimal time duration and an increasing pressure in atmosphere. The very close correspondence between R^1 and T show conclusively that these appear to be related events in each of the 12 species tested. All occurred at pressures between 100 and 450 atms. Absence of a direct relationship between the responses of R^1, T, and LD_{50} and LD_{100} is also evident. The 12 species are arranged on the graph, Figure 11, according to decreasing sensitivity to pressure increase as measured by the LD_{50} and the LD_{100}. From these data it is abundantly clear that a high pressure R^1 or T does not mean that the LD_{50} or LD_{100} will be correspondingly high. It seems apparent that the pressure of LD_{100} is only slightly higher than the pressure of LD_{50}.

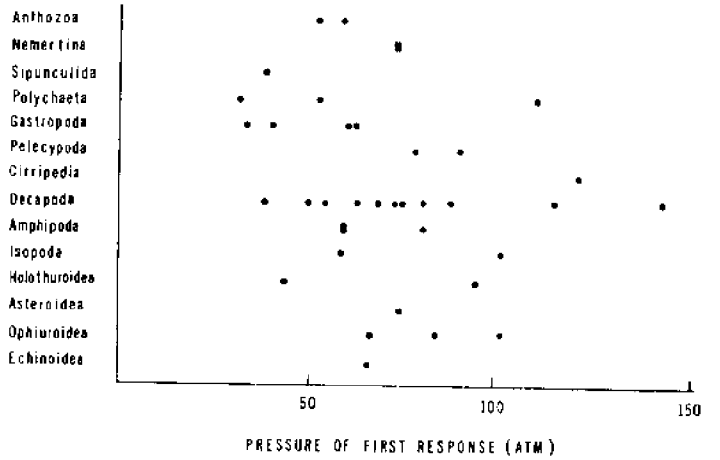

Figure 10. Mean pressure of first response in atmospheres plotted against organic complexity of invertebrates ranging from Anthozoa (least complex) to Echinoidea (most complex). The lack of correlation is immediately obvious.

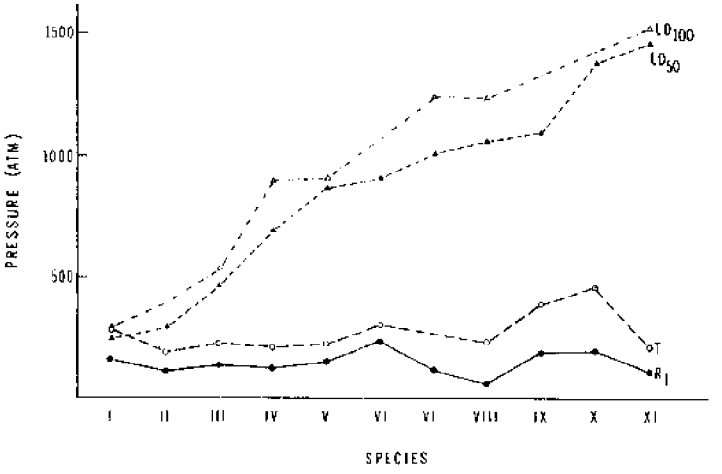

Figure 11. First response of increased activity (R^1), tetany (T), 50% kill (LD_{50}) and 100% kill (LD_{100}) of twelve investigated marine species arranged in the order of increasing hydrostatic pressure resistance. I. *Tozeuma carolinense*, II. *Petrolisthes armatus*, III. *Eurypanopeus depressus*, IV. *Uca pugilator*, V. *Sesarma reticulatum*, VI. *Donax variabilis*, VII. *Talorchestia* sp., VIII. *Amphioplus* sp., IX. *Nereis occidentalis*, X. *Littorina irrorata*, XI. *Sphaeroma quadridentatum*.

The Influence of Method on Results

As is seen from Table 1 our LD_{50} pressures are always higher than those of Naroska (1968) strongly suggesting that increased pressure duration has the influence of depressing the pressure required to achieve LD_{50}.

Zobell (1970) has pointed out a similar case for yeasts and bacteria with the kill pressure (LD_{100}) being lower when the duration of the pressure is increased.

	Yeast Cell Kills
5 min	85,000psi—100% kill
10 min	55,000psi—100% kill
60 min	30,000 psi—100% kill

Table 1

Influence of Methodology on LD_{50} Pressures

Animal	Pressure Duration 1 hr. (Naroska, 1968)	<10 sec.
Mollusca		
Littorina littorea	750	
L. irrorata		1361

Donax variabilis		902
Mytilus edulis	800	
Modiolus modiolus	750	
Mya arenaria	750	
Cyprina	750	
Polychaeta		
Nereis diversicolor	780	
N. occidentalis		1089+
Arenicola marina	520	
Decapoda		
Carcinus maenas	340	
Eurypanopeus depressus		459
Uca Pugilator		694
Sesarma reticulatum		883
Crangon crangon	230	
Tozeuma carolinensis		245
Eupaguras bernhardus	120	
Petrolisthes armatus		285
Isopoda		
Jaera albifrons	770	
Idotea baltica	500	
Sphaeroma quadridentatum		1400
Limnoria tripunctata		1400

Duration of pressure application also influences the pressure of increased activity (R^1) as well as tetany (T) again with longer pressure duration depressing the pressure required to achieve a given response.

We have tried two methods and have found the response to occur at a different pressure depending on the duration of the experiments and especially on the duration of a given pressure (Table 2).

Discussion

Sufficient data are not yet available to substantiate, or deny, the following claims or speculations which have been made, but evidence is accumulating which suggest none is correct.

1. Pressure resistance is a species or genetic property
2. Pressure resistance is related to organic complexity
3. Pressure resistance is related to penetration into the deep-sea

1. Pressure resistance is a species (genetic) property

Recently Naroska (1968) put forth the idea that pressure resistance is a species (or genetic) property and there is considerable evidence favoring this idea. For example each of Naroska's species of fishes showed a different

LD_{50} in atmospheres. On the other hand his three species of clams showed identical LD_{50} of 750 Atms (Table 1). Thus it seems likely that there exists a group sensitivity with shrimps and anomuran crabs more sensitive to pressure than bivalves as well as a species sensitivity. The data suggest that species belonging to a pressure sensitive group (shrimps) show a lesser ability to withstand pressure than those in a pressure insensitive group.

Table 2

Influence of Methodology on Pressures of First Response (R^1)

Species	Method (Average Rate of Pressure Increase) 6.8 atm/min	200 atm/sec
Littorina irrorata	81.0	196
Donax variabilis	54.4	235
Talorchestia sp.	49.6	128
Tozeuma carolinense	42.5	160
Petrolisthes armatus	63.0	106
Eurypanopeus depressus	63.5	129
Uca pugilator	72.2	122

Figures represent pressures of first response in atm

Similarly we have found the R^1 of brachyuran crabs to be remarkably similar with three species having a psi of R^1 between 3250-3290, but the LD_{50} was markedly different with each species.

2. Pressure resistance is related to organic complexity

Regnard (1891) and Ebbecke (1935) put forth the view that resistance to pressure is related to organic complexity with the least organically complex organism being able to withstand a higher pressure than the most organically complex organism. The problem with this idea resides with the difficulty in establishing the organic complexity of a species. The idea seems to have some merit when one considers the extreme ends of the phylogenetic scale. Thus bacteria are among the least pressure sensitive organisms, and birds and man among the most sensitive, but this comparison is not entirely justified owing to the air breathing nature of birds and man.

A more justified comparison is the shrimp with a fish, and when this is done one finds the less complex shrimp much more sensitive to pressure than certain fish and less sensitive than other fish (Naroska, 1968).

Species	LD_{50} atm. Fishes	Shrimp
Zoarces vivparus	370	

Pleuronectes platissa	150	
Platichthys flesus	130	
Crangon crangon		230

We found Hippocampus to experience tetany at 3160 psi whereas the crab Petrolistes experiences tetany at 2750 psi. More data need to be accumulated but it seems likely that a pressure sensitivity gradient will bear little relationship to organic complexity.

The pressure of first response R^1 has no bearing on organic complexity. Thus the coelenterate (anthozoa) showed a similar R^1 to other organisms (Figure 10) including crabs and fishes.

3. Pressure resistance is related to penetration into the deep-sea.

Schlieper (vide, Flugel and Schlieper, 1970) regards pressure sensitivity as being related to penetration into the deep-sea. This is an attractive idea but one lacking in proof. All marine orders of the animal kingdom have one or more species in the deep-sea. Macruran shrimps, anomuran crabs which show the least resistance to hydrostatic pressure are represented in the deep-sea. They are not common but they are there and penetrate to depths in excess of those penetrated by the more pressure resistant Brachyura (true crabs). Isopoda, according to Naroska's results and ours, show the greatest pressure resistance and indeed do penetrate to the greatest depth of the sea (10,000 m) but the genera thus far tested (Jaera, Idotea, Sphaeroma, and Limnoria) are all in the main shallow water genera with no species known from a depth greater than 200 m. The crucial genera remain yet to be tested.

The gastropod genus Littorina is similarly highly resistant to pressure yet is entirely intertidal in depth distribution. This is not to deny that gastropods are found in the deep-sea because they are found there. Littorina however is not so distributed.

Our own view is that resistance to hydrostatic pressure is most likely related to long term ecologic adaptation, with deep-sea species having a greater pressure tolerance than shallow-water species. For this idea there is little evidence although the data of McDonald tend to support the idea. We plan in the future to pursue this hypothesis further.

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Discussion Following Menzies, George and Avent Papers

ZOBELL: For what duration do they tolerate 1,000 or more atmospheres?
MENZIES: We didn't do durational work.
ZOBELL: I don't think we've ever found a bacterium that's killed in an hour at 1,000 atm. But in several hours or a few days, cultures are disinfected at 300 to 500 atm.
MENZIES: The longer the duration of the high pressure, the greater the kill, right?
ZOBELL: Yes.
MENZIES: We approach this question from quite a different angle. We thought we would try to find out if there are any anatomical changes that an animal might undergo with reference to depth. The most obvious anatomical change was reduction in eyes, or loss of eyes. So we plotted what we called the ocular-index system which could be presented as "has eyes"—10; "does not have eyes"—0. Then you find values in shallow water in the Antarctic which as you reach the tropics get deeper and deeper, then in the Arctic occurring in shallow water again. By the way, the deep sea isopod genera are all blind—there is not one that has eyes. So, the greatest depth in this particular instance at 20°N is 1200 m, and this falls very nicely within this arctic-benthal zone of transition where the decapods are present. Many of them do have eyes. They don't have any eye pigments, or they show reduction in this. What this essentially says is that if you really want to find deep sea types, the place to go is to Antarctica, or the Arctic. Another excellent place to go is the Mediterranean, where is it possible to find species with wide depth ranges. These data would tend to sugget that the resemblence between these organisms if not really a pressure relationship

but more temperature-related. This is not to deny that there may be some very serious pressure effects.

SCHELTEMA: You are talking about the adult forms of organisms. Do they move much during their adult cycle, and do they gain eyes, say at an early stage and lose them at a later time?

MENZIES: We are talking about adults. The young don't have eyes; the adults don't have eyes. They are eye-less.

(Question): Do they migrate much during their lifetime?

MENZIES: In this case the adults brood their young in a brood pouch, so there is no pelagic stage—they are obligatory benthos. In general, the pelagic species—this probably applies also to these—have appeared to have a wide power of depth adaptation.

LOWENSTAM: You say they have brood pouch development?

MENZIES: Right. These are isopods. All the isopods have a brood pouch.

(Question): Couldn't you get quite a nice level in upwelling areas?

MENZIES: Certainly, in areas of upwelling we break down the temperature structure. The probability of finding a deep water animal in shallow water is very good. That would be a prediction rather than based on any firm evidence.

MACDONALD: What combination do you find between depth and size?

MENZIES: You are probably aware of the concept of abyssal gigantism? Some people believe that animals in the deep ocean can become giants. Among the organisms I am familiar with, and I observe this from all I have learned in general, the deep ones are quite small on an average. On occasion you find large ones. The classic example in which abyssal gigantism has been based is a group of isopods with which I am familiar. The more recent data we have from the *Eltanin* cruise show that the largest ones are in shallow water, and they are in Antarctica. They are not in deep water.

(Question): Is that true also of the giant squid?

MENZIES: I don't know.

BRAUER: Is this for giants within a well established species in each case? In other words, the one in the Antarctica that you used as a weight to keep your desk down, is that really a member of the same species that Bierstein would have liked to have looked at some place in a treanch?

MENZIES: This is not a case of the same species. It's a case of a species within a genus, and you are looking at the largest species known in the genus—from any depth. On the evidence available now, we find no relationship between gigantism and depth.

MACDONALD: Do you think it would be worth looking at some measure of the quantity of materials that make up an organism in relation to its volume, rather than purely its size? Its density or its dry weight? I would hope it was selection pressure in favor of making an animal make do with as little material as possible.

MENZIES: Why?
MACDONALD: So that it can engulf anything that is available in the way of food.
(Unidentified): Yes, that does work. It works quite well for pelagic animals. They tend to get less dense as you do down in depth. In other words, for animals of the same size, as you go down in depth, they tend to have less material.
(Unidentified): Possibly that's related to flotation rather than feeding.
BRAUER: In relation to the size problem, whether the gigantism story holds or not, I thought I might like to throw on the screen Dr. Zharkova's illustration of the histology of this, because it poses another question that perhaps one might want to pursue. These are two very closely related species. They are the foregut of closely related crustaceans in each case. The one on the right from shallow water in the Kurile-Kamchatka area; in the one on the left, from deeper water—I gather on the edge of the trench. Not in the trench. Zharkova insists, and Bierstein concurs, that the significant point here is that the total number of cells in this cross section is approximately the same, but that the average size of the cells is enormously increased, as you can see, and so are the nuclei. I tried to get Zharkova to make some commitment about whether she thought the DNA content in these nuclei was high, in other words, whether we might be looking at polyploids, but not unexpectedly, she wouldn't be cornered on this point. It poses not only illustration of what that particular group thinks of, in terms of gigantism, but I think it poses a general question that some of you who work with invertebrates perhaps have thought about, namely, the question of the nature of growth in some of these species: Are we looking here at growth in terms of cell replication, in which case we would expect to see hydrostatic pressure effects, which in terms of the effects we see in other systems, might well play a role in modifying size; or are we typically looking just at normally large cells which could then be interpreted as reflecting another kind of adaptation? This might for instance represent the kind of adaptation you would expect in a form that makes its living in rather cold waters, and as a result, at relatively low metabolic rate. Under such conditions, the ratios of diffusion rates to the rates of chemical reactions would represent the kind of conditions that you might expect theoretically should favor large cell size. This is the type of question that it would be fun to answer if you had any material in your hands you could play with, rather than having to fish up an occasional specimen now and then.
LOWENSTAM: Could it be that left sample is full fed?
BRAUER: It could be, but it does not come from the region where it should be best fed, from the shallower waters. Furthermore, I am told that these are not isolated specimens, but that this is typical of the population.
(Unidentified): I just thought maybe the relationship between temperature

and depth for one of these wide-ranging species may be a constant. However, the answer appears to be no.

MENZIES: My answer would be no, because I was only giving the minimum depth for their occurrence, not the mean or maximum depth, in which case, instead of having 4,500 m you'd have 10,000 and the temperature doesn't change.

THEEDE: Some people here emphasize that the abyssal gigantism be due to retarded initial development. Can you comment on this?

MENZIES: It may be due to the longer period of growth if you make the assumption that things that live in Arctic cold temperatures take a longer time to grow, and this is where you find the largest ones. But this has serious problems. The largest isopod in the world was found in the Gulf of Mexico at 600 m, which gives you a fairly warm temperature. I just think the generalization is wrong.

BRAUER: Do we have any data at all about ages of cyclic phenomena analogous to the growth rate on fish scales in deep sea fauna?

(Unidentified): No, except for Scheltema's babies!

(Unidentified): I'd like to hear one more comment on something that Bob Menzies brought up. That is the point that the Mediterranean might be a very good spot to work because the bottom temperature is very similar to the temperature at the surface. What he didn't emphasize is that the temperature he is talking about is a minimum of 12°C and that's quite warm. So, potentially, the Mediterranean is a very exciting place to work, because here you have animals living at relatively high temperatures and abyssal pressures.

GEORGE: When we examine the distribution of species in a region where the water column is isothermal, as in the Mediterranean, where there is no pronounced temperature gradient, and it is warm from the surface to the bottom, we find that several species which are pelagic in the Atlantic, have a tendency in the Mediterranean to penetrate deep down. These species are universal in the Mediterranean, whereas they are pelagic in the Atlantic. In similar isothermal conditions we have shallower species penetrating down to 2000 or 3000 meters.

REPRODUCTION AND DISPERSAL OF BOTTOM DWELLING DEEP-SEA INVERTEBRATES: A SPECULATIVE SUMMARY[1]

Rudolf S. Scheltema
Woods Hole Oceanographic Institution
Woods Hole, Massachusetts 02543

The stability and physical monotony of the deep-sea presents a remarkable environment where bathyal and abyssal benthic organisms successfully live and reproduce. To elaborate here about the nature of this deep-sea environment is unncessary (vide Bruun, 1957) . Suffice to say it is one of great hydrostatic pressure where temperature is constant and uniformly cold (less than 4°C), where day and night are indistinguishable, and where as a consequence there are no obvious seasonal or diurnal cyclic phenomena. Moreover the amount of available food is very low relative to that found in shallow, inshore-waters. It is particularly interesting to know therefore how living creatures manage to maintain themselves in this seemingly unpromising habitat, presenting such unusual physiological demands. It is my purpose to review here what little is known about one basic biological process of the deep-sea benthos, namely reproduction.

There are three aspects of reproduction that I will consider briefly; (1) the reproductive potential or fecundity, (2) the periodicity of reproduction, and (3) the mode of development; that is, whether deep-sea species have direct development without a dispersal stage or, alternatively, if there is some sort of free-drifting larval form. I will try to point out in a very general way how these three aspects of reproduction are related to the dynamics of the deep-sea community.

Reproductive Potential

The reproductive potential or fecundity of deep-sea benthic invertebrates is known in only a very general way. For example, among the isopod crustacea, in particular the Asellotes, a few relatively large eggs are carried by each female. On the other hand, some species of ophiuroids or brittle stars are believed to have large numbers of eggs because "...deep-sea samples usually have a large ratio of young specimens to adults, implying that few adults produce a great many young..." (Schoener, 1967, p. 646). Between these extremes lie many bivalves, and in particular the protobranchs.

[1]Contribution No. 2669 from the Woods Hole Oceanographic Institution. This research was supported by the National Science Foundation Grant GB 6027X.

Perhaps the most useful way to understand the fecundity of deep-sea forms is to compare their reproductive potential with that found among species inhabiting the continental shelf. Recent preliminary data make such a comparison possible among various species within the bivalve genus Nucula found off the northeastern coast of the United States (Scheltema and Sanders, unpublished manuscript). All members of this genus insofar as known are infaunal deposit feeders; seven New England species occur from the shallow sub-tidal to the abyssal depths. Three, Nucula proxima, Nucula annulata (vide Hampson, 1971), and Nucula delphinodonta, are known only from the continental shelf (i.e., down to 200 m). Three other species, Nucula subovata, Nucula granulosa (= corticaa), and Nucula cancellata, are characteristically found on the slope from the edge of the shelf to 2000m. However, Nucula subovata is largely an upper slope species, while Nucula cancellata occasionally extends into the abyss. Finally, the single remaining species, Nucula verrilli is restricted entirely to abyssal depths.

Among these seven species of the genus Nucula, two differ from all the rest in having larger eggs, and these quite clearly have direct development (See Fig. 12); the other five forms appear to be lecithotrophic, presumably having non-feeding, drifting, larval stages of short duration (*vide* Chanley, 1968).

As will be seen later, this predominance of lecithotrophic development is a general characteristic among deep-sea bivalves.

If only the lecithotrophic species are considered, and if these are ordered in accordance with their depth range, it can be seen that the gonads of the shelf forms have a larger volume than the species found along the slope and abyss. Some account should be taken of difference in size of the various species, and this can be done by comparing the ratio of gonad volume and shell length (Table 1). This quotient gives a value for the volume of the gonad relative to the total size of the organism and can be used to compare various species of different dimensions. Its biological significance is that it gives some notion of the relative biomass devoted to reproduction. The shell length rather than body volume has been used because the former is much easier to measure.

Looking now at our specific examples of the genus Nucula (Table 1), the average "gonad volume to shell length ratio" for the two shelf species, Nucula proxima and Nucula annulata, was 0.28 whereas that for the slope and abyssal forms, Nucula granulosa, Nucula cancellata, and Nucula verilli, was only 0.06. The proportion of biomass being devoted to reproduction by lecithotropic shelf species at any one instant is larger, approximately three times greater, than that of slope and abyssal forms.

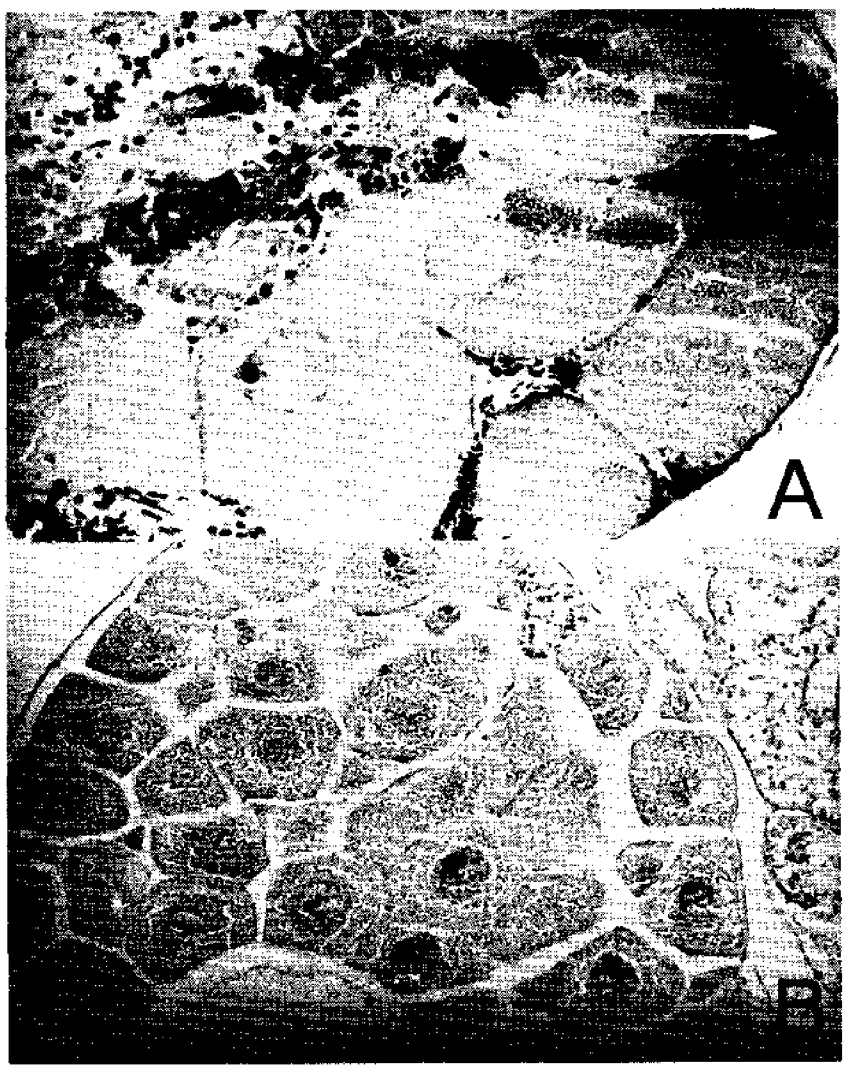

Figure 12. Histological sections of gonads from bivalves belonging to the genus *Nucula*. Both figures were photographed at the same magnification (160X).
a) *Nucula subovata:* section showing large eggs typical of direct development. Note also darkly staining sperm indicated by arrows. Shell length of specimen 1.9 mm.
b) *Nucula annulata:* sections showing eggs typical of species with lecithotrophic development. There is no evidence of hermaphrodism in this form. Shell length of specimen 2.2 mm.

Table 1

Comparison of Reproductive Potential Between Shoal and Deep-water Lecithotrophic Species of the Genus Nucula from off the Coast of the Northeastern United States

Species	Vertical distribution	Shell length mm	Gonad volume mm^3	Gonad volume Shell length mm	Total number of eggs	Eggs Shell length mm
Nucula proxima	Shelf	6.6	2.06	0.31	4120	624
Nucula annulata	Shelf	3.3	0.80	0.37	1233	374
Nucula granulosa	Slope	2.2	0.11	0.07	217	99
Nucula cancellata	Slope	3.3	0.24	0.15	194	59
Nucula verrilli	Abyss	4.3	0.24	0.11	260	60

The number of eggs produced by lecithotrophic species of the continental shelf is also considerably more than slope and abyssal forms. If the number of eggs is expressed as "eggs per millimeter of shell length," then a seven fold difference is seen between shelf and abyssal species (Table 1).

The significance of these differences in *fecundity* between shoal-water and deep-sea species of Nucula becomes evident if one considers the reproductive requirements of any stable or steady-state benthic population. If a bottom population is to maintain itself, it is necessary on the average that each individual be replaced once during its lifetime. But species that produce small numbers of eggs must offset this low reproductive rate either by (1) better survival or (2) a longer period of reproduction during the course of its life.

If the former is true then deep-sea species of bivalves may be expected to have *survivorship curves* with a high initial survival expectancy (vide Deevey, 1947, p. 285, approaching type I curve), whereas shelf species will have a relatively lower initial expectancy of survival (approaching type III curve, Deevey, op. cit.). Stated differently, the minimum average required survival for a shelf species to maintain itself will about 0.05 percent, whereas the average for slope and abyssal species will need to be 0.4 percent. The two groups thus tend toward different reproductive "strategies". It has been shown that frequently these differences in "strategies" are related to other characteristics of *species*, the so- called "K" selection or biologically controlled as contrasted with the "r" or physiologically selected species (vide MacArthur and Wilson, 1967; Sanders, 1968; Pianka, 1970).

If the latter or second possibility proves true, that is if deep-sea forms have a longer period of reproduction, it means either (a) that the species must live longer or (b) that they spawn more frequently or perhaps continuously, rather than periodically as is the case for most shoal-water boreal species. Considering the energy required for continous spawning, the

latter alternative seems rather less likely in the relatively impoverished deep-sea environment.

Periodicity and Reproduction

Periodicity in reproduction is advantageous to a species because all members of a population can then spawn synchronously. This doubtless enhances the likelihood of fertilization. Among shoal-water species having phytoplanktotrophic development, seasonal reproduction is required in order that larvae may take advantage of the high seasonal phytoplankton productivity. As scarcely any of the deep-sea species are thought to have feeding-larvae, the latter advantage evidently does not apply.

The work done thus far on this aspect of reproduction in the deep-sea has been somewhat controversial until now because the data are scanty and difficult to obtain. George and Menzies (1967, 1968) suggested that certain deep-sea isopods have a seasonal cyclic-reproduction, but the data are so meager that their conclusions must be regarded as largely speculative. The evidence used was the occurrence of gravid females.

Sanders and Hessler (1969) found no evidence for reproductive periodicity in an unidentified species of isopod from the genus Ilyarchna. Dredge samples taken in August had 24% of all specimens with eggs in their marsupium, and 27% were in a reproductive condition during December.

Schoener (1968) studied two species of brittle stars collected on a transect between Woods Hole and Bermuda and concluded that their reproduction was periodic and seasonal. Her evidence was based upon the occurrence of sexually mature females and also on length-frenquency analysis. She found that deep-sea dredge hauls made in summer months yielded large numbers of young brittle stars, whereas in winter and spring few small specimens were taken. On the other hand, winter hauls had adults with well developed gonads, whereas samples taken in May lacked any such development.

Scheltema and Rodman (1966) found in a number of slope species of polychaetes that at least some individuals were in a reproductive condition at all times of the year.

Preliminary evidence from samples of Nucula cancellata collected at different times of year show that some portion of its population is in a reproductive state throughout the year. The importance of knowing the duration of reproduction was already pointed out in the previous section on reproductive potential.

Mode of Reproduction

Thorson (1950), on the basis of very meager evidence, ventured the opinion that most deep-sea invertebrates would prove to have direct development and that consequently they would lack a planktonic dispersal stage. Though direct development is now known to be the rule for some

deep-sea taxa, this is clearly not so for many other invertebrate groups. Thus, whereas deep-sea isopods are known to develop directly, some bathyal ophiuroids (Schoener, *in litt.*) and many bathyal and abyssal bivalves (Ockelmann, 1965) have eggs of moderate to very small size, indicateing a lecithotrophic or, more uncommonly, a planktotrophic development. Ockelmann (1965, p. 33, fig. 5) after carefully studying the prodissoconchs and egg size of a large number of species along the Atlantic coast of Europe, was able to demonstrate a positive correlation between increasing depth (up to 400 m) and the percentage of bivalve species having lecithotrophic development. The results of his study further showed that it was only among bivalves of the arctic shelfwaters that direct development was commonly found.

Ockelmann's concept is further extended to much greater depth by recent new data from two deep-sea expeditions. Knudsen (1967) has shown that of the 26 bathyal bivalve species collected during the John Murray Expedition, 58% had lecithotrophic development, 24% had direct development, and 8% are believed to produce planktotrophic larvae. The percentage of lecithotrophic development at abyssal depths is even higher. Results from the Galathea Expedition show that, among the 23 abyssal bivalve species for which the mode of reproduction is known (Knudsen, 1969, 1970), 78% had lecithotrophic development, 13% develop directly, and surprisingly, 9% appear to have planktotrophic larvae (i.e., two species, *Dacrydium panamensis* and *Abra profundorum*).

Turning now to the genus *Nucula* off the northeastern coast of the United States, a similar trend can be seen. Here, again, the difference in the mode of reproduction is evident from the characteristic size of the eggs (Fig 12). Two species, *Nucula delphinodonta* and *Nucula subovata*, have markedly larger eggs and direct development. The five remaining species including *Nucula proxima*, *Nucula annulata*, *Nucula granulosa*, *Nucula cancellata*, and *Nucula verrilli* have eggs ranging in diameter from 100 to 135 μ (Table 2). All five species apparently have lecithotrophic development. Thus among the four deep-water species, one has direct development; on the shelf one of three develops directly.

In general the evidence cited above tends further to strengthen Ockelmann's hypothesis that "lecithotrophic development with a short pelagic larval stage predominates among bivalves of the deep-sea" (Ockelmann, 1965, p. 35).

To date, however, there is but little direct evidence for the occurrence of larvae in the great depths of the ocean. Mileikovsky (1968) has recorded lamellibranch larvae from 1500 to 2000 meters in the region of the Kurile-Kamchatka Trench (p. 214, Table 4). Samples taken with closing nets from the deep-sea submarine *Alvin* have also contained lamellibranch veligers downto 1900 meters. According to the data of Mileikovsky, polychaete larvae can be found at depths exceeding 4000 meters.

Table 2

Relationship between mode of reproduction and geographic range among species of the bivalve genus Nucula known from off the northeastern coast of the United States

	Vertical distribution (m)	Egg diameter μ	Probable development	Geographical distribution
SHELF SPECIES				
Nuculas proxima	<200	100	Lecithotrophic	Western Atlantic: N E coast of U.S.
Nucula annulata	<200	120	Lecithotrophic	Western Atlantic N E coast of U.S.
Nucula delphinodonta	<200	190	Direct	Western Atlantic: N E coast of U.S.
SLOPE SPECIES				
Nucula subovata	530-1500	270	Direct	Western Atlantic N E coast of U.S.
Nucula granulosa	530-1500	120	Lecithtropic	Western Atlantic E coast of U.S. Eastern Atlantic: Bay of Biscay
Nucula cancellata	530-3860	135	Lecithotrohic	Western Atlantic: E coast of U.S. Eastern Atlantic: Bay of Biscay off W. Africa
ABYSSAL SPECIES				
Nucula verrilli	1960-3860	125	Lecithotrophic	Western Atlantic: N E coast of U.S. N E coast of Brazil Eastern Atlantic: off W. Africa

The widespread occurrence of lecithotrophic development in deep-sea benthic species can be significant to their dispersal. Even if the duration of larval development is only one or two days, the larvae may be transported for several kilometers. The velocities of deep-sea currents off the northeast coast of the United States have been investigated in recent years by Knauss (1965), Webster (1969), and Schmitz et.*al* (1970). Measurements on the slope and under the Gulf Stream have given values between 3 and 44 cm/sec. This means that during the course of 24 hours a neutrally buoyant particle may be carried from 2.6 to 38.0 kilometers per day.

A compliation made from the data of Clarke (1962) shows that 35% of all Atlantic bivalve species from depths of over 2000 m have an amphi-Atlantic geographical distribution. The figure is, however, heavily biased toward species with a restricted range because so many bivalves are known from only a single collection (vide Knudsen, 1970, p. 185). When more data are available they will probably show a greater percentage of deep-sea bivalves having wide geographical distributions. On the other hand the Asellote

isopods, reproducing exclusively by direct development, will probably have less than five percent of their species with amphi-Atlantic geographical distribution.

Returning now to the genus Nucula off the northeastern North American continent (Table 1) it is known that all shelf species from this region are confined in their distribution to the western Atlantic. Because of their depth restriction it is apparently not possible for lecithotrophic species to disperse across the Atlantic Basin in a stepwise fashion. Of the deep-water species, however, all with lecithotrophic development are found both in the eastern and western Atlantic Ocean and apparently extend their range by means of pelagic larvae. Only the one directly developing species is restricted to the western Atlantic.

Summarizing, although the data on the mode of reproduction among deep-sea benthic invertebrates is quite scanty, there is enough evidence to conclude that a positive correlation exists between the wide geographic range of taxa and the occurrence of a larval dispersal stage.

It should be very evident from what has been written in this brief discourse that there are very few facts known concerning the reproduction of life history of deep-sea benthic organisms. Yet such knowledge is basic to further understanding the dynamics of bathyal and abyssal communities!

Summary

1. There is a wide range in the reproductive potential among deep-sea species, ranging from but a few eggs as in the isopods to many thousands of eggs as found in some echinoderms. Among protobranch bivalves of the genus Nucula there is evidence suggesting that deep-sea species must either survive better, live longer, or reproduce more continuously than their counterparts on the shelf.

2. Practically nothing is known concerning periodicity or synchrony of reproduction in more deep-sea forms.

3. There appears to be a positive correlation between the geographic range of species and the occurrence of a larval dispersal stage.

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Discussion Following Scheltema Paper

MENZIES: Relative to the isopods, it is true there is a low percentage of similarity between one side of the Atlantic and the other side of the Atlantic with deep sea benthic forms, but there are many species in common that are mesopelagic or bathypelagic, and this accounts for the commonality. In this case the adult itself is swimming in the water and is carried by the water currents.

LOWENSTAM: I wonder that you didn't mention anything about Torstein's shell apex theory. Has anybody in your group tried to apply this to the size of the brood column and the size of the initial?

SCHELTEMA: That's precisely it: what we are saying here with bivalves, doesn't work too well in gastropods. It's not as reliable.

LOWENSTAM: That's too bad. I would like to apply that to fossils. I think that in bivalves in very well preserved fossils, you could tell what kind of development they had.

HESSLER: Where it looked as though there was a deep water bivalve, with a planktotrophic larva isn't possible that an alternative conclusion is that in some cases shell size isn't a good indicator of larva type?

SCHELTEMA: This was originally suggested by Ockelmann, but I rather disbelieve it. I looked at the shells, and indeed it does have a type of larval shell that would indicate the type of development by which it came about. I've dissected quite a few specimens but I haven't got any that show that they have any eggs. If it has small eggs, I can't conceive of how larvae can swim 4,000 m up or down.

GORDON: In this connection, perhaps it's worth mentioning that on the basis of rather smiliar kinds of circumstantial, inferential, indirect evidence, some ichthyologists think that a number of families of fishes have the same kind of life cycle. Some of them have pretty small larvae and they travel a very long way. I know the anglers, for instance, which even as adults are very, very lethargic and probably don't get around very much at all. Yet supposedly they go to 3000 to 4000 m vertically and come back down again.

MENZIES: There is another example in a species of brachiopods which lives in a red clay environment on manganese nodules.

SCHELTEMA: As I recall, these were reported by the German deep sea expedition. I don't think they were using a closing net either. I don't believe it.

GEORGE: There is a particular reproductive phenomenon Galo described as poecilogony. There are some particular species which have a wide geographical distribution they tend to change their reproductive mode when they go to colder waters from planktotrophic to direct development. Are there such cases in bivalves?

SCHELTEMA: I don't think there are in bivalves.

BRAUER: Would it be in order to ask those of you concerned with larval

development of deep-sea forms, how valuable would be the information that one might be likely to get, it someone could provide facilities to keep some of these species? This is the kind of thing we need to determine just how useful deep sea aquarium systems might be.

(Unidentified): I've grown mostly larva of shallow water forms, and I wouldn't have much idea of how to go about this. I have worked with larvae from mid-Atlantic waters and I kept them for years. but I can't figure out how to catch them. I think the way to go about it would be to bring the adults up to spawn.

BRAUER: That's fine—you want the adults topside; you want them long enough so that they could spawn. If you succeed in this, what would you wish to do with the spawn?

SCHELTEMA: I think if his development is fairly short it might tell you how short or how long, and then we could get some notion of how far this creature could be carried. You know our physical oceanographers don't really know too much about bottom currents, where these things are likely to be. They're likely to be fairly near the bottom—the first few meters—where we caught them when we worked from Alvin.

ADAPTATION AND ACCLIMATIZATION TO HIGH PRESSURE ENVIRONMENTS

H. Flügel
Institut Für Meereskunde, Kiel.

Problems and questions related to adaptation and acclimatization of marine organisms to high hydrostatic pressure environments have been a matter of investigations in our laboratory. In spite of these efforts our recent material dealing directly with adaptational phenomenon is still rather limited because of the enormous technical difficulties involved.

The scientific terms 'adaptation', 'acclimation' and 'acclimatization' are used in different ways and, therefore, need to be qualified. Prosser and Brown (1965) define 'adaptation' with the following words:

> "Physiological adaptation refers to conformity and regulation of internal state as well as compensation by long-term acclimation or acclimatization."

Acclimation, on the other hand:

> "refers to the compensory changes in an organism under maintained deviation of a single environmental factor (usually in the laboratory). If acclimation is complete, a measured rate function is the same under one environmental condition as under another. Acclimatization refers to those compensatory changes in an organism undergoing multiple natural deviations of milieu —climatic, physical and biotic."

The definition of the term adaptation given by Kinne (1962,1963,1964, 1971) agrees, on the whole, with that presented by the authors quoted above. Kinne visualizes adaptation:

> "as an ecological phenomenon comprising adjustments of organisms to alterations in the intensity pattern of variables in their environment, which ultimately result in a relative increase in their capacity to survive, reproduce or compete under the new conditions."

The term 'response', also used in connection with pressure experiments, refers to any reaction to an environmental stimulus whether it is adaptive or not. Furthermore, the adaptation of an organism to a changed environmental situation requires usually several days or weeks to develop and persists beyond the environmental stimulus. In most cases, adaptation or acclimatization to ecological alterations are measured by survival tests of whole intact organisms or excised tissues and by measurements of rate functions such as heart beat rate, ciliary movements or metabolic processes.

From these introductory remarks it is quite clear that hitherto in the laboratory merely responses and acclimations of marine animals to elevated hydrostatic pressure were investigated. Genetic adaptation of organisms to

the hydrostatic pressure prevailing at the deep sea floor require long- lasting exposure to the elevated pressure. It is, however, possible to study the physiological adaptations to the abiotic, environmental conditions of the marine habit at the cellular, organic and individual level by resistance tests. These adaptations are genetically fixed by natural selection and—in many cases—may reflect stages or steps of the evolutionary development of the species considered. The resistance adaptations of excised tissues and intact animals to hydrostatic pressure were studied in our laboratory by several authors (Schlieper, 1963, 1968; Ponat, 1967, Naroska, 1968). Ponat examined the resistance of excised tisseus of Cyprina islandica, Mytilus edulis, the sea-anemone Metridium senile and the sea-star Asterias rubens after exposure to hydrostatic pressures up to 900 atm for 6 hrs and after a 1 hr recovery period at atmospheric pressure. The tissues of Asterias rubens survived compression up to 700 atm, those of Mytilus edulis pressures up to 600 atm. Lower pressure tolerances were found in tissue pieces of Cyprina islandica and Metridium senile. Maximum tolerance of both C. islandica and M. senile amounts to 300 atm (Fig. 13). The pressure tolerance of whole

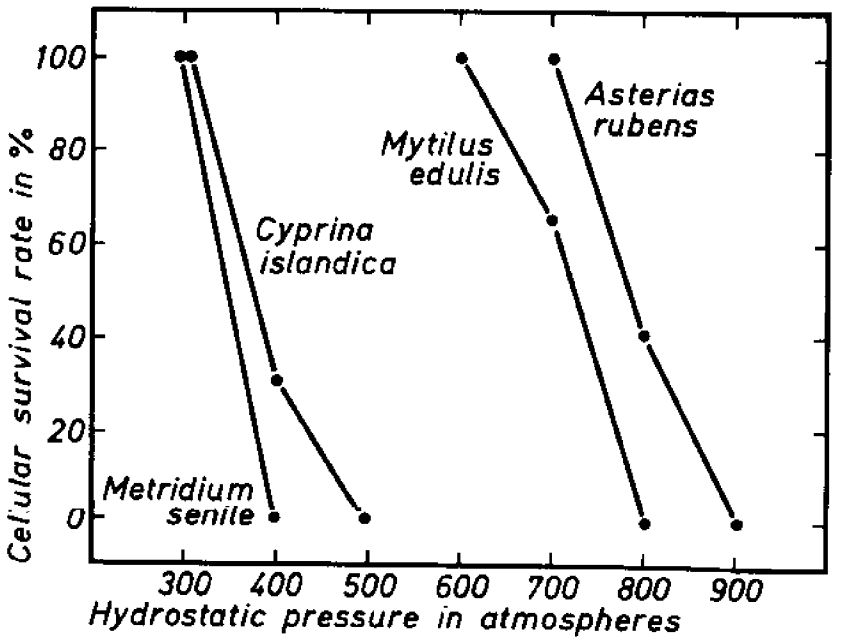

Figure 13. The effects of hydrostatic pressure on the cellular survival rate of ciliated tissues of the sea anemone *Metridium senile* (tentacles), the bivalves *Cyprina islandica* and *Mytilus edulis* (gill epithelium), and the star fish *Asterias rubens* (papulae). Prior to the experiments the animals were acclimated to the experimental temperature (10°C) for 5 days. (After Ponat, 1967; modified).

intact animals was measured by Naroska (1968). The specimens were compressed for 1 hr and the LD_{50} values determined after 24 hrs recovery at normal atmospheric pressure. The mysid Neomysis vulgaris and the decapod shrimp Crangon crangon proved to be significantly less pressure tolerant than the larvae of the teleost Zoarces viviparus and the isopod Idothea baltica (Fig. 14). From his work it is quite obvious that, on the whole, decapods, some teleosts, and mysids exhibit a smaller pressure tolerance than bivalves, isopods, and amphipods.

In many cases the excised tissues as well as intact healthy animals from the same shallow environment, the seashore of the Baltic and the North Sea, show rather different resistance to elevated pressures. Many species survive compressions they could hardly have encountered during their life time. Our observations have shown, that genera whose members tend to occupy deep areas of the ocean show a high pressure resistance. In some species, however, the resistance to compression seems to be a non-specific by-product of euryoecious species.

The pressure resistance of marine organisms has been tested also as a function of temperature, salinity, pH and oxygen tensions. Instead of the whole animal, small, excised pieces of gill tissue were used in many experiments. Such ciliated epithelia can be kept alive for several days if the environmental conditions are favorable. The general physiological condition of the epithelia can be assessed in terms of the degree of the ciliary activity shown. Normal healthy cells show high ciliary activity (classified as '3'); cell death leads to complete standstill of the cilia (classified as '0'); intermediate stages are classified as 1 and 2, respectively (Ponat, 1967; Ponat and Theede, 1967; Theede and Ponat, 1970).

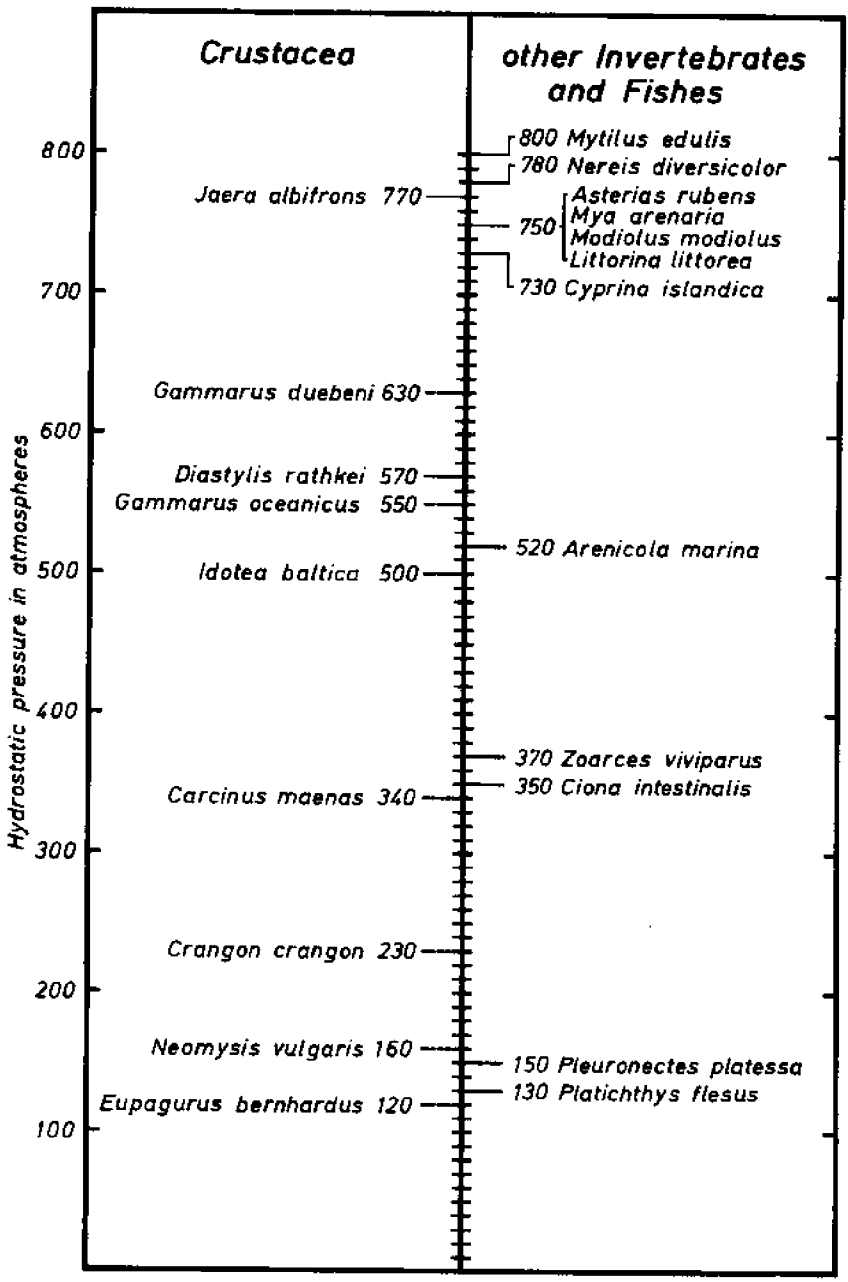

Figure 14. The lethal pressure which killed 50% (LD_{50} data) of the specimens of various marine invertebrates and fishes from the Baltic Sea and the North Sea *(Mytilus edulis)*. The specimens were exposed to the pressure indicated for 1 hr and the LD_{50} data determined after a 24-hr recovery period (Experimental temperature: 10°C). (After Naroska, 1968; modified).

Fig. 15a shows the pressure tolerance of the gill tissues of Mytilus edulis diegensis tested after acclimation to 10° and 20°C. The pressure which significantly reduces the ciliary activity is 300 atm at 10°C and about 400 atm at 20°C (Schlieper, 1963).

Schlieper (1968) has emphasized that marine organisms are genetically as well as non-genetically or individually acclimated (adapted) to their habitat temperature, It is apparently for this reason that the pressure resistance of the eurythermal blue mussel Mytilus edulis decreases in the cold when the specimen are acclimated to low temperatures prior to the experiments.

Fig. 15b shows a contrary pnysiological behavior. The shrimp Crangon crangon from the Baltic survives hydrostatic pressures between 100 and 300 atm somewhat better if acclimated to its optimum temperature. 5°C seem to be closer to this temperature than 21°C (Naroska, 1968).

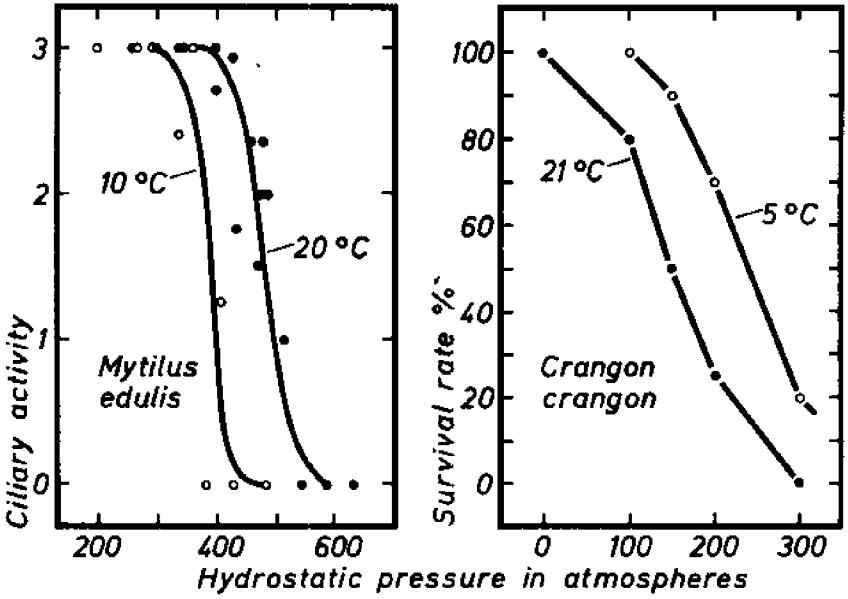

Figure 15. The effects of the acclimation temperature on the pressure resistance. a) Relative ciliary activity of the terminal cilia of *Mytilus edulis diegensis* after 24 hr-exposure to the pressure indicated and acclimation to 10° and 20°C, respectively. (After Schlieper, 1963; modified). b) Survival rate of the shrimp *Crangon crangon* (LD_{50} data) after acclimation to 5° and 21°C, respectively, for 8 days (After Naroska, 1968; modified).

The relative ciliary activity of gill epithelia was also tested after compression for 24 hrs in brackish water and sea water of full strength (Fig. 16). The pressure resistance of ciliary activity of the gill tissues of Mytilus edulis was significantly higher in sea water of 30‰ than in brackish water

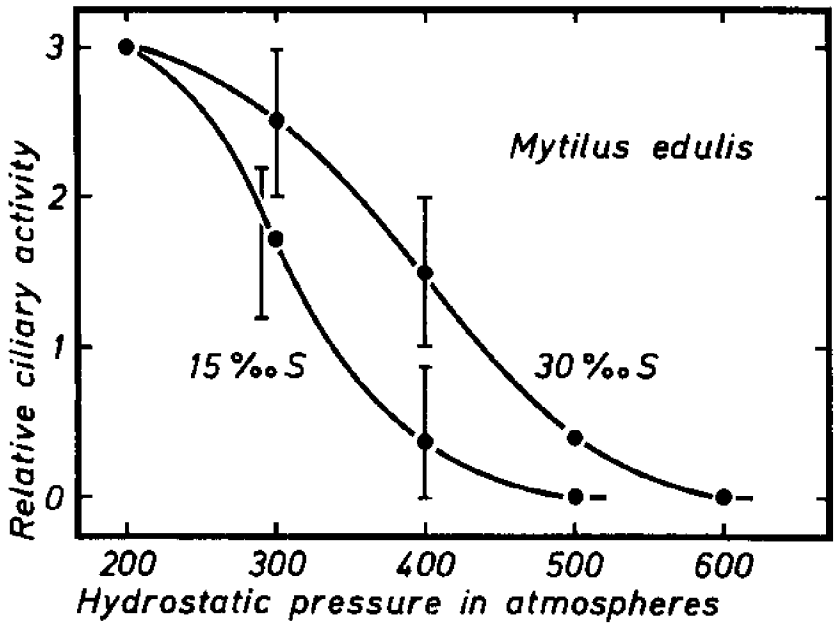

Figure 16. The effects of the osmoconcentration of the external medium on the pressure resistance of the gill epithelium of *Mytilus edulis* from the Baltic Sea ($15^0/_{00}$ S) and from the North Sea ($30^0/_{00}$ S). The whole animals were acclimated to 10°C for at least 5 days (After Ponat, 1967, modified).

of $15^0/_{00}$ S (Ponat, 1967). Various authors have shown that the maintenance of marine organisms in sea water of reduced salinity reduces the cellular resistance to physiological stresses. It has been suggested that this general unspecific decrease of the cellular resistance indicates a weakening of sensitive protoplasmic components (Schlieper, 1966; Theede, 1965; Theede and Lassig, 1967).

The effects of pH-values between 3 and 10 on the pressure resistance were studied by Ponat and Theede (1967) in our laboratory. As shown in Figure 17, the optimum of the ciliary rate shifted from pH 8 at 400 atm to pH 6 at 600 atm. This reaction is not yet understook, but it is suggested that it is evidence of responses at the enzyme level. Apparently, these changes do alter the chemical activity as well as the molecular structure of cell enzymes and other macromolecules . It is for this reason that these authors have begun to study the activity of pressurized enzymes isolated from marine organisms. The alkaline phosphatase of the homogenized gill and mantle epithelium of the bivalve Cyprina islandica was exposed to pressures up to 800 atm at 5°, 15°, 25°, 35°, 45°, and 55°C. The activity of the pressurized enzyme was increased above the optimal species-specific temperature (about 17°C) and retarded below this temperature. This

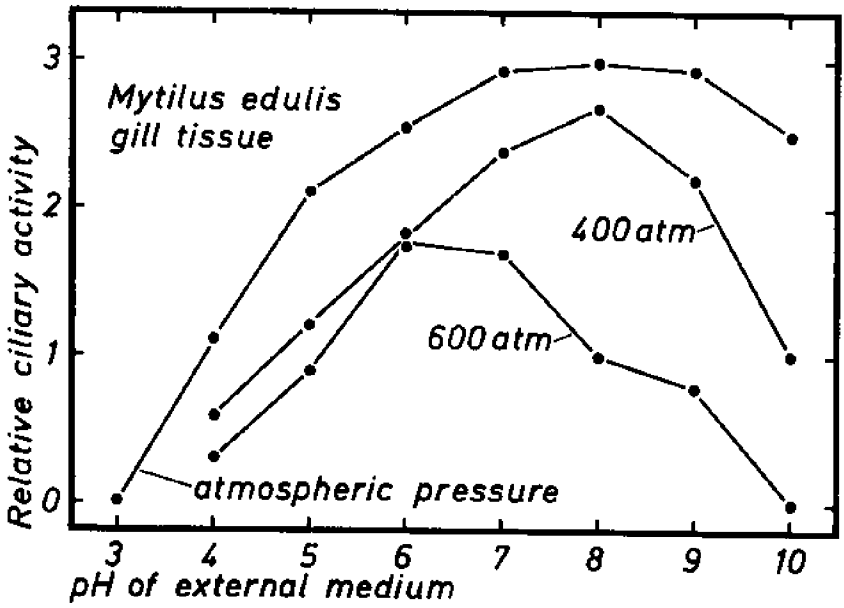

Figure 17. The effects of various pH values on the ciliary activity of the compressed gill epithelium of *Mytilus edulis* (Experimental temperature: 20°C; Salinity of the external medium: 15‰ S) (After Ponat and Theede, 1967; modified).

experiment confirms earlier findings on temperature-pressure relationships. Johnson and Eyring (1970), for instance, report on the luminescence of three species of bacteria at elevated hydrostatic pressure and at various temperature levels above above and below their optimum temperature for brightness. These experiments were summarized with the following words:

> "The important point in the present context is that in all three species the effect of pressure on the intensity of luminescence in cell suspensions is to decrease it at temperatures below the optimum, to have relatively little effect at the optimum, and to increase it at temperatures above the optimum."

Recently the effects of pressure and temperature on enzymes of marine poikilotherm fishes were reported by Hochachka, Schneider and Kuznetsov (1970). Fructose disphosphatase (FDPase), extracted from the liver of the benthic fish Coryphaenoides acrolepsis, is reversibly inactivated by exposure to 340 atm if the experiment is conducted at low temperature (3°C) and in the absence of substrate and cofactor. At 1361 atm and the same low temperature the enzyme is irreversibly denaturated. When however, cofactor and substrate are present, the enzyme is protected against denaturation. Catalysis does not occur at pressures below 680 atm and at low temperature (3°C). The homologous enzyme of a surface dwelling fish, Pimelometopon

pulchrum, on the other hand proved to be strikingly more sensitive to the combination of pressure and low temperature.

The existence of at least two forms of liver FDPase, cold and warm, and the conversion of the enzyme into the cold form by low temperatures and into the warm form by high temperatures may be interpreted in terms of an adaptational phenomenon at enzyme level. The structural stability of pressurized epithelia and cells should also be tested at the sub-microscopical level. Such experiments have already been conducted on amoebae, protozoans and the germ cells of echinoderms (Landau and Thibodeau, 1962; Tilney and Marsland, 1965; Tilney et al, 1966; Kennedy and Zimmerman, 1970; Tilney and Cardell, 1970).

I would like to draw your attention to a few recent experiments. We tested the form stability of epithelial cells of various bivalves under pressure (Flügel and Fritsch, 1971). The pressure chamber used was basically patterned after the device designed by Landau and Thibodeau (1962), modified for the fixation of excised tissue pieces and small intact animals. Our hypothesis is that the cells and their organells from barophilic-eurybathic animals are more resistant to pressure induced disintegration than those from barophobic-stenobathic animals. Indeed, the first tests seem to indicate that epithelial cells of Mytilus edulis are slightly more stable than similar cells of Modiolus modiolus. The pressure induced effects at the ultrastructural level consist in the disintegration of the formerly well organized brushborder of epithelial cells (Fig 18,19). Furthermore, the microtubular elements of the cilia begin to disintegrate at pressures in excess of 300 atm. Protoplasmic material from the sheath of the cilia is discharged in the form of small vesicles (Fig.20). Upon release of the pressure there is regeneration of the microtubular elements of the cilia and reestablishment of the microvilli.

The interesting question as to the acclimation of marine organisms to elevated pressure under laboratory conditions has inspired a series of investigations suggested by Dr. Schlieper. Naroska (1968) was able to keep marine animals pressurized for more than 24 hrs in running seawater. He showed that the oxygen consumption of eurybathial echinoderms dropped slightly upon compression, followed by a rather stabilized state. In some cases, Naroska was able to measure even a slight increase of the metabolism in prolonged experiments (Figs. 21,22). He interprested his experiments in terms of non-genetic, individual acclimation to pressure. We hope to continue such experiments for at least a few days. We then may be able to decide if this stabilized state is followed by an acclimated state. The improvement of our pressure devices as well as the gear used in the field for capture and recovery of animals from deep sea floor is highly desirable. We hope that this conference will stimulate the efforts in this field.

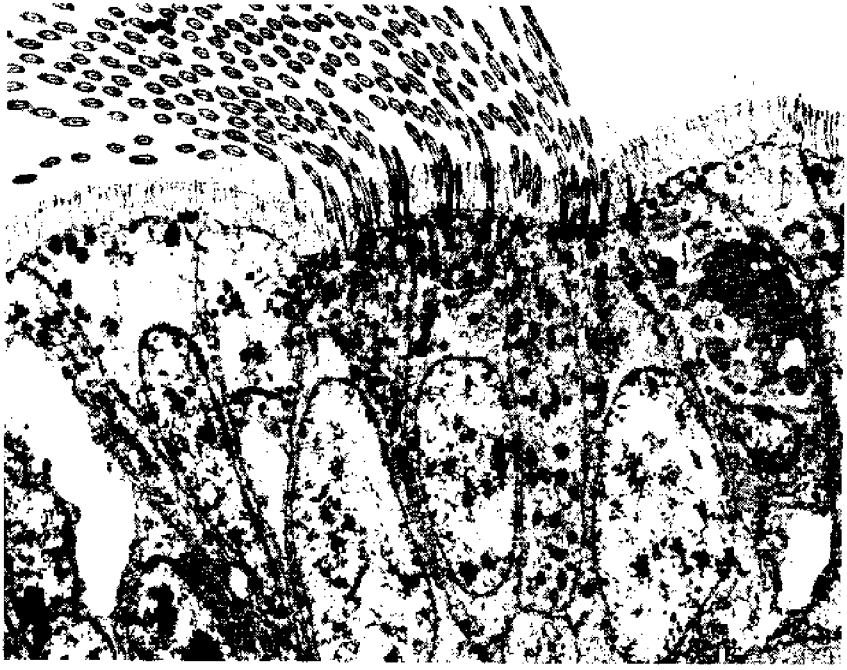

Figure 18. Electron micrograph of the gill epithelium of the bivalve *Modiolus modiolus* at normal atmospheric pressure. Note the well arranged microvillous border. Magnification: x 5,400. (After Flugel and Fritsch, 1971).

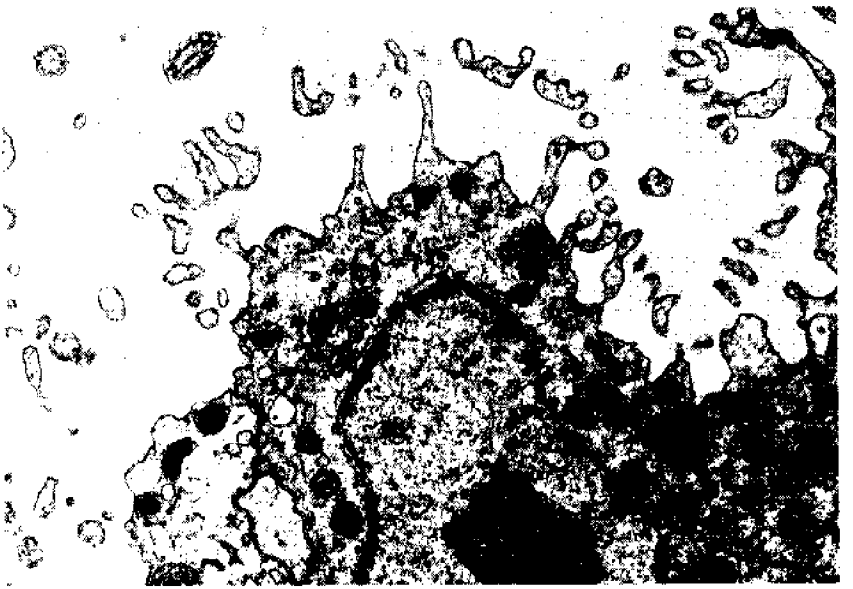

Figure 19. Electron micrograph of the microvillous border of an epithelial cell of *Modiolus modiolus*. The cells were fixed at 300 atm after a 30 mins exposure to that pressure. Note the pressure induced disintegration of the microvilli. Magnification: x 18.000 (After Flugel and Fritsch, 1971).

Figure 20. Electron micrograph of longitudinal and oblique sections of cilia of *Modiolus modiolus* fixed at 700 atm after a 30 mins exposure to the pressure indicated. Note the protoplasmic vesicles external to the cilia. Magnification: x 18.000 (After Flugel and Fritsch, 1971).

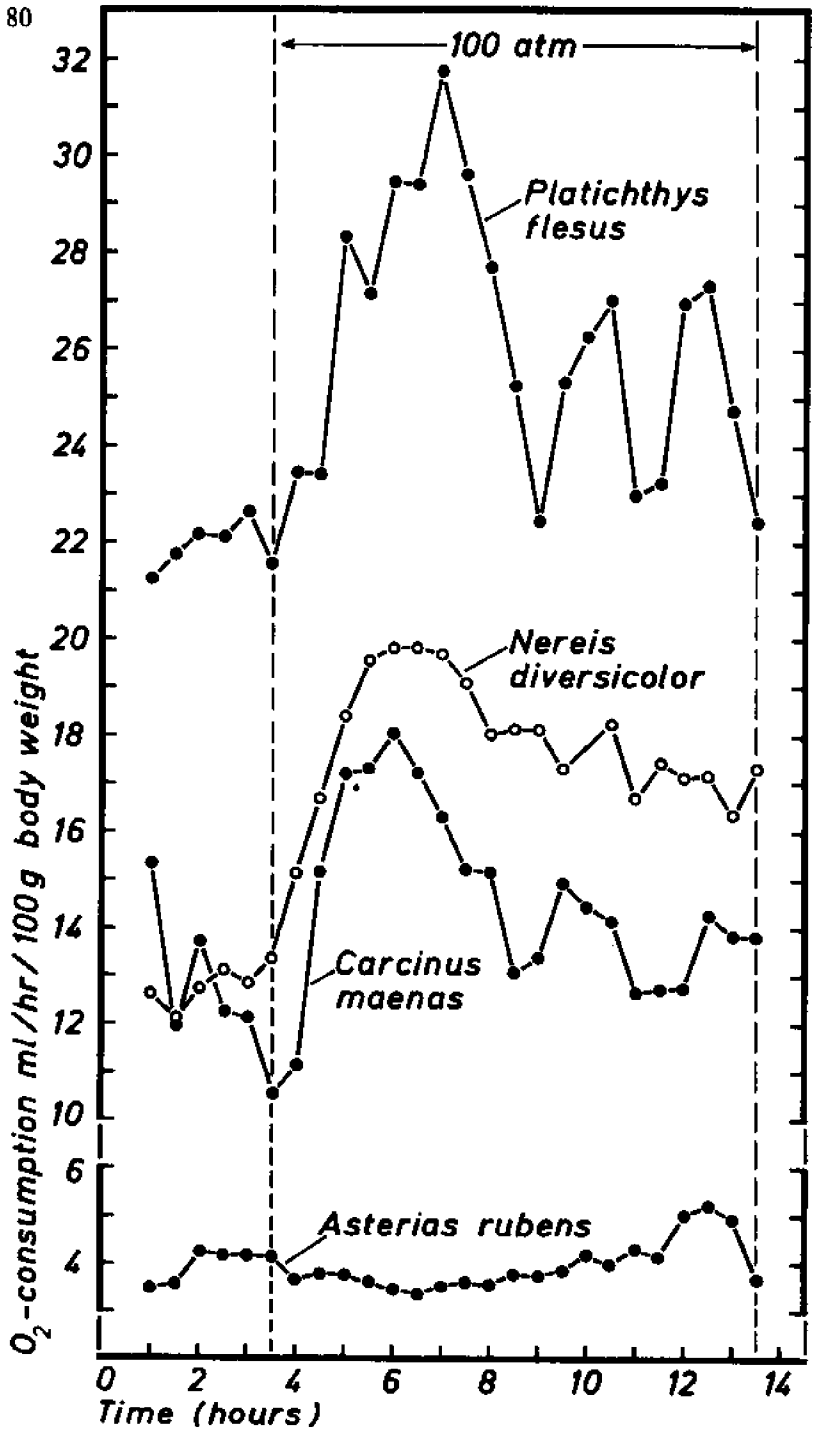

Figure 21. Oxygen consumption of various marine invertebrates and fish at 100 atm (After Naroska, 1968; modified).

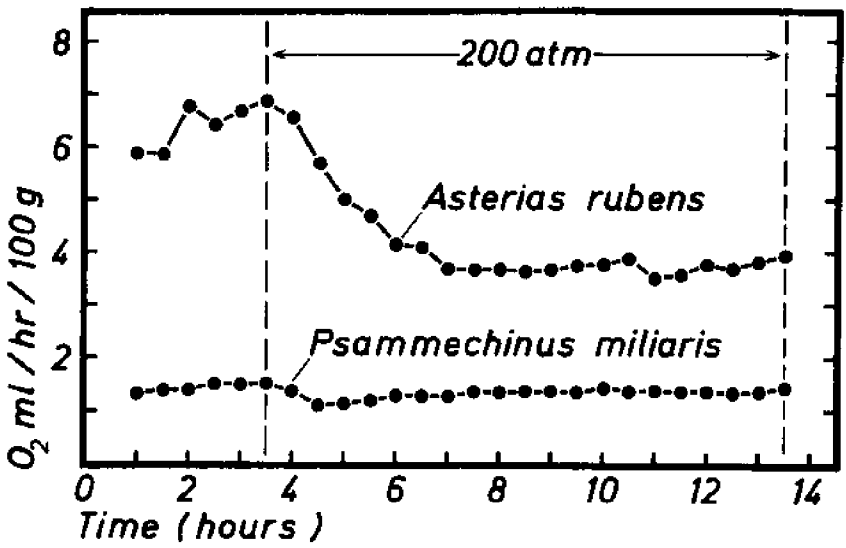

Figure 22. Oxygen consumption of the star fish *Asterias rubens* and the sea urchin *Psammechinus miliaris* at 200 atm (After Naroska, 1968; modified).

ACKNOWLEDGMENT

The author's participation in the workshop conference at Wilmington was made possible through financial support from the 'Deutsche Forschungsgemeinschaft'. Their assistance is gratefully acknowledged. The author feels also obliged to thank Professor R.W. Brauer for his hospitality and constant enthusiasm during the conference. I am particularly indebted to Professor C. Schlieper who stimulated the author's interest in pressure research.

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Discussion Following Flügel Paper

KANWISHER: These are fascinating electron-micrographs. Is there any possibility of doing this while they are in the net in place at depth?

FLÜGEL: We would like to do those in the field. We should have little trouble on one station for a couple of days.

ZOBELL; These were very short-term experiments, only two hours. Could you really interpret them as being acclimatization to increased pressure, or could it be interpreted as an initial pressure shock and recovery from pressure shock to normal metabolism after a few hours?

FLÜGEL: In my opinion, these last few physiological data you refer to are just a recovery to a normal state. We need definitely longer experiments to make the distinction sharply.

BRAUER: Haver you any observations on the behavior of these animals while this is going on? The respiratory rate of an animal that is flexing its

muscles and standing on its tail would differ from one that is resting in dejection.

FLÜGEL: There are many observations available using the pressure microscope chamber. However, to my opinion, mainly protozoans and very small animals have been used. Regnard in 1891 looked upon decapods and I think cyclops while being pressurized, and gave an exact description how the animals behaved. But we didn't use highly organized animals. We were limited always by the volume of the pressure chamber.

MACDONALD: I was interested to look at the central fiber in you cilia. I think this must be the first case of pressure apparently depolymerizing. Have you any information about this?

FLÜGEL; If I'm not mistaken, Zimmerman has made similar observations when he invevestigated tetrahymena.

ZIMMERMAN: The central fibers of the tetrahymena do become dissociated at pressures in the range of 7500 psi. In the next issue of the Journal of Cell Biology, Dr. Kennedy and I have a paper which shows this material. What we've been able to demonstrate though, and what surprises me is that the cilia continued to move although we've lost the intact central fibers. Last month, a colleague, Catherine Henley, informed me that she's been collecting data that indicated that the central filaments are not essential, and that many organisms do not have the central filaments that we usually assume most flagella and cilia have. So apparently they can move without an intact central filament.

FLÜGEL: Dr. Schlieper has looked at the ciliary activity many times, and he observed that the movement of the cilia was at least retarded at the pressures he applied, and ceases around 800 or 900 atm. So there is certainly a relation between the disintegration of the filament and the movement.

ZIMMERMAN: In tetrahymena, though, it can be disorganized and still move.

MENZIES: At the pressures you're describing, in almost every instance, the organisms would recover. We have just been engaged in testing if the cellular structures are disintegrated, or what.

ZIMMERMAN: My answer is: Yes, they can recover at the pressures we've used. There's a sequence which they go through, a vacuolization after about 5 minutes, after ten minutes post-decompression, they start to swim away. Most of them will look normal. In many cases, immediately upon decompression, many of the cells will show movement almost instantaneously.

BRAUER: With the Asteroids, you have a somewhat different sequence, do you not at the pressures used here? The animals go into rigor, and then as you decompress, and no matter how you decompress, they will persist in rigor for fairly extended periods of time, on the order of one to three hours.

Eventually, when you've just gotten ready to throw them in the can, they relax and crawl away. Were the respiration data you have here on your asteroids obtained during rigor? And have you or has anybody else taken any look at the pyruphosphates or at the contractile apparatus under those conditions? It seems to me high pressure contracture should have a biochemical counterpart.

LANDAU: Several years ago, testing the concept in amnion cells, and then also working in Arbacia eggs, on release of pressure you generally get a contraction. And you get a deepening of the furrow if it's part well into the dividing egg. In a couple of the slides which I was going to show before, I have some of these ATP data we were testing out with this contraction. With proper treatment, you can find at this time an increase in the ADP in some cases, and of ATP in another case. This disappears within 30 sec. after relief, so that you must freeze before you assay. The interesting thing is that when it disappears, the disappearance of ATP or ADP is not reflected in changes in ADP or ATP levels. What we're dealing with there so far we haven't any idea.

BRAUER: You have these very interesting data, Dr. Flügel, on cell survival. I tend to agree with Menzies that under many of the conditions you use, I would expect an animal as a whole to recover when you decompress. How would you reconcile this discrepancy, or would you conclude that the cells, once you have isolated them, are rather different beasts from what they were in the animal?

FLÜGEL: Well, I think we know that these isolated excised cells can survive for at least a week or two, I think they must function quite normally.

BRAUER: But they do die when you expose them to pressure?

FLÜGEL: Yes.

BRAUER: Still the intact animal would not die. Yet an animal with 60% of his cells dead is not very likely to survive. There appears to exist a significant and very interesting difference between the resistance of the cells in situ, and the isolated ones.

GORDON: To go back to the question that was raised earlier about behavior: As far I am aware, the only behavioral observations that have been made on whole animals under high pressures were made by Fontaine back in 1929 and by Ebbecke actually in 1932 or there about. They both looked at several kinds of shallow water fishes and at a few invertebrates. You will remember that they found that as the pressure went up to a range of about 120, from 100 to 125 to 150 atm., the aminals became much more active. Then they finally became narcotized, and would survive up to pressures of about 300 atm. and then they would die.

BRAUER: May I rise to a point of semantics. We should not use the term "Narcosis" in this context. In relation to pressure effects this is a source of an incredible amount of confusion. The mere fact that the animal lies there

with all fours stretched out tempts one to say that it's "narcotized", but this still merely means that it lies there with all four legs stretched out, and so why not say so in the first place. There is, I think, a very exciting area of interaction between genuine narcosis as pharmacologists understand it, such as the inert gas type of phenomena, and high pressure effects, and this seems to be true at the level of cell nuclei as well as at higher levels of organization. For the time being, I suggest that we should keep the work narcosis out of the literature on pressure effects until we understand the underlying phenomena a bit better.

(Question): I would like to ask question as to the pressure tolerance of the various species. I wonder if you found out that decapods do not have a high pressure resistance comparable to mollusks?

FLÜGEL: Yes, but these were acclimated and tested at 15°C. They are eurythermal species. But if these decapod shrimp were acclimated to a lower temperature level, would it be possible to raise the pressure resistance? If the temperature is close to a species' optimum, it seems certainly possible to raise the resistance. Dr. Schlieper and some of his students found this in many experiments. I would like to add remarks on pressure adaptation on the macromolecular level, such as the enzymes that Morita and Gordon told about. We made some experiments with alkaline phosphatase of different species. Comparing the activities of a given enzyme at 1 atm. and 800 atm., at low temperatures it was reduced and at high temperatures activity was increased. With the alkaline phosphatase of Cyprina islandica from the Baltic Sea, the temperature where nothing happened was at about 15°C. With bovine alkaline phosphotase, the same point was at 25°C, and with enzyme from Arctic fishes which have very low temperature resistance, this point was even lower, about 8°C (not a point, more a range where nothing happens under pressure).

ALBRIGHT: It appears from one of the slides you showed that as you increased the salinty of the medium, the pressure resistance was increased. When I have worked with bacterial cells, I used the marine psychrophile Vibrio marinus. If I grow the cells in 35‰ sea water salt, the culture grows to 420 atm. approximately. If I lower the salinity to about 3 or 4‰, it will not grow above 80 atm. So there is quite a pressure difference depending upon salinity. I've checked this out with four more marine bacteria—vibrio, chromobacter, serracia, and micrococcus, isolated from marine environments. It seems to be a general phenomenon that as one lowers the salinity of the incubation medium, the maximum pressure to which the cells can go is lowered correspondingly.

D'AOUST: I wonder if that can be simply explained by osmotic effect, or could there be a water pressure effect or change of activity of the water. This is the way it should work if water activity is changed as reported.

ALBRIGHT: It may be. What we've done also is to look at different salts.

Sodium has the greatest effect and potassium has the least effect. We've looked also at a terrestrial E. coli and a micrococcus, and they don't seem so much affected by salinity, vis-a-vis pressure applied to the system. I think this is a phenomenon that should be emphasized. People talk about pressure and temperature; perhaps we should think rather of pressure/ salt/temperature.

YAYANOS: I'd like to say something about fixing tissues under pressure. Glutaraldehyde, for instance, is going to cause reactions with the materials themselves, and these reactions can be pressure dependent. It may be important to try two or three different methods of fixation before one concludes that a particular method can be used. I have one particular piece of evidence that things under pressure aren't always as they may first appear to be. If one measures the activity by the amount of substrate decomposed in a reaction, say trypsin plus benzoyl arginine to give you paranitroaniline plus benzoyl d-arginine. What you measure is the appearance of this color, as a way of saying whether this enzyme is working. At one atmosphere, as a function of time you get so much paranitroanaline formed. At 500 atm. you get the same curve. At 1500 atm., you get the same curve. This is bovine tryspsin. So it's working just as well at 1500 atm. as at one atmosphere. There are a number of ways of following the reaction. Generally, what you do is that you add acetic acid to this reaction to quench it. Then you can measure the amount of paranitroaniline that develops. At one atmosphere and at 500 atm. the paranitroaniline that is formed is stable for more than a week in the presence of this acetic acid. You can read your tube right away, or a week after. But, if you add paranitroaniline at 1500 atm. it disappears, presumably in a reaction between something in this reaction mixture and the paranitroaniline. So we can't even rely on an assay at 1 atm. to extrapolate to 1500 atm.

ZIMMERMAN: How does this apply to fixation with glutaraldehyde?

YAYANOS: Pointing out that reactions can take place at high pressures, we can't assume that what is happening at 1 atm. is happening the same way at 1500 atm or at 2,00 atm.

ZIMMERMAN: But if fixation is good, what are you worried about?

YAYANOS: The fixation itself can be a reaction with the membrane. What is fixation? The fixation itself can be different by freezing, with formaldehyde, or different than fixation with glutaraldehyde.

GORDON: One thing which I think is important to point out here as long as we're talking about adaptation, acclimation, whatever you want to call it, is that in trying to make comparisons between shallow water and deep-sea organisms, the kinds of things we've been discussing so far in no way exhaust all possibilities or relevant variables. The kinds of things that have been mentioned so far have been temperature, and pressure and salinity, specifically. Looking at it a little more broadly, from a more biological point of view altogether, rather than a physiological point of view, I think it's

very important to remember that deep sea organisms as a group have had very different evolutionary histories and occupy very different phylogentic positions in terms of the evolutionary histories of the various groups to which they belong, than do shallow water organisms. Other kinds of things are also going to be important: In terms of metabolism in fishes, things like the nature of the environment from whch the animal comes are very important. And I don't mean just necessarily temperature in terms of the environment, but also, for example, whether the organisms are littoral organisms living in-shore, whether they are epipelagic organisms, and so on. It's also very important to know something about body size and activity patterns of the animals, because all of these affect metabolic activities on a weight specific basis anyway, and presumably also when you look at the whole organisms themselves.

If you cannot do the kinds of experiments discussed by Flügel, where you have an individual animal being its own control, essentially, you must start off with a measurement at 1 atm. You make measurements on two different individual organisms that come from two different places (i.e. shallow and deep sea), and then any difference that you find between these two is not necessarily only associated with the pressure difference or the temperature difference, or what have you. You have to take into acount all these other things. This is just to reinforce the kinds of comments that Lowenstam made earlier where he refused to say that it was just pressure that produced the difference to be found, but would rather correlate with depth until we can actually show that pressure is the ecological variable.

We did metabolism measurements last spring on some lantern fish tissue, and also in the hatchet fish. They were tissue metabolic rates in well-fed, very fresh hamburger from fishes caught in mid-water trawl hauls and measured at three different temperatures. The three species of lantern fish had quite different vertical migration patterns. There are rather important differences between the shapes of the curves for the different individual species. Now in particular, this solid line here is this species of myctopid fish that come right to the surface at night. You can pick one of them up in dip-nets which you put over the side of the ship at night with lights. And it goes down to depths of at least several hundred meters during the day. So it's probably the fish of this group that we looked at here which has the widest range of temperatures, as well as pressures that it gets exposed to during the course of each day. And it has the most stable metabolic rate, or one of the most stable metabolic rates in this group. Another fish which almost never comes close to the surface at all, apparently stays at much lower temperatures all the time, generally in the range below 15°C—seems to regulate its efficient metabolic rate reasonably well at lower temperatures, but shows no control, essentially at the higher temperatures. So there are differences, apparently, in terms of the shapes of the tissues metabolism curves which correlate with the habitat of the animal.

BIOLOGICAL AND OCEANOGRAPHIC PROBLEMS REQUIRING HIGH PRESSURE AQUARIA—

BIOCHEMICAL ASPECTS[1]

Richard Y. Morita
Departments of Microbiology and Oceanography
Oregon State University
Corvallis, Oregon 97331

Introduction

The "state of the art" in the field of high pressure research leaves much to be desired. As a result of this situation, there will naturally be many questions that arise for which there are no answers as yet. The discussion presented here will deal with general concepts of the physiological and biochemical aspects of hydrostatic pressure. For the latest, detailed literature review on the effects of hydrostatic pressure on biological systems, Zimmerman (1970) should be consulted.

All organisms in the marine biosphere live under various levels of hydrostatic pressure. The rule of thumb is to increase the pressure one atm for every 10 m of depth.The average and the deepest depths of the oceans are approximately 3,800 m, and 10,915 m, respectively, equivalent to 380 and to 1091 atm. Actually, the latter is closer to 1,100 atm due to the increased density caused by the compression of the sea water. In the deep sea, the main environmental variable is the pressure. The temperature is more or less constant, varying between approximately 3° and 5°C. Since the temperature is uniformly cold, the test organisms that we are currently employing are termed "obligate psychorophiles," (Morita, 1966) and it is only logical that such organisms be employed in studies dealing with life in the deep sea. Many of our past experiments, as well as those of others, have not been done with the obligate psychrophiles, since their existence in pure culture has only recently been scientifically established. However, many of the past experiments do shed light on the mechanisms of action of hydrostatic pressure. From the Ideal Gas Law ($PV=nRT$) we realize that there is an interaction between pressure, temperature and volume. Molecular volume changes (partial molecule volume changes) are very important in the interpretation of data. This subject will be discussed in more detail by my colleague, Dr. Robert Becker. Salinity is another important variable in the marine environment, and when one throws salt into the above Ideal Gas Law, then things can become extremely complicated. In other words, there are interrelationships between salt effects and pressure (Palmer and Albright,

[1]Published as Special Report No. 315, Oregon Agricultural Experiment Station.

1970), temperature (Stanley and Morita, 1968), and molecular volume (Kettman, *et al.*, 1965).

Complicating Factors Involved in Pressure Research

When one applies pressure to marine organisms, the data are usually interpreted in terms of the pressure applied to the system. However, when doing so, the investigator should always bear in mind that there are many factors to be taken into consideration. Some of these complicating factors are listed in Table 1. The pH changes brought about by increased pressure depend on the buffer system employed. pH changes also take place in sea water and this subject will be discussed later by Disteche. Molecular volume changes caused by hydrophobic bonding or by electrostriction will also be

Table 1

Factors Complicating the Effects of Pressurization on Biological Systems.

pH	Buch and Gripenberg, 1932; Johnson et. al., 1954; Disteche, 1959; Pytkowicz and Conners, 1964.
Ionization of water and water structure	Owen and Brinkley, 1941; Hamann, 1963; Horne and Johnson, 1966 and 1967.
Chemical reaction rates and ionization of various substances (inorganic)	Owen and Brinkley, 1941;Ewald and Hamann, 1956; Disteche and Disteche, 1965; Hamann and Strauss, 1965; Pytkowicz and Fowler, 1967; Pytkowicz et al., 1967.
Aggregation of macromolecules and conformational changes of macromolecules	Linderstrom-Lang and Jacobsen, 1941; Gill Glogovsky, 1955; Kettman, et al, 1965; Murayama, 1966; Josephs and Harrington, 1966 and 1967; TenEyck and Kauzmann, 1967; Kegeles, et al., 1967; Murayama and Hasegawa, 1969; Morita and Becker, 1970.

discussed by Becker. Nevertheless, all the complicating factors, as listed in Table 1, should be taken into consideration.

Life in the Deep Sea

During the Galathea Expedition, life was demonstrated in the various hadal portions of the oceans. This was visually verified by the descents of the bathyscaph *Trieste*. It appears that the number of species existing in the deeper portions of the ocean decreases with increasing pressure. The inquiring mind must ask how these forms exist under the conditions that prevail in the depths of the ocean—especially when it appears that pressure adveresely affects enzyme reaction rates and macromolecular synthesis.

Instrumentation

Although bacteria have been isolated faom the various deeps and trenches (ZoBell and Morita, 1957), these types of organisms still remain an academic curiosity because of the difficulties encountered in isolating them in pure culture, and in obtaining sufficient numbers for biochemical studies. In other words, our instrumentation is not adequate to grow the cells with a constant supply of air and to bleed off the carbon dioxide resulting from respiration. Even after we have grown the cells, we would encounter difficulties because the material would need to be transferred to the pressure reaction vessel for biochemical studies. In other words, a single depressurization step in the entire procedure may allow the biochemical system to change its conformation so that it will no longer resemble the original form under pressure. It is well known that the conformation of macromolecules will change depending on the perturbing forces applied.

As a result of our present types of instrumentation, most of our pressure research is done with forms that can be grown at 1 atm. Such studies will give us some insight as to how pressure can act on metabolic systems.

The various methods presently employed to investigate the effects on microbial systems under pressure are described by Morita (1970).

Species Difference

ZoBell and Johnson (1949), and Oppenheimer and ZoBell (1952), subjected various marine bacteria to various pressures (1,200, 400 and 600 atm) in media. Depending upon the pressure employed, some of the bacteria were killed, some did not multiply, while others were not affected (Table 2). In other studies, ZoBell and Oppenheimer (1950) noted that *Serratia marinorubra* formed long filaments (growth but not reproduction) and this has been noted with other forms subsequent to their studies of 1950 (ZoBell and Cobet, 1964). Why various species within a single genus are different is still not known.

Table 2

Relative turbidity caused by the multiplication of marine bacteria in nutrient broth for six days at 20 C, four days at 30 C, or one day at 40 C at different hydrostatic pressures

(All cultures listed below, except those marked with an asterisk, which failed to grow at 40 C, showed four plus (++++) growth in the controls incubated at normal pressure

CULTURE	300 ATMOSPHERES			400 ATMOSPHERES			500 ATMOSPHERES			600 ATMOSPHERES		
	20 C	30 C	40 C	20 C	30 C	40 C	20 C	30 C	40 C	20 C	30 C	40 C
Achromobacter fischeri	++++	++	*	++	—		—	—		—	—	
Achromobacter harveyi	++++	++++		+	++++	—	—	—	—	—	—	—
Achromobacter thalassius	—	++++	++	—	—	+++	—	—	—	—	—	—
Bacillus abyssus	+	++++	++++	—	++++	++++	—	+	++++	—	—	++++
Bacillus borborokoites	++	++++	++++	—	++++	++++	—	++	++++	—	—	++++
Bacillus cirroflagellosus	++	+++	*	—	++		—	—		—	—	
Bacillus submarinus	++	++++	++++	+	++++	++++	—	++++	++++	—	++++	++++
Bacillus thalassokoites	+++	++++	++++	++	++++	++++	—	++++	++++	—	++++	++++
Flavobacterium okeanokoites	++++	++++	++++	++++	++++	++++	++	++++	++++	—	—	++++
Flavobacterium uliginosum	++++	++++	++++	++++	++++	++++	—	++++	+	—	—	—
Micrococcus infimus	+	++++	*	—	+		—	—		—	—	
Photobacterium splendidum	++++	++++	*	++	++++		—	+		—	—	
Pseudomonas pleomorpha	++	++++	*	++	++++		—	++		—		
Pseudomonas radosa	++	++++	++++	++	++++	++++	—	++++	++++	—	—	++++
Pseudomonas xanthochrus	++++	++++	*	++++	++++	+	++	++	++	+	+	—
Vibrio hyphalus	—	—	*	—	—	—	—	—		—	—	
Mixed microflora from mud	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Reprinted by permission. After ZoBell and Johnson, 1949.

Pressure Effects on Enzyme Reaction Rates

The majority of enzymes studied under pressure are affected adversely. All of the dehydrogenases thus far studied, whether in cell-free form or in intact bacterial cells, display decreased activity with increased pressure (Morita, 1967; ZoBell, 1964). An example of this is shown in Fig. 23. Much of this can be attributed to the molecular volume change of the enzyme. At constant temperature, pressure decreases the molecular volume of the enzyme so that it will not accept the substrate. As a general rule, the enzyme must undergo an increase in molecular volume to be able to accept the substrate if the enzymic reaction is to occur (Laidler, 1951). With this in mind, one begins to wonder how the organisms in the deep sea carry on their metabolic activities, and the questions that naturally come up are the following:

1. Are the enzymes different in deep sea forms compared to those at the surface?
2. If so, how are they different?

The answer to these questions will have to await further studies when instrumentation becomes available.

However, if surface bacterial forms (ZoBell and Johnson, 1949; Oppenheimer and ZoBell, 1952) were investigated as to their pressure tolerance in terms of their enzymes, this investigator would venture to say that they would be very similar in terms of the amino acid composition, temperature characterisitcs, behavior to pressure, etc. The difference between organisms may lie in the particulate enzymes such as those attached to membranes or other cellular structure. Being associated with cellular structures, the enzymes could then undergo concomitant conformational changes, since the associated structure would undergo a conformational

change. For instance, it is known that the mitochondria possess many hydrophobic groups and are responsible for the binding of phosholipids to the protein of the mitrochondrial membranes (Lenaz, *et al.*, 1970), and therefore they would naturally be influenced by both temperature and

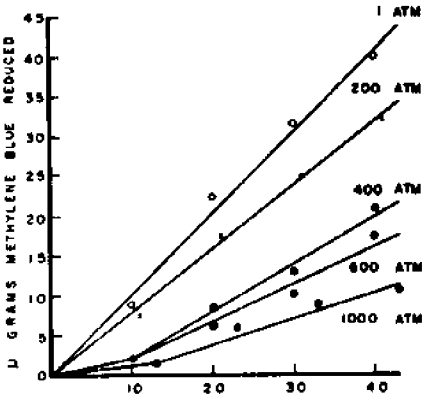

Figure 23. Effect of hydrostatic pressure on oxalosuccinic dehydrogenase activity by mitochondria of *Allomycetes macrogynus*. The reaction was run at room temperature (ca. 26.5°C). All values are corrected for controls. Time in minutes. (After Hill and Morita, 1964).

pressure (see discussion by Becker in this book). If the lipids of the membrane undergo hydrophobic changes due to temperature and/or pressure, it can readily be visualized that the enzymes associated with the mitochondira would undergo certain changes also, or have their active sites hidden. In our laboratory, we have been doing some studies which appear to bear this concept out a little more. When certain bacterial cells (we have not tried all of them) are subject to cold temperature (below its minimum temperature for growth) the cells cease to be permeable to certain of the amino acids. The same phenomenon happens when the cells are subjected to elevated pressures. My guess is that the differences between species with reference to pressure and temperature tolerance lies mainly in the membrane. In other words, membranes possess lipoproteins and the lipid portions are hydrophobic.

Influence of Temperature on Pressure Reactions

There is a temperature-hydrostatic pressure relationship in terms of the maximum temperature for growth under pressure (Table 2) and enzyme reactions under pressure. In the study of pressure effects on luminescence (bacterial bioluminescence), a good correlation between temperature and pressure is noted. An increase in luminescence is noted when the temperature is 35°C (Brown, *et al.*, 1942). At 15°C there is a progressive decrease in luminescence with increased pressure, while at 0°C the decrease in luminescence with increased pressure is very great. At the lower temperature, two forces (pressure and low temperature) combine to bring about a decrease in the molecular volume, hence a rapid decrease in the reaction rate.

This temperature-pressure relationship is further illustrated in the data of Haight and Morita (1962). In Fig. 24, it can readily be seen that between the temperatures of 37° and 45°C, the Q_{10} rule is affecting the reaction at all pressures employed. These data were obtained with washed cells of *Escherichia coli*. Between 45° and 50°C, there is a drop in the reaction rate for the pressures of 100, 200, 300 and 400 atm. However, the reaction rate still increases when the pressure is 500, 600, 700, 800, 900 and 1,000 atm. Between the temperatures of 50° and 53°C, all the reaction rates are decreased with the exception of the 900 and 1,000 atm rates. At 56°C, the highest rate of reaction is shown under a pressure of 1,000 atm and the lowest at 1 atm, indicating the reversal of everything that happens at 37°C. The 1 atm curve in Fig. 24 illustrates the effect of temperature on the system. An increase of temperature from 37° to 45°C results in an increase of activity as one would expect according to Vant Hoft's Law. However, above 45°C, the 1 atm curve shows a decrease in activity with temperature, thereby demonstrating that thermal inactivation has taken place. When the data of Fig. 24 are replotted as shown in Fig. 25 using pressure as the abscissa instead of temperature, one can see the effect of pressure upon the enzyme reaction rates for any given temperature. The 37°, 45° and 50°C curves illustrate the effects of pressure on the reaction rates: the pressure decreases the reaction rate at temperatures near the optimum for the organism, or even at the enzyme's optimum temperature for reaction. However, if the temperature is above the maximum for the reaction mixture at 1 atm, a different picture unfolds. The 53°C curve demonstrates that the effects of temperature are counteracted by pressure equally, and as a result there is no decrease or increase in reaction rates. When the temperature is raised to 56°C, the reaction rate increases with incresed pressure. In other words, we are beginning to see a pressure-temperature interrelationship where the pressure counteracts the effects of temperature so that the enzyme does not undergo inactivation, and therefore can carry out its catalysis at temperatures above its maximum at 1 atm.

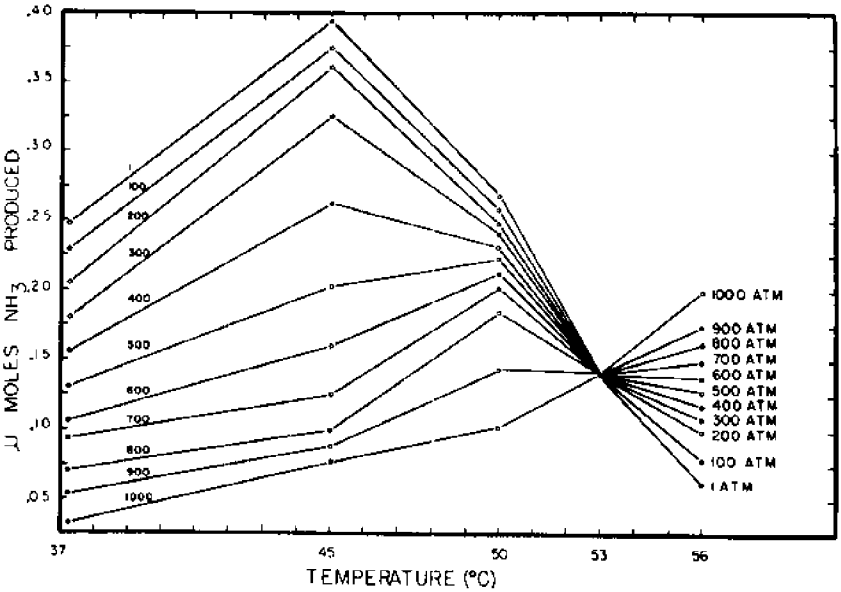

Figure 24. Effect of temperature and pressure on the deamination of L-aspartic acid by washed cells of *Escherichia coli*. (After Haight and Morita, 1962).

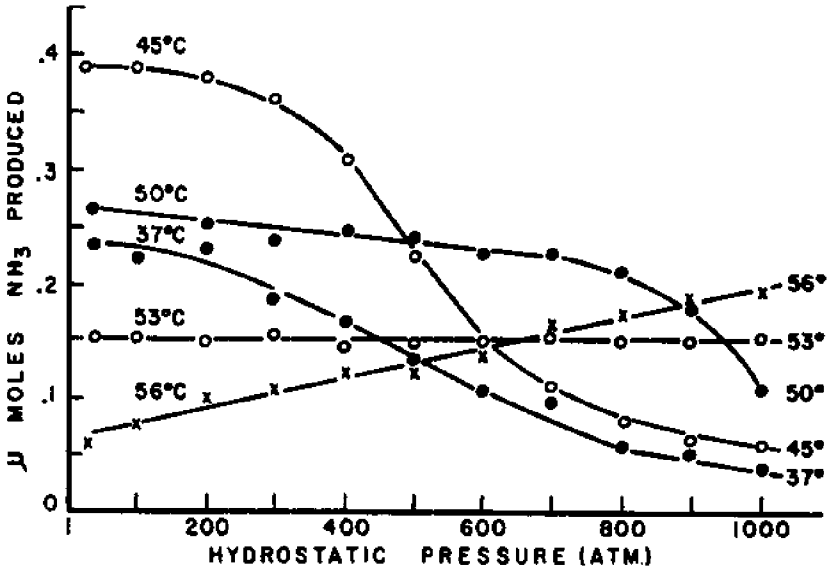

Figure 25. Temperature—pressure effects on the deamination of L-aspartic acid by washed cells of *Escherichia coli*. Replot of Fig. 2, using pressure as the abscissa instead of temperature. (After Haight and Morita, 1962).

Haight and Morita (1962) demonstrated that there is a stimulation of activity above 45°C for the cell-free system, and 53°C for the washed cell preparation. There appears to be a difference between cell-free systems and whole-cell systems which might be explained partially by the fact that in the cell-free system the enzymes are not attached to any particulate substance of the cell. If an examination of the aspartase activity in Fig. 26 illustrates the

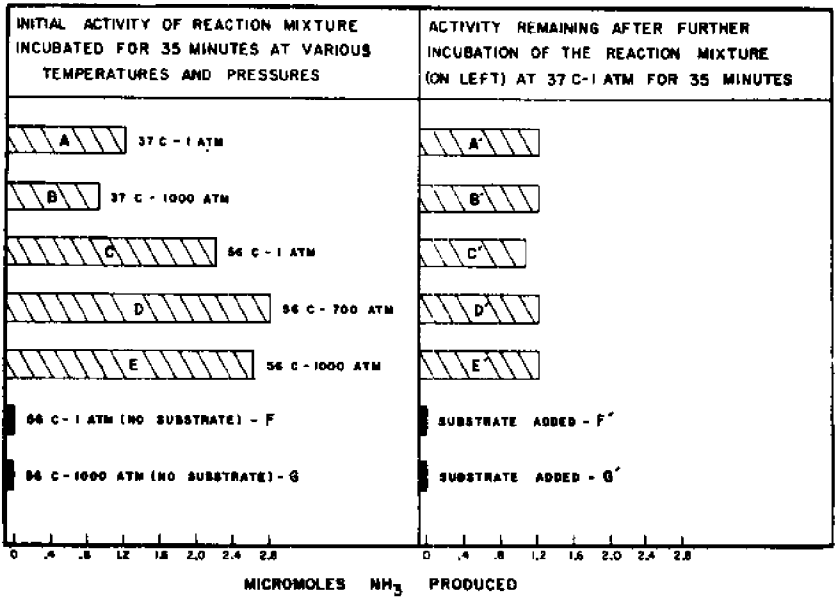

Figure 26. Activity of aspartase remaining after treatment at various hydrostatic pressures and temperatures in the presence and absence of L-aspartic acid. (After Haight and Morita, 1962).

effect of temperature on the inactivation of the enzyme, pressure then can actually counteract this thermal inactivation. There is no substrate limitation in the experiment, and as a result, the amount of ammonia produced at 37°C at one atm and 35 min of incubation is represented by Bar A of Fig. 26. Further incubation of the same reaction mixture under identical conditions for an additional 35 min produces approximately the same level of ammonium as indicated by Bar A'. Bar A' indicates that there is no inactivation of the enzyme during the total 70-min incubation period. However, at 37°C and 1,000 atm, the reaction does not take place so readily as indicated in Bar A of Fig. 26. However, Bar B' is equal to Bar A', indicating that the enzyme has not undergone any inactivation due to the pressure to which it was subjected. From Fig. 24 and Fig. 25, we recognize that temperature of 56°C does inactivate the enzyme. Bar C represents a temperature of 56°C and one atm and indicates the amount of aspartase

activity is quite great in the first 35 min. Further incubation of the same reaction mixture indicates that the enzyme does undergo thermal inactivation when subjected to 56°C at one atm for a total of 70 min., which is represented in Bar C'. Bar D, which is a reaction mixture at 56°C and 700 atm, does illustrate that it is much better than Bar C, indicating that within the first 35 min the pressure has prevented the enzyme from undergoing inactivation. And as a result, when the pressure is released Bar D has the same amount of activity as Bar A and B. The same can be said for Bar E and Bar F. In other words, in Bar C, for the first 35 min of incubation there is some inactivation of the enzyme taking place, which is further illustrated by Bar C'. This thermal protection by pressure is not noted unless the substrate is present in the reaction mixture (Fig. 26 F and F' and G and G').

This concept can be carried further, and from an academic viewpoint we have checked to see whether or not we could make an enzyme reaction take place above 100°C. Fig. 27 illustrates the effect of pressure on the malic dehydrogenase activity (Morita ahd Haight, 1962). The 56°C, one-atm curve in Fig. 27 illustrates an optimal condition for the malic dehydrogenase taken from a thermophile. However, reaction does take place at 101°C at 1300 atm. No activity can be seen at 101°C at one atm. Again, the data illustrate that pressure can counteract the effects of temperature on an enzyme. A pressure of 1300 atm does not allow the enzyme to undergo complete thermal inactivation. Since complete thermal inactivation is not brought about, there is reaction at 101°C; whereas, at 101°C at one atm, no reaction can be detected. Further evidence of this temperature-pressure relationship is illustrated by Morita and Mathemeier (1964). In this case, inorganic pyrophosphotase activity was shown to take place at 105°C. However, in this study, it appears that the metal cofactor is more important than the substrate in protecting the enzyme against heat inactivation when pressure is applied to the system. Morita and Haight (1962) found that substrate was essential in the protection of the enzyme against thermal inactivation at elevated pressure. However, another important fact should be recognized: When enzymes are subjected to elevated temperatures the cofactor can change. With inorganic pyrophosphotase, cobalt replaces manganese as the cofactor when temperatures above 80C are used (Mathemeier and Morita, 1964). Whether or not there is an inner change of cofactors when pressure is applied to the system is still not known. Harold Evans, on our campus, is investigating whether or not sodium or potassium can be interchanged in sodium and potassium activated ATP-ase under pressure.

The temperature at which bacterial cells are grown does influence the action of pressure in intact cells. For his master's thesis, Albright (M.S. Thesis, Oregon State University, Corvallis, Oregon; Albright and Morita 1965), studied the effect of pressure on *V. marinus* MP-1 cells grown at two different temperatures, harvested and then tested at two different tempera-

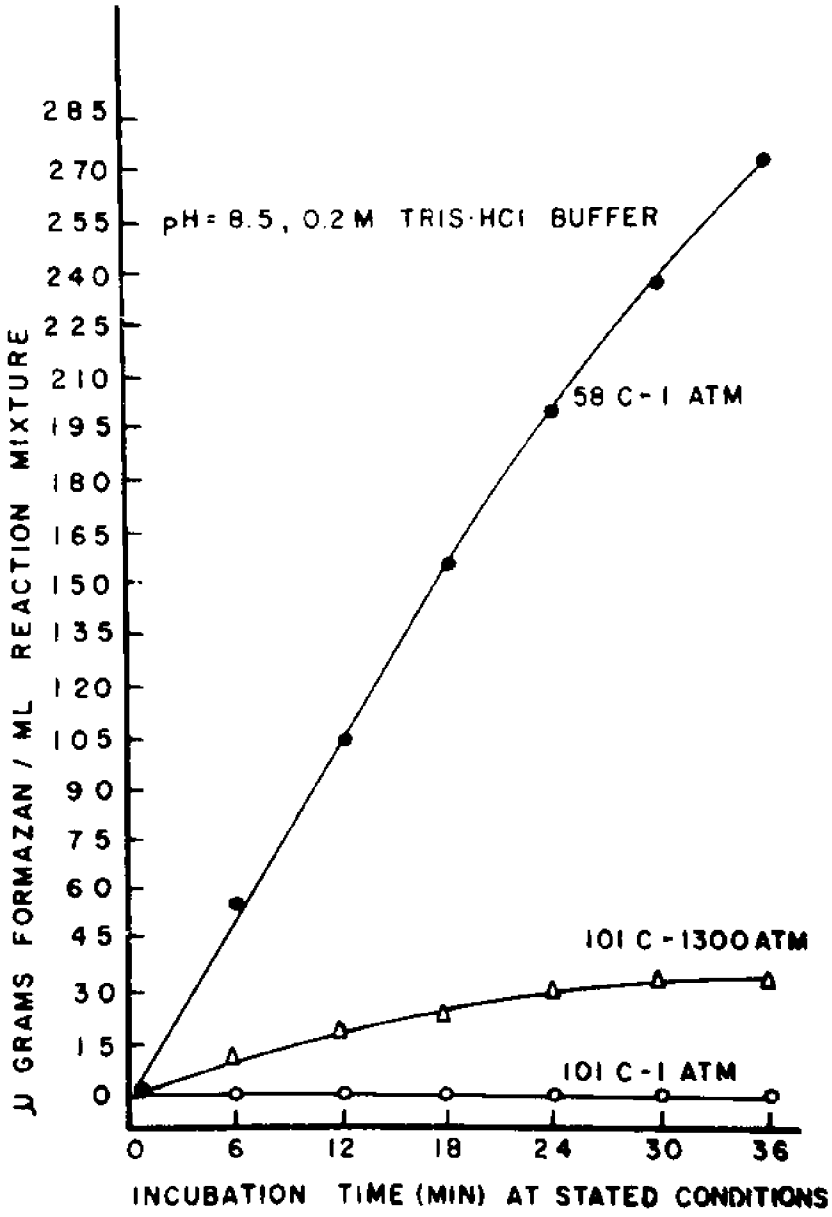

Figure 27. Rate of malic dehydrogenase activity. The curve of 101°C and 1,300 atm is corrected for the 6 min. period required for the initial pressure to reach the final pressure. Values are corrected for controls. (After Morita and Haight, 1962).

tures. In Fig. 28 the effect of hydrostatic pressure on the deamination of L-serine by cells of *V. marinus* raised at 15°C is shown. When the reaction mixture is tested at 15°C, there is an optimal activity at approximately 300 atm and a decrease in activity with increased pressure. On the other hand, when the 15°C-grown cells are tested at a temperature of 4°C, no optimum is observed when pressure is added to the system. As a result, there is a linear

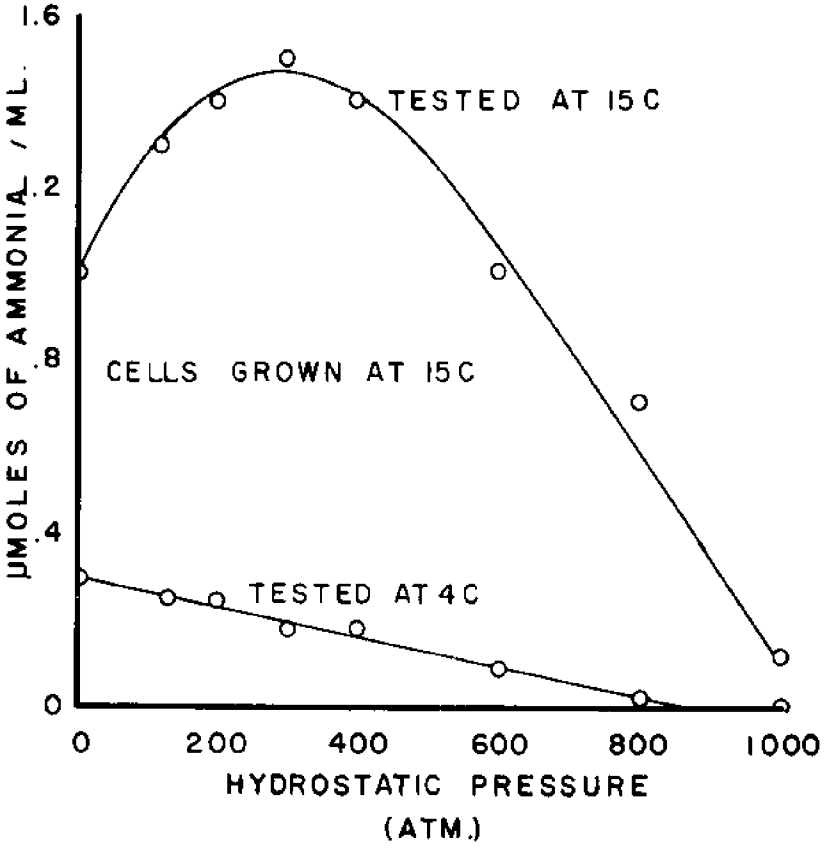

Figure 28. The effect of hydrostatic pressure on deamination of L-serine by 15°C-grown washed cells of *V. marinus*.

drop of L-serine deaminase activity with increased pressure. In Fig. 28 and 29, the effect of pressure is shown on cells grown at 4°C. When these cells are tested at 15°C at various hydrostatic pressures, again it is noted that there is an optimum activity at approximately 300 atm. When 4°C-grown cells are placed in a reaction mixture which is subjected to 4°C-grown, we also see an optimum peak of activity. However, this optimum activity occurs at approximately 150 atm.

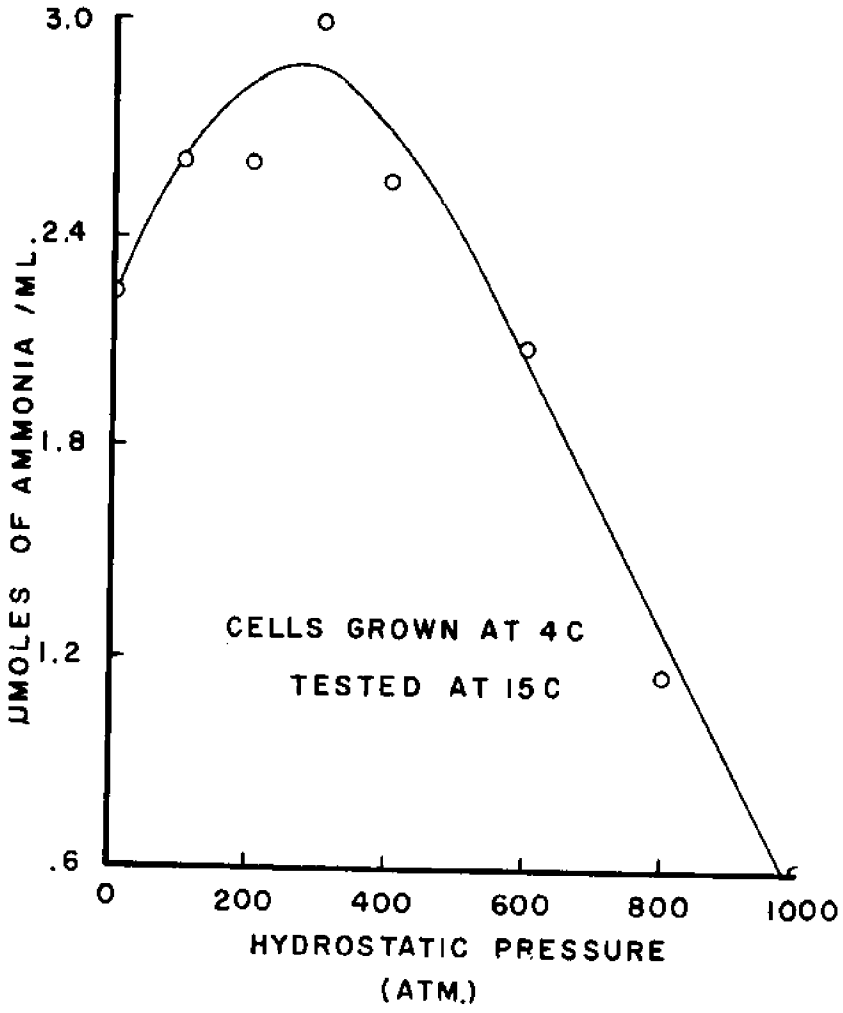

Figure 29. The effect of hydrostatic pressure on the deamination of L-serine by 4°C-grown washed cells of *V. marinus*. Incubation period was 1 hour at 15°C at various hydrostatic pressures.

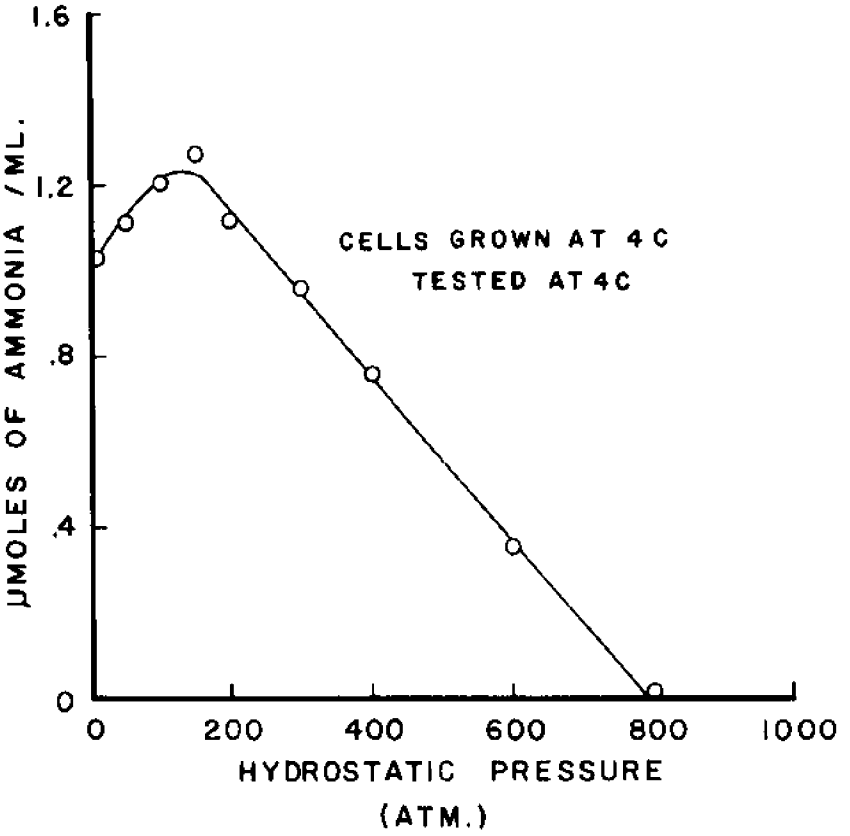

Figure 30. The effect of hydrostatic pressure on the deamination of L-serine by 4°C-grown washed cells of *V. marinus*. Incubation period was 1 hour at 4°C at various hydrostatic pressures.

The above concept applies not only to bacterial cells but to other organisms. Brown (1934) noted this phenomenon when working with muscle contraction on the pectoral fin of the red grouper *(Epinephelus mario)*. This concept should not be overlooked when animals are observed in high pressure aquaria. In other words, the temperature from which the animal is taken will govern the pressure response of the organism. In Albright's studies with L-serine deaminase, whole cells were employed. If the L-serine deaminases isolated from cells grown at 15 C and at 4 C were compared, in all probability the enzymes would react similarly to temperature and pressure.This investigator would again venture to state that one of the main reasons why intact cells function differently when grown at two different temperatures and subjected to various temperatures of reaction is that membrane permeability probably played a great role. In this case, it

may be that the ability of the serine to be transported into the cells through the membrane of 4°C-grown cells is different than that of 15°C-grown cells.

Synthesis of Macromolecules

The synthesis of protein RNA and DNA was commenced in our laboratory by L.J. Albright (Albright and Morita, 1968). It is well recognized that pressure does inhibit the synthesis of these macromolecules and, generally, the greater the pressure applied, the greater the decrease in synthesis of these molecules. Since both Landau and Albright are here, I think it is best to allow them to present their investigations on this subject matter.

ACKNOWLEDGMENTS

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HYDROSTATIC PRESSURE INHIBITION OF RIBONUCLEIC ACID SYNTHESIS IN HELA CELLS

by
J. V. Landau
Rensselaer Polytechnic Institute
Department of Biology

It has been shown that protein synthesis in bacterial systems as well as HeLa cells (Landau, 1970) is affected in a precise predictable manner by the application of hydrostatic pressure. Similar responses have been recorded for the synthesis of ribonucleic acid (RNA). The data on HeLa cells indicate that pressure effects on RNA synthesis differ in magnitude from those on protein synthesis. Figure 31 indicates the effect of increasing pressures on the incorporation of ^{14}C uridine into the RNA of HeLa cells at 37°C. The inhibition of such incorporation is completely reversible upon release of pressure. When the data are plotted as in Figure 32, i.e., incorporation over a 10-min period against a specific pressure, it may be seen that a bimodal curve is presented at 37°C. However, should the experimental temperature be decreased to 22°C a straight line inhibition is indicated and this line parallels the larger portion of the 37°C inhibition. It would seem probable, therefore, except for a temperature interdiction at the lower pressures at 37°C, that the inhibitory effect of pressure on RNA synthesis may be expressed by the slope of the major portion of the curve.

The question is then posed as to exactly what this straight-line inhibition is indicative of. Is it possible that pressure prevents the entry of the labelled uridine into the cell? Is it possible that pressure prevents the phosphorylation of uridine, thus preventing its incorporation into RNA? Does pressure interfere with the action of RNA polymerase or other factors directly involved in the transcription process? Experiments were undertaken to answer the first two of the three questions posed.

In the first series of experiments ^{14}C uridine was added to the cells at 37°C after a pressure of 670 atm had been applied. The cells were kept at this pressure and temperature for 10 min and the entire chamber chilled to a point where the cells were at 1°C before pressure was released. The chilling process took about two min. The cells were then collected and washed several times by centrifugation in the cold. Finally the cell pellet was treated with cold perchloric acid. The supernatant, after neutralization, was then assayed for radioactivity. An atmospheric pressure control was run concurrently, the procedure being identical except for the application of pressure. Also, a low temperature control experiment was performed to indicate whether the uridine would enter the cells after release of pressure at

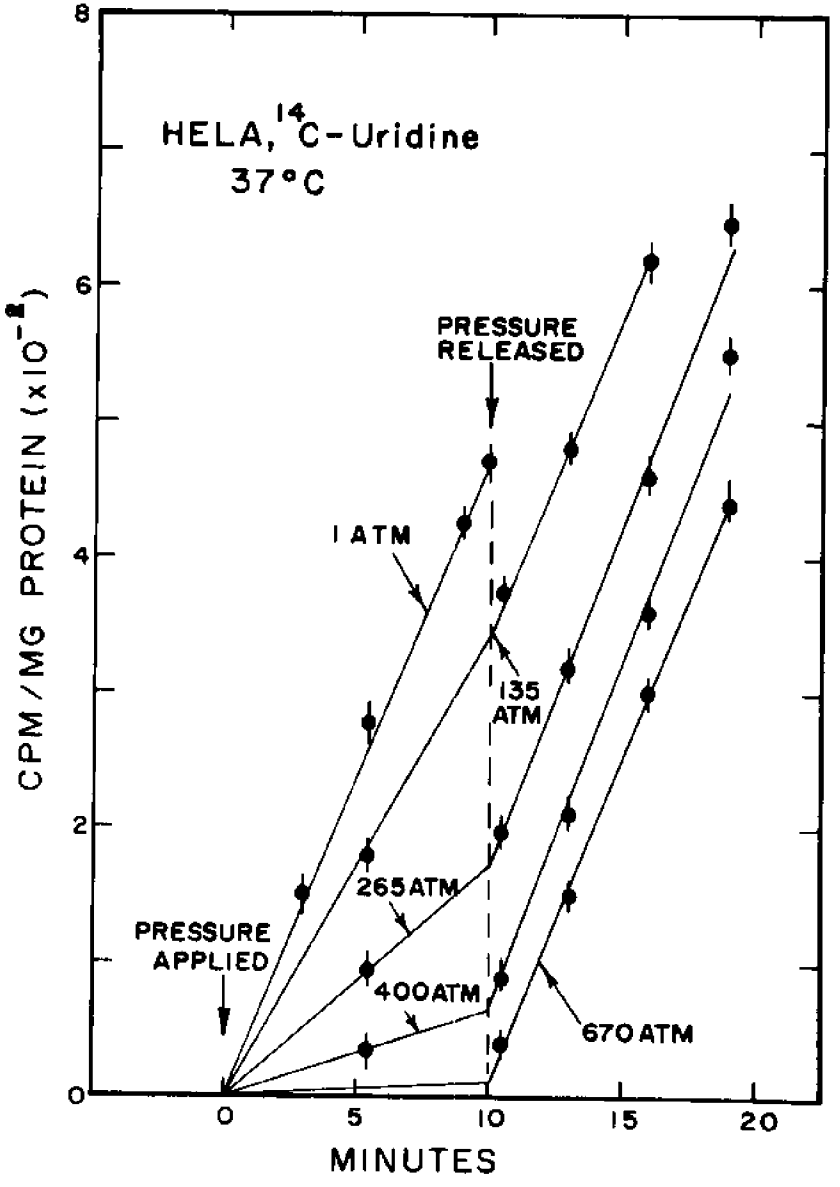

Figure 31. The incorporation of ^{14}C uridine into RNA as a result of pressure application at 37°C. A normal rate of incorporation is resumed immediately on release of pressure (Landau, 1970).

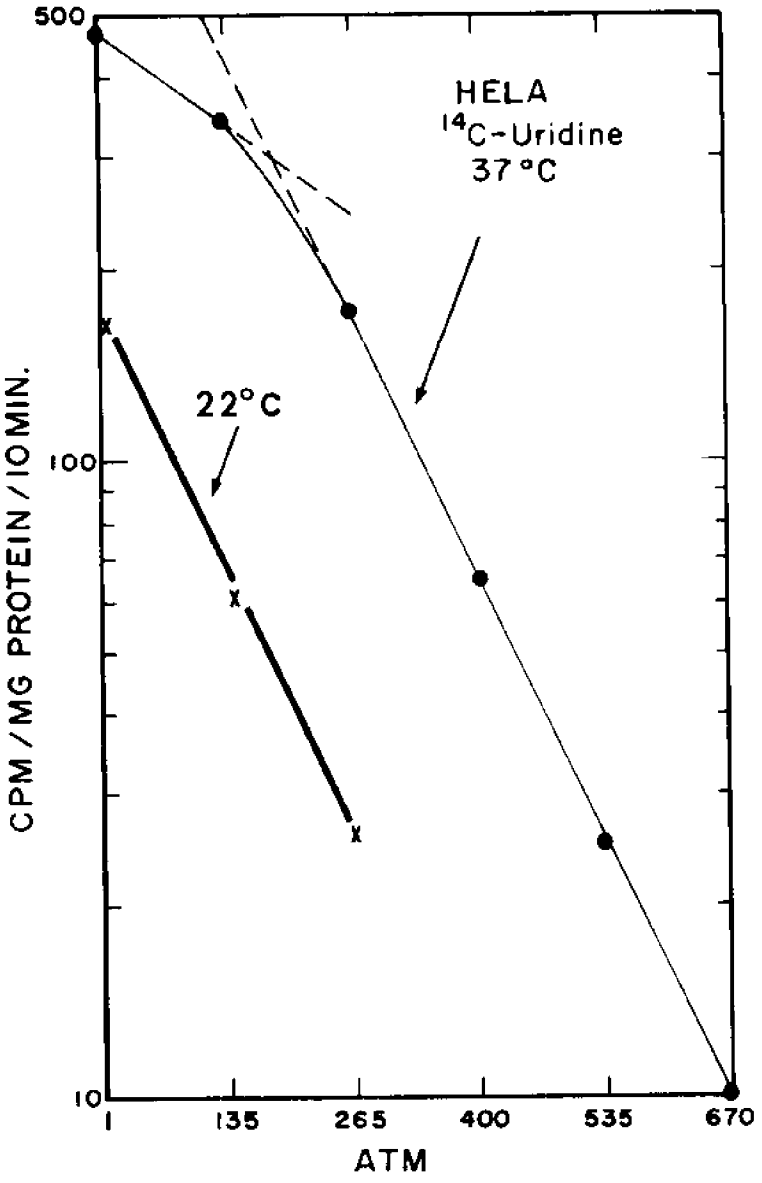

Figure 32. The effect of pressure on the rate of ^{14}C uridine incorporation at 37°C and 22°C. Note the change in character of the curve at 22°C and the parallel nature of the main portion at both temperatures.

1°C, or during centrifugation. The results indicated that 1) the technique was valid, i.e., only a negligible amount of uridine entered the cells at 1°C, 2) the acid soluble content of the cells contained the same amount of ^{14}C uridine whether addition was at 670 or 1 atm. Therefore it would seem that the almost total inhibition of uridine incorporation into the RNA of the HeLa cell at 670 atm and 37°C could not be attributed to a decrease in the entry of the uridine.

In a second series of experiments, the aforementioned acid soluble fraction was placed on a Dowex 1 X 8 - 400 mesh column and eluted with hydrochloric acid for identification of the presence of phosphorylated uridine. The procedure was tested utilizing purchased uridine, UMP, UDP, and UTP as markers to indicate the pattern of elution. Uridine has no affinity for the resin and is eluted by water. The elution pattern for the nucleotides was as shown in Figure 33. The data on the HeLa cell extracts

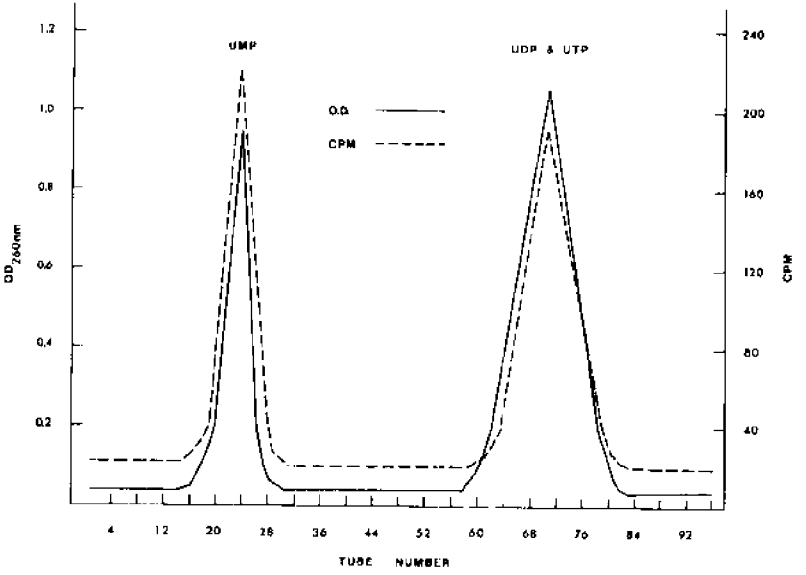

Figure 33. The identification of the acid soluble HeLa cell component as nucleotide. The elution patterns are identical for both pressure treated cells and atmospheric controls.

indicated that 1) most, if not all, of the label within the acid soluble extract is in the nucleotide form, since water elution yielded negligible radioactivity, and 2) the amount of phosphorylated ^{14}C uridine is identical in both the cells subjected to 670 atm and those kept at atmospheric pressure. Figure 33 shows the same radioactivity pattern for both atmospheric controls and pressurized specimens (since they are identical) and the marker optical

density patterns. Therefore it can be stated that pressure does not inhibit RNA synthesis as a result of interference with a nucleotide phosporylating mechanism.

The answer to the third question posed, that of how pressure might directly affect the process of transcription, requires a somewhat more sophisticated approach and is now being actively pursued.

ACKNOWLEDGMENTS.

The author wishes to express his appreciation to Paul Levine, Wm. Wetzel, Paul Schneider and Ronald Scheinzeit for their invaluable laboratory assistance in various phases of this work.

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REFERENCE:

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Discussion Following Landau Paper.

(Question): How did you prepare your profiles—by centrifugation or by column techniques? Centrifugation would probably produce in itself pressures as great as 200 atm.

LANDAU: That is true, but we have tested that and the profiles are no different. In fact, we add the salt after the centrifugation and all of this would affect control and pressurized material in the same way. This of course raises the general point that a lot of our biochemical techniques include a centrifugation stage and fail to prove categorically how this affects our experiments. We thought some time ago of doing the control experiments for this but have not yet.

ZIMMERMAN: I just wanted to mention another well known deep sea form besides the *E. coli* — the tetrahymena. This unusual organism seems to contradict my colleagues who work with bacteria. It has been documented that the polysome patterns are markedly altered when cells were subjected to pressures as low as 5,000 psi. Furthermore, it has been recently reported that the ribosomes from cells subjected to very high pressures have the competence to synthesize polyphenylalanine as readily as those from cells at atmospheric pressure. So that here obviously there are species differences, as Morita pointed out earlier—here there is a completely different system where the microsomes are good from pressurized cells yet the polysomes can be disorganized by high pressure *in vivo*.

(Question): This is interesting. If now we look at marine bacteria, and that in itself is quite a mess—in very simple experiments—such as incorporation

of radioactivity, incorporation into protein—you find that the TCA washes that one normally usually calls on are quite inadequate. When you change the washes, you change all your techniques.

WELLS: Very briefly, I would like to consider pressure-related changes in the chemical environment of some of the reactions that we have been talking about, and such secondary effects on kinetics and equilibra. The activity of many of the components of this environment has a direct bearing on what the molecules we're looking at actually do—pH, of course, is an obvious one. The magnitude of the pressure induced change in pH in a system is determined by both the nature of the buffer system and by the contribution of components of the macromolecular themselves.

Or perhaps even greater concern here is the pressure induced change in gas activity in liquid phases. The decrease in the solubility coefficient of gases with increasing pressure would bring about a condition in gas consuming or producing systems which would drastically alter the slope of a gas partial pressure versus time curve describing this system. Gradients within the gills of fish or the time course of oxygen pressure in photosynthesis or respiration measuring systems are examples. Such a change in oxygen gradient along the gills might in fact require some biochemical adaptation of the respiratory pigments of deep sea fish. It is well known that environmental conditions of temperature, pO_2, and pCO_2 alter the properties of respiratory pigments. Why not pressure? So, whenever we apply pressure to a system, we do more than simply squeeze it, and in many of these cases where we saw hydrostatic pressure along the horizontal axis of a graph, we might even forget pressure as such, and plot something like the pressure induced change in pH or pO_2. I am quite sure that some of the "pressure effects" we are looking at are really secondary effects of pressure acting on the chemical environment.

VIDAVER: Wells here has said something, which for a simple physiologist like myself, I find very puzzling. The accent is on lower solubility of oxygen at high pressures. I understand there is less oxygen at lower depths. I'm not quite sure, but the problem I always have had is one of keeping oxygen out of my physiological experiments because of the increased solubility under pressure. Can you clarify this for me?

WELLS: According to Henry's law $A(C = \alpha_p)$ gas concentration is equal to the solubility coefficient times the partial pressure of the gas and in a closed liquid system, naturally the gas content cannot change. Enns, Scholander, and Bradstreet have shown that the equation $A \mid_N (p_1/p_2) = \Delta\bar{V} \dfrac{(P_1 - P_2)}{RT}$ holds very well up to pressures of 100 atm. p_1 and p_2 are the gas partial pressures at hydrostatic pressures P_1 (atmospheric) and at a P_2 (elevated), V is the partial molal volume of oxygen in water (32 cc/mole). For oxygen, an increase in hydrostatic pressure of 100 atm. causes an increase in $p^{O}2$ of 14%, or the

pO_2 of air equilibrated water pressurized to 1,000 atm. would be approximately 1 atm. of O_2. These are rather large changes, and must be considered in any system in which gas partial pressures are important.
BRAUER: The experiment which Vidaver wants to envision but doesn't quite is the experiment in which we have the very high pressure with a stabilized gas space in which there could also be a low partial pressure of oxygen. And, of course, this can be done in the type of experiment where you stabilize a cavity mechanically, for instance by a catheter, so that the hydrostatic pressure is not seen by the gas phase. Now you get just what Wells is describing, namely, the fact that as you raise the hydrostatic pressure, the partial pressure of oxygen that is sustained by a given concentration of oxygen will have to be lowered.
MENZIES: I have a question relative to pH. Measurements that have been made of pH in great depth have suggested very little change in pH, under high pressure.
DISTECHE: It depends on the buffer system you are using. I saw a report that some people use tris buffer. Well, the effect of pressure on tris buffer is completely atypical. On the other hand, if you use phosphate buffer when come to 1,000 atm., you get 0.3 decrease of pH. It all depends on the type of buffer system you use.
MENZIES: What happens when you use sea water?
DISTECHE: You get a decrease of about 0.2 and 0.25 pH units, because this system is governed by the equilibrium of carbon dioxide/bicarbonate/carbonate at high pressure.
GORDON: There are a couple of additional factors which are worth mentioning, besides the purely biochemical ones mentioned by Morita and the discussants. This relates to work that I personally have been involved with and also especially worked by Hochachka of the University of British Columbia, largely on the *Alpha Helix* in last years cruises to the Guadalupe Islands in February and March and to Labrador. Fairly extensive pressure measurements have been done on known purified enzyme preparations from both shallow water and deep sea fishes using equipment borrowed in part from Morita. It turns out that the activity is a variant modulator, or very important in terms of determining the pressure effects of particular enzymes. In other words, you not only have, say, changes of enzymes, from pressure inactivation by the presence of substrate, and changes in the cofactors, as you mentioned, but also by the modulators which apparently structurally modify the active site in some way. Hochachka has found very clearly that enzymes that are isolated, particularly glycolytic enzymes, are quite different in their responses between shallow water things and deep sea things. These "things" we're talking about now are largely *Micrurus*, rat-tail fishes, brought up from depths between 1,000 and 2,000 m with free-vehicle long lines. Another thing that is probably also generally

important is isozymes. The multiple enzyme forms that occur in different tissues of the same animal, are very different in terms of their pressure sensitivities, and the nature of their responses to high pressure. And then, finally, there are clear species differences. My personal interest in this has been not in purified enzyme preparations but in rather crude tissue preparations in the hope that some of these may show a little more about what actually happens in the tissue as it exists in the organism. We've been measuring oxygen consumption rates against pressure in a couple of different tissues—light muscle and liver in various fishes, and I'll just discuss two graphs that come from two different tissues from fishes from two different areas. One from the liver of a surgeon fish in Hawaii. This is a shallow water, reef living fish. The other one is a curve, which comes from light muscle, from a bathypelagic sea bass - a very unusual fish, but very common in the Galapagos. For the surgeon fish, you find that at 25°C—very close to the environmental temperature at which the fish has been living for a long time—there is a very strong pressure inactivation, or pressure inhibition of activity, whereas at 15°C and 5°C if there is any inhibition, it is very slight. With the light muscle from the sea bass, a mesopelagic and probably a very migratory animal (though we really don't know a great deal about it except that it lives in depths of at least 300 or 400 meters), at 25°C there is possibly a very slight activation, while at 15°C there is a very striking activation, and at 5°C there is again only a very slight effect. This fish came from depths where the water temperature was about 12°C.

BRAUER: How do you control the PO_2 in your systems?

GORDON: We don't - we are measuring changes in PO_2 with time, using an O_2 electrode.

BERGER:Several approaches *have* been employed to aerate cultures held under increased hydrostatic pressures. Heden sparged cultures with gas mixtures containing low concentrations of oxygen in aquaria held at pressures up to about 50 atm. ZoBell suggested the use of fluorohydrocarbon fluids in contact with culture fluids as a means to supply additional oxygen to cultures since the solubility of oxygen in fluorocarbon compounds greatly exceeds that of aqueous solutions. We have used silicone membranes to separate cultures from the aqueous external hydraulic fluid. Because of the high permeability of gases through these membranes, the hydraulic fluid has been used as an external reservoir for additional oxygen to and excess carbon dioxide from the cultures. (Limnol. & Oceanogr *15*, 483-485, (1970).

Another technical difficulty is that of mixing several components rapidly inside the pressure aquarium. Landau and later Boatman used two chambers separated by a thin glass cover-slip; one chamber contained the biological specimen, the other contained fixative. Inversion of the apparatus caused a pointed weight inside the lower chamber to rupture the cover-slip between

the two liquids. This method is not amenable to miniaturization: too great a force would be required to break the cover-slip. For separating enzyme and substrate solutions we have employed 8 and 10mm diameter chambers containing 0.5cc or more fluids and separated by a stretched, thin, latex membrane cut from contraceptive condoms. The membrane is ruptured after inversion of the apparatus by the free-fall of a short, weighted, disposable hypodermic needle inside the lower chamber. One defect of this system is that repeated inversions of the apparatus is required to effect thorough mixing of the small volumes.

Landau has suggested an alternative method which appears superior to all previous ones. Landau suggests that aqueous component solutions can be separated from each other in a single tube by a thin layer of inert silicone or fluorohydrocarbon fluid whose density approximates those of the aqueous solutions. Displacement of a small weight by inversion of the tube would effect mixing of the two components. This method could be scaled to most any volume and be used in a large variety of containers.

HYDROSTATIC PRESSURE EFFECTS UPON PROTEIN SYNTHESIS BY *ESCHERICHIA COLI* AND *VIBRIO MARINUS*

Lawrence J. Albright
Department of Biological Sciences
Simon Fraser University
Burnaby 2, British Columbia
Canada

The majority of the marine environment is characterized by relatively low temperatures of 2° to 5° C, an average salinity of 35‰ and hydrostatic pressures varying from 1 atm at the seawater surface to approximately 1,160 atm at 10,915 m, the deepest known part of the oceans (8). Within this environment, non-barophilic microbes are present but are unable to grow and metabolize at pressures equivalent to those of the ocean depths whereas many bacteria isolated from material removed from the ocean depths are barophilic and cannot withstand the lower pressures of the upper layers of the oceans (16). The adverse effects of increased pressures on non-barophiles and decreased pressures on barophiles have been ascribed to several factors, among them, pressure effects on: enzymic activities (16, 20), cell division (1, 20), cell wall synthesis (6), premeability (4,7) and synthesis of macromolecules such as protein, RNA and DNA (1, 5, 12, 19).

The hydrostatic pressure tolerance of many bacteria (both terrestrial and marine) is greatly influenced by physico-chemical factors of the environment such as temperature, salinity and catabolites present.

The interaction of temperature and hydrostatic pressure has been observed with many bacteria and their sub-cellular systems (3, 10, 11, 21). Within limits, increased temperatures generally increase the tolerance of an organism or enzyme to pressure whereas lowered temperatures tend to increase the sensitivity of a system to pressure. All biological systems do not display this phenomenon however (9).

The effect of salinity and various salts upon the pressure tolerance of several non-barophilic terrestrial and marine bacteria has recently been studied (2, 15, 18). A salinity-pressure response is noted with the marine bacteria *Achromobacter aquamarinus*, *Micrococcus sedentarius*, *Serratia marinorubra*, *Vibrio adaptatus* (Table 1) and *Vibrio marinus* (Fig. 34). The pressure tolerances of these marine bacteria increase with increased salinities of the growth medium (Table 1). The maximum hydrostatic pressure for growth of *V. marinus* at 9°C is ca. 420 atm in a medium containing 35‰ seawater salts and ca. 50 atm in a similar broth of salinity 2.5‰ (Fig. 34). The organism also displays a salinity-pressure-temperature interaction since at a lower temperature of 4°C it does not grow above pressures of ca. 275 and ca. 40 atm in a similar medium containing 35‰ and 2.5‰ seawater salts respectively (Fig. 34).

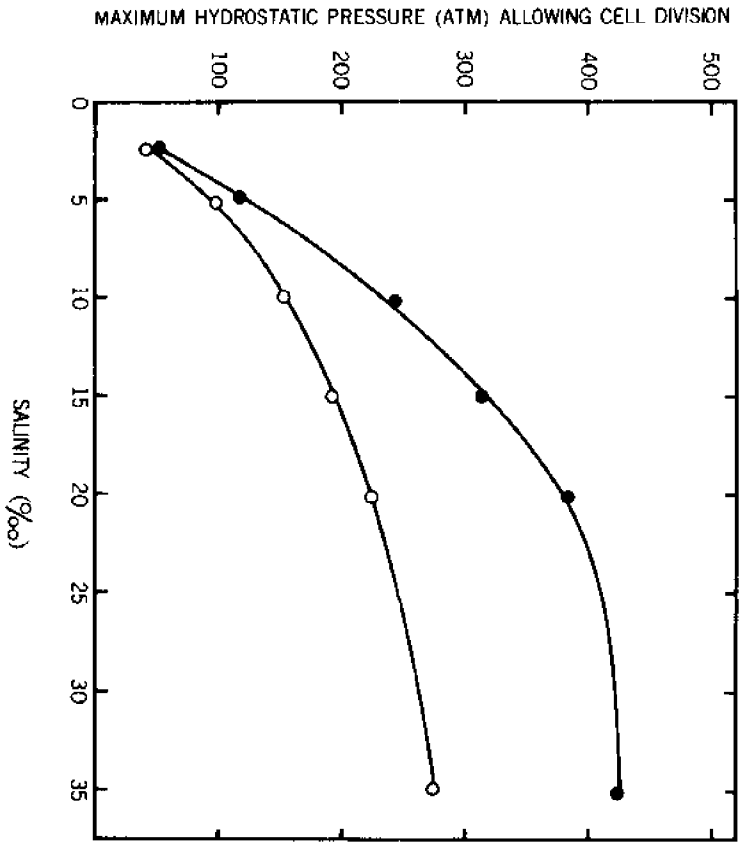

Figure 34. The effect of concentration of seawater salts and temperature on the maxium hydrostatic pressure at which cells of *Vibrio marinus* MP-1 divide. Closed circles indicate incubation temperature of 9 C; open circles indicate incubation temperature of 4 C. From Palmer and Albright, 1970.

Table 1.

Maximum hydrostatic pressure (atm) at which cell division occurs in the defined medium of MacLeod (13) containing the indicated concentrations of inorganic salts.**

Bacterium	Concentration of inorganic salts 35°/₀₀	10°/₀₀	3°/₀₀
Achromobacter aquamarinus	500	350	100
Micrococcus sedentarius	475	—*	—*
Serratia marinorubra	500	425	—*
Vibrio adaptatus	550	350	300
Escherichia coli	300	500	500
Micrococcus luteus	450	400	300

*These cells grew very poorly at 1 atm in the defined medium containing this salt concentration, precluding an assay of cell division under pressure.

**From Albright and Henigman, 1971.

The two terrestrial bacteria tested, *Escherichia coli* and *Micrococcus luteus*, display a variable response to increased pressures as the salinity of the medium increases (Table 1). The growth of the terrestrial bacterium *Streptococcus faecalis* under pressure is influenced by the salt concentration of the medium also. The major cations of seawater responsible for this effect are Mg^{++} and Ca^{++} (15).

Medium catabolites have also been shown to influence the pressure tolerance of bacteria. Marquis, Brown and Fenn (14) have shown that the pressure tolerance of *S. faecalis* is influenced by the catabolites of the medium. These authors attribute this phenomenon to the use by *S. faecalis* of different catabolic pathways depending upon the nutrient metabolized. Each pathway has a unique sensitivity to pressure.

For several years investigations in this laboratory have been interested in the reasons why non-barophilic bacteria cannot grow at increased pressures. In particular, we have been interested in why the terrestrial *E. coli* B/r in nutrient broth at 25°C and the marine *V. marinus* MP-1 in broth containing 35°/₀₀ synthetic seawater salts at 9°C cannot grow above ca. 500 and 420 atm respectively. We are studying this problem with respect to the synthesis of

protein, RNA and DNA by pressurized cells and cell-free extracts of these two species.

The application of 544 atm (8,000 psi) to exponentially growing cells of both *E. coli* B/r (33°C) and *V. marinus* MP-1 (15°C) causes no cellular death within 90 min but slows the rates of cell division and mass synthesis to 3 and 6% respectively of the 1 atm rates (Fig. 35). The synthetic pattern

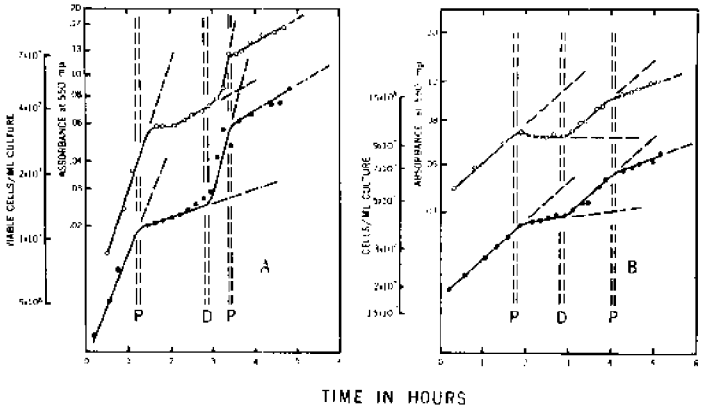

Figure 35. The effect of the alternate application of hydrostatic pressures of 1 and 544 atm upon cell division (solid circles) and culture absorbance (open circles) of (A) *Escherichia coli* B/r and (B) *Vibrio marinus* MP-1. P and D indicate 544 and 1-atm pressure application respectively. From Albright, 1969.

of net protein and RNA synthesis shows a similar response to applied pressure (Fig. 36). The lowering of these synthetic rates is almost immediate (but for net RNA synthesis by *E. coli* B/r) and reversible since both cultures resume synthesis at the pre-pressurization 1 atm rates shortly after depressurization to 1 atm. A second application of 544 atm elicits a similar response. Net DNA synthesis by both cultures slows and ceases with time (Fig. 36). The order of pressure sensitivity thus appears to be protein>RNA>DNA synthesis. Landau (12) found that pressure affects galactosidase synthesis by *E. coli* ML-3 in the order of induction>translation>transcription whereas Pollard and Weller (19) the pressure sensitivity of *E. coli* 15TL to be protein>DNA>RNAsynthesis.

Since protein synthesis appears to be the most sensitive to pressure, in these cells, we are studying the response of the cell-free protein synthetic systems of both *E. coli* B/r and *V. marinus* MP-1 to pressure. We feel that increased hydrostatic pressures may slow or stop protein synthesis at one or several levels of translation: (1) aminoacylation of tRNAs, (2) formation of

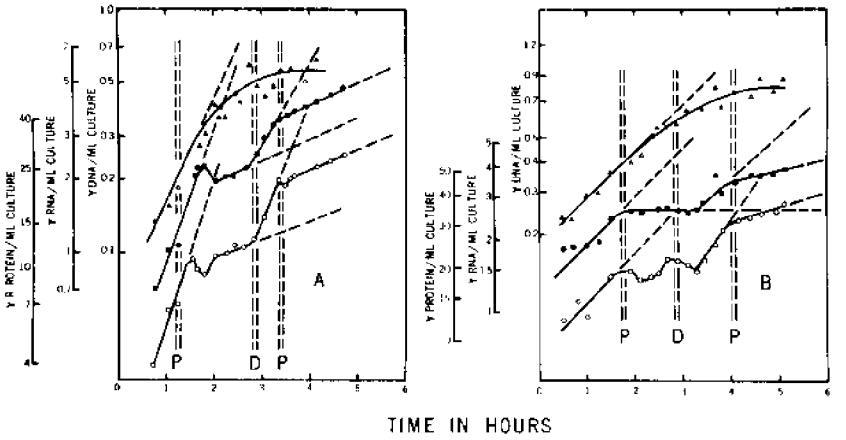

Figure 36. The effect of the alternate application of hydrostatic pressures of 1 and 644 atm upon net protein (open circles), RNA (solid circles), and DNA (triangles) synthesis by (A) *Escherichia coli* B/r and (B) *Vibrio marinus* MP-1. P and D indicate 544 and 1 atm pressure application respectively. From Albright, 1969.

the mRNA-ribosome complex, (3) formation of the aminoacyl-tRNA-mRNA-ribosome complex, (4) formation of the peptide bond and (5) alteration of the fidelity of translation, to name several.

Hydrostatic pressures between 1 and 1000 atm alter the rate of aminoacylation of phenylalanine tRNA, at 33°C, in a partially purified cell-free preparation of *E. coli* B/r (17). However, at 544 atm the level of acvity is approximately 70% that of the 1 atm control. The acitivity of *V. marinus* MP-1 phenylalanyl synthetase at 15°C and at 544 atm is ca. 45% of the activity at 1 atm (17). Since these rates of 70 and 45% are considerably greater than the net protein synthetic rates of 3 and 6% of the *E. coli* B/r and *V. marinus* MP-1 cultures respectively phenylalanyl synthetase activity may not be the prime rate controlling reaction for protein synthesis at 544 atm. This result does not however preclude the possibility that other aminoacyl synthetases are pressure sensitive or that pressure may upset the fidelity of activation and coupling of phenylalanine to its corresponding tRNA. Hydrostatic pressures in the range of 1 to 100 atm cause a marked inhibition of binding of Phe-tRNA to ribosomes in the presence of the mRNA, poly U (Fig. 37). When this curve is corrected for the binding which occurs during the 90-sec period before (8.5 pmoles) and the 35 sec period after (3.5 pmoles) pressurization (the reaction time under pressure was 480 sec) binding is not observed at pressures greater than 600 atm; in fact, dissociation of Phe-tRNA from the complex occurs at pressures in excess of 600 atm. These affects are more apparent upon analysis of the kinetics of binding (Fig. 38). The pressure effects are reversible and binding rates return to the 1 atm rate upon depressurization (cf. Fig. 36).

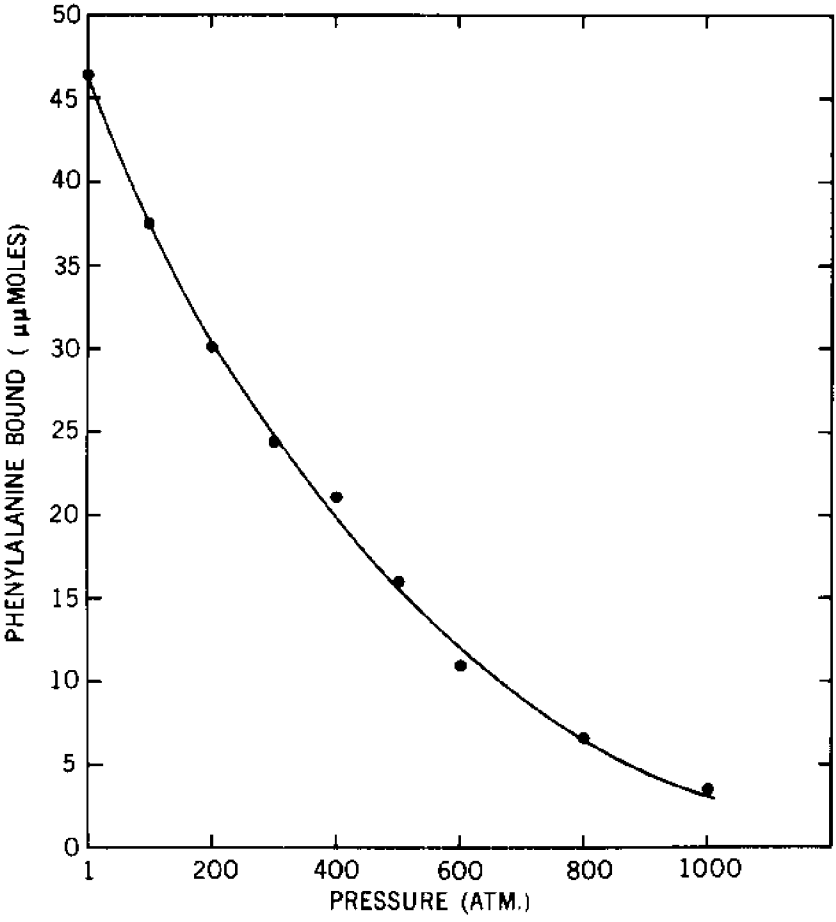

Figure 37. The effect of hydrostatic pressure on the poly U-directed binding of ^{14}C Phe-tRNA to *E. coli* ribosomes. Each point is the mean value obtained from 6 separate reaction mixtures. From Arnold and Albright, 1971.

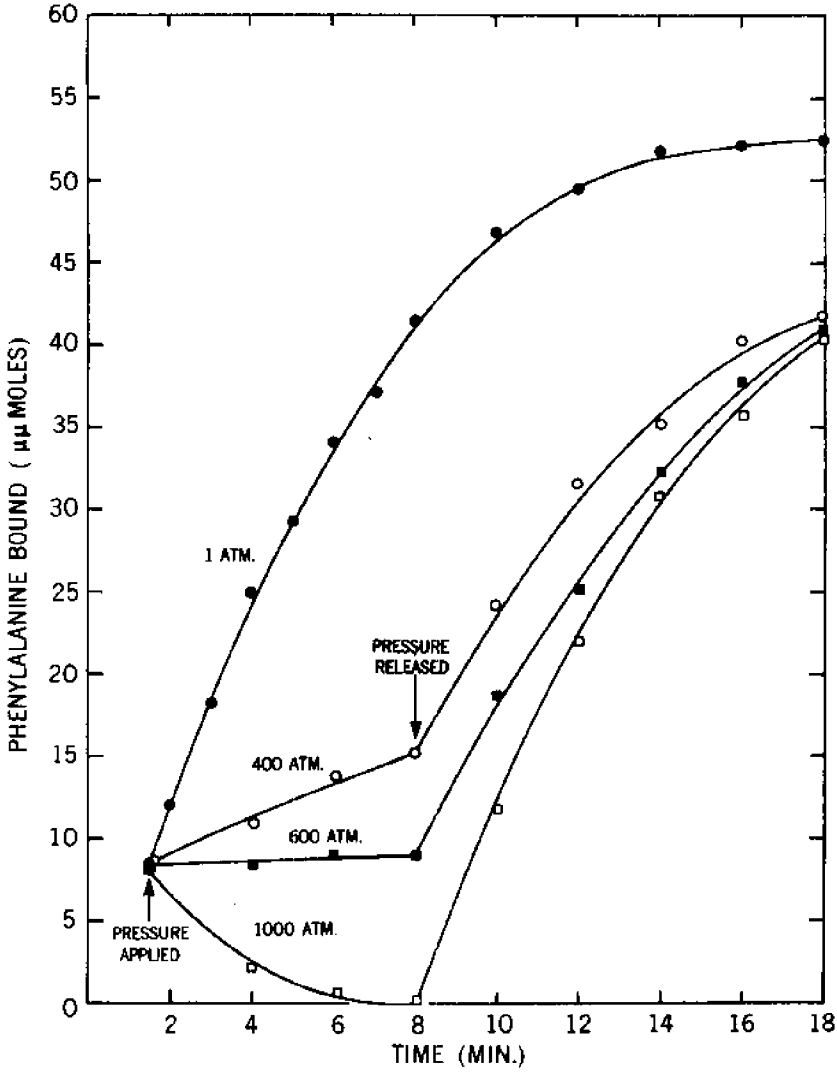

Figure 38. The effect of hydrostatic pressure on the rate of binding of ^{14}C Phe-tRNA to *E. coli* ribosomes. Reactions were pressurized after 1.5 min at 1 atm and depressurized at intervals up to 6.5 min later. Values for binding under pressure have been corrected for binding which occurs in the 35-sec period between depressurization and reaction termination. Each point is the result of a duplicate assay. From Arnold and Albright, 1971.

When we differentiated between the effect of various pressures on binding of Phe-tRNA to ribosomes and the effect on peptide bond formation between adjacent phenylalanine residues, curves, as seen in Fig. 39, were

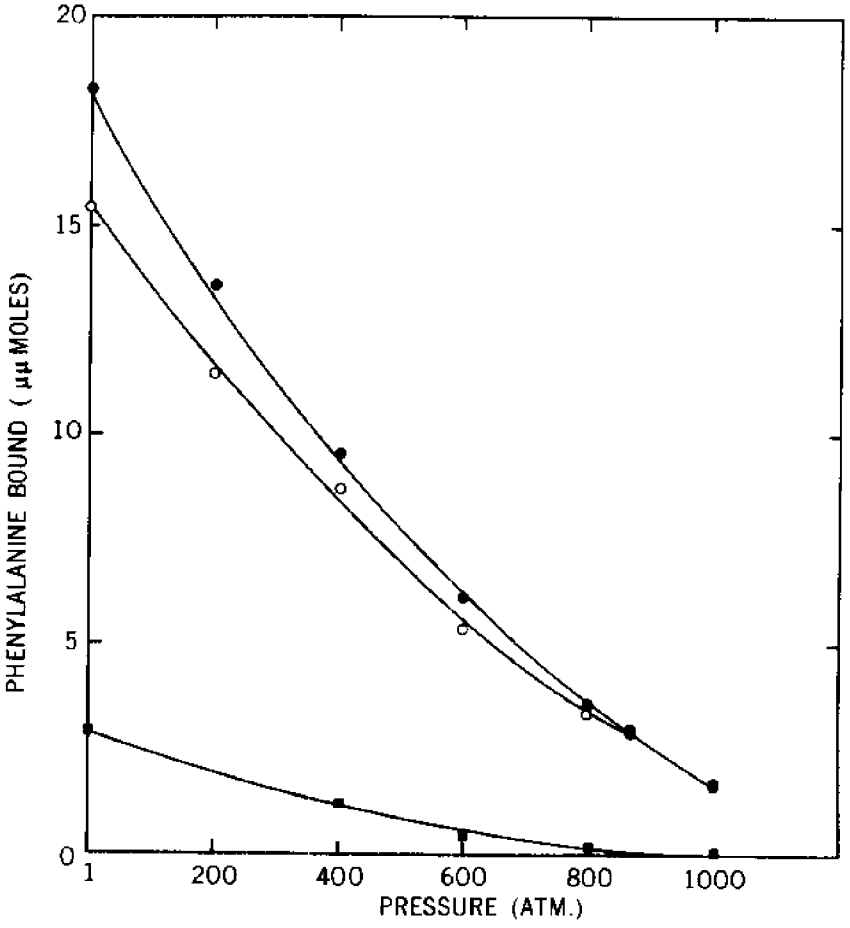

Figure 39. The effect of hydrostatic pressure on the binding of tRNA carrying phenylalanine (open circles) and phenylalanylphenylalanine (squares) to *E. coli* ribosomes. Total phenylalanine binding (solid circles) is expressed as the sum of the other two curves. From Arnold and Albright, 1971.

obtained. The ratio between bound phenylalanine and phenylalanyl-phenylalanine (Table 2) clearly indicates that peptide bond formation is more sensitive to pressure than the aminoacyl-tRNA binding reaction.

Table 2.

The effect of hydrostatic pressure on the ratio of phenylalanine bound to phenylalanine polymerized in an *E. coli* ribosomal system.*

Pressure (atm)	Phenylalanine bound / Phenylalanine polymerized
1	5.3
200	6.2
400	7.7
600	10.2

*From Arnold and Albright, 1971.

The binding kinetics of mRNA to ribosomes are such that the reaction is virtually completed before pressure can be applied to the system. However, when the poly U-ribosome complex is tested for stability under pressure curves such as these are obtained (Fig. 40). Exchange occurs with increased

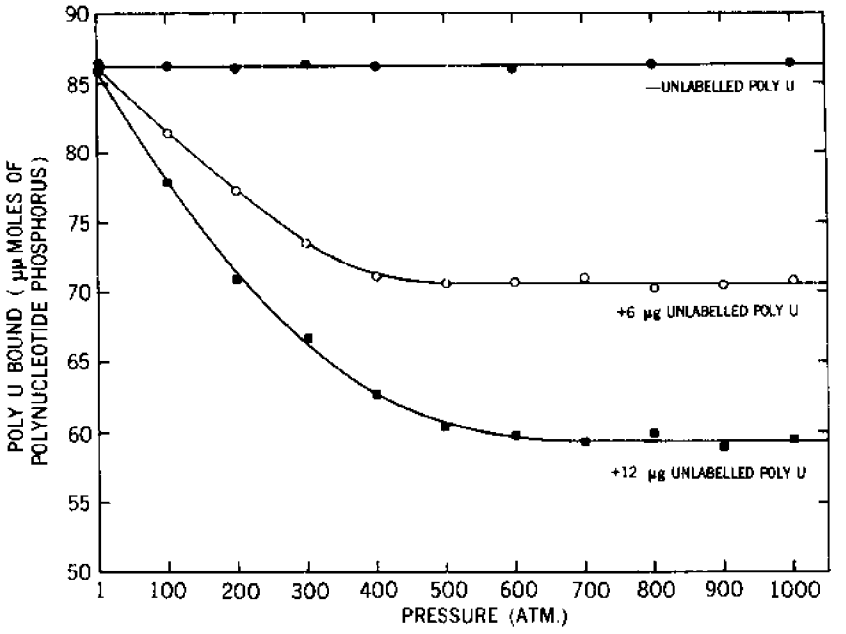

Figure 40. The effect of hydrostatic pressure on the stability of the poly U-ribosome complex using a cell-free system from *E. coli*. Unlabelled poly U, in the amounts indicated, was added 2 min after binding was initiated with labelled poly U, and the reaction mixtures then incubated for 8 min under various pressures. From Arnold and Albright, 1971.

frequency at pressures between 1 and ca. 500 atm. At pressures between ca. 500 and 1000 atm the mRNA appears to be unable to further dissociate from the ribosome whereas these pressures cause a dissociation of the Phe-tRNA from a Phe-tRNA-mRNA-ribosome complex. Perhaps ca. 500 to 1000 atm "freeze" the system so mRNA cannot associate with or dissociate from ribosomes at these pressures. This may be one reason why *E. coli* B/r cannot grow at pressures greater than ca. 500 atm.

Analysis of the kinetics of the protein synthetic reactions discussed here indicates that the order of pressure sensitivities are dipeptide bond formation>formation of the tRNA-mRNA-ribosome complex>aminoacyl synthetase activity. The mRNA-ribosome binding is sensitive to pressure but cannot be ranked in a hierarchy such as this.

The data presented indicate that one reason why the terrestrial bacterium *E. coli* B/r cannot grow at pressures greater than ca. 500 atm may be inability to synthesize proteins at rates commensurate with normal growth and metabolism of this cell. If an isolated protein synthetic system of the marine psychrophilic bacterium *V. marinus* MP-1 shows a similar response to increased pressures, then the sensitivity of the protein synthetic systems of nonbarophilic bacteria to pressures in excess of ca. 500-600 atm may be one reason for the inability of these cells to function in the ocean depths.

ACKNOWLEDGMENT

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HIGH PRESSURE AQUARIA AND PLANT RESPONSES*

William Vidaver
Photobiology Group,
Department of Biological Sciences,
Simon Fraser University,
Burnaby, British Columbia

Most plants, whether limnic, marine, or terrestrial, never experience hydrostatic pressures of more than one or two atmospheres. In the laboratory, however, routine applications of pressures of up to, or even in excess of, 1000 atm yield information about some of the physiological activities of plants that may not be obtainable otherwise. Pressure effects on plant functions as varied as cytokinesis (7, 12), membrane permeability (11), cyclosis (10), growth (5), flagellar activity in algae (3), cytoplasmic sol-gel transformations (3), photosynthetic pigment synthesis (6), photosynthesis and respiration (14, 15), and many more have been described. In my own laboratory we have concentrated on cellular growth and reproduction, photosynthesis, and seed germination, as affected by the application of hydrostatic pressure.

General Experimental Techniques

Several avenues are open to the experimenter who would examine pressure effects on the physiological systems of plants. Approaches to the problems involved vary considerably in technical difficulty and, as might be expected, the more sophisticated techniques often yield significantly more information about the effects of pressure than do simpler methods.

A simple, and perhaps the most widely used approach, requires only an aquarium capable of withstanding internal hydrostatic pressure, a method of opening and sealing the aquarium, and a pump for generating pressure. The sample, when placed in the aquarium, is exposed to pressure for some time period and, upon pressure release, it may be compared with some unpressurized control. Experiments of this type tend to yield *ex post facto* information. Little can be deduced from the experiment as to the actual state of the sample *while* pressure was applied. Apparent exceptions to the limitations of this technique do occur. For example, the difference in O_2 consumed by the sample and control might be extrapolated to an indication of the effect of pressure on the rate of respiration. The extrapolation requires, however, the unwarranted assumption that the rate of O_2 consumption by the pressurized sample remains constant all the while pressure is applied. Finer resolution of the time course of a pressure effect can be obtained by running a series of experiments of various durations, but

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even this modification ignores possible effects of pressurization and subsequent release on the sample. Similar criticism might be applicable to measurements of other kinds of pressure-induced changes.

Another more useful approach to the problem is to monitor one or more aspects of the sample *while* pressure is applied. a 49335 Monitoring may consist of direct micro- or macroscopic visual observation through a window in the aquarium, noting such changes as those in motility, activity, size, shape, or other visible features in relation to pressure. Many less obvious physiological or chemical changes may also be monitored. Introduction of appropriate electrodes into the aquarium can permit continuous recording of O_2 exchange (4, 14), pH, and conductivity. The use of properly designed aquarium windows will permit detection, in the sample, of the effects of pressure on the absorption (14), scattering, and emission of light. Determinations of pressure effects on optical dichroism and light polarization fields should also be possible. Data obtained with the use of monitoring techniques can be related directly, time, to the effects of the application of pressure, of periods of sustained pressure, of changes in the magnitude of pressure, of pressure release, and of variations in other parameters including light and temperature (14). While to do so would introduce complexities of another order of magnitude, it could prove useful in some cases to use monitoring techniques in conjunction with deepsea retrieval aquaria.

The techniques outlined above are primarily useful in carrying out observations on pre-existing biological systems. Numerous studies have been made on the growth and accompanying biosynthetic processes of microorganisms (1, 8, 18), while exposed to pressure. Microorganisms capable of growing in closed culture tubes (those whose metabolism does not require the exchange of large quantities of atmospheric gases) are easily grown in the simplest of pressure aquaria. On the other hand, photosynthetic plants which consume and produce relatively large quantities of O_2 and CO_2 will not grow readily in the small volumes of media enclosed by typical pressure aquaria. To overcome the problems (some of these are discussed below) of massive gas exchange by the organisms it is necessary either to remove and add appropriate quantities of the offending gases, or to use pressure aquaria with sufficient volumes so that changes in dissolved gas concentrations induced by the organism remain insignificant. In many cases it is possible to maintain adequately constant CO_2 concentrations by the use of bicarbonate buffer. No such buffer system is available for O_2 however. Except for microorganisms, in which case cell populations could be kept low enough in a small aquarium to prevent effects of changing gas concentrations on growth processes, an aquarium with a system for circulating and renewing the medium is probably the most appropriate for growth studies. Such systems are described elsewhere in this volume. For growth studies of deepsea microorganisms it would be necessary again combine techniques outlined above with a retrieval system. To use

microbiological culture techniques it would, in addition, be necessary to have a means for subculturing the organisms by transfers from the original to satellite aquaria. At present, no system for subculturing under pressure appears to have been described.

Pressure as a Physiological Tool

In a physiological experiment variations in hydrostatic pressure are analogous to variations in other physical parameters, such as temperature and, perhaps, water potential. Biological systems which normally never experience elevated pressures will nevertheless respond to their application in a characteristic and often predictable way. The probility that any two components of a metabolic pathway will respond in exactly the same way to the application of pressure is probably quite small. Consequently it is sometimes possible to use pressure as a tool in resolving, to some extent, the contribution of component parts to an overall process. In this sense, pressure application can act as a more or less selective inhibitor of metabolic processes. (Pressure as an inhibitor has the advantages of being nontoxic and immediately reversible). Studies show that macro-molecular synthesis in bacteria is affected by pressures, within a certain range, in the order protein > RNA > DNA (1).

In our laboratory (14), with use of an electrode to monitor O_2 exchange by the green alga *Ulva*, it was shown that the water-splitting, oxygen-producing components of the photosynthetic process are essentially unaffected by the application of pressure sufficient to block completely the steady-rate limiting dark reactions. This lack of sensitivity of photosynthetic O_2 production to these pressures suggests that the process is mainly photochemical and perhaps not enzymatically mediated. Another example of a selective pressure effect on components of the photosynthetic process in a green alga is shown in Fig. 41.(15). Current theories of photosynthesis invoke the existence of two light reactions, both of which must function for the overall process to occur. One reaction (PS1), preferentially sensitized by long wavelength red light (>680 nm), is believed to generate ATP and produce a strong reductant. The other reaction (PS2) which can utilize short wavelength red light (.650 nm optimum) is believed to oxidize water to O_2 and supply a weak reductant. In Fig. 41 the upper row of curves represents transient rates of O_2 exchange by the alga in 660 nm light (PS2) as affected by various treatments. The lower row shows O_2 exchange in 707-nm light (preferential PS1 activation). The predominant response to the 707-nm light is a transient O_2 uptake (curve f), which reaches its optimum in the presence of an inhibitor (DCMU) of O_2 production (curves b-g). The effect of pressure is seen in the top and bottom right-hand curves (e, j). Under a pressure of 535 atm they are similar. The O_2-uptake component of the long wavelength curve is missing completely; only O_2-production is seen. An interpretation of this effect is that excitation energy of light, normally

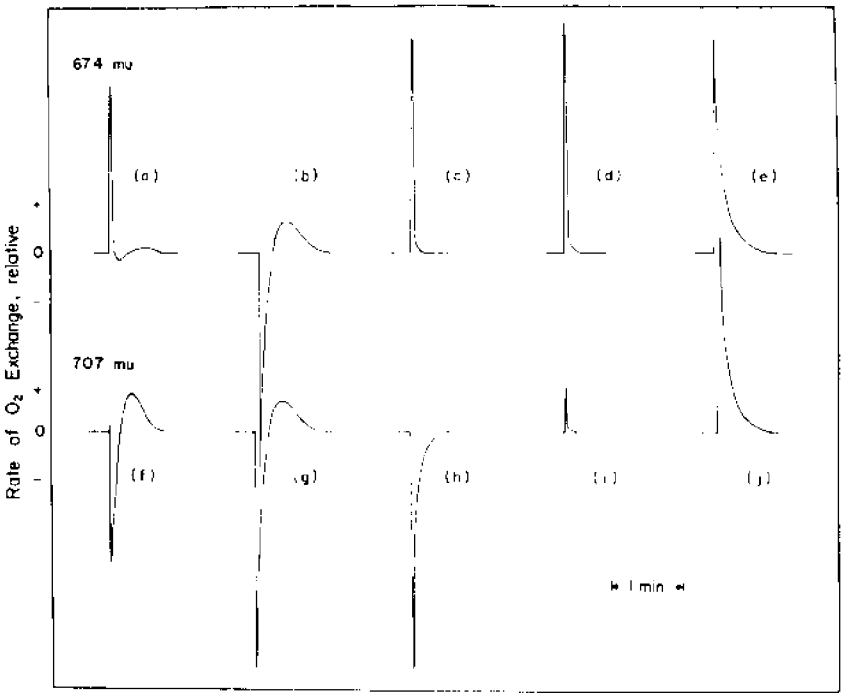

Figure 41. Transients of O_2-exchange in equal quanta (1.1×10^{-8} einsteins cm^{-2} sec^{-1}) 0.5 sec light flashes of two wavelengths as affected by inhibitors. Upper row: 674-nm; lower row 707-nm light. a, f, air, no inhibitor; b, g, 5×10^{-5} M 3, 4-dichlorphenyl methylurea (DCMU), c, h, 7×10^{-3} M salicylaldoxime; d, i, 100% N_2; e, j, 535 atm hydrostatic pressure.

utilized by PS1, when that system is blocked by the application of pressure, is somehow shifted to PS2 where it is used to produce O_2. These experiments also support our hypothesis that the photosynthetic water-splitting process in the alga is relatively insensitive to pressure.

Time-dependent O_2-toxicity effects have been noted, under pressure, on germinating lettuce seeds (16). Exposure to 600 atm for 24 hr was lethal for seeds placed in air equilibrated water; however 24 hrs exposure to 1000 atm in the absence of O_2 had no adverse effect on seeds.

Another pressure effect on seed germination can be an enhancement of germination. When O_2 concentration is optimal lettuce seeds germinate more readily under 200 atm than they do at atmospheric pressure (16). Photosensitive lettuce seeds having a functional phytochrome (2) system are normally retarded in their germination by exposure to far-red (730-nm) light, and enhanced, over controls kept in darkness, by red (660-nm) light. When exposed to 200 arm, regardless of light treatment, these seeds also

germinated to a greater extent than non-pressurized seeds (Figs. 42,43). Presumably, the effect of pressure in these experiments is related to the functioning of the phytochrome system.

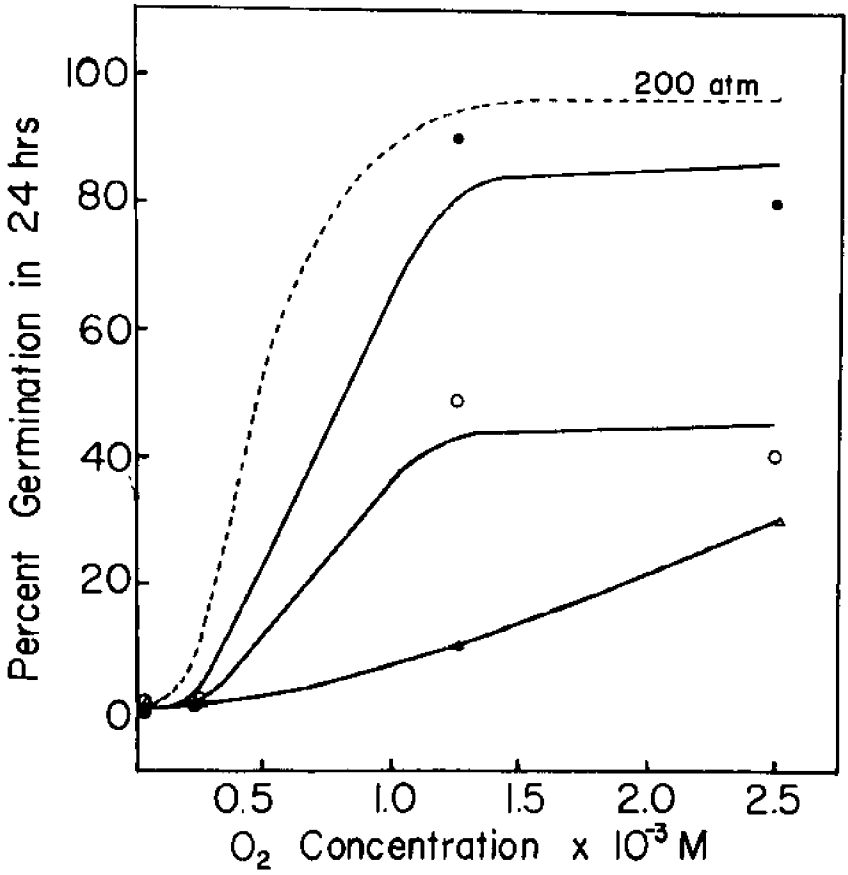

Figure 42. Germination of Grand Rapids lettuce seeds in closed pressure aquaria at atmospheric pressure (solid curves) and 200 atm (broken line). Seeds were given 1 min flashes of red or far-red light 1/2 hr after placing in the aquaria or kept darkened for the 24 hr period. Symbols: (solid circle), red light; (open circle), far-red; (triangle), no light. All germinations of seeds under 200 atm fall on or near the broken-line curve, regardless of light treatment.

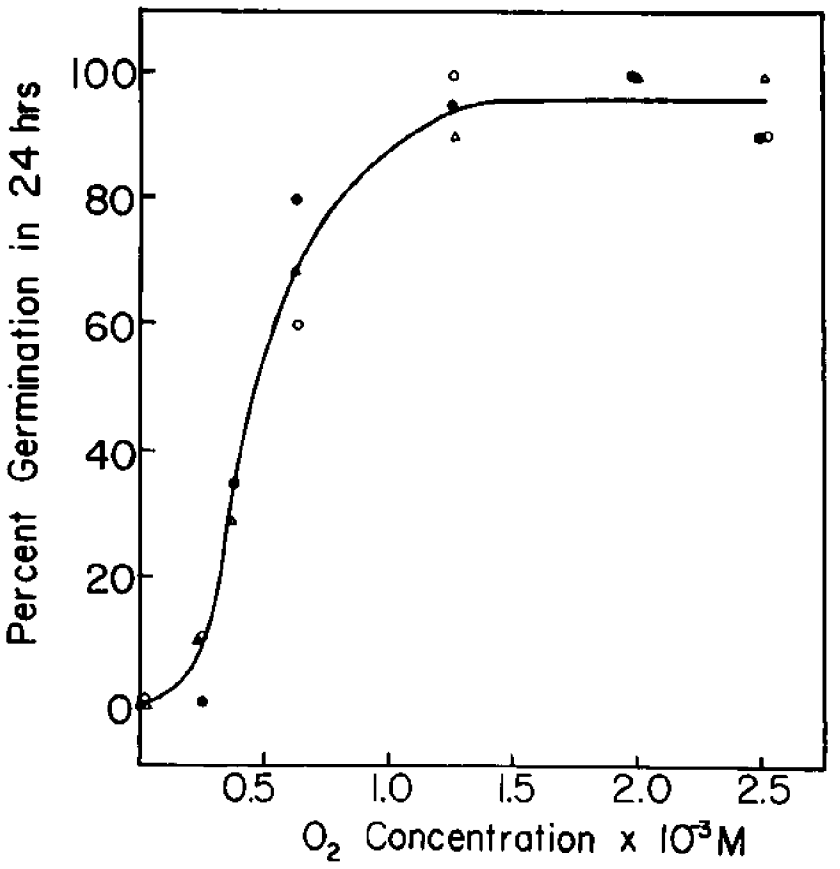

Figure 43. Germination of Grand Rapids lettuce seeds under 200 atm with red (solid circle), far-red (open circle), and no light (triangle). Procedure was the same as described for Figure 42, and the broken-line 200-atm curve, shown in Figure 42, is derived from these data.

Our laboratory is currently engaged in an attempt to grow synchronous algal cultures under hydrostatic pressure. Some algal species are readily synchronized in their life cycle by the proper control of growing conditions and a regulated regime of light and darkness. *Chlorella* cells growing on a 14-hr light and 10-hr dark cycle divide to the extent of about 95% within 2 hr of darkening. Daughter cells produced by this method may be assumed to be all of the same physiological age and at the same stage of their life cycle (13). In a biochemical sense a population of synchronized cells represents an enormous magnification of a single cell. Synchronized cultures are therefore ideal material for studies on the effects of pressure on the synthesis of macromolecules, such as RNA, DNA, and protein. We hope eventually to be

able to observe pressure effects on the synthesis of these macromolecules. Meanwhile, the problem of obtaining synchronous growth in a pressure aquarium has proved to be formidable (9). Being photoautotrophic, the algae produce an O_2 molecule for each CO_2 assimilated. It has been clear since 1920, when Warburg (17) first described the effect, that O_2 is a potent inhibitor of photosynthesis. For some plant species even the 21% O_2 of the atmosphere inhibits the process by as much as 50%. The usual pressure aquarium is closed to the outer atmosphere and O_2 produced by the cells remains dissolved in the medium. Consequently the O_2 concentration increases in the light to the point that cell growth stops, and cell synchrony is lost (see Fig. 44). The problem now becomes one of removing the O_2 which, while technically feasible, is difficult and especially so since we wish to have at least 25 cultures undergoing pressure treatment simultaneously. Our approach is to attempt to achieve a compromise between O_2 production and the volume of the aquaria which will supply sufficient cells for biochemical determinations, by restricting the cell number in each pressure aquarium to the extent that a reproductive cycle occurs before the increase in O_2 concentration disrupts cell synchrony. It has been possible to study the effects of pressure on the cell division in the synchronous cultures. (9). Algal cells are maintained in flasks until the end of the light phase of the growth cycle, at which time they are transferred to the pressure aquaria and pressure is applied. The cells are then allowed to divide in the dark. Figure 45, which shows the relationship between pressure and cell division, also shows that O_2 concentration is highly important. Presumably sufficient O_2 to supply respiratory needs is essential for division. When O_2 concentration exceeds about 40cc/l division begins to be inhibited at 135 atm, and inhibition is complete at 50cc/l.

Future studies should expand the usefulness to the plant physiologist of hydrostatic pressure application as a routine laboratory research tool.

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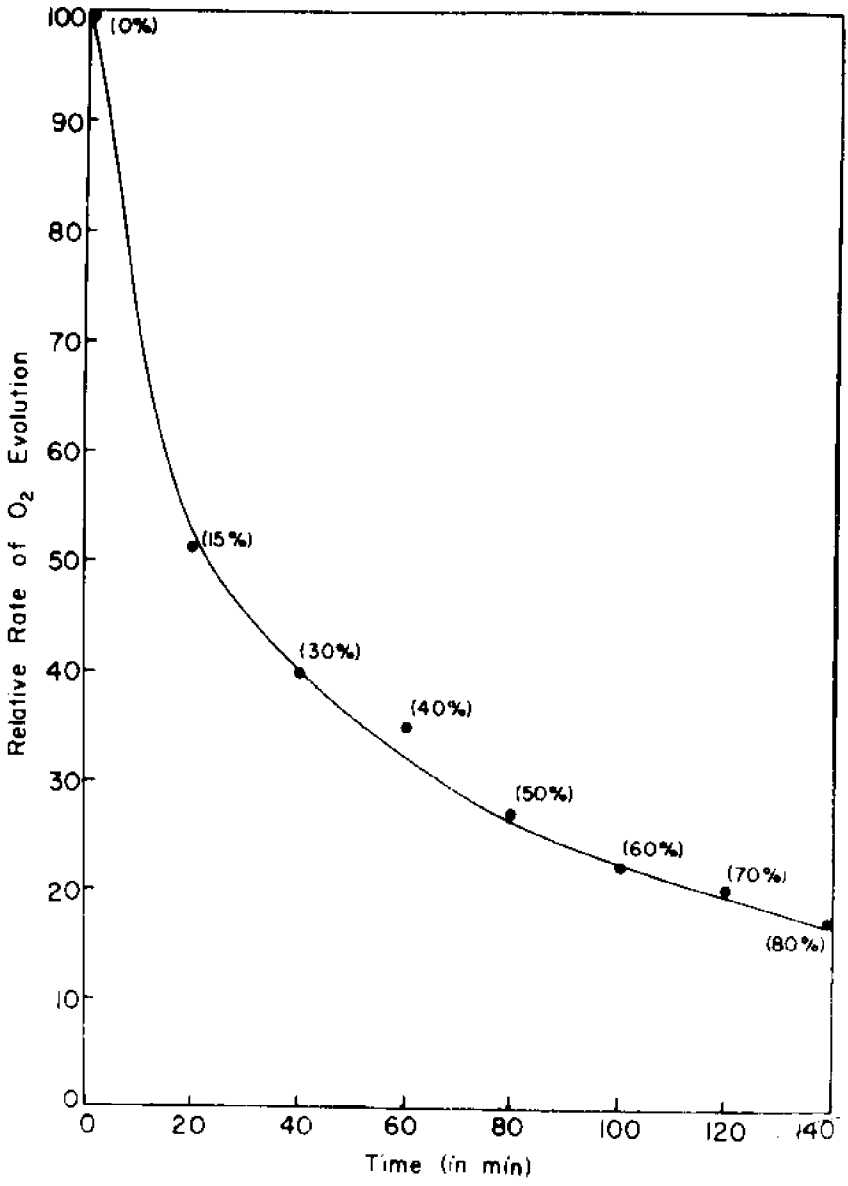

Figure 44. Time course of the autoinhibition of photosynthetic O_2 production of *Chlorella ellipsoidea* cells starting with zero O_2. Cell concentration was 2.5 x 10^7 cells/ml, illuminated with 700 fc, white light. Numbers in brackets refer to percentage of O_2-saturation at one atmosphere. Determinations were made with a Mod. 53 Biological Oxygen Monitor (Yellow Springs Instr. Co. Inc., Yellow Springs, Ohio.)

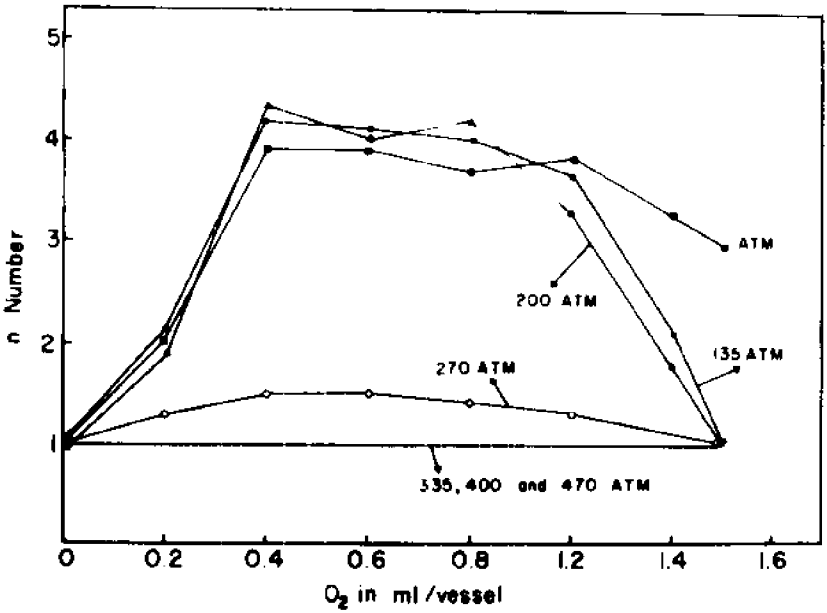

Figure 45. Cell division as affected by variations in O_2 concentrations at various pressures by synchronous cultures of *Chlorella ellipsoidea* grown in culture flasks and transferred to pressure aquaria at the end of the 14-hr light period. Cells remained in darkness in the aquaria for 10 hr.

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Discussion Following Vidaver Paper

SCHELTEMA: There are of course some plants that don't photosynthesize, namely the fungi, I wonder whether there are not deep sea fungi, and wouldn't these be interesting to look at.

VIDAVER: I'd like to. I don't know what I would look for. Are there types of deep sea fungi?

SCHELTEMA: I think there are and I think you would probably find them all the way down.

MENZIES: There have been reports of algae in the deep sea. ZoBell may know much more about it than I do, but one gentleman, Ferguson Wood, has been exceedingly interested in checking out these records of deep sea algae. These are obviously alive, according to him. He recovered them and cultured them. Whether they had chlorophyll or not, I do not know.

ZOBELL: I couldn't add any details without making a speech with regard to the controversial significance of plants, particularly algae, which have been found at sea in many different depths, even in deep sea muds. I think this observation was first made in my laboratory by Ferguson Wood on some of the *Galathea* cores, material from a mud-water interface. Whether these occurred there indigenously or whether they are adventitious species is highly questionalble. Also in many other cases it is highly questionable whether the material was actually collected from depth, because of the difficulties of collecting at a desired depth. Many of the samples have been collected with Nansen bottles, or some type of bottle in general which goes down open with the water flowing through it. It is closed at the desired depth and then brought up. There is always the possibility that some of these

algae have been picked up during the descent. The possibility isn't great. My own considered opinion is that there are algae far below the depth to which sunlight penetrates, but certainly they are not there in very great abundance. But while I have the floor, I would like to comment with regard to fungus question—I am aware of no evidence which satisfies me on the occurrence of active fungi at depths much greater than 200 meters, having been demonstrated there by fungus traps. You probably know what a fungus trap is. You put some food down there—cellulose, starch, or whatever fungi like—and if they grow there *in situ*, then you are pretty sure that they are growing there: however fungus spores, in great abundance, have been found at all depths. Rarely have I collected a deep sea core—and I have collected samples from all of the greatest depths in the world, I believe—in which I cannot demonstrate the presence of mold. These are molds which grow very readily at 25°C to30°C, I have never found one that will grow at 3°C or even 10°C. All this leads me to believe that these molds, along with similar bacterial spores are probably adventitious species which probably have been produced above sea level and fell there.

HESSLER: I would just like to attract attention to Bob Fornier's data wherein he found a small uniflagellar alga in much greater concentration, one order of magnitude greater, at bathyial and abyssal depths than one finds in shallower water. This fact led him to believe that they were probably growing *in situ*, rather than being adventitious.

VIDAVER: Bernard has found a large population around depths of up to 5,000 m. but certainly what they're doing is another question.

MACDONALD: Is it conceivable that you can have a symbiotic alga growing in photophores?

VIDAVER: Oh, this happens all the time.

MACDONALD:Using the lights?

VIDAVER: Well, there really isn't enough light at depths, so I'm told, to permit the reaching of the compensation point by algae.

MACDONALD: But, within the organ itself?

VIDAVER: Oh, I see, you mean in luminous organs? Has anyone looked?

SCHELTEMA: Regarding the question of contamination that ZoBell brought up—this is much more serious than most people realize. George Grice at our institution has put carmine-stained zooplankton in a net, lowered it vertically to 5000 m, and brought it up, and still found some of the carmine-stained animals in it, though he lowered it actually in a way so that it should have washed out.

BERGER: We *have* been able to grow unicellular algae in closed systems under increased hydrostatic pressure. The inhibitions of growth by high concentrations of oxygen produced by photosynthesis and by pH-changes associated with the uptake of bicarbonate ions can be overcome. We have enclosed the cultures in part or totally in silicone membranes through which

gases pass freely. Thus the aqueous hydraulic fluid in the pressurized aquarium serves as reservoir for the excess oxygen produced in the culture and as a source of extra carbon dioxide (Lim. & Oceanogr. 15, 483-485, 1970). The growth rate of *Anacystis nidulans*, an unicellular blue-green alga, was measured over the pressure range 1 to 500 atm. Under our experimenal conditions, no appreciable change in the growth rate occurred at pressures below 150 atm, a 50% decrease in rate was observed at 225 atm, and a very rapid rate of decrease at pressures above 300 atm. We concur with the findings of Vidaver, as with the thallic algae, the oxygen evolving systems of *Anacystis* and *Chlorella* species are essentially unaffected by pressures to 1,000 atm., inhibition of oxygen evolution results from subsequent dark reactions .We have also just devised an automatic system to measure the rate of oxygen evolution while maintaining constant oxygen concentration in pressurized aquaria. Two polarographic electrode systems are used. The first one monitors oxygen concentration and triggers the second system, a large, bare, platinum electrode unit which consumes oxygen when the concentration exceeds the present value.

(Question): I think it is a good point to make sure that everyone understands that oxygen is an inhibitor to all forms of photosynthesis and especially plants. It stresses the same argument as why oxygen is toxic to mammals that simply have never encountered it at much over 0.2 atm. But I wonder about the point that Wells brought up on the hydrostatic effect on the activity of oxygen on the PO_2?

VIDAVER: The experiment that I showed you where the oxygen involved by the cells themselves inhibiting photosynthetic rate? This was done at atmospheric pressure.

(Question): I know that, but the partial pressure of oxygen is not going to be reflected by the content as you squeeze the system under that synthetic pressure. This is the new point, so that if you looked at inhibition vs. PO_2 and then looked at it vs. hydrostatic pressure with a known O_2content, you could calculate the activity increase and see if it was all in the right realm.

VIDAVER: That would mean that we would have to remove even more of the oxygen. I think that if we could keep it at zero, as long as we keep it in the light, we'd be in good shape.

ZIMMERMAN: I'd like to comment that we too have studied pressure and cell synchrony, and attempted to use pressure to synchronize cells in tetrahymena, and we thought we were very successful since we induced as much as 35% of the population to divide synchronously till we realized that was the anoxia.

MACDONALD: You could reoxygenate on the pressurizing side.

VIDAVER: This was Brauer's solution—continuous perfusion.

GORDON: It's expensive and awkward.

KANWISHER: No, it's really very straightforward and simple, because nowadays, there have been a large number of very fancy controlled porosity,

stainless steel, pressure-resistant filters that have been developed for rocket engines. You can use these on the downstream side of the culture flask, and you pump stuff through, and if you have a little pressure valve on the other side you can just have a continuous flow system and keep it going at whatever you like and keep all your cells behind.

(Question):What do you think of the feasibility of a balanced high-pressure aquarium?

DR. BRAUER: Your data, Dr. Vidaver, suggest that this is possible since you can get photosynthesis at a decent rate in high pressure environments. Then we can play the same game that aquarists elsewhere are playing—the people who have been working with closed systems. This would be very nice.

DR. VIDAVER:This would be a feasible thing to do since we are really using pressures as a tool here, and what we're really trying to find out is something about the mechanisms of algal macromolecular synthesis and things like that. One thing I should point out here which I think a lot of people haven't realized, and that is that pressure is a superb metabolic inhibitor. As I have already showed you, it has a differential effect on processes such as the light and dark reaction of photosynthesis. It also has a differential effect on almost any two enzymes. Not a differential effect, but a difference effect. So, consequently, one can use it in this way, but it has other advantages. For one thing, it is essentially immediately reversible, within a certain range, and then, on top of that, it doesn't leave any toxic residue within the system you are working on. So, this aspect of using pressure as a research tool on biological systems, I think is something that hasn't even begun to be developed. It could be very useful.

BRAUER: We've never had a good name for it, but it seems to me that what has been going on between you and Macdonald suggests that it't time we started talking about the "Pharmacology of High Pressure," and I quite agree. They are exquisitely good pharmacologic agents, in a sense. With zero residue.

MACDONALD: Could I just give a practical example of the sort of things I mean. Inhalation anesthetics act in much the same way that hydrostatic pressure does. Inhalation anesthetics, things like halothane or cyclopentane which don't require metabolic transformation to act, but act in just a physical way, affect the microtubules, microvesicles, and things that Zimmerman talks about, in just the same way that hydrostatic pressure does. This would be in sea urchin eggs, and other cellular preparations. Might it not be that these affect the protein/water interactions in some common way?

WELLS: Earlier I suggested that perhaps some of the things we're calling pressure effects are not pressure effects at all but secondary effects of pressure. I think that since you did observe that certain aspects of photosynthesis were insensitive to pressure, you have a very good tool here

for examining this by replotting your data, forgetting about pressure altogether, and replotting those oxygen points to the corrected partial presure of oxygen and plot this against the corrected partial of oxygen alone. You would have to correct for oxygen partial pressures, since the pressure of oxygen would build up more rapidly at 1,000 atm. because its solubility coefficient is much lower than at 1 atm. If you're adding a constant quantity of oxygen, the partial pressure will go up four times as fast at 1,000 atm. than at 1 atm.

COMMENTARY ON HIGH PRESSURE EFFECTS ON CELLULAR SYSTEMS

by
Arthur M. Zimmerman and Selma B. Zimmerman
Department of Zoology,
University of Toronto,
Toronto, Ontario,
Canada.
and
Glendon College,
York University,
Toronto, Canada.

In order to understand the complex physiological activities and the behavior of organisms that reside at great depths it is essential to investigate the cellular systems which are directly influenced by high pressures and low temperatures. In the past, a major problem associated with the collection of such organisms was that of decompression which may have introduced artifacts into subsequent laboratory analysis. At the deepest part of the Marianas Trench, a large fish, approximately the size of a human, would be subjected to an overall pressure in excess of 48 x 10^6 lbs. It is inconceiveable that retrieving an organism at these depths (at temperatures in the range of 3°C) and subjecting it to decompression prior to biochemical and physiological studies would yield meaningful biological information. On the contrary, if means were available where upon organisms could be retrieved, under conditions found in their natural environment, more reliable information would be forthcoming. Perhaps, the best way to illustrate these points is to briefly discuss the morphological, physiological and biochemical changes induced by high pressure in organisms whose normal habitat is at atmospheric pressure.

Morphological Changes

Hydrostatic pressure markedly alters the morphology of cells. Amoebas, which display irregular shapes with active pseudopodia under atmospheric conditions, become spherical and quiescent under high pressure (Landau, *et al.*, 1954). (Fig. 46) *Tetrahymena*, a ciliated protozon becomes tear-drop shaped (Fig.47) under high pressure and loses its ability to display translation movement due to a disruption of ciliary structure (Zimmerman and Zimmerman, 1970). Sea urchin blastula lose their swimming capabilities due to the loss of cilia following high pressure treatment. The surface cells of these blastulae lose their normal flattened shape and become rounded. These morphological changes appear gradually and are dependent upon both the duration and magnitude of the pressure treatment (Young *et al.*, 1970).

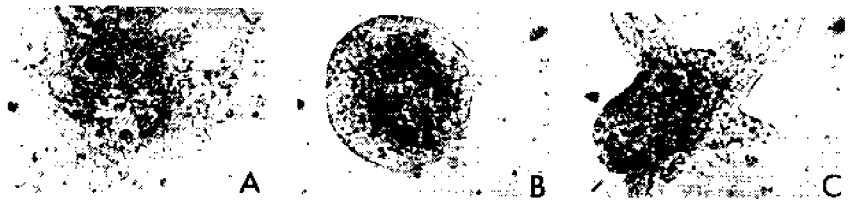

Figure 46. The effects of pressure on the form and activity of *Amoeba dubia* during and after the application of pressure. A) Photomicrographs of *Amoeba* at atmospheric pressure as seen within the pressure chamber. B) The same cell photographed 5 min after the application of 6,000 psi (at 25°C). The cell is quiescent and almost completely rounded. C) The same cell 5-1/2 minutes after decompression. Amoeboid activity is quite vigorous. (From the work of Landau, *et al.*, 1954).

The pressure induced structural changes seen at the level of the light microscope are also readily visible in the transmission electron microscope and the scanning electron microscope. It has recently been reported (Kennedy and Zimmerman, 1970) that the longitudinal microtubules as well as the central ciliary fibers in the *Tetrahymena* cilium are disrupted following short pulses of hydrostatic pressure (Fig. 48). Surface changes which are reflected by a shortening and thickening of cilia have been seen in the scanning electron microscope (Berger and Zimmerman, unpublished). In marine eggs high pressure destroys the integrity of microtubules associated with aster formation. A most dramatic effect of high pressure on microtubules was shown by Tilney, *et al.*, (1966) in the axonema of the heliozoan *Actinosphaerium*. The double spiral array of microtubules was dissociated into a fine granular mass following high pressure treatment.

It is of interest to note that in all the forms studied the structural changes were reversible upon decompression and the cells were able to resynthesize the dissociated or lost cellular structure.

Physiological Changes

Physiological alterations which result from high pressure treatment encompass a wide spectrum of cellular activities. Protoplasmic streaming is markedly affected by high pressure in both animal and plant cells (e.g. amoebe and elodea). The heart rate in marine animals (Naroska, 1968) and in frog tadpoles (Landau and Marsland, 1952) respond dramatically to pressure treatment. Ciliated tissue from numerous marine organisms (Ponat, 1967; Pease and Kitching, 1939; Kitching, 1970) as well as swimming of ciliated protozoa (Kitching, 1957) are also affected by pressure. Pressure exerts an effect on the electrical activity of muscles and nerves which modifies the duration, magnitude and threshold of response (Cattell and Edwards, 1928; Spyropoulos, 1957a,b). Recently it has been shown that the activity of *Tetrahymena* in an electrical field is markedly affected by hydrostatic pressure. *Tetrahymena* normally display cathodal galvanotaxis;

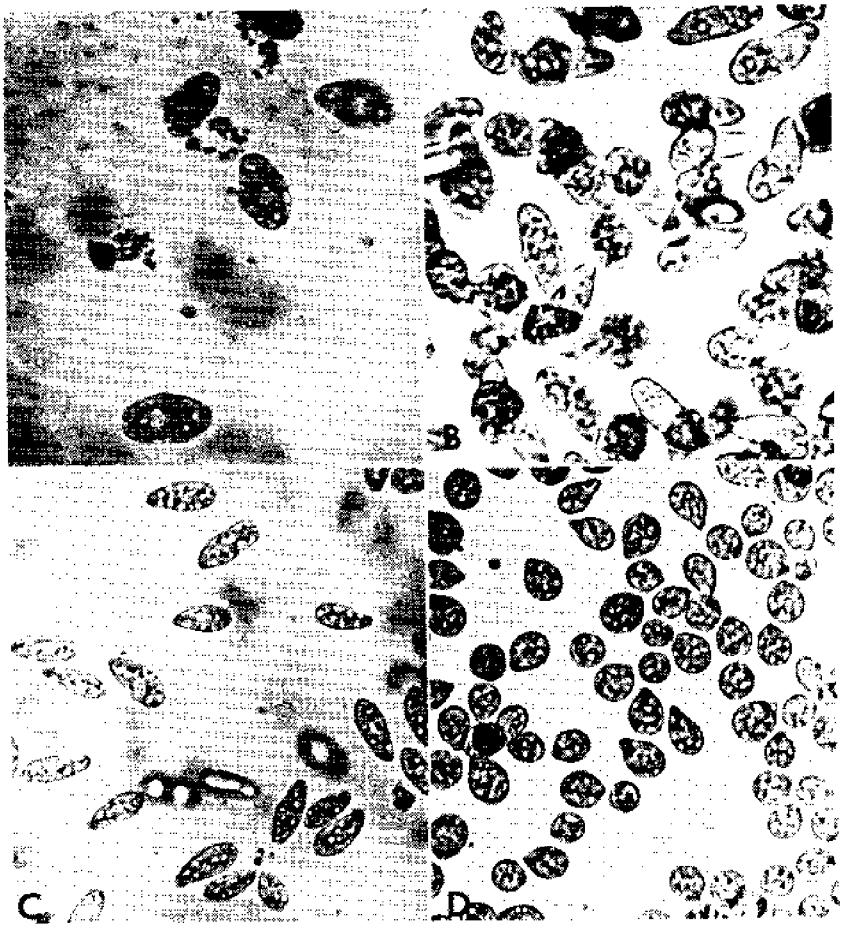

Figure 47. Pressure effects on a synchronized culture and log growth culture of *Tetrahymena pyriformis* GL. A) Heat synchronized control cells at atmospheric pressure. B) Synchronized cells following compression of 10,000 psi (at 28°C) for 10 minutes. Cellular activity is markedly decreased and some cells tend to become spherical while other cells are bulbous at the posterior. C) Photomicrograph of a log phase culture of *Tetrahymena* at atmospheric pressure. D) Log phase cells subjected to 10,000 psi for 10 minutes (28°C). The cells become tear-drop shaped displaying uncoordinated ciliary activity. (Zimmerman, 1969).

Figure 48. The effects of pressure on the fine structure of *Tetrahymena pyriformis*. A) Electron micrograph of a cilium from untreated control cell at atmospheric pressure. The central ciliary microtubules (CT) and the axosomal granule (AX) are labelled. X 52,000. B) This electron micrograph is from a cell exposed to 10,000 psi for 5 minutes. The initial breakdown of the central ciliary microtubules (CT) just above the axosomal granule (AX) is illustrated. The granular mass (GM) of the basal body does not appear to be affected. X 52,000. C) This section is from a *Tetrahymena* following a treatment of 7,500 psi for 10 minutes. Central cilium microtubule (CT) degradation progresses distally (arrows) from the axosomal granule. Some longitudinal and transverse microtubules (MT) persist at these pressures. X 72,000. D) Further cilia degradation (arrows) is illustrated in this section following a pressure of 10,000 psi for 10 minutes. Granular mass of the basal bodies is absent or displaced. X 52,000. E) Further cilia degradation results in what appears to be a swelling of the cilium shaft (arrows). The insert shows a similar cilium in cross section with the central tubules missing. The cells were treated for 10 min at 10,000 psi. X 52,000. (Kennedy and Zimmerman, 1970).

with increasing pressures this response is diminished and at 8,000 psi there is a reversal and the cells then display anodal galvanotaxis (Murakami and Zimmerman, 1970).

Perhaps one of the most dramatic effects of high pressure is the inhibition of cytokinesis and mitosis. It has been well documented that high pressure prevents cytokinesis in a variety of marine cells (Fig. 49), as well as disrupts

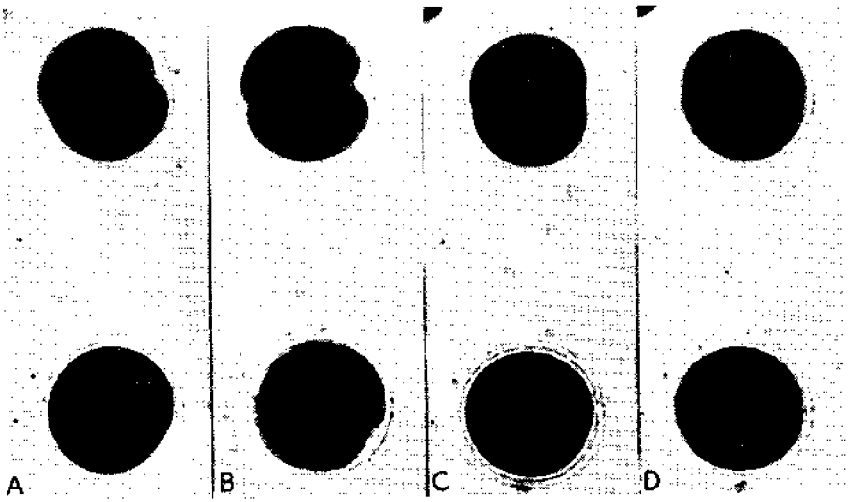

Figure 49. Pressure effects on cleaving eggs of the sea urchin *Arbacia punctulata*. A) Fertilized eggs, at atmospheric pressure. The cell in the upper portion of the photo displays an incipient furrow. The other cell has not yet started to cleave. B) Photograph 1/2 minute after application of 7,000 psi. The furrow continues to progress in the upper cell. The lower cell now displays a shallow furrow on one side. C) Three minutes after compression. At this stage furrows in both cells are receding. D) Four and a half minutes after compression furrows in both cells have completely aborted. (Zimmerman, 1968).

the mitotic structure (Fig. 50) essential for movement of the chromosomes (Marsland, 1970, Zimmerman, 1971). An interesting corollary to this may be found in ZoBell (1970) who reported that many marine microorganisms will develop at faster rates at atmospheric pressures than at the great oceanic depths where they are normally found.

Biochemical Changes

The structural and physiological effects of high pressure previously discussed are probably a consequence of more subtle changes on the biochemical level. Kinetics of chemical reactions are markedly altered by high pressure (cf. Johnson, Eyring and Pollisar, 1954; Johnson and Eyring, 1970). The confirmational alterations in macromolecules are no doubt

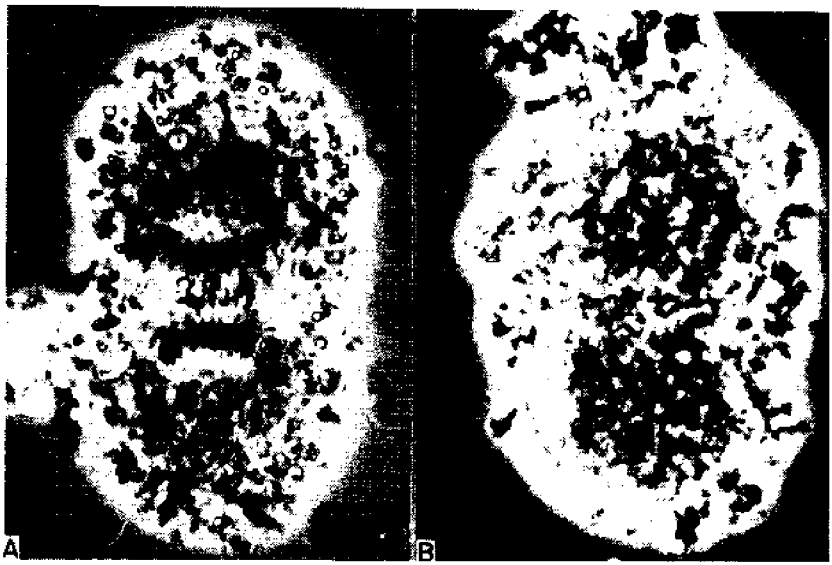

Figure 50. Pressure studies on the mitotic apparatus. The mitotic apparatus was isolated from *Arbacia punctulata* eggs at metaphase. A) An isolate from control egg at atmospheric pressure. B) An isolate from an egg subjected to 7,500 psi for one minute. This egg shows a loss of linear and radial orientation in the spindle and aster. (Zimmerman and Silberman, 1965).

responsible for numerous cellular effects which culminate in physical and structural changes. It is not the purpose of this commentary to cite all the biochemical changes which result from high pressure. However, in general (although there are several exceptions) increasing hydrostatic pressure inhibits metabolic activity (Fig. 51); this is reflected in the synthesis of RNA, DNA and protein in numerous marine microorganisms (ZoBell, 1970; Morita, 1967), *E. coli* (Landau, 1966; Pollard and Weller, 1966), protozoa (Zimmerman and Zimmerman, 1970), marine eggs (Zimmerman, 1970; Marsland, 1970) and in cultured mammalian cells (Landau, 1970).

Concluding Remarks

The previous comments reveal the diverse effects that high pressure exerts on cellular systems. The data support the idea that in order to obtain meaningful biological information on organisms living at great depths, they should be retrieved under temperature and pressure conditions which are indigenous to their normal habitat. The ecosystems at these great depths are quite distinct from that found in the laboratory. Thus it is essential that temperature and pressure controlled retrieval, transfer, and compression chambers be developed in which organisms can be brought from the ocean depths to the laboratory for analysis. At present these developments have

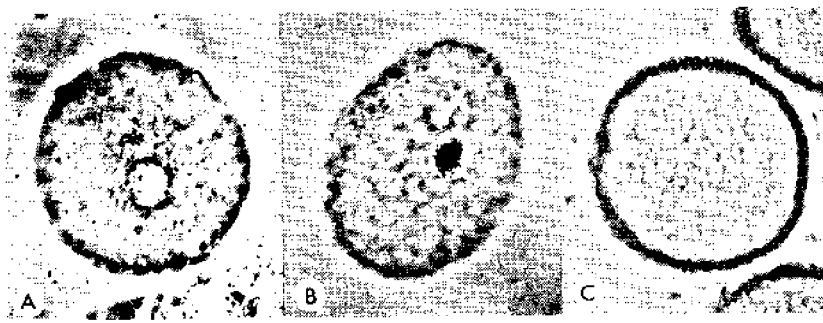

Figure 51. Autoradiographs showing the effects of pressure on the incorporation of ^{3}H-thymidine into fertilized eggs of *Arbacia punctulata*. A) Cells placed into ^{3}H-thymidine 5 minutes after insemination (just prior to the union of the male and female pronucleus) and subjected to 5,000 psi for 60 minutes. The autoradiographic silver grains are localized above male and female pronuclei. B) Eggs were placed into isotope at 45 minutes after insemination (prophase) and subjected to 5,000 psi for 60 minutes. Autoradiograph prepare from cells treated similarly to the previous (B) however pressure was increased to 7,500 psi. There is no incorporation of ^{3}H-thymidine at this pressure level. (Zimmerman and Silberman, 1967).

lagged behind biochemical and physiological techniques and it is hoped that with the recent renewed interest in oceanography, high pressure technology will proceed more rapidly.

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Dicussion Following Paper

SCHELTEMA: I think we naturalists aren't as hopeless as you think we are. I think we are all aware that the animals that arrive at the surface are not the normal animals that the physiologist wants to work with.
ZIMMERMAN: Well then, perhaps we should design a piece of equipment that would permit us to retrieve animals from great depths, and then we would be able to study them in the comfort of our own laboratory.
MENZIES: The alarming thought occurs to me that maybe the hundred or so

new species I have described are really not new species—it's a shame that I brought them up!

ZIMMERMAN: I doubt that very much; you're obviously speaking with tongue in cheek—when you look at pictures of cells under pressure, the shapes change so markedly that you wonder if after the application of pressure you are studying the same cell that you started out with. Of course, I am referring to organisms at the microscopic level; however one could question whether or not similar effects occur in large multicellular organisms.

LOWENSTAM: We keep saying pressure, but the kind of pressure we discuss is important. We are dealing with cells in fluids, and there is osmoregulation, and the very fact that people have brought up, as we have heard today, animals alive from depths of more than 220 atm. ambient pressures raises the question is it the experiment or is it real?

ZIMMERMAN: Yes, I understand your point and the results induced by pressure are quite real. We are all interested in the same general problems. What we wish to know is how does pressure alter cells, or organisms (excuse my "cells"). Our specific interests differ only in the level of organization studied. Whether one is looking at ecological aspects, cellular aspects, or biochemical aspects, we're all studying the effects of hydrostatic pressure. I have studied one level of organization and I am able to predict pressure induced changes in certain systems. One of our major interests is a study of cell division. If we pulse a cell (sea urchin egg or synchronized *Tetrahymena*) for a short time, for example, 2 minutes, at what we consider a modest pressure say, 5.000 psi there will be an effect on cell division that will delay the population of cells from dividing by as much as 15 to 20 minutes during a 60-75 minute cell cycle. I consider this most dramatic. Looking at these cells at the level of the light microscope as well as electron microscope we observe marked structural changes. What I'm emphasizing is that we have to be aware of pressure-induced changes.

BRAUER: In this area the experimentalists are in the unenviable position that they actually have experimental observations to restrain their speculations. So far as I am aware, the marine ecologists as a group have failed almost totally to produce any useable histological data. To argue, then, what these things do or do not look like on the bottom to me is a bit futile at this point, and what is needed may be development of techniques that do produce valid material, preserved in some fashion perhaps by fixation on the bottom, so that we may then be able to analyze its morphology with confidence. I would like to illustrate the tantalyzing possibilities held out by this kind of study by mentioning to you some observations I came across while visiting our Russian colleagues in Moscow last fall. Dr. Zharkova, a member of Professor Bierstein's group, showed me the first histological material purporting to represent deep-sea fauna I have been able to talk out

of anybody. The most interesting material was sections of the foregut of two closely related isopod species, one a relatively shallow water and the other a very deep-water species from the general area of the Kurile Kamchatka trench. As you might expect, the prime interest to the workers from the University of Moscow resided in the fact that the deep-water form was indeed a giant when compared with the shallow water form, and that the gigantism appeared to be associated overwhelmingly with an increase in cell size rather than in cell number in homologos cross-secions. To my mind, however, far more exciting thing to note was that the deep-sea specimen revealed a very high incidence of mitotic figures, contrasting sharply with the shallow water material. Since these speciments were not fixed until they were brought to the surface, the finding of a very high mitotic indices in the deep-water form could connote any one of several alternatives: Inherently high mitotic rate on the bottom; conditions on the bottom which inhibit mitosis and which are released on transit to the surface, giving rise to an intense wave of mitoses; a distribution in the time division of the cell cycle such that the stages entailing chromatin agglomeration and karyokinesis occupy an exorbitantly large fraction of the total time; or finally, an intense wave of mitoses brought about in cold acclimatized cells during transit through the relatively warm layers at the surface. In all, the second and third of these options, seem to me a bit more likely than the first and last. However, whichever way the final decision goes, if this kind of observation could be confirmed and expanded, I think it would make an important contribution to our understanding of tissue growth and development, mutation rates, and the like, in the deep sea environment. All told, it seems to me that the problem of cell population dynamics with all its ramifications is among the most fundamental problems of deep sea biology, but can become accessible only after we shall have developed adequate methods to collect and preserve the tissues we propose to study.

MACDONALD: I think a useful way of looking at the question of evolution today might perhaps be to consider a surface organism, perhaps some intermediately complex, and trying to find out what the most pressure-sensitive component of his physiology is. And if you apply a gradually increasing pressure, the first change that I think you could detect is an excitatory effect, and although I go along wholeheartedly with the sentiments expressed by Zimmerman it's quite possible that the ultimate effect in pressurization begins with adjustments in the neurophysiological balance. At the neurophysiological level, I am suggesting that moderate pressures can, in fact, be useful for exploring bonding effects between the water environment and protein, and perhaps the membrane structure to which that protein is contributing. I find intriguing similarities between inhalation anaesthetics which are non-active metabolically, and pressure, and this is why I think it may be a nice--though not the only—approach, to

look at the integrative level of the animal, and not necessarily the lowest level of organization. Molecular biology is extraordinarily sensitive but the system is different.

GORDON: In terms of the kinds of cytological changes we've been discussing, are the pressures that interest you in the range of, say, 50 to 100 atms?

ZIMMERMAN: They usually aren't much greater than that. We get some changes as low as 2,000 psi, and most often they run at 5,000 psi.

GORDON: The reason that I ask this, is for the benefit of those who haven't spent much time at sea. There are these organisms that we've mentioned several times in passing--all these deep scattering layer things that are vertically migratory, and in particular there's quite a variety of these which come right to the surface and which prowl at night and which during the day are probably down between 100 and 1000 meters. They go twice diurnally through this kind of change. In many cases you can get them and keep them alive for quite substantial periods of time, especially invertebrates. This is the place to begin anyway to look at some of the non-completely shallow water things.

ZIMMERMAN: I should like to make one concluding statement. Although we are all interested in the effects of pressure on organisms that reside in the ocean at great depths, hydrostatic pressure may also be used as an analytical tool for studying cellular processes. Just as we use temperature or any other physical agent, we can employ hydrostatic pressure for analytical studies. This use of high pressure has not been extensively discussed, but I would like to bring this to your attention at this time.

CHAPTER TWO

DEEP WATER ORGANISMS FOR HIGH PRESSURE AQUARIUM STUDIES[1]

by
Robert R. Hessler
Scripps Institution of Oceanography
LaJolla, California 92037

Introduction

On the surface of it, this question of what deep-water animals are available for high-pressure aquarium studies requires an enumeration of the kinds of animals that live in the deep sea. However, this listing is neither possible nor necessary in a treatment of this length. We all know that the same major environments exist in the deep sea as exist in shallow water. One can find both rocky bottoms and soft substrates for benthic organisms, and an unrestricted water column for the pelagos. In these environments live the same higher taxa that exist in shallow water: polychaetes, bivalves, gorgonians, amphipods, fish, sponges, and so on.

Also, it is well known that as one goes into deep water, relative importance of these taxa often changes, and usually lower taxa which are not so important and commonly not even present in shallow water become major components of the community. For example, among the isopods (which are present in both shallow and deep communities), the subtribe Paraselloidea is relatively unimportant in shallow water communities, but it is a dominant element in the deep sea. Within this subtribe, the family Haploniscidae is limited to the deep sea, except rarely at high latitudes.

All of these generalities are easily seen in the literature, particularly in the Soviet compendium on the Pacific Ocean (Zenkevich, 1969) and the extensive literature searches that play such an important part in the Galathea Reports.

Viewing the subject from the more restricted point of view of the workshop, the question becomes: what is there for the experimenter to work on? The problem then becomes what animals would be interesting subjects, and what animals are feasible to study. The question concerning interest is an easy one to answer. At this stage, there has been so little work with deep-water organisms that almost any animal would be interesting.

It is the second question that deals with the real limiting factor. Feasibility is a composite of the characteristics of the animal and of the community in which it lives, all viewed through the filter of our technical

[1]This contribution of Scripps Institution of Oceanography was supported by NSF grant GB14488

abilities. If the animal can't be caught, or if it is always dead upon retrieval, it doesn't matter how interesting its physiology might be.

The remainder of this discussion will treat aspects of deep-water organisms which affect the feasibility of their utilization in pressure aquarium studies. Toward this end, we should consider our goal to be healthy animals. Obviously, moribund animals will be much easier to obtain, and correspondingly less interesting. A further assumption will be that our goal is truly abyssal and hadal forms. It is much easier to get animals from shallower depths, but once again, this is less interesting.

Difficult Aspects of Deep-Water Organisms

The most serious problem in collecting deep-water organisms is their fragility. Generally, by the time one has focused on an individual for study, it has been mechanically damaged. Its limbs are broken. Its body is ruptured or torn. Spines and scales have been knocked off. Delicate mantle cavities are clogged with mud. For some taxa, undamaged specimens are almost unknown.

Fragility is particularly troublesome with the deep-water pelagos. Food is rare for deep-water organisms, so that energy expenditures have been reduced wherever possible. Because maintaining a position in the water column can be a significant source of energy consumption, many mechanisms have evolved to approach a state of neutral buoyancy. Often the problem is solved through incorporation of buoyant materials. Crustaceans frequently contain lipids whose low densities help buoy the animal. Fish use swim bladders, but this organ tends to be lost in abyssopelagic fish. To minimize the need for buoyant materials there has commonly evolved a reduction of high density tissues, notably within the skeletomuscular system. Both bone and muscle have significant negative buoyancy, and a common trend among deep pelagic fish is a lessening of both of these tissues (Marshall, 1960). Often there is an increase in water content. Deep-water animals are often gelatinous in consistency; the abyssal octopods are a striking example of this. Here, the body fluids may also be positively buoyant as a result of different ionic concentrations (Denton, 1964). Many abyssoplanktonic crustaceans have voluminous bodies in which most of the body cavity is empty space.

The result of most of these mechanisms to increase buoyancy is that the animal tends to be flabby and fragile, making mechanical damage a serious problem.

Of all the presently employed sampling techniques, trawling (a term which lumps dredging, trawling, and plankton netting) obtains the most animals. But by the very nature of the technique, it submits the animals to the most severe mechanical damage. To solve this, one could use a grab, corer, or large volume water sampler. These minimize mechanical and

physiological damage, but because of the two following features of deep-sea communities, there are serious problems with these devices as well.

Deep-sea animals are sparsely distributed. Programs which have studied standing crop both in shallow and deep water typically report that the abundance of benthic life is two or more orders of magnitude less in the latter environment (Sanders & Hessler, 1969). Once again, grabs, cores, and volumetric water samples are put at a disadvantage. In any such sample, the few animals obtained are not so likely to belong to the same species as would be the case in shallow water, and in repeated tries, the same animal may not appear again. Thus, one will commonly not have the benefit of replicate runs to give one's studies statistical reliability.

Deep-sea animals tend to be small. The average benthont is less than 5 mm long. Few are larger. Thus, finding them becomes a major problem once the sample is on the deck. The usual solution is to wash the sample, retaining the animals on a screen of appropriate mesh. The result is massive mechanical damage unless extraordinary care is used.

Washing the sample will usually result in extensive physiological damage as well, because the organisms tend to be stenothermal. Abyssal temperatures are 3°C or less, and at any one spot exhibit little variation. Thus there is little selective pressure for maintaining temperature tolerance. Most work on the deep sea has been done either at low latitudes or in the warmer months. Since samples usually need to be washed, the animals are bathed in the warmer surface waters for at least short periods of time. Even brief exposure to such temperatures seems to cause severe thermal shock or death. Such exposure is difficult to avoid. If the sample does not need washing, it is probably because it was winnowed by water on the way up. Without the insulating effect of the cold bottom mud, the animals were exposed to the warmer ambient water, and they will have been killed before they reach the investigator. The situation with deep-water pelagic organisms is especially difficult because, by the netting technique, organisms are exposed to ambient water conditions from the moment they are caught.

The problem of thermal shock can be minimized by either working in high latitudes, or in mid latitudes in winter months; however, the frequency of bad weather in such circumstances creates many other disadvantages. Refrigerated sea water may be used in washing, but it is usually not feasible to make more than small quantities.

In concluding and summarizing this list of vexing characteristics, one must emphasize the quandary they put us in. None of the standard techniques now used by deep-sea biologists is satisfactory. Each is strong where the other is weak. Later, a more promising approach will be mentioned.

Positive Aspects of Deep-Water Organisms

Many deep-sea animals have a depth range of 2000 m or more. Thus, a species whose maximum depth is abyssal may have a minimum depth in the bathyal or less. Some species (or higher taxa) with a deep depth range at low latitudes may live much more shallowly at high latitudes. This is the phenomenon of equatorial submergence (Ekman,1953).

The result is the same in both cases: individuals of deep-sea taxa may be collected at shallower depths. The main difficulty with this is that individuals living at shallower depths may not have the genotype or phenotype of deep representatives, as a result of restricted gene flow or acclimatization. The answer to this must await actual measurements.

A second feature that offers hope deals with escape reactions. Most people who have been on the bottom in deep submersibles have been surprised by the fact that most larger benthonts are not at all or are only slightly disturbed by the presence of the submarine. This means there is some hope of catching single individuals for *in situ* experimentation or for recovery in special containers.

One of the most promising aspects of the mobile component of the deep-water fauna is that it is attracted to bait. This should be no surprise. In an environment where food is scarce, one would expect that animals would be adapted to home in on food with exquisite sensitivity.

John Isaacs and the Marine Life Research Program (sponsored by the State of California at Scripps Institution of Oceanography) have developed a deep-sea camera, called the Monster Camera, which takes advantage of this attribute. A can of bait (spoiled fish, etc.) is photographed at 5- to 15-min intervals using a time-lapse camera and strobe light. The device is free-fall, and at the end of a preset time, its weights are released, and the camera returns to the surface.

Figures 52A-D are a selection from a series taken at Station Victor, at a depth of 5850 m in the northwestern Pacific (34°03'N, 163°59'E). Soviet studies in standing crop (Filatova, *in* Zenkevich, 1969) rate this area as supporting a benthic invertebrate biomass of 0.5-1.0 g m^{-2} (wet weight), a very small amount compared to shallow-water bottoms. The first picture (fig. 52A), taken within 15 min of contact with the bottom, shows a typical manganese nodule-red clay bottom, barren of life, as is characteristic of deep-sea photographs from those depths. Thirty minutes later, two natantian decapods have come into view (fig. 52B). An hour after bottom contact, the first fish was recorded. At 3-3/4 hr (fig. 52C) there are about 10 fish. The orientation of the fish suggests the presence of a current, which will be a crucial physical feature in determining the degree to which mobile scavengers can find food. At 7-1/4 hr (fig. 52D) the number of fish that have collected is truly remarkable, in view of the initial indication of barrenness.

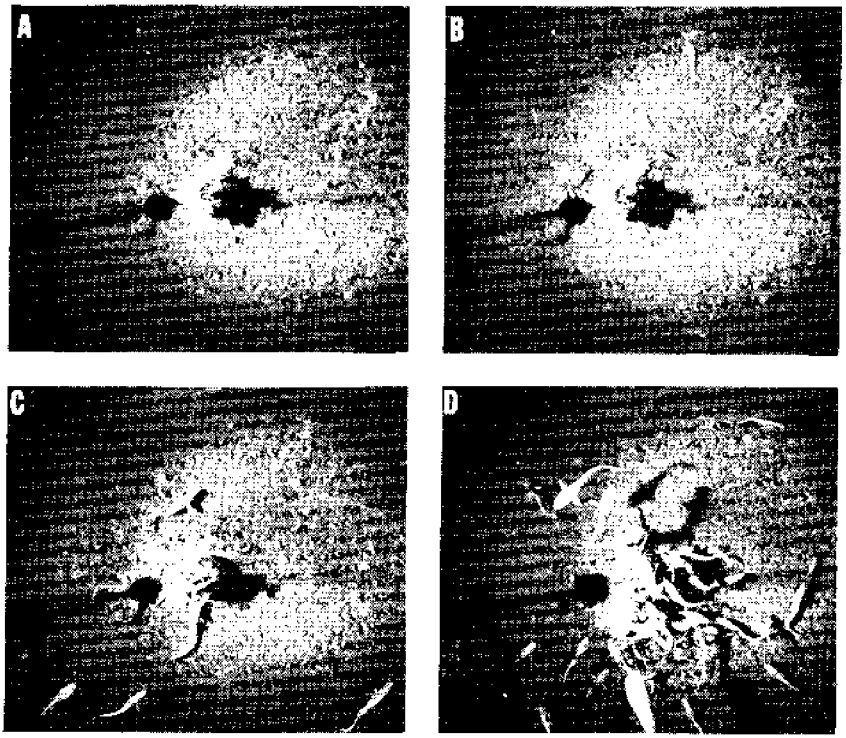

Figure 52. Monster Camera studies of the attraction of animals to bait. The locality is "Station Victor", 34°03'N, 163°59'E, 5850 m. A, within 15 min of placing the bait on the bottom. B, 45 min after placement. C, 3-3/4 hours after placement. D, 7-1/4 hours after placement.

Fish dominate most of the photographs, but a wide selection of invertebrates is also attracted, as shown in figures 53A-D. The Monster Camera has revealed that natantian decapods (usually the first animals to appear), crabs, amphipods, holothurians, echinoids, ophiuroids, cephalopods, and polychaetes can be attracted by bait. We must note that most animals are too small to be recorded in such photographs. Undoubtedly there is a large variety of small things also being attracted.

The pictures suggest that no matter how barren the bottom or sparse the fauna, there are always some organisms that will be attracted unless some physical factor, such as anaerobic conditions, restricts the availability of life. These records give a firm foundation for believing trapping techniques could be developed to yield suitable aquarium materials. By using traps, the problem of mechanical damage should be largely eliminated.

Even if one could capture animals and not damage them mechanically, physiological stenotopy may still be a serious barrier to success. The

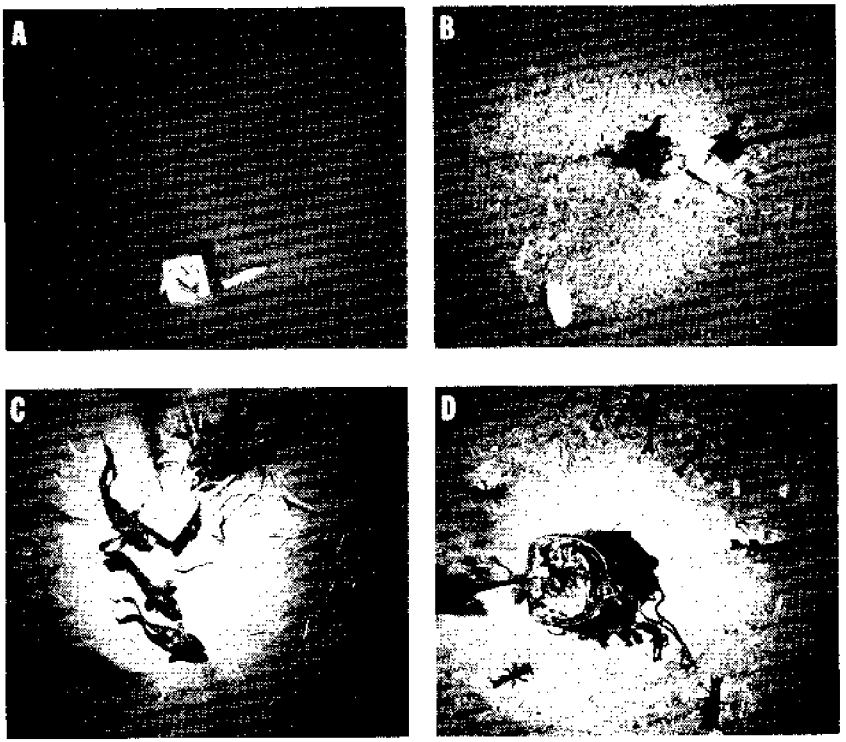

Figure 53. Monster Camera photos showing the attraction of invertebrates to bait. A, three gammaridean amphipods. These amphipods, about 28 cm long, are far larger than any that have ever been reported. 23°54.5'N, 144°04.9'W, 5300 m. B, holothurian, Station Victor. C, the polychaete *Hyalinoecia tubicola* in great abundance and one lithodid crab. 30°53'N, 116°45'W, 2000m depth. D, natantian decapods. 21°14.5'N, 158°14.5'W, 2000 m depth.

problem of temperature is solved by using appropriate insulators, by working where or when surface waters are cold, or by using refrigerated water on deck. The problem of pressure effects is more difficult to solve, but this may not be serious over short periods of time, as indicated by the following anecdotes.

In February, 1969, the deep-sea program at Woods Hole Oceanographic Institution collected a large benthic sample from 2000 m (bottom temperature 2.5°C) on the Gay Head-Bermuda Transect. (Sanders & Hessler, 1969). The sample was washed in the cold North Atlantic surface water. Some of the animals (bivalves, polychaetes, and a tanaid crustacean) were put in dishes of seawater in a refrigerator at 5°C. Taken in part, the animals were transferred to the laboratory's circulatory seawater system. In each group, there were a few animals alive after a month. A few bivalves had put out their siphons. The tanaid lived even though it had lost a limb.

Sanders (personal communication) feels bacterial activity, against which no preventative measures were taken, may have participated in the animals' unhappiness and eventual death.

Eve Southward of the Marine Biological Association of the U.K. (personal communication) kept a pogonophoran from 1700 m alive in refrigerated sea-water for 18 months. Andrew Carey at Oregon State University (personal communication) kept a gastropod from 2800 m alive for three months.

The lesson from such incidents is that if at least rudimentary care is taken to protect the animals from major temperature changes, one can keep the animals alive long enough to get them back into *in situ* pressure conditions. This is not to say that these animals have not suffered permanent pressure damage, but at least we can say it was not immediately lethal. For the early stages in high pressure research, that may be good enough. It will not be too difficult to work out ways to protect animals from major temperature changes, but to do the same for pressure is more difficult, and certainly a lot more expensive.

Conclusion

To evaluate these lists of the refractory and the hopeful aspects of deep-sea organisms, it appears that there is good reason to be optimistic. Given the present technical abilities, a little luck, a fair amount of money, and a lot of patience, one should be able to retrieve animals alive.

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Discussion Following Hessler Paper

ZOBELL: I would like to point out that there is a terrific difference between the effects of pressures on the order of 200 atm. (which occur at 2000 m) that you talk about, and pressures on the order of 500-1000 atm. that you'll

have to deal with if you are working with truly hadal deep sea organisms. Our single cells indicate that we find only negligible effects—biochemical or physiological effects from pressures on the order of even 200 or 300 atm. Where we start to get the discontinuity is between 400 and 500 atm., and 600 or 700 atm. usually have lethal effects on organisms that are normally barophobic. I would like to share your optimism for dealing with organisms from depths down to even the average depth of the sea, 3,800 m, but I believe that the problems are going to be of a different order of magnitude when you start to collect invertebrates or even vertebrates at depths of 5,000 to 10,000 m.

HESSLER: I think your point is well taken.

BRAUER: The infaunal and the average epifaunal component you are looking at in these pictures, I think is the concentration from a very wide area over the bottom. The mobile component of the deep fauna is not basically different, but at an extreme in terms of the average fauna composition. But these are the animals you will have to work with. Is anybody anticipating trying to get an idea of the density of these things by using several traps simultaneously, and comparing the results to what you get with a single trap by looking at the numbers you get at a certain time?

HESSLER: There are people who are trying to do this. I don't view their attempts with any degree of optimism because there are some imponderable variables that are difficult to deal with. You must know the current speed, you must know how fast they swim, you must know how sensitive they are to the odors and dilution effects of the bait odors as they work. And so I don't think that using these attraction techniques they are going to be able to find the density. I would much rather see them just use camera surveys in which they have a camera floating along and taking picture after picture, and then counting fish. I think that this is more likely to tell what the density of these creatures are.

SAUNDERS: Well, but camera surveys, you know, have never revealed, for example, Greenland sharks and things like that.

HESSLER: I think that exactly reflects how rare they are. I think that's a real thing. I don't think it's the cameras that scare the animals away, because in my experience with submarines, submarines don't scare fish.

SAUNDERS: But you've never seen the big fish until .....

HESSLER: In a way my evidence is not simply negative evidence. When you are down in a submersible, you can tell when a fish has been at a spot because there is a cloud of sediment hanging from where the fish darted away if he was sitting on the bottom, or even if he was close to the bottom.

GORDON: There is a fair amount of progress being made in keeping some of the more mesopelagic kinds of animals alive in capacity and really doing some fairly sophisticated work with them. Dr. Childress at Santa Barbara has had a couple of species of mid-water (less than 1300 m) crustaceans in culture for some time, and specifically, a thing called *Macrophausia india*

which he keeps in large numbers, moulting, growing, and feeding, and reproducing in the laboratory. It's very hardy. It's a very large, bright red euphausid, and he's able to have the thing going in the lab for a year at a stretch or more. And there are a very few of the mid-water fishes that we know about, so far anyway, which you can trawl in an Isaacs sled trawl or other kind of mid-water trawl and bring up to the surface and keep alive for some time. Lantern fishes and things like this are very, very fragile, and very difficult to handle. The same thing is true for the *Gonyaulix*, and a number of others. And the ones that you can keep alive, in general are rather rare, so that they're very hard to work on. For example, there's a thing which occurs in mid-water trawls occasionally off Southern California, and if you go out and trawl for a month, going every day, several times a day, you might catch three of them. But when we do catch one, it's possible to keep it alive for some period. Childress on the *Alpha Helix* earlier this past year caught three of these during the course of a month's trawling, and was able to keep one alive for almost two weeks, in a tank on board, and finally brought them back to his laboratory in Santa Barbara. The fish that I mentioned yesterday from the Galapagos, this mid-water sea bass, is potentially one of the best candidates that way. It is a very unusual mid-water fish. It's small, very solid, it does not get damaged very much in the trawl. On the *Alpha Helix*, we had one on board for three days, just keeping him in refrigerated sea water.

SCHELTEMA: With reference to the question of the fragility of these deep water animals, even with these relatively hardy benthic creatures, a general tendency is that by the time they reach the surface they've been flayed. The scales come off very easily. It's just a question of what kind of animal do you want to work on. Do you want to work on an animal that is suffering from severe wounds or do you want to work on an animal that is basically healthy? I think it's difficult to use data from animals that are badly wounded.

(Question) Are those polychetes in the picture—tubiculous polychetes?

HESSLER: Yes, they make a tube which they carry around with them.

FLUGEL: Is it possible that those numbers directly underneath the bait were just exposed by the activity of the fish swimming around?

HESSLER: No, they move on the surface.

FLUGEL: I didn't see that many tracks converging towards these traps.

HESSLER: They actually swim. They hop up to the water.

LOWENSTAM: That 2000 m bivalve and polychete sample—was it taken with a dredge or was it taken with one of the bottom sediment samples?

HESSLER: It was taken by a trawl technique with the epibenthic sled. The animals came up with a large volume of mud, and then they were washed in the surface water.

BRAUER: It might be worth mentioning that the fascinating picture Hessler just showed may have some relation to an extension of work on artificial

reefs which is currently of interest to us. As you know, with these structures the key question as yet unanswered is whether they merely pirate fish from the surroundings and concentrate them or whether they actually enhance productivity in the region in which they are placed. There is some theoretical reason for feeling that they might be more likely to enhance productivity in the sub-photic zone (where they've not been tested to date, so far as I know) than in the shallow water where they have been deployed hitherto. We are currently exploring designs involving several artificial reefs spaced in appropriate geometric patterns in an attempt to study this question. If that technique works, it may well be applicable also to the deep ocean bed situation where some sort of pattern of serial photographs, or serial sampling along lines connecting two or more garbage cans, may provide analyzable data bearing on the question of population composition and population density.

HESSLER: On this point, I would like to report that on the West Coast they are already fishing sablefish (a bathyial fish) in commercial quantities. I think they are in for a rude shock, because I don't think the turn-over rate is going to be high enough to support that fishery very long, but they think that life is golden now.

ZOBELL: During your erudite discussions on the properties of deep sea fauna, I understood you to say that many of these forms were filled with empty space. What does the zoologist mean by "empty space"?

HESSLER: Nothing very complicated. If this is a cross-section of the body, there will be a little muscle here—there may be a digestive gland here—a gut here—and a nerve cord here and a little muscle here. And this is filled with fluid.

ZOBELL: Oh, it's water then, not empty space?

GORDON: Have you ever tried to hook them? Put hooks on them?

HESSLER: This has been done. The people at Scripps have done it. I think people elsewhere are using the technique of free-fall long lines. They have the same basic technique with an automatic magnesium release and then a long string of hooks, and you routinely catch large quantities of fish in this fashion.

GORDON: We've gotten very large numbers of rat-tails and things like this down on Guadalupe Island that way, and you get three-foot-long fish. The only trouble is they all come up fairly dead. They die fairly rapidly on the way up. There are some people who've proposed having, say, a very long lead from the float so that the animals themselves never come up right near the surface, and then sending the diver down and trying to put them in a box of some kind that would be insulated while they're still down at some depth. But the indications are that even at depths of 30-50 m they are already dead.

LANDAU: How long does it take the bait to get down to the bottom, and is it open at the bottom or is the bait exposed as it is going down?

HESSLER: The bait is exposed as it is going down. Its residence time at any point in the water is not sufficiently high—I doubt it if any pelagic organisms could take advantage of it. These things have sinking rates of many meters per minute, so it's a fairly fast swim for anything that wants to keep up with it.
FLUGEL: The giant brachiopods that you photographed at 6,000 m—did you capture them in your trawl?
HESSLER: No one has ever captured that, and I don't think anyone ever will. That beast looks like it is very fast, very competent to take care of itself. It could be captured in traps easily.
MACDONALD: Has anybody tried to anesthetize this material and bring it up in a relaxed condition?
HESSLER: Not to my knowledge. I see no reason why it wouldn't be possible. I envision a trapping technique whereby at a given point in time a release not only drops the weights but closes the entrance to the trap and at the same time could break a vial of anesthetic to be released into the pressure chamber or into the temperature chamber. All of these things would be feasible.
MENZIES: Really, all you have to do is to make that can big enough, or have a lid on it to bring it back. Instead of bringing back the camera, bring back the can full of fish. On that light that you used for the camera—was that kept on constantly or was it just triggered with the camera to take the picture? Could it have been a means of attracting the fish to the bait?
HESSLER: No, it was a strobe light. It just flashed at the time the picture was being taken.
MENZIES: Have you ever tried strobing twice quickly? I mean, a few seconds apart for possible movement perhaps?
HESSLER: I don't know whether they've done this or not.
REYSS: The Mediterranean could be an interesting field of investigation. In this Sea, benthic animals, in depths between 2000 and 3000 m are, of course, small, fragile and sparse. They are stenothermal too, but in winter the sea surface temperature is the same as in deep sea, i.e., 13°C. An important fact is that for some groups (annelids, crustaceans, bivalves) only 2 or 3 species can represent 80% of the population, and some species are represented in all samples by hundreds of specimens, so we can study them, and collect them when we need them.
(The following comment was contributed by Dr. Beatrice R. Richards after the conference by invitation to bring out what appears to the editor a most important potential source of deep sea fauna suitable for use with high pressure aquarium systems).
RICHARDS: There are very little data available on deep-sea fouling per se. A great amount of information has been published about the engineering and types of materials to be used for deep-sea structures, as well as a

considerable number of studies made of the fauna living on the ocean floor. One work in particular that may be of use to you is "Ecology of the Deep-Sea Benthos", by H. L. Sanders and R. R. Hessler, Woods Hole Oceanographic Contribution No. 2171, 1970. This is a very inclusive study of the transect of the ocean floor, between New England and Bermuda in Science, 28 March 1969, Volume 163, pp. 1419-1424.

James Muraoka, Naval Civil Engineering Laboratory, Port Hueneme, California and John DePalma, U.S. Naval Oceanographic Office, Washington, D. C. are the two I mentioned who are perhaps the most knowledgeable about deep-sea fouling. Muraoka's work is concerned with deep submersible test units in the Pacific and DePalma's with the Tongue of the Ocean in the Atlantic. Much more fouling attached to rocks on the ocean floor was recorded from the Pacific area. Specially mentioned were Foraminifera, Bryozoa, glass sponge, serpulids, brittle stars, chiton and hydroid. The assumption was made that it would be possible for these organisms to become attached to material submerged.

Anaerobic, aerobic and sulphate-reducing bacteria as well as polychaetes, nemerteans, small crustaceans, pectens, limpets, sea cucumbers, and brittle stars were found in the sediment.

Algae, hydroids, filamentous bryozoans, molluscs, and barnacles were found on panels 14-m deep in the Tongue of the Ocean but none at the 1722-1737m depth. The marine borer, *Xylophaga,* was present at both deep-sites.

Polypropylene lines were used at both locations. Off Port Hueneme, a dense growth of hydroid plus slime, annelids, star-fish, and sea anemones was recorded, but no delineation was made as to the kinds of growth at various depths. Hydroid was reported growing on the STU frame on the bottom.

DePalma gives an excellent chart of the fouling limits on the propylene mooring line in the Tongue of the Ocean. The fouling was primarily restricted to the upper 300 m (dominantly hydroids, bryozoa, and barnacles). Slight fouling to 1600 m and below 1600 m.

Dr. Bryce Prindle, Woods Hole Oceanographic Institution, has made numerous observations on the distribution of the fouling off Bermuda, Florida, and New England at depths to 5500 m. In addition to the fouling reported by Muraoka and DePalma, Prindle noted that Radiolaria (?) were present from 2000-3500 m, and that wireworms (white and pink) were found from 500-4500 m on most materials. Slimes were also found. The radiolaria clung tenaciously to nylon. They do not cause "bulk" fouling but would be very detrimental to optical surfaces.

Muraoka's report R-329, November 1963, includes an experiment of submerging *Limnoria tripunctata* removed from coastal piling to a depth of 1,700 m and successfully retrieving them. This experiment was also

performed under simulated laboratory conditions. I do not know if this experiment was ever repeated but feel that a one-shot deal of 5-min exposure is not conclusive.

William F. Clapp Laboratories
Duxbury, Mass.

CURRENT DEEP BENTHIC SAMPLING TECHNIQUES FROM SURFACE VESSELS*

By
Robert J. Menzies**

Introduction

The standardized and current apparatus for deep-sea collecting of marine organisms have been described in a variety of review articles by Holme (1964) and Hopkins (1964). The techniques of deep-sea trawling utilizing accumulative tension for bottom contact indication and a review of reasons for trawl failure were described by Menzies (1964). The use of acoustic pinger data for bottom contact information has been developed and described by Rowe and Menzies (1967) and Wall and Ewing (1967).

The interest in obtaining reliable on-deck information about bottom contact has come about from the realization that over *50%* of trawl failures are due to faulty bottom contact information (Menzies 1964).

Deep-Sea Trawl Behavior

Heretofore there has been no visual record of what a trawl was doing on the bottom in the deep sea (depths greater than 2,000 m), although divers have recorded the action of trawls in shallow water (Holme, 1964). On the R/V *Eastward* Cruise (E-36-67) a 5-ft Blake trawl was outfitted with a time-activated Edgerton-Germalshausen-Grier underwater camera that was placed inside the trawl looking directly out the mouth (Figure 54, A-B). It took pictures at 3 to 5-sec intervals, and the photographs tell approximately what the trawl was doing on the bottom. The Loran "A" fixes suggest that the trawl was towed between 32° 31' N, 67° 41' W and 32° 34'N, 67° 45'W, trawling a rough distance of 4.4 n. mi, or 8 km over the bottom during the total 1 to 1-1/2 hours on bottom. Bottom contact was observed using a pinger and a strip chart tension recorder.

The camera took pictures first at 3-sec intervals but by the end of the run, 67 min later, it had slowed to 5 sec per exposure. In all, 192 frames were taken during the trawling. In order to understand the information presented in the photographs, it is essential to know the rigging of the trawl and the placement of the camera and strobe light (Figure 54, A-G).

Photographs of the first bottom contact showed a bent, mud-covering trawl pipe with the weight hanging down below the apex of the trawl bridle. Obviously, the trawl wire was then on the bottom (Figures 54-C, 55-D). The trawl bridle pipe was probably bent during descent, or when it hit the

*Contribution No. 276 from the Department of Oceanography, Florida State University.

**Department of Oceanography, Florida State University, Tallahassee, Florida, 32306.

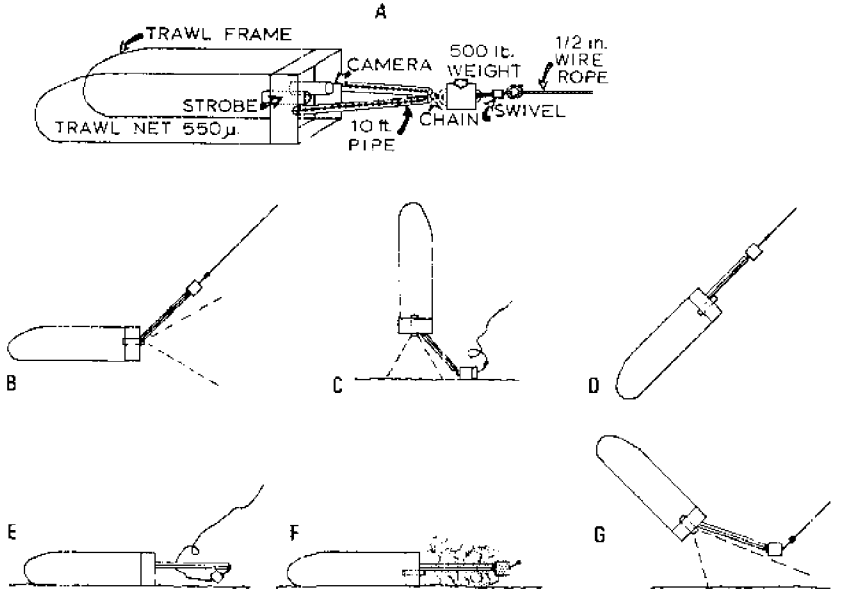

Figure 54. Deep-sea trawl behavior. A. Diagram of 5' beam trawl showing position of camera and strobe. B. Trawl being lowered, photos taken show clean water and no bridle in picture. C. Trawl upset, camera faces bottom, takes photo view perpendicular to bottom. D. Trawl on way up, bridle and clean water or mud falling off show in photo. E. Trawl on bottom and weight drops and bridle toward trawl mouth. F. Trawling, mud clouds in photo, no bridle. G. Trawl oblique, camera takes oblique view of bottom.

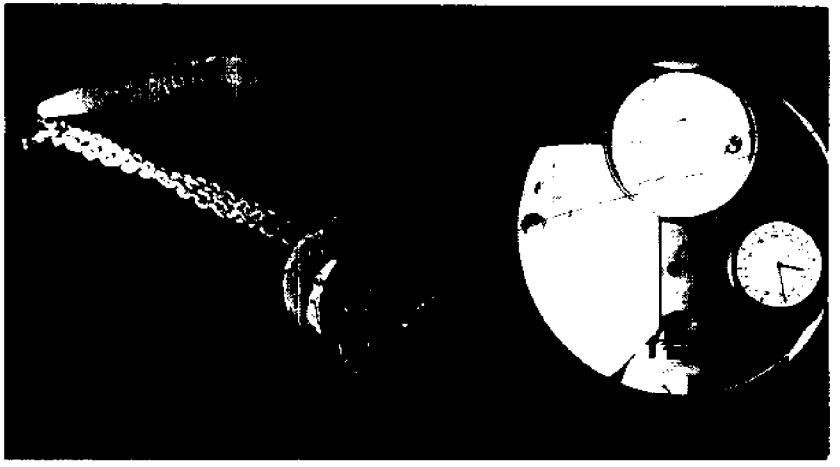

Figure 55. Underwater trawl camera photograph showing trawl behavior on bottom at 5,050 m. (Eastward Sta. 7799). Trawl on bottom with weight dropping toward trawl mouth (ref. Photo Ser. II-8).

bottom. A cloud of mud shows (Figure 54-F, Figure 56) in front of the trawl. Figure 57 (XV, 13-frame) show muddy water but no mud clouds and suggest the camera face was covered with a thin layer of mud. Figure 58 (XV-8 frame) shows the bent trawl pipe and a little of the bottom at an oblique angle. Figures 54-D and 59 (XV-frame 5) show the bridle and weight being lifted off the bottom with a cloud of mud coming off the junction of the bridle and the weight. Photos showing mud clouds and no bridle within the photo frame suggest trawling (Figure 53=F, Figure 56) (ser. XV-2). These figures, not arranged in time sequence, show information characteristic of deep trawling that is now available through photographs and information which formerly was based on imagination.

Analysis of the photos tells the following story: (Series refers to the sequence of strips of film, each with around 20 frames.)

Series I. First 15 frames clean water, trawl being lowered.

Series II. Six photos show mud on camera front, and photos 7-10 show bent pipe. The relationship of the bent pipe to the weight in Figure 55 (II-8) suggests that the trawl has flipped over sideways.

Series III. First 18 frames show mud on front of camera, next frame (19) shows bent pipe and frames 20-28 show trawl being raised under tension—up to now the trawl has done nothing but hit bottom, flip sideways, and bury mouth in the mud—this has taken three minutes.

Series IV. Frames 1-13 show trawl being raised and dropped. Frame 14 shows overhead bottom photo as trawl aims at bottom, mouth-first (Figure 54-C). Frame 15 shows camera covered with mud. Frames 16-20, covering 12 seconds, show trawl trawling.

Series V. VI. VII. Twenty-five frames each, all show mud clouds suggesting active trawling (Figure 56).

Series VIII. Frames 1-20 show clouds of mud but there was excess wire on the bottom, hence little actual trawling. Frames 21-26, with camera face dirty, suggesting trawl dug into mud.

Series IX. Frames 1-3 still dirty camera front; frames 4-23 clean water, suggesting trawl aimed upwards, absence of bridle from photo frame suggests trawl now on its side.

Series X. Frames 1-25 show same as IX.

Series XI. Frames 1-20 with mud on camera face, trawl dug into mud. Frames 21-28, mud clouds and active trawling.

Series XII. Frames 1-24 mud clouds, trawling.

Series XIII. Frames 1-16 mud clouds and trawling; frame 17-19 face of camera dirty; frame 20 clear water, frame 21 mud clouds and trawling; frames 22 and 23 with front of camera dirty.

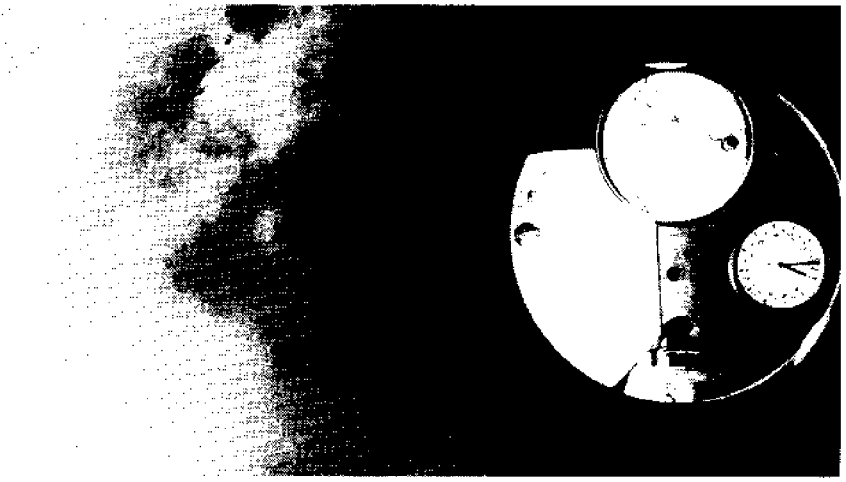

Figure 56. Mud cloud, trawl probably working (ref. Photo Ser. XV-2).

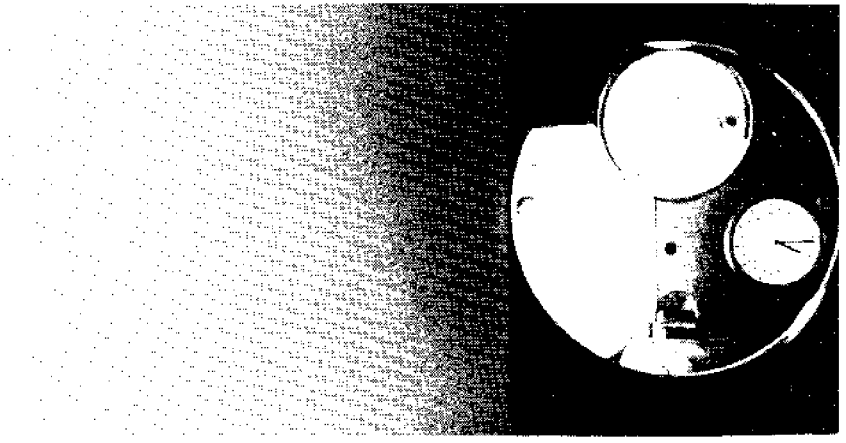

Figure 57. Camera face covered with mud, no photo of bottom.

Figure 58. Camera takes oblique view of bottom (ref. photo Ser. XI-8).

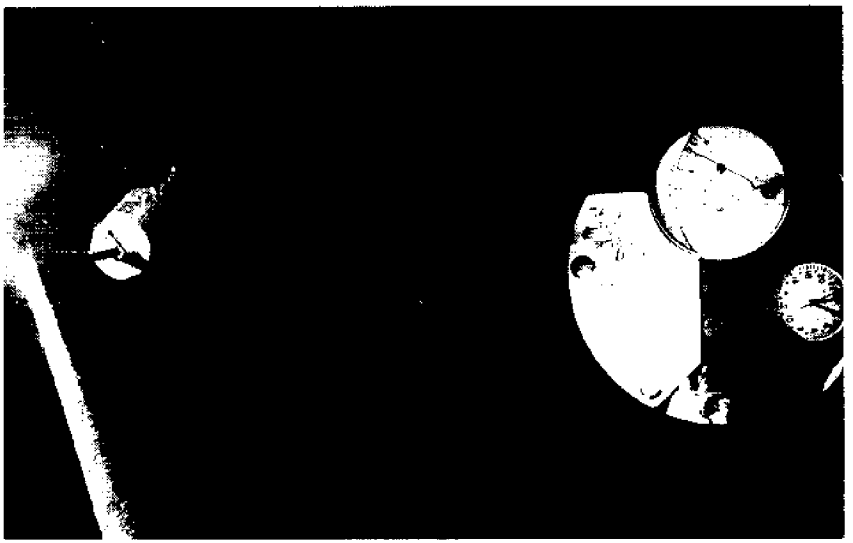

Figure 59. Trawl leaves bottom, note mud falling from bridle (ref. Photo Ser. XV-2).

Series XIV. Frames 1-3 camera face dirty; frames 4-12 clean water; frame 13 trawl pipes show; frames 14-17 camera face dirty, frames 18-22 trawl pipes show; frames 23-24 camera face dirty; frames 25-26 show trawl bridle and weights being lifted, frame 27 trawl off bottom. (This sequence suggests jumping or skipping into and out of the mud.)

Series XV. Frame 1 trawl off bottom; frame 2 trawl trawling; frames 3-6 trawl off bottom (Figure 59); frame 8 oblique view of bottom (Figure 58); frames 7, 9, 11, mud clouds, trawling; frame 10, 12, 13-24 front of camera dirty (Figure 57).

Series XVI. XVII. XVIII. XIX. Twenty-five frames, each of trawl coming up.

If the above interpretation is correct, the trawl spent a cumulative period of 252 seconds or roughly four minutes of its 90 supposed minutes of trawling doing what it should have been doing. The bottom trawling efficiency (not its catching efficiency) amounts to less than 10%. It would appear from the photographic evidence that most of the time was spent twisting, flipping, and flopping over the sea bed, spilling its contents back onto the sea floor, or being buried mouth first in the mud. This trawl captured 1/3 gal. of the maganese-encrusted pebbles, debris and animals as indicated below:

R.V. *Eastward* Station No. 7799

Porifera - 2	Bryozoa - 3	Polychaeta - 3
Nematoda - 0	Echinoidea - 0	Cumacea - 0
Alcyonaria - 1	Brachiopoda - 1	Isopoda - 7
Actiniaria - 8	Mollusca - 8	Amphipoda - 20
Tanaidacea - 1		(some pelagic)

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Discussion Following Menzies Paper

REYSS: I think that decompression does not affect animals, or affects them less than mechanical damage due to the dredging. In samples, from between 4,000 and 5,000 m depth, with an epibenthic sled, we could observe some living animals e.g. Cumaceans, and Polychaetes, when coming to the surface. These living animals were always in the upper part of the sample, i.e. the last dredged. Techniques like large cores (Reineck corer) could permit collecting some living animals, in undisturbed samples, if conditions of temperature are good. And then these animals could be recompressed in high pressure aquaria on the ship.

SCHELTEMA: I would like to suggest that if we start looking at animals that are easily brought up alive, we shall be looking at the unusual rather than the usual deep sea animal, and that this is what bacteriologists have been doing. I guess there is nothing else to do, but I think that we should strive to get animals which are typical deep sea animals because ultimately what we want to know is what the deep sea community is like - not what an isolated animal that happens to be very resistant to pressure and temperature changes.

GORDON: Perhaps it might be useful from the standpoint of people who aren't sea-going types and who haven't had too much personal experience with some of these collecting problems, just to mention the kinds of difficulties that Menzies skirted around a bit. He showed us some that you encounter if you hit the bottom. There are plenty of others on the way down and the way back. One of the main problems, even though it may sound in principle to be a really simple one, is the question of where you actually caught any particular organism. This is something that marine biologists have been complaining about for the last hundred years, and they still do. As far as I am aware, despite considerable amounts of recent engineering efforts by quite a few people, there still is not available any really very reliable net that will let you say, "I caught this particular organism, whether its a fish or invertebrate, in the depth range from, say, 700-800 m," or something like that. There are nets that are compartmentalized, where you can get some rough notion as to whether you caught it when the thing was going down, or at some point while it was being towed at, and most of the time whether it was being stable while it was being towed, or anything like that. And then perhaps you can get some compartments that say, "Okay, this was caught at....." whatever its maximum depth is, but it might also have caught something else on its way back up again. None of these mechanisms are reliable. The failure rate in most opening and closing nets, as far as I am aware, is at least 50% and probably more like 70 or 80% of the time.

TEAL: I think that we do have closing nets that work better now. I think of the nets that George Gregson uses which he calls "bongo" nets.

GORDON: Oh yes, we've seen bongo nets, too. They fail as well.
TEAL: They fail too, but when they work, they bring up a catch from known depths. In any case, what we've been talking about here is things on the bottom, and if you're over 5,000 m of water and you bring up clams, you can be pretty sure that you've been on the bottom.
VIDAVER: Sometime, if anyone really wants to see what's happening in the high pressure environment of the deep sea, and wants to see it on the surface here, someone's going to have to devise an aquarium and a trap which will maintain the pressure for the duration of the trip from the bottom of the sea to the surface, and not only that, but to maintain it in such a way that we can observe what's going on. This is a difficult technical operation, but it cannot be solved with any kind of trap which allows the pressure to decrease in it during the ascent. There must be some value in doing this because this is the only way some of the answers are going to be obtained. And I think it is not possible to discount the need to eventually do this.
TEAL: I'm going to talk about the value of doing things *in situ,* which is, of course, one way of making sure that you haven't reduced the pressure.
VIDAVER: I think that's worth reiterating the point that Dr. ZoBell made before, and that is that, despite Bob Menzies heroic isopods, very few animals from more than 6,000 meters are likely to survive to the surface in any kind of a device that does not take care of the pressure, and, as Menzies, and others have pointed out in the past, of the temperature problems. We are going to have to resolve these two if we want really deep animals. Scheltema's point seems to me pertinent: the fact that we have a few athletes that will make it ot the surface does not answer our questions. The animal that is stupid enough to not know that it is too deep or too shallow, and to die as a result, I find both unintelligent and unattractive. I want the ones that area bit more sensitive in order to know what to worry about.
GEORGE: Deep sea data gathering has more than one objective. One objective is to know what exists in the deep sea by just collecting it—the trawling may be quite okay for that. Second purpose is to quantify, to know the biomass, density, species distribution and so forth. For that the grabs, or the corers have been quite nice. The third is to bring them up under pressure, and again recollect the temperature gradient involved is tremendously important. In my own experience, I found no problem at all collecting deep water organisms at 8° over 2,000 m bottom and I kept them alive for two or three weeks.
HESSLER: When I first started working with deep-water organisms, I had the idea that it would be impossible to bring them up alive from any so-called deep-sea depth, and I was surprised to find that very commonly when you are washing the animals you found some living animals, albeit briefly, in the screen. These were animals from 2,000 or 3000 m. I was then

surprised by this event. I am surprised today when Reyss talks about commonly getting deep water animals alive from 500 m. What I would like to suggest is not if we don't attempt to get animals from deeper, we'll never find out whether what we are going to be surprised by. All of the physiologists in this room say that from 6000 m, or deeper, you are not going to get living animals. I would like to suggest that you may be surprised.

ZOBELL: With regard to the possibilities of collecting deep sea organisms of any kind, I am in complete concurrence with the view expressed by Hessler and the view expressed by Menzies that I think it is highly probable that we will be able to bring to the surface alive virtually all kinds of organisms in permitting normal reduction of the pressure that would take place during the time they are being brought up, considering that the time is not too long. I say this because I have yet to see any adverse effect of pressure such as occurs in the sea from physical pressure alone of short duration. When I say that we are going to get into the threshold at pressures of the order of 500, 600, or 700 atm. this has to do with prolonged pressure, such as would interfere with the biochemistry, the physiology, the biochemical reaction rates. I would qualify further what I said with regard to any adverse effects of physical pressure. I would have to exclude here the effects of dissolved gases. Here we get into a different situation which has not been discussed at all. Carbon dioxide, oxygen, nitrogen, possibly even other gases, here we may find some very dramatic effects. But, usually in the sea, gases that are dissolved are at partial pressures no greater than the partial pressure of gases that are in the atmosphere. Also, the rate of decompression is a very important fact. Instantaneous compression, meaning "milliseconds", I think, is going to be injurious to lots of organisms. It is to our bacteria. We have yet to find bacteria that will stand instantaneous compression. But if we compress them slowly, slowly being a matter now of a thousand atmospheres in a second, most of our bacterial cells tolerate this. I recommend a somewhat slower rate of decompression. Usually we attempt to decompress from 1,000 atm. in about a minute, rather than in a second, in order to avoid some of the mechanical things that happen, such as blowing out piston stoppers and breaking the apparatus itself because it is being compressed so rapidly. But as far as all the work I have done on single celled organisms, including more than 200 species of bacteria, yeasts and molds, as well as a few observations on a few simple invertebrates, I have yet to find any that will not withstand being compressed to 1,000 atm. in a matter of one to five minutes, in a virtually gas-free system, and then being decompressed in a matter of one to five minutes from 1,000 atm. to 1 atm.

Temperature, of course, is tremendously important, and as I said yesterday, in the introductory remarks, we are talking in a vacuum if we

ever talk about the effects of pressure without qualifying the temperature because if the temperature changes, too, then there are some effects on large molecules, on enzymes and so on, which are irreversible. So, I believe that it is going to be possible to recover organisms—deep sea organisms—without the necessity of keeping them at *in situ* pressures, provided, I would repeat, the gas pressure is low or normal, provided provisions are made for maintaining temperatures which are near those *in situ*, and provided too much time is not involved, because even with some of our deep sea barophilic bacteria, those which will grow only at high pressures, we find that after several hours they gradually die off at 1 atm. And if these die off, then most likely more complex multicellular organisms are going to die off too, in a matter of 12, 18, 36 hours or so. But if we're talking in terms of what is going to happen in a matter of 2 or 4 or 6 hours, about the time it takes now to elevate a payload from the Challenger Deep to the surface, I'm very optimistic about finding living invertebrates, even vertebrates, if they occur there. We've been finding living bacteria and other micro-organisms at such depths do survive this rather sudden change in environment from 1,000 atm. to 1 atm. even though the temperature has been changed, from let us say, 3°C to 8° or 9°C, as often times we find in our samples by the time we get them to the surface.

VIDAVER: I see no basic reason why decompression effects should vary significantly from compression effects. I think Menzies film yesterday, showing us rather drastic physiological compression effects on several organisms which even varied with time, suggest that this may not be exactly so. In my own limited experience with compressing invertebrates, I have found rather drastic effects, such Nudibranchs excising the serration on their backs. I don't know anything about the Nudibranch anatomy, but at about 500 atm. they just drop off completely. It doesn't kill the animal. He will recover, grow new serrata, and swim around for several weeks all by himself.

BRAUER: All the evidence we have from more complex organisms like the ones Vidaver mentioned, or like the beautiful films that Menzies showed, or like some of the stuff we've got, and a good many others indicate that the pressures we've got at the end of our line are such that only very few, and very specialized, beasts can survive. I would like to take a little bit of exception with your permission to your making a difference between physical compression and physico-chemical effects. I do not see, and indeed I think that you do not, that we are talking about anything in any of these organisms that does not involve in some way precisely the effect of high pressure on the thermodynamics of the various chemical reactions which keep our systems together. Undoubtedly the pressure effects we are talking about are not physical compression in the sense that the thing is crushed by the pressure of many tons, as the newspapers used to put it 30 years ago, but are in fact the chemical consequences of the alteration, displacement of equilibria, or of rates of the reactions which hold our systems together.

These changes are very important and very large. I think you're right that given a little more time some of these can be corrected - how much time, and how much increase in tolerance is a subject we here are studying right now.

YAYANOS: I have to take exception to the point of view that physical compression cannot be an important factor, because the compressibilities of all the different parts of an organism aren't quite the same. Shedding of the shell of a Nudibranch while the remainder of the structure survives may very well be not a chemical effect, but a physical effect due to the difference between the compressibilities of the shell and the rest of the organism. There is a perfect example at the molecular level when you have association between a membrane and a nucleic acid, and what is the compressibility at a lipid as against that of a nucleic acid—you have a physical dissociation not a chemical one.

ZIMMERMAN: I have no experience with marine organisms that are indigenous to these great depths. But for many years, I have been looking at invertebrates in pressure chambers, and what I'm amazed at is that anything can be brought up from any great depth without proper decompression. Whenever we have subjected things to high pressures, even in a variety of invertebrates, everything seems to stop. Not usually 100%, but usually there is a 50% decrease, and especially in the range of 10,000 psi, but if you look at the biochemistry you wonder how in heavens' name any organisms at 10,000 psi can not stop RNA, DNA, or protein synthesis. How can you take a system that is existing at these high pressures and expect to operate at a completely different pressure level and have them grow for as long as three days as we have heard they do in this morning's discussion. I think the approach has to be to develop instrumentation to collect at these depths, in such a fashion as not to have to worry about collecting, if you want to do physiological studies. I would criticize very strongly any studies in which people bring up organisms that are decompressed and attempt to do a meaningful physiological analysis. We know from the muscle work that there are spike potentials, that the heart rates change, as Landau and Cattell showed years ago, that there is a marked effect on the rhythmic activity; we know from studies on fine structure that when you compress there are alterations—its the same thing with decompression. What you are doing at one level is reversing at another pressure.

HESSLER: Most of the successful work on bringing things up has involved decompression from 200 atm. or less.

ZIMMERMAN: Yes, well, Dr. ZoBell has clearly pointed out that this is below the critical pressure. But when we go beyond this to 400 to 600 atm, this is where we have the really strong biochemical alteration, and I should think we ought to look at these.

LANDAU: It is much more serious because we work with tissues used to 37°, and we are talking about no effects up to 6000 psi. But when we go down to 3° we get effects right off the bat.

DEEP-SEA TRAWLING AND DREDGING USING ULTRASONIC TECHNIQUES

by
Lucien Laubier,
Jacques Martinais
and
Daniel Reyss
Centre Oceanologique de Bretagne,
B.P. 337 - 29 N. Brest
France

During the past ten years, the use of pingers has profoundly modified methods of work on the deep-sea bottom.

At first used to localize, in relation to the bottom, instruments dropped vertically from a ship (Edgerton and Cousteau, 1959, Edgerton, Cousteau, Hersey and Backus, 1960, for deep-sea cameras; Nalwalk, Hersey, Reitzl and Edgerton, 1962, for rock dredges) pingers have also been used with success to survey the descent of otter trawls (Backus, 1966) or beam trawls (Rowe and Menzies, 1967).

In all these methods, the pinger essentially gives knowledge of the precise instant of contact with the bottom, and the length of time the dredge or trawl works on it. In general, the assistance of a pinger substantially increases the chance of success in abyssal dredging, (see Menzies, 1964).

The diagrams provided by Kullenberg (1951) according to his theoretical calculations on the behavior of a cable towed in water, can be adapted, with respect to the length of cable paid out for a given depth, by using weights fixed on the wire, just in front of the dredge, a process which significantly reduces the length of wire needed to reach bottom (Rowe and Menzies, 1967).

These important improvements on techniques of work on the deep-sea bottom remained relatively empirical, and on the other hand, all the possibilities of the pingers had not yet been exploited.

During the Noratlante cruise on the N.O. *Jean Charcot*, August-October 1969 in the North Atlantic, we had the opportunity of making numerous abyssal operations which permitted us to improve techniques previously employed.

Theoretical Shape of the Wire

The theoretical considerations of Kullenberg in 1951, in preparation for the *Galathea* expedition, are an excellent starting point (see Appendix 1, where we summarize Kullenberg's reasoning leading to the equations for a wire in water).

First it is appropriate to insist on the basic simplifying hypotheses: movement is described in a vertical plane and it is assumed that the shape of the cable in the water remains the same. This assumption supposes that there is no paying out nor heaving in of the wire and that the unit ship-wire-trawl forms a stable geometric figure which is displaced horizontally at a constant speed. This approach to the problem permits, at least as a first approximation, a determination of the important parameters. Experience will show if this model can be applied to other cases.

The theory shows that in the very simple case of the cable alone, i.e., without any trawling apparatus, the shape is linear. Thus the angle β defined between the cable and the horizontal plays a very important role generally and will be called the asymptotic angle of the wire. It is solely dependent on the characteristics of the cable and the speed of the ship.

In general, with the model under consideration, the shape of the wire is given by a second order differential equation expressing the relationship between the differential logarithm of radius of curvature R and the curvilinear coordinate *s*.

The family of curves which forms the general solution of this differential equation, depends thus on two parameters determined by the boundary conditions at a particular point on the wire.

This particular point can be none other than the outboard end of the cable. At this point, the force necessary to make the trawl undergo a horizontal movement of constant speed, equal to that of the ship, is entirely determinate. Its vertical component is equal to the weight of the apparatus, its horizontal component to the resistance of the apparatus to advance in the water. Equations 9 and 10 thus permit determination at this point of angle α_o, the angle of the cable with the horizontal and the radius of curvature R_o.

Integration of the differential equation (8) along the wire proceeding towards the ship allowed us to show that the angle α tends toward the asymptotic angle β whatever the value of α_o. But if α_o is larger than β, i.e. if the relationship of weight to resistance of the trawl in water is greater than that of an element of the cable, the concavity is turned downwards. In the opposite case, if the traction essentially consists of the resistance of the apparatus to advance as would be the case when, for example, a net or dredge is in contact with the bottom, the angle α_o approaches 0 and the concavity of the cable is turned upwards.

Appendix (II) shows that during this integration of the differential equation along the cable, the angle β and the curvature 1/R tend toward 0 when the abcissa tends toward infinity.

Consequently if we give the angle α_o (different from β) the value $\alpha = \beta$, 1/R 0 would never be reached for a finite length of the cable.

In other words, it is not possible to conceive of a shape of a wire bearing a point of inflection (at a point of inflection, the curvature cancels out and the sign changes). This must be kept in mind in schematizing a cable in water.

Practical Consequences

The methods elaborated permit dredging or trawling on a given bottom with the least possible wire. In fact, taking into account the speed of the winch, the time necessary for paying out and heaving in is considerable when working at great depths, and therefore the station time can be significantly reduced.

Fig. 60 shows a family of curves representing different possibilities for the shape of the cable in water for a given speed and a given length. If the independent variable is assumed to be the vertical component of the traction of the wire on the apparatus, and we observe the position, in the vertical plane, of the apparatus, we notice the depth of the apparatus increasing regularly with its weight.

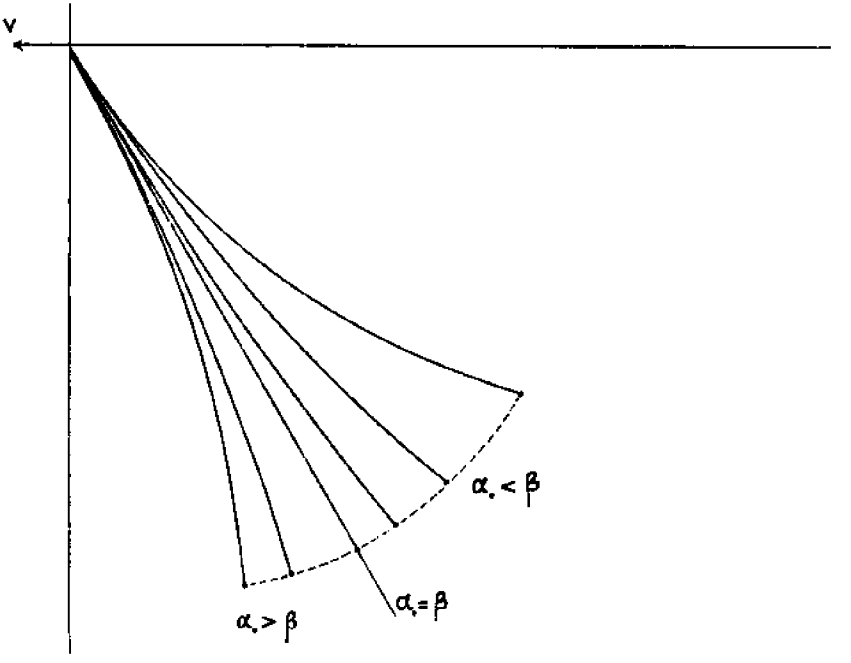

Figure 60. Family of curves showing possible wire shapes. Wire angle measured from horizontal at sea surface is α. Angle between outboard end of wire and horizontal is β.

The process consisting of artificially weighting a trawl by attaching weights to the wire permits a considerable reduction in the length of wire needed to reach bottom. Rowe and Menzies, 1967). It is necessary that these weights act effectively and, therefore, that they do not touch the bottom. It is not sufficient merely to use such weights; it is also necessary to be sure that, throughout the trawling operation, the conditions are maintained. To watch the distance between the weights and the bottom we have been led to use pingers.

Other Advantages of the Utilization of Pingers

In its classical application, a pinger fixed to an apparatus dropped vertically from a ship permits an evaluation of the distance of the apparatus from the bottom. If the apparatus, a trawl for example, is not located vertically beneath the ship, simple geometric considerations (fig. 61 and 62) show that the difference of the sonar paths between the pinger and the ship, and between the pinger and the ship after reflection on the bottom of the ping, is equal to 2 H sin Θ , H being the distance of the pinger from the bottom and Θ the angle defined in figure 60.

We have attempted to use pingers to evaluate these two sonar paths not solely in terms of their difference. In fact, knowledge of these two lengths permits a determination of the exact position of the pinger in relation to the ship in the vertical plane of operation. The pinger is found at the intersection of two circles centered, one on the ship, and the other on the "image" or reflection of the ship in relation to the bottom, whose respective radii are l_1 = direct path and l_2 = reflected path.

These circles intersect in two points, both symmetrical with respect to the vertical plane of the boat; the good point to note is obviously that which is located astern the ship.

In the strictest sense, to evaluate these sonar paths with precision, it would be beneficial to use ultra-sonic transponders, that is to say generators of pulses, triggered by a preliminary pulse emitted from the ship. It was, however, simpler to use existing pingers whose emissions were triggered by an internal clock on the condition that the stability of the latter was sufficient.

If, for example, a precision of 15 m along the path for the entire operation (6 hrs) was desired, it would be necessary to have a stability of the clock better than $\frac{15M}{1500\ M/S} = \frac{1}{100}$ S. OR : $\frac{1}{6 \times 3600 \times 100} = 0.5 \times 10^{-6}$ (relative value).

This was the case for the pingers used and for the time base of the recorder, both crystal = controlled.

There is no reason for the recorder and the pinger to be synchronized from the beginning. The echo is recorded as a straight line parallel to the

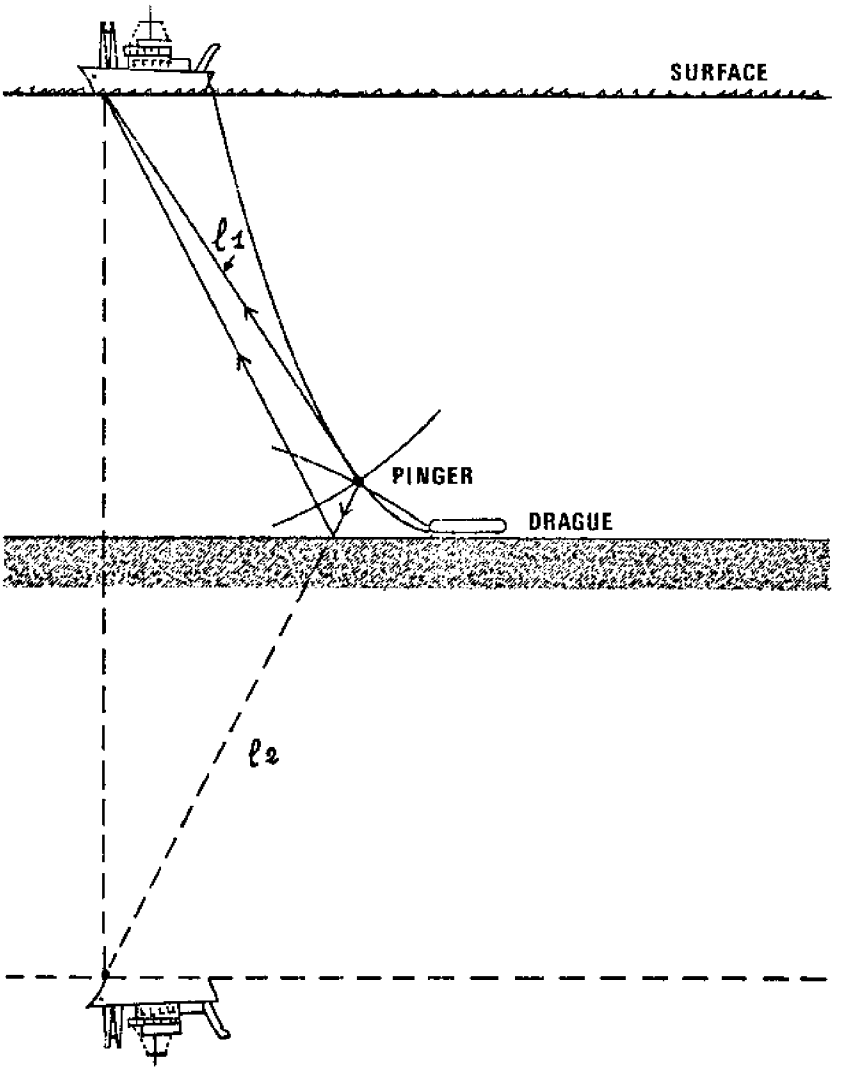

Figure 61. Sound trajectory of a pinger-fitted trawl.

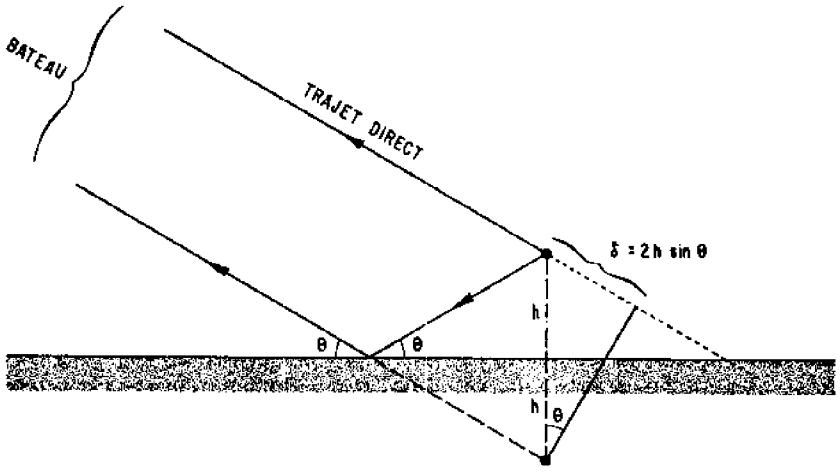

Figure 62. Detail of sound trajectory from pinger.

axis of the recording if the distance partnership is constant. If the pingers move away or approach, the trace of the echo deviates to one side or the other of this straight line.

Unfortunately, even if the stability of the two clocks is sufficient, their frequencies of recurrence may not be sufficiently close together. One of the time bases then drifts with respect to the other in a linear fashion. It is easy to take this into account.

From this the position and speed of the trawl can be watched constantly in the water and, consequently, the validity of the mathematical model calculated previously can be tested, especially making use of the different parameters at our disposal: movement of the winch and speed of the ship. Such is the case for a trawling operation using a large otter trawl: experience has led us to follow these steps:

1) The trawl is paid out into the water with the ship proceeding at the nominal speed for trawling, such that the otter boards diverge normally and orient properly.

2) Paying out the wire begins the moment the weights and the pinger are in the water.

This paying out increases progressively until the maximum speed permitted by the winch is reached. The speed of the ship is then lessened to a third or a half of its former speed. The trawl descends almost vertically.

3) When the trawl reaches 3/4 of the depth, one can evaluate within a good approximation the point where it should touch the bottom and the moment of contact. The speed of the ship is therefore increased to make the arrival of the trawl on the bottom coincide with the arrival of the ship in the position

corresponding to the beginning of trawling under normal conditions. The wire approximates a straight line inclined with an angle β to the horizontal line.

The increase in speed of the ship is progressively translated into an increase of horizontal speed of the trawl and a decrease of its vertical speed in spite of paying-out speed remaining maximum. A few minutes before arriving at the bottom, this rate of paying out is progressively reduced to avoid fouling the net, the otter boards, and the span.

4) When the trawl touches the bottom, the speed of the ship is reduced to the nominal speed for trawling and, while the figure formed by the wire stabilises, the length run out is controlled with precision so that the height of the weights above the bottom stays constant and is located between 5 and 10 m.

5) In preparation for heaving in the trawl, the ship is stopped and the winch starts slowly. The distance between the weights and the bottom remains constant for a while and the trawl continues to operate. When the trawl approaches the vertical of the ship, one can heave in swiftly. For the arrival at the sea surface, the ship slightly increases speed, so that the trawl is extended horizontally astern until it is hauled in. The diagram representing the path of the trawl in the vertical plane will give precisely the length of area sampled and will thus permit a quantitative evaluation of yield.

Concrete Example and Remarks

Table I represents a complete record (on EDO 333 PSR) of one operation with a double-beam trawl, whose course was diagrammed by the method indicated above (fig. 63).

A first remark concerns reception: for significant horizontal distances between the ship and the pinger, reception of the ping becomes impossible with a horizontal transducer. Backus (1966) underlined this problem and proposed the use of a non-directional hydrophone for reception. An orientable transducer would be more practical, and we are working at Brest in this direction.

A second remark concerns the amount of weight to be placed on the wire for a specified apparatus: the exact traction force of each apparatus, as a function of the speed must be known in order to calculate the weight necessary to obtain a straight wire, the optimal condition with respect to the duration of the operation (appendix I). Finally, the important gain in length of wire must be noted in relation to classical techniques.

Conclusions

The technique which has been described permitted us to considerably reduce the length of time for abyssal oparations while increasing the distance covered on bottom.

In addition it permits an estimation of the bottom surface covered by the trawl or the dredge, thus allowing deduction, as to biomass, bearing in mind the selectivity of the apparatus. In fact, as Backus (1966) has established, it is possible to make routine use of otter trawls or epibenthic dredges in the abyssal depths, an indispensable technique for sampling benthis fauna and deep-sea fishes.

Table 1.

Trawl double beam (Blake)
B012—Station 035—Sample 084

Time	Top	length wire meters	wire angle	Speed ship knots	distance† log	direct†† path Fms	reflect* path Fms
23h35	0	0	90°	0.6	0	0	3180
23h45	1	681	90°	0.6	19	315	2850
23h55	2	1325	83°	0.8	35	725	2500
0h05	3	2006	83°	2	54	1025	2145
0h15	4	2690	80°	2	99	1390	1830
0h25	5	3043	70°	2	162	1565	1865
0h35	6	3145	60°	2	215	1625	1960
0h45	7	3325	50°	2	279	1735	2060
0h55	8	3511	50°	2	339	1825	2235
1h05	9	3699	53°	1.8	406	1925	2225
1h15	10	3816	55°	1.8	470	1990	2335
1h25	11	3867	55°	1.6	529	2010	2355
1h35	12	3926	55°	0.5	584	2050	2385
1h45	13	3679	55°	0	600	1930	2275
1h55	14	3407	55°	0	609	1785	2125
2h05	15	3110	60°	1	613	1650	2020
2h15	16	2600	60°	1	630	1370	2135
2h25	17	2036	60°	1	654	1060	2340
2h35	18	1469	60°	1	680	740	2570
2h45	19	894	60°	1	713	430	2800
2h55	20	335	40°	1	741	125	3080

*Unit of length for log readings 0.05 n. mi.
fully corrected (Matthews, D.J.1939)
Trawl on bottom
36° 21'3N
8° 42'6W
Trawl leaving bottom
36°22'8N
08°45'7W

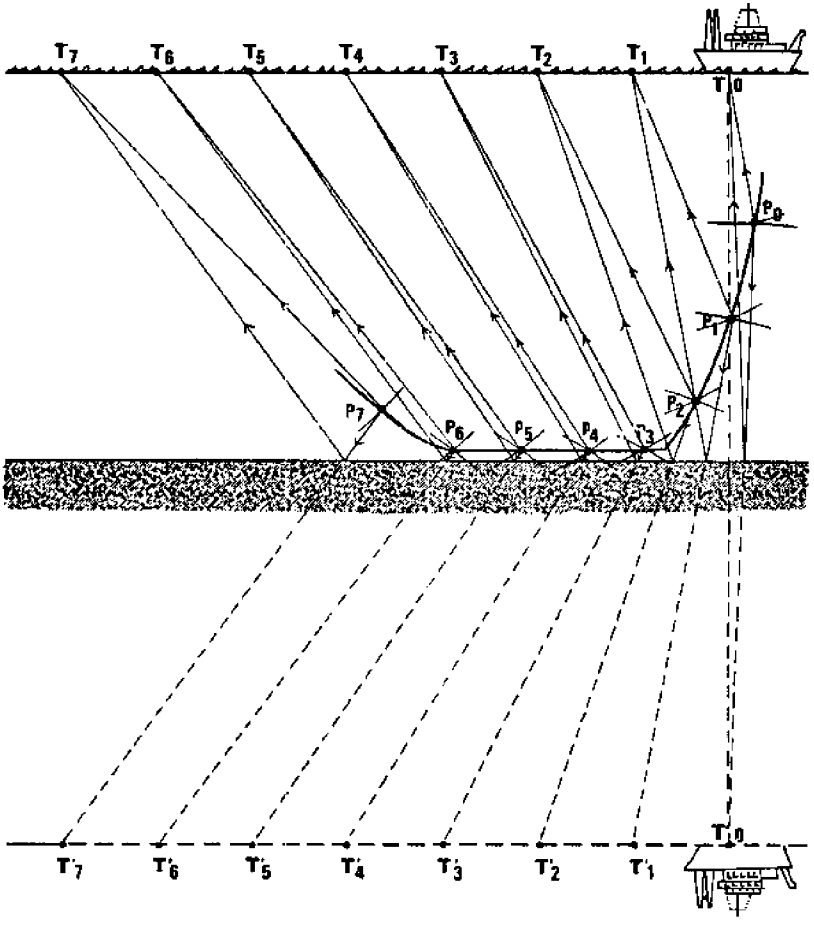

Figure 63. Progressive positions of the ship (To, T_1...) and a pinger-controlled trawl (Po, P_1...) during one operation.

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Appendix I

Considérons (figure 64) un élément de câble MM' de longueur ds, faisant l'angle α avec l'horizontale.

Si le mouvement est stationnaire, les forces agissant sur cet élément de câble s'annulent. Ces forces sont :

—les tractions T et T' = T + dT transmises par le câble aux points M et M'.

—le poids de l'élément ds dans l'eau : $p\ ds$ si l'on désigne par p le poids par unité de longueur du câble.

—la résistance hydrodynamique dont nous noterons les composantes normale et tangentielle : $F_N \cdot ds$ et $F_T \cdot ds$.

L'équilibre de ces forces s'exprime par les équations :

$$(1) \quad p\ ds \cos \alpha - F_N ds = T\ d\alpha$$

$$(2) \quad p\ ds \sin \alpha + F_T ds = \alpha\ T$$

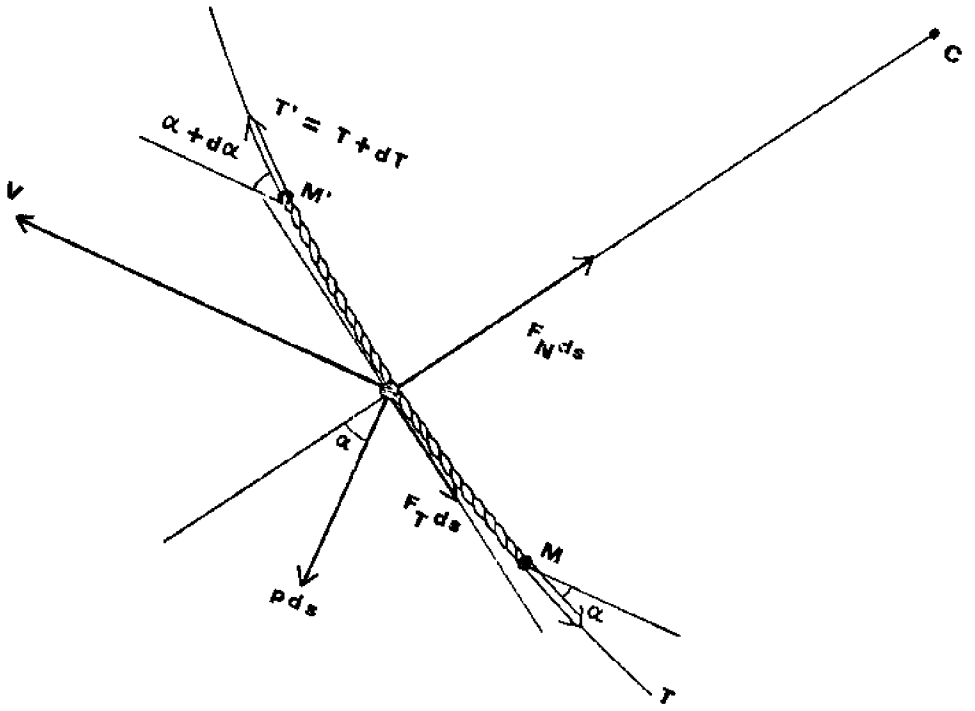

Figure 64. Forces on a trawl warp.

La vitesse de l'élément de câble, égale à la vitesse de l'ensemble du câble et donc du bateau est horizontale. Donc :

(3) $F_N = k_N \cdot D \cdot (V \sin \alpha)^2$

(4) $F_T = k_T \cdot D \cdot (V \cos \alpha)^2$

D : diamètre du câble. k_N et k_T étant deux coefficients hydrodynamiques indépendants du câble.

En introduisant le rayon de courbure $R = \frac{ds}{d\alpha}$, les équations deviennent :

(5) $R[p \cos \alpha - k_N \cdot D \cdot (V \sin \alpha)^2] = T$

(6) $R d\alpha [p \sin \alpha + k_T \cdot D \cdot (V \cos \alpha)^2] = dT$

L'équation (5) montre que pour une certaine valeur de α, le [ ] s'annule et donc, T gardant une valeur finie, non nulle, $\frac{1}{R} = 0$ et le câble est rectiligne. Cette valeur particulière de α est mommé anglec asymptotique du câble β.

Pour éliminer T des équations (5) et (6), il faut différentier (5) et :

(7) $$dT = [p \cos \alpha - k_N D \cdot (V \sin \alpha)^2]\, dR - R\, [p \sin \alpha + 2k_N \cdot D \cdot V^2 \sin \alpha \cdot \cos \alpha] \cdot d\alpha$$

(8) $$\frac{dR}{R} = \frac{2p \sin \alpha + 2k_N \cdot D \cdot V^2 \sin \alpha . \cos \alpha + k_T \cdot D \cdot V^2 \cos^2 \alpha}{p \cos \alpha - k_n \cdot D \cdot V^2 \sin^2 \alpha} \cdot d\alpha$$

Cette équation différentielle détermine complètement la forme du câble, à condition de se fixer en un point, par exemple au point d'attache de l'engin tracté, l'angle et le rayon de courbure initiaux, α_o et R_o.

Si l'engin a un poids P dans l'eau et une trainée Tr à la vitesse considérée, nous avons :

(9) $$\operatorname{tg} \alpha_o = \frac{P}{Tr}$$

(10) et d'après (5) : $$R_o\, [p \cos \alpha_o - k_N D\, (V \sin \alpha_o)^2] = \sqrt{P^2 + Tr^2}$$

Appendix II

Il est intéressant de se demander si une courbure avec un point d'inflexion peut représenter un câble ayant un mouvement stationnaire.

Nous avons vu que (Appendix I—éq. 8) : $\frac{dR}{R} = f(\alpha) \cdot d\alpha \cdot$, ou $f(\alpha)$ est une fonction dont le numérateur est fini et dont le dénominateur s'annule pour $\alpha = \beta$ avec :

$$f(\alpha) \sim \frac{A}{\beta - \alpha} \quad \text{quand } \alpha \to \beta$$

$$R = e^{\int f(\alpha . d\alpha)} = e^{F\alpha} \qquad F(\alpha) \text{ étant la primitive de } f(\alpha)$$

qui s'annule pour $\alpha = \alpha_o$

$$F(\alpha) \sim \int_{\alpha_o}^{\alpha \to \beta} \frac{A}{\beta - \alpha} \cdot d\alpha = -A \left[\operatorname{Log} | \beta - \alpha | \right]_{\alpha_o}^{\alpha \to \beta} \to +\infty$$

$$R = \frac{ds}{d\alpha} = e^{F(\alpha)} \sim \frac{1}{\beta - \alpha}$$

Nous pourrions également déduire cette équivalence de l'équation (5) en admettant que T doit rester finie.

Nous en déduisons que si $\alpha \to \beta$, $s = \int ds$ n'est pas convergente et donc le point ou l'égalité $\alpha = \beta - x$ lieu n'est jamais atteint.

SUBMERSIBLES AS SAMPLE-COLLECTING DEVICES AND AS POSSIBLE PLATFORMS FOR *IN SITU* EXPERIMENTS.

by
William Saunders
Lockheed Ocean Systems

Man's extension into the ocean's depths to further his knowledge is greatly aided today by deep submersibles capable of supporting complex scientific missions involving sample collecting and *in situ* experiments.

The history of deep submergence can be traced to its beginning in 1930 with Beebe and Barton's bathysphere. Two major technical milestones have occurred in the short history of submersibles. First was the decision to break with direct surface support and second, the deviation from the "elevator" approach of up and down operations in a narrow water column.

Beebe and Barton's experience with their tethered bathysphere serves to illustrate the problems of a surface dependent submersible. "At 1,950 feet they had a scare. The bathysphere began to pitch, banging them about. For a fraction of a second, which seemed an exceedingly long time to us, it felt as if we had broken loose and were turning over" Beebe wrote. Every two or three minutes the bouncing started up again. It was an unavoidable part of going into the depths like a yoyo on a string. As the tender rolled, the bathysphere bounced".[1] The safety and comfort of the observers were greatly affected by the conditions at the surface.

Piccard's bathyscaph Trieste reveals the limitations of the elevator approach to submersible operation. "It must be remembered that the bathyscaph is not a submarine. It has neither the mobility nor the controllability of a submarine. Whereas a submarine may be regarded as analogous to a dirigible, the bathyscaph may be considered to be a lighter-than-water free balloon. The craft is at the mercy of currents and is limited mostly to 'elevator' type operations, such as investigations of the water column from the surface to the seafloor and detailed studies of the sea floor atthe base of the water column. The bathyscaph type of configuration does not lend itself to survey work".[2]

These remarks are intended to point up the emergence of a new breed of submersibles that are reliable, mobile and able to accomplish a wide variety of missions. Contributions by Beebe, Barton, and Piccard paved the way.

The French effected a quantum jump in submergence capability with Cousteau's *Diving Saucer*, a small, highly maneuverable vehicle, which marked the transition of man's role in the ocean from that of passive observer to active participant.

Lockheed's submersible *Deep Quest* is an example of the advanced "active" underwater workboat which evolved from the earlier models. It provides a shirtsleeve environment for a crew of two plus two scientists for 48 hours at depths to 2,400 m. In addition to its depth and life support capability there are other aspects of the vehicle that make it an "active" workboat.

After launching from its mothership, *Deep Quest* is physically separated from surface support. It carries electrical power in the form of lead acid batteries capable of delivering 230 KWH, as well as two emergency batteries which can provide 3.6 KWH of emergency power.

The power for escape from the small diameter water column is provided by two 7-1/2-hp axial thrusters, two 7-1/2-hp vertical thrusters and two 7-1/2-hp lateral water jets. These propulsion units, in conjunction with sternplanes and a rudder, provide an exceptionally good five degree of freedom dynamic maneuvering capability throughout the speed range from 0 to 4.0 knots.[3]

Two manipulators are mounted on the bow of the submersible. Each can be individually controlled in all its movements, including telescoping and lateral translation. The manipulator "hands" can be interchanged with alternate tools to perform specialized functions. The unique dual installation permits greater versatility and increased lift capacity. Each manipulator can lift 225 kg wet weight in water for a vertical lift or 45 kg horizontally with the arm extended at a radius of 1.8 m. The gripping force of each hand is 225 kg with a maximum jaw opening of 20 cm.

Another important capability of the Deep Quest is the location or acquisition of underwater objects and the ability to consistently return to this site many times. In the past, this has been a problem.

Deep Quest's role in the successful recovery of the flight recorders of two crashed airliners serves to illustrate the ability of the advanced submersible to work in the ocean. First, the site of the crash had to be surveyed until the plane was located and then the submersible had to sift through the wreckage; in some cases stripping metal panels away from areas that the flight recorder was thought to be in. This meant extensive use of the manipulators and all the fine maneuvering available. In both cases, which included return dives to the same location, the flight recorders were recovered.

The use of a submersible as a collecting device is not a new concept. Many submersibles have been equipped with plankton nets as a secondary mission feature. Some have brought back samples of slower fish or animals from the depths by using the manipulator as a capture tool. These attempts have, for the most part, been clumsy, and most samples collected were badly damaged. It is hard to pick up animals with a manipulator and have a "soft glove" grip.

Another approach that would be effective would be the use of traps similar in style to those used by a fishing boat and planted by the

submersible in much the same way as commercial traps are set. These could be simple open traps or designed for pressure and temperature so that the sample could be returned safely to the surface under the conditions of its environment. In this approach to sample collecting, Lockheed has developed a Large Object Recovery device, which is capable of lifting a 1.5 m diameter, 6 m long object weighing as much as 1360 kg in water. This device is mounted on *Deep Quest* on the surface by divers. The unit has been tested to retrieve various shapes and sizes such as spheres, cones, and odd shaped scrap iron. This method could be considered the direct approach to retrieval.

For larger objects or traps, the indirect approach can be used. In October, 1970, *Deep Quest* was instrumental in raising a World War II fighter plane from 1040 m of water. This was achieved by attaching a lifting line to the aircraft. The line was then payed out from a bow-mounted spool back to the surface, and then the line was transferred to a support ship which in turn winched the plane to the surface.

The flexibility provided by *Deep Quest's* large payload, long endurance, high and low speed maneuverability and wide selection of equipment mounting locations make her ideally suited for *in situ* experiments.

Deep Quest can be equipped with a six-barreled core which can be used at any selected location. The coring process may be directly observed by the scientist. The large payload capacity available on *Deep Quest* will allow the acoustic properties and layering of the main sediments to be studied concurrently utilizing velocity-attenuation probes and sub-bottom acoustic reflection equipment along with the coring operations.

The manipulators may be used to perform simple work tasks or to obtain samples of the bottom sediment or rock outcroppings at selective points. Control of the manipulators is accomplished from the forward viewport position; operation of the manipulators is performed directly within the field of view from this station.

Continous measurements of optical, chemical and acoustic properties of the water mass may be made concurrently with other missions allowing a much greater accumulation of supporting data. *Deep Quest* is equipped to measure both sound velocity, water temperature and depth. These can be augmented with customer supplied nephelometers, oxygen and CO2 sensors, salinometers, etc. or could be replaced entirely with customer supplied sensor packages as desired. A number of parameters could thus be routinely recorded throughout the water column on every dive, correlated with both time and depth.

The inherent maneuverability in conjunction with precise control of buoyancy also permits operation within close visual range of the seafloor at all speeds, including hover without disturbance of the sediment. This capability together with selective control of lighting and use of the viewing ports, television, sonar and cameras provides an excellent platform for stationary or moving observation missions on the ocean bottom.

The location of this observation platform is important, for if we cannot dictate its position, we have become passive once more. With its maneuvering motors, trim system, variable ballast and maindrive, *Deep Quest* can operate in any area of its depth range of 2,400 m. It can cruise, hover, turn or lie motionless on the bottom or at any level in the water column.

Experience has shown that the novice observer has the most faith in direct viewing. However, as the vehicle and its mission become more complex, the direct viewing method is not sufficient. The distance of view is also affected by water conditions at the site. Operations have shown great advantages in indirect viewing with optics, mirrors, binoculars, television and even image sonar. Some scientists have worked with underwater television and have been disappointed with the results. This, in most cases, can be traced to the late 1950's when underwater television fell into disfavor due to poor resolution, poor equipment stability, and poor equipment reliability. In the 1960's, high quality equipment became available and this overcame many of the problems. Some of the advantages of the television approach are portability, the wide selection of lens from wide angle to zoom, and the ability to work under extremely poor lighting conditions. (Divers who could not see what they were doing, have on occasion, been directed in their work from the surface by the use of television.)

Along with methods of viewing, a major advancement is the ability to permanently record what is viewed. Some methods are fairly simple, such as an observer with a hand held camera snapping pictures through the viewport. Others such as video tapes are a little more exotic. Still and movie cameras mounted externally can pan and tilt and then focus over a wide range, which is a vast improvement over using fixed cameras with fixed focus, where the observer had to sit patiently until a target happened into his field of focus.

There are many submersibles in operation today and their abilities are as varied as their design depths. Most will allow direct observation, but researchers soon tire of passive observation. The answer lies in the utilizing of the active "workboat" submersible. These submersibles in many cases have more capabilities than any one scientific mission requires. However, the successful use of a submersible as a platform for *in situ* experiments and as sample collecting devices is the result of the mission planner and his understanding of this research tool and utilizing its abilities to accomplish his needs.

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Discussion Following Saunders Paper

(Question): What are the current operation costs on some of the commercial submersibles?

SAUNDERS: *Deep Quest* is $8,000 a day. The *Alvin* costs about $15,000 a day, $2,000 an hour. You must keep this in mind—doing biological research from a submersible is extremely expensive. But, then of course, there are some things you can do no other way.

(Question): Along these lines, would it be possible to envision, instead of using and operating a submersible every day, to have a more permanent station, located at 2,400 m and use a submersible to shuttle people to it?

SAUNDERS: Everyone forgets that your big cost with a submersible is that, regardless of what the submersible itself costs, if you have a support ship, that's your basic cost, and you all know that in sending a surface ship out you have to pay a crew, fuel, food, and all these things probably add up to $5,000 a day. Then you would add on the cost of the submersible.

SCHELTEMA: It strikes me that one of the biggest limiting factors in submersibles is the difficulty of launching them and the dependence on sea surface conditions. Bill might want to make some statements as to how this affects the number of days you can actually dive.

SAUNDERS: We've worked in Southern California and we got away with working in large sea states. We came around to the Atlantic Coast and tried to work in half of these sea states and got killed. It was just rough—the periodicity is different. So, I think the conditions of the water are things you have to look into. But as your operators become more efficient, you learn little tricks. Like on one coast you can back into the swells; on this coast we went head on into them. And that was lifting with a crane. Well, now I'm working with a ship that has an elevator, so we can virtually drop the submersible right into it, and then bring it up on the elevator. This has given us another added advantage. But, there is no getting out of it, you do lose days to weather. I think the number has gone down and we get better operating records. And also, in this beginning work, I think you might pick a spot that has a history of good weather. You would not go right to where bad weather is to start with.

GORDON: Even out in the open sea in the tradewind areas, where you've got very steady winds coming across thousands of kilometers of open sea surface, and you've got a steady 6 to 8 foot swell going, it's possible to launch these things. It's being done for instance, on the windward side of the Hawaiian Islands, from a submersible platform. You can have that towed out from a shore base, then sink it below the surface and then you have skin divers release the submersible and have it just go off right there.

Then all you need is a little boat to tow it out of the rafts, and then it comes back and lands on the rafts under the surface, comes back up by floating, you tow it back in shore again. This is being done regularly now.
SAUNDERS: Once again, you have to trade this off. That approach is very good. It takes a lot of the surface action out. But sooner or later you have to bring it up to the surface and tow it ashore, and if you don't have interface problems with your submersible, you might consider interface problems with your divers who get kicked around quite a bit there.
LOWENSTAM: What is the range and speed of your submersible?
SAUNDERS: The endurance is somewhere on the order of 18 hours and 2 knots and that decreases significantly as you increase up to our maximum speed somewhere on the order of 4 or 5 knots.
TEAL: John, (Kanwisher) you have been down in a couple of them yourself, haven't you? And I know Rudy has. Maybe you can tell us a little bit about some of the biological uses that have been made.
KANWISHER: Go down and look at things. That's what people have mostly done, and George Graves has gone down and done some sampling of epibenthic copepods, a few meters off the bottom, using a closing net.
GORDON: I'll ask the question differently. Have you done anything that couldn't have been done without it?
KANWISHER: Yes, positioning the nets right above the bottom is something which could hardly be done in another way. You can imagine doing it in some other way, but that is the way it was done. It is very difficult to get a sample net very near the bottom.
TEAL: I have tried to think of things I could do with a submersible in conjunction with a fellow that works with Eric Barham, and I have been unable to come up with anything that I really needed that instrument for, and it is partly lack of imagination on my part, I'm sure. That's why I ask the question. You have had experience with it.
SAUNDERS: I think that one big advantage of going down in one of the submarines is to get a visceral feeling for what is at the bottom of the ocean and what it looks like. This is important to an ecologist, I think. But, also, you see things that you have never brought up.
TEAL: Yes, Eric Barham pointed that out very well. And I think that is important. For things that go on land, we have looked at them for a million years and we have a great deal of experience about the way things happen on land and in the air. But we have relatively little experience with the way things happen in the water.
KANWISHER: If as an ecologist you want to find out what birds are on land and where they are, you don't have to swoop a net. You would like to be able to see a huge area, and I guess a submersible could do that—a wide survey of things that are there.
TEAL: I think it's very easy to design experiments which can be readily done from submarines and not really done in other ways. We have designed

such experiments. Unfortunately, as you know, our submarine sank a couple of years ago and we haven't been able to do anything in the last couple of years. Therefore, we haven't any results. John doesn't want to say it, but he told me when it started: "I'm going to wait until they can really run this, and then I'll start using it." And he said, "After a while, I'll be afraid they'll get too contemptous and careless and then I'll stop." John made the last dive in it! He didn't mean to cut it that close.

SAUNDERS: I think an important thing to emphasize about a submarine's ability that can't be done easily any other way is its ability to return to any one spot over and over again. This means that you can carry out long-term experiments and do things that cannot be done easily on the surface.

KANWISHER: How about the exploration of underwater canyons? Has that been tried? Underwater caves?

TEAL: One of the things it has been used successfully for and will be successfully for when we get it going again, as far as *Alvin* goes, anyway, is the exploration of the canyons. It's hard to get nets or other kinds of gear into a canyon—I shouldn't say it's hard to get nets into canyons, but it's hard to get them out.

GORDON: Dick Rosenblatt has spent a lot of time in Baja California, doing exactly this kind of thing. The number of kinds of animals that they didn't even know existed in these areas, and which turned out to be very abundant all of a sudden, is really amazing—all simply because of these sampling problems—without having the ability to get in there and look yourself, chances are you would never find out about them.

KANWISHER: As you can gather, I'm somewhat anti-submarine, which isn't very popular, I'm told. But there are these accidental things that happen which are very hard to view in retrospect. For instance, some of this microbiology which presently is going to be mentioned here, I think, is one of the most stunning results in biological research in years, and is completely accidental fallout. The fellows' lunches were good after a year on the bottom at 1,500 m, or: we had a swordfish attack the submarine at 550 m. He drove its bill into it, and the poor guy was caught, and we killed it and brought him up and ate him. It's not what we had in mind for an experiment in the mid-water, looking at the fish distribution! But, if you were to balance it, say, at the oceanographic laboratory in Woods Hole, *Alvin* had two or three times the volume of all the rest of the budget put together, and even at that, it wasn't enough. Even though they had that vast budget, there wasn't any budget for adapting the submarine for scientific use, and therefore, it was never used to real advantage. Undoubtedly, the thing is that submersibles are here to stay, whether it is for biology or not it doesn't matter. But if you want to have hydrostatic aquarium and sampling methods, you'd better go to your own lab and make them up, and you can give them to some of your rich friends with submersibles.

SAUNDERS: That is perfectly true. We have submarines and to some

extent the money which is available to run submarines will not be available if you don't run submarines to do something else. So we may as well plan to take advantage of them.

GORDON: Well, I think biological oceanography has long been used to riding piggy-back on other programs, and I suspect that the same thing is going to continue to be true, especially in this kind of case, and there have to be many training dives that people have to make in order to get their own expertise up in operating these things, and you can take advantage of those, if you're lucky.

KANWISHER: There is one problem that submersibles solved that really couldn't easily be done any other way, and that was to get to the bottom of the deep scattering layer story. That's a problem we worked on something like 13 years or so, and in one dive it was essentially solved.

ISOLATION AND CHARACTERIZATION OF BACTERIA FROM THE DEEP SEA

BY
R. C. Kettling, Jr., R. R. Colwell
and G. B. Chapman
Department of Biology
Georgetown University
Washington, D. C. 20007

It is unnecessary to justify exploration of the ocean depths to such a group as that gathered at this conference on high pressure aquarium systems. The motivation arising from the "need to know" and the potential application of any discovery are too obvious. Only a brief comment as to why the interest in deep sea bacteria is offered. There is pitifully little information about the bacteria inhabiting the ocean depths, and yet the prevailing belief is that, as in terrestrial and aquatic ambient pressure systems, the bacteria will solubilize and mineralize wastes and pollutants rapidly and efficiently in their role in the natural cycles of the ocean. The bits of evidence coming to light are not, in fact, supporting this notion. The few studies which have been done on marine bacteria, predominantly the pioneer work of ZoBell and Morita, suggest that complex metabolic activities do occur at *in situ* pressure and temperature in the deepest parts of the oceans but the rate and total efficiency are significantly slower.

ZoBell and Morita clearly demonstrated the existence of bacteria in the sea depths of 6,000 m (1). Our own interests in deep sea bacteriology are several. The isolation, identification, and classification of pure cultures of deep-sea bacteria, their relationship to terrestrial forms and metabolic capabilities in the natural habitat form the main theme of our research activity. The question of barophilism, i.e., obligate barophiles as a portion of the natural microbial ecology of the sea, is a dominant and and persistent directive to the experiments which we have done and are presently carrying out.

Our research in deep-sea bacteriology was initiated by careful examination of pure cultures of bacteria provided by Dr. Morita. These cultures were isolated from sediment samples obtained from the Philippine Trench (9,854 and 9,443 m) and the Challenger Deep (10,373 M) in the Marianas Trench of the Pacific Ocean. The 31 isolates were subjected to extensive testing and a total of 116 characteristics were compiled for each isolate. The data were analyzed by computer and five distinct taxonomic clusters were identified: four *Pseudomonas* spp. and an *Aeromonas* sp. One of the *Pseudomonas* spp. was identified as *Pseudomonas fluorescens*, a common species of the genus found in water and soil. Puzzling features noted for these deep-sea sediment bacteria were lack of an absolute requirement for natural or artificial seawater and eurythermic rather than obligately psychrophilic nature.

Quigley and Colwell (2) suggested certain explanations for these observations. First, these deep sea sediment bacteria may indeed be valid representatives of the deep ocean microbial ecosystem. Second, the isolates may be those which survive the long passage from surface waters, either as animal or plant commensals and sink to the ocean floor after the death of the host, remaining essentially "dormant" in the sense of being viable but metabolically inactive. Finally, the isolates may be both eurythermic and eubaric, active under deep ocean temperature and pressure and able to survive the decompression and heating which occur in passage from the ocean bottom to the surface during the sampling procedure while their less sturdy brethren expire under the stress. *In situ* pressure and temperature studies of the metabolism of these bacteria, i.e., their ability to grow at 0°C under 1,100 atm of pressure, support this hypothesis. However, whether these strains are dominant in the deep sea sediment ecosystem or merely represent opportunist bacteria is a question which we are now pursuing.

One of the new species of bacteria isolated from the deep sea sediments identified by Quigley and Colwell (3) as *Pseudomonas bathycetes* has been studied extensively in our laboratory. Electron microscopy (Fig. 65) reveals *P. bathycetes* to be typically Gram-negative, without unique or unusual subcellular structures. A variety of fixation, embedding and straining techniques have been applied. Inclusion of 2.3% NaCl and 0.4% $MgCl_2$ provided good fixation, a salt concentration which approximates that in seawater. The ultrastructural features of this species include cell wall, plasma membrane, ribonucleoprotein particles and nuclear material similar, if not identical to, the Gram-negative terrestrial pseudomonads.

The enzymes of the deep sea sediment bacteria have been investigated using electrophoretic methods, and the DNA strains has been chemically characterized (4). The objectives of this were to investigate enzyme heterogeneity and overall DNA base composition as a means of estimating molecular evolution among these forms and to correlate the data with the computer taxonomy.

Results, in the form of an esterase zymogram, are shown in Figure 66. Groupings of strains, according to electrophoretic profiles, were observed on polyacrilamide gel columns after appropriate straining. The groups A and B showed the typical patterns for strains identified as *P. bathycetes* . The groups separated by computer, thus, also yielded specific enzyme patterns. The overall DNA base composition of the deep-sea bacteria fell into a narrow range of 56-59% G+C (Table 1). Thus, the eurythermic, barotolerant bacteria isolated from the deep sea sediment of the Philippine and of the Marianas Trenches of the Pacific Ocean possess remarkably similar overall DNA base composition. The zymogram data also provide evidence for homogeneity of the taxonomic groups identified among the deep sea sediment isolates.

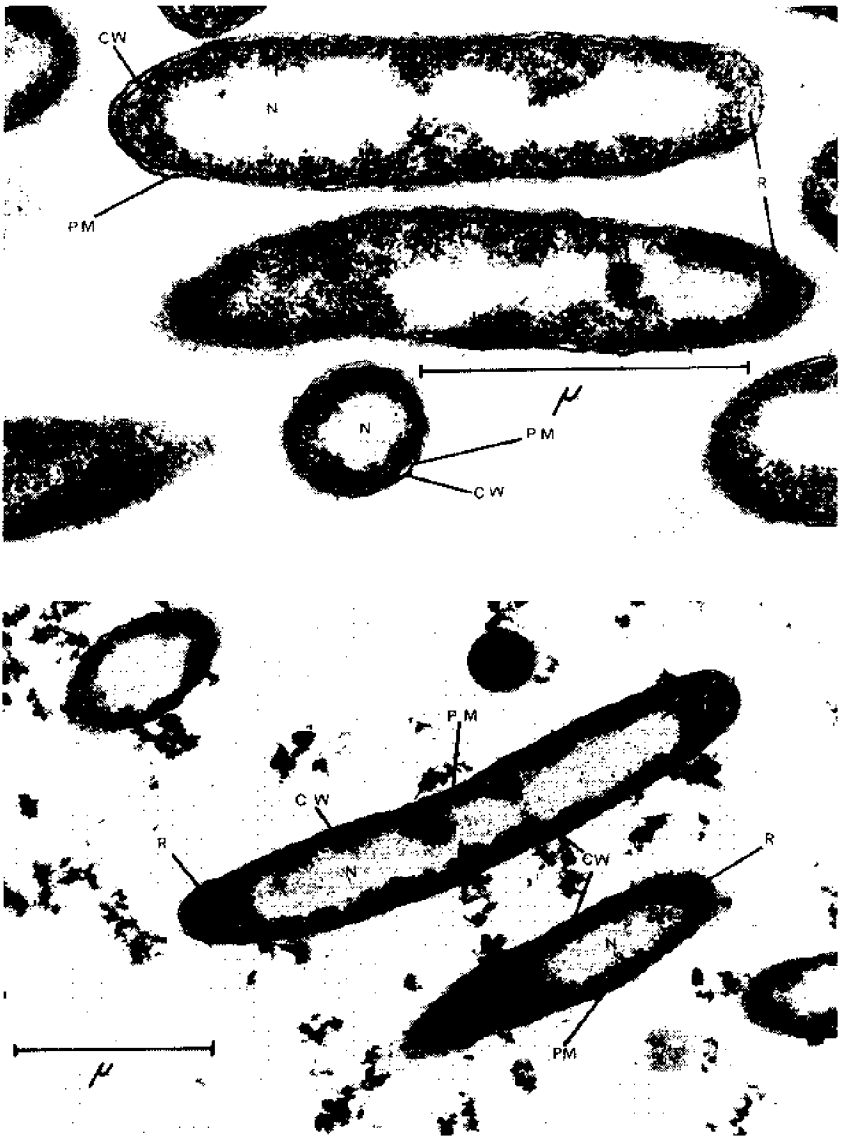

Figure 65. Ultrastructure of deep-sea isolate, *Pseudomonas bathycetes* strain C6M. The ultrathin sections reveal the typical Gram negative structure not unlike that of terrestrial Gram negative bacteria. Distinct nuclear region (N), granular cytoplasm with numerous ribosomes (R), plasma membrane (PM) and cell wall (CW) can be seen.

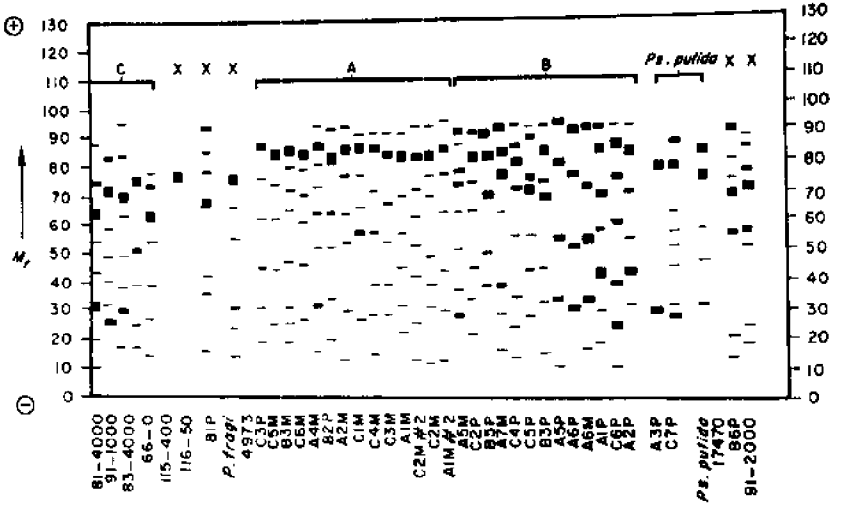

Figure 66. Esterase zymogram of deep-sea bacteria. Also shown are patterns for reference strains of terrestrial bacteria, *Ps. putida*, *P. fragi*. (Hogan and Colwell, 1969).

While the sample of sediment bacteria included strains requiring seawater for growth, the majority did not require addition of natural or artificial seawater. It has been hypothesized by Belser (5) that marine bacteria possess DNA base composition values tightly clustered around a mean of 44%+2% and, therefore, the Gram-negative rod-shaped bacteria with an absolute requirement for seawater salinities may arise by stringent selection of factors under polygenic control. Our data do not support this hypothesis. We have found DNA base composition values for obligate marine bacteria, specifically *P. bathycetes*, in the range 56-59%. In fact, the esterase pattern of one of the other deep sea bacterial species, identified as *Pseudomonas* species, was similar to the terrestrial species, *Pseudomonas putida* biotype B of Stanier, Palleroni and Doudoroff (6). Thus, we conclude that many taxonomic groups are represented among the obligate marine bacteria.

Our current research work involves two lines of investigation: the development of sampling techniques for the isolation of bacteria, maintaining *in situ* pressure and temperature during and after recovery, and the extension of the deep sea sediment bacteria studies to strains isolated from the Virgin Island Basin of the Atlantic Ocean.

The development of a system capable of retrieving samples at constant temperature and pressure and manipulating the samples under these conditions after retrieval is being done in cooperation with the Naval Research Laboratory in Washington, D. C. (7). The Deep Ocean Environmental Sampler (DOES) has been designed, constructed and subjected to preliminary testing.

Table 1.
Overall DNA base composition (moles % GC)
of DNA of deep sea bacteria

Strain No.	T_m°C	GCmoles%
A1M	93.0	57.8
A1M2	92.7	57.1
A2M	92.4	56.4
A4M	92.4	56.4
A5M	93.3	58.5
A6M	92.9	57.6
A7M	91.0	52.9
B3M	92.4	56.3
C1M	92.8	57.4
C2M	93.0	57.8+
C2M2	93.0	57.8+
C3M	93.0	57.8
C4M	92.5	56.6
C5M	92.6	56.8
C6M	92.7	51.1+
C2P	93.0	57.8
C3P	92.5	56.6
C4P	92.7	57.1
C5P	92.6	57.0
C6P	92.7	57.1
C7P	93.0	57.8
A1P	92.4	56.4
A2P	92.2	56.0
A3P	92.4	56.4
A5P	93.0	57.8
A6P	92.4	56.4
B1P	92.3	56.1
B2P	92.3	56.1
B3P	92.6	57.0
B5P	93.2	58.0
B6P	92.5	56.6
66-0	93.0	57.8
81-4000	91.5	53.9
83-4000	89.9	50.0
116-50	86.2	41.2
1150400	92.5	56.6
E. coli W1485F-*		

* The Control strain which T_M°C determined routinely with each sample.

+Buoyant density, 1.716g/cm^3; GC, 57.1%.

The DOES is shown in Figure 67. It consists of an outer cylindrical jacket, double-walled and pressure resistant, with an insulating air space between the walls, and a shaft with pistons fixed at each end (Fig. 67-1). When the sample is taken, a pin is pulled, and the outer jacket falls by gravity and traps the water sample between the pistons. The O rings on the piston complete a pressure resistant seal (Fig. 67-2). When the sampler is hauled to the surface and preparatory to insertion into the transfer aparatus, a restraining device is removed from the lower piston, which is then free to move up the shaft. After valve blocks are attached to each end (Fig. 67-3), the sampler can be inserted into the transfer apparatus which is essentially a high pressure circuit, as shown in Fig. 68. The DOES is designed to retrieve samples to 10,000 psi.

The DOES set up for the first sea trial, November, 1969, is shown in Figure 69. A J-Z microbiological sampler was used for simultaneous non-pressurized sterile sampling. A modification of the sampler for taking samples aseptically is shown in Figure 70.

Preliminary sea trials have been run aboard the USNS *Mizar* in November, 1969, and April, 1970, and aboard the R/V *Eastward* in July, 1970. Sampling closure problems have been the main obstacle to complete success in retrieving samples under pressure. A hydrographic wire triggering modification is also being developed.

Bacteriological analysis of a sample taken aboard the R/V *Eastward* in July 1970 from 3,800 m demonstrated the presence of 10^2 viable, aerobic, heterotrophic bacteria per cc, after incubation at 0°-5°c. The triggering modification operated properly on this occasion, but a valve leaked and the sample was retrieved in an unpressurized condition.

A sediment sample taken by Shippex Grab aboard the USNS *Mizar*, November, 1969, in the Virgin Island Basin at sonar depth of 3,950 m has been subjected to bacteriological study. Isolates from seawater-yeast extract agar plates at 25°c have been tested for identification and classification and for hydrostatic pressure tolerance. The pressure studies in this case were undertaken using the apparatus designed and published by Oppenheimer and ZoBell (8) (Figure 71). Preliminary results reveal the dominant bacteria are *Vibrio* spp. growing in the range of 15°=5°c and lacking an absolute requirement for salt. The pure cultures demonstrated the capacity to survive and reproduce under various regimes of pressure and temperature similar to the *in situ* conditions.

In summary, the study of deep sea bacteria in our laboratory to date reveals the presence of a variety of taxonomic groups which are capable of survival and growth under *in situ* conditions of pressure and temperature but which are in some ways dissimilar to those organisms which might be considered "typical" marine forms. This fact raises the very important possibility that we may be studying only opportunist bacteria in the deep ocean environment when conventional sampling techniques are employed.

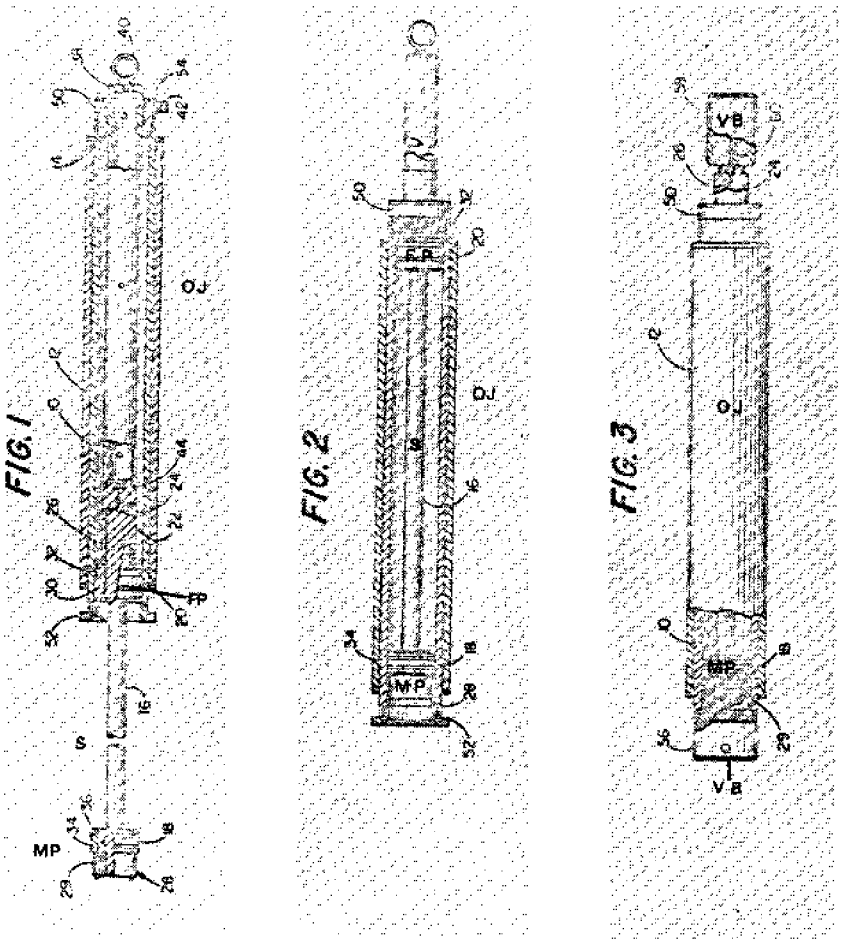

Figure 67. The Deep Ocean Environmental Sampler (DOES)
(1.) The sampler ready for taking a sample. An outer jacket (OJ) is suspended above a shaft (S) with a movable piston (MP) attached to the lower end and a fixed piston (FP) at the upper end. When the sampler is triggered the outer jacket drops over the two pistons trapping a water sample.
(2) Closed position of sampler.
(3.) The sampler after it has been brought to the surface; valve blocks (VB) are attached to each end. The sampler can be connected to a high pressure circuit for transfer to storage vessels. Thus, the sampler can be used repeatedly with samples maintained under pressure.

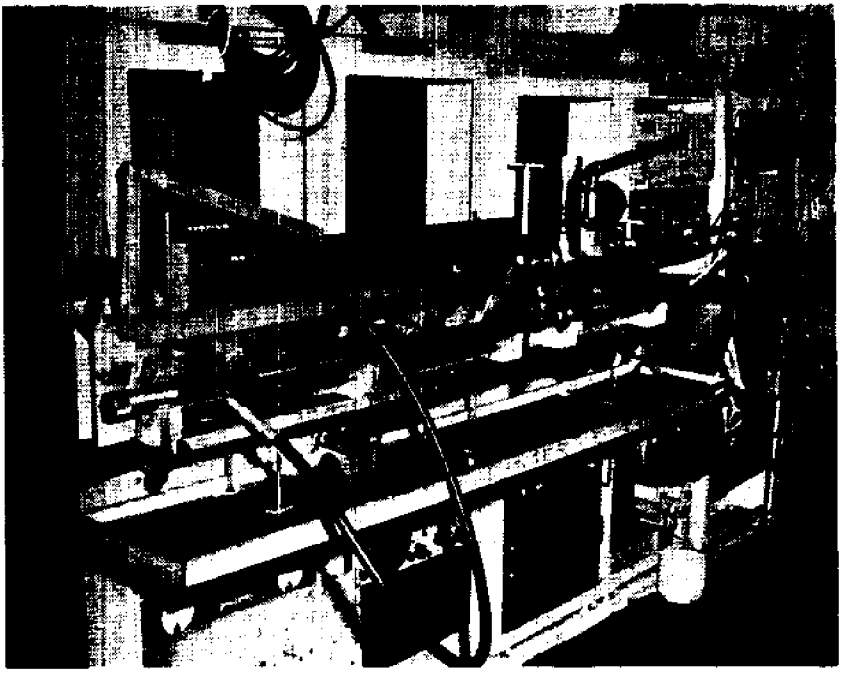

Figure 68. Transfer apparatus used for transferring water samples under constant pressure. The transfer device consists of a high pressure circuit to which the sampler (S) and the transfer vessel (TV) are connected. Pressure is equalized throughout the circuit by means of two manual pumps. The valves are opened and a slight positive pressure, applied to the piston, causes it to move up the shaft, forcing the water sample into the transfer vessel. The sampler shown here is a "bottom hole" oil well sampler originally used in the research but replaced by the DOES.

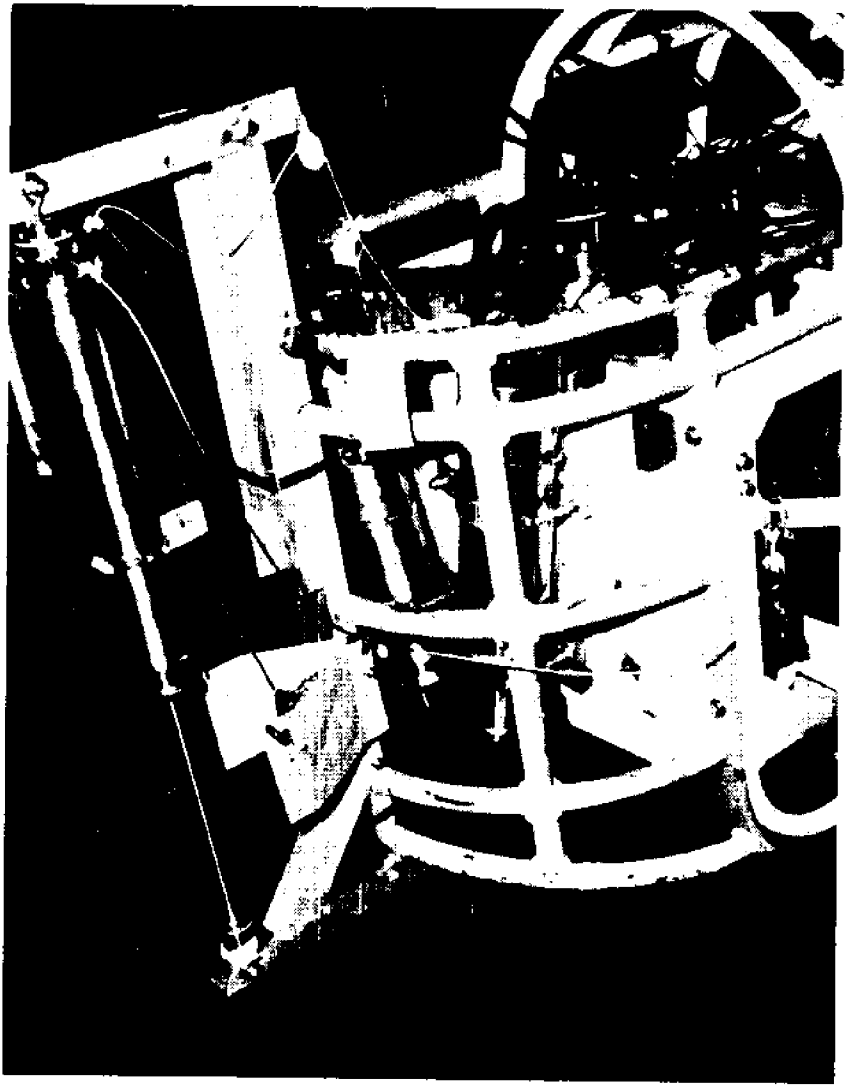

Figure 69. The sampler as set up for the first sea trial aboard USNS Mizar, November 1969, mounted on an undersea platform "fish" (F.). The outer jacket (OJ), shaft (S), and movable piston (MP) are shown. In this photograph the fixed piston (FP) is obscured by the outer jacket. A IZ bacteriological sampler bulb (JZ), shown attached to the fish, is activated, when the DOES closes, by action of the chopper blade (C) cleaving the glass tube and allowing simultaneous non-pressurized sterile sampling.

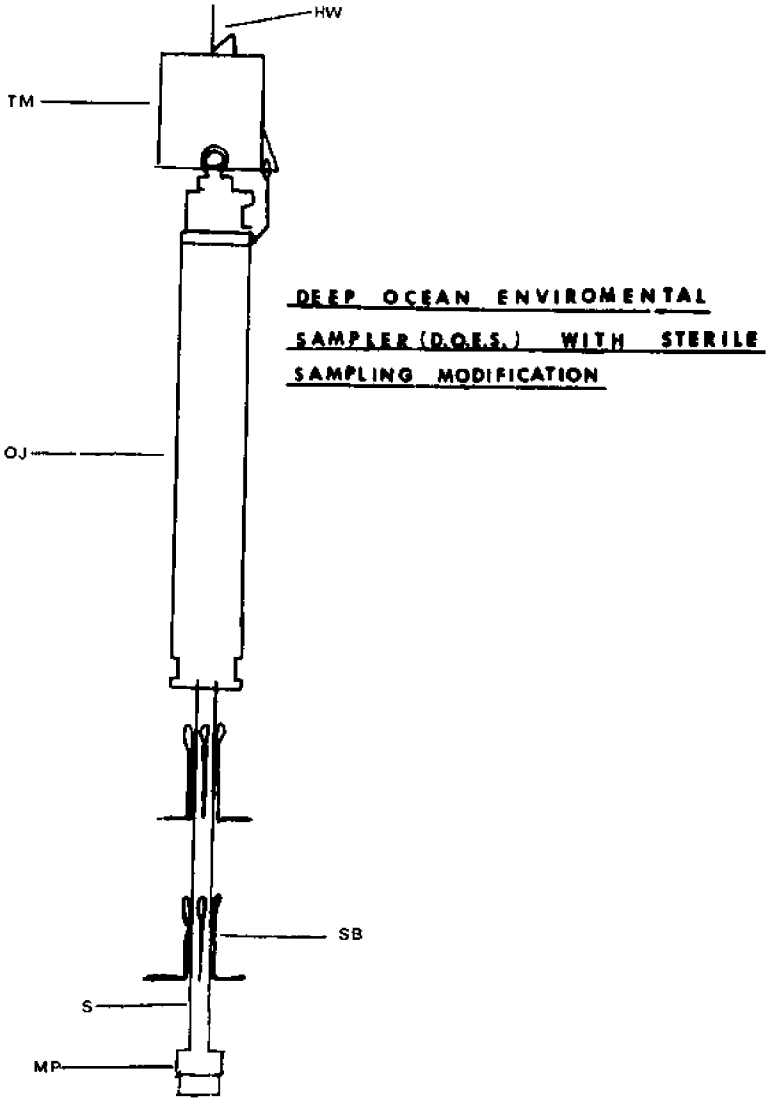

Figure 70. DOES shown with a modification for taking samples with sterile materials for bacteriological study. The sampling bulbs (SB) are attached to the shaft of the DOES. When the sampler is tripped, the outer jacket drops, breaking the tips of the sampling bulbs and drawing a water sample. The bulbs contain sterile enrichment media for growth of deep-sea bacteria. Also shown is the tripping mechanism (TM) which was used successfully in July 1970 on a standard hydrographic wire (HW).

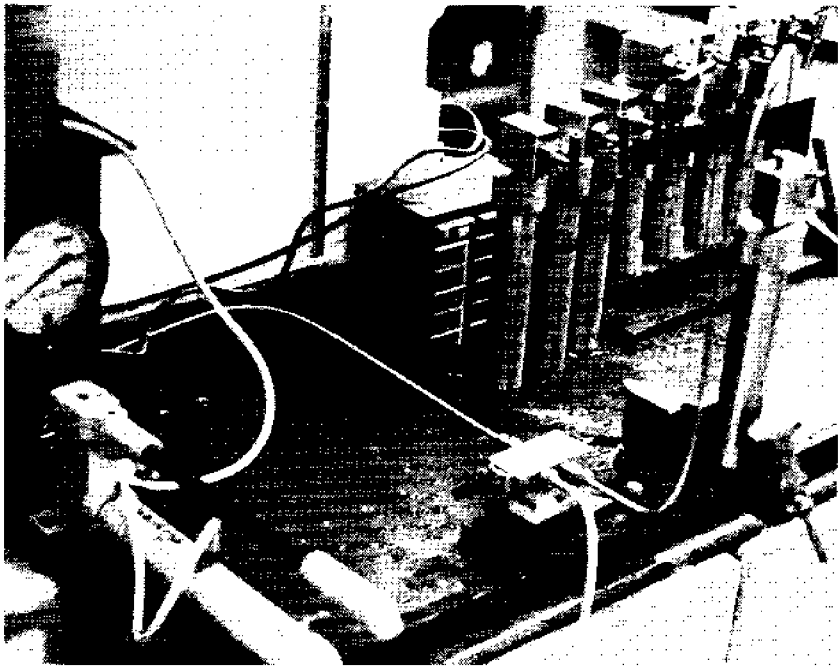

Figure 71. Apparatus for culturing bacteria under hydrostatic pressure. (After ZoBell and Oppenheimer 1950).

Because of this possibility, devices for retrieving and maintaining biological specimens under pressure will have to be developed before definitive statements can be made concerning the structure, function, and taxonomy of deep-sea organisms.

Acknowledgments

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DISCUSSION FOLLOWING KETTLING PAPER

ZOBELL: Did the sampler provide for collecting sediments or just water?
KETTLING: Well, when we were attempting to take the sample with the fish, we attempted to stir up a sediment, slurry, by dragging across the bottom. Unfortunately, we were unable to get samples.
ZOBELL: What was the maximum depth that these species of protozoa came from? A vibrio, was it?
KETTLING: The vibrio was taken at 9,100 m.
ZOBELL: I am very much interested in, and also very much concerned with, the preponderance of organisms which you said grow at 15° to 45°C. This is not an uncommon observation. I have been perplexed for about a quarter of a century now, by making similar observations. Especially puzzling are some cases when like a few other people we actually found obligate thermophiles, bacteria, which would grow at temperatures no lower than 45°C and up to 60°C. I ask you ecologists, what is an organism doing on the deep sea floor which will grow only at 45° or 60°C, when the temperature it is found in is normally around 3°C? I believe that most of these organisms that you have been getting here, as well as many or most of the organisms that we have been getting for years, are not indigenous to the marine environment. I am inclined to believe that they are organisms which grow and develop in shallow water, or maybe even in soil, in fresh water, or in sewage, and are transported by air, water, birds, man, or other means, and settle to the sea floor, where refrigeration coupled with high pressure (which has a comparable and even a synergistic preservative effect) has kept the organisms alive. If this is true, we may have a possible record of micro-organisms, but not much of a record of micro-organisms which are contributing to the food cycle and to the biology of the deep sea at the present time, and this is one of the big unanswered questions.

This does not belittle the excellent work that you and Colwell and many other microbiologists have been doing. Indeed, we have found obligate

eurybaric, psychrophilic organisms, some of which will grow only in sea water medium. Many of these bacteria that we get, just like those that you got, grow just as well in fresh water medium as they do in synthetic or natural sea water. Thus, we are finding some which I believe are truly indigenous to the deep sea environments; but the way I interpret our results in recent years, we probably are finding far more adventitious species than autochthonous or indigenous species. This is very disturbing because it makes it very difficult for us to interpret their significance quantitatively. Could you comment upon any of these problems?

KETTLING: I think this points very directly to the fact that at least for the marine microbiologists, there is a need to increase controlled experiments involving different pressures and temperatures. Another thing strikes me as important is the fact that we find out that if we take core samples, the concentration of bacteria drops off rather markedly, within a meter or so. And this might lead us to question whether these might be fossil remains, these are cultures arising from single organisms that have been preserved.

SCHELTEMA: Do these decrease or do they drop off to zero?

KETTLING: The majority drops off to zero in a couple of feet.

SCHELTEMA: Is it possible that some of these organisms which grow at high temperatures are contaminants?

ZOBELL: This is what I thought when I first started to find them, because even in the best of families, there are breaks in technique that result in contamination. In fact, it is extremely difficult in normal sampling manipulation in a marine laboratory at sea to avoid contamination for various reasons. Much more difficult than in a stationary laboratory on land where you can work in laminar air flow under well controlled conditions. For many years, I charged this to contamination, but when we start to find more of these than the number of apparently indigenous species, I feel quite convinced they were indeed present in the samples, and that relatively few of them are contaminates.

With regard to finding that the population of viable bacteria does indeed drop off very rapidly with core depth in the topmost meters, I couldn't concur with you that down to zero. I have yet to obtain a sample from core depths as great as 5 or 6 meters where, if we use large samples, perhaps as much as 100 grams, I haven't been able to detect some viable bacteria. Very few indeed. I concur to say that the number does drop off with depth so that you may find only about 10% as many at a depth of 10 centimeters than at the mid-water interphase, and at 100 cm. only 1% or even fewer. This suggests that they are dropping off rapidly with depth. But how rapidly is this in the time scale? My geology friends tell me that some of the cores that we have collected from some of the deeps, which are also the ones that I think you and Colwell have worked with sedimentation occurs on an average rate of a millimeter per thousand years. If we find a drop-off in 10 cm. or

100 cm., we are still talking about rather many years, though not necessarily geological time. I still think that these are living fossils if we find them at even 10 cm. or 100 cm. At an average rate of a millimeter per thousand years in that position, 10 cm. represents quite a long period of time.

Another question that gets into this which some of you other biologists can help us microbiologists with, is what is the possible effect of burrowing organisms in the transfer of materials? To what extent might they be responsible for the bacterial population we find at 10 cm. and even 100 cm.? I have asked many zoologists about this and they all seem to differ in their opinion. Most of them are quite convinced that they know what happens in shallow water but they don't know a thing about burrowing organisms at great depths. This is only one mechanism, of course. There are others, seismic disturbances and turbidity currents and things like this that can be responsible locally for the burial of large bacterial populations of recent origin. But these things occur so regularly that I don't believe that seismic disturbances or volcanic eruptions or anything like that could account for all of them.

LOWENSTAM: You might get at this statistically if you compare sedimentation which is undisturbed, either on a basis of percentage or the number of organisms per unit volume, to one which has been homogenized by detritus feeders for instance. That ought to give you some idea of whether or not the mucus for instance acts as an additional substrate of deposit feeders. You could incubate the bacteria below the surface. Has that been done?

KETTLING: Not to my knowledge.

SCHELTEMA: In a similar fashion one could test sediments from various cores by these various techniques by growth. It is quite possible to look at them and be able to tell where they were disturbed by biological activity. So there is a way of getting at this too.

MORITA: To help the microbiologists, I think we have to really ask about the source of the marine microbes. At stations spread all the way across the Pacific we found quite a few that do not require any salt. When it comes to defining what is a marine micro-organism, most of us use a working definition: If it'll grow, and multiply, and catalyze the right reactions, it's more or less a marine micro-organism. If you think about cultures that Dr. Colwell played around with from the Challenger Deep and Philippine Trench, the odd thing about them is that if we isolate them and subject them to the isobaric and isothermal conditions from which they come, they expire. So we get about working on the problem to try adapt them back to these original isobaric and isothermal conditions, and were very successful. The unfortunate part is simply a question of money: Whenever we have a strain that liked what you might call hadal conditions, every time it was taken back to 1 atm. for transfers, we lost it. But there was one odd thing about it—my technician made a mistake once and she pulled off some media

for halophilic bacteria, and she then subjected originally barophile isolates to the halophilic media, and not only this one, but any others we have tried since survived. There are still too many riddles left for easy generalization.
LOWENSTAM: In terms of water activity, deep salt water is fresh water with salt in it. You take an organism from, say 6,000 m and remove the pressure in the same water, that's an osmotic shock added to the temperature and the pressure shocks. It is a horrible thing to work with I am sure, but in terms of classical effects of pressure on water activity and the osmotic resistance of organisms, this may be why the experiment with halophilic medium worked.
TEAL: Look at some of Ralph Horne's work: He tells you how pressurized things lose structure, and how, if you put in salt, they gain their structure back.

In situ Respirometry in the Deep Sea

J. M. Teal
Woods Hole Oceanographic Institution

The bottom of the ocean with the animals and bacteria living on and in it is a system of considerable interest. It is the final sink for organic matter in the sea. It is the last place where organic material can be remineralized before it is removed from the oceans by burial.

Except for a few anoxic basins the water above the sediment contains sufficient oxygen in all parts of the oceans to support respiration, but the oxygen content in the mud itself typically disappears a very short distance beneath the surface. Aerobic metabolism occurs above that depth; anoxic metabolism processes below. But the total oxygen uptake by the sediments and the organisms therein, across the sediment surface, represents a combination of oxidative metabolism on the part of the animals and the oxidation of reduced compounds produced in the metabolism of organisms living in the anoxic layer.

The biological oxygen uptake is directly related to the activity of aerobic organisms but the chemical oxygen uptake is probably also a fairly good measure of the expense of activity on the part of anoxic organisms (Teal and Kanwisher, 1961). The activity of these bottom systems has been studied very little. Howard Sanders and his co-workers, among others, have made extensive studies of the distribution and abundance of benthic animals (Sanders and Hessler, 1969), but the rates of activities of these animals are virtually unknown. The supply of food is certainly limiting in the deep oceans since it must all come from the surface; therefore the activity of the animals living at the bottom of the sea may be low compared to the levels of activity at the same temperature of animals in shallow water. We don't know whether or not bacteria are as important in the deep sea benthic communities as they are in shallow water.

To provide information on points like these we would like to measure the respiratory rate of the benthic system at the bottom of the deep sea. It would be possible to make such measurements from core samples brought to the surface as has been done by Pamatmat (1971). But with *in situ* measurements we can make certain that the system has not been exposed to such changes in either temperature or pressure as will certainly occur with samples raised to the surface, at least with apparatus presently available.

With *in situ* measurements one can also be fairly certain that the minimal amount of disturbance is made of the delicate surface layer of the bottom. Although it is possible that measurements made on bottom cores raised to the surface and repressurized will show the same rates of oxygen uptake as measurements made *in situ*, one can have no confidence that this will be true until the *in situ* measurements have been made.

We measure the changes in oxygen concentration under a bell jar placed on the sea bottom and interpret the decrease in oxygen concentration as a measure of the integrated activity of the organisms on and under the portion of the seabed enclosed by the bell jar.

It seems fairly obvious that the ideal way to place the bell jar is with a submersible from which the operator has the maximum ability to see what is taking place during the measurement. But submersible costs are at least 3 to 4 times greater than ship costs, and many more measurements could be made if they could be done from shipboard rather than from submersibles.

We plan to build both types of apparatus to take the maximum advantage of opportunities for making measurements.

The measurement of oxygen concentration in sea water with good sensitivity and precision can readily be done by the use of oxygen electrodes, although more information could be obtained if other variables were measured as well. In view of the added complexity of instrumentation that would be necessary, it hardly seems worth while in the first trials to go beyond the measurement of oxygen.

The mechanical arrangements of the experiment are relatively simple. We need a stirring device to stir the water being sampled at the electrode surface and to stir the water under the bell jar so that the entire volume is sampled by the electrode. We use a small magnetic stirrer in a case filled with light oil and closed with a rubber diaphragm which allows the pressure to equalize. It would be relatively simple to build a valving arrangement by which the electrodes could alternately look at water enclosed by the bell jar and water outside the bell jar on the assumption that oxygen concentration in the bottom water during the period of the experiment would remain constant. This would provide a calibration point. For our first tries, however, we have not built such a valve because our experience indicates that oxygen electrodes are sufficiently stable so that a calibration at the beginning of the experiment and one at the end of the experiment. just before the bell jar is put in place on the bottom and after it is removed from the bottom, i.e., in both cases when it is seeing bottom water, will be sufficient for calibration.

In a long term experiment it might become desirable to have a pumping mechanism to exchange water in the bell jar when it reached certain oxygen concentration, for example a 10% reduction from the concentration originally enclosed with the water under the bell jar. Such exchange would both assure that the oxygen concentration under the bell jar never fell far from that that the organisms experience naturally and provide periodic recalibration of the electrode. An alternative method would be to open the top of the bell jar and allow currents to exchange the water.

The bell jars themselves are of very simple construction, a piece cut from a plastic cylinder with a flat piece of plastic glued to one side to form a top.

It has a light rubber flap valve in the top to avoid pushing water sideways and thereby stirring up sediment when the bell jar is placed on the bottom. A flange around the cylinder allows it to be pushed a known depth into the sediment. Power supplies for the stirrers and measuring and recording apparatus are either lead acid or alkaline batteries; zinc-carbon or mercury batteries are not adequate because of their poor performance at low temperatures.

Because of the difficulty of knowing the history of batteries we feel it is particularly important to have an excess of power in any instrument that one is going to the expense of placing on the bottom of the ocean. It might be pointed out to those unfamiliar with the characteristics of batteries that two half power batteries used in parallel are not an adequate substitute for one full power battery because of the virtual impossibility of matching batteries so that their potential will change at the same rate.

Since the pressure case enclosing the measuring and recording apparatus may be positively buoyant the use of a lead-acid battery may in some cases have the added advantage of supplying the weight necessary to anchor the apparatus to the bottom. The simplest sort of measuring circuit is to drop the current from the electrode across a temperature compensating thermistor and measure the resulting voltage.

We use the thermistor in the feedback loop of an operational amplifier biased at the electrode's polarization voltage of 1 volt to give us an output suitable for driving the movement of a hundred microamp galvanometric recorder; these constitute together our measuring and recording system. An inplace recorder has the advantage of supplying a permanent, accurate record of the changes in oxygen during the course of the experiment. It has the disadvantage that the system must be retrieved before the results are known and the further disadvantage that you don't know until the experiment is finished whether or not the apparatus was functioning properly.

Telemetering the data overcomes those disadvantages by telling you how the experiment is going in real time. John Kanwisher, Ken Lawson, and I have used a simple radio link in shallow water. We plan to use a sonar link in the deep sea. It is possible to treat the data in a number of ways—making a pulse rate proportional to oxygen—a frequency shift proportional to oxygen—or to do a complete analog to digital conversion and send the data digitally. The disadvantage in using a telemetering system is that you must be within a few miles to receive the signal, especially if we are talking about a relatively small weak power system that we envision and use. Because of the relative advantages and disadvantages of the two kinds of systems we have decided to combine them and use both in place recording and telemetering of the data so that we can know how the experiment is going as well as have an accurate record of the results at its conclusion.

We plan to place the bell jars on the bottom first by use of a submersible which will allow us to pick the site for the placement and to observe characteristics of the bottom. Because of the difficulties of obtaining diving time on submersibles and the great expense in their use we also plan to try bell jars that can be lowered from the surface or allowed to fall free from the surface until they hit the bottom. We have not yet tried any such device but are building a prototype consisting of a cylinder with an opening top. This will fall to the bottom with the top open in order to penetrate sufficiently to get a seal around the edges. The top will then slowly close to avoid disturbing the sediments as much as possible.

We have successfully used our bell jars in shallow water but have yet to try them in the deep sea. To get some idea of the length of time that may be required to make a measurement, I have calculated minimal rates of oxygen uptake based on the data of Emery (1960) for the rate of deposition of organic matter off Southern California. His data indicate that only 1/3 of the organic matter in the Santa Barbara basin is oxidized, but I have assumed a value of 1/2 for the continental slope and 1 for the deep sea floor. Assumptions of the oxygen uptake in sediments of the Santa Barbara basin will be 4 $mm^3cm^{-2}day^{-1}$. The value for the continental slope would be 1/4 of that, and the value for the deep sea floor would be 1/4 of that for the continental slope or 0.25 $mm^3cm^{-2}day^{-1}$. Using a bell jar 5 cm deep and requiring a 10% change in the oxygen to have a reasonable experimental change, the experiment would have to last about 18 hr in shallow water, on the order of three days on the continental slope, and 12 days in the deep sea.

These values do not take into account oxidation that occurs right at the surface of the sediments since they are based on accumulations found in cores taken from the bottom. I expect that actual oxygen consumption would be higher than these minimal values. Pamatmat's, (1971) measured values off Washington are about 2.5 $mm^3cm^{-2}day^{-1}$ or about 10 times the values calculated from Emery's data. However, these values are for a portion of the abyssal plain that is close to a productive shore and we might expect that other values for abyssal plains would be lower. In any case it seems likely that experiments will have to run with periods of time of several hours or days to get a significant measurement.

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Discussion Following Teal Paper

MACDONALD: Can I ask John Teal how he is going to express his results?
TEAL: Oxygen uptake per square something on the bottom.
MACDONALD: I am not quite clear what the significance of such a unit is.
TEAL: Well, the measurement of oxygen by an area of bottom is presumably a measure of the oxygen consumption of the things that live in that area of bottom.
MACDONALD: That somehow seems to me incomplete. I should like to see a measure of the amount of biomass that is there.
TEAL: Yes, you can core it. If you use a submarine, you can take the device up and core the bottom under it. That's easy.
MACDONALD: You think that can be done from the surface?
TEAL: It can be done. I am not sure that I have a mechanism for doing it in exactly the same spot. That would be one of the disadvantages of doing it, but you could think of something to do in exactly the same spot but it would be easy to do a whole series of them round about and then do more measurements under the bell dirt like this.
KANWISHER: If the experiment lasts for more than day, and if you were to use a submarine, and it is going to cost $10,000 to come back and get your tools, and if we paid $200 for a transmitter, it would be throwing good money away just to retrieve it. We'd best just leave it there.
SCHELTEMA: Can I ask a question about your way of expressing the situation inside there? It strikes me that this is a source of confusion with different people doing the same thing in the same way with different shaped bell jars and this sort of thing. The oxygen consumption might be rather dependent upon the exchange across the surface. Is there any way of physically describing it in terms of the stirring rate?
TEAL: What we did was put some ink in water and watch it.
BERGER: We have been up against the same sort of thing in a pressure chamber. I have never been able to demonstrate a relationship between the rate of stirring and the oxygen uptake, within certain limits. It depends on having an oxygen uptake fast enough so that you make an appreciable oxygen gradient there that's different from the one that exists normally.
PAMATMAT: As far as benthic community metabolism is concerned, I have always believed that the most reliable estimates of its natural rates are to be obtained by means of *in situ* measurements. After hearing some of you yesterday, this belief has become unshakeable.

However, as Kanwisher has stated concerning the use of a deep-sea submersible, *in situ* measurements will be prohibitively expensive for routine use in the oceans. In fact, for routine application, all the *in situ* techniques that we have either used (Pamatmat and Fenton 1968; Pamatmat and Banse 1969) or considered using appear to be much more expensive than shipboard measurements. It needs to be emphasized that the key consideration is "routine application", for the oxygen consumption by the seabed is of such great importance in understanding the marine food chain that it should be measured in many parts of the world ocean.

The value of *in situ* respirometry in the deep sea, as I see it now, lies in the hope that it will show us how accurate our shipboard measurements are, and if the latter are in error, enable us to identify the sources of error and to design appropriate corrective measures. It should now be obvious to you that in my opinion the ultimate test for the accuracy of a shipboard method is by comparisons with *in situ* measurements. In our present state of knowledge, we do not know with what accuracy our shipboard experiments will allow us to extrapolate metabolic rates under simulated *in situ* pressure to naturally occurring rates in the deep sea. This is primarily because of the unknown effect of temperature increase and decompression. Properly designed experiments comparing *in situ* and shipboard data should show us whether resubjecting sediment cores to simulated hydrostatic pressure and temperature *in situ* will reproduce their *in situ* respiration.

Fortunately, in shallow coastal waters to 180 m depth the effect of pressure or decompression appears to be negligible (Pamatmat, 1971). Comparisons between *in situ* and shipboard measurements have shown no difference between the two methods; experiments with sediment cores in a pressure bomb consistently showed no effect of pressurization and decompression. On the other hand, core samples from about 2700 m off the coast of Oregon and Washington showed ambiguous results in the same type of experiments in a pressure vessel. The difference between consistently negative results in shallow water and ambiguously positive results in deep water might be an indication that more experiments will eventually show a significant effect of pressure on benthic respiration at depths as shallow as 2700 m.

Finally, I would like to say that there is a limitation, too, within the *in situ* techniques mainly in performing experiments where you want to change the conditions. For instance, in my work on oxygen consumption in Puget Sound I found a seasonal cycle which coincides with the temperature cycle. So, in order to isolate the effect of temperatures I had to do my measurements at the same temperature every season. It seems to me it would be very difficult to change the temperature *in situ*

MENZIES: Even harder to change the pressure.

PAMATMAT: Yes. Other manipulations like varying oxygen concentrations

or the addition of inhibiting or stimulating substances I think are in order, and will be called for in the future, and these are a little bit more feasible with the *in situ* method.

THEEDE: When you use this chamber on the bottom of the sea you make water movements near the electrode, and then I could imagine that the water movements in the whole chamber will differ from the normal water movements on the ocean floor. If that is the case, then the condition of the bottom tested will differ from the normal condition of the bottom. What is the influence of the oxygen tension on the bottom on your observations?

PAMATMAT: I made my measurements in Puget Sound where strong tidal mixing makes the water very homogeneous over a great depth above the bottom of the sediment surface, and so, in order to get a good estimate of the naturally occurring rates of oxygen consumption, you should mix the water thoroughly as in Puget Sound. In other cases where there might be gradients in the oxygen concentration above the bottom, you should modify your experiments, or at least in order to simulate the natural conditions.

TEAL: It was interesting in those movies this morning: In every one of them the particulate matter in the water even though the apparatus had been in place for sometime and the bottom water should have been undisturbed by the general apparatus, showed constant random movement or convectional movements. It's not just "still".

PAMATMAT: No, I don't think so. In fact, there are more and more measurements on the currents in the deep sea. But there may not be a gradient immediately above the bottom of any great magnitude to affect the rate of oxygen consumption.

HESSLER: Is it possible to measure that gradient?

KANWISHER: Yes. It would be possible if you had something like a submarine to put down there to do it with; if you had you could stir up that stuff on the bottom. You have obviously achieved something in that case which is as good as or better than whatever occurs naturally as far as destroying the gradient.

THEEDE: Were these long term measurements or short measurements?

TEAL: I have only succeeded so far in having a measurement that lasted for half an hour which shows anything dramatic.

THEEDE: And the oxygen content of the water—did it decrease greatly this time?

TEAL: It didn't decrease at all. I don't remember what our sensitivity was the way we had the electronics set up.

LOWENSTAM: What was the dimension of the bell?

TEAL: Something like 30 cm. in diameter.

MACDONALD: Can't physical oceanographers calculate the kind of experiment you are trying to do?

TEAL: Yes, I can calculate it. In fact, I had the figure on that piece of

paper. But the kind of figures you get are subject to monumental uncertainties. You could do it on the basis of the oxygen disappearance from the bottom waters, for example, if you knew the time it takes the bottom water to flow across the bottom, and at the same time you make the assumption that the oxygen disappearance from the bottom water all occurs at the water/mud interphase. That may be fairly true—there aren't many animals that lift off the bottom. But, the numbers you have to go to for age in the bottom water are the basis of the monumental uncertainty I was thinking of.

MENZIES: I don't think so. 500 years for the Atlantic and a thousand years for the Pacific, and I'll bet you probably find you won't be off by a factor of more than two.

TEAL: But that's on the basis of C-14 measurements

MENZIES: No, they're on water turn-over, too. We know the amount of Gulf Stream pouring more into the system.

TEAL: That's the basis on which I did it to get the kind of numbers that would come pretty close to these same kinds of things that I was talking about—oxygen uptake on the order of a cubic millmeter of oxygen per square centimeter.

MACDONALD: I find it very difficult to imagine a square centimeter consuming oxygen.

TEAL: Well, just think of the number of animals in a square centimeter of bottom. If you make a measurement over an area of the bottom that's the kind of way you can put the results. The same thing as a city based on the number of people prorated over that area.

PAMATMAT: There is obviously a certain amount of oxygen consumption. If you can show that this consumption is due to respiration, regardless of the unit of area or volume that you use, this is equivalent to the oxidation of a certain amount of organic matter, assuming certain respiratory functions, it doesn't matter how you express it.

MACDONALD: How many cells do it?

TEAL: That is yet another matter that is also of interest. I'm sorry I didn't mention that, but I sort of assumed it.

HESSLER: Are there any estimates of chemical oxidation—inorganic oxidation?

TEAL: Yes, Mario Pamatmat has some estimates which I don't trust.

PAMATMAT: Yes, this is commonly done, the rate of total oxygen uptake of the sediment is the sum of respiration and inorganic chemical actions, because of the presence of reduced substances below the surface of the sediment as a result ultimately of the anaerobic metabolism of the bacteria below the surface. The total uptake is commonly partitioned into respiration and inorganic chemical oxidation by poisioning the overlying water after measuring total uptake so that the residual uptake after poisoning is a measure of the inorganic chemical uptake.

HESSLER: Is this 10% of the total or 1%?
PAMATMAT: In the stations that are regularly occupied for one year in Puget Sound there are differences with these things.
BERGER: But that doesn't matter, you're still getting oxidation, and it's really a reflection of anaerobic microbial metabolism which in turn is biological activity.
PAMATMAT: The total oxygen uptake by a unit area of sediments, as Teal and Kanwisher proposed at one time, is an integrated measure of the total amount of organic matter in the sediment core. That is the best that we can say at this time.
BRAUER: The reason Teal's topic was brought up for discussion is frankly not just an arbitrary whim. So far as we could find out this is about the only kind of experiment *in situ* that anybody has done with any remote semblance of success. Now, I admire some of Menzies' experiments with things which run up and down on a wire—but they don't affect this argument. We desire to place before this meeting the question of whether in fact it is merely by coincidence that nobody *happens* to have done any other experiments *in situ*, or whether this is an indication of the fact that the range of kinds of experiments which in fact you can do *in situ* to resolve biological questions of deep sea biology are extraordinarily limited, and despite gallant attempts there are only one or two very specific questions which are at all appropriate for work *in situ*.
GORDON: I'd like to comment on that because I think that there are quite a few kinds of experiments that can be done, where we don't have to worry about all the fancy stuff which you have been talking about because most of the things which we have been discussing so far are the things that are really dependent upon having a live system left when you're through, essentially, or trying to hoist a system up to the surface. There are lots of things that really don't require that. Certainly, for the kinds of things that Lowenstam is concerned about, he really doesn't need to have a live animal—all he needs to have is a bone, or teeth, and things like that back at the surface after some period of time. For various experiments that we've done previously, going way back now, but at much shallower depths. A number of years ago, Scholander, Kanwisher, and I spent a summer up at fjord in Labrador worrying about the nature of the mechanisms of freezing resistance in some Arctic fishes. One of the experiments that we did was to lower a trap full of shallow water fishes down to the bottom of this fjord in several hundred meters of depth, and keep them there for a period of ten days to two weeks, and then bring them back up again to measure changes in the composition of the body fluids in these animals after that period of exposure to very low temperature and somewhat increased pressure. You can certainly do things like that very readily.
KANWISHER: And I'll give you the heart beat of the fish, or tail beat frequency, or almost anything you want.

GORDON: In connection with this last thing that John Teal talked about, I'm very glad to hear that he mentioned something about a free vehicle. I think that is a classical case of a beautiful system to use with free vehicle recovery system. You can do a great deal of what you want to do with a syntactic foam float and a pinger, and the main piece of technological improvement that would be required is getting a release that would drop the weight off so it would work when it's supposed to work rather than only 50% of the time.

HESSLER: We've got better releases than that now—they are not quite as bad as you make them out.

GORDON: Well, all the better. We can do many kinds of things with devices you put down and bring up.

KANWISHER: Looking at the movies this morning, I would never have entertained the possibility of measuring the respiration of fish down there. But the ability to attract them to a bait brings that well into the realm of possibility.

WELLS: We got some fish today at a pressure of about 100 and some odd atmospheres which were brought to the surface and their guts everted through their mouths and they had very large gas bubbles in their eyeballs. Now these gas bubbles were of interest to me sometime ago, because it has been postulated there were mechanisms of concentrating gas in the eyeballs and in addition they have counter-current mechanisms similar to the ones in their swimbladder. So I took a bunch of gas samples out of the eyeballs and this gas was of a very similar composition to that of the swimbladder. Then Bryan D'Aoust and I were given the opportunity during an *in situ* exposure, at pretty shallow depths, at Sea Lab II where I removed samples under pressure from vitreous humors of fish, sealed them in pressure chambers, and sent them up to Bryan who analyzed them on the surface. This was not published in the National Geographic—it was in Life Magazine. We found that there was no great super-saturation of this vitreous humor, at least in the species of fish we worked with. But here again, this technique had been mentioned earlier, and in such studies as this, where we have a relatively small pressure gradient that is a great barrier to any of these gas-containing creatures, it is entirely possible to send men down—and we can send men down almost to 40 atm. now—to meet these fish down there, and stuff them in a pressure chamber after catching them on a hook, and decompress them. Bryan and I have also met fish who could be brought up 100 m. This is also a lethal transition for fish with swimbladders—100 m vertical excursion kills them. But if you swim down to 30 m and stick a needle into their swim bladders and pull the gas out, they will be very happy when they come to the surface. These same things do require high pressure aquariums to study these fundamental physiological problems.

ZOBELL: Maybe, relevant to this discussion are the classical observations by the primary production people which have been made for more than 50

years, worldwide in fresh and sea water alike, with dark and light bottles; the light bottles to make measurements the rate of photosynthesis *in situ*, the dark bottles to measure the rates of respiration in a closed system, sometimes closed and sometimes piston-stoppered, so that it would be subject to the same pressures and the same temperatures—to all the environmental conditions. I think this is an elementary example of a kind of *in situ* measurement which has been made very extensively. I have made many observations myself, borrowing this technique, using dark bottles to eliminate the effects of light—to determine the rate at which various organic compounds, various sugars, various amino acids, and other things that may be attacked by bacteria, consume oxygen. Submerging these bottles at different depths in the sea in piston-stoppered bottles, and after two days, or five days, or whatever time seems to be appropriate, determine the oxygen uptake by knowing how much gas was there originally and how much is there at the end. I think the same thing could be done at great depths.

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CHAPTER THREE
PRESSURE-INDUCED STRUCTURAL CHANGES IN AQUEOUS SOLUTIONS AND THEIR POSSIBLE PHYSIOLOGICAL SIGNIFICANCE*

by
R. A. Horne
and
B. A. Courant
Chemistry Department
Woods Hole Oceanographic Institution
Woods Hole, Massachusetts U.S.A.

*Woods Hole Oceanographic Institution Contribution

Introduction

When first we heard that a workshop on "High Pressure Aquaria" was being planned we were immediately filled with enthusiastic interest. We had long felt the need of some kind of a confrontation between physical chemists on the one extreme and physiologists on the other, with perhaps molecular biologists somewhere in between, to explore together the effects of moderately high hydrostatic pressures on biological material and processes. Of the two basic needs for such a meeting, one is now well-recognized but the second is not so clearly appreciated, yet in the long run it may prove to be far the more important in contributing to our fundamental understanding of biological processes. First, when dealing with the deep ocean, clearly we are entering one of the most extraordinary environments in the whole universe—a closely regulated thermostat (in contrast to the widely fluctuating temperatures which are the rule in the solar system, including the terrestrial land masses) consisting of an aqueous electrolyte solution (in contrast to the gaseous mixtures obtaining near universally elsewhere) under moderate high (~1000 atm. max) hydrostatic pressure (in contrast to the rarefied gaseous planetary atmospheres). And secondly, beyond our immediate, as it were "in hand", confrontation with a real high pressure biotic environment in the deep sea, is the utilization of high pressure as a research tool for the exploration of the most delicate and intricate, not to say profoundly important, biological processes. Pressure is a controllable variable in biosystems in the same sense as are temperature and ionic strength, and in this connection it is rapidly proving itself just as useful.

The effects of temperature, pressure, and solutes on biological systems, in particular on the stability in solution of protein material, parallel one another and are in many respects interchangeable. While pressure effects are usually small compared to temperature effects, requiring hundreds of

atmospheres to accomplish the work of a few degrees celsius, they may nevertheless be important, and better yet, they are often revealing.

The Structure of Liquid Water, Aqueous Solutions and the Hydration Atmospheres of Solutes

In order to understand the effects of pressure on the biomaterial in the most fundamental molecular terms, it is necessary to review briefly the structures of liquid water and solutions and the effect of pressure upon them. To simplify the discussion we will adopt the following system of nomenclature:(1)

Water-i	The monomer
Water-ii	Small polymers $(H_2O)n$ of n 2 to 4
Water-iii	Large polymers of n 4 a. Randomly hydrogen-bonded b. Hydrogen-bonded with at least non-ice-I-like near-neighbor order
Water-iv	Ice-I-like
Near Solutes	
Water-v	Electrostricted water of hydration
Water-vi	Enforced water structures near ions (except water-v)
Water-vii	Broken water structure near ions
Water-viii	"Icebergs" or clathrate structures near nonpolar solutes or nonpolar segments of macromolecules
Near Interfaces	
Water-ix	Near neutral and nonpolar interfaces
Water-x	Near silica
Water-xi	Absorbed or chemically bound water

Following the Frank-Wen model (2), the structure of pure bulk liquid water consists of flickering H-bonded clusters (an average cluster contains about 40 water molecules at room temperature) or water-iii surrounded by more-or-less "free" or monomeric water-i (Figure 72). The size and concentration of the water-iii aggregates depend on temperature and, as we will see, upon pressure. The thermodynamic effects consequent to the addition of a neutral solute to pure water indicate an ordering of the water molecules to form "ice-bergs" about the solute. In the case of polar or ionic

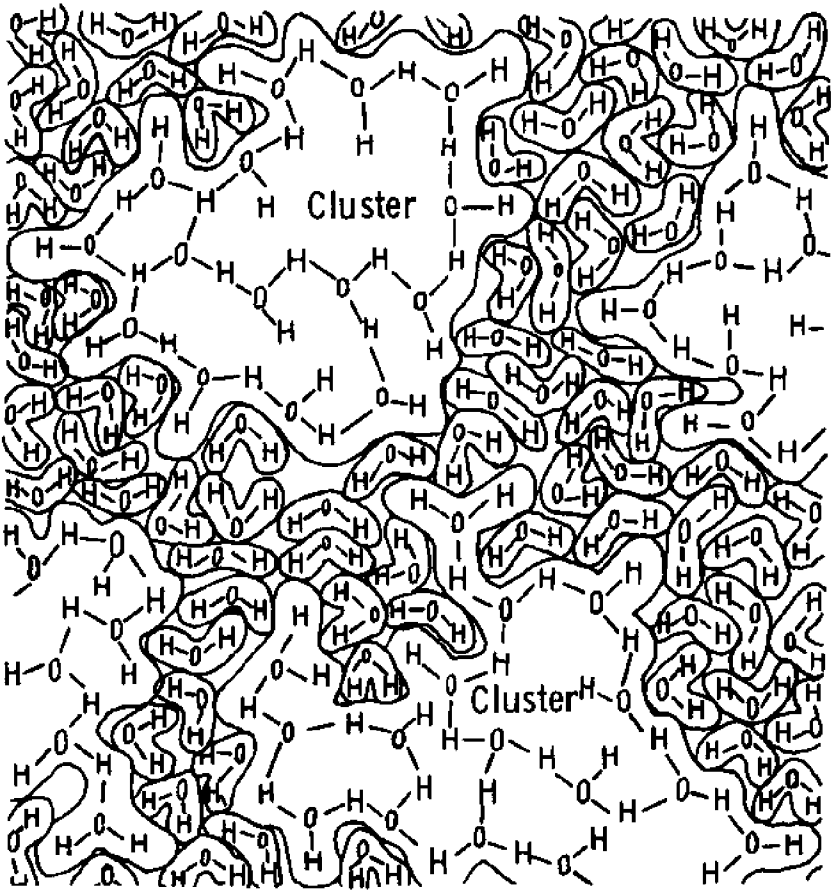

Figure 72. THE STRUCTURE OF PURE, BULK, LIQUID WATER. (From R. A. Horne, *Adv. Hydrosci.*, *6*, 107 (1970))

solutes even more complex changes occur in local water structures near the solute species. Viscosity changes indicate that some ions are water structure-makers and others structure-breakers. In order to explain this complexity of behavior Frank and Wen (3) proposed a two zone model for the hydration atmosphere of ions in aqueous solution, while we have elaborated this structure in even greater detail (4). The primary hydration (Figure 73) consists of tightly bound, electrostricted water-v surrounded by an H-bonded region (water-vi) which appears to be in essence a Frank-Wen cluster (water-iii), and this in turn is surrounded by a region, water-vii, of broken water structure where the coulombic field of the ion is strong enough to disrupt the order of bulk water but still too weak to re-orient the water molecules into some new pattern.

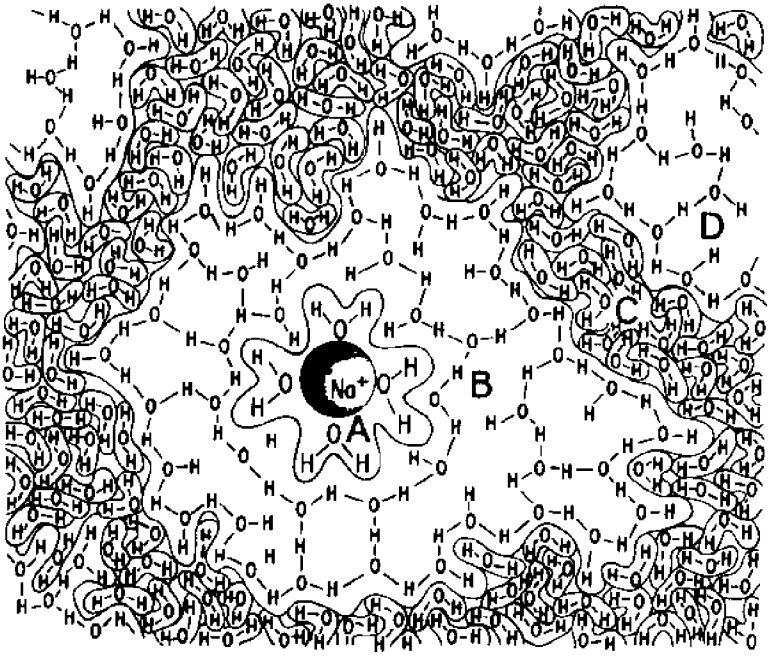

Figure 73. THE LOCAL WATER STRUCTURE NEAR A CATION IN AQUEOUS SOLUTION. (From R. A. Horne, *Adv. Hydrosci.*, *6*, 107 (1970))

The water structure near a non-polar solute appears to be entirely different from that near polar and ionic solutes, thus we can distinguish two distinct forms of local water structure—*coulombic hydration* (water v-vi-vii) of ionic and polar solutes and *hydrophobic hydration* (water-viii) of non-polar solutes. In this connection the tetra-alkyl-ammonium cations, $(C_nH_{2n+1})_4N^+$, are of particular interest inasmuch as their hydration runs the spectrum from coulombic to hydrophobic as n increases (5). A complex molecule having both hydrophobic segments and polar sites (Figure 74) such as a biomacromolecule will have a hybrid hydration sheath and we may speak of the molecule as having so much coulombic and so much hydrophobic character just as we speak of a chemical bond as having so much ionic and so much covalent character.

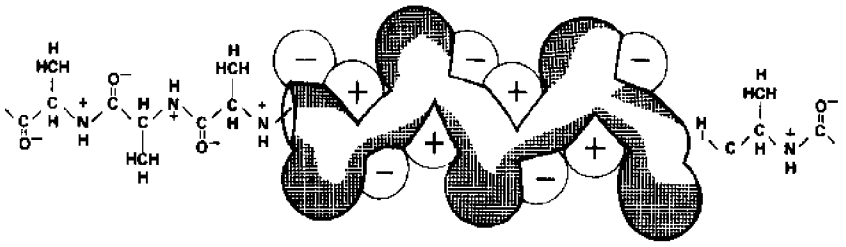

Figure 74. HYBRID HYDRATION SHEATH WITH COULOMBIC HYDRATION (OPEN CIRCLES) SURROUNDING CHARGE SITES AND HYDROPHOBIC HYDRATION (SHADED CIRCLES) INCASING HYDROPHOBIC SEGMENTS

Water near a neutral interface such as the air/water interface will also have a structure different from that of the bulk liquid. This layer of vicinal water (water-ix) may be quite thick, extending hundreds, even thousands of Å's into the bulk liquid. If the layer is indeed thick, the biological implications are enormous for nearly all the water in living cells and tissues would be "abnormal" water-ix (and waters v, vi, vii and viii). Inasmuch as there is no *a priori* reason for postulating that the water structure surrounding a dissolved neutral gas molecule (such as Ar, O_2 or N_2) is different from water structure at the interface of the bulk phase of this same gas, water-viii and water-ix may very well be synonymous.

Having reviewed the many kinds of water structure we will now turn our attention to the effects of hydrostatic pressure on the different water structures.

The Effects of Pressure on Water Sturctures

The structure of the Frank-Wen clusters (water-iii) is not known. The H-bonding may very well be more or less random and the structure is probably *not* that of Ice-I. However, like Ice-I, it is a low density (or high volume) form, thus we expect it to be relatively unstable with respect to the application of hydrostatic pressure. The relative viscosity of pure water exhibits a minimum with increasing pressure (Figure 75). The initial decrease in $\eta_p/\eta_{1\ atm.}$ arises from the destruction of the water-iii aggregates. Somewhere between 500 and 1000 kg/cm² the destruction is complete, and $\eta_p/\eta_{1\ atm.}$ begins to increase with increasing pressure as a "normal" liquid should. Notice that at temperatures and pressures corresponding to the bottom of the deep ocean the relative viscosity of sea water may be as much as 2% less than at the surface.

The effect of hydrostatic pressure on coulombic hydration is less clear. The electrical conductivity of aqueous NaCl solutions increases

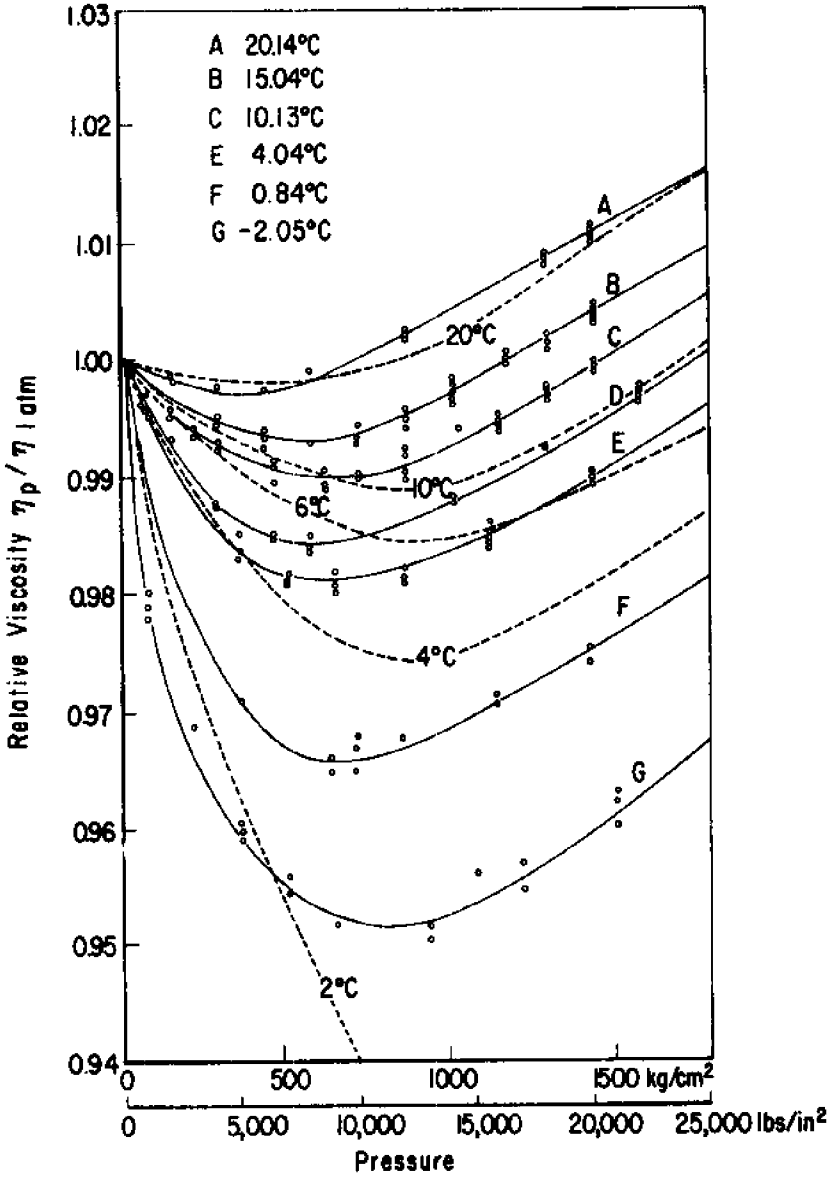

Figure 75. RELATIVE VISCOSITIES OF PURE WATER (DASHED LINES) AND $35^0/_{00}$ S WATER (SOLID LINES) UNDER PRESSURE. (From R. A. Horne and D. S. Johnson, *J. Geophys. Res.* 71, 5275 (1966))

more rapidly with increasing hydrostatic pressure than one expects on the basis of the volume decrease and initial viscosity decrease. Following Zisman(6) this anomalous increase increment is attributed to pressure-

induced dehydration of the ions resulting in a decrease in their effective radio(7), but Gancy and Brummer(8) following Adams and Hall(9) take exception to the commonly held view that NaCl is a strong electrolyte at 1 atm and attribute the increased conductivity to increased dissociation of that electrolyte under pressure. Adopting the former view, as the hydrostatic pressure is increased first the water-vi surrounding an ion (Region B in Figure 73) is destroyed, then, when the density of the bulk solution exceeds the density of the incompressible electrostricted water-v (Region A in Figure 73), even this hydration envelope is broken down. Now this is an important point in the light of our earlier remarks concerning pressure as a research tool, for here we can do something with pressure that we cannot do with temperature. Temperature, like pressure, breaks down the Frank-Wen clusters, but temperature, unlike pressure, has little effect on the effective radius, ϑ, of ions in aqueous solution, as evidenced by the operancy of Walden's rule $\lambda\eta \neq f(T)$.

Unfortunately, for these are the water forms of greatest biological interest, the effect of pressure on vicinal water (water-ix) and hydrophobic hydration (water-viii) is even less clear. Their structures may be of a clathrate type but there is some evidence that they are a relatively high density form (10) in which case the holes in the clathrate cages would have to be occupied by further water molecules in order to achieve the high density. Such a high density form would be relatively stable with respect to pressure (and we would expect biological pressure effects to be small). Our own view, based upon such observations as the ability of pressure to denature proteins, is that while more stable under pressure than coulombic hydration, water-viii and water-ix are still less dense than water-i, and thus still pressure sensitive, although less than water-iii. This view keeps open the possibility of important biological pressure effects.

Pressure Effects on Biological Systems

We are happy to say that we do not feel obliged to dwell on the importance of water in biosystems! This finally seems to be getting the appreciation it deserves. Our views, however, do seem to remain more radical than those of the rest of the scientific community, for we have become convinced that water structure, in particular the balance between coulombic hydration and hydrophobicity, has been the principal chemical factor determining the origin and direction of the evolution of life on this planet (11). But we would like to conclude with some frankly speculative remarks upon possible physiological effects based on pressure-induced changes in water structure.

If pressure can indeed dehydrate ions it should be possible to reverse the selectivity of Na and K by biomembranes by the application of pressure.

The sizes of organisms with non-pumped respiratory pigments depend upon circulation by diffusional processes. If the viscosity of biowater decreases with increasing pressure, should there be a tendency for the size of such organisms to increase with depth in the sea?

If there is any validity to the Miller-Pauling clathrate theory of anaesthesia (12) and if water-viii is affected by pressure, then the influence of dissolved gases such as 0_2, N_2 and Ar on the nervous system should exhibit a depth-dependence in the sea.

Biologically speaking there appears to be something crucial about a temperature somewhere between 30 and 40 C. (13). The association of reported "kinks" in the temperature-dependence of water properties with vicinal rather than bulk water (14) has magnified rather than diminished the potential biological importance of these higher transitions. Living things appear to utilize some sort of structural transition in either a "third state" (water-ii, water-iii b, or water-iv) in the bulk liquid or in the vicinal water and/or hydrophobic hydration (water-viii and/or water-ix), as the basis of regulatory functions (15), for example temperature control (near 37C) in homeothermic animals (16). Just as the density maximum at 4C is displaced by the application of pressure, this structural transition equilibrium might be expected to be displaced by pressure, and significant biological pressure effects could arise in this manner.

In conclusion we would like to repeat our earlier statement that we have barely begun to explore the potentialities of pressure as a research tool for exploring biological form and function.

Support of the National Science Foundation and the Office of Naval Research is gratefully acknowledged.

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Discussion Following Horne and Courant Paper

(Question:) Could you help me out a bit on the differential effects of pressure on the Na^+ and K^+? One of the botanists at our place has studied the effects of pressure on beta galactosidase which has two substrates, one of which is Na^+ activated and the other is K^+ activated. We find an increase in the prescence of Na^+ and a decrease in the pressence of K^+. You made some comments about denuding the ions under pressure. What sort of pressure ranges are you talking about?

COURANT: To completely strip the ion of all the water around it, you have to go to quite high pressures, 2,000 atm. or so. But up to a relatively modest pressure, all you are doing is stripping away the second hydration sheet of the ions. There should be a difference here, now, because of the differences in the sizes of the K^+ and Na^+. Li^+ of course, is extremely inert.

LOWENSTAM: How do you distinguish between hydroxyl and molecularly bound water? We have many molecules like SO_2, H_2O, and in that case you have hydroxyl bonded water and you have rates which depend in part on the structure. Does this apply to sea water?

COURANT: The ions do not take part in an equilibrium process.

BRAUER: Many of us are intrigued by the interaction between the effects of high hydrostatic pressures, and the effects of some of the metabolically inert anesthetic agents. You made reference to this very briefly, and of course, we're familar with the Pauling concept of the relation of the clathrates to this biological effect. Pharmacologists are split into a pro-Pauling and an anti-Pauling factions on this, and I think at the moment the antis rather have it. Would you choose to spell out for us a little bit here the type of effect you might anticipate from the presence of, let us say, something like xenon, in the medium?

COURANT: I was at a meeting once and I was sitting next to Dr. Eagan from the Chemistry Department at Woods Hole, and somebody raised the question "what is the mechanism of anesthesia", so I happily started explaining things in terms of clathrate type model, and as I was talking, Dr. Eagan was shaking his head and I felt more and more stupid as I was going

along. But when I finished, I turned to him and asked him to explain what he thought was the case, and he said, well, for instance, laughing gas could not answer because the pressures at which it acts are too low. That's all I can say about it.

MACDONALD: You don't believe in the Pauling model, What do you believe in?

BRAUER: There are at least twenty different ones that one can play with. The so-called theoretical apparatus that anesthesiologists have used, in fact consists of setting up series of agents, looking at some of their physical properties, and expressing one's satisfaction when particular values for a physical property line up in about the same way that the anesthetic potencies do. I think it was Pauling himself who pointed out for the first time that, for the case of the so-called inert gases, if any one property lines up, just about all the others must do so as well, and so the probative value as to any particular mechanism disappears. You can escape from this by choosing special gases. We've worked with the hydrogen-helium relation. In that case there is a separation of size related at "interaction related" properties (Brauer, J. Applied Physiol. 29:23-31, July 1970). Miller and his colleagues have worked at the other end with some of the perfluoro compounds. We can throw out some of the possible interpretations, but you're still left with large bundles, and while there was a time that I enjoyed immensely writing differential equations and talking about quantum mechanical interpretations, since I've grown older I find this is less amusing, and I prefer to try to get more incisive experiments. The interplay that Courant is talking about, it seems to me is likely to be one of the tools we haven't exploited fully so far. As MacDonald pointed out, some very startling interactions are beginning to show up in relation to the effects of high pressures on the central nervous system, and on various forms of anesthesia. We're beginning to find similarities as well as differences, and each time we find these, the cells within which theories will have to be confined begin to narrow. This is why I think this is an important thing, but I'm not about to give you any theory, not because it can't be done, but because it's much too easy.

MACDONALD: Dr. Smith in Ottawa has been looking at the effects of some of these known specific anesthetics on membranes using the spin resonance technique. Just putting a spin needle in the membrane and then applying the anesthetics, he sees a change in the spin needle spectrum. What this means I don't know.

(Question): I think there is one more of the twenty theories that has a little more respect than Brauer has implied. That's the lipid solubility. This presentation has suggested that the war here may be solved as clathrates become more important at the higher hydrostatic pressures. As Brauer said, the lipid solubility and clathrate properties correlate very nicely.

HORNE: The Pauling-Miller theory of anesthesia is one of those ideas which, despite ingenuity and a great deal of merit, appears to have fallen

into dispute because one of its brother ideas is untenable. In their quantitative analysis of Pauling's (1) theory of the clathrate model for bulk liquid water Frank and Quist (2) were able to account for many of water's properties. However, the model was incompatible with the observed X-ray radial distribution (3) and was subsequently abandoned. Nevertheless, my own view is that while absent in the bulk phase clathrate-like structures still are probably important in the hydrophobic hydration surrounding non-polar solutes (such as the noble gases, N_2, O_2, etc.) in aqueous solution and the hydrophobic segments of biomacromolecules, and in composing the layer of "vicinal" water near neutral interfaces. The clathrate-structured hydrophobic hydration can give rise to many phenomena (such as the "kink" properties of vicinal water (4), including biological ones such as anesthesia. The various mechanisms for anesthesia that have been proposed need not be alternatives, but rather different aspects of the same process. For example, inasmuch as lipid solubility depends on hydrophobicity, I would not be surprised if solubility, clathrate characteristics, and anesthesic action were not all correlated.

You might be interested to know that Miller has recently extended the water-clathrate theory to a number of geological observations, including the absence of bubbles below a certain depth (i.e., greater than a certain pressure) in Antarctic ice and the chemistry of the polar caps of Mars [5,6].

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ELECTROCHEMICAL DEVICES FOR *IN SITU* OR SIMULATED DEEP SEA MEASUREMENTS

by
A. Distèche,
Laboratory of General Biology,
University of Liège, Belgium

There are many electrochemical devices which can or could be used to control simulated marine conditions at normal pressure (1,2), but unfortunately only a few have been shown to operate when pressure is applied. Salinity sensors and oxygen probes are commercially available for deep-sea oceanographic work and can easily be fitted to pressure aquaria. They will not be described here. Some ion-selective electrodes have been tested at high pressure. Solid ion-exchangers (glass) sensitive to H^+, Na^+ give reliable results, and have been successfully used in several laboratories (3 to 11). Liquid ion-exchange electrodes sensitive to Ca^{2+} show poor response at high pressure (10). The cells used for these measurements necessarily include reference electrodes (Ag-AgCl, Calomel-Hg) the performances of which have to be tested under pressure. Their behaviour is of interest for physiological work since they are used in a variety of ways to pick up electrophysiological signals.

A. *Construction of high pressure ion selective electrode cells.*

a. *Liquid piston electrodes*

Glass electrodes can be used under pressure provided pressure is equally applied to both sides of the membrane. This can be achieved by using a light non-conducting fluid (silicone (3), n-heptane (10)) which acts as an insulating layer between the internal and external reference electrodes as shown schematically in Figure 76 for a cell to be used in the ocean.

Figure 77 shows the electrode system used by Distèche (8) on the French bathyscaphe *Archimède* for measurements down to 10.000 m. (a) represents the glass electrode with its internal Ag-AgCl electrode and (c) the Ag-AgCl outer reference electrode. They are attached to a Perspex holder (2)(3) which fits into a cylindrical sleeve (1). The upper compartment is filled with silicone-oil and communicates with the exterior (bore (4)) for pressure equalization. The under compartment is perforated to allow the passage of water and is fitted with a conical deflector to force water through the cell when it moves upwards. A steel cylinder, not shown, with openings and deflectors for the water flow protects the cell. A container (7) can be fitted for laboratory tests. The outer reference electrode is made of silver wire

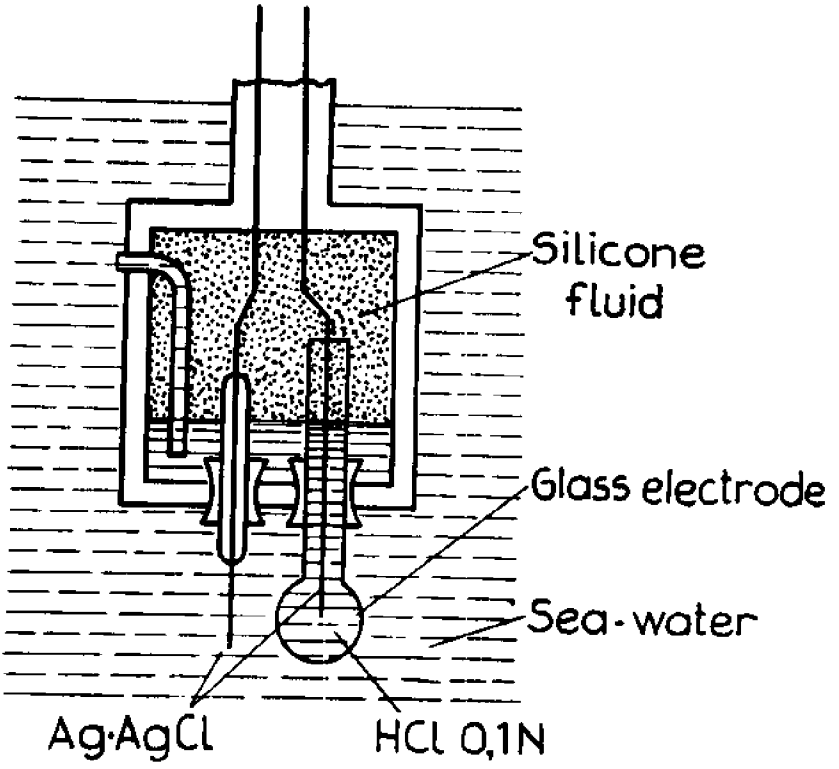

Figure 76. Schematic glass electrode assembly for measuring pH at great ocean depth (A. Disteche (3), by courtesy J. Electrochem. Soc.).

embedded in Araldite, protected by a sleeve with a porous tip, filled with sea-water. The gap between the halide and the metal is protected with silicone grease. The electrode is connected to a junction box (Fig. 78), attached to the submarine. The electrode can be fitted or removed under water without loss of insulation. The box is filled with silicone oil which is pressure equilibrated (bore (7)). The oil, because of its buoyancy, stays trapped in the box when the lid (8) is removed to push the electrode in. The female ends of the electrode leads are protected with "Parafilm" sleeves which are pierced when the electrode is screwed into position. The electrical connectors are machine-shop made and can easily be taken out for inspection and cleaning. Other types of connectors can be used. Magnesia insulated cables with copper sheating are available in various sizes and types (coaxial, multilead (3, 7, 12)) and one can also use plugs designed for oceanographic equipment. Such electrode systems could easily be adapted for work in high pressure aquaria, and deep-sea habitats. The oil box can then be omitted.

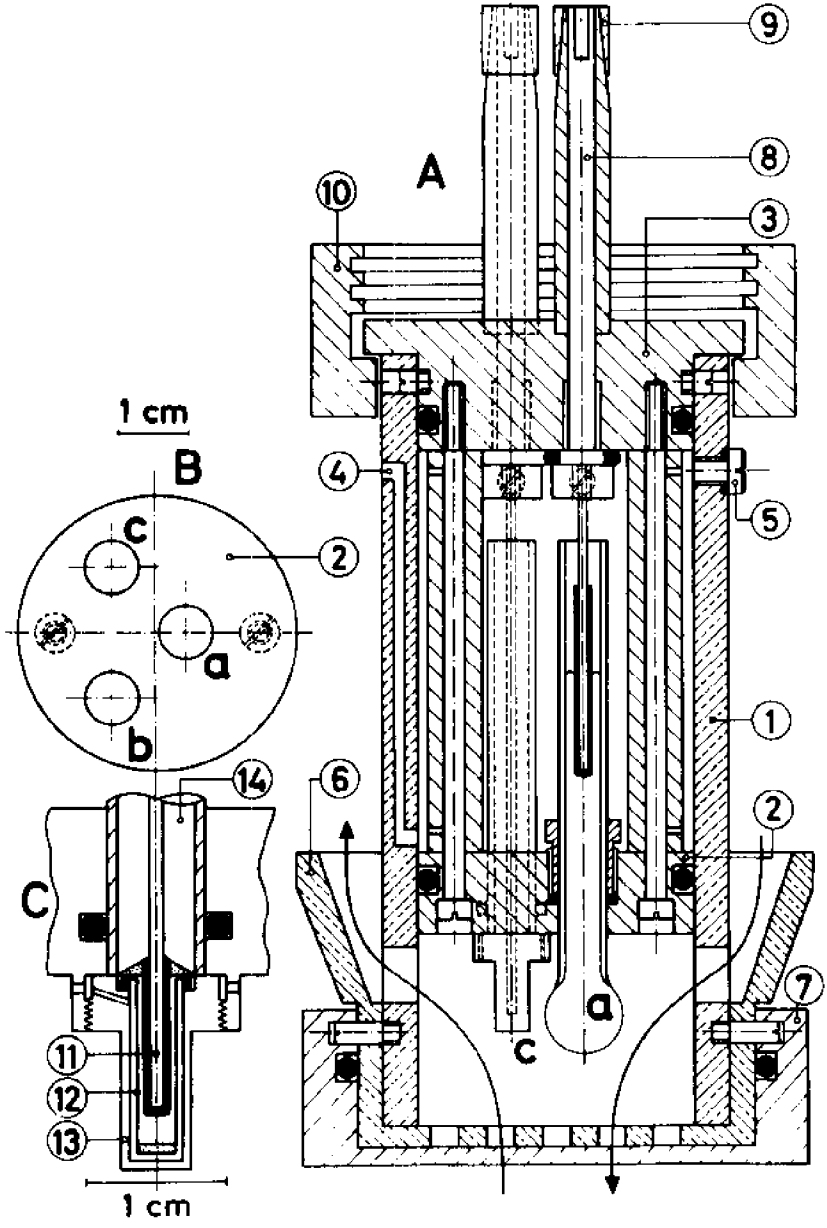

Figure 77. Deep ocean pH sensor (A. Disteche (8), by courtesy Bull. Inst. Oceanogr. Monaco).

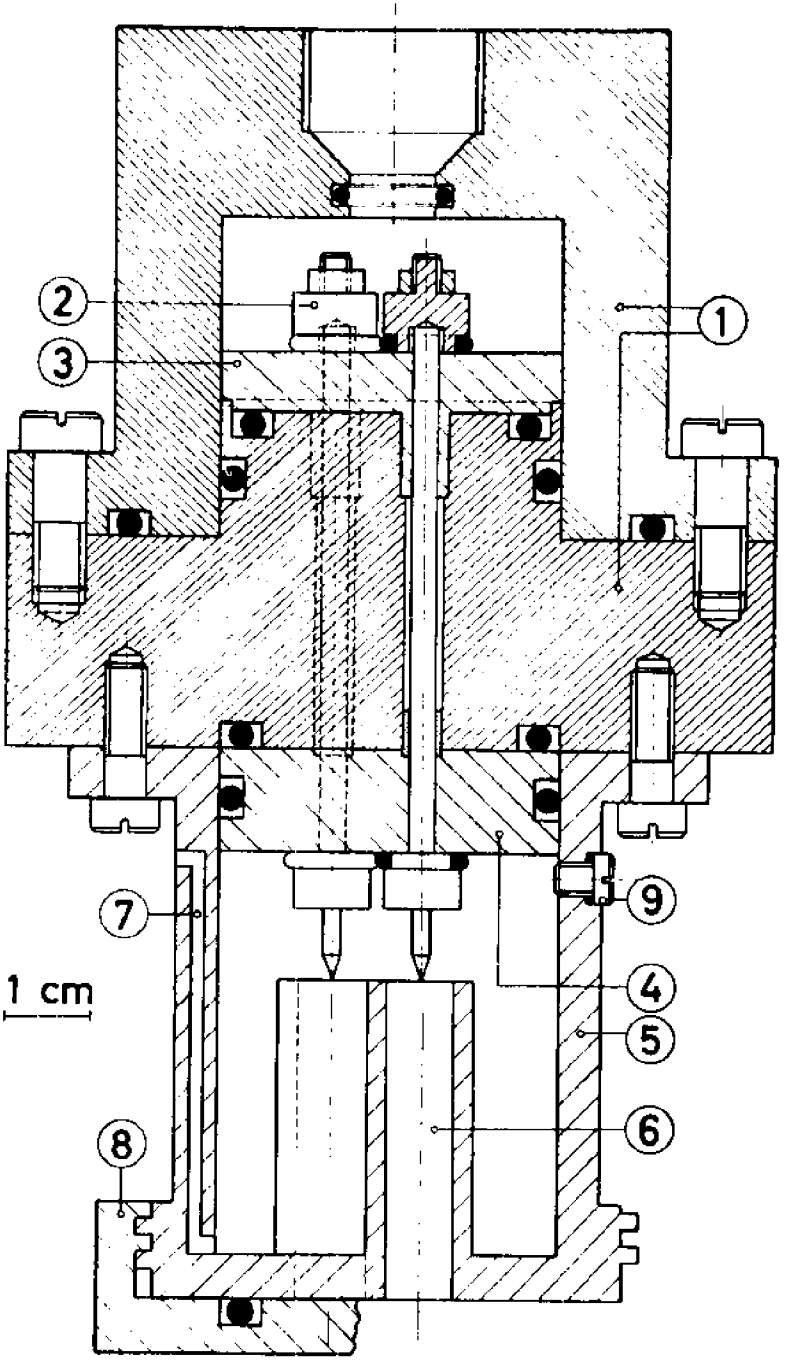

Figure 78. Silicone oil junction box for deep ocean pH sensor (A.Disteche (8), by courtesy Bull. Inst. Oceanogr. Monaco).

The laboratory version of the cell is shown in Figure 79. The outer Ag-AgCl electrode simply passes through the water-oil interface, eliminating the difficulty of the metal-halide gap.

The assembly is fitted in a pressure vessel (Fig. 80) filled with water and silicone oil. The steel stopper is of interest because it can be

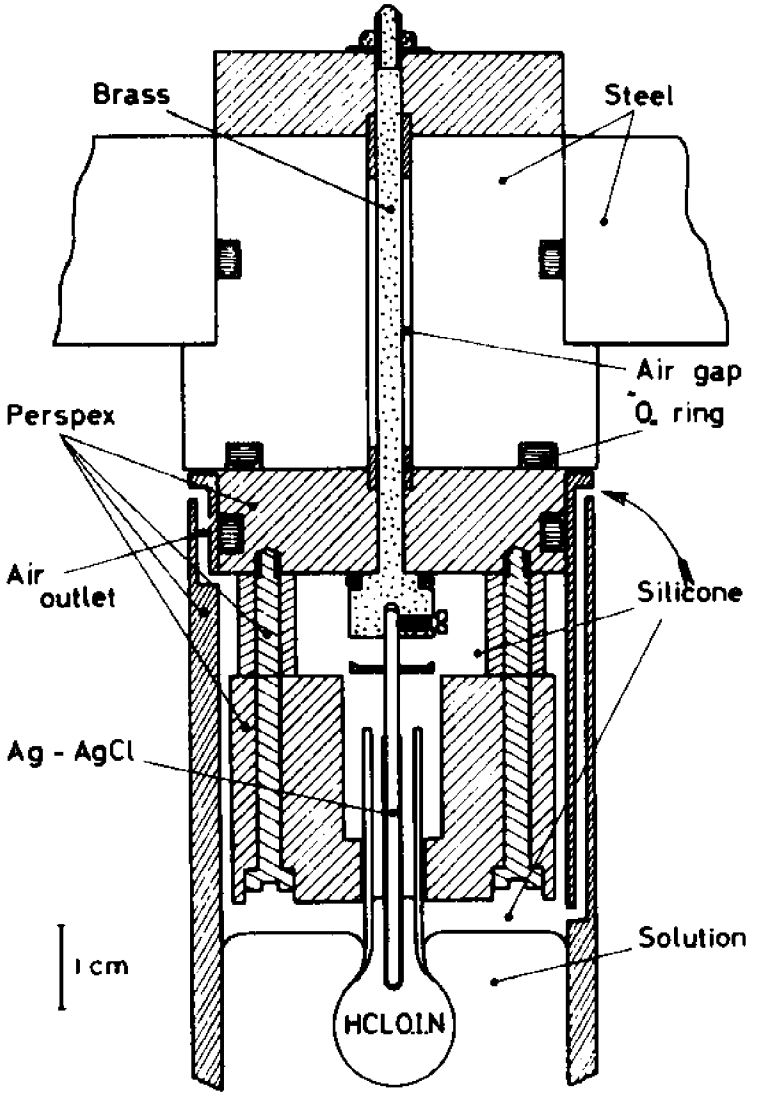

Figure 79. High-pressure glass electrode assembly for laboratory measurements (A. Disteche (5), by courtesy J. Electrochem. Soc.).

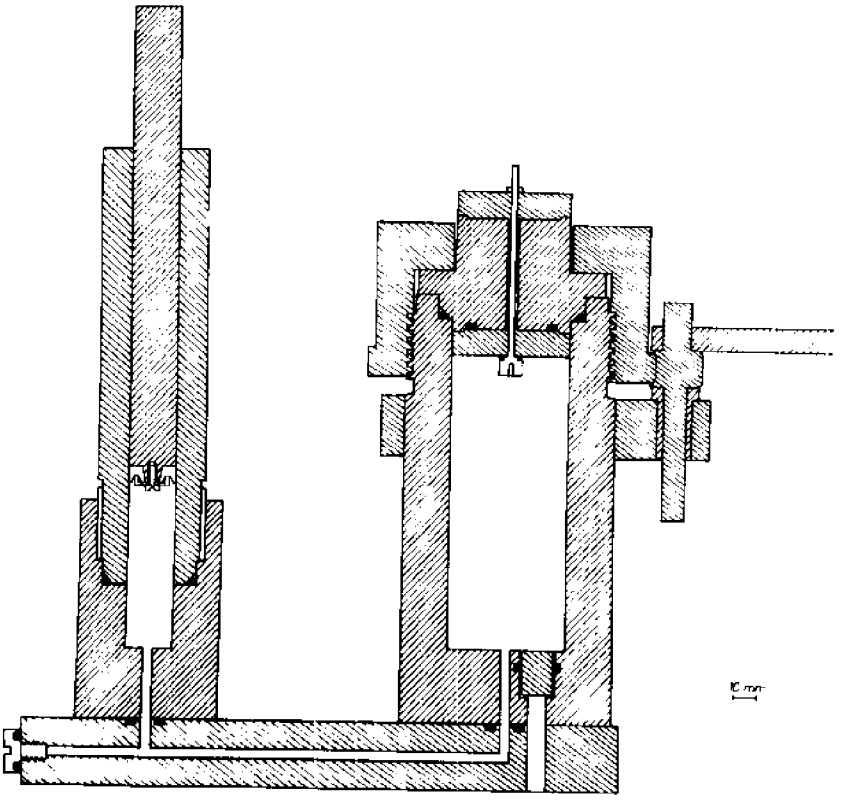

Figure 80. High pressure vessel for testing electrochemical devices.

removed very quickly and easily. The "O" ring seal is trapped without piston action. To avoid displacement under pressure the thread of the sealing lid is made square and the lid is screwed tight with the help of a gear and lever mechanism. More sophisticated laboratory cells have been designed by Whitfield (10). n-hexane is used as liquid piston and pressure is applied to the electrode compartment through a teflon plug or a mercury trap. Figure 81 shows the cell assembly for low pH measurements (Corning-015 glass), Figure 82 is a general purpose cell and Figure 83 a calomel-liquid ion-exchanger cell.

Figures 84 and 85 show a high pressure pH-sensor designed by Ben-Yaakov and Kaplan (11) for oceanographic application in the 300 m depth range. The reference electrode is filled with 2.7 M KCl compressed by a spring-loaded syringe to ensure an outflow of the KCl solution at the porous tip of the electrode. Ag-AgCl electrodes

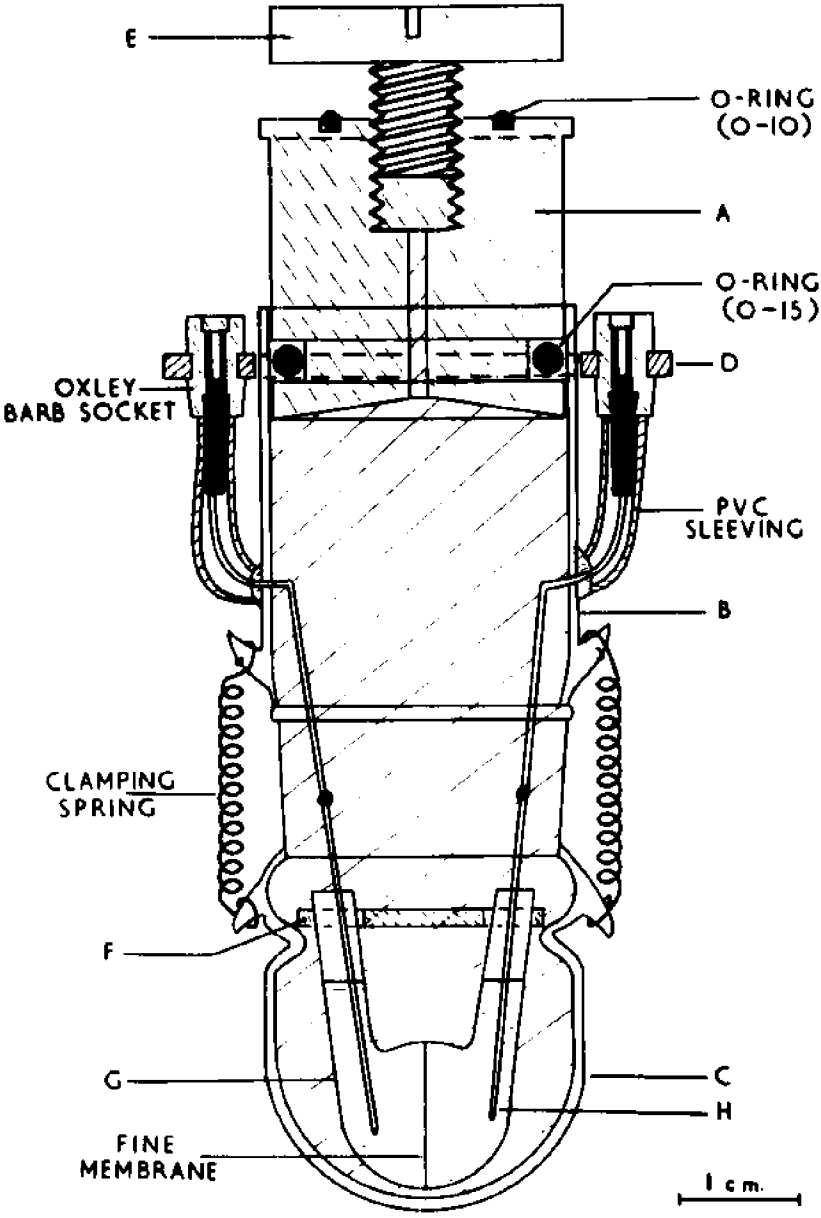

Figure 81. Low pH high pressure cell : A-gastight piston (Teflon), B-lead tube (Pyrex), C-electrode holder (Pyrex), D-fiberglas collar, E-bleed screw (Teflon), F-Teflon former, G-glass electrode (Mouquin-Garman), H-silver/silver chloride electrode. A second glass electrode is stacked alongside the one shown. After the electrodes have been placed in position, the whole cell is filled with n-heptane. (M. Whitfield (10), by courtesy J. Electrochem. Soc.).

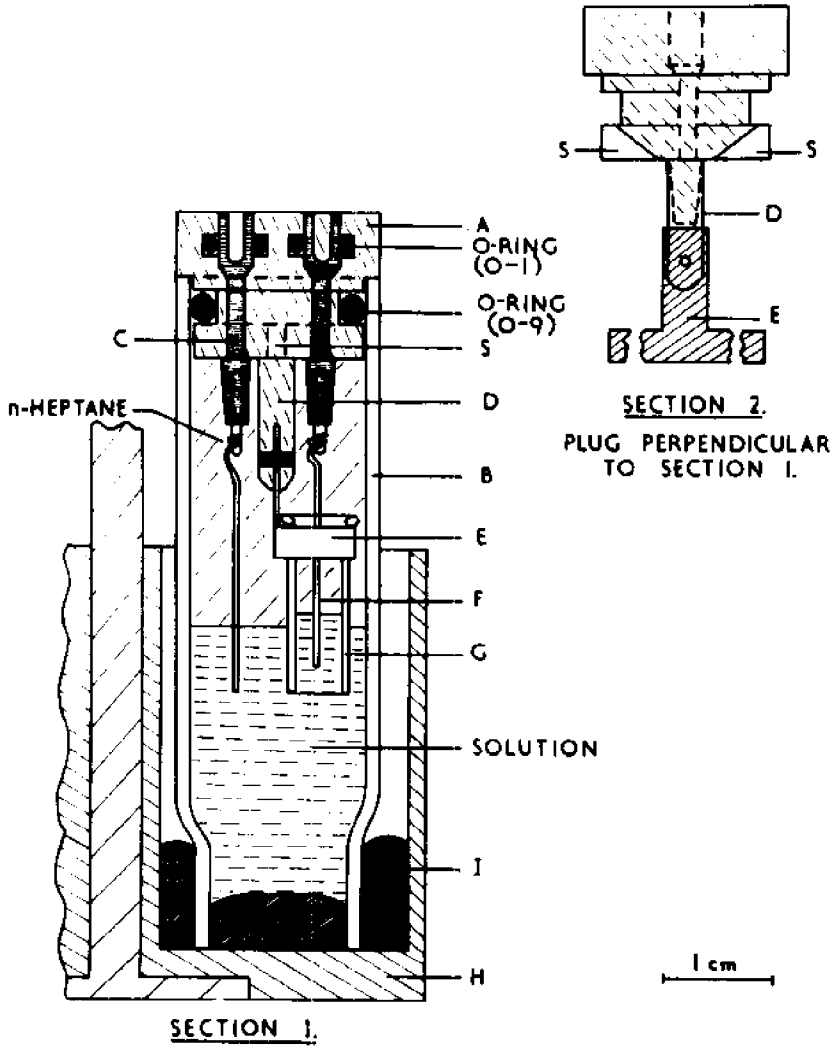

Figure 82. General purpose high pressure cell : A-Teflon plug, B-cell body (Pyrex), C-platinum contact, D-Teflon rod, E-platinum stirrup, F-silver/silver chloride electrode, G-glass electrode, H-holder, I-mercury trap, S-slots in piston. Redistilled silicone oil (MS 200/5 cs) may be used instead of n-heptane in the insulating layer. (M. Whitfield (10), by courtesy J. Electrochem. Soc.).

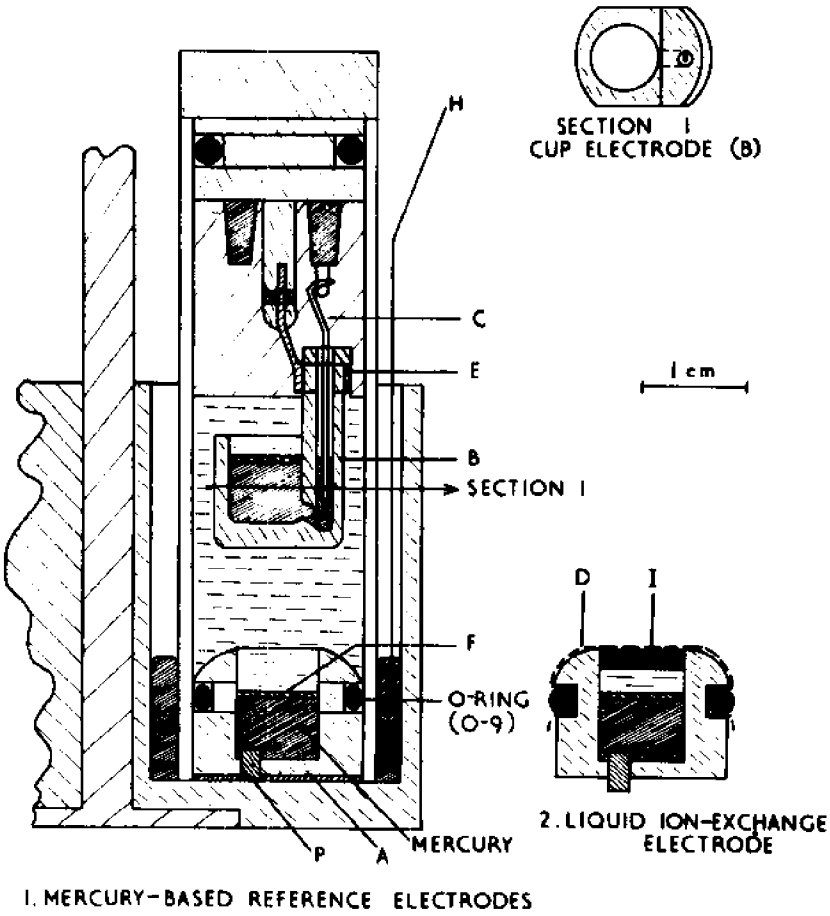

Figure 83. Calomel/liquid ion-exchange high pressure cell : 1. Mercury-based : A-combined electrode/piston (FEP polymer), B-cup electrode (FEP polymer), C-contact wire, E-platinum stirrup, F-calomel layer, P-platinum, H-contact to mercury reservoir. E. Liquid ion-exchanger D-sheet of dialysis membrane, I-ion-exchange oil (other components as in 1). (M. Whitfield (10), by courtesy J. Electrochem. Soc.).

are of the thermally-coated bead type. Pressure compensation is maintained by silicone oil and a rubber cap.

b. *Rubber piston electrodes*

The simplest glass electrode assembly is that of Culberson and Pytkowicz (9) (Fig. 86). Pressure is transmitted to both sides of the glass membrane by a rubber stopper. The Ag-AgCl electrodes are attached to cables terminated by Mecca-plugs and are insulated with silicone rubber. Silicone grease protects the halide-metal gap. The system has the advantage of simplicity, avoids eventual exchanges

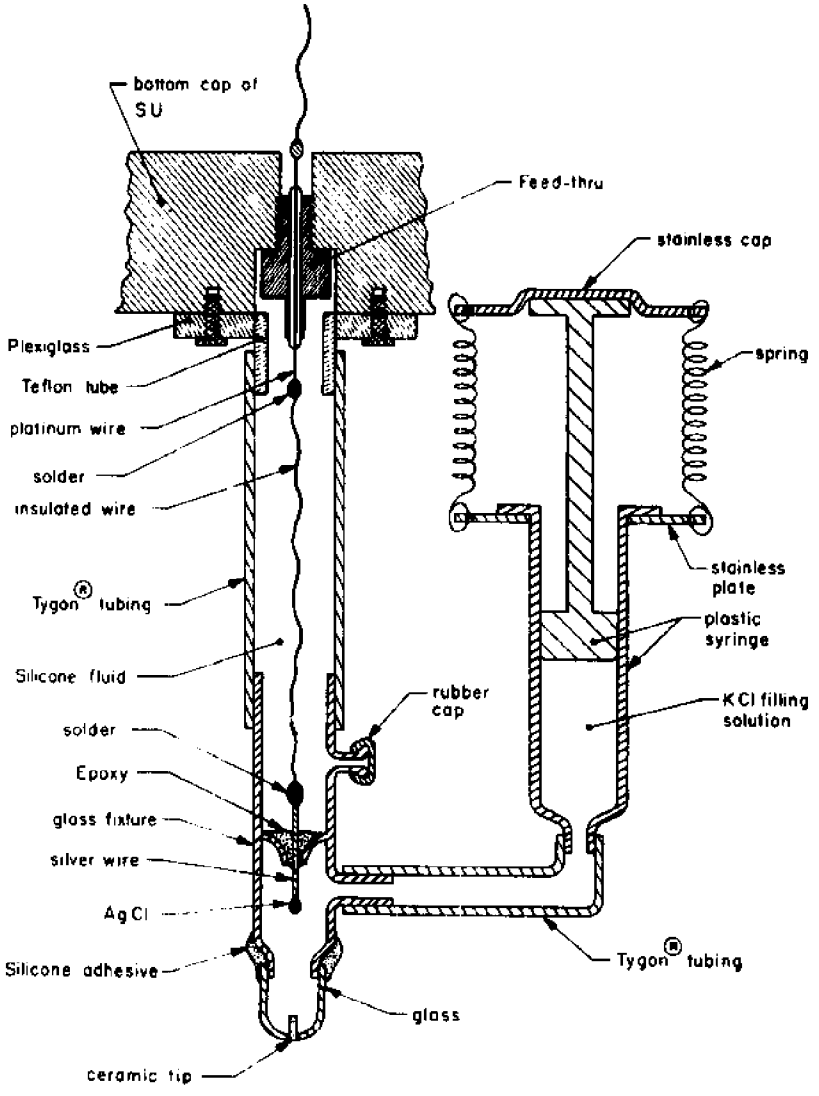

Figure 84. KCl liquid junction reference electrode for oceanographic applications. (S. Ben Yaakov and I. R. Kaplan (11), by courtesy Rev. Sci. Instruments).

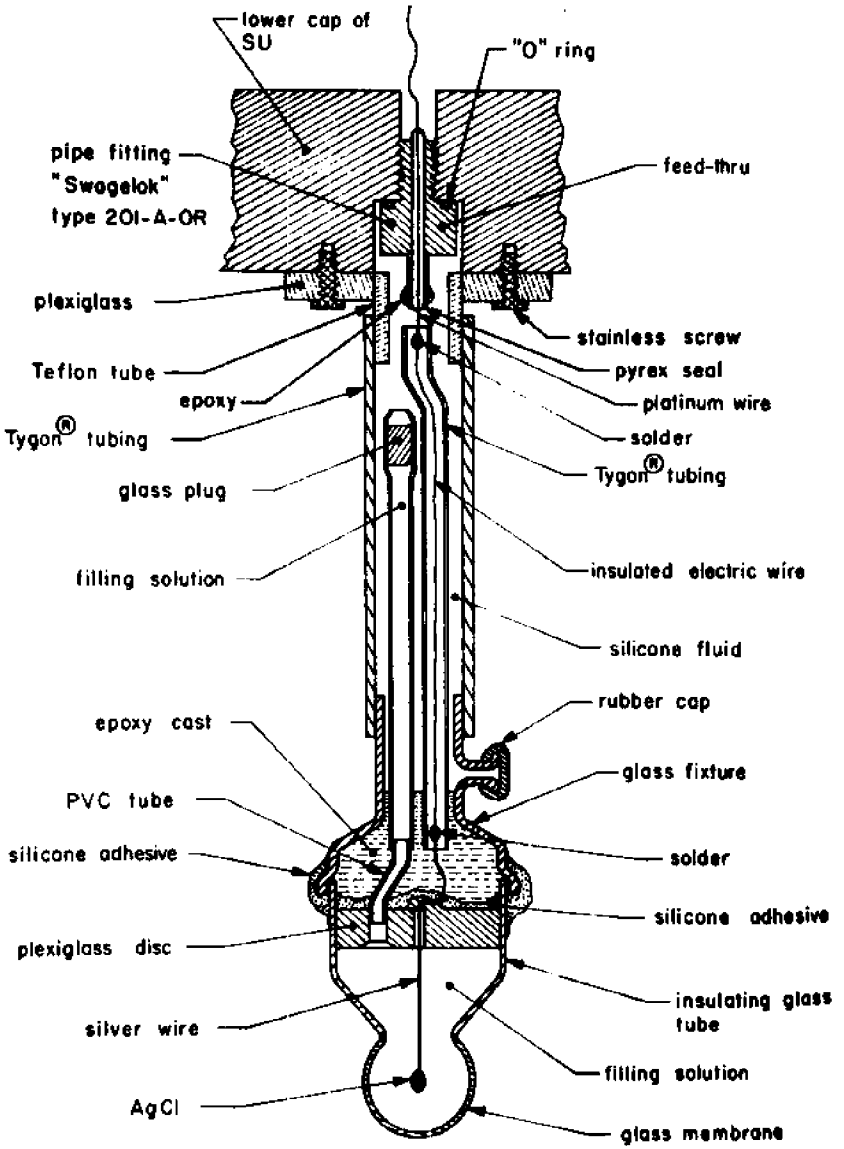

Figure 85. Glass electrode pH sensor for oceanographic work. (S. Ben-Yaakov and I.R. Kaplan (11), by courtesy Rev. Sci. Instruments).

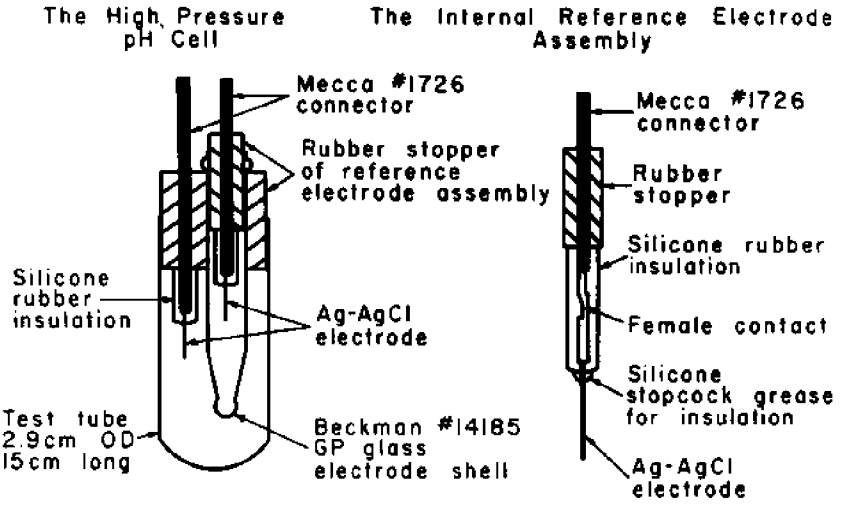

Figure 86. The Culberson-Pytkowicz high pressure pH cell. (C. Culberson and R. Pytkowicz (9), by courtesy Limnology and Oceanography).

between solution and silicone oil or n-heptane, although possible exchanges with rubber cannot be overlooked. The cell is cumbersome to use for general laboratory work when both inside and outside solutions of the cell have to be changed often. It can however easily be adapted to aquarium work, care being then taken to use sealed pigtail-ended connectors on the inside of the pressure vessel, to insure sufficient insulation.

c. *Glass membrane geometry, glass types, reference electrodes*

Extensive investigations by Whitfield (10, 13) have shown that flat membrane electrodes show the smallest asymmetry potential and hysteresis effects both at atmospheric pressure and at high pressures. Strains in the membrane seem to be the important factor, but surface leaching of alkali-oxides during manufacture, and hydration rate are to be taken into account. Asymmetry is reduced by aging in aqueous solutions and this process is more rapid, the thinner the membrane. Only Corning-015 glass can be supplied in quantities for workshop manufacture of electrodes. Large sized bulbs (1 or 1.5 cm ϕ) can easily be blown with relatively low electrical resistance and small asymmetry. They are well suited for oceanographic work (7, 8) where general insulation, and electrical noise pick-up are always very difficult problems. Corning-015 glass can be used up to pH 8.2 without alkaline correction in sea-water at high pressure (6). At pH 8.2 commercial high pH electrodes must be used, which can be reblown if a flat or thinner membrane is desired. Prolonged aging and alternating pressure runs generally reduce the asymmetry of commercial bulb electrodes

to a reasonable level, often less than 1mV for a 1000 bar pressure step. The special flat Mouquin-Garman membrane electrodes designed by Whitfield have asymmetries as low as 30 μV at 1 atm and 0.5 mV at 2000 bar. The remarkable degree of precision thus achieved is best illustrated by Figure 87 showing the effect of pressure on the emf of a cell containing carbonate-bicarbonate buffer in KCl.

Great care should be devoted to the preparation of the silver-halide electrodes (3, 9, 10).

The slightest scratch or uneveness in the coating makes the behaviour of the electrode erratic and contributes heavily to the asymmetry and hysteresis of the cell. If used in the open sea or in aquaria the outer electrode should be protected with a sleeve terminated with a porous pad. Fouling of glass and junction electrodes after prolonged use in seawater seems unavoidable but might perhaps be reduced by silicone coating of the glass. Ben Yaakov and Kaplan (11) use thermally coated bead type Ag-AgCl electrodes which they claim to be very stable in 2.7 M KCl. The asymmetry of their glass electrode and liquid junction cell assembly is however 0.6 mV at 150 bar, which is very high compared to the performances of the other cells reported in this paper. Calomel electrodes, at first sight promising, are sluggish in response and require about one hour to stabilize after a pressure step (3, 10). Obviously the reference electrodes are a weak link in high pressure cells.

B. *Some results from work with high pressure ion selective electrodes*

a. *Hydrogen ion electrodes*

Since the initial work of Distèche published in 1959 (3), the effect of pressure on the dissociation of many weak acids and corresponding buffers have been investigated using the junctionless glass-Ag-AgCl electrode system (3 to 6, 9, 10, 14).

Weak electrolytes dissociate under pressure because the ions occupy a smaller volume in the solution than the undissociated molecule. The volume change $-\Delta V^0$ at infinite dilution and pressure p is related to the shift of the dissociation constant K by the well known equation:

$$\left(\frac{\sigma \ln K}{\sigma p}\right)_{m,T} = \frac{-\Delta V^0}{RT}$$

The e.m.f. E of the cell at 1 atm. or at pressure p is given by

$$EF/2.3\,RT = \log m_H^{ref.} - \log m_H^{X} + 2 \log \gamma_{HCl}^{ref.} / \gamma_{HCl}$$

where X and ref. correspond to the two compartments of the cell containing equal Cl^- concentrations; m_H is the hydrogen ion concen-

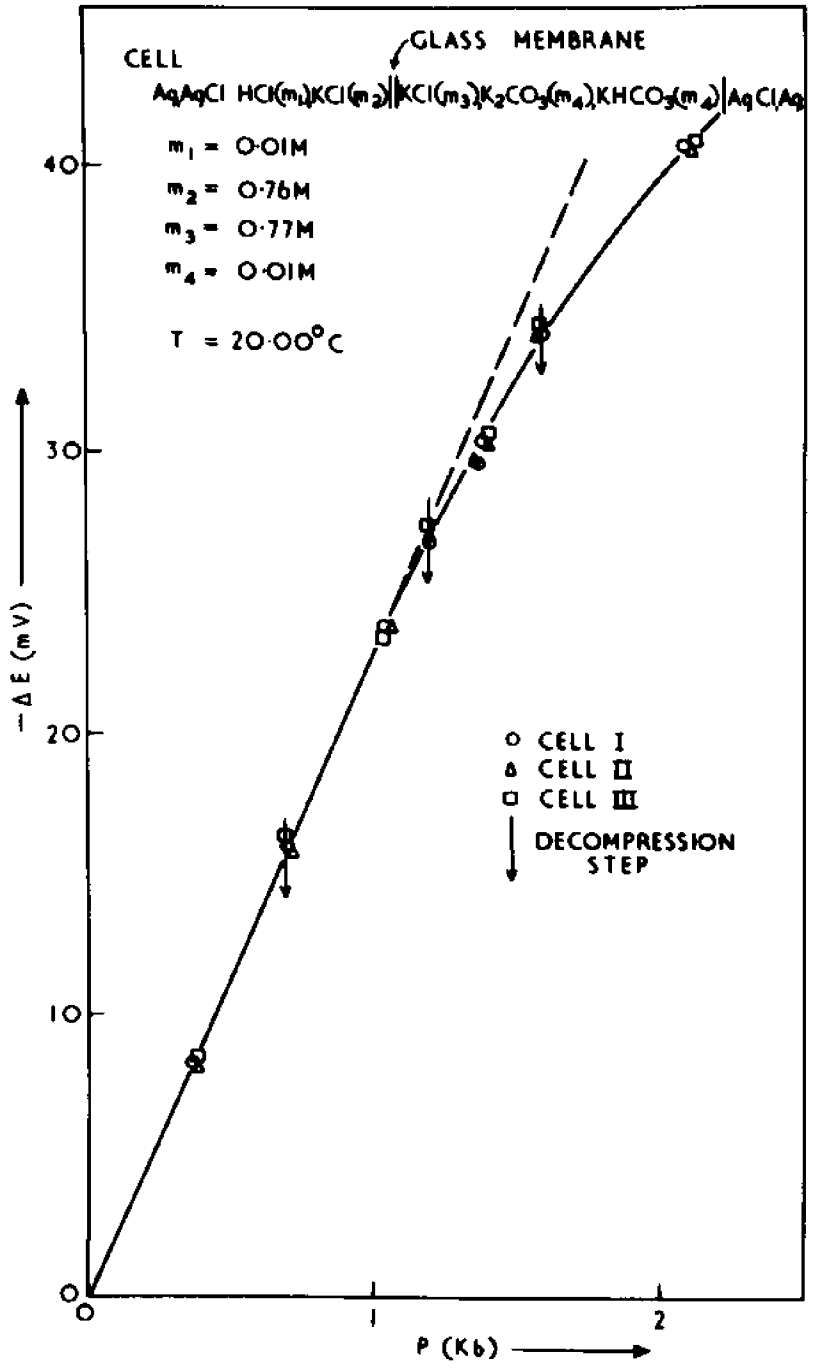

Figure 87. Reproducibility of pH measurements on a carbonate bicarbonate buffer in KCl. (M. Whitfield (10), by courtesy J. Electrochem. Soc.)

tration on the molal scale ($pmH = -\log m_H$) and γ HC1 the mean activity coefficient of HC1.

One will notice that these cells do not measure pH but $pwH = -\log m_H \gamma_H \gamma_{Cl}$.

It can be shown that in buffers where m_H is negligible compared to the buffer concentration, and if γ_{HCl} can be made to cancel in both cell compartments, that:

$$(E_I - E_p)\, F/2.3\, RT = \log Kp/K_I + 2 \log (\gamma A)_I/(\gamma A)_p = \log k'_p/k'_I$$

where γ_A is the mean activity coefficient of the weak acid HR considered and k' the ionization function $m_H \times m_R/m_{HR}$. Thus if both cell compartments contain the same ionic species at the same ionic strength (except for the buffer species usually replaced by HC1 in the ref. compartment) $\log k'_p/k'_I$ can be obtained at any pressure p as a function of the ionic strength. Extrapolation to zero ionic strength and zero buffer concentration is then possible and yields $-\Delta V^0$ at pressure p or 1 atm. If p is equal to 1000 $kgcm^{-2}$, $(E_I - E_p) \times 0.016$ corresponds to ΔV^0 in cm^3mole^{-1}, provided that $(E_I - E_p)$ varies linearly with pressure, which is usually the case.

We will now describe some results of importance to biologists and oceanographers. Figure 88 shows the effect of pressure on phosphate buffer (2^d step). Double extrapolation yields $\Delta V^0 = -24.0$ cm^3mole^{-1} (5). ATP gives the same results; phosphorylcreatine -21.2 cm^3mole (5). Figure 89 gives the effect of pressure on Tris-buffer (tris (hydroxymethyl) amino methane). The effect is extremely small, which can be explained by the fact that $K_{bh} = K_w/K_b$ (K_{bh} = acidic dissociation constant for reaction : $BH^+ + H_2O \rightleftharpoons H_3O^+ + B$; K_b = basic dissociation constant for reaction : $B + H_2O \rightleftharpoons BH^+ + OH^-$; K_w = dissociation constant of water). The effect of pressure on water, measured by Hamann (14) with a glass electrode, involves $\Delta V^0 = -20.4$ cm^3mole^{-1}. This is of the order of magnitude of what is to be expected for amines : for trimethylamine $\Delta V^0 = -28.1$ cm^3mole^{-1} from density measurements (15). The Tris-buffer at pH 8.44 has been studied in the range of the ionic strength of sea water, since pH-standards for sea water prepared with Tris-buffer instead of bicarbonate buffer are now available (16). The half-solid square is obtained in presence of 0.030 M SO_4^{2-}, no SO_4^{2-} ions being added to the reference compartment. It is obvious that the effect of this ion is merely an ionic-strength effect. Ca^{2+}, Mg^{2+} and SO_4^{2-} at concentrations found in sea water have a negligible medium effect as shown by the solid square. This buffer of special interest since it provides a buffer system in the physiological and oceanic range for

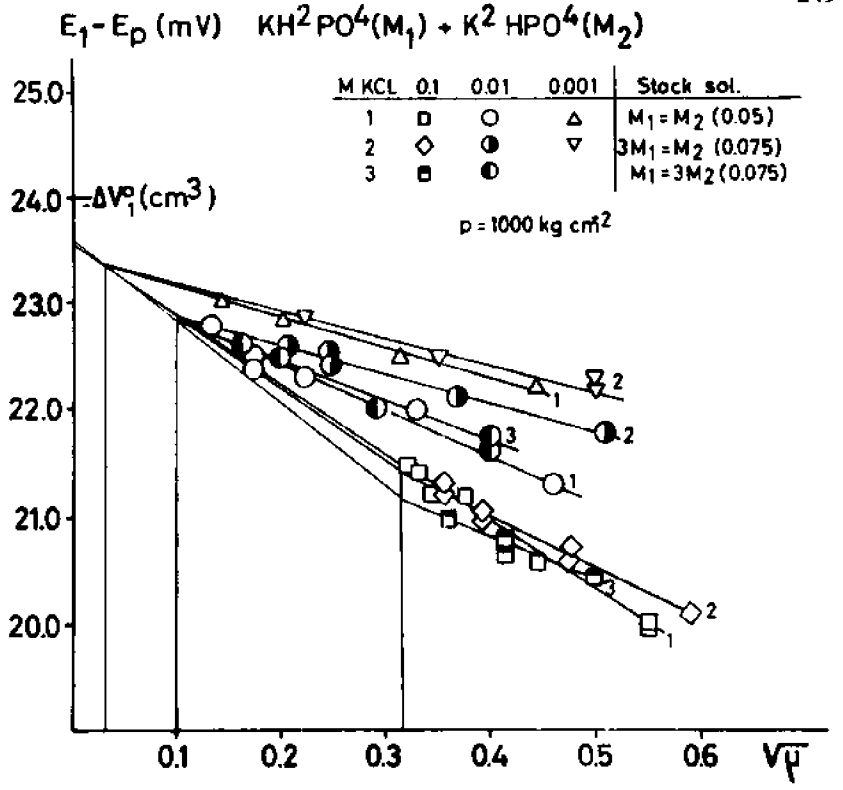

Figure 88. Effect of pressure on phosphate buffer ($K_{(2)}$) in 0.1, 0.01 and 0.001 M KCl. Extrapolation of $(E_1\text{-}E_p) = 2.3\ RT/F \log k'_p/k'_1$ to zero KCl and buffer concentration at three different buffer ratios: $-\Delta V_1 = 24.0$ cm^3 mole^{-1} (22°C). (A. Disteche (5), by courtesy J. Electrochem. Soc.).

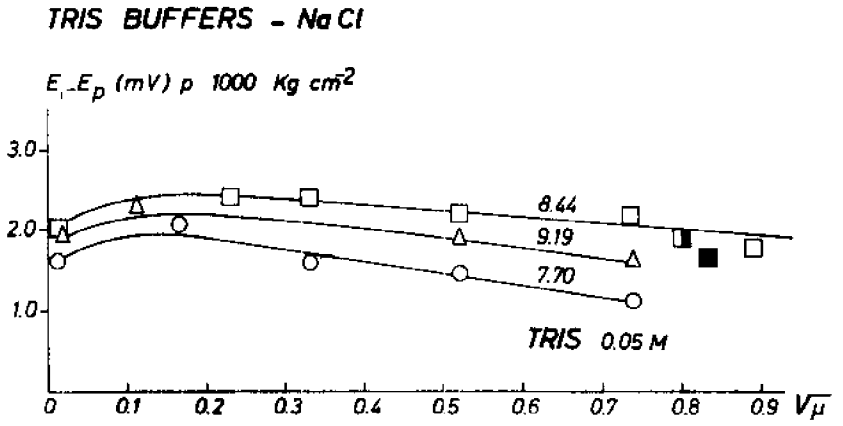

Figure 89. Effect of pressure on Tris-buffer; 0.030 ▮ M Na_2SO_4, ■ $CaCl_2$ 0.010 M, $MgCl_2$ 0.026 M, $MgSO_4$ 0.030 M. Ref. solution HCl 0.01 M NaCl and $CaCl_2$ 0.010 M, $MgCl_2$ 0.056 M when required (22°C).

which the effect of pressure on the pH is very small. It could be used in comparison with other media in biological work and provides, for pH determinations in seawater, a useful reference solution with identical ionic composition, except what regards the buffering species. The effect of temperature on the pH of Tris-buffer is however rather high : d pH/dt (pH units/degree) $= -0.028$ in the pH range 7-9 (17). Figure 90 shows the effect of pressure on bicarbonate buffer and the same type of results could be shown for carbonate buffer. Extrapolation in both cases yields $\Delta V^0 = -25.4$ cm^{3}mole^{-1} (6).

Figure 91 and Table I give a more detailed study of what happens at $\mu = 0.75$ in NaCl when the buffer ratio is modified in bicarbonate and carbonate buffers (total CO_2 : 0.0025 M). It includes results from earlier work (6) and more recent unpublished ones. Curve (4) shows the effect of pressure on the e.m.f. of the cell for pure bicarbonate and carbonate buffers in NaCl between pmH 5.0 to 9.5. The circled croses, curve (4), show the effect of adding 0.030 M Na_2SO_4 in the buffer compartment. Curve (2) gives the effect of 0.026 MMgCl_2, 0.010 M $CaCl_2$ and 0.030 M $MgSO_4$. The solid circles refer to measurements made with Tris-sea water in the reference compartment, the other symbols refer to measurements against 0.01 M HCl

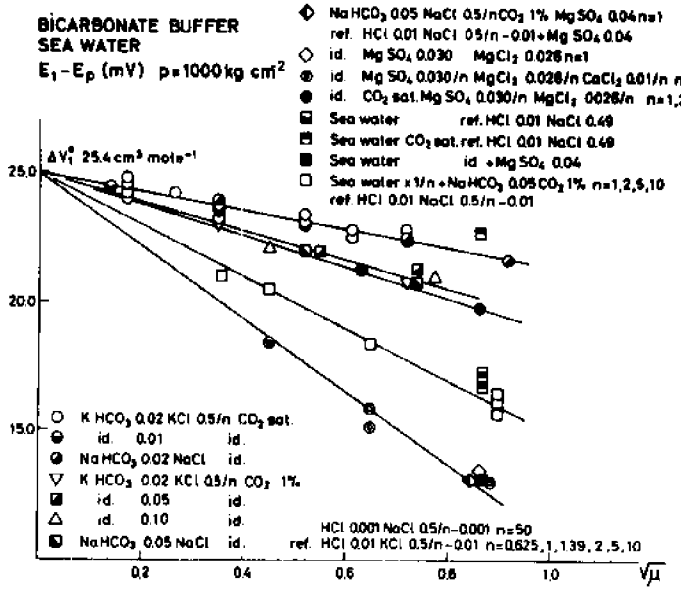

Figure 90. Effect of pressure on bicarbonate buffer (22°C), at different buffer ratios as a function of $\sqrt{\mu}$ in NaCl, KCl, in presence of Mg^{2+}, Ca^{2+}, SO_4^{2-} ions, and in sea water. (A. Disteche (6), by courtesy J. Electrochem. Soc.).

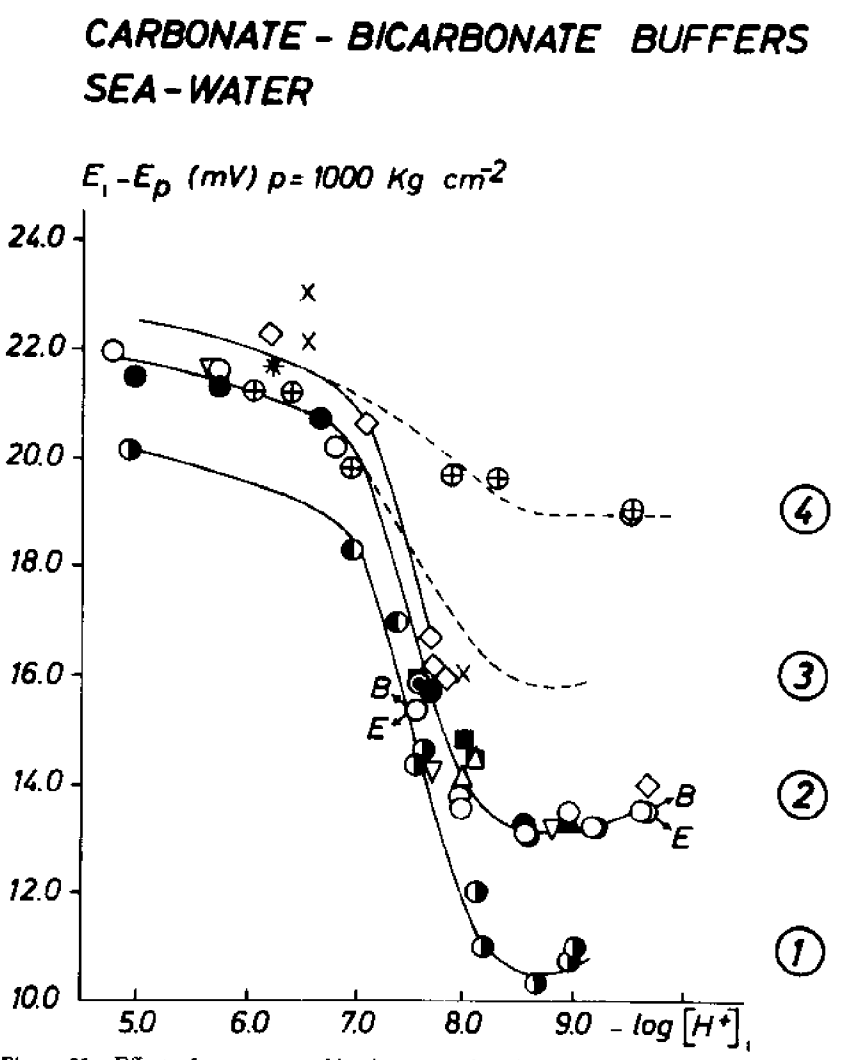

Figure 91. Effect of pressure on bicarbonate and carbonate buffers in NaCl and on artificial sea water at 22°C at μ 0.75 as a function of pmH_1 (see Table I).

plus the same salts as in sea water except SO_4^{2-} and the carbonate species. Different types of artificial sea water have been used (see Table I). Curve (1), from previous work (6), shows the shift resulting from the introduction of SO_4^{2-} in the HCl-compartment, resulting in the formation of HSO_4^- which dissociates under pressure. A rough correction for this unwanted effect was proposed, but the present results, especially those with Tris-buffer sea water as reference, are much more accurate. They show that SO_4^{2-} ions have only a small

Table I

Molar composition of the solutions used in the experiments reported in Figure 91

Curve Number	Symbol		HCl	NaCl	$MgSO_4$	$MgCl_2$	$CaCl_2$	KCl	$NaHCO_3$ Na_2CO_3	Na_2SO_4	Tris
2	Open circle	ref.	0.01	0.49		0.026	0.010				
		Buf.		0.50	0.030	0.026	0.010		0.0025		
	Solid circle	ref.	0.02	0.48	0.030	0.026	0.010				0.05
		Buf.		0.50	0.030	0.026	0.010		0.0025		
	Open triangle (point up)	ref.	0.01	0.49		0.056	0.010				
		Buf.		0.50	0.030	0.026	0.010		0.0025		
	Solid triangle (point up)	ref	0.01	0.71							
		Buf.		0.50	0.030	0.026	0.010		0.0025		
	Solid dot	ref.	0.01	0.4009		0.05299	0.01015	0.00911			
		Buf.		0.4606	0.02821	0.02478	0.01015	0.0091	0.002338		
	Solid square	ref.	0.01	0.4009		0.05299	0.01015	0.0911			
		Buf.		0.4109		0.05299	0.01015	0.00911	0.002338	0.02821	
	Star	ref.	0.01	0.71							
		Buf.	same as previous (prolonged equilibration)								
	Cross	ref.	0.01	0.71							
		Buf.	same as two previous (prolonged equilibration, Culberson and Pytkowicz (9))								
	Diamond		NaCl, $MgCl_2$ and buffer only (6)								
	Open triangle (point down)	ref.	0.01	0.49		0.056	0.010				
		Buf.		0.50		0.056	0.010		0.0025		
3			Boric acid effect (0.0043 M) on curve 2 (6)								
4			NaCl and buffer only (6)								
	Circled cross	ref.	0.01	0.65							
		Buf.		0.66					0.0025	0.030	
1		ref.	0.01	0.49	0.030	0.026	0.010		0.0025	(see (6))	
		Buf.		0.50	0.030	0.026	0.010		0.0025		

The E.m.f. values in Figure 91 are corrected at $\mu = 0.75$ (6)

medium effect on bicarbonate buffer in the acid range and none at all in the alkaline range. Curve (3) now indicates what happens when boric acid ($4.3\ 10^{-4}$M) is added to the artificial sea water, which then very closely simulates and pmH changes observed in natural sea water submitted to pressure.

The effect of Ca and Mg can be understood if one bears in mind that Ca and Mg form undissociated carbonate complexes at 1.0 atm. pressure at pH>7.08 (18). This is the reason why the apparent dissociation constant used by oceanographers pK'_2 is 9.12 instead of 9.60 as in NaCl-carbonate buffers of the same ionic strength. This apparent constant is defined as $K'_2 = a_H \times [CO_3^{2-}]_T/[H\ CO_3]_T$ where T means total concentrations (free and bound) and a_H the hydrogen ion activity. Under pressure the carbonate complexes dissociate, and the

freed CO_3 ions compensate the acidification due to the enhanced dissociation of HCO_3^- and H_2CO_3. The dissociation of boric acid, however, further acidfies the medium. The X in Figure 91 refer to measurements by Culberson and Pytkowicz (9). These authors use HCl 0.01 N + 0.71 NaCl as reference solution, which makes their results 1m V higher than ours; for they include in the emf shift, due to the dissociation constant change, the shift related to the effect of pressure on γHCl, not equal in both cell compartments. Even if one takes this into account, there still remains a difference of 1.0 mV between their results and ours, for which we have no explanation except the fact that the pressure runs made by Culberson and Pytkowicz take many hours and that we operate by pressure steps of 250 kg cm^{-2} lasting about 30 min each. We estimate that this is the time required for temperature and emf stabilization (3). We found that prolonged equilibration often results in unexplained irreversible changes which become obvious when pressure is released and which depend on the type of glass electrode used.

Table II

Shift of true ($pK'_{(1)}$, $pK'_{(2)}$ and apparent ($pK''_{(2)}$ ionization functions at 1 K bar (Δ 1 K bar) of carbonic acid in sea water

22°C	$pK'_{(1)}$	$pK'_{(2)}$	$pK''_{(2)}$	
1 atm.	6.00	9.60	9.12(*)	
Δ 1 K bar	0.350 (at pmH_1 7.0)	0.330	0.230	A. Distèche, this paper
	0.365 (at pmH_1 7.0)	0.328	0.226(*)	A. Distèche and S. Distèche (6)
	0.365 (at pmH_1 7.0)	—	0.245	C. Culberson and R. Pytkowicz (9)
	0.375 (at pmH_1 6.5)	—		
	0.48	—	0.18	K. Buch and S. Gripenberg (19)

(*) corrected for unwanted $S^2O_4^{=}$ ion effect in ref. cell, see (6).

From the curves of Figure 89 it is possible to calculate (Table II) the shifts of the following ionization functions at 1 K bar : $pK'_{(1)}$, $pK'_{(2)}$ and $pK''_{(2)}$ (the last one being the apparent ionization function relating m_H and the total $CO_3^{=}$ and HCO^{3-} concentrations, see (6)).

These pK shifts are calculated from the well known equation and other related relations :

$\Sigma CO_2/C.A. = (1 + K_{(2)}/[H^+] = [H^+] K_{(1)}/(1 + 2K_{(2)}/[H^+])$ assumed to be valid for $K'_{(1)}$, $K'_{(2)}$ and $K''_{(2)}$, and which reduces in bicarbonate solution to $[H^+]^2 = K_1K_2$, when $\Sigma CO_2 = C.A.$ (C.A. = $[HCO_3^-] + 2 [CO_3^{2-}]$ = carbonate alkalinity).

At 1/2 $(pK'_{(1)} + pK''_{(2)})_1 = 7.56$, it can be checked at 1000 kg cm^{-2}, that 1/2 $(pK'_{(1)} + pK''_{(2)})_{1000} = 7.273$. The difference of 0.287 fits with the observed value : 0.288 (16.8 mV). Besides $\Delta pK''_{(2)} = 0.230$ is constant within ± 0.002 in the pmH interval 7.56 — 9.0, taking into account the reaction $2 HCO_3^- \rightleftharpoons H_2CO_3 + CO_3^{2-}$. All this shows that the data fit the above equation between pmH 7.0 and 9.0. If however one takes ΔpmH_{1000} observed at 6.5 for $\Delta pK'_{(1)}$, as Culberson and Pytkowicz do, the value of $\Delta pK''_{(2)}$ calculated at pmH_1 7.56 increases to 0.253, but keeps the right value at $pmH_1 > 8.0$. It is obvious that the condition $[H^+]^2 = K_{(1)} K_{(2)}$ in bicarbonate solutions with a value imposed by the dissociation constants at 1 atm, requires the curve giving the pmH pressure-induced shifts in function of pmH_1 to be symmetric with respect to the value obtained at pmH_1 7.56. This is the reason why we take pmH_1 7.0 instead of any other to calculate $\Delta pK_{(1)}$, from the pmH shift induced by pressure. This also means that the equation $\Sigma CO^2/C.A. = f(K_{(1)}, K_{(2)} [H^+])$ does not represent what happens at $pmH_1 < 7.0$ where the Δ pmH curve should reach a plateau much more quickly than it does, if the equation were valid. Therefore some arbitrariness remains in these calculations which to our present stage of knowledge cannot be removed. One way out will probably be to include in the calculations the effect of HCO_3^- and CO_3^{2-} trapping by Na^+ ions.

However the actual estimates are certainly much more accurate than the early values of Buch and Gripenberg and we now begin to fully understand what happens in sea water when pressure is applied. Culberson and Pytkowicz (9) have obtained the temperature dependance of the shifts induced by pressure between 22C and 2C in natural sea water:

$$\Delta pK'_{(1)2C} = 1.154\ \Delta pK'_{(1)22C},$$
$$\Delta pK''_{(2)2C} = 1.142\ \Delta pK''_{(2)22C};$$
$$\Delta pH_{8.6,\ 2C} = 1.16\ \Delta pH_{22C},$$
$$\Delta pH_{7.6,\ 2C} = 1.18\ \Delta pH_{22C}$$

If the temperature change were also known for the true ionization function $pK'_{(2)}$ which relates the concentrations of H^+, CO_3^{2-} and HCO_3^- free plus bound to Na, it would be possible to calculate at all pressures and temperatures, with reasonable accuracy the CO_3^{2-} bound to Mg and Ca, the CO_3^{2-} and HCO_3^- free plus bound to Na, the CO_2 concentration, from one pH measurement and the total CO_2 content of the water.

Pytkowicz and coworkers (20 to 25) made successful use of their data to reevaluate the carbonate cycle in the ocean and to determine the solubility of various types of $CaCO_3$ (aragonite, foraminifera, calcite, oolites). They use the glass electrode as a saturometer, calculating the amount of CO_3^{2-} released from the samples after each pressure step. Their results will not be discussed here; they lead to a much better understanding and evaluation of the saturation and compensation depths for carbonates in the ocean.

Distèche and Dubuisson (7, 8) used the glass electrode cell to determine, from the French Bathyscaphes FNRS III and *Archimède*, pH profiles in situ in the Mediterranean sea (Fig. 92). More recently, partial profiles have been recorded from the *Archimède*, by a French team, in the Pacific Ocean, in the Japan Trench (Fig. 93). The slope between 5000 and 8,800 meters corresponds to the expected effect of pressure on sea water of constant composition. Figures 94 and 95 correspond to pH-profiles obtained between the surface and 280 m in Southern California by Ben-Yaakov and Kaplan (11).

b. *Na^+ and Ca^{2+} ions electrodes*

Figure 96 shows the excellent response obtained by Whitfield (10) with a Na-sensitive glass electrode. The slope of 120 mV for each 10-fold change in activity for 1-1 electrolyte is not affected by pressure. With a liquid ion-exchanger electrode sensitive to Ca^{2+} ions, the slope at 1 atm. is 90 mV as required for a 2-1 electrolyte, but the response to 500 and 1000 bar steps is but poor.

c. *CO_2-sensitive electrodes*

Preliminary experiments have been undertaken in our laboratory with a flat membrane hydrogen glass electrode (MacInnes type) in contact with a thin film of bicarbonate solution separated from the surrounding fluid by a Teflon membrane permeable to CO_2. So far poor results have been obtained. The amount of fluid under the electrode is so reduced that the possibility of pressure sensitive ion exchanges with the membrane, and even with the Perspex electrode holder cannot be excluded. If such an electrode could be made reliable it would, coupled with a normal hydrogen ion electrode, give a unique cell from

which the complete equilibrium of carbonic acid could be calculated without the need for any chemical analysis.

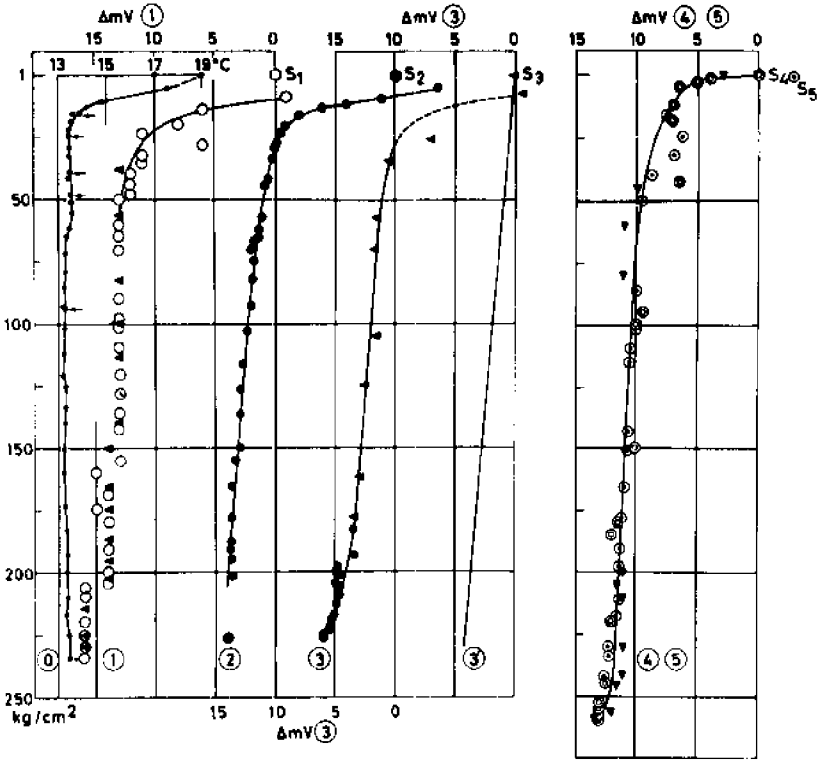

Figure 92. pH profiles in the Mediterranean Sea (French Bathyscaphes FNRS III and Archimede) (Δ pH in mV, uncorrected for temperature changes; 3′ shows the effect of pressure on water of constant composition; S_1, S_2, S_3, S_4 surface values: pH 8.12-8.17). (A. Disteche and M. Dubuisson (7,8), by courtesy Bull. Inst. Oceanogr. Monaco).

C. *Cells for living membrane potential determinations at high pressures*

Figure 97 shows a cell for measuring the potential between the outer and inner faces of frog or fish skin (26). The skin is fitted in a window which separates two compartments in which the potential is measured with two Ag-AgCl electrodes. The upper part of the cell is filled with silicone oil, and the whole assembly is introduced in the pressure cylinder described earlier. Brouha, Pequeux, Schoffeniels and Distèche have shown that a pressure step of 100 kg cm^{-2} on frog skin produces a short depolarization followed by an important hyperpolarization (Fig. 98). The effect of pressure is reversible. Using different bathing fluids and drugs affecting active transport of Na or the

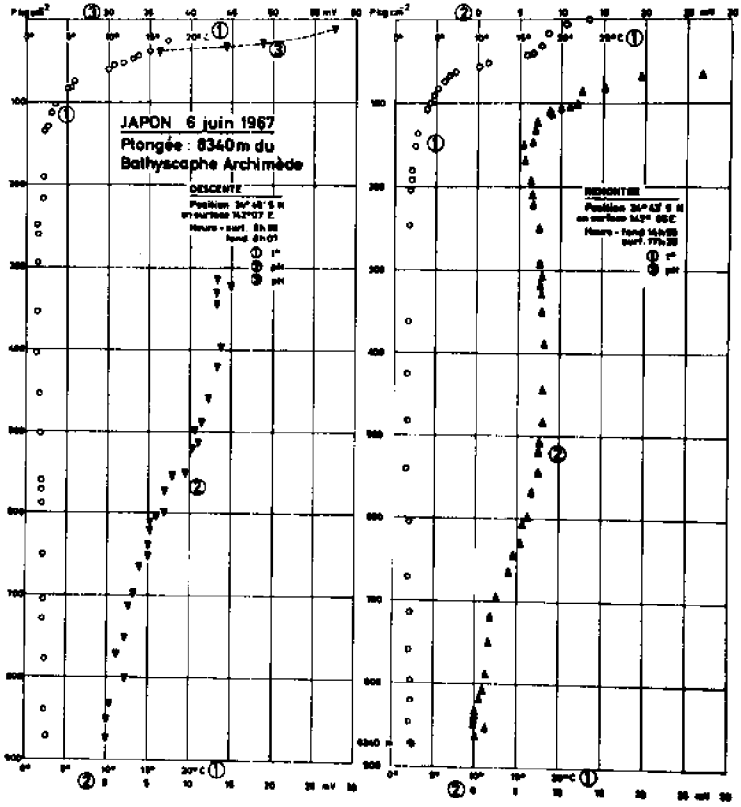

Figure 93. pH profiles in the Japan Trench (French Bathyscaphe Archimede (Δ pH in mV, uncorrected for temperature changes; arbitrary zero on the bottom).

permeability of this ion, it is possible to show that pressure enhances selectively the permeability to Na and has no effect on K, which means different permeability mechanisms for these two ions. Unpublished results (Pequeux) indicate that Mg-activated ATPase extracted from gills, kidneys of frog and teleosts is more active under pressure (60% increase at 250 kg cm^{-2}) but Na- and K-activated ATPase seems partially inhibited, and we try to link these observations with the pressure response observed on the skin. Pressure affects the ionization pattern of proteins and complexes like those formed by Mg and ATP.

Fish skins show a very small response to pressure, and this could be explained by the fact that Cl^- ions short-circuit the membrane potential due to other ions. If $SO_4^=$ ions are substituted to Cl^- ions the effect described for frog skin is also observed on fish skins.

D. *Conclusions*

The array of electrochemical devices available for experiments in high pressure aquaria or the ocean is but poor, and a great deal of work

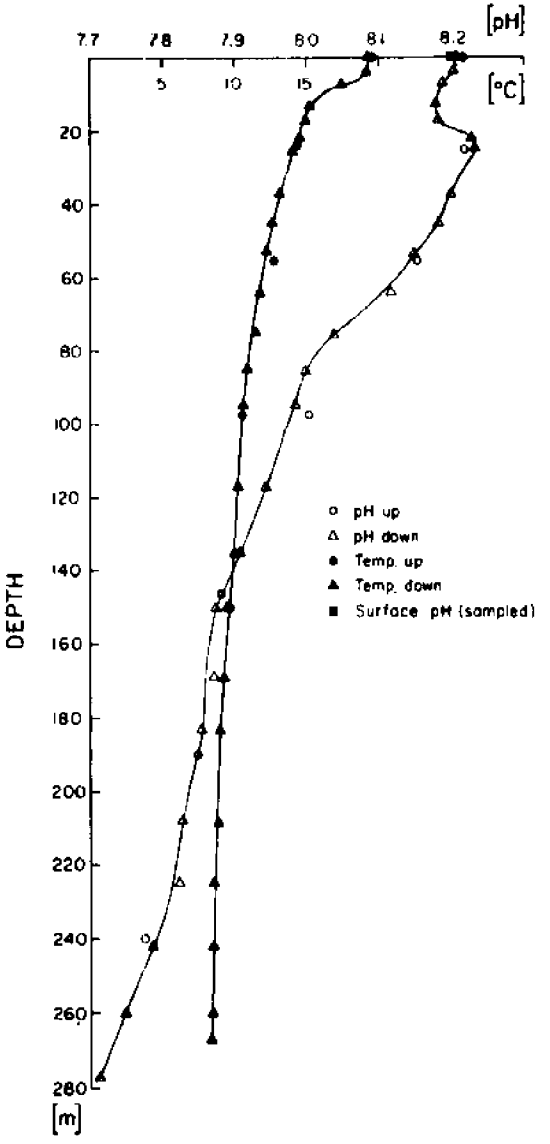

Figure 94. Temperature and pH versus depth (San Pedro Basin, 3.5 km from shore off Southern California). (S. Ben-Yaakov and I. R. Kaplan (11), by courtesy Rev. Sci. Instruments).

has to be done to increase their number and their reliability. Excellent results have been obtained with hydrogen glass electrodes and Na-electrodes, and probably any other type of ion-sensitive glass will be satisfactory. The problem of reference electrodes is solved, but Ag-AgCl electrodes appear as the fragile link in the system. Their

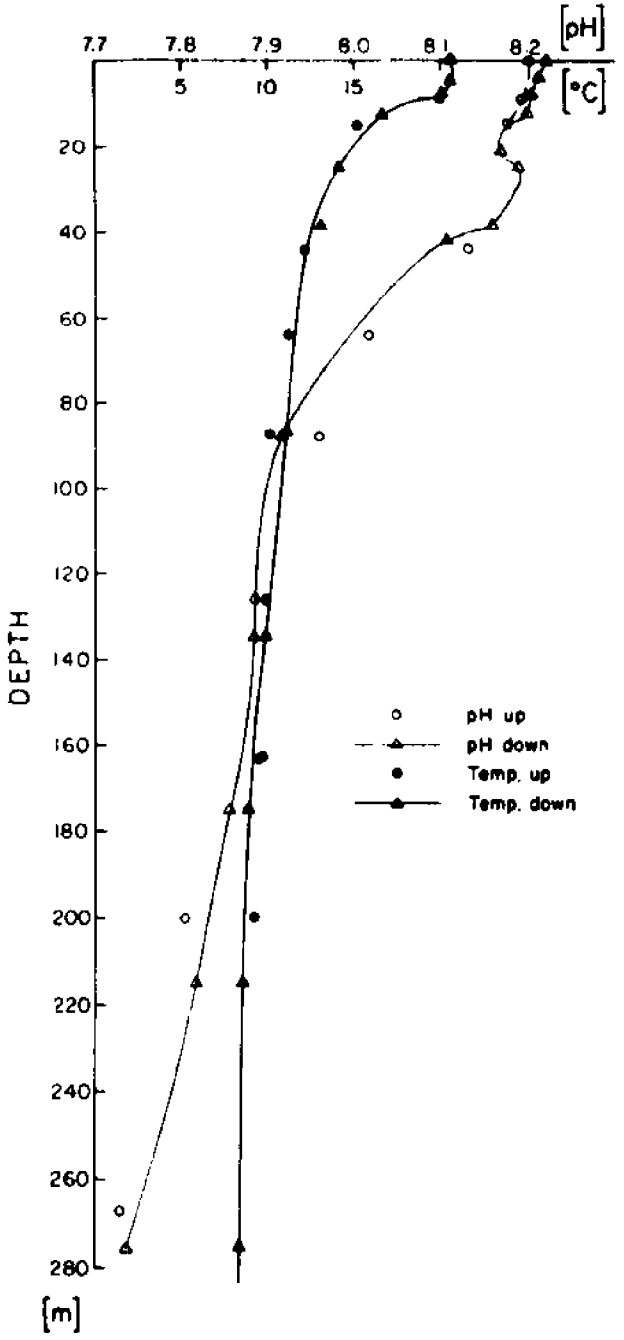

Figure 95. Temperature and pH versus depth (San Pedro Basin, 8.6 km from shore off Southern California). (S. Ben-Yaakov and I. R. Kaplan (11), by courtesy Rev. Sci. Instruments).

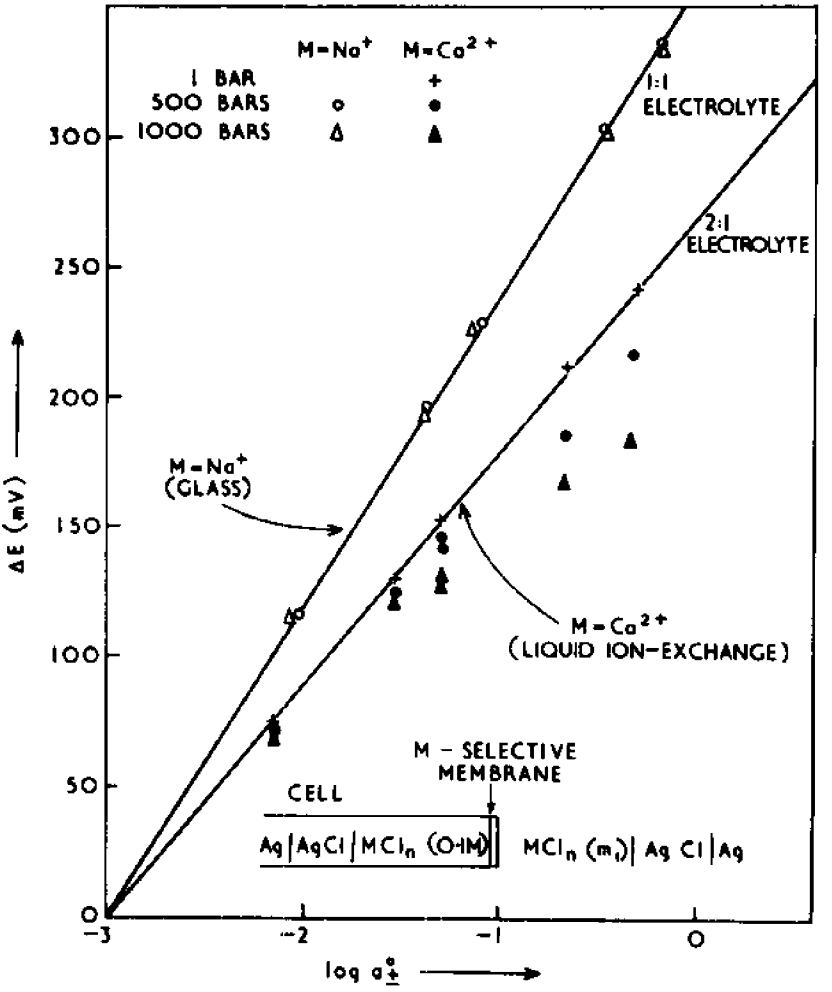

Figure 96. Response of cation selective electrodes as a function of pressure at 25°C. Solid lines indicate theoretical slope. (M. Whitfield (10), by courtesy J. Electrochem. Soc.).

response under pressure, in good conditions, is however such that one can expect microelectrodes commonly used in electrophysiology to behave properly under pressure.

Measurements of pH at high pressures have made it possible to unravel the very complex pressure induced effects on the main ionic equilibria in sea water : acidification due to the enhanced dissociation of carbonic acid and boric acid is partly compensated by complex Mg and Ca-carbonate ion pair dissociation. Similar problems will certainly be encountered in body fluids. Tris-buffer shows how the dissociation of water can, under pressure, compensate the dissociation of amino groups.

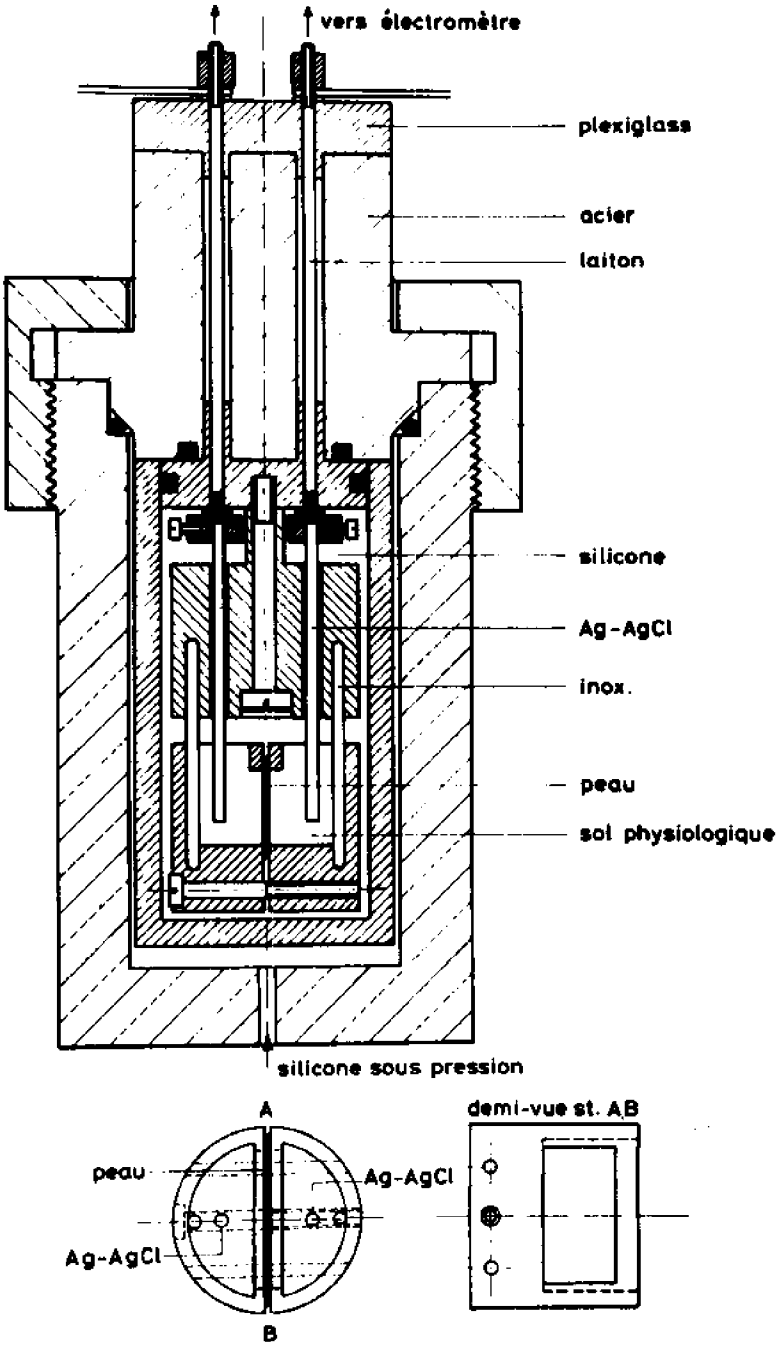

Figure 97. Cell for measuring the potential across fish or frog skin. (A. Brouha, A. Pequeux, E. Schoffeniels and A. Disteche (26), by courtesy Biochim. Biophys. Acta).

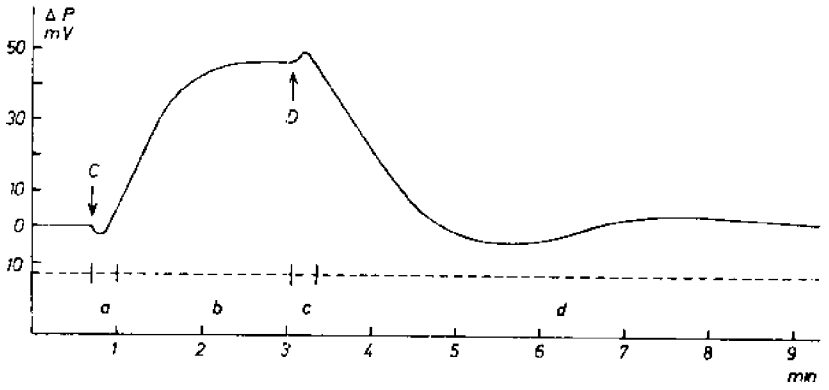

Figure 98. Effect of pressure on the potential of frog skin. (A. Brouha, A. Pequeux, E. Schoffeniels and A. Disteche (26). by courtesy Biochim. Biophys. Acta).

Similiar effects will be met with on proteins. Simple physiological experiments on frog and fish skin potentials show pressure to have quite different effects, fish skin being rather insensitive because heavy shunting of Cl^- ions. These few examples roughly indicate some possible mechanisms to explain why certain types of animals withstand high pressure without damage.

Glass electrodes already suitable for monitoring high pressure aquaria and to obtain pH-profiles in the ocean, might be tried further in a number of interesting experiments.

A phytoplankon suspension introduced in a glass electrode will produce or remove CO_2, and this can be recorded by the electrode if it is illuminated periodically. Electrodes in contact with bacteria or located in small chambers containing benthic species might be used to measure metabolism. Changes in pH at interfaces between mud and water or in sediments could be recorded in smiulated and controlled environments before being tried from a manned vehicle. Electrodes stacked at intervals could detect pH-gradients. Divers operating from underwater habitats and equipped with portable pH-meters might collect a great deal of information regarding microclimate and marine life, and perhaps it will be possible to carry out experiments in high pressure aquaria to refine their observations.

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Discussion Following Distèche Paper

LOWENSTAM: I have been wondering, in your buffering experiments, have you checked silica as to buffering?
DISTÈCHE: I have not, but tried to add silicate ions to sea water and there were some differences, but no one could interpret them. One of the reasons is nobody could find exactly what the silicon looked like from the phase rule

point of view and what changes there would be when pressure is applied. Apparently, there is a small change. When you make up sea water you get the type of change I showed you. If you use natural sea water, there is always a slight movement in a very fast interval, and you don't ordinarily get any idea of why it is like that. So a final interpretation has to rely on very precise knowledge of the chemistry to know what's in sea water.

LOWENSTAM: I feel that in order to determine whether or not Sillen's basic idea is any good, one that should at least try to avoid the effect of the silica.

DISTÈCHE: Yes, try the simpler systems first and then complicated ones, just as we did with the problem of sea water. The problem of sea water is really extremely complicated and carries a warning for people who are going to try to interpret biological fluids, because the biological fluids are even worse than sea water.

ZIMMERMAN: You mentioned ATP. Would you please give some additional information about that?

DISTÈCHE: Well, we tried ATP and it behaves exactly like phosphate. But that doesn't mean that we know what happens when ATP splits. You then get a water molecule coming, then the splitting, the ionization of one of the groups, and this is not so easy to judge.

ZIMMERMAN: Would this also apply to NAD or NADP?

DISTÈCHE: Yes, As soon as you are in the proper pH-range where you can discount the pH 3.8 you get the same result. If you are so acid that you get the first constant of the phosphoric acid, you get another result. The first constant is a difficult one to examine because the amount of hydrogen ion is comparable to the concentration of the acid in the buffer so you have to make a fantastic amount of correction and calculation in order to find out what the constant really is. I've only given you the formula where the pH is sufficiently high so that the amount of hydrogen ion is completely negligible compared to the buffer.

SOME CONSIDERATIONS CONCERNING STRATEGIES FOR THE STUDY OF THE CHEMICAL COMPOSITION OF DEEP OCEANIC WATERS

By
R.M. Pytkowicz
Oregon State University

Pressure affects chemical equilibria and the rates of reactions. Therefore, the distribution of chemical species which are involved in chemical reactions will be different at in situ pressures than it is after decompression. A knowledge of the *in situ* speciation is important because it may affect biological availability, properties of geochemical interest such as degrees of saturation, and physiochemical properties of seawater such as the osmotic pressure and the attenuation of sound.

Three alternative strategies are possible to determine the chemical composition of deep oceanic waters.

Firstly, one may in some cases use *in situ* probes. Examples are the pH probe of Disteche, the *in situ* carbonate saturometer of Ben-Yaakov, and a variety of oxygen electrodes. Such probes have two advantages: their directness and their capacity to determine temporal and spatial fine structure through continuous scans in space and time. Their drawback is that they may be expensive single-purpose probes which require special ship time.

The second strategy is to bring samples to shipboard, make measurements at one atmosphere, and use pressure correction tables determined in the laboratory to correct the data to *in situ* values. Actually, there are three possibilities in this second strategy.

(a) If one is determining total concentrations such as dissolved oxygen, total inorganic phosphate, and total carbon dioxide in waters away from mineral phases and one expresses the concentrations in weight per weight units then no pressure correction is necessary.

(b) If one is studying the products of fast reactions which were at equilibrium at the pressure *in situ* and which reach a new equilibrium at atmospheric pressure then laboratory measurements of the equilibrium shifts upon decompression will yield correction tables which can be used on the ship.

An example of this approach is the determination of the *in situ* pH and of the concentrations of the carbon dioxide components such as bicarbonate and carbonate in deep waters from pH and alkalinity measurements at one atmosphere. This problem was solved by Disteche at the University of Liege and by Culberson in my laboratory.

(c) The reactions which control the species under consideration are slow, may or may not have reached equilibrium at pressure, and will shift by a time dependent amount upon decompression. This shift may be the case when sediments and pore waters are brought up together. Nothing has been

done about this problem except for the tacit assumption that there has been no shift upon decompression.

I strongly advise the development of *in situ* squeezers to separate pore waters from sediments before decompression.

The third strategy is to simply simulate the deep-sea environment in a pressure bomb and infer the course of reactions in the deep oceans from the laboratory work. This approach is best when the natural system is too complex for in situ probes or for simple correction tables. Examples of such studies could be the study of physiological reactions, of the structure of biological macromolecules, and of ion-association and of metal-organic complexes in seawater under pressure.

Next, I will examine some specific problems in greater detail. The determination of the effect of pressure upon the pH hinges upon the fact that the pH is uniquely determined if the total dissolved carbon dioxide and the titration alkalinity are determined, provided that the dissociation constants of carbonic acid are known at all pressures. Buch and co-workers and Lyman determined these constants at atmospheric pressure, and Disteche and Culberson determined them at high pressures. As the carbon dioxide system is described by 4 equations in 6 unknowns, any two pertinent measurements completely specify it. Thus, if the pH and the alkalinity are measured at one atmophere, the total carbon dioxide can be calculated. The total carbon dioxide and the alkalinity are invariant with pressure and, therefore, the pH can be determined at any pressure from two measurements at one atmosphere and from the laboratory determined equilibrium constants at pressure. Through these considerations Culberson and I prepared pH pressure correction tables. We found that pressure acidifies the pH, and the effect may be as large as 0.4 pH units for a depth of 7 to 10 kilometers. The ecological implications of such an acidification as well as the geochemical ones have not been fully exploited.

It is tempting in this correction table approach to the chemical speciation to use partial molal volume data, as the shift in equilibrium constants with pressure is a function of the partial molal volume change during the course of the reaction. I should point out, however, that volume data in dilute solutions do not apply to seawater because they yield only the shift of the thermodynamic equilibria with pressure. To go from thermodynamic constants to concentrations it is necessary to know the stoichiometric activity coefficients as a function of temperature, salinity, and pressure. These are not known in general because they depend not only upon the ionic strength but also upon the formation of ion pairs. Therefore, it is better to determine stoichiometric equilibrium constants directly by techniques such as potentiometry.

D.R. Kester and I applied the third strategy to the study of ion-pairs in seawater at pressure. Many ions in seawater are not completely free but

form electrostatic pairs with other ions to some extent. This association affects the solute charge and size distribution and, therefore, affects most chemical properties of seawater such as the colligative ones, the electrical conductance, the attenuation of sound, and the solubilities of minerals. Pressure in general tends to promote the dissocation of these ion-pairs. We measured the effect of pressure on sodium sulfate pairs by the use of sodium glass electrodes in a pressure bomb but were forced to estimate the pressure effects on calcium and magnesium sulfate pairs from partial volume data because calcium and magnesium electrodes are not yet sensitive enough.

I would like to wind up this talk with a few recommendations:

1. The high pressure equilibria of phosphoric acid need to be studied to understand the chemistry of formation and dissolution of apatites and phosphorites. Hydrogen sulfide should also be studied because of metal-sulfide complexes.

2. The interaction of trace metals with dissolved organic matter and clays should be studied at pressure because such interactions affect their biological availablity and geochemical fate.

3. Physiological equilibria should be studied at pressure.

4. The field of reaction rates in sea water at pressure has hardly been touched.

5. Better ion-selective electrodes are needed to determine the chemical speciation at pressure by potentiometric methods.

6. The study of water structure at pressure may have strong biological implications, especially near membranes.

PRESSURE EFFECTS ON THE PROPERTIES OF HIGH MOLECULAR WEIGHT SUBSTANCES IN SOLUTION

by
R.R. Becker
Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregonn 97331

Introduction

The purpose of this paper is to give a brief and rather restricted discussion of the effects of hydrostatic pressure on proteins in solution. In other parts of this book are recorded effects of hydrostatic pressure on whole organisms, and it may seem inappropriate to talk about effects on protein molecules and aggregates of molecules when in fact many of the effects seen (including death) are undoubtedly related to more highly organized and complex systems. The justification for selecting proteins as the models is two-fold. Firstly, successful adaptations to unusual environmental conditions must produce functional proteins. The broad and important subject of adaptation to extreme environments has been discussed in some detail by Hochachka (1971). Secondly, much is known about protein structure, and it is hoped that some of the knowledge, with special reference to those forces responsible for maintaining the native structure of proteins (Kauzmann, 1959), can be related to the effects of hydrostatic pressure on proteins, other macromolecules and more complex structures. Thus, an attempt will be made to describe relevant studies in simpler systems that provide at least some basis for an understanding of pressure effects on organisms.

As pointed out by Johnson and Eyring (1970), the large size and complex structures of biological macromolecules result in molecular volume changes of activation and of reaction that can be quite large. In this regard, Harrington and Josephs (1968) have found a volume change ΔV of 380 cc per monomer unit in the association of the muscle protein myosin. Such large volume changes would lead to measurable effects on biological systems at quite low pressures (50 atmospheres or less). These pressures are within the range encountered by a variety of living organisms.

In the case of individual enzymes isolated from organisms that live under relatively high hydrostatic pressure, Hochachka (1971) emphasizes that the control of reaction rates is not determined mainly by energy volume relationships since enzyme-substrate and enzyme-modulator affinities are largely pressure insensitive. On the other hand, maximal velocities have been found to increase, decrease, or remain the same when hydrostatic

pressure is applied (Hochachka 1971). Penniston (1971) has attempted to explain some of these variations on the basis that in general the activities of monomeric enzymes are stimulated by applied hydrostatic pressure, and those of multimeric systsms are diminished. That the situation is exceedingly complicated when reaction rates are considered is further evidenced by the finding of Becker and Evans (1969) that the β-galactosidase of *E. coli*, a multimeric enzyme, shows a reversal of the effects of pressure depending on whether the monovalent cation present is K^+ or Na^+.

Since the effects of hydrostatic pressure on individual enzyme reactions are usually relatively small, and at best difficult to interpret, it appears reasonable to center this discussion on the large volume changes that occur in many aggregation reactions of proteins. Obviously, any effect that is important at low pressures must involve reactions in large volume changes. These are certainly not restricted to protein systems, and are known to be of great importance in lipid interactions in aqueous systems. (Hamann, 1962) Hopefully, however, it will be possible to extrapolate in at least a general way from the aggregating protein systems to the more highly organized cellular components such as organelles, membranes, etc.

Stabilizing Forces in Proteins and Associating Protein Systems

Four levels of structure in proteins are commonly used as a convenient basis for discussion. The primary structure is that involving covalent bonds (about 50 kilocalories per mole) which can be broken only under drastic conditions (acid- or enzyme-catalyzed hydrolysis). The secondary and tertiary structures involve the folding of the polypeptide chain into a specific conformation, the interactions being non-covalent and very weak (a few kilocalories per mole). The same weak interactions are involved in the quaternary structure, the term used to describe the packing of monomers into polymeric or aggregated structures. As a result of these weak interactions, proteins and protein aggregates are delicately poised systems whose structures and interactions may be influenced by small perturbations of pH, temperature, ionic strength, and pressure.

The weak interactions are categorized as the hydrogen bond, the ionic bond and the hydrophobic bond. An excellent discussion of the important features of these intereactions, including the crucial role of water, is found in the book by Jencks (1969). The hydrogen bond in macromolecules is most familiar in its participation in the α-helix and β-structures in proteins, and in the double helical structure of DNA. It is not clear if there is a volume change in the formation or breaking of hydrogen bonds involved in macromolecular structure. A second type of stabilizing force is due to the interaction between oppositely charged groups, the electrostatic interaction commonly referred to as the ionic bond. In pressure studies, this interaction is very important in that a volume decrease (about 10-20 cc per bond) occurs when the ions are separated, and a corresponding increase occurs in the

formation of an ionic bond. The third type of interaction is the hydrophobic bond, which has been described as the tendency of non-polar side chains to associate when in an aqueous environment (Kauzmann, 1959). An important feature of the hydrophobic bond is that its formation leads to a large volume increase (again about 10-20 cc per bond) due to the release of oriented water surrounding the separated apolar groups. Thus the breaking of either hydrophobic bonds or electrostatic bonds is accompanied by a decrease in volume. Therefore, an increase in hydrostatic pressure would be expected to weaken and disrupt structures stabilized by these interactions. It should be pointed out that this oversimplified view of the effect of hydrostatic pressure appears to apply only to aggregating systems involving polymerization of subunits to relatively high molecular weight aggregates. These systems appear to behave in a predictable manner, probably because the interacting groups are on the surfaces of the polymerizing species.

As mentioned above, the two stabilizing interactions in aggregating systems most likely to be affected by hydrostatic pressure are electrostatic and hydrophobic bonds. The muscle protein myosin appears to be an example of an aggregating system in which the electrostatic interactions are more important in maintaining the polymeric structure (Harrington and Josephs, 1968). In contrast, the aggregation of polyvalylribonuclease (PVRNase) as detailed below, appears to be due largly to hydrophobic interactions. This was expected since the protein is prepared by attaching numerous apolar (in this case valyl) residues to the surface of ribonuclease A.

The Poly-L-Valyl Ribonuclease System

Polyvalyl ribonuclease (PVRNase) offers a convenient model for the study of the effects of hydrostatic pressure on aggregation reactions in that non-polar side chains occupy a sizeable proportion of the surface of the molecule (Kettman *et al.* 1966). It has been noted that polypeptidyl proteins containing added non-polar residues aggregate at much lower temperatures than their respective native proteins. (Krausz and Becker 1968). Although native ribonuclease (RNase) aggregates only at high temperatures, PVRNase solutions can show a turbidity at temperatures as low as 30°. This low temperature aggregation must be due to the increased apolar interactions afforded by the polyvalyl chains atached to the enzyme, since polyglycyl derivatives are actually more thermally stable than the native enzyme. The aggregation of PVRNase at 39° as influenced by salt concentration and hydrostatic pressure has properties indicating participation of both electrostatic and hydrophobic bonds. Thus the aggregation of PVRNase was found to be sensitive to pH (maximal between pH 6 and pH 7) and occurred at a lower temperature when the salt concentration was increased. These results would suggest that while the aggregation of PVRNase may be due primarily

to attractive apolar interactions, there are also repulsive forces involved which can be diminished by higher ionic strengths.

The effects of various solvents and solutes on the aggregation reaction of PVRNase at atmospheric pressure have been studied in detail (Nishikawa, Morita and Becker 1968). In the usual procedure for measuring the rate of aggregation, a cuvette containing suitable solvent was equilibrated at 39°C, the cold protein solution added, and the increase in absorbance at 320 nm used as a measure of aggregation. The rate of aggregation is defined as ΔOD_{320nm} per minute, and is plotted against salt concentration in Figure 99. The figure shows the effect of increasing sodium chloride concentration on the aggregation rate at pH 7.4. The nonlinear pattern in the rate change suggests two regions of salt effects. In the low salt region (0-0.3 M) an increase in salt elicits a rapid rise in the aggregation rate which levels off. The high salt region (above 0.3M) shows a logarithmic rise in the rate with increasing salt concentration.

In addition to sodium chloride, three other electrolytes have been examined with respect to their effects on the aggregation rate. It is seen in Figure 99 that all of the electrolytes studied cause a sharp rise in the rate at low salt concentration. Above 0.15 M, however, the effects on rate vary markedly with the type of electrolyte. While increasing concentrations of NaCl increase the aggregation rate, the tetraalkylammonium salts show different effects depending on the length of the alkyl groups. Tetramethylammonium chloride increases aggregation only at somewhat high concentration compared to NaCl, while tetrabutylammonium bromide distinctly decreases the rate at very low concentrations. Of the reagents examined, guanidine hydrochloride is the most effective in diminshing the aggregation rate.

The two distinct patterns of the response of the aggregation rate to salt concentration indicate the presence of at least two kinds of interactions of the polyvalyl chains on the surface of the protein. In the lower salt concentration range it appears that electrostatic repulsions between the positively charged ends of the added polyvalyl chains are being dimished by the added salt, resulting in an increased rate of aggregation. At the higher salt concentrations, the change in rate depends much more upon the nature of the salt. The more "normal" electrolytes such as sodium chloride or tetramethyl ammonium chloride cause an increased aggregation rate by strengthening hydrophobic interactions, while guanidine hydrochloride, an excellent protein denaturant (Tanford, 1968) prevents the formation of the aggregate. It is then, most likely that the aggregation reaction of this modified protein involves hydrophobic interactions which exceed the repulsive electrostatic forces that would tend to keep the system monomeric.

Thus, on the basis of what is known about these interactions, one would predict that hydrostatic pressure would slow the aggregation reaction. To test the effect of pressure, studies, were conducted in a stainless steel

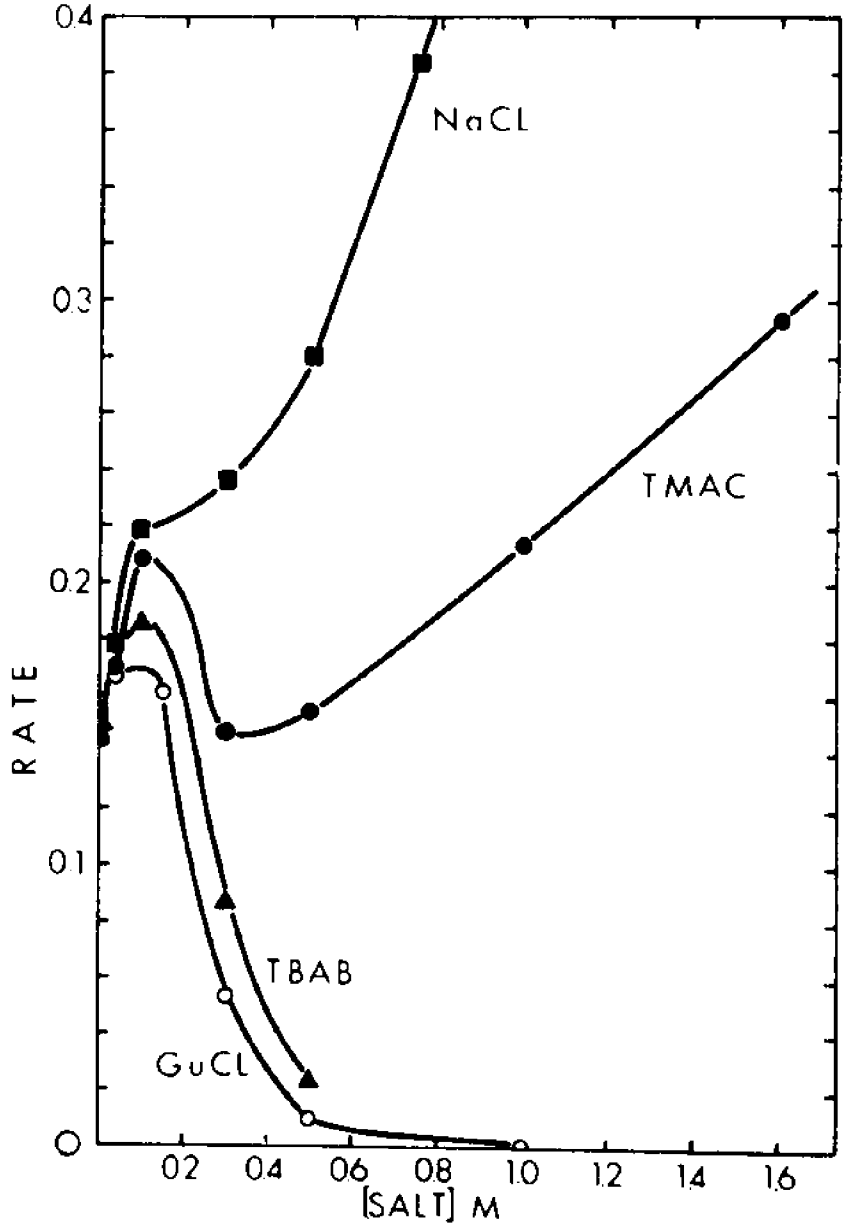

Figure 99. Effect of electrolytes on the rate of aggregation of PVRNase. The reaction was in 0.01 M TES, pH 7.4 at 39°. TMAC, tetramethylammonium chloride; TBAB, tetra-n-butylammonium bromide; GuCl, guanidine hydrochloride.

high-pressure cell with white sapphire windows and with an optical path length of 1 cm (Morita, 1957). The rate of aggregation was again defined as the change in OD_{320mm} per minute. The effect of hydrostatic pressure on the rate of aggregation at 39° is shown in Figure 100. The aggregation rate is greatest at 1 atm. and decreases markedly at 150 atm. and 300 atm. (The time scale reflects the minutes elapsed after placing the PVRNase solution into the high pressure optical cell at 39°. The non-zero intercepts for the OD changes determined at 150 and 300 atm. are due to the aggregation which takes place in the 7 or 8 minutes required to attain the desired pressures.) When the hydrostatic pressure was released, the rate of aggregation abruptly increased to that at 1 atm., showing distinctly the pressure dependence of the aggregation rate.

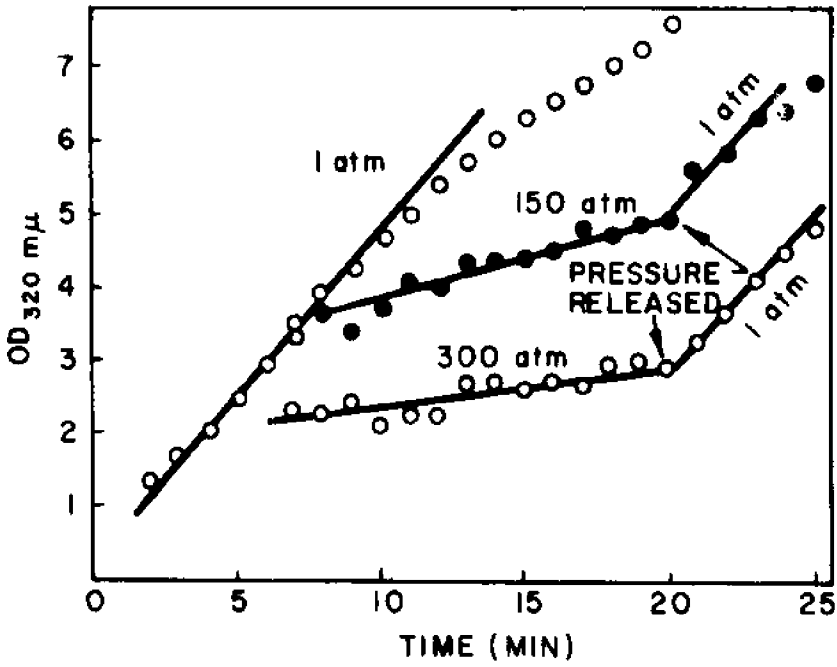

Figure 100 Effect of pressure on the rate of aggregation of PVRNase. The reaction was at 39°in 0.6 M NaCl.

The diminution of the aggregation rate by applied hydrostatic pressure suggests that the transition state of the rate-limiting step exhibits a positive volume change of activation (ΔV^*). This volume change for the aggregation reaction can be estimated using the assumed relationship (Hamann, 1963) between rate constants and pressure:

$\frac{\partial \ln k}{\partial P} = \frac{-\Delta V^*}{RT}$ where R is the molar gas constant, T is the absolute temperature and k is the aggregation rate. ΔV^* values of over 200 cc per mole were found in the pressure range up to 300 atmospheres.

The very large size of the positive ΔV^* can be interpreted as the release of a large number of solvent molecules from the monomer's polyvalyl chains in the rate determining transition state of the aggregation. Kauzmann (1959) has pointed out that the process of transferring hydrocarbons from water solution into nonpolar solvents is accompanied by a volume increase (e.g., +18.1 cc per mole for ethane into hexane). This rather large volume change has been interpreted as being due to the release of water molecules "bound" about the alkane molecules. The reaction occurs spontaneously at room temperature in the direction of hydrophobic bond formation (i.e., non-covalent alkyl-alkyl interactions with the exculsion of solvating water molecules). While the magnitudes of the ΔV of reactions cannot be directly compared to their ΔV^*, the values found would suggest that the aggregation of PVRNase is due mainly to apolar interactions of the alkyl side chains of the attached polyvalyl residues. Further observations which support this notion are that the aggregation of the polyvalyl derivative occurs at rather moderate temperatures and that such aggregations can be reversed in the cold (0—4°).

Myosin Self-Association

The importance of pressure on the sedimentation properties of associating systems has been emphasized by several authors (Josephs and Harrington 1967; Kegeles, *et al.* 1967; Ten Eyck and Kauzmann, 1967), and estimates of the pressure change required to bring about an observable change in the association reaction has been made. These authors point out that the extreme sensitivity to pressure is due to the large molar volume of proteins such that a small fractional change in volume will result in a large molar volume change. Harrington and Josephs (1968) have calculated the change in equilibrium constant for three hypothetical reactions using a molar volume change of 0.5%. Large effects are obtained at quite low pressures. For example, the ratio of the equilibrium constant for a polymer product of molecular weight of 105 at 1 atm to that at 80 atm is 3.6. If the molecular weight of the product is 106, then the ratio is calculated to be 4 x 105.

Much of the earlier work on the effects of pressure on the association of the muscle protein myosin has been summarized by Harrington and Josephs (1968). Studies of the monomer-polymer equilibrium in the analytical ultracentrifuge provided information about some of the essential features of these interactions. The ionic strength dependence is illustrated by the fact

that when myosin solutions in 0.5 M KCl are dialyzed to lower the salt concentration, high molecular weight components are observed in sedimentation patterns. (For more detailed studies of the effect of ionic strength, see Godfrey and Harrington, 1970a and 1970b) The molecular weight of the polymers formed at pH 8.3 and 0.137 M KCl is about 50×10^6. Assuming a monomer molecular weight of about 600,000, the number of monomer units in the aggregate is 83.

The pressure dependence of the equilibrium constant was evaluated in experiments in the ultracentrifuge in which the pressure was varied by varying rotor speed or by layering mineral oil over the protein solution in the cell. The equilibrium constants obtained in 0.18 M KCl and pH 8.3 were of the order of 10^{70} at 1 atmosphere and around 10^{45} at 50 atmospheres. From such experiments a $\Delta\bar{v}$ of 6.4×10^{-4} cc per gram or a ΔV of about 380 cc per mole for association reaction was obtained (Harrington and Josephs, 1968). Since the effect of increasing salt was to destabilize the polymer, it was concluded that electrostatic effects were important in the aggregation. This is to be compared with the opposite effect of relatively high salt concentrations in the PVRNase system where hydrophobic interactions dominate. However, in both cases (myosin polymerization and PVRNase aggregation) increasing pressure destabilizes the polymer with a resultant large positive volume change. The explanation for the volume increase is the release of water from the monomer upon polymerization. In the case of myosin the water is apparently mainly from that associated with ionic groups and in the case of PVRNase the water apparently is from that associated with non-polar groups.

Recently, Dreizen and Kim (1971b) have initiated studies on the subunit structure of the myosin isolated from the skeletal muscle of a benthic fish, *Coryphaenoides* species. However, detailed studies of the type described for the rabbit protein have not yet been reported. Of interest also is the fact that the myosin ATPase activity of the benthic fish is "relatively insensitive to pressure and temperature under conditions encountered by the living fish."(Dreizen and Kim, 1971a p.513).

Discussion

For any system to be influenced by relatively low hydrostatic pressures (a few to several hundred atmospheres), large volume changes must occur. Some of the properties of two such systems, one prepared by chemical modification of a native protein (PVRNase), and one naturally occurring (myosin) have been given above. Properties of a number of other self-associating systems have previously been summarized and some of their common features discussed (Harrington and Josephs, 1968; Morita and

Becker, 1970). Perhaps the system studied in most elegant fashion is the protein from tobacco mosaic virus (Lauffer and Stevens, 1968). In these studies, the release of water upon association of the protein was measured directly and found to be about 150 moles per so-called A-protein unit (molecular weight 10^5). Other self-associating systems studied include hemocyanin (Van Holde and Cohen, 1964; Morimoto and Kegeles, 1971; Tai and Kegeles, 1971), collagen (Cassel and Christensen, 1967), flagellin (Gerber and Noguchi, 1967), and Actin (Ikkai and Ooi, 1966). All of these systems involve volume increases in the association reactions, and molecular weights greater than several million for the polymerized products.

In addition to pressure, the forces involved in the aggregation reactions may be influenced by salt concentration, pH, and temperature. To have similar degrees of association in a system at, for example, 200 atmospheres as compared to 1 atmosphere, it would be necessary to change one or more of these factors, or the nature of the interaction. Therefore the question as to which type of interaction dominates for a given system becomes important. Although this question is difficult to answer for most naturally occurring systems, it does appear that the aggregation of PVRNase is dominated by hydrophobic interactions. TenEyck (1970) has employed the ultracentrifuge in studying the effects of varying pressure and temperature on the association of polyvalyl-chymotrypsinogen, a system similar to PVRNase. His results indicate both a positive volume change and a positive temperature coefficient for association as expected for hydrophobic reactions (Kauzmann, 1959). As models for protein aggregation reactions, both PVRnase and the polyvalyl-chymotrypsinogen system suffer from the fact that uniform polymers are not produced, and the type of specificity observed in the naturally occurring systems is lacking. But they have been useful models in demonstrating that interactions involving apolar patches on the surface of molecules are possible, and the properties shown are consistent with what has been predicted from other model studies. Although in many of the association reactions, it is possible to determine whether or not hydrophobic interactions or electrostatic interactions dominate, it is doubtful if a detailed understanding of the processes can be gained until specific interactions are studied by more direct means (Richards *et al*, 1972).

In considering the effect of pressure, little emphasis has been placed here on the influence on individual enzymes since the interpretation of these results is difficult at best (Brandts *et al.*, 1970). However, for associating systems that have been studied in detail the behavior can be interpreted, at least in a qualitative way, in terms related to better understood model systems. The question as to the differences between self-associating systems from organisms that live in the depths compared to those from near the surface is certainly an intriguing one. Until more is known about these systems, but especially until several from different sources have been

compared in some detail, one can only speculate about the adaptive changes that occur.

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Discussion Following Becker Paper

DISTÈCHE: Is any analytical work being done to compare the types of amino acids in deep sea tissues with those of shallow water forms?
MACDONALD: That might give a clue as to what types of amino acids are chosen, to engineer resistant organisms.
BECKER: Yes, this would be the first place to start. I've been that route with thermophiles and halophiles. In halophiles there's a suggestion that there might be more acidic residues—they're loaded with aspartic acid, glutamic acid, and so on. If you take a computer program to compare composition of the aldolases, or the isocitric dehydrogenases, from bacterial sources of mesophiles and thermophiles, I think it would be very hard to detect differences. Still, it is the first thing you have to do anyhow, even if you want to look at the hamburger that Malcolm Gordon works with, for example. I would be certain that you would find such differences. You can take such work down to the Keilin-Hartree preparation which carries electrons, and you will still find the differences, especially when you get to the cytochrom form of it. But in general analyses you'll not see much, and I would not be surprised if you would not find a great deal of difference in overall composition. I don't expect these things to be any more different than they have to be to survive, and that doesn't have to be very much. If you compare normal adult hemoglobin to sickle-cell hemoglobin, one replacement in the B-chain suffices. A more general approach, such as calculating hydrophilia and so forth is fun, but I don't think it means very much. One of the changes that's interesting is in lactic acid—some of these organisms will use it as an energy source. I don't know whether the change of lactate production occurs at the aldolase point where intuitively you might think it would, but which as mentioned was not sensitive at all. I wonder if that means something.
ZOBELL: You gave us a pretty good diagram and word picture of your concept of the molecular configuration changes which occur when a large molecule such as protein is denatured. Would you elaborate briefly upon the irreversibility.
BERGER: I believe that most of the denaturation procedures which are

irreversible involve a covalent change. Now there are always people who titrate proteins between pH 3 and 10; you are usually safe unless you are titrating pepsin. What happens with ribonuclease about pH 10 is a chemical change where you actually eliminate sulfur. Of course when you hydrolyze this, you get out an amino acid which nobody had ever seen before,just by holding at high pH. I think that most irreversible bonds are somewhat covalent changed. Now, I can conceive of denatured states in which there is no way of easing them back. It's not unrelated to this problem of Winston Atkinson's, which I mentioned earlier and I think this is what makes believers out of a lot of us. If he took the ribonuclease chain and reduced the four SS bridges and let it air-oxidize back, it came back right. The people who synthesized insulin made the two chains. This is the obvious route to take. One of them has 31 residues and one of them has 20.

SPECTROSCOPIC INVESTIGATION OF THE EFFECT OF HYDROSTATIC PRESSURE ON PROTEINS AND MEMBRANE STRUCTURE.

John R. Rowlands
Southwest Research Institute,
San Antonio, Texas.

I would like to briefly outline the results of preliminary investigations that we have conducted on the effects of pressure, up to 2500 psi on protein solutions and erythrocyte ghost suspensions using the electron-spin-resonance spin-label technique.

Spin labels are stable free radicals containing functional groups that are chemically bound to known functional groups of molecules of biological interest such as proteins. The use of spin-labeling technique in studies of biological macromolecules was recently introduced by McConnell and co-workers[1] (1965). Although it is relatively new, sufficient studies have been conducted to demonstrate conclusively that the technique has great utility in studies of conformations of proteins and membrane constituents in solution.

The basic principle that is utilized in the spin-labeling technique is that the electron-spin-resonance spectrum of a free radical is sensitive to its environment.

The spin-labeling technique makes practical use of this fact by utilizing nitroxide radicals as probes of macromolecular structure.

These nitroxide radicals have a sufficiently simple electron-spin-resonance spectrum that extremely small changes in their rotational freedom can be easily detected and interpreted. McConnell and co-workers[1] (1965) in their pioneer development of the spin-labeling technique, showed that the electron-spin-resonance spectrum of a spin label is influenced in an extremely sensitive manner by the nature and extent of bonding of the label to a macromolecule. Hence, conformational changes of the macromolecules have a profound influence on the mobility of the spin labels. Typical spectra of nitroxide spin labels exhibiting various degrees of immobilization rangine from totally immobile to freely rotating are illustrated in Figure 101.

In preliminary experiments to study the effects of pressure at the cellular level, erythrocyte ghost membranes were chosen, since they offer an excellent model system for membrane studies.

Erythrocyte membranes were prepared from fresh rabbit blood by the method of Dodge, Mitchell and Hanahan[2] and the ghosts were suspended in 20mM phosphate buffer at pH of 6.8. The steroid analog

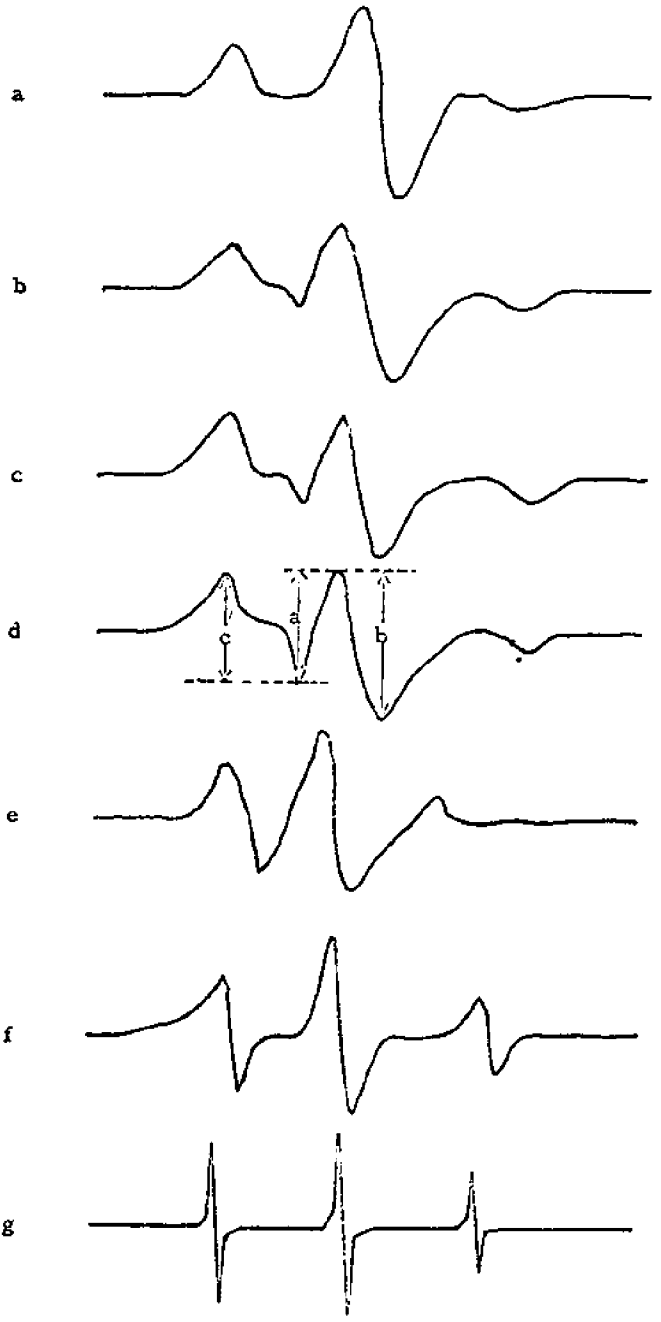

Figure 101 ESR Spectra of a "Rigid Glass" Spectrum (a) to a slightly immobilized freely rotating label in (g) including intermediate transitions. (From Snipes & Keith and also Griffith & Waggoner 3, 4).

17β-Hydroxy-4′,4′ dimethylspiro [5α-androstane-3, 2′-oxazolidin] -3′-yloxyl (Fig. 102) was then added to the mixture. This lypophilic label is a derivative of the steroid 5α-androstan-3-one-17-ol, and is readily incorporated into membranes. After an incubation period of approximately 4 hrs the suspension was dialyzed for a 24-hr period for removal of excess label.

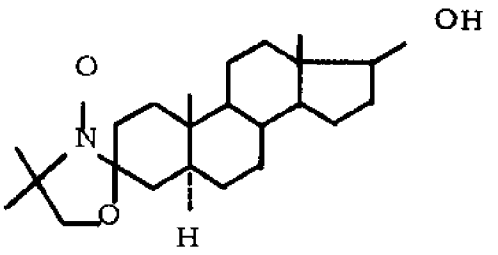

Figure 102

The apparatus designed and constructed for the high-pressure experiments is shown schematically in Figure 103. It was found most convenient to use commercially available dry nitrogen to pressurize the system. As obtained from the manufacturer (Linde Gas Company), the K cylinder contains 220

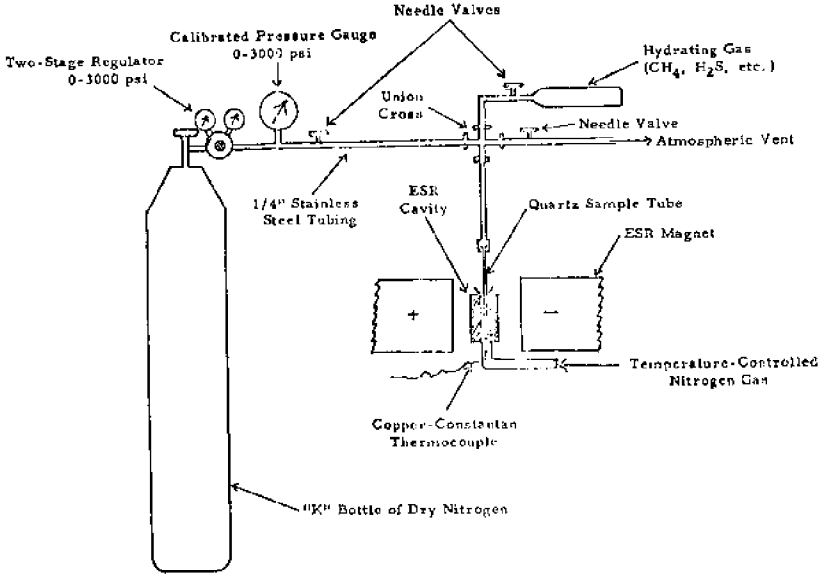

Figure 103 Schematic of High Pressure Apparatus

cu. ft. of oil-pumped nitrogen at a pressure of about 2700 psig. A special two-stage pressure regulator with an adjustable valve-controlled diaphragm provided a convenient means of increasing the pressure from 0 psig to 2500 psig in measureable 10-psi increments. Pressures can be reduced in the system by closing the diaphragm on the regulator and slowly opening a vented needle valve, allowing gas to escape until the desired pressure is obtained.

Entrained in the pressure line is an additional calibrated (dead weight-tested from 0 to 3000 psi) gauge, which provided pressure readings with an accuracy of ± 5 psi.

At a junction approximately 6 ft. from the regulator, a Swagelok union cross allowed interconnection of the system to: (1) a needle valve serving as a vent, (2) an ESR sample tube, and (3) an additional line to allow a hydrating gas to be introduced into the system for certain experiments. These connections are shown schematically in Figure 103. All tubing used in the system is 1/4'' stainless steel, while connecting unions are Swagelok brass. The 1/4'' stainless steel tubing leading to the ESR tube terminated approximately 2 inches above the cavity. A 1/4'' to 1/8'' adapter was precision bored to 3/16'', such that it tapered from 1/4'' to 3/16'' to 1/8'' to allow free passage of a 1/8'' tube, but retaining an extruded region of the tube. This adapter is shown schematically in Figure 104. A Teflon ferrule

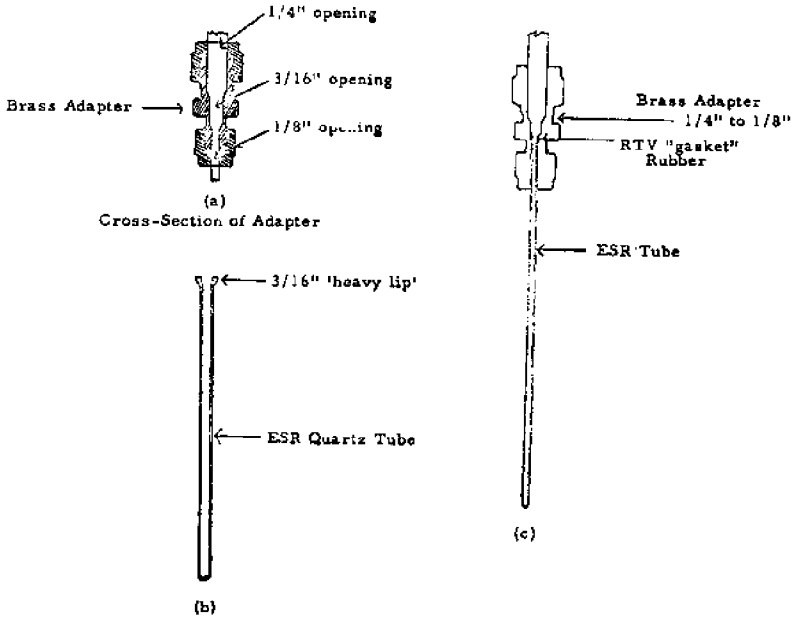

Figure 104 (a) Swagelok Adapter connecting ESR sample tube to pressure line. (b) Quartz sample tube. (c) Schematic of sample tube positioned in modified adapter.

formed a seal between the quartz tube and brass adapter on the 1/8'' side. A seal and cushion was formed around the lip of the quartz tube by applying RTV self-curing rubber around the 3/16'' opening, such that high pressures did not force the tube out of the adapter. The sample tube is pure quartz with 1-mm walls and an I.D. of 1 mm, which was modified by extruding one end to form a heavy walled lip and sealing the opposite end. The length of the tube is approximately 15 cm and is shown in the diagram in Figure 104. The extruded outer lip can pass through the 1/4'' adapter, but not the 3/16'' opening; hence, high pressures cannot force the tube out of the adapter as depicted in the adapter in Figure 104.

The volume of solution effectively observed spectroscopically is approximately 0.01 cc in the quartz tubes used in this system. Once the tube is placed into position in the ESR cavity, the temperature can be readily controlled using a modified Varian V-4547 variable temperature-controlling unit. In this unit, dry nitrogen is passed through a closed stainless steel coil immersed and maintained in liquid nitrogen which, in turn, passes through a heater coil which is controlled by a variable potentiometer. By varying both the N_2 flow and the potentiometer, temperatures in the cavity can easily be controlled from -190°C to + 250°C ± 0.1°C. As the gas passes around the sample tube, it also comes into contact with a copper-constantan thermocouple, which is positioned to the center of the sample cavity. Temperatures were monitored by directly observing a Hewlett-Packard digital voltmeter, which was connected to the thermocouple.

An ESR spectrum of the labeled ghosts at 37°C and 0 psig is presented in Figure 105 and indicates a strongly immobilized species with a correlation time considerably less than 10^{-8} sec. Once the system reached thermal equilibrium the labeled membranes were alternately subjected to pressure of 0 and of 2000 psig while recording the ESR spectrum immediately after changing the pressure and achieving thermal equilibrium.

The amplitudes of the high and low field hyperfine components designated a and b in Figure 105 were measured for each scan and their ratio calculated as a function of pressure.The data for these measurements are presented graphically in Figure 106.

No detailed effort to explain the origin of the differences in a/b ratios observed at atmospheric pressure and 2000 psig will be offered at this time since further studies both on this system and oriented lipid bilayers will be performed in the immediate future. It seems, however, that relatively low hydrostatic pressures are capable of inducing changes in membrane structure which are observable by this technique. We have also conducted studies of several proteins including hemoglobin, hemocyanin and bovine serum albumin using the technique. However, no obvious changes were observed in the spin label spectra at pressures up to 2500 psig. It is our intention to extend these measurements to include pressures up to 5000 psig during the forthcoming year. Possible pressure-induced conformational changes will be

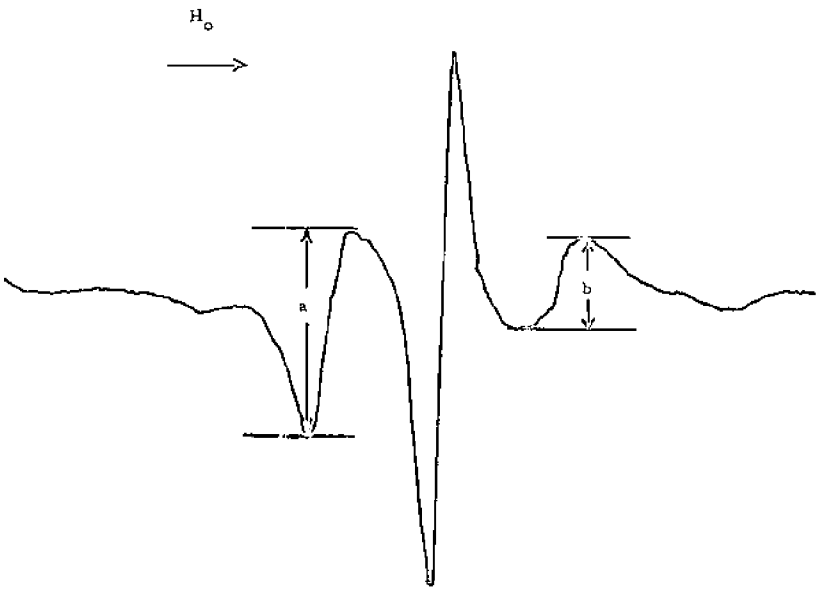

Figure 105 ESR Spectrum of Erythrocyte Membranes and Steroid Spin Label

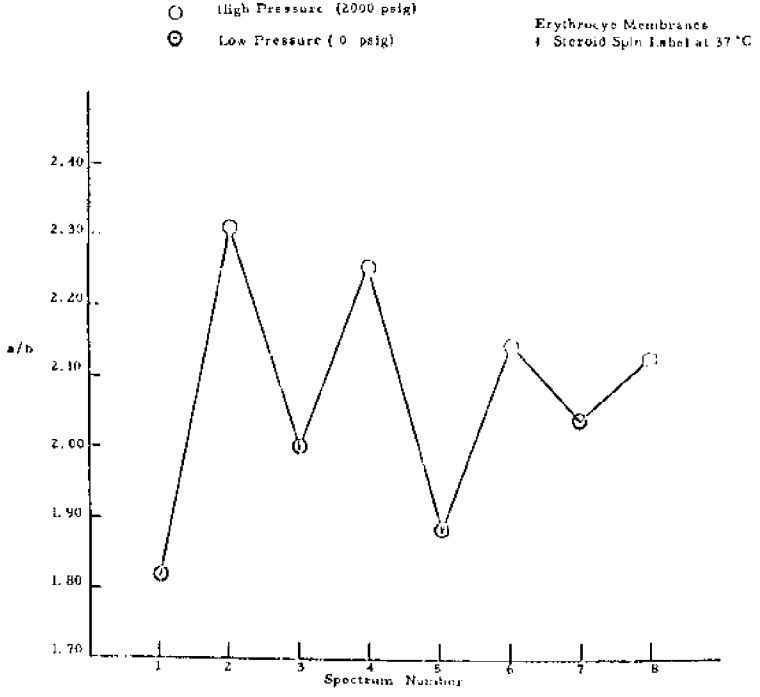

Figure 106 Ratios of a to b from Spectra in Figure 105

studied both by electron spin resonance spin label and fluorescence singlet-singlet energy transfer studies.

I would like to acknowledge the support of the Department of the Navy, Naval Ship Systems Command, under whose sponsorship this work was performed. (Contract No. N00140-70-C-0275).

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Discussion Following Rowlands Paper

MACDONALD: I should like to quite briefly point out that I think it has been shown that membrane permeability changes at 70 atm. are in the ions and not in the membrane.
ROWLAND: Well, I am a bit out of my field in this. I am just a chemist who happens to have done some work on this.
MACDONALD: The point is the whole red cell is quite pressure-sensitive. (Question) Have you used any substrates in your system that produce free radicals naturally such as chlorophyll.
ROWLANDS: No. To be sure of what you are seeing is quite time-consuming. We have done some work on the effect of free radicals in photosynthesis for some other purposes—air pollution—and you have to be pretty careful about your experimental conditions to make sure you get reproducibility. To add the effect of pressure to that, we felt would be prohibitive.
(Question) Is this a saline system?
ROWLANDS: Yes.
(Question) What would be the effect of subjecting this to the pressure of, say, oxygen? One of the suspected effects of hyperbaric oxygen is free radicals. Have you tried to follow the effects of these pretty fast acting free radicals?
ROWLANDS: No. We didn't pressurize the unlabeled membranes since we don't see anything anyway.

CHAPTER FOUR

HIGH PRESSURE ENVIRONMENTAL CHAMBERS

by
Robert C. DeHart
Southwest Research Institute

Abstract

In this paper, materials suitable for use in high pressure environmental chambers are discussed and design considerations for the chambers are given. Some of the hazards associated with high pressure environmental chambers are described.

I. Materials for High Pressure Environmental Test Chambers

A. General

Materials suitable for use in high pressure environmental test chambers must meet several requirements before they can be considered acceptable. The most important of these requirements are:

(1) High yield strength
(2) No change in strength with age
(3) Adequate resistance to cyclic loading
(4) High ressitance to corrosion
(5) Availability of sections having adequate thickness and size
(6) Completely developed fabricating techniques
(7) Good quality control
(8) Acceptable price

B. T-1 Steel (ASTM A-514 or A-517)

This low-carbon quenched and tempered alloy steel is weldable and has exceptional strength and toughness.The principal alloying elements are manganese, nickel, chromium, and molybdenum. Water is used in the quenching operation, and the quenching temperature is in the 1,650°F to 1,750°F. range. Tempering is accomplished at from 1,100°F to 1,275°F.

Plate thicknesses ranging from 1/4" to 6" are available in widths up to 136". The yield strength of plates thicker than 4" is considerably less than the yield strength of thinner plates. At the present time, United States Steel Company, the developer of T-1 steel, (ASTM A-514 or A-517) does not recommend its use in pressure vessels in thicknesses over 4". Depending upon the requirements of the application involved, T-1 steel can be heat treated to give either of two different sets of mechanical properties.

Strength, toughness, and weldability are the properties of most importance for the material from which the vessel under consideration is to

fabricated. The mechanical properties for T-1 steel plate heat-treated to provide suitable material are shown in Table I.

Table I.

Tensile Properties of T-1 Steel

Thickness	3/16 in. to 2-1/2 in. incl.	Over 2-1/2 in. to 4 in. incl.	Over 4 in. to 6 in. incl.
Yield strength ext. under load, min, psi	100,000	90,000	90,000
Tensile strength, psi	115,000 to 135,000	105,000 to 135,000	105,000 to 135,000
Elongation in 2 in., min. percent	18	17	16
Reduction of area, min, percent	50	50	45

A typical S-N curve, which illustrates the fatigue characteristics of T-1 steel, is shown in Figure 107.As indicated in this figure, an endurance limit of 67,000 psi was obtained with a rotating beam type test. The results of a recent fatigue test on a vessel fabricated from T-1 steel have demonstrated that extreme care must be taken to eliminate defects in the weld and base material if fatigue failures are to be prevented. In addition, as is the case for almost all pressure vessels fabricated from high-strength materials, careful attention must be given to nozzle design.

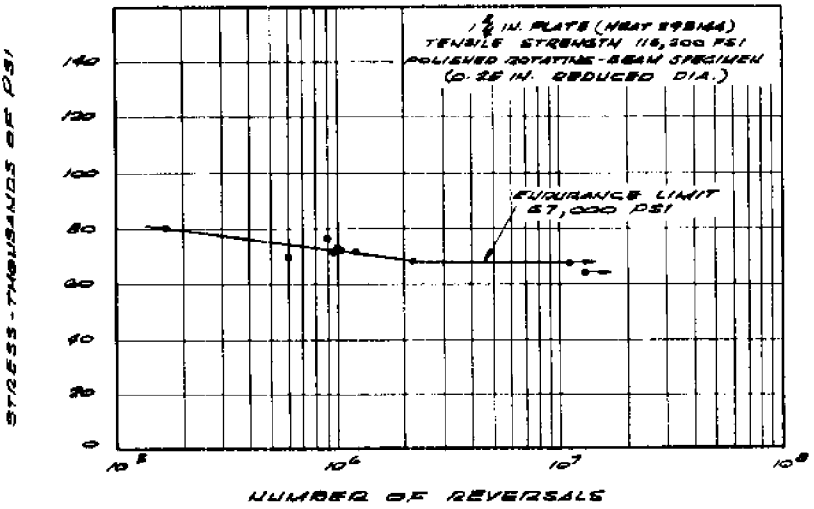

Figure 107 Results of Polished Rotating-Beam Fatigue Tests on T-1 Steel

One of the principal advantages possessed by T-1 steel is its low cost. For plate thicknesses such as those required for a vessel of the size under consideration,T-1 steel can be obtained at a cost of about $.20 per pound.

C. Titanium

Titanium is becoming more extensively used in pressure vessels, particularly in those cases where a high strength-to-weight ratio is needed. Most of the impetus to develop titanium for pressure vessels has come from the missile industry. Of the several titanium alloys currently available, the 821 alpha titanium alloy, which can be manufactured with a yield strength of 120,000 psi, appears to be the more suitable for pressure vessel work. This alloy has been manufactured in plate thicknesses of 1'' which, of course, is not adequate for the size vessel under consideration in this study. According to the titanium prlducers, however, the thickness of plate that can be rolled at present is limited only by melting capacity. The 821 alpha titanium alloy is weldable.

Even if plates of titanium alloy having sufficient thickness could be produced, the high cost of the material makes it an unlikely candidate. For example, the cost of titanium plate is $4 per lb at this time and is not expected to go below $2 per lb within the next five years.

D. Maraging Steel

In the past year, International Nickel Company has introduced several steels, which rely on high nickel content for the martensitic transformation rather than on the presence of carbon. These steels become martensitic on air-cooling from elevated temperatures. On subsequent aging at intermediate temperatures, intermetallic compounds such as nickel aluminide and nickel titanide are precipitated. Tensile strengths of 300,000 psi can be obtained with a ductility in the range of 40 to 60% reduction of area and toughness values of 18 to 25 ft-lb as measured in the Charpy V-notch test. It is believed that the low carbon content of these steels is responsible for their unusually high degree of ductility and toughness. Their ultimate strength is essentially the same as their yield strength, and the elongation in a 1-in. gage length is about 12%.

The heating and cooling cycles involved in the treatment of these materials are quite similar to those used in conventional heat treatment. In fact, the types of metallurgical reactions involved bear an analogy with those which occur in the hardening of tool steel and stainless steel which is hardenable by precipitation of martensite. The first step in the heat treatment is a solution anneal at 1,500°F. The steel is then air-cooled to room temperature. The final properties are developed by aging at 900°F for three hours after which the steel is air-cooled. A subsequent bake at 300°F. for 16 hours minimizes the possibility of hydrogen embrittlement.

The 25% nickel steel requires one additional treatment. This alloy does not transform to martensite on cooling to room temperature from 1,500°F because its martensile transformation temperature is too low. It must be

reheated to 1,300°F for about four hours in order to precipitate some of the nickel, aluminum, and titanium. The precipitation of these alloying elements raises the transformation temperature above room temperature. This alloy has the advantage of being in the form of soft ductile austenite after the 1,500°F treatment. In this condition it can be cold-worked to as much as 90% reduction in area.

These steels have a distinct advantage in that they can be welded after heat treatment without severe loss of strength in the heat-affected zone of the weld and in the weld itself. At the present time, there are no commercially available electrodes for the maraging steels; however, the laboratory experimental work needed to develop a means of manually welding the maraging steels with coated electrodes or by the inert-gas-shielded process has been completed. Thus, it is expected that suitable electrodes will be commercially available in the immediate future. The International Nickel Company reports that preheating in general is not he required.

Weld fabrication of the vessel may be difficult in that the completed weld must be either solution treated and aged or given a low-temperature aging after the weld is completed. In the case of solution treatment plus aging, it is possible to provide a weld joint efficiency of 100%; however, this in all probability may not be economically feasible. In the case of low-temperature aging treatment, it is expected that it will be possible to obtain a 90% joint efficiency with the strength of the weld deposit being slightly lower than the strength of the base metal.

While there is little previous experience in fabricating thick plates of maraging steel, it is believed that this material can be produced for about $2.75 per pound.

E. HY-80 and HY-100 Steels

The HY-80 steel, which is presently used in submarine construction, is available in any desired thickness and can be welded in any thickness. Usually no heat treatment is required after welding. This steel has a carbon content of about 0.15% and has a yield strength of approximately 80,000 psi. The main alloying elements are nickel, chromium, and molybdenum.

The HY-80 type steel can be heat-treated to a yield strength of 100,000 psi. In this case, the steel is known as HY-100 and can be obtained in thicknesses up to 2-1/2".

HY-80 and HY-100 are weldable steels and have been welded without heat treatment for several years. The quality control and procedure required for the successful welding of these steels have been well established.

F. Glass-Reinforced Plastic

Glass-reinforced plastic has been extensively used in the manufacture of solid propellant rocket motor cases. The design strength for these materials is approximately 90,000 psi; however, they do not have to withstand this stress for long periods of time. Glass-reinforced plastics are viscoelastic. They are different from the metals or glasses in that they exhibit a nonlinear

stress-strain behavior at all loads except at extremely high rates of loading. Their mechanical properties are rather sensitive to fabricating techniques, and they creep under sustained loads. They exhibit no clear-cut yield point and have no elastic range. The modulus of elasticity is usually computed by taking the slope of the stress-strain curve at the origin. At this point the viscous effects are small. These tangent moduli are of doubtful value in calculating deformations at higher stress levels or after protracted loading. The reinforced glass plastics are anisotropic, and their elastic and plastic deformations under combined stresses are complicated.

The literature is replete with data on the tangent moduli of reinforced plastic. These moduli are a function of the ratio of fiber to resin, orientation of fibers, curing cycles, moisture content, temperature, and previous history of loading.

Industry has had considerable experience with reinforced plastic pipe and fittings. Thousands of cyclic and sustained pressure tests have been conducted. Also extensive field trials are under way. In these tests and field trials when failure occurs, it appears generally as a delamination failure. Recommended working pressures for reinforced plastic pipe result in hoop stresses less than 5,000 psi. Also in the design of end fittings it is usually required that the interlaminar shear stresses be maintained at less than 500 psi.

The principal disadvantage of glass-reinforced plastic material is its tendency to creep under long-term loading conditions. Efforts are under way in many laboratories to eliminate creep and to increase the permanency of elasticity of glass reinforced plastics.At this time, however, no firm values for long-term effective moduli have been established for any of the many possible combinations of fiber and plastic.

II. HIGH PRESSURE ENVIRONMENTAL TEST CHAMBER DESIGN CONSIDERATIONS

Because of the wide variety of structural, material, and equipment evaluations that must be made, external pressure test chambers must be able to apply pressures ranging to 30,000 psi. In some cases, they must be able to withstand a large number of pressure cycles. They must also be easy to open and close; and it must be possible to bring instrumentation leads from the test article through the test chamber walls to data recording equipment. Operating temperatures as low as 32°F are also frequently necessary.

The unique demands on the pressure vessel used for an external-pressure testing facility make it impossible to find a code such as the Unfired Pressure Vessel Code of the ASME that is applicable. For example, the end closure for the test chamber usually incorporates quick opening features. One type of quick opening end closure is shown in Figure 108. In this case, interrupted threads transfer the hydrostatic pressure load on the closure head

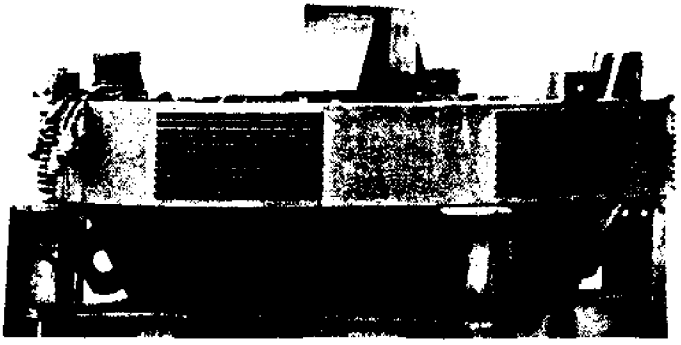

Figure 108 Interrupted Thread End Closure

to the cylinder. Penetrations into the interior of the test chamber are usually placed in the end closures, where the stresses around the penetration can be kept at a low level without introducing complicated reinforcing details such as are frequently required if penetrations are placed in a cylindrical shell.

While a sphere provides the lightest-weight test chamber, this shape is not generally used. Most of the items evaluated in the test chambers have one dimension greater than the others. For this reason, cylinders are a more suitable shape.

One of the operating conditions frequently encountered is that sea water must be used as the pressurizing medium. The corrosive nature of sea water makes it necessary to protect the inner surface of the test chamber. Stainless steel and Monel metal liners are usually used. In some cases, a polymer coating has been found to be effective.

With the advent of high-strength materials in submarines and other submersible vehicles, it has been found that fatigue failures are possible. External-pressure test facilities are used to evaluate the effect of cyclic loads on structures, materials, and equipment. Because of this use, the test chamber itself may be subject to many pressurizations, introducing fatigue considerations into the design of the chambers.

As was discussed earlier, some fatigue tests on hull structures can be run without cycling the test chamber itself.

At the closure for the test chamber, seals must be provided. These seals vary with the size and type of test chamber; however, in almost every case, a rubber O-ring is the principal part of the seal.

Some of the models used in the experimental stress analysis of stiffened cylinders and in the evaluation of materials under external pressure have a maximum dimension of 6 to 23 inches. For this type of work, a small-diameter, high-pressure test chamber such as the one shown in Figure

109 is very useful. For small size test chambers, a seal of the type shown in Figure 110 is suitable. (3) This seal consists of a split ring which moves on a tapered surface as pressure is applied to the rubber O-ring directly beneath it. The split ring thus fills the annular space that develops as the cylindrical shell moves radially outward. Instrumentation leads are brought through either closure head. There are numerous small test chambers and they are not expensive.

Intermediate-size test chambers are considered to be those having a 30-to-60 inch diameter. Chambers of these diameters having working pressure

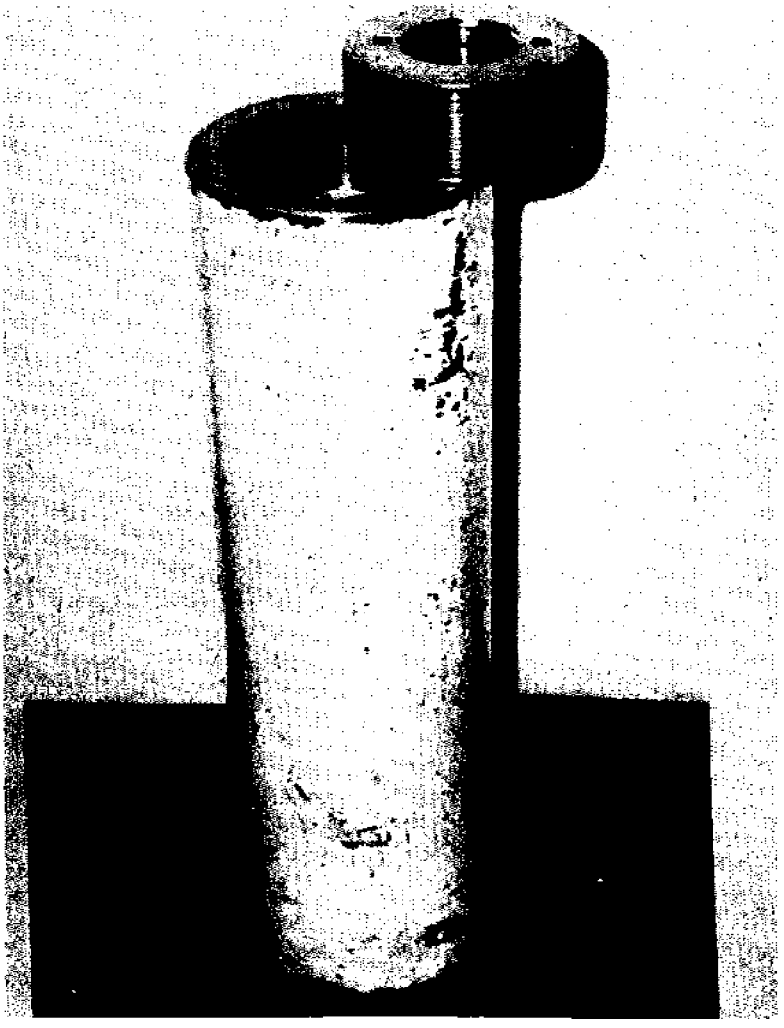

Figure 109 10" Diameter Test Chamber

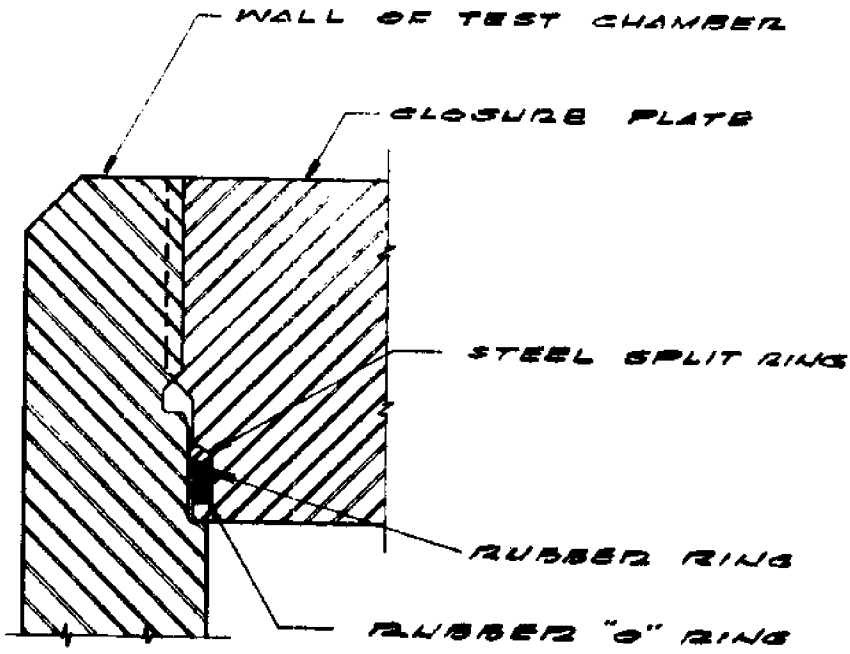

Figure 110 Diagram of a Split Ring Seal

of 12,000 to 15,000 psi are available at Navy laboratories and at some industrial laboratories. Threaded or breech-lock end closures can be used for test chambers having diameters in the 30 to 60 inch range. Wall thicknesses for the test chambers of these diameters range from 5 to 10 inches, depending upon the material and safety factor used. The split-ring type of seal previously shown can also be used on vessels having diameters as large as 48 inches. Another suitable seal is the Bridgman type shown in Figure 111. For this seal, the gasket does not need to be subjected to an initial pressure higher than the internal pressure it is to seal. The area of the gasket is smaller than the area of the piston under it, thus, the pressure on the gasket is at all times greater than the pressure in the chamber.

Large test chambers are those having diameters in the 7-to 12-foot range. Test chambers of this size are located at Navy laboratories and at some industrial laboratories. These test chambers are utilized in the testing of large models and hulls for oceanographic research vehicles. Breechlock end closures can be used for large-diameter test chambers that have operating pressures in the 3,000 to 5,000-psi range. For higher pressures, flanged and bolted or yoke-type end closures are more suitable.

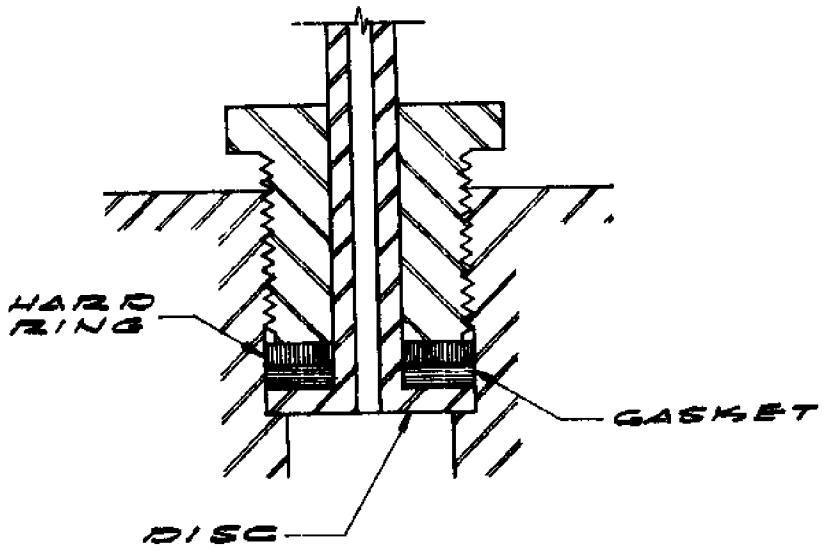

Figure 111 Diagram of a Bridgman Seal

III. PRESSURE TESTING HAZARDS

In hydrostatic pressure testing, care must be taken to eliminate all air from the system. If this is not done and a failure of the piping or test chamber occurs, the energy stored in the air can drive components of the system at high velocities. For the large size test chambers, i.e., those containing more than about 10 cubic feet of liquid and for pressures in the range of 10,000 psi, large motions of the test chamber and components of the pressurizing system will result if a failure of the test chamber occurs even though absolutely no air or gas is present in the system.

As an illustration of the hazard involved, when the screwed head of a two-foot diameter test chamber that was nine feet long failed abruptly at a pressure of 13,000 psi the entire vessel and its contents were propelled 20 feet vertically into the air.

Frequent inspection and testing of piping, hoses, valves, fittings, pressure gages, pumps, and the test chamber itself are necessary if accidents are to be kept to a minimum. The use of fluids with low compressibilities is also helpful in minimizing the hazard.

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of Complex Welded Structures Involving Metals of Low to Ultra-High Strength Levels," Naval Research Laboratory Report No. 6300, (June 1965).
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Discussion Following DeHart Paper

SAUNDERS: I'd just like to pass on some thoughts on these windows, to give you some numbers to work with. Someone mentioned optical qualities. When you start getting up in the high pressure ranges we found that you probably look at T over D ratios greater than or equal to about two. If you want to maintain this optical clarity, and an included angle of at least 90°, I would caution you to become very familiar with the failure modes of these windows. They do give you some indications; for instance, the optical clarity around the outer edge may start to go. When it starts extruding you should be way ahead of it, actually; you should know before it happens. Another thought is, try to arrange it so no direct viewing is ever done. This can be done quite simply, using mirrors, cameras, anything of this nature. One advantage in using optical methods is that you can reduce the size of your window, and consequently reduce the size of the insert in your pressure chamber, and substantially reduce the cost of fabrication.
DISTÈCHE: May I ask you if fiber optics have ever been used in high pressure vessels?
SAUNDERS: We have looked at fiber optics for control. This would mean that we could have introduced the hull penetrator, viewport and the electrical controls through the same area. But I'm afraid that's as far as we've gotten.
DISTÈCHE: That would be quite interesting, especially if you were looking in corners of the hull as for instance,where the animal is.
SAUNDERS: You have to put optics on it. One of the big problems you have is getting the bundle through the chamber walls.
DISTÈCHE: Well, you might not have to take the bundle through. In other words, you could use it in conjunction with a viewport, and have optics at the end to really focus.
VIDAVER: I have used very crude fiber optics to transmit light down to the edge of the pH electrode. It wasn't real fiber optics because it was simply a little button of plexiglass and another rod attached to it, and then glued to the side of a plexiglass cup, but the light did have to bend a little. It worked quite well, enough for photosynthesis to occur in different places in the chamber right under the meter at 100 atm.
SAUNDERS:We don't know if there is going to be any deformation of the bundle at higher pressures.
BRAUER: I have two questions. One on windows: How useful is the application of polaroid films in connection with periodic surveys of stress

relationships, extrusion liability, and failure liability. Has it been used? On the subject of crack failure: I think it would be extremely useful to many in this room if we could have the advice of some of our engineer colleagues about the testing procedures they use. Do you use penetrant dyes? How useful are x-ray techniques? If many of these things are going to be used, and we do not have decent testing programs, sooner or later some of these are going to produce a catastrophic failure. While I realize there is an over-population problem, it is not in the sciences. Could we have some advice on that?

DEHART: I think you're referring to photoelastic characteristics, using them for stress. The difficulty is that there are large residual stresses in plastic windows, and you don't have any baseline from which to measure stress increase, to tell whether things are in difficulty. The stress levels are usually quite low. It's rather an extrusion problem that's more severe. Our recommendation is to pressure test the window to 30-40% more pressure than you intend to use. That's the best procedure to guarantee safety.

BRAUER: Except that we have a cycling problem, don't we? All of our systems are subjected to multiple cycles through which these things are being carried, and I think it can't be enunciated strongly enough that if you operate with these things, unless you have regular, firmly established testing programs, in which these things are looked at periodically, you're sooner or later going to be in trouble.

DISTÈCHE: I think that it is possible to detect the deformation of a viewing port using a sensitive enough strain gage. There are now strain gages extremely sensitive to deformation.

DEHART: But the strains in a window that has a thickness to diameter ratio of one and a half to two are so small, that I don't think you could really use that as a means for detecting failure. Shear is more of a problem, but it's pretty hard to measure shear internally.

SAUNDERS: Have you ever examined the outer edges of a viewport that is starting to extrude? Do you find a sort of cloudiness or crazing?

DEHART: We did find some rather large striations. It is difficult to look through it, and it usually gives you a little bit of lead-time on it. We never found anything that was what we would call an abrupt, catastrophic failure. There was a large deformation before it let go. I agree with you that it's best not to use direct viewing at these high pressures, although I'm forced to do this in a submarine hull.

MENZIES: You took a picture of a failure. You said you had it under pressure for some time. How did you decide when you were going to take that picture?

DEHART: Well, there had been some other failures prior to this one. This was just luck. It was not in the precise instant, it was about 1/2 second after the flowing occurred and we knew it was going.

TEAL: In *Alvin*, when that swordfish stuck its sword into its side, you

might suppose he could have aimed for the window as one of the eyes. If he'd struck that straight on, what kind of shock can it take?
DEHART: Not very much. Shock resistance of plexiglass is not very good. It will fracture. Polycarbonate has much greater impact resistance, but we have found polycarbonate in 1/4 or 1/2 inch sheets, but nothing like 2-1/2 or 3 inches. You could possibly build it up in layers.
GORDON: We have obtained some 2 inch polycarbonate, but the optics were a problem. You said that none of the acrylics are very good above 25°C. What do you do if you want to work in the tropics with things that live at 30°C or more?
DEHART: But you don't find these higher temperatures at the depths?
GORDON: But there are lots of animals that migrate into these warm layers, or in the Red Sea and other places where there are quite high temperatures at depth, and there is a whole set of fascinating problems to be studied!
DEHART: If you are interested in higher temperature applications, I come back to a reinforcing technique, but I'd test it to a higher temperature than you propose to use.
GORDON: Are there any other materials than plexiglass to which you could turn?
DEHART: I don't know of any, unless it's glass, but that is a poor last resort.
GORDON: The second question—about lights. A lot of us are interested in observing these animals under pressure. Is it always better to have the lights outside—say two ports, one for the light and one to look through? Are there any lights you can build flush? How about heating?
DEHART: The outside lights are in a good state of development. We have tested EG and G lights at 6,000 psi very successfully.
GORDON: They don't give off too much heat?
DEHART: They get faintly warm, but you see there is the water around them.
GORDON: That's okay in the ocean, but how about other chambers?
(Question): You could use a 35 mm projector outside with a filter that abosrbs the infrared.
BRAUER: We are using simple automobile headlight bulbs inside the pressure envelope in a secondary pressure type housing. They give off some heat, but at our circulation rates this has been no problem.
ZOBELL: Regarding window materials for optical work—for several years, for our spectrographic analyses on relatively small volume systems, where the optical properties are of prime importance, we have been using synthetic sapphire which has been quite useful at pressures up to 1800 atm. Could you comment on the applications of these? Of course, they are economically feasible only for quite small windows, certainly less than one centimeter in diameter.
DEHART: No, I'm sorry.

DOGGETT: If your design called for an 8-inch thick window and you could only get the 4-inch plexiglass sheet, could you laminate two thicknesses of this to make your 8-inch window? Do you have any indication how lamination would affect the deterioration of such windows compared to single thicknesses?

SAUNDERS: We built one 5-inch window in two 2-1/2 inch layers, and it worked very successfully. We found that the bond between them behaves essentially like the material itself.

CORROSION BEHAVIOR OF HIGH-STRENGTH MATERIALS IN SEA WATER

by
W. W. Kirk*
The International Nickel Company, Inc.,
Francis L. LaQue
Corrosion Laboratory,
Wrightsville Beach, N.C.

Abstract

Various ocean environments near the surface and at the bottom are characterized with respect to factors that might affect corrosion of materials. Reviews of data obtained to date conclude that, for most materials, corrosion behavior in deep ocean environments is very much similar to that near the surface, so that the volumes of data collected in surface waters are valid for most ocean locations. The development of high-strength steels is reviewed, since high operating pressures in new aquarium systems are desired. Wire rope materials and research are also reviewed for interests in applications involving deep water sampling and capturing devices and chambers.

Finally, reference is given to corrosion products from various alloys and the resulting contamination effects in closed aquarium systems.This information leads to the logical consideration of materials resistant to corrosion in sea water.

Introduction

Material selection and design considerations for hardware that will handle or be operated in sea water require a knowledge of the corrosion behavior of many metals and alloys and the environmental factors that can affect that behavior [1].

The corrosion of materials in marine environments has been recognized for hundreds of years, but not until the past fifty years were any systematic evaluations conducted that led to the useful documentation of results [2]. Much of that early work was based upon the impetus of military needs for better performing materials. This prompted active participation and support from many Navy research and development personnel. The metals producers obviously became involved, since this new research led to the development of new alloys and a pressing need to evaluate the performance of existing materials in sea water and the chloride-bearing atmosphere.

The International Nickel Company, Inc. undertook many longrange corrosion programs[3] and established a laboratory specifically for the study

of marine corrosion. This facility, the Francis L. LaQue Corrosion Laboratory, is shown in Figure 112 as it now exists at Wrightsville Beach, N.C. The extensive marine atmospheric test lots operated as a part of this laboratory at Kure Beach, N.C. are shown in Figure 113.

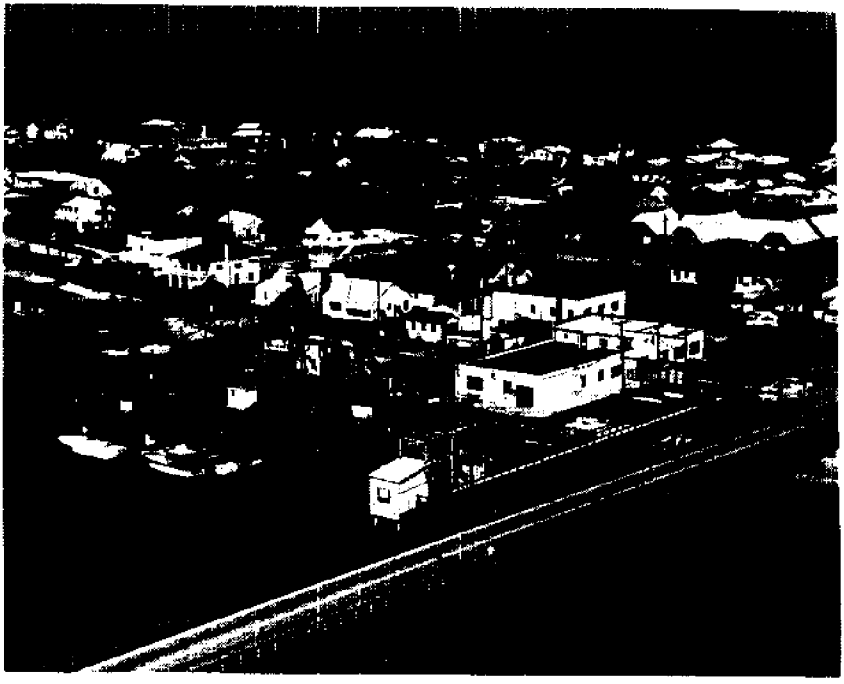

Figure 112 Francis L. LaQue Corrosion Laboratory at Wrightsville Beach, N. C.

The first of these testing programs in natural sea water involved ship hull, shafting, propeller, piping, and piling materials [4,5,6,7]. Continuously changing requirements and new applications demanded newer and better materials until, even today, better performing materials for these same applications and many others still are being sought. The corrosion researchers accepted the objectives of guiding research in alloy development by more extensive electrochemical and metallurgical research. Equipment such as the polarization test cell shown in Figure 114 has been used to measure corrosion potentials and the polarization characteristics of many materials [2,8].The galvanic series of metals and alloys in sea water as given in Table I was derived with this type of apparatus. This work is expanding to apply known corrosion principles to develop new alloys, new techniques and new ideas that will be useful in ocean exploration in and above surface water, as well as in the deep ocean.

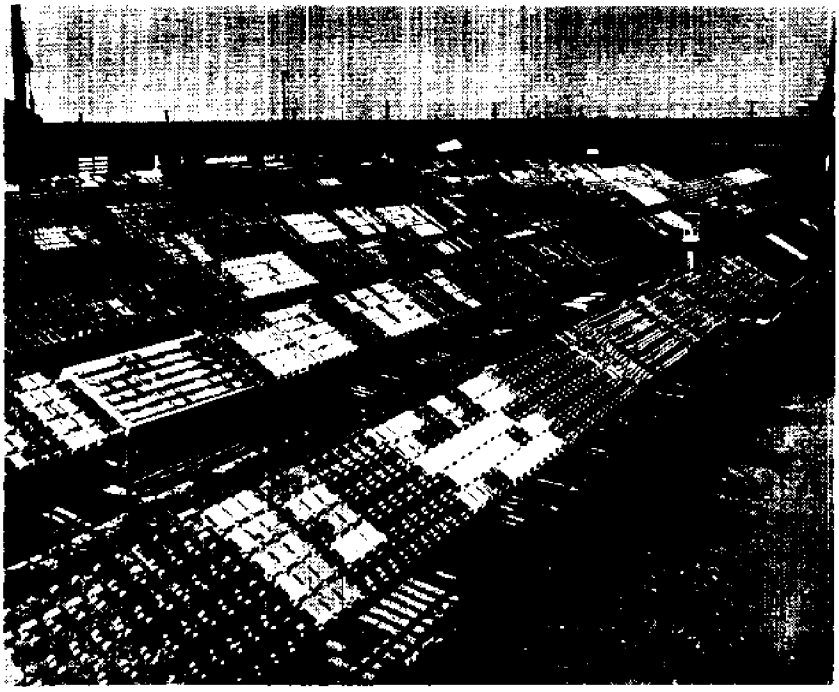

Figure 113 Marine Atmospheric Test Lots at Kure Beach, N. C.

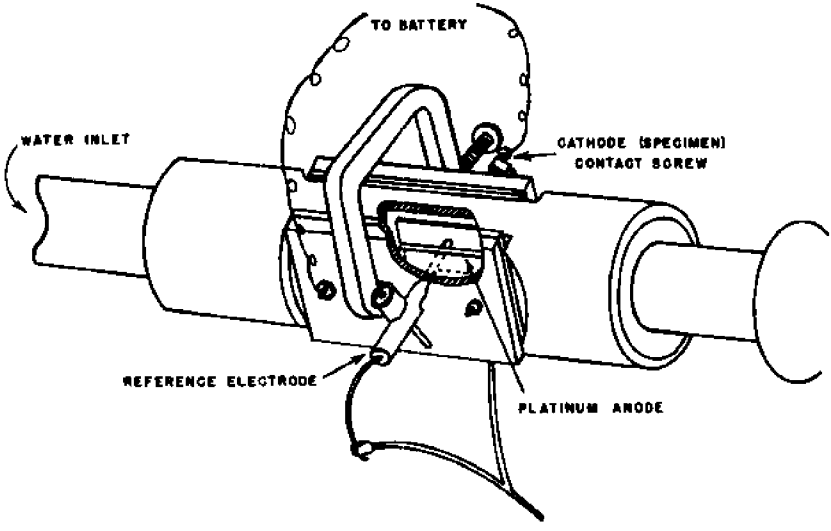

Figure 114 Schematic Diagram of Polarization Test Cell.

Table I

Corrosion Potentials in Flowing Sea Water
(8 to 13 ft./sec.) Temp. Range 5-28°C

Alloy	Volts, vs. Sat. Calomet Half-cell**
Magnesium	0.98-1.30
Beryllium	0.96-0.98
Aluminum Alloys	0.76-1.00
Cadmium	0.70-0.74
Mild Steel, Cast Iron	0.60-0.71
Low Alloy Steel	0.58-0.62
Austenitic Ni Cast Iron	0.43-0.53
Aluminum Bronze	0.31-0.41
Naval Brass, Yellow Brass	0.30-0.40
Tin	0.31-0.33
Copper	0.30-0.37
Pb-Sn Solder	0.28-0.36
Admiralty Brass, Al Brass	0.27-0.35
Manganese Bronze	0.27-0.33
Silicon Bronze	0.26-0.29
Tin Bronze	0.24-0.32
Stainless Steel-Types 410, 416*	0.25-0.35
Nickel Silver	0.24-0.28
90-10 Copper-Nickel	0.22-0.28
80-20 Copper-Nickel	0.21-0.27
Stainless Steel-Type 430*	0.20-0.27
Lead	0.19-0.24
70-30 Copper-Nickel	0.17-0.23
Nickel-Aluminum Bronze	0.14-0.22
Nickel-Chromium Alloy 600*	0.13-0.17
Silver Braze Alloys	0.10-0.20
Nickel 200	0.10-0.20
Silver	0.10-0.15
Stainless Steel-Types 302, 304, 321*	0.05-0.10
Nickel-Copper Alloy 400	0.03-0.13
Stainless Steel-Type 316*	0.00-0.10
Alloy "20" Stainless Steel	0.05-(-0.03)
Nickel-Iron Chromium Alloy 825	0.04-(-0.02)
Ni-Cr-Mo-Cu-Si Alloy "B"	0.04-0.01
Titanium	0.06-(-0.04)
Ni-Cr-Mo-Alloy "C"	0.09-(-0.03)
Platinum	0.25-0.19
Graphite	0.30-0.20

*Alloys in low-velocity sea water may exhibit a potential near -0.5V.
**All values negative except where indicated as positive.

Factors Affecting Corrosion in Sea Water

The constituents and properties of sea water and local conditions under which it may be found govern the corrosion performance of metals and alloys in that water. Each of these must be understood, measured and documented before one can predict the performance of any metal or alloy or combination of materials in sea water. Comments concerning the more important factors that can affect corrosion of metals and alloys in sea water are as follows:

1. Oxygen

Oxygen dissolved in sea water plays a role in most corrosion reactions. It is believed that many materials would not corrode in the complete absence of dissolved oxygen in the sea water. This condition rarely occurs in nature, and it has not been found practical to eliminate all oxygen from sea water in industrial processes. Less than one part per billion of dissolved oxygen in sea water has been shown sufficient to sustain corrosion reaction on some materials [9].

2. Temperature

An increase in water temperature generally increases the rate of reactions, and such effects on corrosion reactions are governed by the laws of thermodynamics. When corrosion is controlled by the diffusion of oxygen to a corroding surface, the corrosion rate for steel has been found to double with approximately every 30°C rise in temperature[10]. Such effects cannot be generalized, however; and certain temperature phenomena have been found quite complex and to vary from one material to another.

Temperature effects can be indirect also, such as the effects that may occur on the life and growth of marine organisms and upon the amount of oxygen or other gases that may be dissolved in the water.

3. pH

Sea water is a naturally buffered solution, so there is usually not a significant change in pH under natural conditions. Usually any change from the normal range of about 7.5 to 8.4 is associated with a departure of other environmental variables from ambient conditions.

4. Salinity

Natural sea water rarely varies out of the range of 32-36 ppt salinity at any location in the world except, of courese, in harbors or estuaries, where fresh water streams may create local dilution. Variations in the small ambient range given have no effect on corrosion reactions.

5. Water Velocity

This factor can be significant in that an increase in sea water velocity can increase the corrosion of active materials such as steels [11]. Conversely, an increased water velocity can decrease the corrosion of passive materials, such as stainless steels. The latter materials, for instance, ususlly perform well at sea water velocities of 5 FPS or greater; but caution must be

exercised in the use of such materials in stagnant or slowly-moving sea water, where crevice corrosion or pitting can occur.

6. Fouling

The attachment and growth of marine organisms on metal surfaces can be protective to some degree on uniformly-corroding materials. On passive materials, however, it can shield local areas, break down the protective or passive films and induce severe pitting or concentration cell corrosion, similar to that shown in Figure 115.

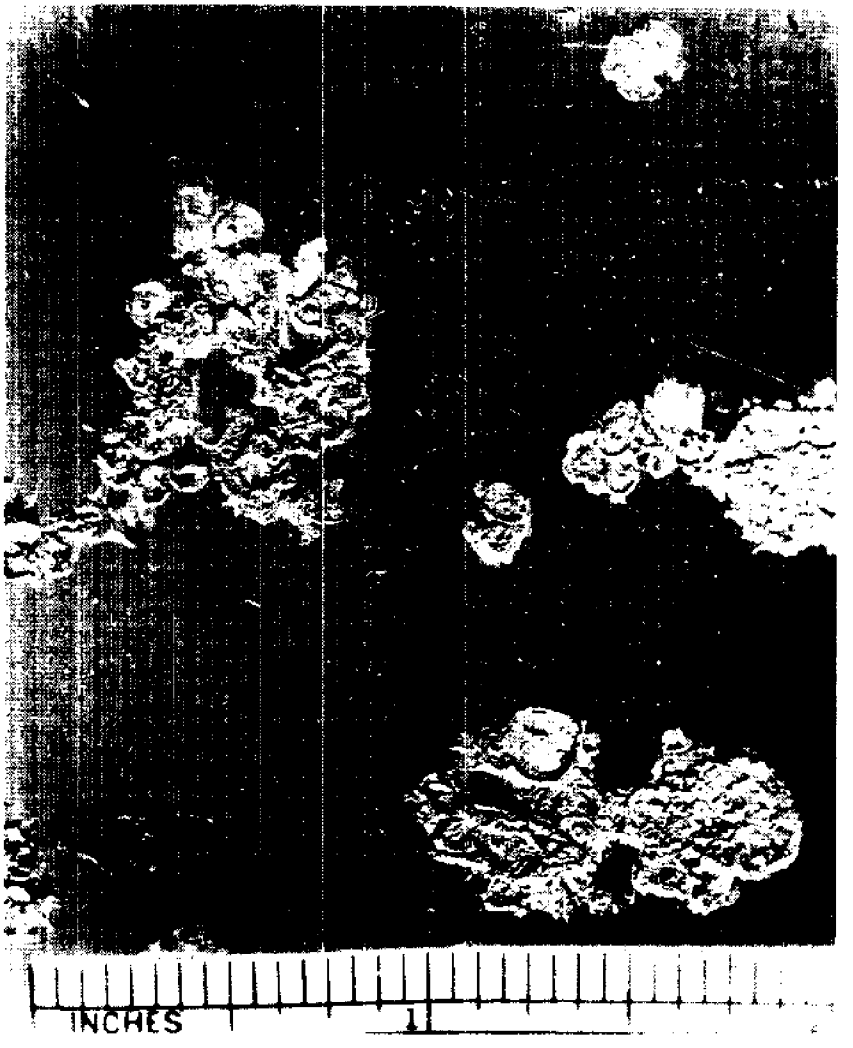

Figure 115 Concentration Cell Corrosion on Type 304 Stainless Steel Caused by Attached Marine Organisms.

7. Contamination

Any alteration of the chemistry of sea water may be expected to affect corrosion reactions in some way. Such effects have been encountered where water is obtained from a polluted harbor or possibly in or near bottom sediments. The primary contaminants that affect corrosion reactions are sulfides, ammonia, and carbon dioxide.

8. Galvanic Contacts

Any electrical contact between two or more dissimilar metals in sea water can alter their normal corrosion potentials and, therfore, their expected corrosion behavior. A galvanic couple involving two materials close together in the galvanic series in sea water, as shown in Table I, would usually not cause any accelerated corrosion of either member of the couple. A contact between two materials far apart in this series, however, can result in accelerated corrosion of the more active member, often to a very large extent. Such an effect is shown by the data in Table II, where the galvanic

Table II

Galvanic Corrosion of Steel in Contact with Different Metals in Sea Water [1] at 10°C for 16 Days

Couple	Corr. Rate of Steel MPY[2]	Galvanic Effect MPY
Steel to steel	30[3]	—
Steel to copper	96	66
Steel to 18-8 stainless steel	35	5
Steel to titanium	41	11
Steel to nickel	111	81

(1) Sea water velocity of 7.8 feet per second.

(2) MPY: mils penetration per year.

(3) This corrosion rate is high, since the test was conducted for a short period. A corrosion rate of 5 MPY is generally accepted as typical for carbon steel in sea water.

effect, or increase in corrosion rate, of carbon steel is shown when the steel is galvanically coupled to an equal area of several other materials. Small anode-to-cathode area ratios would accelerate corrosion of the anode even more. It is interesting to note that the galvanic effect on steel when coupled to 18 Cr-8 Ni stainless steel or to titanium is very small. These two materials polarize very easily in sea water and, therefore, cause less of a galvanic effect on the anodic member of the couple than might be expected by their relative positions in galvanic series. Various tables [12] and indicators have

been devised to assist the designer and materials selection engineers to understand better the expected performance of certain materials and material combination in sea water.

Characterization of Environments

The properties of sea water have been measured and documented at many sites throughout the world, including shoreside laboratories as well as in the open ocean. Such data measured at one of the corrosion testing sites used by the U.S. Naval Civil Engineering Laboratory in the Pacific Ocean off the coast of California are shown in Figure 116[13]. It is interesting to note the minimum in dissolved oxygen content found at this site at a depth of 2500 feet (800m). This minimum does not appear to be typical of all deep ocean sites, since investigations at the Tongue of the Ocean[14] have found that the oxygen level near the bottom did not vary from that near the surface at the same site.

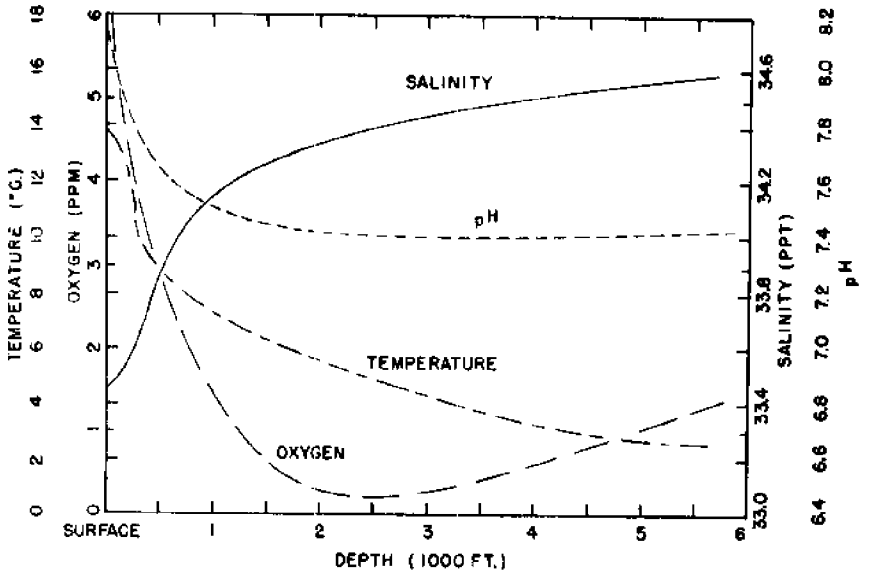

Figure 116 Hydrology Data from Pacific Ocean Sites[13].

Other characteristics of the ocean which are of interest in research efforts include, of course, pressure, which may reach 1000 atmospheres in the deepest locales of the ocean. Water velocity near the ocean floor is generally recorded at less than 0.1 knot compared to variable velocities up to several knots near the surface. Fouling organisms growing on solid surfaces in deep water at most sites have been found to be very slight, compared with normally heavy growths of organisms in surface waters.

Bottom sediments can differ widely in their chemistry and mechanical and physical properties and, therefore, affect corrosion in different manners. In some areas bottom sediments have been found to contain rather high levels of hydrogen sulfide, excluding oxygen from the local environment and having some adverse effects on the corrosion behavior of many materials.

Comparison of Corrosion Results in Deep Ocean and Surface Waters

Most of the deep ocean corrosion studies to date have been conducted by the U.S. Naval Civil Engineering Laboratory and the U.S. Naval Research Laboratory. Other laboratories have been invited to expose samples on these deep ocean corrosion racks, and most of the data have been published along with observations made from each of the test sites [13]. Typical corrosion data for several materials exposed in the Pacific Ocean at several sites and for several different times are shown in Table III and are compared with

Table III

Corrosion Rates of Materials
in Deep Sea Water
MPY [1] for Exposures (Days)
in Pacific Ocean

Material	123	197	403	751	1064	MPY at Wrightsville Beach (2)
Mild Steel	2.0	1.7	2.3	0.8	0.9	5.0
Ductile Iron	3.2	2.1	3.2	1.0	0.7	5.1
90-10 Cu-Ni	0.8	0.8	0.6	0.6	0.7	0.3
70-30 Cu-Ni	1.1	0.9	1.2	0.9	0.6	0.2
"G" Bronze	0.4	0.2	0.7	0.7	0.3	0.3
Zinc	5.9	2.3	5.9	3.6	0.7	0.7
Depth, Ft.	5640	2340	6780	5640	5300	Surface
Temp., °C	2.8	7.2	2.7	2.3	2.5	5-30
0_2, PPM	1.2	0.6	1.7	2.1	1.8	5-10

(1) MPY: mils penetration per year.
(2) Exposure for one year.

corrosion rates in the surface water at Wrightsville Beach. For the corrodible materials such as mild steel, corrosion rates found in the deep ocean are slightly lower than that found in the surface water. This result is probably attributable to the lower temperature and lower oxygen found near the floor of the ocean. For the copper-base alloys, however, very little difference can

be seen in the corrosion behavior near the ocean floor and at the surface. The corrosion rate of zinc varied considerably from one deep ocean site and exposure time to another, but the long-term rate is identical to that measured in surface water.

Results from an inspection of materials for localized corrosion in deep sea water are shown in Table IV. All the materials listed have some degree of susceptibility to localized corrosion. Obviously, Incoloy* alloy 825 and Carpenter** alloy 20Cb-3 in this list have the greatest resistance to localized corrosion in sea water. The results shown are very much typical of the performance of the same materials, found on numerous occasions in surface sea water. One notable exception, however, is the increased corrosion noted for aluminum alloys in deep sea water. Several of the 5000 and 6000 series aluminum alloys have good resistance to corrosion in shallow waters but have been found to suffer very severe crevice corrosion or pitting in deep sea water [15].

Table IV

Localized Corrosion of Materials
in Deep Sea Water

Material	Max. Depth of Pits or Crevice Corrosion (mils) for Different Exposures (Days)				
	123	197	403	751	1064
410 S.S.	50(p)	50(p)	50(p)	50(p)	50(p)
304 S.S.	Inc.	Inc.	2	Nil	Inc.
316 S.S.	Nil	Nil	Inc.	Nil	1
Carpenter [1] alloy 20Cb3	Nil	Inc.	Inc.	Nil	Inc.
Incoloy [2] alloy 825	Nil	Inc.	Inc.	Nil	Inc.
Monel [2] alloy 400	5	7	40(p)	40(p)	40(p)
Aluminum alloy 5052	70(p)	70(p)	70(p)	70(p)	70(p)
Depth, Ft.	5640	2340	6780	5640	5300
Temp., °C	2.8	7.2	2.7	2.3	2.5
0_2, PPM	1.2	0.6	1.7	2.1	1.8

(p)-perforated.
Inc.-incipient.

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(2) Registered trade marks of The International Nickel Co., Inc.

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In addition to the corrosion rates and penetration depths, the pattern of corrosion on most materials in deep sea water is also typical of that found in surface water. In spite of the low oxygen levels near the bottom, localized corrosion on passive materials, which is often dependent upon oxygen concentration cells, proceeds at relatively rapid rates.

These comparisons emphasize the fact that results from surface sea water studies appear to be valid for most deep ocean use. One can logically base design and material selection for a wide variety of marine environment applications on corrosion data obtained in the much less costly surface sea water programs. This statement does not imply that the extensive deep ocean tests conducted to date are not of extreme importance and value. Obviously the environment in which much of the ocean research, exploration, and exploitation of future years will take place must be characterized. This research also led to the development of unique and successuful emplacement and retrieval techniques for ocean floor experiments.

High-strength Materials

High-pressure research involved in deep ocean retrieval chambers and high-pressure aquaria require stronger materials or extremely thick wall construction. Several reviews of the development of very high-strength steels have been published recently [16,17,18]. Some of the materials studied in marine corrosion programs are identified in Table V. These are all corrodible steels as shown by the corrosion rates in quiet sea water given in Table VI. It should be noted that, in spite of the relatively high alloy content of the maraging steel, the resulting sea water corrosion behavior is only slightly better than that of mild steel.

Table V

Identification of High-strength Steels
Used in Sea Water Corrosion Tests

Alloy	0.2% Offset Y.S., KSI	Nominal composition							
		Ni	Co	Cr	Mo	Ti	Al	C	Mn
18 Ni maraging	200	18	8	-	3	0.15	0.08	0.01	0.06
steel	255	18	8	-	5	0.40	0.08	0.02	0.05
HY80	95	2	-	1.3	0.3	-	-	0.15	0.25
4340	240	2	-	0.8	0.3	-	0.06	0.35	0.75

Table VI

Corrosion of High-strength Steels in Quiet Sea Water of Temperature Range 5-28°C

Alloy	Corrosion Rate, MPY [1] 6 Mo	1 Yr.	3 Yrs.
18 Ni maraging steel	2.4	2.5	2.0
HY80	2.6	3.7	3.6
4340	2.8	4.3	4.8
Mild steel	5.0	4.0	3.0

(1) MPY: mils penetration per year.

The harmful effect of increased sea water velocity on corrodible steels was previously referenced. This fact is confirmed by the corrosion rates shown in Table VII. In sea water moving at only two feet per second, corrosion of all alloys was significantly greater than in quiet sea water. This deleterious effect continues with further increases in water velocity, with prohibitively high corrosion rates (100 mils per year) suffered at high tangential water velocities over 100 feet per second. The stainless steels are virtually untouched at velocities of this magnitude.

A problem identified in the development of high-strength steels is the increasing susceptibility to stress corrosion cracking with increased material strength levels [19]. The cracking behavior of maraging steels in marine environments has been covered in the literature [18]. Some results from the exposure of stressed U-bend specimens are given in Table VIII. Of those materials listed the 18% Ni maraging steel at a yield strength level of 200 KSI exhibits the most useful stress corrosion cracking resistance at a relatively high strength level. This characteristic, along with the alloy's

Table VII

Corrosion of High-strength Steels in Sea Water Flowing at 2 Ft./Sec. (Temperature Range 5-28°C)

Alloy	Corrosion Rate, MPY [1] 6 Mo.	1 yr.
18 Ni maraging steel	11	7
HY80	-	12
Mild steel	12	9

(1) MPY: mils penetration per year.

Table VIII

Stress Corrosion Cracking Behavior
of U-bends Immersed in Sea Water
of Temperature Range 5-28°C

Alloy	0.2% Offset Y.S., KSI	Total Testing Period, Days	Failure Ratio*	Days to Failure
18 Ni maraging steel	200	1100	0/2	-
	255	-	2/2	90-97
HY80	95	180	0/6	-
4340	240	-	2/2	6

*Ratio of number of specimens failed to number exposed.

other useful mechanical properties and fabricability, renders this alloy an attractive choice for a wide variety of marine application. Furthermore, the alloy can be protected from corrosion and cracking by conventional cathodic protection methods, using either impressed current or galvanic anodes.

In any review of the corrosion behavior of high-strength steels, the corrosion fatigue characteristics should be pointed out. Results from rotating beam tests at a cyclic frequency of 1400 RPM in sea water are shown in Figure 117. These data indicate a corrosion fatigue strength at 100 mc of only 5000 psi for the 18% Ni alloy. For comparison, the corresponding

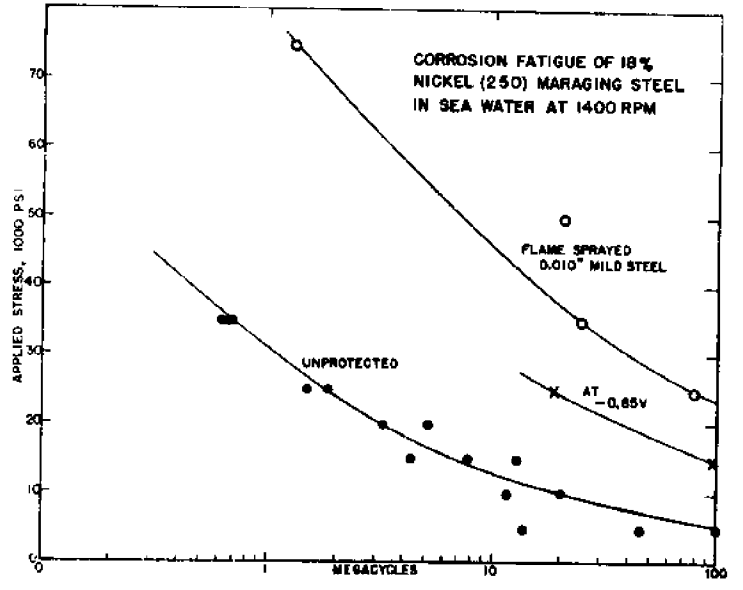

Figure 117 Corrosion Fatigue of 18% Nickel Maraging Steel in Sea Water.

corrosion fatigue strength can be obtained through use of cathodic protection (at -0.85 V. vs. a saturated calomel half-cell), but the strength achieved still remains at only a small fraction of the useful mechanical strength of the alloy. A further slight improvement was gained with a flame sprayed steel coating, offering both cathodic protection and a barrier to corrosion of the base alloy steel.

These corrosion data illustrate that, for most applications where steels are in direct contact with sea water, some degree of protection other than simple coatings would be necessary. It is not known whether conventional cathodic protection schemes would be successful under high-pressure conditions. Perhaps the specific vessel designs and requirements would dictate the type of protection needed, should steels be used for construction.

Wire Rope Materials

Another area of corrosion research that should be mentioned is the evaluation of wire ropes that may be used to lower and retrieve instruments and vessels in the ocean. In recent years oceanographers have utilized every known metallic and non-metallic rope construction for both working and mooring applications [20,21]. The sea has wrought its toll, as few ropes have survived even a year of exposure in sea water, Many have failed in 30 days or less, leaving expensive equipment on the ocean bottom. These rapid failures of marine wire rope at sea promoted a major sea water test program on marine wire rope materials [22].

A constant load test device designed to test wires or small diameter strands or wire ropes is shown in Figure 118. Several such units installed in a single tank of sea water permit a simultaneous comparison of several different wire materials and/or several different stress levels. Data from such tests have been useful in screening alloys and protective schemes to increase wire rope life.

Results of these sea water tests on several marine wire rope materials are summarized in Table IX. These observations indicate that zinc and aluminum sacrificial type coatings are most effective in the bottom mud and in quiet sea water, but that any water motion sharply reduces their useful life. The uniformity of the coating and its thickness appear to be directly related to the service life of the wire rope in sea water. Once the zinc or aluminum cladding has corroded away, exposing the bare steel wire beneath, general rusting begins and the rope is virtually at the end of its useful life.

The 90-10 Cu-Ni cladding was found to provide only minimal protection to the Type 304 stainless steel wire below the water line. This wire, with or without cladding, however, has been found useful for applications above the water line.

STRESSED WIRE CORROSION TEST

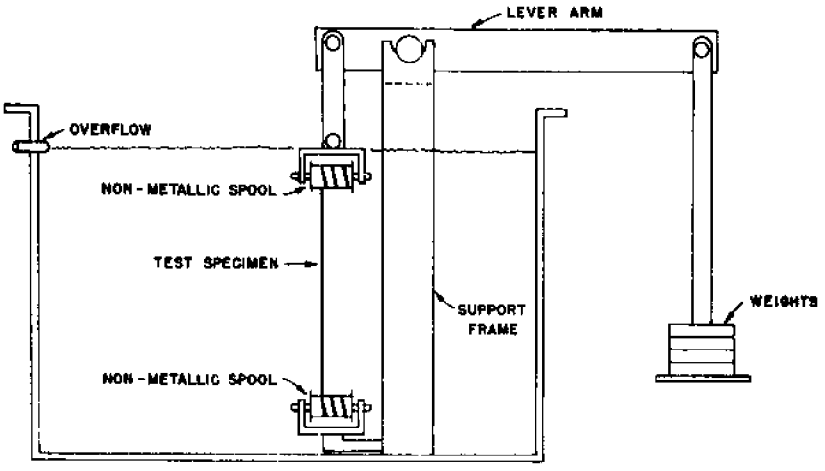

Figure 118 Stressed Wire Test Apparatus.

Table IX

Life[1] of Wire Strand and Wire Rope in Sea Water Tests

20-100 mil diameter wires stranded into rope

Coating or Cladding			Life in Months			
Wire	Type	Thickness in mils	Quiet S.W.	Flowing S.W. 2 Ft./Sec.	Splash Zone	Sulfide Mud
IPS[2]	Zn	.3-.5	12	6	12	12
IPS	Zn	.5-1	24	12	18	24
ISP	Al	.3-.5	24	9	12	12
IPS	Al	.5-1	36	12	18	24
304 S.S.	90-10 Cu-Ni	.2-.4	9	6	60	6
304 S.S.	90-10 Cu-Ni	.5-.8	24	6	60	6
304 S.S.	None	-	12	6	60	3

(1) Time to initiation of significant corrosion of base metal.
(2) Improved plow steel.

These evaluations indicate that some of the existing materials would be useful for short terms in sea water, but that more durable wire rope alloys or positive protection will be required for long-term immersion. It has also become evident that service failures due to mechanical factors occur well before the predicted corrosion lives as determined by laboratory tests. For this reason the users and manufacturers of wire rope are concurrently developing wire rope constructions that will reduce twisting, spinning, kinking, unlaying, and other mechanical problems with both instrument and mooring-type wires.

Corrosion Products

Analyses of corrosion products on many different materials exposed in sea water have been conducted. These materials include most of the commonly used alloys such as steels, stainless steels, and alloys of nickel, copper, aluminum, and zinc. All of these develop oxides, hydroxides, and chlorides along with traces of sulfides, phosphates, and sulfates. Complexes of these anions, such as oxychlorides and hydroxychlorides, are also frequently identified. Metallic cations, of course, are found also in all of these corrosion products.

These various constituents found in corrosion products can contaminate sea water often to an intolerable extent, depending upon the application in question. Toxicity levels of many constituents of sea water are now being determined with an attempt to arrive at maximum levels permissible for biological purposes. Significant levels of many metallic and non-metallic ions are measured in natural sea water; but what levels may affect biological demand, organic chemistry, or electrochemistry are not known to date. Owing to the variations of the minor constituents in sea water, much of the aquarium and biological laboratory work conducted to date has made use of synthetic solutions rather than natural sea water. For the retrieval of deep ocean organisms and their transfer to laboratory aquaria, however, it may be desirable to have a supply of clean, fresh sea water available.

Corrosion-resistant Alloys

Sufficient evidence has been accumulated to demonstrate that extreme care must be exercised in the selection of materials for high-pressure aquaria and deep ocean retrieval chambers. These applications require a combination of good mechanical properties, fabricability and excellent corrosion resistance of the materials. Recognizing these needs, a major alloy development and corrosion research program has been underway in the laboratory to develop alloys with virtual immunity to corrosion in sea water. Many of these are identified in Table X. All of the alloys listed in this table possess excellent corrosion properties in marine environments, although there are environmental alterations that can destroy the corrosion immunity

of some of these materials. Additional experimental alloys are now in the laboratory, being developed for specific applications in marine environments.

Table X

Corrosion Resistant Alloys

Alloy	Nominal Compositions					
	Ni	Cr	Mo	Co	Fe	Others
Inconel[1] alloy 625	Bal.	21	9	1	5	4 Cb
Inconel alloy 718	Bal.	19	3	1	19	5 Cb, .9 Ti, .5 Al
Hastelloy[2] alloy C-276	Bal.	16	16	1	5	.004 C, 4 W
Elgiloy[3]	15	20	7	40	Bal.	--
MP-35N	35	20	10	35	-	--
Rene[4] alloy 41	Bal.	19	10	12	.5	1.5 Al, 3 Ti
Ti alloys	- - Various Alloys - -					

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(2) Registered trade mark of Cabot Corporation.
(3) Registered trade mark of Elgin National Industries.
(4) Registered trade mark of Allvac Metals, A Teledyne Co.

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Discussion Following Kirk Paper

CARR: By trade I'm really a chemical oceanographer who works in trace elements, and I can assure you that some simple-minded calculation, using Kirk's figures, indicate that iron, and chromium, and cobalt, and some of these minor constituents in the metal under still water corrosion rates will load a small vessel, such as I think you are interesed in working with, at an unbelieveable rate. In fact, it loads it so fast that even a relatively insensitive detector like our atomic absorption spectrophotometer can be used directly

on a 1 cc water sample drawn from the chamber, so that there's no question that this is extremely serious. There is one good side to it. I think if we can promote some cooperation between the biologists and the chemists for once—if we can ride piggy-back with you and do sampling, where you also pull your samples, so we can characterize the *in situ* environment, we'd then be able, very easily, to monitor it for you when you brought it back to the laboratory and started to draw it. I don't think it's necessary to belabor the point. The equipment is there, and the techniques are available. They're pretty standard, and things such as neutron activation require a cubic centimeter sample, so we're not going to run your aquarium dry for you either while we're helping. I think it's a problem that could produce good results for almost all concerned.

MENZIES: Could you give us some idea of the relative ease of maching some of these nickel alloys?

KIRK: Not very well. As I said, some of these are at various stages of development. I think most of the ones that I showed are available in some form or shape today. As to their machinability, weldability, fabricability, for any particular alloy, I think I could give you appropriate names that you might contact, if you would like to find more information about it.

VIDAVER: Can you say anything about the effect of the surface of the material on corrosion rates? Contrasting highly polished surface with a relatively rough machined surface.

KIRK: In water, I don't think there would be a great deal of difference on most materials. When you come down to some of the detailed questions of possibilities of the initiation of stress corrosion cracking, or fatigue cracking, then yes, I agree with the previous speaker that the degree of surface roughness can have a very significant effect. What roughness factor would apply for a given application, I just couldn't say because I think it would depend on what you want to do with it. Probably the smoother the surface the better the behavior you're going to get in general. But you have to hit a happy medium and not go overboard with maching costs, to get an extremely polished surface, if you don't need it.

GORDON: Are there any readily applied, easily replaced, very inert coatings that you can put on some of these surfaces that will stand lots of cycles up and down?

KIRK: I wish I could say "yes" but I'm afraid that I can't. I don't know of any.

GEORGE: I performed a number of test panel experiments in tropical warm water, using steel cages, and the major failure was corrosion. As you pointed out, stainless steel is not stainless steel in sea water. Are you applying any kind of greasing to protect the franes?

KIRK: If you are talking about stainless steel, I would suggest you attach a couple of carbon steel anodes to your frames, just bolt them on. Depending on the size of your frame or its geometry you'll probably want to attach

more than one, but the increased or accelerated corrosion rate of carbon steel when coupled to stainless steel is very slight, so that if you could put a piece of steel in sea water and have it last for the length of time you want, just choose that thickness and bolt it to your stainless steel frame and you'll be all right. I would not coat it, though.

KALBER: Our work is entirely in closed systems, some under high pressure and some not, in large and small volumes for long and short times. The comment that Mr. Kirk has made about the problems of accumulation of potential toxins in organic and inorganic corrosion products is a very considerable one to us. I would like to make a suggestion to those of you who anticipate doing work and having to deal with corrosion by-products, particularly for long periods of time: It's been successful in our laboratories. One of these has been with biological filters which operate very nicely in closed systems. Second, what we call autotrophic conditioning, using photosynthetic organisms, mainly green plants, for the purpose of removing undesirable products. As Mr. Kirk pointed out, they have an uncanny ability to remove things, some of which we want them to remove, and we find out that some species, and some species combinations, are highly successful here. They will work under pressures. We have used them from 300 to 1,000 psi. A third possibility is, of course, ion exchange. We have used this selectively to remove some of our problem ions. In making up our sea water, for instance, it comes especially high sometimes in iron and copper, and so we process this automatically. It is possible to do this in relatively large volumes and recirculate it in a thousand gallons and over daily.

(Question) There seems to be an even more obvious solution to some of these vessels which are usually cylindrical, and it's just a simple lucite sleeve. Diffusion is kind of slow, and it would take an awful lot longer to accumulate the iron in the area where it counts.

KIRK: As far as corrosion resistance is concerned, particularly if you are interested in either an extremely heavy walled vessel for high pressure use, or using one of these higher strength steels for the advantage of the high strength, but where the steel itself might corrode, there is one other possibility. I'm sure that this has been done using some sort of metallic overlay, weld overlay, of one of the corrosion-resistant materials, Kastalloy-C, for instance, or Inconel, You can weld overlay this to the cover or line your vessel with a corrosion-resistant material, but your strength is obtained from the high strength steel which is now not in contact with the water. This might be an economical solution to the corrosion problem, and still be permitted to use the attractive strength of levels of the steels.

GORDON: I think one of the problems with that kind of thing is that all the times the compressibilities of the coatings are very different from the compressibilities of the materials, and your vessels may creep, and you end up getting things in all sorts of odd places, and also the coatings are very uneven in thickness, and you can't get them into odd corners and all this

kind of thing. They just don't work.

KIRK: This is true, and of course, if the coating might be too thin, or should ever be damaged and expose even a little fingertip-size point of the steel, you're in trouble. This is a little anode that is going to try cathodically to protect your entire vessel, and it's going to corrode right through.

(Question) Just a point, a little bit off the subject. In what you have to say, Mr. Kirk, you indicated that you recognized fouling as a major corrosion source, or a corrosion effect. Now, I gather that most of the long term data you have shown us have been derived from your field-testing operations such as you have here. In what way do you allow for the corrosive effect of fouling mechanism?

KIRK: We don't. We just let the fouling occur naturally. I don't know whether I mentioned it or not, but on corrodable material, such as steels, for instance, some fouling organisms can be helpful in reducing corrosion, because they protect the surface, but on other materials, such as stainless steels, or the nickel-copper alloys which are susceptible to localized corrosion because of passive film breakdown, the fouling organisms can be very detrimental.

KANWISHER: It would seem to me that the variability that you found in rates of pitting of some of these strips could be explained just by fouling.

KIRK: Oh, yes, probably they could.

ZOBELL; A statement, perhaps of trivial interest. There has been some comment with regard to the appearances of fouling organisms at appreciable depths in the sea. I believe that a benthic biologist will agree that there are certain fouling organisms that occur at all depths in the sea. In this connection, it may be of historical interest to point out to you that on the *Galatea* Deep Sea Expedition the first invertebrate that was found at a depth greater than 7,000 meters was a barnacle on a stone about as big as two fists brought up from a depth of 10,260 meters in the Philippine Trench on July 21, 1951. While I'm on my feet, I would like to ask a question which I hope has more significance, that is, the effects of having unlike steels mated in the same equipment. Specifically, an instrument company, for example, on occasion has provided me with high pressure valves in which the body is made of one type of steel, the needle valve of another type of steel, and the handle of a third type of steel, all of which they sell for $85 as stainless steel.

KIRK: Stainless, or steel?

ZOBELL: Well, let us say steel, but I think it's usually specified that it is 18-8 type 300 steel. We've got into considerable difficulty with the corrosion of these things. You have to replace them at fairly frequent intervals. In correspondence with the company, I've got the information that maybe one was type 303, one was type 304, and then the handle may be a case-hardened steel or something like that. Now, mating these together,

would this have a galvanic effect? Would this contribute to the corrosion, to have these different alloys mated?

KIRK: They would be close enough in the galvanic series. With the possible exception, maybe, of a ferritic or a martensitic type of stainless steel of the 400 series, or some type other than the 300 series. This might have enough of a galvanic potential difference. But the 300 series mainly are so close together, that differences in their corrosion potentials can't be measured. We can't get them any more accurately than 25 millivolts, and they're that close. The odd thing, of course, about stainless steels, is as I've mentioned, they're easily protected—so easy to polarize. One pit on a relatively small surface of stainless steel is enough of an anode in itself to protect the rest of the surface. So that perhaps the less corrosion resistant stainless that you might have in a valve, if it begins to corrode a little bit and it's in electrical contact with all the other components, can cathodically protect them, where otherwise they might corrode also.

MACDONALD: What about titanium?

KIRK: Generally at room temperature or in a cold sea water, we have not seen any of the titanium alloys corrode. However, in the stress corrosion work that has been conducted over the past few years, particularly in the application of fracture-mechanics work to stress corrosion studies when initiating a notch or with a crack which may be present in the surface of a thick plate when it comes from the mill, titanium alloys are notoriously susceptible to crack propagtion in the presence of any corrosive environment. They'll do it in distilled water, in fact. So this, I think, is the problem there. General corrosion or pitting—no.

APPENDIX

This Appendix was assembled at the suggestion of Dr. Claude ZoBell who recommended that a summary of the key sources utilized by various laboratories might be useful to other workers, both those now active and those about to enter the field. This recommendation was unanimously adopted by the conference.

U.K. Suppliers (MacDonald)

Pressure pump—
Olin Mathieson Ltd., North Hylton Road, Sunderland, U.K.

Pressure transducers Type BP6—
Either-Hadyn Ltd., Caxton Way, Stevenage, Herts, U.K.

Long range stereobinocular microscope—
Meopta DM 23 (Czech).

Pressure fittings and gauges—
Pressure Products Industries Ltd., Stanley Green Industrial Estate, Cheadle Hulme, Cheshire, U.K.

Solenoid valves for pneumatic control Type AC.1403E—
Enots Ltd.,Aston Brook Street, Birmingham, U.K.
Dead weight tester—
Budenberg Gauge Co. Ltd., Broadheath, Manchester, U.K.

U.S. Suppliers of High Pressure Apparatus, Instruments, and Materials with particular reference to high pressure aquarium systems and barobiology (ZoBell)

1	Alleghany Plastics, Inc. Route 51, Thorn Run Road Coraopolis, Pa.	O-rings, Teflon bearings, sheets, tubes, etc.
2	American Instrument Company 8030 Georgia Avenue Silver Springs, Md. 20910	Barokams, Check Valves, Couplings, Fittings, Flexible hoses, French pressure cells, Gages, Line filters, Optical cells, Pressure balances,Pressure generators, Regulators, Rupture disks, Steel tubing, Tools, Valves
3	Atkomatic Valve Co., Inc. 141 South Sherman Drive Indianapolis,Indiana 46201	Solenoid valves
4	Autoclave Engineers, Inc. Box 4007 Erie, Pa. 16512	Barokams, Check valves,Couplings, Fittings, Pumps, Rupture disks, Steel tubing, Tools for preparing tubing, Valves
5	Beckman Instruments 2500 Harbor Blvd. Fullerton,Calif. 92634	All kinds of electrodes, Optical cells
6	Becton, Dickinson & Co. Rutherford, N.J.	Needle tubing
7	Benthos, Inc. Edgerton Drive North Falmouth, Mass.02556	Deep-sea corers, Pressure-testing chambers and service, Depth-recorder

8	Bethlehem Corporation Hyperbaric Oxygen Therapy Div. 225 Second Street Bethlehem, Pa. 18016	Hyperbaric chambers, Hyperbaric oxygenation
9	Cary Instrument Company 2724 S. Peck Road Monrovia, Calif. 91016	Optical windows
10	Chicago Rawhide Mfg. Co. O-ring Division 900 N. State Street Elgin, Illinois	O-rings, Synthetic rubber products
11	Conax Corporation 2300 Walden Avenue Buffalo, N.Y. 14225	Thermistors
12	Corborundum Company Electronic.Devices PlO. Box 337 Niagra Falls, N.Y. 14302	Thermistors
13	Enerpac Test Systems Butler, Wisconsin53007	Couplings, Gages, Pumps
14	Fred S. Carver, Inc. One Chatham Road Summit, N.J. 07901	Hydraulic presses, Gages
15	General Electric Company Medical Development Operation Dept. K, Bldg. 5, Room 105 One River Road Schenectady, N.Y. 12304	Permselective membranes
16	Harry Miller Corporation 4th and Bristol Streets Philadelphia, Pa. 19140	Hydraulic fluids and additives
17	Harwood Engineering Co., Inc. South Street Walpole, Mass.	Pressure intensifiers
18	Haskel Engineering & Supply Co. 100 East Graham Place Burbank, Calif.91502	High pressure hoses, Pumps, Switches

19 Heise Bourdon Tube Co., Inc. South Main Street Newtown, Conn. 06470	Gages
20 High Pressure Equipment Co. 1222 Linden Avenue Erie, Pa. 16505	Barokams, Check valves, Couplings, Gages, Pressure generators, Pumps, Relief valves, Rupture disks, Steel tubing, Tubing tools, Valves
21 Hoke Incorporated One Tenakill Park Cresskill, N.J. 07626	Couplings, Needle valves, especially for gases
22 Kistler Instrument Corporation 8989 Sheridan Drive Clarency, N.Y. 14031	Transducers
23 Leeds & Northrup Sunneytown Pike North Wales, Pa. 19454	Electrodes
24 Linde Air Products Co. 30 East 42nd Street New York, N.Y.	Optical windows
25 Marsh Instrument Company P.O. Box 190 Wilmette, Ill. 60091	Gages
26 Minnesota Mining and Mfg. Co. 367 Grove Street St. Paul, Minn. 55101	Fluorochemicals for gas reservoirs
27 Parker Appliance Company 17325 Euclid Avenue Cleveland, Ohio	O-rings
28 Parr Instrument Company 211 53rd Street Moline, Ill. 61265	Barokams,Reaction vessels
29 Plastic and Rubber Products Co. 2100 Hyde Park Blvd. Los Angeles, Calif.	O-rings

30 Pressure Products Industries Box 277 Hatboro, Pa. 19040	Barokams, Couplings, Gages, Line filters, Pressure intensifiers, Pumps, Steel tubing,Thermocouples, Tools for preparing tubing, Transducers, Valves
31 Quartz Scientific,Inc. 989 Commercial Street Palo Alto, Calif. 94303	Optical windows
32 Sno-Trik Company 31755 Aurora Road Solon, Ohio 44139	Fittings, Valves
33 Stupakoff Ceramics & Mfg. Co. Latrobe, Pa.	Electrical connectors, Kovar-glass terminals
34 Tem-Pres Research 1401 S. Atherton State College, Pa. 16801	Barokams, Pumps
35 Tubesales 175 Tubeway Forest Park, Ga. 30050	Steel tubing
36 United States Gauge Division of American Machine & Metals, Inc.	Gages
37 Vacudyne Corporation 375 E. Joe Orr Road Chicago Heights, Ill. 60411	Hyperbaric chambers
38 Victory Engineering Corporation Victory Road Springfield, New Jersey 07081	Thermistors
39 Wallace & Tiernan Division of Pennwalt Corporation 25 Main Street Belleville, New Jersey 07109	Gages
40 Wm. I. Mann Company 116 W. Foothill Blvd. Monrovia, California	Optical windows

41 Kahl Scientific Instrument Corp. P.O. Box 1166 El Cajon, Calif. 92022	Bacteriological samplers, Bottom corers, Reversing thermometers, Pressure-testing chambers

High Pressure Apparatus, Instruments, and Supplies with particular reference to high pressure aquarium systems and barobiology (Numbers in parentheses after each item indicate its source or supplier)

Accumulators (18)

Barokams (pressure chambers, cylinders, vessels, "bombs" etc., from baro-plus kamara, Greek for chamber) (2, 4, 20, 28, 30, 34)

Blowout disks (2)

Couplings (2, 4, 20, 30)

Depth-recorder (7)

Electrical connectors for barokams (33)

Electrodes, glass (5, 23)

Electrodes, ion-selective (5,23)

Electrodes, oxygen-sensing (5)

Electrodes, reference-cell (5)

Filters, line (20, 30)

Fittings, barokam (2, 4, 30)

Fittings, tube (2, 32)

Fluorochemicals (FC compounds for gas reservoirs) (26)

French pressure cells (2)

Gages, Bourdon (2, 4, 13, 19, 20, 25, 30, 36, 39)

Gages, Dead-weight (2, 30)

High pressure hoses (18)

High pressure testing devices (7)

Hydraulic fluids and additives (16)

Hydraulic presses (14)

Hyperbaric gas apparatus (8, 37)

Needle tubing, steel (6)

Optical windows, glass (40)

Special Sources of Supplies/Wrightsville Marine Bio-Medical Laboratory

BLH Electronics,Inc. Waltham, Mass. 02154 617-894-6700	Pressure transducers
Carolina Valve and Fitting Co. P. O. Box 15116 Charlotte, N.C. 28201 704-523-3747	Swagelok tube fittings, Whitey valves
Climax Molybelenum Co. One Station Plaza Greenwich, Conn. 06830	High pressure vessels
Electro Oceanics, Inc. 2000 S. Sante Fe Avenue Compton, California 90221 213-537-2430	Underwater electrical connections
Glenair, Inc. 1211 Air Way Glendale, California 91201 213-247-6000	Underwater connectors and cables
H.M. Harper Co. 8200 Lehigh Avenue Morton Grove, Ill. 60053 312-Y06-6000	Fasteners
Mecca P. O. Box 36993 5919 Jessamine Houston, Texas 77036	Single pin underwater connectors
Pelagic Electronics, Inc. 174aake Shore Drive East Falmouth, Mass. 02536 617-540-1200	Deep sea solenoids
Roylyn Inc. P. O. Box 3685, Grand Central Station Glendale, California 91201 213-245-8521	Gauges

Victor Equipment Co.
2336 Auburn Blvd.
Sacramento, California

Pressure Regulators

SOURCES OF REFERENCED PRODUCTS (KIRK)

The International Nickel Co., Inc.
Huntington Alloy Products Division
Huntington, West Virginia 25720

Elgin National Industries,
Elgiloy Co.,
853 Dundee Ave.,
Elgin, Illinois 60120

Carpenter Technology Corp.
P. O. Box 662,
Reading, Pa. 19603

Allvac Metals Corp.,
Teledyne-Allvac,
P.O. Box 470,
Monroe, N.C. 28110

Cabot Corp., Stellite Division,
1020 W. Park Ave.,
Kokomo, Indiana 46901

Standard Pressed Steel Co.,
Highland Ave.,
Jenkintown, Pa. 19046

OTHER MATERIAL AND PRODUCT INFORMATION SOURCES (KIRK)

Alloy Casting Institute
405 Lexington Ave.,
New York, N.Y. 10017

American Iron and Steel Institute
633 Third Avenue,
New York, N. Y. 10017

American Society for Metals
Metals Park, Ohio 44073

American Society for Testing and Materials
1916 Race Street,
Philadelphia, Pa. 19103

Copper Development Association
405 Lexington Ave.,
New York, N.Y. 10017

Metal Powder Industries Federation
201 East 42nd St.,
New York, N.Y. 10017

National Association of Corrosion Engineers
2400 West Loop South,
Houston, Texas 77027

Hi-Pressure Penetrator Vendors (Saunders)

Viking Industries
Chattsworth, California

Bendix
Sidney, New York

Burton Electrical Engineering
El Segundo, California

Electro Oceanics Inc.
Compton, California

Hi Rel Connectors Inc.
Irwindale, California

Gulton Industries, Inc.
Newport Beach, California

D.G. O'Brien Inc.
Framingham, Mass.

Vector Cable Company
Houston, Texas

Conax
Buffalo, New York

Joy Manufacturing Co.
New Philadelphia, Ohio

Rodchester Corp.
Culpepper, Virginia

Kemlon connectors
Houston, Texas

Kintec, Inc.
Chattsworth, California

Sources for Special Equipment Used in CIT High Pressure System (Westphal)

316 stainless high pressure valves-
P23-406 10,000 PSI, Circle Seal Co., Anaheim, Calif.

Constant pressure fixed displacement water/oil pump-
Model 5216-CS-10055, Sprague Engineering Co., Gardena, Calif.

CHAPTER FIVE

PRESSURE EFFECTS ON MARINE INVERTEBRATES IN AN OPEN-SYSTEM HIGH PRESSURE AQUARIUM*

by
H. A. Lowenstam and James A. Westphal
California Institute of Technology

Abstract

An open-system high pressure aquarium is described. Data are presented on experiments performed with this equipment on marine vagrant benthic invertebrates derived from the surface waters and dredged from depths between 46 and 64 meters and 560 and 730 meters. The suitability of the equipment for long-range pressure studies is demonstrated by maintaining experimental animals at elevated pressures for periods up to six months.

Introduction

Deep sea expeditions and bottom photography of abyssal and hadal depths have recovered in the last three decades benthic invertebrates from all depths of the oceans (Bruun, 1957; Zenkevitch, 1963). A number of benthic species belonging to 4 invertebrate phyla have been reported from the deepest oceanic trenches. This indicates that hydrostatic pressures slightly in excess of 1000 atmospheres are no hindrance to metazoan life, its growth, development and reproductive processes. However, there is a decrease in species diversity and class representation in the benthic communities starting at about 5000-m depth and becoming more pronounced at depths greater than 8000 m (Zenkevitch, 1963).

There has been a question whether hydrostatic pressure or temperature is of primary significance in controlling the depth ranges of deep sea species. Of further interest is the interaction of these two ecologic factors on biochemical processes. Another ecologic factor, tending to obscure the precise role played by hydrostatic pressure on the depth distribution of benthic deep sea deposits and filter feeders, has been the availability of adequate food resources. Lastly, the possibility of salinity changes of various magnitudes as a limiting ecologic factor in shallower and deep sea organisms in relation to pressure has been considered. Temperature has been stressed as a major factor in controlling the depth distribution of a variety of marine invertebrates, based on the observation that a number of eurybathic species range from shallow bathyal depths at high latitudes to deep abyssal and hadal depths at low latitudes where the habitats have low temperatures in common. Support in favor of temperature as a major controlling ecologic

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factor has come also from the observation that benthic invertebrates living at depths between a few hundred meters to as much as 2000 m can be recovered alive when taken in large sediment grabs. The large sediment volume provides insulation to maintain the temperatures at the depths of animal recovery. Aiding in the recovery of live organisms has been the practice of sampling in mid-latitudes during the coldest months of the year when the temperature differences between surface and bottom are greatly reduced compared to those of the summer months.

Some laboratory and *in situ* experiments have investigated the pressure tolerances of a variety of marine invertebrates which are limited in nature to shallow waters, and in one case extend from deeper to shallower waters (summarized in Schlieper, 1968). Major interest in these experiments was to determine the lethal hydrostatic pressure on these experimental organisms. It was generally found that the lethal pressures were far in excess of the hydrostatic pressures at the maximum depths where the species are found in nature (summarized in Flugel and Schlieper, 1970). Among the most striking examples are the intertidal to very shallow water bivalve *Mytilus edulis* and one of its subspecies *diegenesis*, and the intertidal gastropod *Littorina littorea*. The lethal pressure for 50 percent of the individuals of *M. edulis* from European waters was found at 800 atmospheres, of *L. littorea* at 750 atmospheres, and of all individuals of *M. edulis diegenesis* from the West coast of North America at 350 atmospheres (Naroska, 1968; Menzies and Wilson, 1961).

The laboratory experiments were carried out in closed pressure systems which, as recognized by the investigators, posed problems of possible toxic effects of the metabolically raised pCO_2, resultant changes in pH and accumulated metabolic waste products in the aqueous medium as well as by respiratory induced reduction of dissolved oxygen in the water. In the case of the *in situ* experiment, the rate of adaptation or adjustment of the experimental animals to accelerated compression or decompression was noted as a possible factor in affecting the survival curves which the investigators established (Menzies and Wilson, 1961). As recognized by all earlier investigators, there was a need for developing an open-system, high-pressure apparatus for experimental studies, to reassess the published data obtained in closed systems, and to extend the investigations to long-range growth experiments.

Scope of Our Investigations

We have been interested in determining whether hydrostatic pressure limits by itself, or through interaction with other ecologic factors, the depth distribution of stenobathyal and eurybathyal invertebrates in the oceans. The data derived from previous pressure experiments tend to suggest that the pressure tolerances of the invertebrates investigated are greater and, in many

instances, vastly in excess of those which correspond to their known depth distributions in nature. There is the possibility that hydrostatic pressure tolerances may be responsible only for the upper or only for the lower depth limit of a species and, in some cases, of neither one. We would like to know further whether in eurybathyal species the mineralogy and trace element chemistry of their hard tissues are affected by hydrostatic pressures over the range of pressures in which the organisms live in nature. We have some data to show that in some eurybathyal species the trace element chemistry of their skeletal minerals changes with increase in depth in a manner which cannot be related to temperature either over part or over their entire depth range. We have found the same to be true in samples of species which have limited depth ranges but whose congeneric species occupy consecutively greater depth ranges. It is of interest to determine experimentally whether an increase in hydrostatic pressure in these organisms is partially or entirely responsible for the observed changes in trace element contents. The answer to this question should interest ecologists and paleoecologists, and equally biochemists, for in case the particular changes are related to the effect of hydrostatic pressure on the physiology of these organisms, they can serve to direct biochemical research of specific high pressure phenomena. To investigate these questions requires a system with flowing sea water at high pressure, and one which permits observation of the behavior of the experimental invertebrates. When samples of suitable species can be obtained from nature, extended observations and possibly growth experiments could be initiated, provided that food sources can be injected continuously into the high pressure chamber.

Let us assume these conditions can be met. The initial question concerns the criteria used to define pressure tolerance limits of a species in relation to the depth ranges which bracket their distribution limits in nature. Previous experiments indicate that with increase in hydrostatic pressure, the experimental animals pass through stages of excitation followed by a phase of "tetany" prior to reaching lethal pressures (summarized in Flugel and Schlieper, 1970). Following depressurization after "tetany" sets in, recovery and apparently unimpeded activity for a few hours have been commonly reported. From the ecologic point of view, only the definition of the pressure range at which "normal activity" is maintained is of significance. The ranges of pressures bracketing the states of excitation, tetany and death are of interest to assess biochemical responses of the species and, except for those inducing death, have meaning with respect to recovery ranges following depressurization to the state of normal activity.

In the following, we describe an open, high pressure system, which we have developed. Next, we describe pressure experiments performed on selected invertebrates with this equipment.

Open-System High Pressure Equipment

As discussed above, properly to study the effects of environment on certain chemical and morphological characteristics of marine animals which normally live deep in the ocean, it would be useful to have available controlled environment aquaria in the laboratory. The major difficulty in the past has been to assure aquarium environments with the proper pressure, temperature, and chemistry for long time periods. We describe in this paper one approach to this problem which has proven successful for several years.

The fundamental controlling factor in the design of such aquaria is the need to maintain, for long periods, a constant high hydrostatic pressure in a container that does not contaminate the sea water and that allows visual observation and photography of the animals under study. To accomplish this, we have chosen to make an "open" system in which the sea water, with the proper chemical modifications for a given experiment, is made to flow continuously through a plastic-coated stainless steel pressure vessel. The flow rate is adjusted to reduce any internal chemical contamination of the sea water to an insignificant level, thereby relieving one of the most serious difficulties of such high pressure systems.

AQUARIA. For most of the invertebrate animals which we have been studying, an accessible volume of about one liter is sufficient to contain several specimens without crowding. Since the sea water is flowing continually through the aquarium, contaminants from the metabolic products of the animals are carried away quickly with no difficulty. For pressures up to 5000 m equivalent depth, cast 316 stainless steel cylinders with external end caps containing the windows have proven quite satisfactory. We believe that cast cylinders with somewhat thicker walls and smaller windows will prove satisfactory for pressures up to 10,000 m equivalent.

Figure 119 shows the details of one of our standard aquaria which has been used for pressures up to 3000 m equivalent. The cylindrical section is centrifugally cast 316 stainless steel (Vollrath Co., Sheboygan, Wisconsin) which has been machined inside and outside, then passivated. The end caps are machined from 316 stainless steel bar stock and passivated. A tapered hole (15° total angle) has been bored in one cap and a "Lucite" plug, with polished faces, has been inserted. During the initial pressurizing of the aquarium the window will seat permanently, with no leakage, if the surface of the hole and the plug are reasonably congruent.

The surfaces of the inside of the aquarium, which are exposed to sea water during use, are coated with a thin layer of epoxy material (PC-621, Carl Biggs Co., Santa Monica, California) especially designed for corrosion protection. Water flows into the chamber from a port in the front cap and is exhausted through the back port.

PUMP SYSTEM. To produce a continuous flow of sea water at constant pressure with minimal contamination, we have chosen a system which

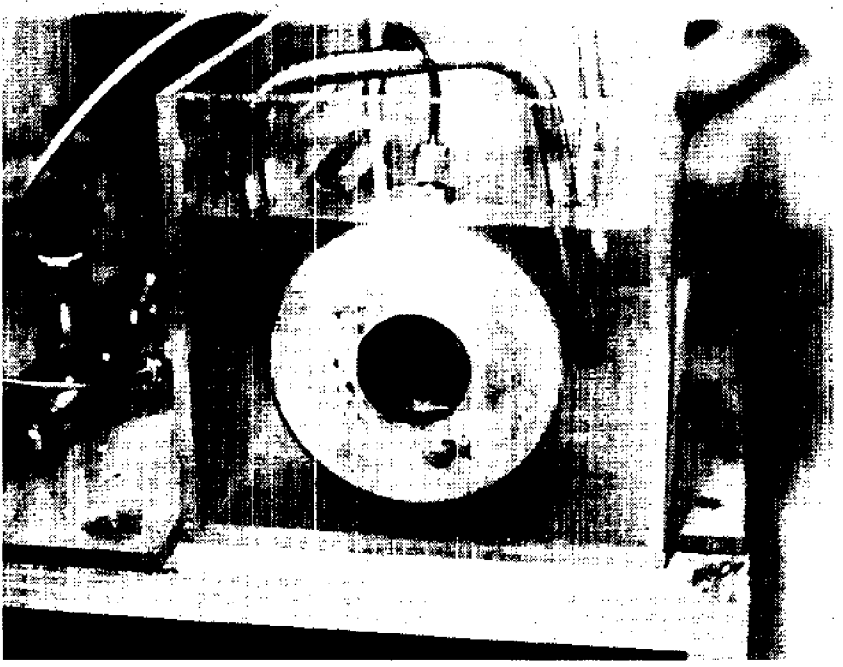

Figure 119 High Pressure Aquarium

prevents the sea water from coming in contact with the pump by use of a pressure interchange chamber. The system has essentially two separate branches interconnected only through a limp rubber bladder in the interchange chamber. This technique allows the sea water branch to be totally isolated from the pump and from the majority of raw metal surfaces.

Seawater, appropriately modified for a particular experiment, originates in a sea water sump. From the sump, it flows into the interchange bladder during an appropriate time of the running cycle and hence to the aquaria. Each aquarium has a stainless steel capillary tube with a bore of 0.0045-inch attached to its exhaust which regulates the flow of the sea water through that individual aquarium. The flow rate is a function of the aquarium pressure and length of capillary, and can therefore be different for each aquarium attached to the system.

To see in some detail the operation of the system, referring to Figure 120 and starting in an initial condition, sea water has filled the bladder in the interchange chamber and has been pressurized by the pumping of distilled water into the interchange chamber around the outside of the bladder. Valve (5), at the outlet of the system is open, and valve (8) in the distilled water branch is closed. Under these conditions, sea water will flow through the aquaria from the interchange bladder and out through the capillaries to waste.

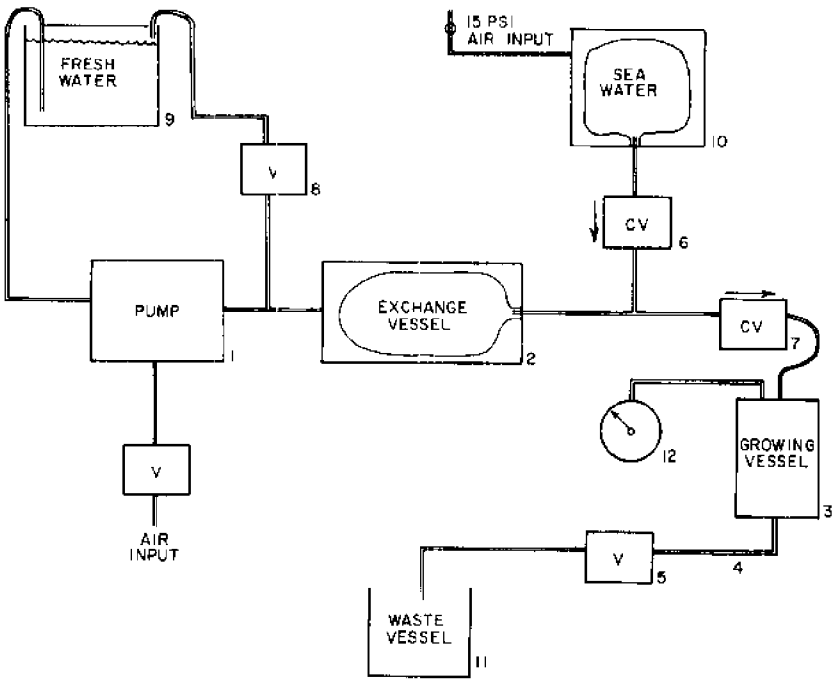

Figure 120 Schematic Arrangement of High Pressure Aquarium

The pump in this system is a positive-dispacement constant-pressure type, which will be described in detail later. By counting the number of strokes of this pump we can ascertain when the sea water supply in the interchange bladder is exhausted, i.e., the bladder is nearly collapsed. At this point, the valve (5) in the exhaust line is closed, stopping the flow in the aquaria. The pump is stopped and the valve (8) in the distilled water return line is open, relaxing the system pressure upstream from check valve (7) but retaining the pressure on aquaria. The sea water supply is pressurized with about 1 atmosphere of air or other appropriate gas and this pressure now forces new sea water into the interchange bladder through check valve (6), and displaces the distilled water outside the bladder back into the distiled water sump through open valve (8). A fixed time is allowed for this recharging step, then valve (8) is closed again and the pump started, repressuring the system. Finally, valve (5) is open and flow through the aquaria is reestablished. During the whole recharge cycle, which takes about three minutes in the present system, pressure has been retained in the aquaria.

The pump (Sprague Engineering Co., Gardena, California, Model 5216-CS-10055) is basically an air-driven hydraulic system which automatically produces a constant output pressure. The pump consists of two piston cylinder chambers; one, which is driven by air pressure, acts as the

"motor", the other pumps the distilled water. The pressure produced in the output is determined by the input air pressure which can be continually adjusted to any desired value and by the ratio of the area of the "motor" piston to the area of the pump piston. In the case of one of our presently operating systems, the ratio of areas is 100:1; thus 1 atmosphere of air applied to the motor will produce 100 atmospheres of pressure in the distilled water outlet. This capability, to smoothly change the pressure is essential for "pressuring up" animals which are obtained at one atmosphere, by dredging or other means, from the ocean. The use of distilled water in the pump branch of the system prevents serious corrosion problems in the pump which occur if sea water is used.

In this short description of the system, many details have been ignored. However, of these few are very important to successful operation. It is important that any particulate material, larger than the bore of the capillaries, be removed before the sea water is allowed to pass through the capillaries. We use a small filter made from Dacron wool and a 0.45 μ Millepore filter disk. This is particularly important when carnivorous animals are fed live food.

If some failure of the timing or pumping system allows the bladder to collapse completely, the thin rubber will be extruded into the sea water pipeline and allow distilled water, which would be deleterious to the animals, to be pumped into the aquaria. To prevent this, a small valve has been installed immediately in front of the inlet-outlet port which is mechanically closed by the collapsing bladder. If a failure occurs, a pressure loss occurs in the aquaria, but this condition is more desirable than fresh water flooding.

To allow introduction of food or other materials, while the system is running, a "feeding loop" has been installed. This consists of an arbitrarily long length of tubing with the appropriate fitting which can be inserted into the sea water feed line with manually operated valves without loss of pressure.

RELIABILITY. Most studies of marine animals with this system require that the environment be constant over periods of months. To achieve such reliability in an unattended system requires close attention to many small details of construction and operation. As in all marine aquarium systems, the less metal that is in contact with sea water, the more reliable and "healthier" the environment. Great care has been taken to coat all accessible metal surfaces with epoxy. However, in valves and other rubbing parts, the metal surfaces are necessarily exposed. We have found that 316 stainless steel is marginally satisfactory for most uses. It is very difficult to obtain commercial valves and other parts that are *all* 316 stainless; in particular, valve seats are often made from copper or its alloys, which is especially toxic. We have found that, after a short aging period of a few days in sea water, most plastics and rubbers are non-toxic and may be safely

used. Special care must be given to exposed electrical wiring to prevent damage from sea water, as well as exposed metal parts of valve actuators and timers.

Derivation and Species Composition of Experimental Animals

The ability of our equipment to maintain ambient pressures in the living chamber up to about 300 atmospheres provided a means of carrying out pressure experiments on marine organisms from depths up to about 3000 m. Attempts were made to recover deeper invertebrates by dredging with the USC research vessel *Velero IV* in the San Pedro Basin. Dredging was carried out during the winter months when temperature differences between the surface and bottom waters are at a minimum. Despite this precaution, we were able to recover alive only a limited number of different invertebrates from depths up to 845 m. Moreover, we found that only individuals of 4 gastropod and 1 ophiuroid species taken between 560 to 730 m depths maintained normal activity in our observation tanks under atmospheric conditions; two other gastropod species showed signs of severe muscular impedance under these laboratory conditions. These assemblages were dredged from the deep part of the San Pedro Basin at 33°25'42''N.L.; 118°14'45''W. Recorded bottom temperatures at these depths in the San Pedro Basin range from 5° to 6°C (Emery, 1960).

Repeated dredging off the east coast of Catalina Island near Ship Rock provided us with living specimens of a diverse benthic fauna from depths between 46 and 64 m. Bottom temperatures measured at these localities during the coldest months of the year range from 12° to 13° C. To evaluate more precisely the mean annual temperatures, we determined with the aid of O^{18} measurements the average temperature recorded by the carbonate of several year-old shells of articulate brachipods, taken alive earlier from the same dredging stations. The isotopically determined temperatures ranged from 11.3° to 12.9°C(Lowenstam, 1961).

Samples of surface water species were collected from the intertidal zone to depths of about 3 m south of Corona del Mar and off Palos Verdes Hills. Individuals of the species derived from the surface waters, 46 to 64 m, and 560 to 730-m depths were used for the pressure experiments described below.

Pressure experiments were performed on individuals of 1 chiton, 2 bivalves, 4 gastropod, 1 circipedian, 1 isopod, 2 crustacean, 1 ophiuroid, and 1 echinoid species from surface water environments. Experiments on invertebrates recovered from 46 to 64 meter depths were carried out on individuals of 6 gastropod, 2 ophiuroid, 1 echinoid and 1 holothurian species. Of the invertebrates dredged from 560 to 730-m depths, individuals of 6 species of gastropods, and of 1 ophiuroid species were subjected to the pressure experiments.

Maintenance and Behavior of the Organisms Prior and After the Pressure Experiments

The invertebrates obtained from the surface waters and from greater depths were kept initially for observation in tanks in our laboratory for periods ranging from one week to several months. This holding allowed us to determine whether the individuals were adaptable to laboratory conditions and to study their behavior and feeding patterns. Thirty-liter tanks made of Lucite were used for this purpose. Filtered sea water of 33.5‰ salinity was obtained from the intake of Marineland at Palos Verdes Hills and replaced in the tanks every 4 to 6 weeks. Water temperatures were kept uniformly at 15°C in the tanks containing invertebrates from the surface waters and recovered from depths between 46 to 64 m. The animals obtained from depths of 560 to 730 m were maintained in water cooled to 7°C to 9°C. *Dunaliella tertiolecta* and *Artemia gracilis*, cultured in our laboratory, were used for feeding the animals..

The vagrant benthic invertebrates, derived from the intertidal zone to a depth of 64 m, showed normal behavioral activity and fed actively on the food supplied to them.

Of the invertebrates recovered from 560 to 730 m in depth, the 4 individuals of the gastropod, *Borcotrophon bentleyi*, were highly active and moved most of the time in search of food on the bottom and walls of the tank. By comparison, *Calliostoma platinum*, *Trophonopsis tolomius*, and an unidentified gastropod were less active, but frequently moved up on the tank wall, thus indicating no muscular impedance. The two unidentified ophiuroids, when observed in light, were usually stationary and only occasionally moved slowly to hide under rocks. To judge from changes in tank position, following periods of darkness, their range of activity seemed limited, and only 1 individual survived the first month in the tank. Two individuals of *Trophonopsis lasius* and one of *T. tolmius*, recovered with a broken shell apex, remained stationary but attached firmly to the substrate most of the time. *Bathybembix bairdii* was the most abundant gastropod in the dredge hauls from 560 to 845 m. Of the 8 individuals kept in the tank for observations, 5 had broken shells. The individuals were lying most of the time on their sides with their bodies extended into the water. Occasionally, slow movement of the foot and head was observed. Despite their obvious muscular impedance, the animals remained alive for periods of from 1 week to about 2 months.

All animals subjected to the pressure experiments were returned after decompression to the tanks where they were kept from a few days to a week for further observations of possible depressurization induced effects.

Initial Pressurization Tests on Intertidal Invertebrates

To determine the suitability of our pressure equipment for experiments on marine invertebrates, the following tests were carried out. Ten individuals of 6 intertidal species were subjected to hydrostatic pressure increases of 500 psi at a time, up to a maximum of 3000 psi or about 200 atmospheres. The experimental animals remained at each pressure level for a period of one hour. After reaching the maximum pressure of 3000 psi, depressurization was carried out by the same procedure. After completion of these experiments, the animals were kept for observation in an aquarium under normal atmospheric conditions for a period of one day. Temperature was kept constant at 15°C in the pressure equipment during the experiments, and in the tank where the experimental animals were kept prior and following the experiments.

Of the 6 species, all individuals of the 4 species of mollusca, namely, *Nuttalina california*, *Acmaea scabra*, *Tegula funebralis* and *Mytilus californianus* survived the experiments. Of the crustaceans, 70% of the individuals of *Pollicipes polymerus* and 90% of those of *Pagurus samuelis* remained alive following the experiment.

Observations on the behavior of the experimental animals during the experiments were made on some of the species. The individuals of *Nuttalina californica* and *Acmaea scabra* remained stationary but firmly attached to the wall of the pressure vessel throughout the experiment. *Pollicipes polymerus* remained open up to a pressure of 1500 psi. At 3000 psi, the individuals were closed and their peduncles were rigid. During depressurization, pedunclar rigidity was lost in those individuals that survived at about 1800 psi. In the case of the hermit crab, *Pagurus samuelis*, the individuals were found to be hyperactive at about 1000 psi, but showed movement and good reflexes up to 1500 psi. After further increase in hydrostatic pressure, the individuals withdrew into their shells and remained inactive. In the course of depressurization, movement resumed only after the pressure had been lowered to about 700 to 800 psi.

Pressurization experiments were carried out next on samples of the wood-boring isopod *Limnoria lignorum*, again in water with a constant temperature of 15°C. Four wood blocks were obtained from Dr. D. J. Reish; each was infested with 10 individuals of this species. The organisms in the first block were pressurized to 1500 psi, in the second to 2000 psi, in the third to 3000 psi and in the fourth to 3600 psi, each for a period of 7 days. Folowing depressurization, the isopods were removed from the wood blocks, examined individually and then kept under observations for another 7 days. All individuals pressurized to 3100 psi or less were found to be alive after completion of the pressure experiments and remained alive during the observation period of one week thereafter under 1 atmosphere of pressure.

Of the 10 individuals subjected to 3600 psi, only 50 percent survived the experiment.

The data obtained from these initial experiments indicated that our equipment was basically suitable to conduct pressure experiments over extended period of time. These experiments served further to define the kinds of observations on the behavioral responses we wished to obtain with reference to our objectives and in turn the kinds of organisms likely to provide the information desired. Examples of the questions which arose were the uncertainty about whether the individuals of *M. californianus* which survived pressurization to 3000 psi maintained normal activity in the upper pressure range or went into a state of "tetany" from which they recovered following depressurization. In the case of *L. lignorum*, it remained uncertain whether at the maximum pressures which all or most individuals survived, their boring activity was reduced or stopped altogether. The individuals of *Nuttalina californica* and *Acmaea acabra* showed no movement during the experiments. Firm attachment to the walls of the pressure vessel suggested that muscular control of their foot was not seriously impaired, but we were not able to ascertain the upper pressure limit at which normal movement would cease in the organisms.

The behavioral responses of the hermit crab, *Pagurus samuelis*, to pressurization and decompression were the most instructive for application to subsequent experiments. The organisms showed some movement and apparently good reflexes up to 1500 psi during the pressurization phase. Clear signs of hyperactivity at a pressure 1000 psi and recovery from "tetany" between 700 and 800 psi, following depressurization tend to suggest, however, that these organisms are able to function normally only at maximum pressures in the range between 500 to 1000 psi.

A. Surface Water Invertebrates

Individuals of 9 species taken from interidal habitat at a maximum depth of 3 m were subjected to rapid pressurization and decompression experiments in waters of constant temperature of 15°C. The experimental animals were represented by 1 bivalve species, 3 gastropod species, 1 crustacean species, 1 ophiuroid species and 1 echinoid species.

Two separate pressurization experiments were carried out on single individuals of the bivalve *Leptopecten latiauratus monotimeris*. In the first experiment the pressure was raised by increments of 200 to 250 psi every 5 to 10 minutes until an ambient pressure of 1000 psi was reached. The individual was kept at this pressure for a period of 30 minutes. Increase in pressurization was then resumed every 15 minutes in increments of 250 psi until a maximum pressure of 3000 psi was attained. Depressurization was carried out at 5-minute intervals at a rate of 250 psi pressure reduction.

During pressurization, the individual remained stationary with the valves open most of the time until a pressure of 1000 psi was reached, after which it moved briefly. At a pressure of 1250 psi the individual opened and closed the valves rapidly and attempted briefly to swim, but unable to do so, finally became motionless. At a pressure of 1500 psi, the animal closed the valves and remained in this state until the pressure was lowered to 750 psi. From there until depressurization was completed, the animal reopened its valves but no movement was registered.

The second experiment involved a uniform increase of pressure of 250 psi at a time, at 15-minute intervals and decompression at the same rate in steps of 250 psi. Signs of normal activity were registered until the pressure reached 750 psi. The individual kept its valves open intermittently, closed them rapidly and finally swam to the wall of the vessel where it attached itself by byssus strands. During further increase in pressure to a maximum of 2000 psi, and thereafter until the pressure was lowered to 750 psi, the animal remained motionless with its valves closed. From then on until decompression was completed, the individual reopened its valves but closed them again rapidly whenever the wall of the pressure chamber was tapped.

There is close agreement in the second experiment in the behavioral responses of the individual to increase and decrease in ambient pressures. 750 psi or about 50 atmospheres appear to be the upper pressure limit for normal adductor muscle control. The same was found to be true for the individual in the first experiment during the depressurization phase. This individual was shown further to register impeded muscular activity up to 1000 psi or about 67 atmospheres of pressure and signs of nervous hyperactivity at 1250 psi or about 80 atmospheres of pressure. To judge from these limited data, it would appear that the *Pecten* subspecies could potentially range to depths up to about 500 meters.

Pressurization and decompression by 250 psi increments at 15-minute intervals was applied similarly to individuals of 3 gastropod species. Three individuals of *Olivella biplicata* were placed together into the pressure chamber. Active movement of the individuals occurred until the pressures were raised to between 500 and 750 psi. Loss of muscular control was registered, starting at 1000 psi. The individuals were lying on their sides with the foot extended intermittently. Hypernervous activity was registered by jerky random movements of the foot intermittently up to the maximum pressure of 2500 psi. During the depressurization phase, 2 individuals were incapable of righting themselves until they were returned to the observation tank. The third individual regained impeded muscular control at 1500 psi, and at lower pressures remained stationary until it, too, was returned to the observation tank. Here, all 3 individuals recovered and resumed normal activity.

Another pressurization experiment was performed on a single specimen of *Tegula funebralis*. Normal movement was registered up to 750 psi.

Further elevation in pressure up to a maximum of 2500 psi caused the animal to withdraw into the shell, and it remained in this state until depressurization was completed. This individual also recovered after being returned to the observation tank.

An individual of *Archidoris montereyensis* was subjected next to the same experimental procedure. The individual moved actively on the walls and roof of the pressure chamber up to 1000 psi. At increased pressure, which was raised to a maximum of 3700 psi, the animal moved only occasionally and the body became compressed. At 3500 to 3700 psi, the rynophore remained extended and slight head movements were observed. No observations were made during the depressurization phase. It would appear that normal activity occurs up to 1000 psi and impeded muscular control extends at least to 3700 psi.

One individual of the rock crab, *Pachigrapsus crassipes,* was pressurized to 2500 psi. Normal activity was registered up to 500 psi. From there on until 1000 psi was reached, the animal appeared to be stiff-legged and moved more slowly. Free movement stopped from 1250 psi on up. Hypernervous activity was registered intermittently by occasional jumps followed by involuntary twitching of the legs and claws up to 2000 psi. In the range between 2000 and 2500 psi, the animal became motionless. During depressurization, slight nervous hyperactivity of the legs was resumed starting at 2000 psi. Mouth movement started at 1500 psi and increased as the pressure was reduced to 500 psi. The animal remained immobile until depressurization was completed but recovered shortly thereafter in the observation tank.

Another experiment was performed on an individual of the ophiuroid *Ophioneris annulata*. Normal behavior was registered until 750 psi was reached. At this pressure the arms became humped, and the movement started to be jerky. Intermittent slow movement, interrupted by hypertension of the arms, continued clear up to 2500 psi, after which depressurization was initiated. Following the lowering of pressure to 750 psi, the arms became relaxed and showed a firm grip on the vessel wall. The animal resumed normal activity after being returned to the observation tank.

Pressurization of an individual of the echinoid *Strongylocentrotus purpuratus* showed active movement on the wall of the pressure vessel up to 500 psi. Between 750 and 1750 psi, the animal was stationary most of the time, but occasionally moved very slowly on the vessel wall. At 2000 psi the animal fell off the wall, its tubefeet retracted and no further motion was registered. Very minor spine movement was noted, however, until the pressure reached 2500 psi. During depressurization, only active spine movement was observed, and the tubefeet were shown to be attached to the substrate. The individual recovered in the observation tank.

Of the 7 species tested in the foregoing experiments, only 2 were represented by more than one individual. The question arose whether the

recorded behavioral responses of a single individual were representative of the species from a given habitat. Also, one may ask whether the pressure increments and the length of time at which the organisms were kept at a given pressure may affect the maximum pressure at which normal activity can be maintained. To answer these questions a series of three experiments was performed with four individuals of *Tegula funebralis* in each experiment. Of the four, two individuals were of average adult size and two of smaller size. Two pressure chambers were employed in the experiments, placing two experimental animals into each chamber.

In the first experiment the pressure was raised and lowered in increments of 50 psi, and the animals were kept at each pressure level for a period of 15 minutes. The animals were kept at 900 psi for 15 hours. Next the pressure was increased until 1050 psi was reached, and then lowered to 750 psi. The animals were kept at this pressure for 18 hours. Thereafter, they were gradually repressurized again to 1050 psi followed by depressurization to atmospheric conditions, starting at 400 psi in 100 psi steps.

In the course of the initial pressurization phase, individual differences in behavior were noted. One of the smaller animals fell off the wall at 400 psi but regained muscular control of the foot within 15 minutes and climbed back up the wall of the vessel. The other smaller individual fell off the wall at 750 psi but kept moving slowly on the bottom of the pressure chamber. One of the two larger ones slowed down its movements starting at 600 psi, whereas the other one moved actively around until 800 psi was reached. At this pressure all 4 individuals showed reduced activity, and that only on the bottom of the vessel; one of the larger individuals continued limited movement there until 850 psi was reached. At this pressure, the 3 other individuals stopped moving altogether, one of the smaller ones showed signs of hypernervous activity, and one of the larger ones withdrew into its shell. During the period of extended pressurization of 900 psi, 3 of the individuals remained motionless through the 15-hour period and the fourth,a small one, moved once and then became immobile also. The animals remained in the state of tetany after resumption of pressure increase to 1050 psi and following decompression to 950 psi. At this pressure, one of the smaller individuals extended its foot and tried unsuccessfully to move. A similar unsuccessful attempt was made by one of the larger individuals between 900 and 850 psi. Limited movement was registered in the 3 other individuals starting at 850 psi, and normal activity was resumed by all 4 individuals at a pressure of 750 psi, at which point they were kept for 18 hours. The same behavioral patterns were repeated in the following repressurization phase to 1050 psi and the succeeding decompression phase. After the 4 individuals were returned to the observation tank, they maintained normal activity for a period of 4 days, when the experiment was terminated.

In a second experiment, 4 similarly sized *Tegulas* were pressurized and depressurized in increments of 100 psi and kept at each pressure level again

for 15 minutes. Pressures in this experiment were carried to 1500 psi, and when the decompression phase reached 800 psi, the *Tegulas* were kept at this pressure for 16 hours before depressurization was resumed.

Despite the higher maximum pressure, the behavioral responses of the experimental animals differed only slightly from those of the previous experiment.During the initial pressurization phase, one large and one small individual fell off the wall at 800 psi. Limited movement continued or was resumed by all 4 specimens until pressures between 1000 and 1100 psi were attained. At this point, one small individual retracted into its shell and one large one fell off the wall of the pressure vessel. "Tetany" set in at higher pressures, and 3 of the animals remained in this state until the pressure was reduced back to 1100 psi, after which they resumed slow movement. The fourth, a large individual, did not resume slow movements until the pressure was lowered to 900 psi. After the animals were kept at 800 psi for a period of 16 hours, two kept moving actively and two others were found to be stationary but with the foot extended. During the final decompression phase, all 4 individuals showed active movement and maintained normal actvity in the observation tank where they were kept for two days. Lack of ill effects of the pressure experiment was in evidence, in that the animals were actively moving up and down the walls of the observation tank.

In the third experiment, 4 similar-sized *Tegulas* were pressurized in steps of 200 psi, and the animals were kept at each pressure level for 30 minutes. The animals were pressurized to a maximum of 1600 psi. During decompression, the individuals were kept, as in the previous experiment, at a pressure of 800 psi for 16 hours. During pressurization, initial impedance of muscular control was noted in one of the large and one of the small individuals at 800 psi and in the two others after reaching 1000 psi. Tetany extended from 1200 psi to 1600 psi and lasted during the decompression phase until the pressure was lowered to 800 psi. The reversing motor controlling the water flow of the first pressure chamber stopped during the 16-hour period during which the pressure of 800 psi was maintained. Yet, the individuals in this chamber as well as those in the other one where normal flow rates had been maintained showed normal behavior, and the same was true after one day in the observation tank.

Invertebrates from Depths of about 50 Meters

Pressure experiments were performed on specimens of 6 gastropod species, 2 ophiuroid species, 1 echinoid species, and 1 holothurian species, which were taken from depths between 45 and 65 meters. Of these, 3 species, namely *Olivella biplicata, Conus californicus, and Cypraea spealca*, range into the surface waters, as shallow as mean low water to low water springs.

Two experiments were performed on individuals of *Olivella biplicata*. A single individual was subjected to pressure increments of 250 to 500 psi at

10-minute intervals to a maximum pressure of 2500 psi and then depressurized by the same procedure. Normal activity was registered until a pressure of 500 psi was reached. Impeded movement occurred up to 1000 psi. Following increase in pressure to 1500 psi, the animal became first stationary, then lost muscular control of the foot and fell on its side. Unsuccessful attempts to right itself and slow movement of the extended foot and siphon were noted until a pressure of 2250 psi was reached. At 2500 psi the animal retreated into its shell and remained in this state until the pressure was lowered agin to 2250 psi. Throughout the remainder of the decompression phase the individual remained lying on its side but extended its foot and siphon. Hypernervous activity, indicated by jerky foot and siphon motions, was registered until the pressure was lowered to 1000 psi. At 750 psi the individual attempted unsuccessfully to right itself. Following return to the observation tank, the individual regained, with 30 minutes, full muscular control and moved rapidly back up on the wall of the tank.

Two individuals of the same species were next pressurized together in the same chamber. Pressurization was carried out uniformly in increments of 250 psi, again at 15-minute intervals to a maximum pressure of 2500 psi. One of the individuals lost muscular control of the foot when the pressure reached only 250 psi. The animal showed violent hypernervous activity of the foot while lying on its side until a pressure of 1500 psi was reached. It then withdrew into its shell and remained in a state of tetany in the course of increased pressurization and throughout the decompression phase. Indications that the abnormal behavior of this animal in the experiment was due to poor physical conditions were confirmed by its death following depressurization. The second *Olivella* showed normal activity until 500 psi was reached. At 750 psi, its movement became less frequent and then only at reduced speed. In the succeeding phase of further increase in pressure to 2500 psi and during decompression up to 1200 psi, the animal alternately withdrew into its shell or extended its foot and head and then showed hypernervous activity. From 1200 psi down until decompression was completed, the individual regained muscular control of its foot and moved actively around again. The animal survived the experiment and behaved normally in the observation tank.

An individual of Cypraea spadica was pressurized similarly in increments of 250 psi at 10 to 15-minute intervals to 2500 psi. The animal moved initially to the roof of the pressure vessel where it moved around intermittently until a pressure of 500 psi was reached. It then became stationary and had its mantle expanded until the pressure reached 2000 psi, when failure of muscular control caused the animal to fall off the vessel ceiling. The body remained extended and the foot moved slowly while the animal was lying on its side during further pressurization to 2500 psi and until the pressure was lowered again to 2000 psi. Partial muscular control was regained at this state, as indicated by the ability of the individual to

right itself again, though it remained stationary until the pressure was lowered to 1500 psi. Thereafter, until the depressurization was completed, the animal moved intermittently, slowly, but only in the floor of the vessel, indicating that muscular control was still partially impeded.

A similar experiment on an individual of *Conus californicus* gave the following results. Normal activity was registered until a pressure of 1500 psi was reached. Highly impeded movement was noted up to a pressure of 1750 psi. At 2000 psi, the animal fell on its side. The body remained extended and the foot and siphon showed limited movement. At 2500 psi and during the following depressurization phase down to 2000 psi, the animal righted itself and tried, without success, to move slowly back up on the wall. Limited muscular control was thereby indicated. In the course of lowering the pressure to 750 psi, the animal remained motionless with the foot attached to the vessel floor. Thereafter, the foot became partially retracted until decompression was completed. After the animal was returned to the observation tank, it remained motionless for about 25 hours but thereafter resumed normal activity.

An individual of an unidentified species of *Nassarius* was pressurized to 2000 psi. Pressure was increased by increments of 250 psi every 15 minutes and depressurized at 5-minute intervals. Normal activity was registered up to a pressure of 500 psi, followed by extreme hypernervous activity of the body up to 1250 psi. During this phase the animal attempted to move to the wall but fell sideways. From 1500 psi on and until the pressure was lowered to 1750 psi, the animal remained motionless with the body appearing compressed and with the proboscis retracted. During further depressurization, the animal extended its body out of the shell, made unsuccessful attempts to walk and then withdrew back into its shell. The animal recovered in the observation tank and resumed normal activity.

A specimen of *Nassarius fossatus* was pressurized to 2500 psi, by pressure increments of 250 to 500 psi at 10-minute intervals. The animal moved intermittently and slowly on the roof of the pressure vessel until 1500 psi was reached. Between 1500 and 1750 psi, it fell off the roof and showed hypernervous activity by rapid, poorly controlled movement on the floor of the vessel. During further elevation of pressure to 2500 psi, the animal lay on its side. The body was greatly extended, and slow free motions were registered by the foot and siphon. The animal withdrew into its shell and remained retracted until the pressure was lowered to 2000 psi. From there on until decompression was completed, the individual first extended its foot and siphon slightly, and, starting at 750 psi, the animal became fully extended and began slow body motion but was unable to right itself. Within 5 minutes after the animal was returned to the observation tank, normal activity was resumed.

An experiment was performed on a specimen of *Megasurcula carpenteriana*. Pressure was raised in increments of 250 psi at 5-minute intervals.The

animal was initially retracted into its shell. Starting at 750 psi, the animal became active and moved slowly on the bottom of the pressure vessel until a pressure of 1500 psi was reached. At 1750 psi, the individual moved excitedly up the wall of the e pressure vessel, then stopped and fell and remained motionless on the floor of the vessel. It remained in this state until the pressure was lowered from 1500 to 1200 psi. Within this pressure range, the animal extended its foot and moved very slowly up on the wall and then retracted again into its shell. It remained motionless thereafter until decompression was completed. Normal activity was resumed in the observation tank.

Two pressure experiments were performed on unidentified ophiuroids. One was conducted on a single individual. Pressure was increased in increments of 250 psi at 5-minute intervals to a maximum pressure of 2500 psi. Initially motionless, the animal started to move slowly in the range of pressures between 750 and 1500 psi. The motion was laborious, and some of the arms showed signs of muscular contraction. Thereafter, until a pressure of 2000 psi was attained, the animal showed rapid horizontal arm movements in response to a strong light. Attempts to move away from the light were extremely slow and jerky with arched arms indicating muscular contraction. At higher pressures the animal went into a state of tetany and remained so until the pressure was lowered again to 2000 psi. Partial muscular control of the arms was regained at 1500 psi, and slow movement with still recurved arms started in the range from 1500 psi to 800 psi. The individual died after being returned to the observation tank.

The pressure response of one large and one small individual of another unidentified ophuiroid species was tested in another experiment. Pressure was increased in increments of 200 to 300 psi at 5-minute intervals to a maximum pressure of 2000 psi. The two animals moved occasionally in a normal way in the pressure vessel until 1000 psi was reached. Involuntary jerky motions of the arms were registered at 1300 psi. Both individuals became immobile at 1500 psi. At 1750 psi, the smaller individual showed impeded movement of the arm tips and slid off the wall, whereas the larger one remained immobile. At 2000 psi the arms of both animals became arched and rigid and remained in this state until the pressure was lowered to 1000 psi. At this pressure the larger individual started to move around rapidly and the smaller one slowly without any evidence of muscular impedance of their arms. Only limited movement was registered by both animals in the final phase of decompression.

Three experiments were performed on individuals of *Lytechinus anamesus*. In the first, a single animal was pressurized in increments of 250 and 500 psi at 10-minute intervals to a maximum of 4000 psi. The individual moved actively on the wall and roof of the pressure vessel up to 1000 psi. From 1500 psi to 2500 psi it remained stationary, attached to the wall of the pressure vessel, and showed reduced movement and pedecilaria

of the spines. At 2750 psi, the animal fell off the wall, moved very slowly to the lower part of the wall, reattached itself there with its tubefeet to the vessel floor and then became stationary. It remained motionless in the course of further pressurization to 4000 psi and during decompression until the pressure was reduced to 1500 psi . At this stage the animal slid to the vessel floor, moved slowly the length of the floor, and then became stationary again. At 600 psi it attempted unsuccessfully to climb the vessel wall. Following transfer to the observation tank, the animal moved around fairly actively on the aquarium floor but was unable to climb up the walls.

In the next experiment two individuals were pressurized in increments of 250 psi at 10-minute intervals to 3600 psi. Both animals showed normal activity until the pressure reached 1000 psi. Slower movement followed until a pressure of 1500 psi was reached. Both individuals became stationary at 1750 psi and remained motionless until depressurization was completed. After transfer to the observation tank, both individuals stayed alive for a period of two days, when the experiment was terminated. During the observation period, the animals lost their color pigment and the spines bordering their corona.

In the third experiment, two individuals were pressurized in increments of 250 psi at 15-minute intervals to 1000 psi and left at this pressure in the pressure vessel for 4 days. Normal activity was registered during the pressurization phase, and it continued through the following 2 days of pressurization. During this time the animals were seen feeding on *Dunaliella*. Thereafter, the 2 *Lytechinus* specimens became stationary at the bottom of the bottom of the pressure vessel and died on the fourth day of the experiment.

A single unidentified holothurian was pressurized in increments of 250 to 500 psi at 10-minute intervals to 4000 psi. Active, normal movement was noted until 1500 psi was reached. At 2000 psi the animal became stationary, but the anterior body portions moved occasionally sideways until the pressure reached 2750 psi when tetany set in. The animal remained in this state at elevated pressures and during decompression until the pressure reached 3000 psi. Anterior body movement in a stationary position was then resumed. Normal activity began again at 1500 psi and was maintained following depressurization in the observation tank in the next two days after which the experiment was terminated.

Invertebrates from Depths Greater than 500 Meters

The behavioral responses of the experimental animals, recovered from depths between 560 and 730 m, are shown in Table 1. The results of the experiments of rapid pressurization and decompression steps are considered first.

The experimental animals, which showed normal activity prior and during parts of pressurization experiments, include three identified species.

Table 1

Species	Pressurization increments at 15 minute intervals	Pressure range of normal activity	Pressure range of impeded activity	Pressure range of observed tetany
Boreotrophon bentleyi	200-250 psi	15-1250 psi	1250-1500 psi	1500-3000 psi
Calliostoma platinum	200-250 psi	15-1250 psi	1250-2000 psi	2000-2500 psi
Trophonopsis tolomius	200-250 psi	15-850 psi	850-1000 psi	----
Trophonopsis lasius	200-250 psi	----	15-250 psi	250-1000 psi
Bathybembix bairdii	200-250 psi	----	15-250 psi	250-1000 psi
Gastropod gen. et sp. indet.	200-250 psi	15-850 psi	850-3250 psi	3250-3500 psi
Ophiuroid gen. et sp. indet.	200-250 psi	15-1000 psi	1000-1750 psi	1750-2000 psi

Data from the literature and unpublished depth records indicate a more extended depth distribution of the three species than would appear from our dredge recoveries of conspecific individuals. Therefore, the experimentally determined pressure ranges encompassing normal activity of conspecific individuals can be assessed with greater confidence in these three species. They are *Boreotrophon bentleyi*, *Trophonopsis tolomius* and *Calliostoma platinum*.

The pressurization experiments performed on 3 individuals of *Boreotrophon bentleyi* showed uniformly normal activity between 1 and 83 atmospheres of ambient pressure. The experimental animals were found to be most active in the pressure range between 67 to 83 atmospheres. The genotype of the species was reported from a depth of 20 fathoms, equivalent in ambient pressure to about 3.7 atmospheres. However, reliable depth records show that this species occupies depths with hydrostatic pressures between 9 and 73 atmospheres. The range in ambient pressures determined experimentally for normal activity of the organisms agrees closely with that of the unknown depth range of this species.

A single individual of *Calliostoma platinum* registered in our pressure experiment normal activity over the same range of pressures as the samples of *Boreotrophon bentleyi* , viz. 1 to 83 atmospheres. Ambient pressures corresponding to the depth range from which this species has been recovered in the waters off Western North America range from 21 to 76 atmospheres. The upper pressure limit determined for this species experimentally is close to that of its deepest depth record, whereas the lower pressure limit does not. The differences though small, namely 1 atmosphere under experimental conditions and 21 atmospheres in nature, seem real, since the depth distribution of this species is well documented.

In the case of *Trophonopsis tolomius*, there is an overlap of the experimentally determined and of the environmental pressures.However, the minimum pressure marking normal activity, registered by *in situ* pressurization and during subsequent maintenance in our aquaria at 1 atmosphere, is

significantly lower than the ambient pressure of 44 atmospheres at the shallowest depth from which individuals of this species have been recovered. The maximum pressure at which normal activity was observed in the two individuals in our pressurization experiments, i.e., 58 atmospheres, is lower than the ambient pressure of 73 atmospheres at the greatest depth from which individuals of this species have been dredged. Considering the depth range of the dredge haul from which our experimental animal was recovered, no significance can be attached to the slightly lower value of the upper pressure limit tolerated by the individual under experimental conditions.

The experimental data obtained from an ophiuroid and an additional gastropod species which were not taxonomically identified, are limited to comparison to pressures of the depth range from which the individuals were recovered in nature. The individual of the ophiuroid was found under experimental conditions to display normal activity in the range from 1 to 68 atmospheres of hydrostatic pressure. The ambient pressures at the depths of its recovery range from 56 to 73 atmospheres. The upper pressure limit, as determined experimentally, lies close to the midpoint of the pressure range from which the individual was dredged. This seems to suggest that that ophiuroid was recovered close to its deepest depth occurrence in nature. The marked difference between the minimum pressure tolerated by individuals of this species under experimental conditions and the ambient pressure corresponding to the shallowest depth from which it was recovered cannot be properly interpreted without information of its shallowest depth penetration in nature.

In the case of the unidentified gastropod, the individual was found to behave normally from 1 to 58 atmospheres of pressure. The upper pressure limit corresponds to the minimum pressure of the depth range from which the individual was recovered in nature. The range of pressures tolerated by this gastropod under experimental conditions coincides with that of *Trophonopsis tolomius*. As in the case of the ophiuroid, the lack of data on the depth distribution of the unidentified gastropods limits further comparison of the pressure range tolerated by this species under experimental conditions and in nature.

The pressure experiments were extended to individuals of *Trophonopsis lasius* and *Bathybembix bairdii*, which showed impeded muscular control in *in situ* depressurization to 1 atmosphere of ambient pressure. The known depth distribution of *Trophonopsis lasius* corresponds to a range of pressures from 18 to 73 atmospheres and of *Bathybembix bairdii* from 22 to 110 atmospheres. Following elevation of ambient pressures in the pressure chamber to about 15 atmospheres, the individuals of both species became immobile and rigid and remained in the state of tetany thereafter until the experiments at elevated pressures of about 90 atmospheres were discontinued. Following depressurization and return to the tank with 1 atmosphere

of pressure, the individuals of these species, as well as the others discussed previously, remained alive from a few days to two months.

A second set of experiments was carried out to determine whether individuals of some of the species recovered from depths in excess of 500 meters would also behave normally when subjected to constant higher pressures for extended periods of time. We were also interested to find out whether normal activity could be restored in individuals of those species which showed impeded muscular controls following *in situ* depressurization to 1 atmosphere, once ambient pressures of their habitat range were restored for a day or more. These experiments were designed to test the suitability of our open-system pressure device to grow deeper water invertebrates at constant elevated ambient pressures and by feeding them with *Dunaliella tertiolecta*. Individuals of *Boreotrophon bentleyi*, *Calliostoma platimi*, amd *Bathybembix bairdii* were selected for this study.

Two experiments were performed on *Boreotrophon bentleyi*. Initially, one individual was placed in a pressure chamber and later, two individuals were kept together in another one. The rapid pressurization and decompression experiments indicated that individuals of this species were most active in the range of ambient pressures between 67 and 83 atmospheres. Therefore, an ambient pressure of about 75 atmospheres was selected for behavioral studies over extended periods of time. The single individual was kept at this pressure for 2-1/2 months. Periodic checks on its behavior through the plastic window showed active feeding and normal activity, which involved rapid climbing on the walls and movement along the ceiling of the pressure chamber. Figure 119 shows a photograph of the individual adjacent to the window of the pressure chamber while the experiment was in progress. After 2-1/2 months the individual was depressurized and returned to the tank with an ambient pressure of 1 atmosphere. There, it showed normal activity for a period of several weeks and thereafter was preserved in 70% alcohol for shell study.

It seems worth noting that during the experiment, while the animal was checked routinely through the window for its behavior, a temporary failure of the pressurization unit occurred which caused a sudden drop in ambient pressure to 1 atmosphere in the living chamber. Active movement of the gastropod continued during the pressure failure and following repressurization to about 75 atmospheres. This indicates that the sudden depressurization caused no ill effect on the individual. The incident illustrates dramatically that this species is insensitive to rapid changes in ambient pressures over the range encountered in its depth distribution.

In the second experiment, two individuals were pressurized to about 75 atmospheres of hydrostatic pressure to determine for how long they can be kept alive under these conditions in our pressure chamber. The individuals showed active movement and were seen to feed intermittently for a period of six months. One of the individuals died at the end of this period and began

to decompose in the pressure chamber. Evidence of the ill effect of its decomposition products on the other individual were seen in that it stayed thereafter close to the water intake of the chamber and died also a few days later.

The next experiment involved an individual of *Calliostoma platinum*. It had been kept for several months in a tank with a hydrostatic pressure of 1 atmosphere and showed there limited activity although no noticeable muscular impedance. The individual was subjected to 67 atmospheres of ambient pressure, which represents the mean pressure of the depth range from which it had been recovered. For a period of 24 hours the individual registered normal behavior in the pressure vessel, but subsequently went into a state of tetany. Following the depressurization and return to the tank with 1 atmosphere of pressure, the experimental animal stayed alive for a few days but indicated a high degree of impedance of its muscular control.

The final experiment was carried out on two individuals of *Bathybembix bairdii*, which showed signs of muscular impedance in the tank with ambient pressure of 1 atmosphere where they were kept for several months. The individuals were initially subjected to an ambient pressure of 67 atmospheres for 1 day and to 50 atmospheres on the following day. The two individuals went immediately into a state of tetany and remained so throughout the remainder of the experiment.

Discussion

The experimental data indicate that our open-system pressure vessel is capable of maintaining experimental animals at elevated pressures for periods of up to six months. Our experiments were designed to determine basically the pressure ranges in which the vagrant benthic invertebrates from environments of 1 to 80 atmospheres of ambient pressure function normally for comparison with the depth ranges which they occupy in nature. As indicated by the description of the behavioral responses of the animals in our experiments, we have defined the upper limit in pressure tolerance for normal activity as the first sign that movement of the animals became noticeably impeded and was usually accompanied by the inability to climb or stay on the vessel walls. By this definition we have excluded that part of the pressure range for some of the experimental animals where they showed still some muscular control and usually hypernervous reactions, as in nature they are likely to avoid penetration to depths with ambient pressures in this range.

The data for the surface water samples indicate that the upper limit of pressure tolerance for normal functions is largely equivalent to pressures at depths between about 300 and 500 m, the maximum in one case only extending to about 700 m.

In the case of the animals derived from 46 to 64 m, the range of maximum pressures for normal activity for all species spans a depth range from about 300 to 1000 m; hence, is greater than for surface water samples. Closer inspection of the data indicates that most of the gastropod species, namely 5 out of 7, are characterized by lower maximum pressure tolerances than all of the echinoderm species tested. However, two of the gastropod species were found also to withstand a maximum pressure as found at about 1000 m . Three of the gastropod species recovered between 46 and 64 m are common in the surface waters. They are *Olivella biplicata, Conus californicus* and *Cypraea spadica*. Similar results in maximum pressure tolerance were obtained for individuals of *Olivella biplicata* taken from the surface waters and from depths of 46 to 64 m. The upper pressure limit for the surface water individuals was found to lie between 500 to 750 psi, for the deeper water individuals at 500 psi. Our specimens represent the deepest dredge records of this species, known to us. This indicates that the maximum pressure tolerance of this species far exceeds the ambient pressures of its deepest recorded depth occurrence. *Cypraea spadica* has been previously recorded to occur at depths up to 45 m. The maximum pressure tolerance determined in our experiment is 500 psi, equivalent to a pressure at depths slightly in excess of 300 m. This value indicates again that pressure is not the limiting factor of its shallow depth range. The maximum pressure tolerance determined in our experiments for *Conus californicus* is 1500 psi, equivalent to about 1000 m. Yet, we have been unable to find depth records for the species in excess of 50 m.

The invertebrates collected from 560 to 730 m maintained normal activity in the observation tanks, after recovery. Their maximum pressure tolerances were found to fall within the pressure range at depths between 580 and 880m. In these cases, the highest tolerated pressures lie within the range of pressures encountered in their habitats or are slightly below or above those of their greatest known depth recovery. This finding contrasts sharply with the maximum pressure tolerances of shallower water species, which are significantly lower than those of their known maximum depth ranges. It is of further interest to note that of the deepest water animals which we used in our pressure experiments, the shallowest depth record for *T. tolomimius* is 440 m, for *Calliostoma platinum* 215 m and for *Boreotrophon bentleyi* 90 m. Yet, even though we have demonstrated that individuals of the species can tolerate surface water pressure, none seems to be able to penetrate into shallower waters. Again, this result would indicate that ecologic factors other than hydrostatic pressure prevent them from doing so.

Most of our experiments involved rapid pressurization and decompression and further, were performed on only one individual per species. A few longer range pressurization experiments on conspecific individuals gave similar results, particularly with reference to the upper range of maximum pressures at which normal activity was registered in the short range

experiments. Particular examples are the extended pressure experiments performed on individuals of the intertidal gastropod, *Tegula funebralis* , and the long range experiments on the deeper water gastropod, *Boreotrophon bentleyi*. However, additional experiments on larger numbers of individuals per species will be required to determine the validity of the maximum pressure tolerance determined by us for species where only one individual was rapidly pressurized and decompressed.

The implications of the close agreement between the maximum pressure tolerances as determined experimentally, with the pressure of their depth range for species from depths greater than 500 m may be biased. For we have obtained only meaningful data from individuals of species which can tolerate *in situ* decompression without visible effects on their activity. Again, larger series of individuals from other species at this depth range should be subjected to pressure experiments. Concerning deeper water animals, it is of interest to recall that two of the gastropod species survived *in situ* depressurization but showed severe muscular impedance thereafter. Of the likely reasons for this phenomenon we can exclude differences of salinity and water chemistry. The differences in surface and bottom waters at the time of recovery were minor. In the case of *Bathybembix bairdii* it would appear further that at the shallowest record of recovery of this species, the bottom water temperature is close to that at which our experimental animals were maintained. The most conspicuous difference in the ecology of the organisms which we recovered between 560 and 730 m and our experimental conditions may be the low content of dissolved oxygen at the recovery site.

We wish to restate here that with rare exceptions our experimental animals survived our pressure experiments.

Acknowledgment

The project was supported by a grant from the Office of Naval Research, Contract No. NONR-220(46), Project No. NR104-667. We are indebted to Dr. D. S. Gorsline for providing us with ship time on the *Velero IV* to obtain the experimental animals by dredging; Dr. M. Keen for identifying the deeper water gastropods; Dr. J. H. McLean for information on the known depth ranges of some of the deeper water gastropods; N. Shields for technical assistance; and B. Swart for maintaining the experimental animals under atmospheric conditions in the laboratory.

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Discussion Following Lowenstam Paper.

HESSLER: Were the species that did survive all of these manipulations ones that are normally known in environments of reasonable oxygen tensions, or are they also known from environments of low oxygen?
LOWENSTAM: They're all from the same environment. Just a deeper water.
HESSLER: So you are showing that different species of the same genus can behave in quite different fashions in response to your treatments.
LOWENSTAM: In response to my treatments, yes. I'm still dubious that we should go any further. I presume that you must expose the organisms to light in order to observe what they're doing.
HESSLER: Is there any possibility that it's the light they don't like, rather than the oxygen? They come, I presume, from a dark environment. This certainly is true in the case of some of the ophiuroids, I know. They don't like light at all.
LOWENSTAM: That's right. They used to hide under rocks which we put into the aquaria, but that may not shield them. That is a possibility.
VIDAVER: Of course, that wouldn't explain the effect that pressure could have on them.
LOWENSTAM: It would explain it for some species if they were very sensitive. I would expect that more than one factor was involved.
MENZIES: Dr. Lowenstam, I missed the earlier part of your talk and I'm very sorry about that. How long did you keep these animals in the aquarium?
LOWENSTAM: The particular organism that I talked about in detail, up to six months. As to rate of pressurization, we have never gone below 5 minutes in 250 psi steps - where we stop for five minutes and go up, and then 15 minutes, and in some cases an hour, waiting at each level - without finding some real differences in control groups with reference to the maximum pressure tolerance for normal activity. On the other hand, we have found that the slower the pressurization the later the apparent onset of "tetany."
BRAUER: What are your flow rates? What is the water turn-over rate in the

aquarium? And, finally, are you making any provision for stirring these aquaria to make sure the water is mixed in them in any reproducible way?
LOWENSTAM: No, we are not stirring them. Flow rates are such that we use a carboy of five gallons in about three days' time. The alloy 316 is non-magnetic stainless steel, so that it would be an easy matter to stir them. We did some schlieren observations in the preliminary tests, and we felt that there was enough turbulence because these are small cylinders. Thus we felt stirring was not necessary. Maybe we are wrong and should do parallel experiments and find out whether perhaps the animals would do a little better. But even with the slow pressurization of the animals, none of them changed their maximum pressure tolerance for maintenance of normal activity. Even when keeping them close to the upper pressure range where they could move around actively and feed if we injected food into the system through a feeding loop, they didn't seem to show any ill effects at all.

DESIGN AND PERFORMANCE CHARACTERISTICS OF CURRENTLY EXISTING HIGH PRESSURE AQUARIUM SYSTEMS

Hans Theede
Institut für Meereskunde,
University of Kiel, Germany

At our laboratory of the "Institut für Meereskunde" in Kiel, Prof. Schlieper initiated some eight years ago studies on the action of high hydrostatic pressure on marine invertebrates and fishes (Schlieper, 1963). In the following years many short-term experiments with small animals or tissue pieces could be done with a basic pressure apparatus, similar to that designed earlier by ZoBell (1954) and others (Fig. 121). It is inexpensive and easy to handle and still offers many facilities for high pressure studies. It consists of two cylindrical pressure vessels of stainless steel, which are connected over needle-valves (AMINCO) and capillary tubes with a manometer and a hydraulic hand-pump. The test specimens, which are to be studied after compression, are not directly placed into the steel chambers but into small plastic containers, three of which can be introduced into one

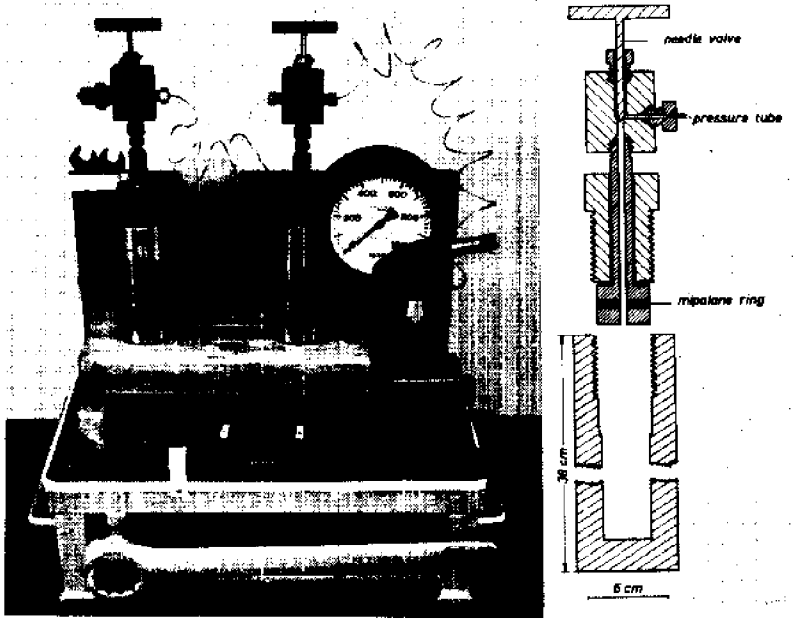

Figure 121 Left side : Pressure apparatus with closed pressure chambers for short term experiments. The pressure vessels are connected by needle-valves and capillary tubes to a hydraulic hand pump, which in turn is coupled with a small water container. Right side : Longitudinal section of a pressure chamber. Length of the chamber 38 cm, diameter 7/8 cm, wall thickness 2 cm, volume about 300 ml. (After Ponat, 1967).

steel-cylinder. Within the plastic test-chambers, factors like salinity, ionic composition, pH, oxygen tension etc. can be varied. Additionally the single steel-chambers can be placed in water jackets with definite constant temperatures, or the whole equipment can be easily placed in a constant temperature room. In this way these chambers permit keeping small organisms for short periods of time at high hydrostatic pressures combined with definite other external factors.

Studies of the pressure resistances of a great number of shallow water species of the North Sea and the Baltic have been performed with the aid of this apparatus. (Schlieper, 1963; Ponat, 1967; Schlieper, Flügel & Theede, 1967; Ponat & Theede, 1967; Naroska, 1968; Schlieper, 1968; Flügel & Schlieper, 1970; Theede & Ponat, 1970).

According to Naroska (1968) among amphipods, isopods, molluscs, echinoderms, and polychaetes, certain species were able to withstand pressures exceeding 500 atm. for one hour, whereas some decapods and teleosts, a mysid, and a tunicate were relatively pressure sensitive. On the whole, he found that species of these systematic groups from shallow waters proved to be especially pressure resistant, just as other species derived from the same groups have proved themselves able to become accustomed to deep sea conditions in nature.

In several invertebrate species the survival capacity proved to be higher in a medium that resembles the ocean depths in that it is not fully saturated with air. The resistance to pressure decreased with increasing oxygen tension. As a reason for this, it is suggested, that oxidation and inhibition of sensitive SH-enzymes and of other organic compounds occurs under pressure (Theede & Ponat, 1970).

The same pressure apparatus could also be used in the study of some enzyme activities under pressure (Ponat, Beress & Theede, 1971). In such experiments several test samples in small plastic jars could be exposed to pressure at the same time.

After decompression the reaction (i.e., phosphatase, aldolase, etc.) was stopped, and the reaction rate measured with a spectrophotometer.

In order to observe the reactions of surviving small animals, isolated organs, or tissues during the action of compression, a steel chamber equipped with hard glass windows is used (Fig. 122). A similar type had been designed earlier by Marsland & Brown (1936) and Marsland (1938), (1950), and several modifications of it are in use by different authors. Light penetrates from the upper side of the chamber through the glass windows. The specimen, placed in a special small plastic chamber or mounted by glass rods and vaseline on the lower window, can be observed with the aid of an inverted microscope. A cross stage allows the field of view (Fig. 122) to be varied readily.

This pressure chamber could be used successfully for observing the activity of small transparent animals, their heart beat, or ciliary movement.

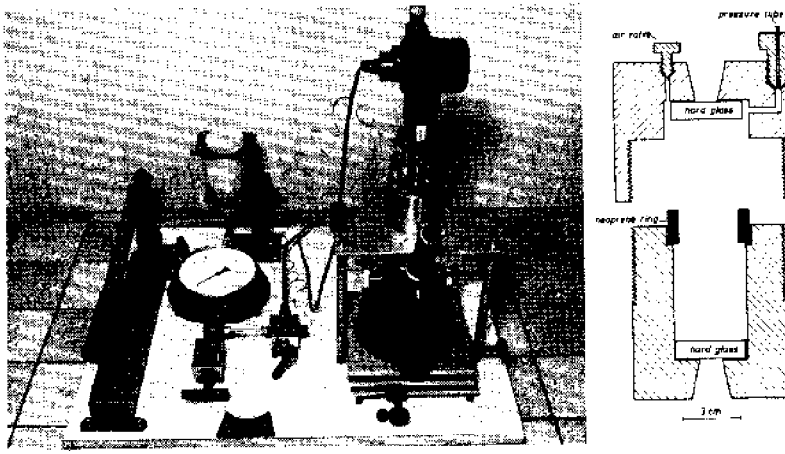

Figure 122 Left side : Pressure apparatus with window chamber and inverted microscope for observation of small animals high pressures.
Right side : Longitudinal section of a pressure chamber with hard glass windows. (After Ponat, 1967).

Results of Naroska (1968) show that the heart frequency of amphipods responded with temporary accelerations to a stepwise increase of hydrostatic pressure. After decompression the heart rate recovered, provided that pressure had not been too high. Similar results were also obtained by Pease and Kitching (1939) with the ciliary rate of *Mytilus* gills.

The time of maintenance of ciliary activity under the influence of high pressure was measured in media of different osmotic strength and ionic composition as well as at different pH values (Ponat, 1967; Ponat & Theede, 1967). At 500 atm. the activity time increased nearly logarithmically over the pH-range from 5 to 9. When pressure was increased up to 600 atm., the sensitivity of the tissue to pressure increased, especially at higher pH values (Ponat & Theede, 1967).

One disadvantage of these two pressure devices is that they only contain relatively small amounts of water. Therefore, most laboratory experiments, using these instruments had to be completed within short periods of time (one or a few hours), since only a limited oxygen supply was available. This disadvantage could be eliminated by an open system, supplying continuously running sea water under high pressure (Fig. 123). With this apparatus, which was assembled with the aid of AMINCO, it was possible to keep animals at high pressures for longer periods of time. Many experiments up to 24 hours and more have been performed with this instrument.

The sea-water is pumped at a constant rate by an electric hydraulic pump from a temperature regulated and aerated reservoir through ball valves into a large stainless steel pressure chamber, which is connected with a manometer. The water passes the steel chamber through a pressure tube, then arrives at a manifold, one side of which is connected to a pressure

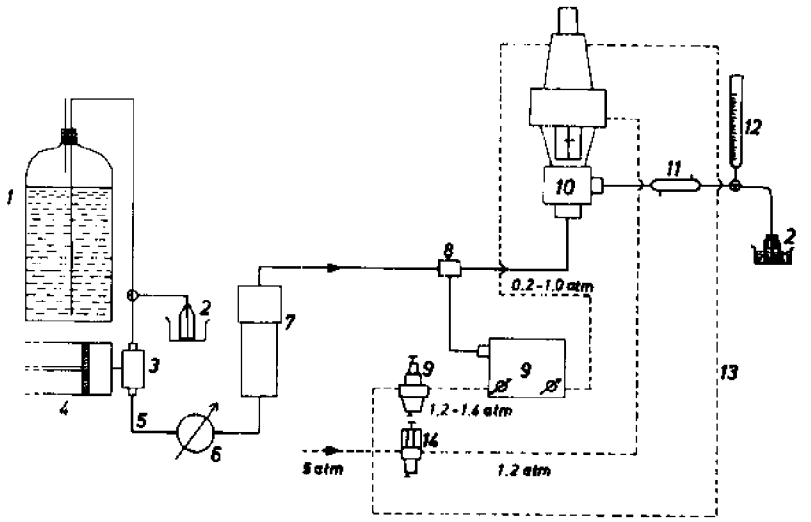

Figure 123 Pressure apparatus for measurement of oxygen consumption of marine animals in running seawater at high hydrostatic pressure. 1 - water reservoir, 2 - Winkler bottle, 3 - ball valves, 4 - hydraulic pump, 5 - pressure tube, 6 - manometer, 7 - pressure container, 8 - manifold, 9 - pressure control unit, 10 - adjustable outlet valve, 11 - cooling coil, 12 - burette, 13 - line for compressed air, 14 - reducing air valve. (After Naroska, 1968).

control unit (Honeywell), the other one to a sea-water outlet valve. The pressure control unit continuously regulates the opening of an outlet valve by pneumatic impulses. In this a way the pressure in the experimental chamber is regulated at a certain level. The pressure fluctuations amount to about 10 atm. When the medium leaves the outlet valve, it suddenly decompresses, and, in connection with this fact, small air bubbles can be formed. Therefore the water passes a cooling coil before the oxygen content in it is measured by the Winkler method. Figure 124 offers a view of the entire running sea water pressure system. Figure 125 shows one part of this equipment.

Many measurements of oxygen consumption over periods of 24 hours have been conducted in Schlieper's laboratory by Naroska (1968). During longer exposure under pressure a certain tendency toward recovery of the respiratory rate could be observed.

In order to study changes in the structure of cells and tissues under pressure, Flügel (1971) designed a pressure vessel basically patterned after the pressure chamber used by Landau & Thibodeau (1962), which allows fixation of a small specimen while under pressure (Fig. 126). The stainless steel pressure chamber is closed by a steel cover and a screw cap and sealed with a neoprene 0-ring. The fixation chamber within the pressure vessel is separated into two compartments by a circular cover glass. One compartment is filled with the fixing agent (glutaraldehyde), the other contains the biological specimen (small marine animals, excised tissue pieces) together

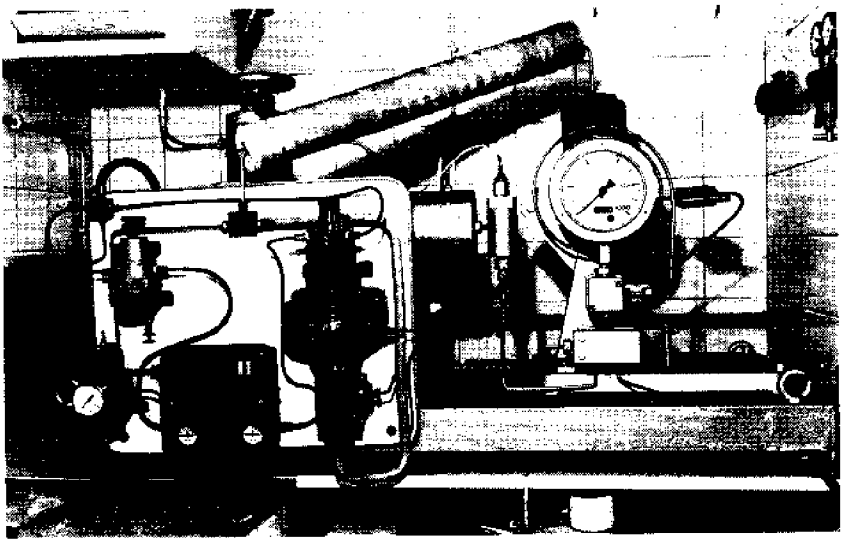

Figure 124 Pressure device with continuously running seawater. In front the regulating system, with the pressure control unit on the left, and the adjustable outlet valve on the right side. In the background a part of the hydraulic pump, and behind the manometer a pressure cylinder. (After Naroska, 1968).

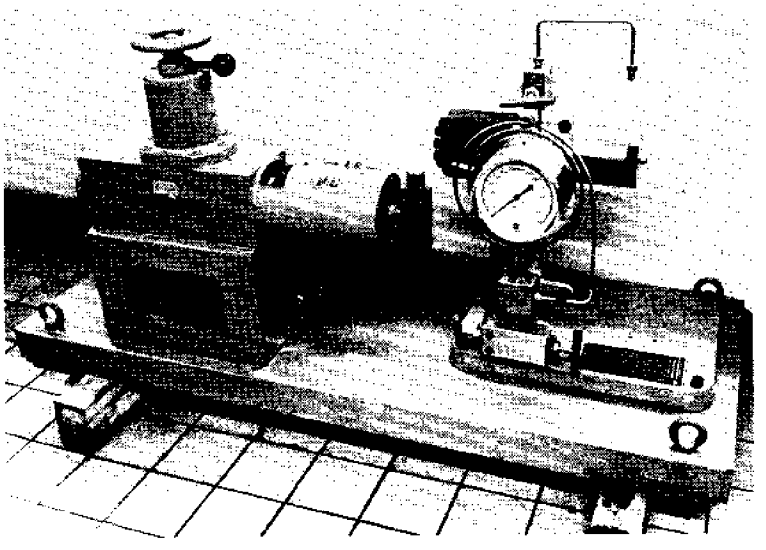

Figure 125 Part of the pressure equipment shown in Fig. 124. On the left the hydraulic pump, on the right manometer and pressure vessel. The pressure chamber has a volume of about one liter.

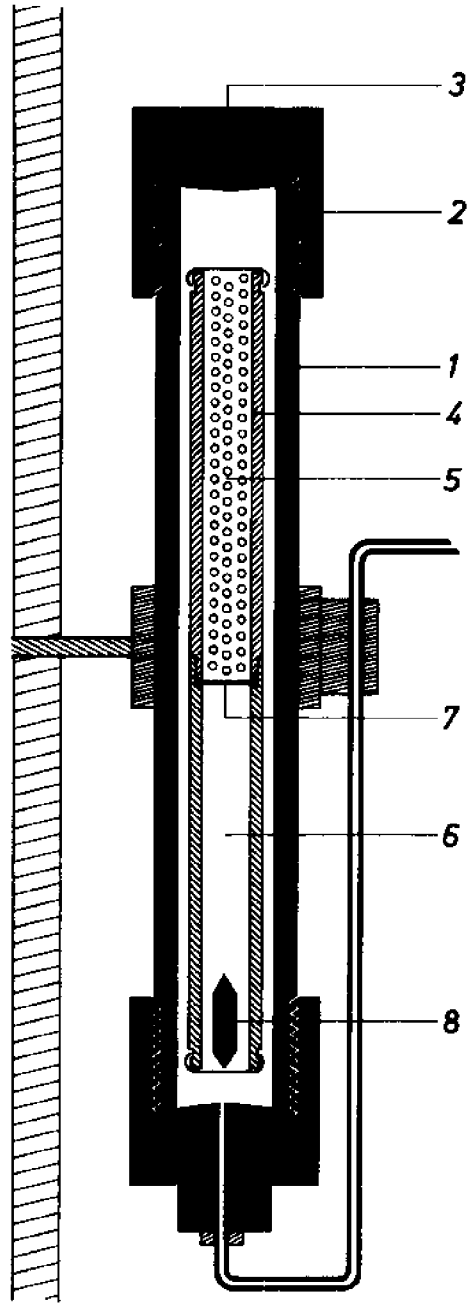

Figure 126 Vessel for fixation of tissues or small animals during the action of compression. 1 - pivoted pressure chamber, 4 - fixation chamber covered by rubber lids, 5 - fixative compartment containing glutaraldehyde of double concentration, 6 - specimen compartment, 7 circular cover glass, 8 - steel bullet tipped at both ends. (After Flugel, 1971).

with a steel bullet tipped at both ends. After the desired period of compression, the pivoted pressure vessel can be released. It is then spun around rapidly by the tension of the coiled pressure tube. The cover glass separating the fixation chamber is broken by the steel bullet and the specimen exposed to the fixing agent. After approximately 10 to 15 min. the pressure is released and the fixation completed at atmospheric pressure.

For optical measurements of enzyme activities under pressure we use a small steel curvette with quartz glass windows (Fig. 127), which is placed in a Zeiss PMQ II—spectrophotometer. Where the optical cell is connected with the pressure tube, a movable piston with double neoprene O-rings provides pressure equilibrium between the inside and outside of the cell and at the same time prevents the mixing of the reaction medium inside the optical cell with the medium of the pressure tube.

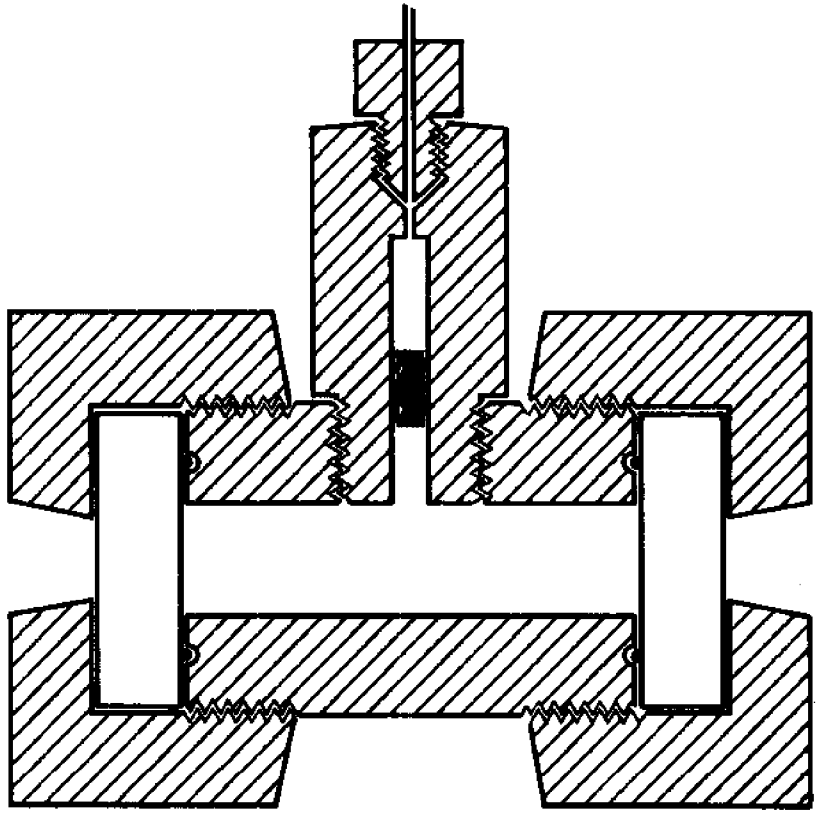

Figure 127 Optical cell for the study of enzyme activities under high pressure.

For further studies such high optical pressure cells will be equipped with temperature jackets. For measurements of metabolic rates of marine animals, which should be extended in future to real deep sea forms, we plan to try to reduce the pressure fluctuations in the running sea water system for long-term experiments, and to equip it with electrodes for continuous measurements of oxygen consumption, and, so far as possible, also of other parameters.

Acknowledgments

I am grateful to Dr. C. Schlieper, who encouraged me to write this report and to Dr. H. Flügel, who provided unpublished data concerning his pressure apparatus (Fig. 126). I also wish to thank the Deutsche Forschungs-Gemeinschaft and Dr. R. Brauer for financial support for my participation in the meeting at Wilmington, North Carolina.

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Discussion Following Theede Paper

MACDONALD: This outlet valve is a critical part for your pressure control-How does it work? Is there an adjustable outlet?

THEEDE: There are an adjustable outlet valve and a pressure control unit supplied with compressed air, coupled to the valve, and then it is mechanically adjusted.

MACDONALD: Is this outlet valve a valve which is continuously being controlled?

THEEDE: Yes, but the pressure changes which are produced by the piston of the pump are not compensated.

MENZIES: At any time did you have any difficulty keeping the system in operation? I am assuming that you might have had some, and I would like to learn about those in particular so that one can avoid the problems.

THEEDE: When the water was dirty, the pump did not function so well at first. Later we only worked with filtered sea water and this was better.

BRAUER: There was one very interesting point I believe I saw in your paper. You made some rather nice differential temperature measurements.

THEEDE: We have water of 15.8 to 16.2° coming to the valve at about 500 cc/hr, and flowing out of it at 18.6 to 18.8°C. The temperatures were measured with a thermistor.

BRAUER: As you are flowing across the particular restrictor used here,

there is enough friction apparently so that you generate heat over and above the adiabatic cooling, so that the water that comes out is perceptibly warmer than the water that comes to the valve. From the point of view of people who do gas analyses this is a catastrophe because it changes the solubility of the gases and you need to exert great caution to not get extremely gross artifacts. I think it is one of the things we'll all need to watch out for very carefully.

VIVADER: Do you regulate oxygen concentration in the flow system?

THEEDE: No. We only worked with air-saturated water, and then measured the decrease in the oxygen content in the running sea water. We never began with undersaturated water.

AN OBSERVATIONAL HYDROSTATIC PRESSURE VESSEL FOR THE STUDY OF THE BEHAVIOR AND METABOLISM OF WHOLE ANIMALS[1]

By
R. M. Avent**, R. J. Menzies**
and
D. Phillips**

Abstract.

A reasonably large volume pressure vessel is described which enables the investigator to simulate a deep-sea pressure environment in the laboratory and monitor its behavioral and physiological effects on whole animals.

Introduction

Investigations on the effects of hydrostatic pressure on biological materials have taken many forms since the completion of Regnard's original studies (1891). Consequently numerous experimental pressure vessels have been designed to meet the requirements of individual investigators (Table 1). The absence of suitable commercially available pressure vessels and components is not surprising in light of the bewildering variety of designs and performance characteristics of pressure equipment presently employed for research purposes.

An experimental system for the study of whole organisms under hydrostatic compression was desired which would combine the advantages of high visibility, a relatively large volume, a high pressure capability and portability. No existing system meets all of these requirements. The pressure vessel described here was designed and constructed to allow monitoring of behavioral and physiological changes of whole invertebrates and fishes at hydrostatic pressure up to 1,000 atm., the pressure equivalent of a 10,000 m deep marine environment.

The vessel is equipped with observation and lighting ports, an oxygen-monitoring system, electronic feedthroughs, and an external integral camera mount. It has a volume of 900 cc, which permits the insertion of large, active animals. Its relatively small size and weight (190 lbs.) allows its operation aboard research ships.

1 Contribution No. 277 from the Department of Oceanography, Florida State University.

** Department of Oceanography, Florida State University, Tallahassee, Florida, 32306, U.S.A.

Table I
A Tabular View of Pressure Vessels Used In Biological Investigations

References to Pressure Vessels	Pressure Capability (psi)	Viewing Ports No. & type	Internal Volume (ml)	Purpose of Pressure Application or Material Tested
Regnard (1885)	12,000	None	ca.150	Observation of small animals.
Brown (1934)	10,000	None	ca.1-2	Centrifugation of cells to measure intracellular viscosity.
Landau & Peabody (1963)	15,000	None	0.4	Cells and cell extracts.
Marsland(1938)	20,000	2/tempered glass	400	Microscipe observation of cells.
Johnson & Lewin (1946);Zobell & Oppenheimer (1950); Zobell (1959);Morita & Haight (1962); Schlieper (1968); Landau (1970)	15,000 to 30,000	None	ca. 150 to 300	Enzyme preparations, bacterial cultures, or small animals. (Several modifications of the same basic design.)
Marsland & Brown (1936)	7,000	2/glass	very small	Microscope observation of cells.
Brown, Johnson & Marsland (1942)	7,000	1/glass	very small	Study of bacterial luminescence.
Morita (1957)	15,000	2/White sapphire	7.5	Spectrophotometric determination of enzyme activity.
Ponat (1967)	15,000	2/hard glass	75	Observation of small animals and tissue pieces.
Naroska (1968); Flugel & Schlieper (1970)	6,000	None	850	Determination of 0_2 consumption of whole animals. (Employs a circulating sea water system.)
Botts, Johnson, & Morales (1951)	5,000	2/Herculite plates	ca. 200	Observation of whole animals.
Sundnes (1962)	150	2/plate glass	360 liters	Study of large animal physiology and behavior.

The Pressure Vessel

Type 303 stainless steel was selected for the fabrication of all major structural components because of its excellent machining qualities and adequate ultimate tensile strength. All points of potential leakage are sealed with hard rubber 0-rings of appropriate dimensions. Low machining tolerances were maintained throughout to minimize the extrusion of 0-rings and acrylic windows.

The pressure vessel (Figures 128 and 129) is essentially a thick-walled cylinder enclosed on either end by heavily threaded end caps, each of which is fitted with a 60° conical "Plexiglas" window. Identification of the end caps assures that they are replaced on particular ends of the cylinder each time the instrument is assembled. This allows the proper seating of the faces of respective cylinder and end caps which become specific for each other because of minor wear and pressure distortion.

One side of the cylinder (the floor in actual operation) is connected to a manually operated, stainless steel, pressure pump (Enerpac Co., Butler, Wisc.) with high pressure cone fittings, adaptors, and tubing. An Enerpac 30,000 psi gauge and valve are included in this system. A bleed valve is mounted in the upper surface of the cylinder through which air bubbles are expelled prior to pressure application.

Two insulated bulkhead connectors have been implanted to one side of the pressure inlet to facilitate the use of physiological transducers, electric stimulators, or other instruments requiring the transmission of electric current to or from the chamber interior at high pressure levels.

Two dissimilar ports are located in the upper surface of the cylinder, one on either side of the bleed valve.

The larger of the two is designed specifically as a lighting port and is enclosed with a threaded steel pressure plug into which is mounted a thick, 20°, conical, Plexiglas window. The light source consists of a rheostatically adjusted, high intensity lamp with an integral heat filter (a rapid flow water pathway about four inches in length). This lighting unit screws directly into the outer end of the pressure plug. The lamp provides sufficient illumination to make motion pictures of animal behavioral patterns under pressure.

The smaller port is closed similarly with another threaded steel pressure plug into which is fitted a long, cylindrical, Plexiglas window. This window is interchangeable with a model 5331, Yellowspring Instruments Co., Ag-Pt oxygen electrode which has been specially repotted in epoxy resin and machined to fit the pressure plug orifice. The electrode is used in conjunction with the YSI model 53 oxygen monitor, which in turn may be connected to any suitable strip-chart recorder. A small flat platform placed on the concave floor of the pressure chamber permits a one-inch magnetic stir-bar to rotate when driven externally by a commercial magnetic stirrer.

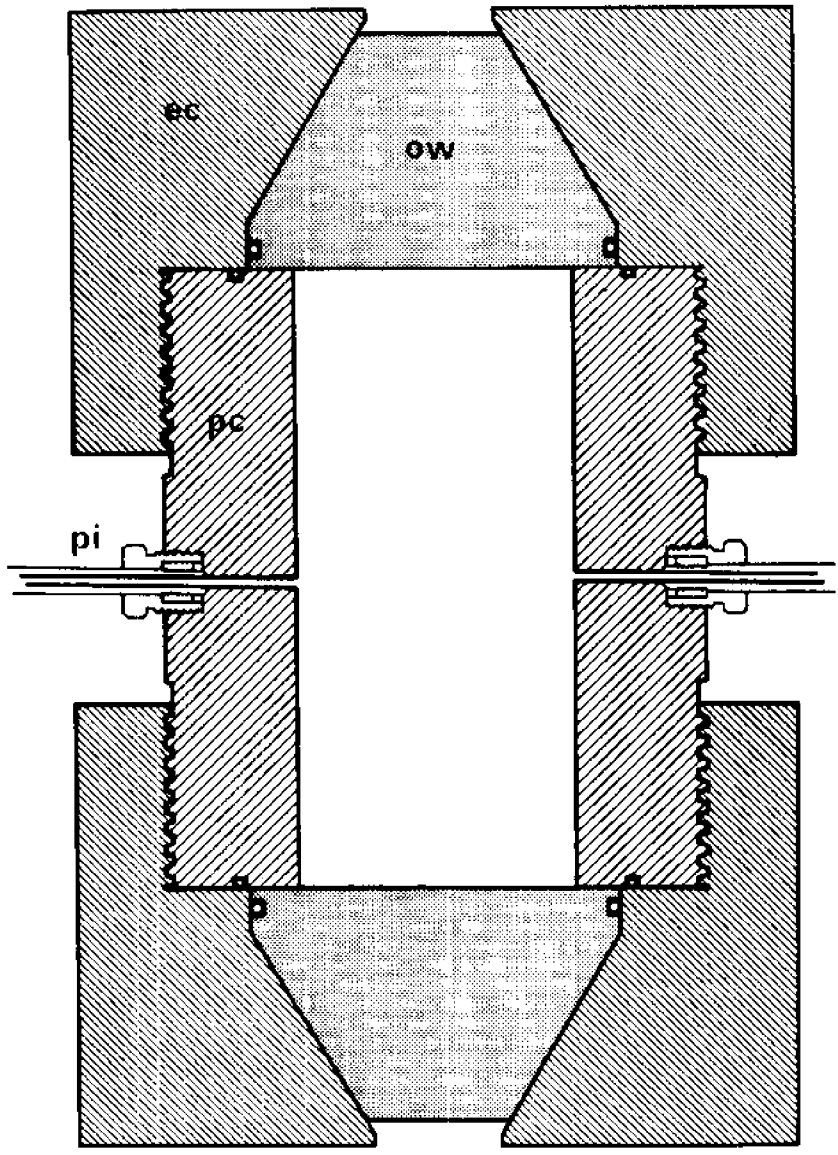

Figure 128 A longitudinal view of the pressure vessel; ec, end cap; ow, observation window; pc, pressure cylinder, pi, pressure inlet.

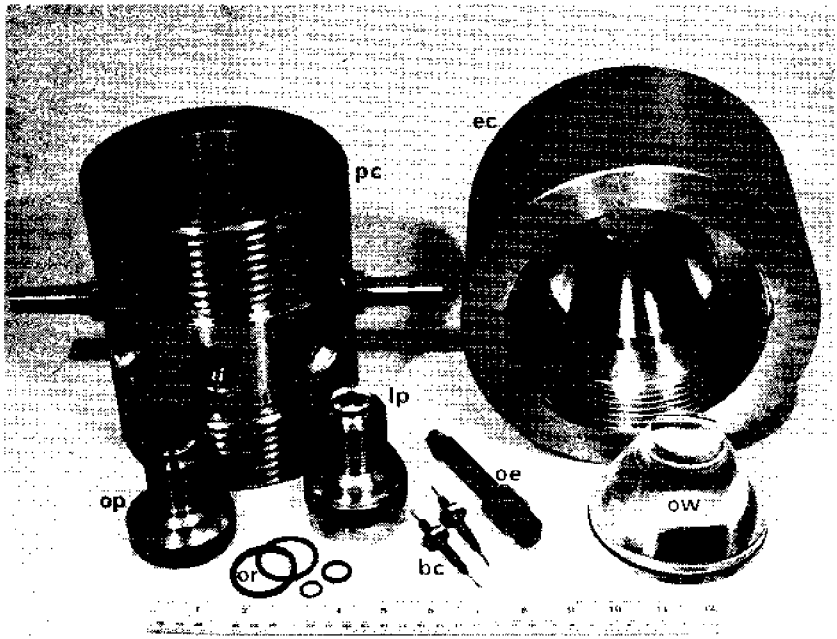

Figure 129 The disassembled pressure vessel. ec, end cap; lp, lighting plug; oe, oxygen electrode; op, oxygen electrode plug; or, O-rings; ow, observation window; pc, pressure cylinder.

The water currents produced by the rotating stir-bar ventilate the electrode and are necessary for accurate oxygen determinations.

Strong pivots are mounted on either side of the cylinder permitting the investigator to rotate it 360° in a vertical place between aluminum standards. The vessel may be secured at any angle by tightening set screws at the top of the standards.

Two holes have been drilled and tapped in each end cap into which lifting rings are screwed to facilitate the handling and securing of the pressure chamber, especially at sea. A photographic mount which also screws into these holes, positions an 8 mm "Bolex" 160 Macrozoom movie camera directly in front of either of the observation ports. This method of mounting effectively reduces camera vibration.

The assembled pressure vessel is seen in Figure 130 with accessory photographic and O_2 recording systems intact. Table 2 gives the design parameters for major pressure vessel parts.

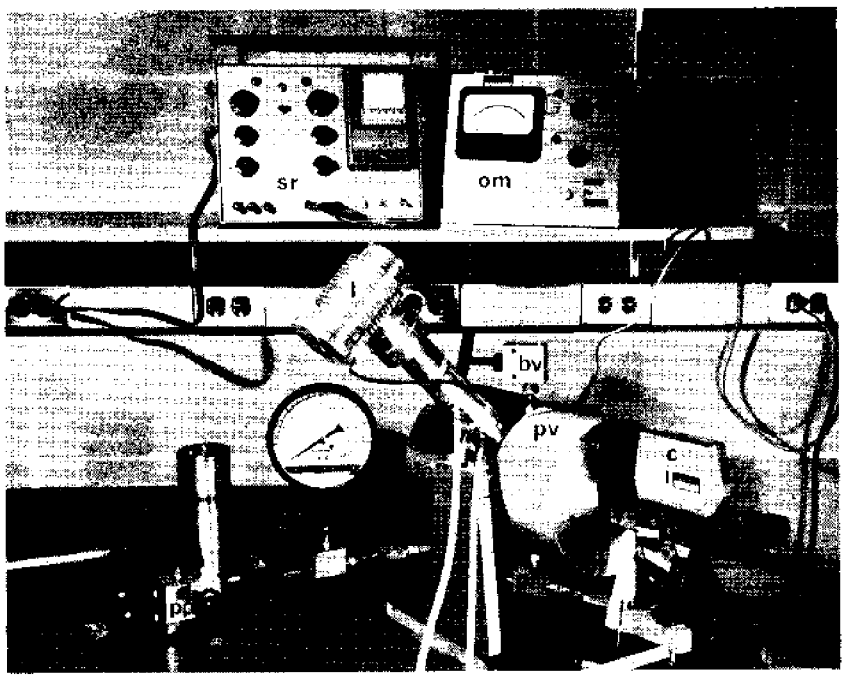

Figure 130 The assembled pressure system. bv, bleed valve; c, camera; l, light source; om, oxygen monitor; pi, pressure inlet; pp, pressure pump; pv, pressure vessel; sr, strip chart recorder.

Table 2

The Pressure Vessel: Design Parameters of Major Parts

Part	Structural Material	Description (All Dimensions in mm unless otherwise specified)
Pressure Cylinder	303 Stainless Steel	O.D. 151; I.D. 82; Length 178; Acme threads 1/8 inch deep, 4 per inch. Perforations for feedthroughs, light and O_2 ports, pressure inlet, and bleed valve.
End Caps	303 Stainless Steel	O.D. 204; I.D. 151; Thickness 126; Machined 6060°, window cavity; D.O.L. 5 flange (Stachiw, 1969). Internal threads matching those on cylinder.
Observation Windows	Annealed Plexiglas	Maj. Dia. 104; Min. Dia. 41; Thickness 68; 60° truncated cone with radial 0-ring groove.
Lighting Plug	303 Stainless Steel	Threaded Dia. 39; Max. Dia. 58; Total length 77; Machined 20° conical cavity to accommodate Plexiglas window;0-ring grooves in 45° lapped face, externally, and near high pressure window face, internally.
O_2 Probe Plug	303 Stainless Steel	Threaded Dia. 32; Max. Dia. 61; Total length 59; Bored for cylindrical Plexiglas window or O_2 probe. 0-ring groove in external 45° lapped face.

Pressure Application

Operating the pressure system is a relatively simple process. Initially, all pressure line, gauge, pump, and valve connections are secured, inspected for leakage, and fitted to the pressure inlet in the floor of the vessel. The system is then purged with well aerated and filtered sea-water or Instant Ocean, an artificial sea water preparation, contained in the pump reservoir. The appropriate physiological recording systems are prepared for operation and connected to the pressure vessel. The vessel is filled with water, and experimental animals are introduced through the main lighting port. Most of the air in the vessel escapes through the open bleed valve in the roof of the vessel when the lighting port plug is screwed in, the remainder of which is expelled by operating the pump handle. The photographic accessories are mounted if a visual record is to be made. The bleed valve is closed, and the pressure is increased to the desired level through manual operation of the pump handle.

Any pressure up to 1,000 atm may be attained and maintained at a constant level for hours or days in a constant temperature environment. The temperature variations accompanying compression or decompression of sea water are relatively small, being a function of the rate of pressure change and the ability of the vessel to dissipate heat to its surroundings. It is unlikely that the internal chamber will deviate from ambient temperature more than ±3°C after rapid compression or decompression to or from 1,000 atm (ZoBell, 1959) and normally it would be expected to deviate much less.

From a safety standpoint, it is imperative that all air bubbles are expelled from the pressure system prior to pressure application. In addition a small air bubble dissolved under pressure will produce large errors in 0_2 recording, and may affect animals' resistances to pressure (Theede and Ponat, 1970).

The light port is constructed in such a way as to assure its failure before the main view ports thus assuring protection to the observer. Thus far no failures have been encountered after 200 cycles of use up to 450 atms.

Acknowledgement.

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Discussion Following Avent Paper

SAUNDERS: I would like to direct these remarks to the actual hardware of the test chamber, especially opening seals. The 45° lap fitting is a good method of sealing. But, it also is an expensive and time-consuming way to go. With clever and careful use of face seals, you can accomplish the same

thing. Another thought concerns the electric feed-through. As these chambers become more complex and you will want to read more items, I think you should think of multipin penetrators. I noticed on this chamber single pins for each electrical feed-through. It is now possible to take as many as 55 pins through a 2-inch hole in one connector, and some of these prototypes go on to 13,500 psi. This means one penetration in your hull versus a multitude of them, so you get more reliability in your test chamber. This particular penetrator is being made by Viking Industries in Chattsworth, Calif. The unit was developed basically with the DSRV. It is their hull penetrator, and I think drawings are available from them to study their approach. I don't recommend necessarily using their hull penetrator because this is man-rated and we get awfully complicated there. Two sets of class A seals, two sets of 0-rings. So you have to have a multitude of failures. I think in a test chamber, you could cut the cost down by going to the single approach, and then having safety devices to ward off anything if it was to go wrong.

VIDAVER: Did you take any precautions in attempting to coat the steel?

AVENT: I'll take the last question first. We were considering applying a coat of teflon to the inside of the chamber, but we haven't talked to anybody who believes that it will work. The type 303 steel we are using has adequate tensile strength, but is not particularly corrosion-resistant. It has fantastic machining qualities, however, and with the tolerances we wanted on our high pressure chamber, we took this into consideration before the other factors.

LOWENSTAM: We had tried initially to use very carefully tooled stainless steel tubes. We always found that there was a considerable oxygen consumption inside the vessel. And if you want to measure oxygen uptake in relation to metabolic processes you would have to determine the noise to get the signal-to-noise raitio. I do believe that you can coat it with plastic because in our experiments, where we carried them on for a six-months period, we have good evidence that they do work despite the general skipticism I have discovered there.

AVENT: I have run some oxygen determinations for periods of several days at 1 atm, and I have seen no great oxygen decrase in the chamber for that period of time.

WELLS: With reference to bubbles in there: I wonder if these bubbles you referred to were oxygen or air bubbles. You see, we have the problem with the increase of the partial pressure of gases due to pressure alone. As an additional problem, if there is an air bubble we could conceivably have nitrogen narcosis.

AVENT: We were always very careful to bleed our valves in our pressure system and to fill everything up with sea water saturated at 1 atm in such a way as not to include any air bubbles.

(Question) In the work here referred to was it really just oxygen, or was

oxygen the only thing measured when you trap a bubble in there?
AVENT: In the cases where we did find out later that we did have a bubble, our oxygen level was just fantastically high—way off scale.
MENZIES: With reference to sea stories—it's legitimate here to bring out some of our failures, and we have had them. The needle valve which you saw in the photograph had been in the system and subjected to pressure as high as 20,000 psi. For some reason this needle valve decided it was going to cease working, and we tried to open it. We couldn't, and so we had to unscrew the entire bolt, and when we did this we found out we were making a new set of threads which we hadn't really expected. What had happened in this instance was the metal of that bolt had fused with the metal of the case around it. I was speaking to Mr. Kirk this morning about this, and he said, "This happens quite often in the case of very similar metals."
SAUNDERS: Do you use grease? We had the same problem of getting connections apart. Once they are put together, they stay together. In most cases, we use some form of silicone grease.
(Comment) Silicone grease is not a very good lubricant compared with ordinary hydrocarbon greases. We destroyed a whole pressure vessel once by using it. Silicone grease is a good thing to avoid.

THE DOUBLE ENVELOPE CONCEPT IN THE DESIGN OF HIGH PRESSURE AQUARIA

By
Ralph W. Brauer and Michael R. Jordan
Wrightsville Marine Bio-Medical Laboratory
7205 Wrightsville Avenue
Wilmington, North Carolina 28401

The preceding presentations suggest that the problem of designing high pressure aquarium systems adaptable for work with nano-plankton and with micro-organisms are coming under control rapidly, even though details of design undoubtedly will change from laboratory to laboratory to accommodate specific experimental requirements. By contrast, the problem of providing for larger animals, especially when one contemplates designs which would allow interphasing the shipboard or laboratory systems with any type of deep retrieval device, is only beginning to be approached actively. Yet, it seems to me that it is in this area that a great deal of scientific work that needs to be done in the near future is likely to be encountered. Whether one looks the problem of histological and cytological studies, or one considers work with excitable and contractile tissues, or whether one is concerned with growth, differentiation, and tissue replication—these and a host of other problems pertinent to the biology of deep sea fauna await experimental study which can only be carried out on specimens of sufficient size and of sufficient complexity to permit the necessary sampling and manipulative procedures.

Based on such considerations, we set out some time ago to explore design principles which might lend themselves to assemblies that would meet the requirements for this kind of work. We felt that initial boundary conditions must include a working volume that could reach several cubic feet, the ability to maintain continuous circulation of water of predetermined ionic and dissolved gas composition, a safe, i.e. contamination-free, environment, maintenance of ambient pressure throughout with fluctuations or pulsations not to exceed 0.5% of mean selected ambient pressure, provisions for controlling temperature to the degree of precision required by any experiments envisioned, means of observing the animals, and systems for bringing out of the high pressure envelope such samples and such chemical information as will be needed to steer the experiments. Additional requirements which for the time being we put aside included mechanisms for introducing food, and for manipulating specimens inside the test chamber.

To meet these requirements we decided upon a basic design consisting of a high-pressure envelope within which the aquarium system proper is to be enclosed. This in turn is to consist basically of a double reservoir system, reservoir A to be essentially a flexible, gas-impermeable, and chemically inert bag, while reservoir B is to be a rigid, transparent vessel made of

material suitable for housing the marine life to be studied. The two reservoirs are to be interconnected by some pliable and chemically acceptable type of hose, and the rigid reservoir in turn is to be linked via a hull penetration to a suitable flow control valve leading to sampling and overflow lines. Within the high pressure enclosure, this dual reservoir system in turn is to be surrounded by a suitable hydraulic fluid (mostly distilled water) maintained at the desired pressure by means of an appropriate pumping system. Temperature control would be maintained by immersing the outer shell in some type of constant temperature bath. In the Mark I version (Figure 131) of this system, we realized this design literally

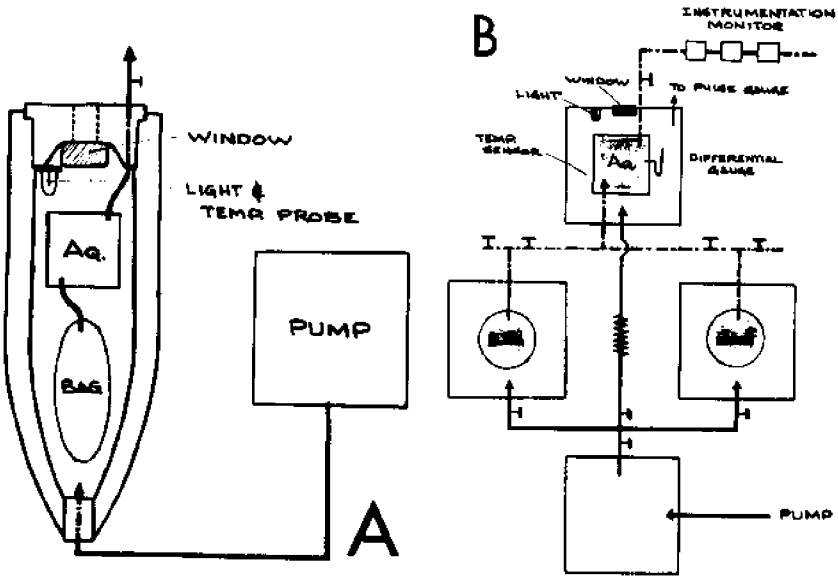

Figure 131 A-Plan of the prototype double envelope high pressure aquarium at the Wrightsville Marine Bio-Medical Laboratory.
B-Plan of Mark II version of the system as currently in use.

in the fashion described above using a modified 16" projectile as the high pressure envelope, with a vinyl chloride plastic bag as the collapsible reservoir and a lucite cylinder with plane windows at either end as the rigid reservoir. Supplied by a solenoid-controlled differential membrane pump this system was found to be operational and to permit us to maintain a number of shallow water species at hydrostatic pressures of the order of 100 atm. for one day. A window inserted into the breech end of the shell provided for direct observation; a tungsten iodine lamp protected by a tempered glass shell provided for internal illumination as required. Chemical control was maintained by leading the effluent from the chamber to oxygen and pH-electrodes for continuous control and comparison with the original

sea water used as the aquarium medium. This relatively simple and small system did not completely eliminate transmission of the pressure pulse from the pump to the aquarium. Furthermore, since the experimental assembly was wholly enclosed within the single shell duration of operation was limited by the volume of sea water contained in the reservoir bag.

These shortcomings were largely eliminated in the second, Mark II, version of the system. In this version we split the pressure envelope into four compartments, each contained within separate 16'' projectiles (Figure 131-B). Shell No. 1 served as a pressure reservoir and was connected directly to the pump. In order to reduce pressure fluctutation to a minimum, a double needle valve resistance was introduced between the high pressure segment of the pump and reservoir No 1, and the low pressure, gas-driven, side of the pump was modified by the inclusion of an auxiliary reservoir that effectively doubled the cylinder volume on that side. The end result of these modifications was to provide a sawtooth pattern of fluid flow providing for continuous flow at a steady rate through 90% of the cycle and zero flow for approximately 10%. By the time this pulse had been transmitted through reservoir No. 1 the flow had been smoothed out so that pulsation was barely detectable, and by the time this pattern in turn had been transmitted to the aquarium receptacle proper, no pulsation could be detected on the strain gauge.

Reservoirs Nos. 2 and 3 contained the two collapsible bag reservoirs which could be inserted into the line alternately so that one of them would be in service supplying high pressure sea water to the aquarium receptacle while the other could be filled. The capacity of each bag as used in that version was 20 liters. The bags in turn were connected via polyvinyl hose to epoxy coated stainless steel lines leading out through hull penetrations in vessels 2 and 3 and led through the bottom of receptacle No. 4 to the rigid aquarium cylinder (ranging from 1 to 15 liters capacity) within that envelope. The aquarium reservoir in turn was connected to the outside through flow control valves to sampling, rate metering, and spillover systems as before. Supplementary connections provided for pressure equalization through adjustable resistances between all four high pressure envelopes. The entire assembly was mounted in a frame and surrounded by a polystyrene-foam lagged stainless steel envelope connected to a circulating constant temperature water bath. Appropriate valving was provided to allow shifting high pressure from pressure reservoir No. 2 to No. 3 or vice versa. Provision for filling the reservoirs was made by suspending polypropylene containers filled with sea water 5 ft. above the top level of the high pressue assembly and connecting these through appropriate conduiting to the inlet of either reservoir. This system was tested at pressures up to 700 atm. and steady flow at rates up to 5 ml/min. for periods which to date have extended to five days.

This system has served us well in a series of studies on the responses to increased pressures of surface dwelling fish. Since the essential data resulting from this work have already been incorporated into Figure 3 they do not need to be elaborated upon here. We would rather turn to the next stages in the development of high pressure aquarium systems as we visualize them based upon such experience as we have gathered.

On the negative side, we have had some trouble in maintaining adequate asepsis in the reservoir systems as presently used when we have tried to employ untreated sea water. Most of this difficulty should be resolved either by turning to bacteriological filtration of sea water, by the use of millipore filters or by the use of some of the new stainless steel screens (provided these prove chemically inert in the presence of sea water), or by utilizing artificial sea water made up from a suitable salt mix. The problem obviously will be less severe when we switch from surface-dwelling organisms, which call for operating temperatures in the neighborhood of 20°C, to deep sea organisms which might be expected to like 4°C or even lower temperatures.

A second problem is the matter of conduits to transport sea water under high pressures from one envelope to the next, a feature which evidently is essential to the functioning of our design. In the first version of our device, we relied either upon stainless steel tubing as such, or upon epoxy coated stainless steel tubing. Since we are dependent upon conduiting over a good many feet, we are not satisfied that coatings can be applied evenly and reliably. In the next generation of our systems, we propose to resolve this particular difficulty by extending the principle of the double envelope to the conduiting. As in the vessels themselves, the conduiting then will consist of a hard high pressure envelope surrounding a relatively soft elastic internal conduit which will carry the sea water or other aquarium fluid. The space between these two will be filled with the hydraulic fluid, distilled water most probably, and will be maintained in pressure equilibrum with the envelopes surrounding the elements of the aquarium system. Using somewhat stiff laboratory tubing for the inside we expect no difficulty in coping with such internal pressure gradients as will be needed to maintain the desired sea water circulation. Preliminary trial shows that such a system can be made to work provided internal and external pressures are balanced carefully. We are currently in the process of testing a number of alternative valving systems which will be compatible with this type of conduit, but at the present, none of these designs can be considered completely successful. In principle, the system will involve coupled needle valves, one of which will be connected to the inert aquarium fluid carrier and the other will provide passage for the envelope fluid. Present indications are that by using especially designed needle valves and a differential strain gauge system, such links, though a bit cumbersome, should be amenable to effective control. As we view it, the requirement for chemical inertness becomes less

critical in the lines leading from the aquarium to the outside since water contained in these lines is being led away from the aquarium system and hence is not likely to come into contact with the animals under observation. Accordingly, the distal segment of effluent line will continue for the time being to remain a conventional stainless steel line controlled by paired needle valves.

Another problem which has concerned us is the matter of maintaining the aquarium water stirred sufficiently to assure uniform mixing so that sampling of the effluent water will provide an effective means of following changes in water composition. A promising appearing solution to this problem is to provide a narrow lateral compartment of the aquarium receptacle with a plastic encased piston with loose flutter valves, and utilizing a high pressure packaged solenoid to activate the piston. Our aquariums are provided with appropriate passages to power such devices, and it is quite easy to turn the aquarium water over once every 5 minutes by this means. We are inclined to think that this also will be the system we shall utilize to provide some supplementary filtration should this become desirable. With regard to visual observation, we are relying for the moment upon lucite windows with a relatively large thickness to cross-section ratio. At the higher pressures, we are supplementing that system with a 45° front silvered plane mirror. We consider this observation system passable, but inelegant, and are exploring possibilities of replacing our plain parallel lucite windows with some type of window providng one or two negative diopters of refraction making the window in effect into the front element of a wide angle lens.

In the course of our preliminary studies with the Mark II high pressure aquarium system described above, we found that this type of system tends to place a bonus on sheer size. The reason for this is that while at low pressures the compressibility of water is quite negligible, this is no longer true at pressures in the range of 500-1000 atm. A liter of water at 1,000 atm. corresponds to nearly 1.08 liters of water at 1 atm. If the total volume of water contained in an otherwise rigid system is increased, a compressibility of this order of magnitude implies that the system begins to behave increasingly as though it contained an elastic fluid. Thus, for instance, a 100 cc sample withdrawn from a 100-liter system under 1,000 atms. will result in a pressure drop of little more than 10 atm. or 0.1% of the ambient pressure. Clearly, such behavior is desirable in a system designed to facilitate chemical and other manipulations. This quality also contributes to damping out any transient pulses imposed upon this system by whatever pumping devices are employed. Because of the soft reservoir element in our dual reservoir aquarium systems, the benefits of a large reservoir of water under high pressure can be exploited even if the actual salt water system has only a very limited volume. These considerations have led us to project a third generation of high pressure laboratory aquarium systems in which the

pressure reservoir will be installed away from the main laboratory, and will consist of an extended tubular element connected to a pumping manifold equipped with appropriate barostats.

Since the principal characteristic essential in this context is the behavior of the system as an elastic high pressure reservoir of reasonable capacity, consideration has been given to using a more highly compressible fluid as the pressure accumulator. Either a hydrocarbon fluid, or a gas-filled pressure accumulator, or some type of mechanically loaded piston could be considered. Of these possibilities, we have rejected the gas-filled piston concept, both because of the danger of diffusion into the aqueous phase across almost any seal at the high pressures prevailing over long periods of exposure, and because of the marked temperature dependence of such a system. Hydrocarbon-loaded systems pose handling problems, including once again the need for mechanical separation from the aqueous phase. It seems to us at the moment that such assemblies provide insufficient advantage over an all-water system to justify the extra trouble involved. A mechanically loaded piston system on the other hand seems to us to hold genuine promise of providing many of the advantages sought with a minimum of mechanical complications. The system currently being evaluated involves an auxiliary piston with a dead weight load. Such a device has many desirable qualities but is limited in its ability to absorb rapid pressure pulses. This characteristic may require some extra precautions in pump design, but this does not appear to pose any extraordinary difficulties.

Returning to the third generation design, from its reservoir, conventional high-pressure conduits can be brought to the laboratory building, emerging at the level of the bench in the form of a series of high pressure valves and connectors into which any desired assemblies can be plugged. The connection then serves to provide hydraulic fluid directly at the maximum pressure of the reservoir or at any desired pressure via a secondary barostat, to some type of membrane enclosed system resembling, in principle, that which is comprised of our present pressure vessels Nos. 2, 3, and 4. This assembly, however, can now work with much smaller reservoirs (No. 2 and 3) for a given aquarium or experimental vessel (No. 4) because the pressure reservoir function now is taken over solely by the external system. Alternatively, using the dual conduit principle, the external pressure reservoir could be utilized to provide high pressure sea water in a manner analogous to what is now provided at the level of the conduit linking our present receptacle 2 and 3 with the aquarium vessel No. 4. This would still further reduce the size and complexity of the system required to be installed in the laboratory area proper. In either case, this type of assembly provides for the first time a relatively low unit cost capability for supplying sea water under high pressures with zero pulsation, contamination-free, and under conditions where the benefits of an elastic system compatible with sample

withdrawal, tracer injections, and the like are made available to the investigator without obliging him to attend a cumbersome and costly high pressure system which must share part of this laboratory space with him.

The ease with which any system can be manipulated which has reasonable pressure volume characteristics has stood us in good stead in connection with the introduction of certain types of instrumentation. For this purpose, we have availed ourselves of small parasite high pressure chambers which can be inserted into the effluent line at will without jeopardizing the main system. In our hands these have been use, for instance, to provide measurement of Po_2 in the aquarium fluid without any worry over possible contamination of the water surrounding our subjects by electrode by-products; the electrode system after all is removed safely downstream from the aquarium compartment proper. Such small supplementary chambers are easily thermostated to the degree of precision required for satisfactory electrochemical measurements. More than one such unit can be inserted in series as needed for a variety of measurements, and different designs could allow for optical, magnetic, or other sensors as desired. At the flow rates we are currently employing, and with the conduiting currently in use, the lag time between sampling of the aquarium water and examination of this fluid in the parasite chamber is less than 5 minutes, quite acceptable for most measurements with which we are likely to be concerned. In future assemblies, this time lag can be reduced to less than one minute, and we currently have designs on the drawing board which should reduce the volume of the sensing chamber to the point where measurements read out of the parasite assembly can be counted upon to faithfully reflect conditions in a well mixed aquarium with a total delay of less than 3 minutes.

A final concept that has grown out of work with the aquarium module described is the concept of container handling of biota in the high pressure aquarium systems. The matter of transferring small or medium size active animals from one compartment of a high pressure assembly to another for observation, surgical procedures, segregation from others, and the like, remains a difficult one so long as the animal itself has to be manipulated inside the heavy high pressure envelope. The use of the double envelope concept which underlies the entire design of our aquarium system as described above, should make it feasible to avoid this difficulty by utilizing container handling procedures much of the way. At an early stage following capture or rearing, individual specimens or groups of specimens would be transferred to separate compartments. From that point on, handling procedures can be reduced to the vastly simpler manipulations involved in mechanically transferring the container enclosing the specimen from one compartment to another while maintaining its connections to the non-rigid sea water lines which provide the necessary circulation. This principle, by very simple extension of well established engineering concepts utilizing such devices as split thread retaining rings converting a closing plug into a piston,

provides a means even now for designing systems capable of transferring specimens of macrofauna from one compartment to another in a complex high pressure assembly, and designs are currently being formulated to expand this basic concept to provide for interlocking of more highly differentiated containers.

To recapitulate briefly, the high pressure aquarium system we have been concerned with is aimed at providing a device suitable for manipulating and handling relatively large sized specimens, i.e., specimens exceeding 20 cc displacement. The basic design concept involves the use of a high pressure enclosure provided with a barostatically controlled pump, and containing, suspended in a suitable hydraulic fluid, a dual reservoir the soft element of which is employed to force water through the rigid element, the latter being the aquarium space proper. A number of designs have been tested in which the pressure-resistant envelope surrounding the entire system has been sub-divided into various communicating elements, and some of the principles governing the behavior of such multi-envelope systems have been discussed. A prototype design was illustrated which is currently in use and has led to a number of studies of the effects of high hydrostatic pressures on shallow water fish and crustaceans. Some guiding principles have been presented which will govern design of the next generation of systems which are to provide for more adequate mixing of aquarium water, separation of the pressure generation system from the laboratory system, and fuller exploitation of the elastic properties of water to facilitate experimental manipulations by providing large volume high pressure reservoirs. The use of satellite chambers for chemical control has been mentioned. The concept of handling biological specimens in high pressure systems in specialized containers discrete from the pressure envelope has been tested in the prototype assemblies, and will be applied more fully in the next generation of systems in the hope that this technique will provide the basis for a safe method of manipulating biological specimens in connection with the retrieval of deep ocean fauna.

Acknowledgment

This work was supported by Research Contract No. 14-16-0008-569 from the National Fisheries Center and National Aquarium of the Department of Interior, and by research grants from the North Carolina Board of Science and Technology, and the United Medical Research Foundation.

Discussion Following Brauer Paper.

LOWENSTAM: I was wondering whether we shouldn't try to come to some kind of terminology agreement on the various stages we have observed in our experiments, and which others will observe. What kind of reactions

would one classify as tetany, or as rigor, etc. I wonder whether we are already at that stage?

BRAUER: This is a point I feel is extremely well taken, and it's come up two or three times in the lovely slide Menzies showed much earlier; and in the discussion that you and I had about what you are calling your cut-off points, and what they mean. In defense of the vague terminology that we're using, may I remind you that our bias as mammalian physiologists makes us acutely aware of the fact that when an animal shows spastic movements of a violent and poorly coordinated type, it does not thereby automatically have a convulsion. For instance, in our early work with the high-pressure phenomena we very carefully refrained from fixing on that particular term until the electroencephalographic data showed that these truly were what is technically known as convulsions. So, perhaps it would be excusable for just a little while longer to abide by a somewhat vague terminology, possibly naming these things as "Stage No. 1, 2, 3, 4, 5," until the physiological experimentation, which is obviously what we are all working up to, will have told us what we are looking at. I am not yet fully convinced that the mammalian phenomena which I know, and to which I am willing to give specific names, are truly comparable to the invertebrate phenomena. Yet, as I listen to your descriptions of them, I am more and more inclined to suspect that in fact they will, to some extent, prove to be comparable. If so, I'd suggest that we adopt well established mammalian terminology to describe analogous events in sub-mammalians and invertebrate species. I'm willing to write stages, for vertebrates, and did so in my introductory comments, and I'd be delighted to see those terms used. For the mammalian situation, there is an early phase which is associated with tremors; there is a second phase of hyperexcitability and convulsions well documented in warm-blooded species, which is a neurological phenomenon at the level of the CNS. Beyond this is a very grey zone that we are just getting into, and I think this is the zone in which much of your invertebrate stuff is going on. We very tentatively refer to this as the "cardiovascular" phase. To put numbers on these: the tremor phase at any reasonable compression rate happens in most species around 40 to 60 atms. The convulsive seizures, as you saw earlier, occur mostly somewhere between 60 and 100 atm., and the cardiovascular phase begins near 90 or 100 atm., and in some species may range to as high as 200 atm. This is the mammalian picture; anything we have carried into the higher pressure range of the third phase dies fairly quickly.

ZIMMERMAN: In pressurization and decompression from the amoeba on to isolated mammalian cells, and to isolated tadpole cells, with just a little stretch I can pretty well match every one of these phases. Even with nothing more than a single cell, therefore, you can pick out almost every one of these with just a slight stretch of the imagination.

BRAUER: I agree; the phenomena, as I listen to them, sound alike. Yet, I

am still most unanxious to give them names which will delude people into thinking that they are, in fact alike. My inclination at the moment would be to suggest that the real record I would like to see is the type that Menzies put out—motion pictures of the typical sequences with stages numbered for the time being—to be unnumbered and named as we learn to understand what each means.

MENZIES: With invertebrates you have such a wide range of forms. It's very hard to tell when a clam's in a tremor stage. It's a little easier with some of the brachiopods—so you really couldn't pick out some of those points, could you? It depends also to a great degree on how clear your optical system is, and how close you can get to the object, whether you can pick out things precisely. With certain organisms we have had as many as eight responses. Let's call them R-1,2,3, and 4. It's perfectly obvious that R-1 in one case would not be R-1 in the next case, but the same kinds of things happen. In one animal the R-3 would be the hyperexcitability; in another the R-1 would be. I think in general this is only due to our inability to see the phenomena.

BRAUER: Well, aren't we really all saying the same thing? That we are not quite ready for a system that admittedly would be nice, but at this point is perhaps dangerous.

ZOBELL: With regard to the limits at which these stages occur, it would certainly have to be specified that this applied to barophobic organisms, because when we start recovering deep-sea fauna, we may find they aren't quite applicable. We may find that the reduction of pressures of these orders or an augmentation to higher pressures may bring about these or similar physiological events.

MACDONALD: In almost every aquarium we've seen this afternoon, an indirect pumping system has been used. I take it that at least some of you are aware of the stainless steel diaphragm pumps used in the chemical engineering industry which cost about $2,000. You can, in fact, get these to function at any pressure that we're likely to be interested in, and the pumping fluid only comes in contact with the stainless steel container. Have you considered using one of these?

BRAUER: We have considered using those, and you have already given the real reason. We can't yet afford them. On top of this, they have some other problems which at this stage still worry me. If you have ever sat inside a pressure chamber, as I have, even though you're protected by a large volume of gas which buffers pulsations, whenever a rapidly reciprocating membrane pump provides the chamber circulation, your eardrums seem to swell up in protest against the hum until they seem too big for the chamber. The type of beat that you get out of these pumps, unless you take great pains to take it completely out of the chamber, is not physiologically neutral. Any kind of system that gets you a slow, quiet stroke delivery is preferrable. This is one reason why we like the gas-driven pump we use. For the moment, we

are inclined to shy away from the heavy membrane pump with its relatively high frequencies.

MACDONALD: I'm not aware that the frequency has to be high, and hydraulic accumulators are smoother anyway. But I'm not really an engineer.

BRAUER: Well, the frequency has to be high if you have a membrane of the thickness of the ones that are being talked about at these pressures, or else your pump becomes enormous.

AN APPARATUS FOR THE RECOVERY AND STUDY OF DEEP SEA PLANKTON AT CONSTANT TEMPERATURE AND PRESSURE.

*A. G. Macdonald,
Department of Physiology,
University of Aberdeen.
**I. Gilchrist,
Department of Mechanical Engineering,
Queens University of Belfast.

In this paper we wish to describe the progress made in the development of equipment which can recover a living plankton sample from the deep sea, with an insignificant change in its hydrostatic pressure or temperature. Following retrieval the plankton sample is transferred, at constant pressure, to an experimental pressure vessel for observation. The equipment is to be used to investigate the pressure tolerance and other properties of those animals which normally live at several hundred atmospheres hydrostatic pressure. There is little doubt that interesting experiments can be carried out on animals recovered from moderate depths in the conventional manner, and preliminary observations which we have obtained in this way will be reported elsewhere (Macdonald, Gilchrist and Teal, in preparation). It is equally clear that certain experiments on animals which normally live at considerable depth can only be satisfactorily carried out if the main physical factors in the environment, such as pressure, light and temperature are controlled.

Experiments carried out at depth by remote means or from a deep submersible represent an important development discussed elsewhere in the conference. The equipment described in this paper should enable the investigator to control the experimental conditions and perhaps eventually bring healthy deep sea organisms into the shore laboratory.

The Equipment

Plankton is filtered using a vertically hauled plankton net fitted with a pressure vessel at the cod end (Figure 132). The vessel is closed at depth and after recovery is coupled to an experimental pressure vessel mounted in the ship's laboratory. The plankton suspension is then flushed from the recovery vessel to the experimental vessel at constant pressure. To recover and study the animals successfully, it is required to overcome five basic problems. These are;

1. the filtration of plankton with minimal mechanical damage
2. the retention of high hydrostatic pressure in the recovery vessel

* Presented by A.G. Macdonald.

** Present address—Brunel University, Uxbridge, Middlesex.

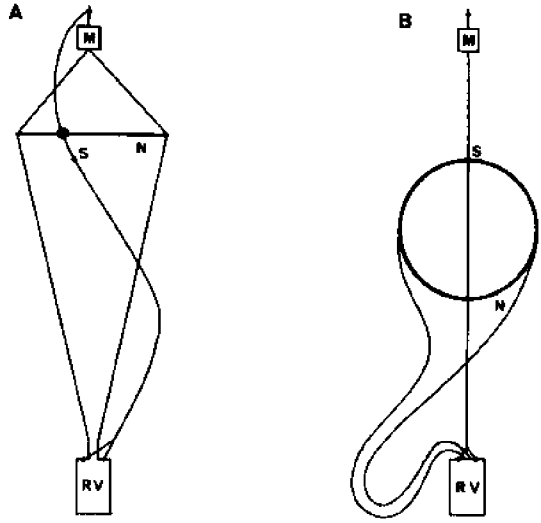

Figure 132 A diagram of the recovery apparatus. A in the filtering condition, B when collapsed for hauling in.
N, a 2 m diameter plankton net.
M, a messenger activated release device.
S, the stopping point of the net ring when the weight of the recovery apparatus is taken by the second circle.
RV, recovery vessel.

3. the maintenance of constant temperature
4. the circulation of seawater at controlled pressures in transferring the plankton to the experimental vessel.

The fifth problem is that of designing and carrying out experiments on the retrieved organisms. We have given consideration to this aspect, but the work has not progressed to the point where discussion is appropriate.

1. The Filtration of Plankton

Since plankton is sparsely distributed at depth a large diameter net is required. A 2-m diameter conical net is used to filter the plankton sample into a 2.5-cm diameter opening in the recovery vessel. The 2.5-cm diameter entry was the largest that could conveniently be provided. Clearly the conditions for the capture of undamaged plankton are far from ideal, and particular care must be taken to smooth and control the flow of water close to the recovery pressure vessel. The vessel can be used with water flowing through it during filtration or in other ways illustrated in Figure 133. The most promising (and complicated) configuration is shown in Figure 133c. To filter animals from a specific depth band we use reciprocating vertical hauls. A flap valve on the recovery vessel prevents the sample from being washed out during the downwards portion of the cycle, and tests with mock-up equipment show that we can collect some planktonic animals in good condition.

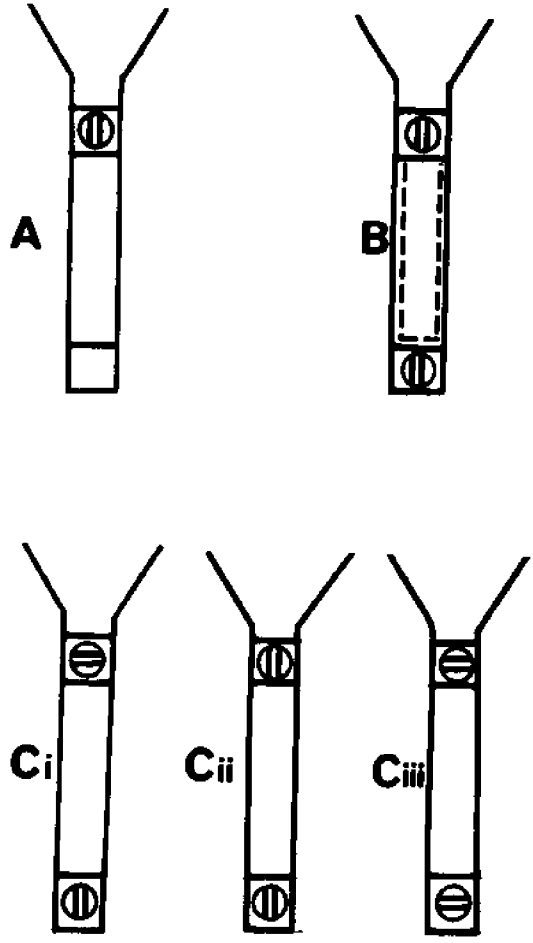

Figure 133 Diagram showing various methods of retaining plankton inside the recovery pressure vessel.

A. Shows the globe valve open to receive plankton continuously during filtration. The end of the pressure vessel is permanently closed.

B. Shows a cylindrical filter inside the pressure vessel and open valves at each end allow a stream of water to pass through during filtration.

C. Shows the front globe valve closed, causing plankton to accumulate in a zone of still water at the cod end of the net. After filtering for a period of time the valves are opened and closed as indicated by Cii and Ciii, allowing only a short period when water flows through the pressure vessel.

The flap valve referred to in the text is not shown in these diagrams.

As in many fields of biology our proposed experiments will be considerably aided by convenient animals. From our experience so far we think that Ostracods, Amphipods and Copepods survive capture relatively well.

2. The Retention of Pressure in the Recovery Vessel

The recovery vessel is a thin cylindrical vessel of 55 cm internal length and 5 cm bore fitted at each end with globe valves which can be closed remotely at depth (Figure 134). Sea water of salinity 35‰ at 0°C undergoes a volume decrease of only 4.01% on being compressed to 1000 atmospheres. Consequently it may be shown that an increase in the volume of the recovery vessel of only 0.25% of the total volume is sufficient to cause a pressure drop of 68 atm. (1000 psi).

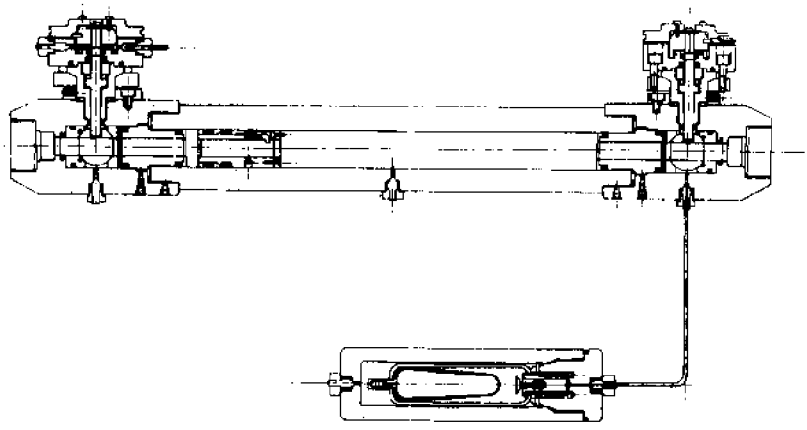

Figure 134 The recovery pressure vessel. The vessel consists of a 55-cm long, 5-cm bore cylinder with globe valves at each end. From the right hand globe valve a pressure connexion runs to a hydraulic accumulator shown at the bottom of the drawing. The seals are shown in black. In the left half of the recovery vessel body a piston is shown (which is not described in the text) and which may be used to help transfer animals to the experimental pressure vessel.

From this it may be expected that the elasticity of a steel recovery vessel will affect the pressure maintenance characteristics. When a recovery vessel consisting of a simple stainless steel cylinder is closed at depth in an ideally rigid manner and brought to the surface, it may be shown that the pressure in the vessel is 4.9% less than the pressure at closing (Figure 135). This applies even when a vessel having an infinitely large "K" ratio (outside diameter divided by inside diameter) is used. This pressure fall off (ΔP_E) is mainly caused by the elasticity of the vessel walls but also by the fact that

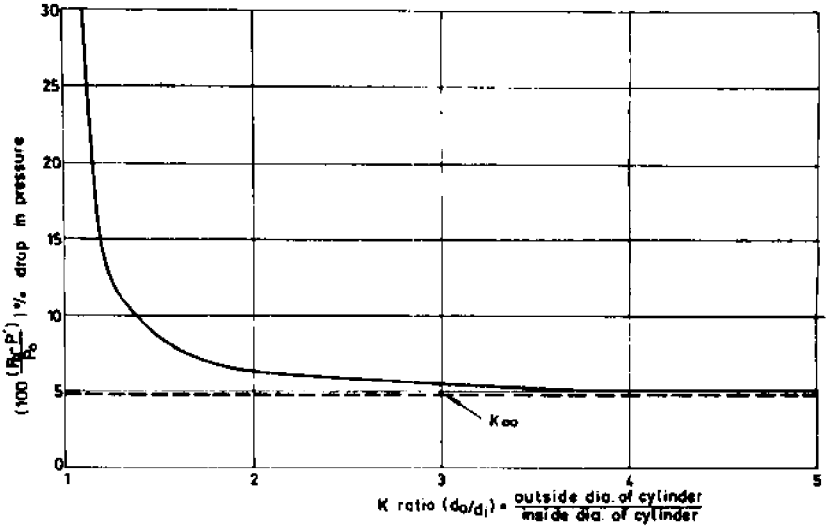

Figure 135 Pressure fall-off in a stainless steel cylinder after it has been closed at depth (pressure P_D) and brought to the surface (pressure P). This pressure fall off (ΔP_E) occurs due to the elasticity of the steel vessel. The pressure fall off is plotted against the ratio of the outside to the inside diameters of various steel cylinders.

steel is itself slightly compressible. (If a peiece of steel is uniformly compressed from 0 to 1000 atmospheres the decrease in the volume of the steel is 0.063%).

Additional factors also affect the pressure maintenance in the recovery vessel. As the vessel is raised from depth after the valves have been closed, the pressure seals will deflect into their sealing position and local distortion can take place where vessel components join. An increase in the temperature of the recovery vessel also increases the pressure of its contents (ΔP_T). For biological reasons however temperature must be controlled to the extent that pressure change with temperature is negligible.

Immediately the recovery vessel has been closed at depth a leak tight seal must be established between the vessel and the surroundings. It has been found that satisfactory sealing can be achieved using standard neoprene seals on the globe valves and recovery vessel body. The volume change in the recovery vessel due to seal displacement however must be kept to a minimum by using seals which fill their sealing grooves as completely as possible. When this is done it can be predicted that the pressure maintenance characteristics of a simple, rigidly constructed steel vessel would be satisfactory for operation at the higher pressures. However it may also be shown that the percentage pressure fall off through seal displacement (ΔP_S) increases as the trapping pressure decreases and at low pressures the performance of a simple rigidly constructed steel vessel becomes inadequate.

Various methods may be used to improve the pressure maintenance of the recovery vessel but the use of a gas accumulator with the vessel was considered the simplest and most effective. This has been adopted in the present equipment. Figure 134 shows the recovery vessel, which has a "K" ratio of 2.25. This gives a minimum pressure fall of 6.3%when the vessel is used without the accumulator. Globe valves of the design shown in Figure 136 were chosen for the recovery vessel since this provides a compact valve having high rigidity. Distortion at the threaded parts in the globe valve and recovery vessel body may be shown to cause negligible pressure loss.

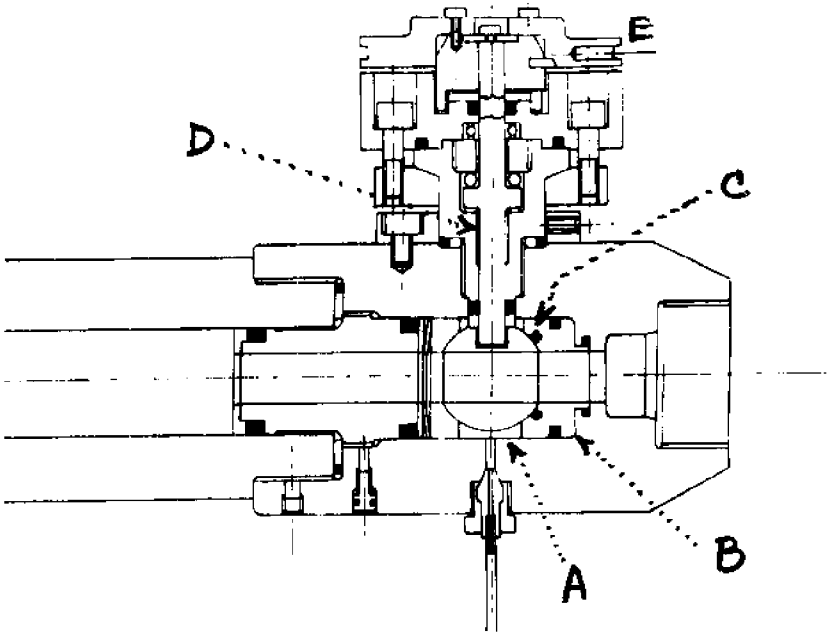

Figure 136 A globe valve used in the recovery vessel, a spherical ball (A) drilled through with a 2.5 cm diameter hole is used in the valve. The ball is mounted against the globe valve seat (B) which contains the pressure tight seal (C). The valve is closed using spring tension on the pulley (E) to turn the ball, which is coupled to spindle (D) through 90 degrees.

Thus far we have evaluated the gross pressure change expected during recovery by calculating from first principles. Experiments simulating the recovery operation have been carried out on the equipment to confirm these data. Figure 137 shows the results of a simulated recovery in which the recovery equipment is immersed in a large pressure vessel and subject to a high ambient pressure. Whilst at pressure the recovery vessel is closed and the large pressure vessel decompressed to simulate hauling the recovery vessel up from depth. The pressure retained in the recovery vessel is then

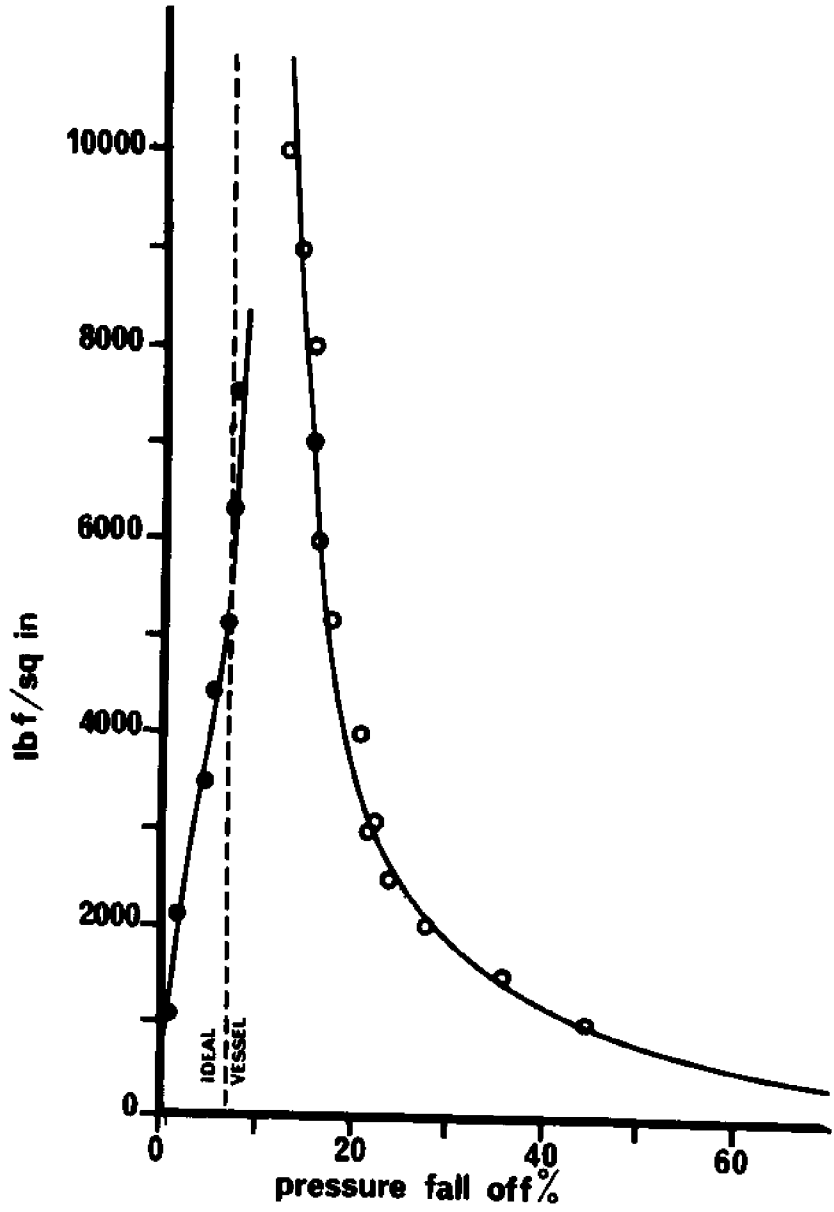

Figure 137 Pressure fall off during simulated recovery experiments. See text.
The dashed line represents the calculated ΔP_E for the test vessel.
The open circles are experimental points obtained from simulated "recovery" from pressures shown on the vertical axis.
Closed circles are experimental points obtained wtih the same test vessel but corrected to a hydraulic accumulator charged at 136 atm (2000 psi.), or less.

measured. A special shortened recovery vessel body is used in these tests and this accentuates the measured pressure fall off. The curves on the graph show typical results obtained from the recovery vessel is used with and without an accumulator. Figure 137 shows that the pressure fall off is 16.5% from a recovery pressure of 500 atmospheres using this arrangement of the test recovery vessel on its own. Of this 7.5% may be shown to be due to the vessel elasticity, the remainder being due to seal displacement. These results also show that at this pressure using an accumulator with the test recovery vessel reduces the total pressure fall off to 7.5%. However after correction to compensate for the larger recovery vessel body in practical use, the results of similar tests show that the recovery operation can be carried out from all depths to 10,000 metres with a pressure loss which is less than 3.5%. A more detailed description of the equipment and tests will be published elsewhere (Gilchrist, Crossland and Macdonald, in preparation).

We have tested the equipment at sea by recovering sea water from depths of 1400 and 2000 m. A depth recorder and a pressure transducer were connected to the recovery vessel pressure chamber. The recovery vessel was mounted in a handling frame (Figure 138). At depth the globe valve was closed by a spring mechanism triggered by a messenger. On recovery the pressure in the vessel was independently read from the "depth recorder" (which gave the pressure-time history of the vessel sample chamber during the recovery operation) and the pressure transducer. The pressure transducer was also used to give a record of the fluctuations in pressure as the sea water sample was transferred to the experimental pressure vessel (Figure 139) [Table 1]. Details of the transfer operation are given below. On recovering seawater from 2000 m a small increase in the recovery vessel pressure (above the pressure at closing) was noted. This increase was probably caused by a rise in the temperature of the apparatus as at that time no attempt was made to provide thermal insulation.

3. Maintenance of Constant Temperature

We propose to insulate the recovery apparatus by mounting it in a tank in which water movement will be limited. Calculation of the temperature rise suggests that it will also be necessary to provide a 10 cm thick layer of insulation round the tank using a mixture of water and plastic granules. Deep sea organisms are likely to be sensitive to a small rise in the temperature of their environment and we anticipate maintaining ambient temperature to $\pm 0.2^{\circ}C$.

4. The Control of Pressure and the Circulation of Sea Water at Constant High Pressure

The experimental pressure vessel is connected to a hydraulic circuit (Figure 140) which consists of an air powered pump, pressure measuring

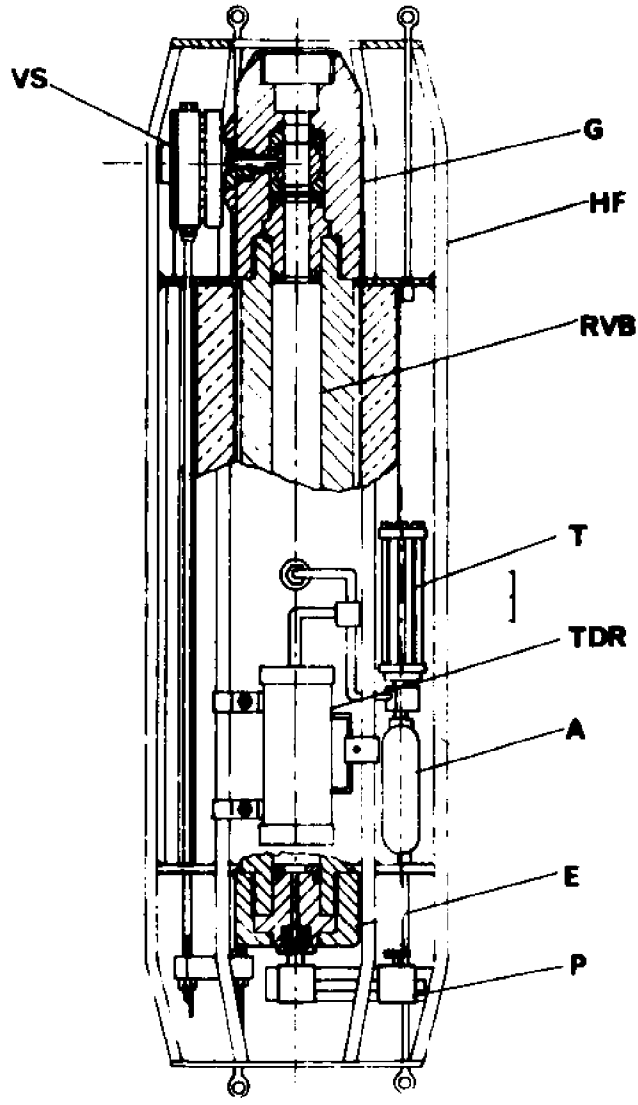

Figure 138 The recovery vessel mounted in its handling frame, H.F.
G, globe valve.
VS, valve spindle, rotated 90°by a spring mechanism.
RVB, recovery vessel body.
E, end cap.
T, housing for a pressure transducer.
TDR, time-depth recorder.
A, hydraulic accumulator.
P, needle valve and pressure inlet where seawater was flushed into the experimental vessel.
The small vertical scale represents 2 inches.

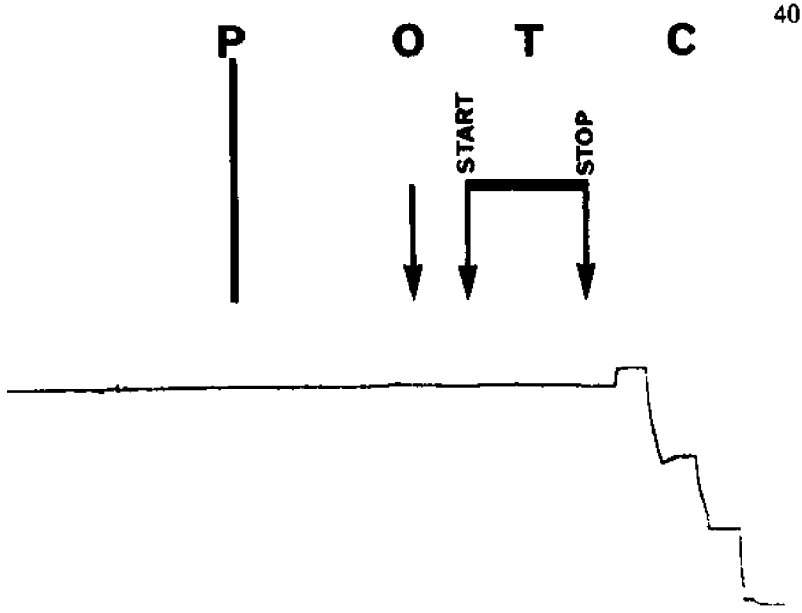

Figure 139 Recording of pressure during the Transfer operation carried out at sea. (See text).
P, constant 144 atm (2116 psi) in the recovery vessel after recovery from 1400 depth, and measured when coupled to the experimental pressure vessel.
O, the point when the globe valve connecting recovery and experimental vessels is opened. This valve can only be opened when pressure on either side of it closely matches.
T, Transfer of deep sea water to the experimental vessel starts and stops, without a fluctuation in pressure. Transfer lasted 4 minutes.
C, calibration steps to zero pressure at the end of the experiment.

Table I

Cast No.	Depth of recovery apparatus from wire paid out and calculated pressure	Pressures at which globe valve closed	Recovery pressure: Depth recorder	Recovery pressure: Transducer	Pressure change during recovery (per cent)	Pressure fluctuation during transfer (per cent)
3	1,400 m 140 atmos[2] (2,058 lbs/in^2)	144 atmos[2] (2,116 lbs/in^2)	144 atmos.[2] (2,116 lbs/in^2)	—	0	—
4	1,400 m 140 atmos[2] (2,058 lbs/in^2)	144 atmos[2] (2,116 lbs/in^2)	144 atmos[2] (2,116 lbs/in^2)	144 atmos[2] (2,116 lbs/in^2)	0	—2.3
5	2,000 m 200 atmos[2] (2,940 lbs/in^2)	200.6 atmos[2] (2,949 lbs/in^2)	211 atmos[2] (3,101 lbs/in^2)	—	—5.5	—
6	2,000 m 200 atmos[2] (2,940 lbs/in^2)	200.6 atmos[2] (2,949 lbs/in^2)	211 atmos[2] (3,101 lbs/in^2)	215 atmos[2] (3,160 lbs/in^2)	—6.5	—2.0

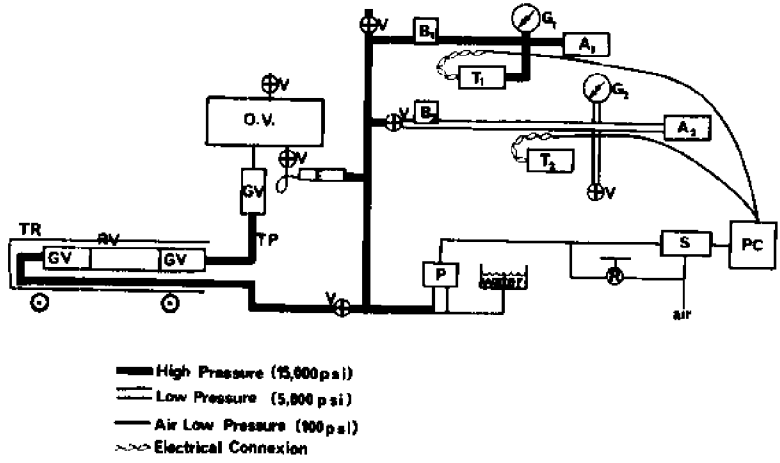

Figure 140 Block diagram of the hydraulic circuit with the recovery vessel coupled to the experimental observation vessel.
A, hydraulic accumulator.
G, pressure gauge.
T, pressure transducer.
B, bursting disc.
V, valve.
OV, observation vessel.
GV, globe valve.
TP, transfer pipe.
RV, recovery vessel.
TR, trolley and handling frame.
P, air powered high pressure pump.
S, solenoid valve on air supply to pump.
R, air regulator (by-passes solenoid valve if required)
PC, potentiometric controller.
F, filter.

equipment, bursting discs and valves. The system pressure and the rate of circulation of sea water are controlled independently. Pressure is generated by the air powered pump and measured using a transducer whose output causes a potentiometric controller to switch off the air supply to the pump at a selected value. An accumulator charged at the appropriate pressure with nitrogen smooths the pulses of pressure generated by the pump. A circulation of seawater through the system is required to transfer the recovered plankton suspension to the experimental vessel and for subsequently maintaining animals in the vessel. The former requires a flow rate of up to 200 cc/min and the latter a flow rate of 1 to 10 cc/min.

The transfer of plankton can be accomplished by first connecting the recovery vessel to the experimental vessel and by raising the pressure of the latter to match the recovery pressure. When the pressures are equal the recovery vessel globe valves can easily be opened by hand to give a one inch bore connection at pressure between the two vessels. Transfer is then accomplished by directing a flow of water through the recovery vessel which

flushes the animals into the experimental vessel. Figure 139 shows a typical recording of pressure variations which are experienced during transfer. The pressure recording in Figure 139 was obtained by controlling the outflow of water through the experimental vessel by manually adjusting the appropriate valves. For slow and prolonged circulation of seawater we have used a pneumatically powered needle valve which releases drops of water to atmospheric pressure at a rate set by a timing mechanism. The arrangement works adequately at moderate pressures but for higher pressures the arrangement shown in Figure 141 is giving promising results. Two pneumatically operated valves connected in series are used to work in a "lock out" fashion giving a discontinuous flow of water which is measured using a drop recorder. A rate meter output from the drop recorder drives a servopotentiometer to adjust the frequency at which the pneumatic valves open and close. This method of control is considered to offer two worthwhile advantages. It uses generally useful and inexpensive items of equipment and because the water flows through relatively large bore valves (3mm diameter) blockage by the cast exoskeletons of crustacea or other debris is unlikely to occur.

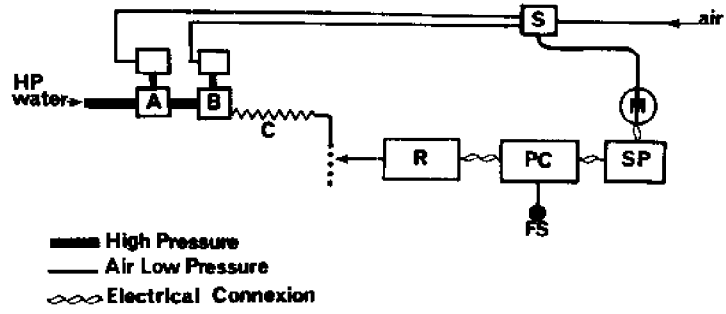

Figure 141 Block diagram of an apparatus under study to control the flow of seawater at constant high pressure. See text. A, B pneumatically operated needle valves. When A is open B is closed, and vice versa. C, capillary. R, drop recorder and rate meter. The arrow represents the drop sensor. PC, potentiometric controller. SP, servo potentiometer. M, timer motor and relay. S, solenoid valve. FS, drop frequency selector potentiometer.

We have had one opportunity to recover plankton at sea. Time was limited so it was decided to filter at a depth of 600m to obtain plenty of organisms, at 5% of the maximum working depth of the equipment. Figure 132 shows the 2-m diameter plankton net before and after a messenger-actuated mechanism collapses it and triggers the globe valve closing mechanism. Our first three attempts to recover plankton failed for trivial reasons but the fourth attempt yielded an excessively concentrated plankton suspension which we transferred and observed at pressure. Most animals were dead, presumably as a result of mechanical damage but several active

copepods and ostracods were observed. The relevant pressure data are listed in Table 2. Although we filtered far too many animals, we take comfort from the fact that the globe valves sealed in the presence of excess plankton and that we have gone through the technical operation of filtration, recovery, transfer, and observation of plankton at pressure.

Recovery of a Plankton Suspension at Constant Pressure

	1	2	3	4	5
	Depth Range from which Plankton was filtered		Pressure within the Recovery Vessel before Transfer		Pressure Fluctuation during Transfer
	measured by wire paid out	*measured by depth recorder	*measured by depth recorder	measured by transducer	measured by transducer
Depth in metres	530-660 m	580 m	—	—	—
Pressure in atmospheres	—	58 atm	58 atm	53 atm	46.6-53 atm
Pressure in pounds per square inch	—	850 psi	850 psi	780 psi	685-780 psi

*The discrepancy between measurements obtained with the depth recorder and the transducer may be attributed to an uncorrected zero error in the former. The data show that pressure within the recovery vessel did not change during recovery; absolute pressure measurements were not important.

The experimental pressure vessel which receives the plankton sample for study consists of a cylindrical stainless steel vessel having a 7.5 cm bore. The vessel has an internal length of 24 cm and may be used at pressures up to 1000 atmospheres. Temperature is normally controlled to ± 0.1°C. Since much of the study of the animals will be carried out by observing their activities, considerable attention has been paid to providing suitable high-pressure windows. A research program, which includes creep and cyclic fatigue tests has been carried out. It has been found that a conical acrylic window having a cone included angle at 60°, and a thickness/viewing diameter ratio of 1.6 is suitable for use in the experimental vessel at pressures up to 1350 atmospheres and temperatures up to 20°C. The results of this research program will be published elsewhere.

Windows of 38 mm viewing diameter are mounted at each end of the experimental pressure vessel. Radial holes in the vessel wall also provide the following

1. Pressure inlet and outlet ports
2. A 2.5 cm bore entry for plankton and a 2.5 cm entry for instrument probes
3. A rotating shaft for stirring purposes.

The contents of the vessel are viewed by a means of a sterobinocular microscope giving a magnification of up to x 32 over most of the length of the vessel.

At the time of writing the equipment is undergoing alterations to simplify its use at sea. We conclude from our results that some progress has been made on the way to carrying out controlled experiments on deep sea animals. We would not wish to anticipate the rate of progress in the future but would emphasize that it may be some time before we get down to interesting biological observations.

Acknowledgments

This work is supported by the Natural Environment Research Council. We are pleased to acknowledge the support given to the project by Professor J. A. Kitching, O.B.E., F.R.S. and Professor B. Crossland, and also the skilled assistance given by workshop staff.

Discussion Following MacDonald Paper.

SAUNDERS: In the initial phase of the study, could this retrieval system and observation system have been combined into one unit?

MACDONALD: Yes, it could, but I chose not to for many reasons. I think the best one is this: When you work with the kind of winch we had, to haul in just a few thousand meters, takes 12 hours. So, while you have one haul going over the side, you are doing the experiments from another. We've got to do experiments that take 12 hours, and that's what we did.

VIDAVER: Can you describe your materials? If anyone does happen to come up with some evidence that one material is better than another, or has some reason why he selects a particular material, I think it's valuable to hear about this.

MACDONALD: Most of the stuff that is machined is FB 520 B, British designation. This is pretty good at not corroding. The corrosion problems we have had with it have been seal corrosion, serious pitting near 0-rings. I just pray about this; I don't do anything about it. Wherever possible, we use a high strength alloy called Hydurex, which is a bronzy sort of thing where we have threads to avoid risk of corrosion. This is messy stuff in sea water. It stains it very easily. Perspex is the perfect window material, completely stress-relieved, carefully machined. In fact, the window was designed

intuitively. The sort of data that Stuckey had out a few years back, and Gilchrist has done an infinite number of creep measurements that will be published soon. But the windows are okay for millions of cycles as far as I can see.

BRAUER: How about the optics in your system? I am impressed particularly with the microscope used with the long focal length. Who makes these?

MACDONALD: The microscope is Czech, the ordinary sort of thing, but when I was setting up this project I got the microscope I could find.

MENZIES: I don't have a question. I have a comment. I think these results are beautiful. They agree perfectly with my own hypothesis and naturally I like them. I don't want to beat that isopod to death or anything like that, but there is a serious point of deciding whether an animal, indeed, is more adjusted to higher pressure than another especially with reference to the technique you use in keeping your pressure and the duration over which it has been done. Then again this is just another plea for some kind of standardization. Obviously each person is going to use a different technique depending upon what he wants to see, but it does seem as though we are going to reach a point sometime where I would like to compare your results with the data that we are obtaining. Unless I know precisely how you do it and copy your method or vice versa I don't think we will end up with comparable results.

MACDONALD: This week it has occurred to me that this is a problem and I failed to consider the obvious thing and that is using a very severe pressure dose and really measuring recovery. And all you need for that is just a very simple steel vessel. It is nice to look at the animal while it is being pressurized, but it isn't essential. The measurement is the recovery rate.

ZIMMERMAN: I disagree. I think that if we are going to do this type of study we should have a window. Even if we are just going on one shot LD 50's or whatever we are attempting to do, we have to watch the cells when they are being subjected to the pressure. It might not have to be as elaborate as the one you have shown us, but it is very desirable. But we should observe them while they are being pressurized if we are going to make any kind of a meaningful result on recovery.

LOWENSTAM: Now I want to comment further on what Menzies suggested. In geochemistry we are accustomed to have some standards we distribute to see how our equipment is performing, and I would suggest that we find some hardy beasts which can be transported under minimum precautions to the different labs, and just see with the equipment of each of the investigators, what type of results we get with the same animals. Obviously it would have to be at least 10 or 15 specimens to be meaningful. Then we might get around this problem. Whoever finds the most hardy beast then distributes it for this purpose.

BRAUER: I would like to add to this one more plea, namely, that if you are

going to compress your beasts in water, then the chemical composition of the water is likely to be relevant to the things you are going to see. If you take surface water and you compress your beasts in that, and you compare with experiments done in water taken from some reasonable depth, the results will not necessarily be the same. So it seems to me that if one is going to do the kind of comparing that I quite agree you need, unless you do this with some attention to the water you use, and to the gas composition, the comparisons are going to be very difficult or they will be pure empiricism. If so, they should be based not upon one species, but on the pharmacologist's minimum series of three standard species, and I remind those of you who might still have the recollection that the pigeon unit for tincture of digitalis was never really satisfactory. This was the amount of tincture digitalis that made a pigeon vomit. Distributing a standard pigeon for this purpose was never a satisfactory solution, and I wonder a bit whether the same might not apply here.

VIDAVER: In response to Brauer's comment on various kinds of sea water being used in supposedly identical experiments; Those of us who routinely culture marine algae get around this rather handily by using something we call artificial sea water.

BRAUER: True, we do this too, but it isn't that it won't do for many experiments until the salt mixture, and the mode of aerating the solution prior to use have been tested and standardized for high pressure work.

SCHELTEMA: Are you able to make anything other than vertical hauls with this device?

MACDONALD: It's a bit heavy to do other than that, but it's a second order of modification only. There's nothing in the basic design that can limit the place and the time to stop it horizontally, but I don't plan to, I am going to stick to vertical haul.

SCHELTEMA: The reason why I would regard it as a good thing in the best of all possible worlds is that you can then at least control the depth at which you did your sample. The shorter the distance, the better since you have to tow in depth for long distances to get much of anything in deep water. If you can make that long distance all at the same depth, so much the better.

VIDAVER: Could you explain the principle of the pressure accumulator?

MACDONALD: It is a bag of gas in a steel chamber. These are commonly used in hydraulic circuits to cushion shocks to store energy. Just a rubber bag which connects to a valve through which you can feed in the correct charge of compressed air.

SCHELTEMA: The whole thing, in fact, works at all because sea water must be more compressible than the steel. Otherwise, you haul it back up and there's not enough pressure left at all. So what you are doing there is making sea water even more compressible. Actually, by having that piston flush up against there you could, as you said, make that change as small as you want.

MACDONALD: I went through the design stages because I think one should know what is going on, and I should very much like to know about the pressure retention of the core well device sampler you described just recently. Do you know what that did?
KETTLING: We haven't got any data now.
MACDONALD: Because if you get a sample from 500 atm., as I said that if the sealed displacement is equal to .2% of the total volume, then you get a 10% pressure drop, and we got changes that are interesting. Did you have any device?
KETTLING: We did use an older model of the same type of thing and that produced changes in that order.
(QUESTION) Did you measure the pressure inside on the bottom?
MACDONALD: Yes, but we haven't been able to recover the pressurizer.
(QUESTION) Oh, I see, very interesting. What goes wrong?
MACDONALD: Enclosure was one of the problems, and limitation of ship time.
(QUESTION) You mentioned you were going to do some insulation. I was wondering what you picked for the material?
MACDONALD: You won't like this. It is a rubber tank with all the pressure vessels wrapped in a 10 cm layer of polypropylene beads. This is my design because that is the most efficient insulation device I could think of which I was prepared to lose.
(QUESTION) Are you having trouble with the weight increase? You are talking about going from 400 to 800 lbs., and I know you are running this on a steel wire. Could you use an insulating layer that would also contribute to the buoyancy.
MACDONALD: It would be nice, but I think the 800 pound figure might be looked at—it ought to be all right.
SCHELTEMA: Is there any material inside which coats the stainless steel so that the sea water does not come in contact with it?
MACDONALD: Not with the recovery vessel, although I have done that with bits and pieces in the pressure circuit. There is quite a bit of piping I am not too pleased with.
FLUGEL: Dr. Macdonald, could the recovery vessel be mounted on a sleigh or a carriage and towed on the bottom and stay at one depth?
MACDONALD: I don't know about these things. Three years ago, I would have said yes. Menzies told us about some data this morning that might have a bearing on that! You used 1500 pounds, didn't you? In principle, I think yes.
ZOBELL: I have two questions please. One about the elasticity factor, and another about the retrieval receptacle. First with regard to elasticity, I wonder whether elasticity does change as a straight line function of pressure, and secondly whether the elasticity will not in addition to depth and pressure also be a function of tensile strength of the material with which you are

pressed by the formula of La May. The second question which I will ask while I am on my feet has to do with the retrieval receptacle. I think all of us will be interested to know, what do you do with material once you have transferred it from the collecting apparatus to the retrieval vessel? What kind of observations do you make at 500 atm. or some other pressure which are meaningful?

MACDONALD: Let me take the second question first. We hope to get measurements of respiration, and we've got a circulator arranged to do this. The trouble will be getting enough material in to give us a decent rate to measure. But I believe that the accurate measurement of metabolic rate is important in this environment, to follow the kind of changes that can occur. Initially, I think we are going to get more information by looking at these. We have a stereo-microscope to look right through this pressure vessel. I think pressure tolerance could be determined by preferably manipulating pressure. For example, the general story is that in my crustaceans that live near the surface you get marked changes with quite small changes of pressure. Now, it is going to be interesting to see if these materials give the same pattern. Eventually, I think it may be possible to use a kind of pharmacologic approach, putting chemicals into sea water in an attempt to analyze the properties of this material. I'm not very competent to answer your first question, but I can show you Gilchrist's work and the calculated data. I've no reason to doubt it. Obviously, the small differences between steels could affect the curve slightly, but the graph which we put out was for a typical high strength steel.

DEHART: Let me comment on that, although I am not competent either to give the details of this elasticity matter. Having been talking with construction engineers about such problems for many years, I don't have any reason whatever for questioning the data which you got. I rather suspect, though, that these apply to your system, to the wall strength, the kind of metal, the particular system, with which you are working rather, than to systems which may be used in general with different wall thickness, different diameters, and different kinds of metal which do vary very greatly in tensile strength depending upon the formula of the stainless steel. As you know, there are considerable differences in the tensile strength of stainless steel and chromel, and so on. I believe this certainly will influence the elasticity.

MACDONALD: I showed you that graph where you have this interesting effect where even with an infinitely thick wall vessel, you still lose 5%, I don't think this will hold true, without too great a change for different steels.

(QUESTION) As regards the contents of the accumulator described, if you knew you were going to sample at 500 atm. you could start your accumulator already at 400 atm.

MACDONALD: That is what we do. I was told by a marine instrumentation

engineer that this was a fundamentally hopeless job because this pressure loss was very much greater, and I thought it worthwhile to tell you that in fact by perfect design you can get down to a few percent, an idealized closed system.

GORDON: Macdonald mentioned the work that Cousteau had been involved with earlier. I corresponded with the man who was specifically doing the testing with Cousteau at the Oceanographic Institute at Monaco; and after some correspondence back and forth about various things, I finally got a very cryptic letter saying it doesn't work, and we are having trouble with welds on our seams, and that was the last I heard. The basic principle that they were working on I think is still an intriguing one, and probably with adequate engineering and maybe some modifications it might work out.

A PRESSURE AND TEMPERATURE PRESERVING SAMPLING, TRANSFER, AND INCUBATION SYSTEM FOR DEEP SEA MICROBIOLOGICAL AND CHEMICAL RESEARCH

by
Kaare R. Gundersen and Carrol W. Mountain
Department of Microbiology, University of Hawaii,
Honolulu, Hawaii

Surface recovery of water samples from the deep sea using conventional water samplers (Nansen bottles, van Dorn samplers, Niskin and JZ bacteriological samplers, etc.) may, unless special precautions are taken, introduce immediate physical-chemical-biological changes in the water sample due to:

1) Decompression (range 1000 to 0 atmospheres of hydrostatic pressure)
2) Heating (range 0° to 30°C)
3) Introduction of light (range complete darkness to full, direct sunlight)
4) Contact with air

It is well established that each of these factors, alone or in combination, may have profound effects on biochemical systems and, hence, experimental analysis of organisms recovered from the deep sea by means of a conventional sampler may well not reflect *in situ* biological function. The same argument obviously holds for purely chemical systems also.

On these assumptions we are presenting here the design of a water sampling device and its associated system for subsequent transfer and experimental manipulation of the water sample while maintaining *in situ* sampling conditions. The first prototype of the basic mechanisms was successfully tested in early 1968. Two improved models were built and extensively tested in 1969. The design below embodies further refinements and a brief description of the entire transfer system.

HIGH PRESSURE SAMPLER (HIPS)

I. GENERAL PRINCIPLE OF OPERATION

Basically, the sampler is a piston, so designed that when the piston rod is permitted to withdraw from the piston housing, seawater is sucked into the piston chamber. This action is triggered and controlled so that the seawater sample is captured at the correct ocean depth and its hydrostatic pressure is retained until removal from the HIPS, or transfer to secondary units, in the laboratory. Reversing thermometers, attached to the sampler, permit exact determination of sampling depth and temperature. The fully assembled sampler (minus support frame and electrical parts) can be autoclaved and consequently provides for fully aseptic sampling.

II. DETAILED DESCRIPTION (see Figure 142)

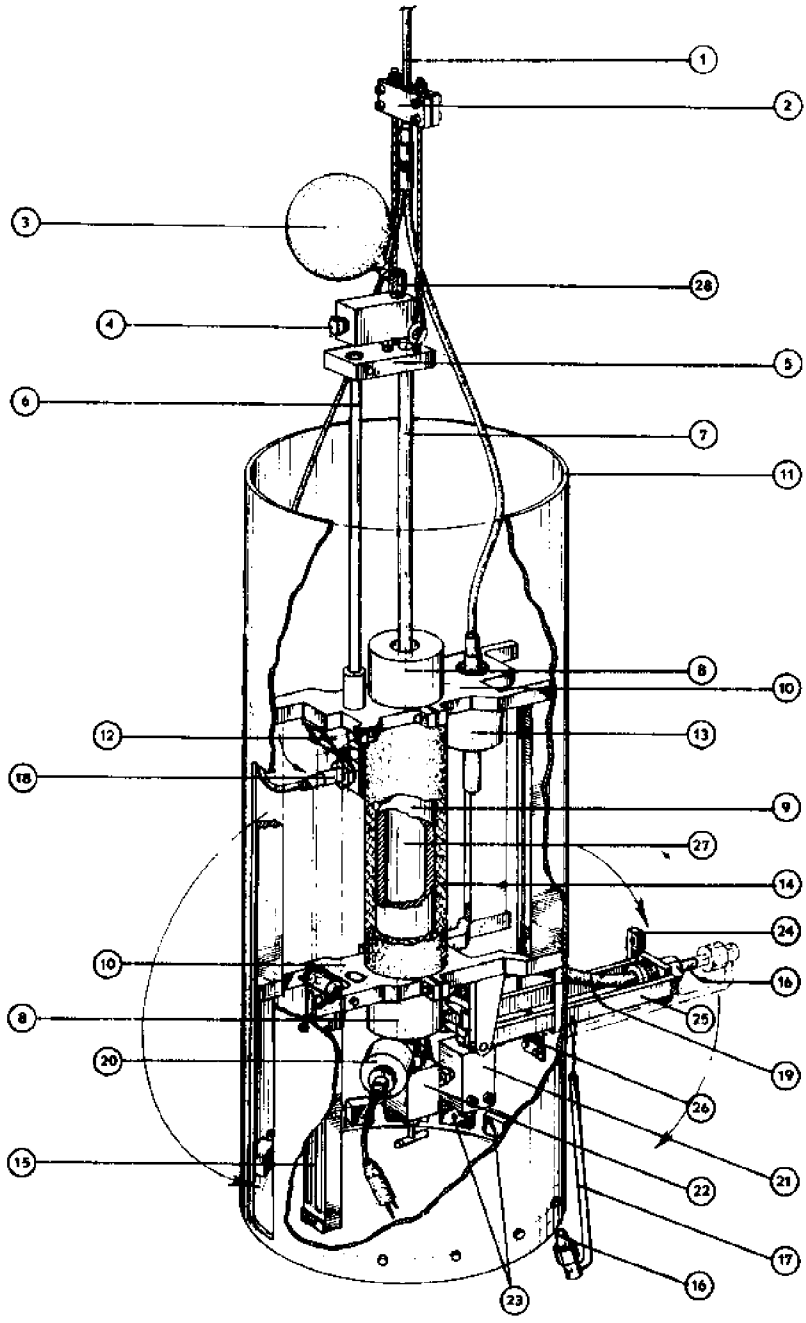

Figure 142 High Pressure Sampler (HIPS) shown after sea water sampling has been accomplished and the mercury switch (18) and the reversing thermometers (15) activated. The mechanical parts are protected by a fiberglass cylinder (11).

The sampler is lowered into the ocean supported by a 2-conductor hydrographic cable (1) which is fastened to the piston rod (7) through the arrangement of the cable clamp (2), hanger cables, eye bolts and piston-rod end plate (5). The piston head (not visible in Fig. 142 but shown schematically in Fig. 143) is at this time, and until sampling begins in the fully bottomed position. When the piston head and its connecting rod are allowed to pull out of the piston housing tube (9), sample seawater is sucked into the piston chamber through the filling system. The filling system consists of (at terminal end) a breakaway glass tube (16) connected with a filling hose (19) which is connected with a ball check valve (21) in line with a needle valve (22) which is fitted into the piston end plug (not visible in Fig. 142) with port directly into the piston chamber. The entire filling system is evacuated prior to the sampling. Its internal volume is less than 1% of the total volume of the water sample.

The pull-out action of the piston rod cannot start until the breakaway glass tube is snapped and the vacuum broken. When this has occurred, the piston housing is free to fall away by the force of gravity, assisted by the weight of the various parts and lead trim weights (23) and seawater will enter. The snapping of the breakaway glass tube (16) is accomplished in the following manner: Activating of the solenoid (13) which withdraws its plunger from the latch bar (24) hole frees the filling system terminal which is carried by two hinge bars (25) allowing it to swing out, powered by a torsion spring. The terminal end is fitted with a breaker rod (17) which swings into sharp contact with the breaker rod stop (26), thus snapping the breakaway glass tube after it has completed its full 90° swing out away from the device.

The piston housing, instead of being provided with a fixed upper end cap, is provided with a floating plug (not visible in Fig. 142 but shown schematically in Fig. 143) which is free to slide up and down the housing cavity and serves as a pressure compensator. The cavity (27) between this plug and the piston head is filled with chemically pure water (glass distilled) which has been introduced, before sterilization of the sampler, through the distilled water inlet (4), bypassing the ball check valve (28), and the hole down through the piston rod. When the sampler is first lowered into the ocean, this floating plug is in its extreme upwards position. As the external hydrostatic pressure builds up, the plug is forced downwards to compensate for any lower internal pressure. In this way, the external pressure will not affect the ball check valve (28) because the internal pressure will always equal the external pressure. Should this for some reason not be the case, as for example due to sluggish movement of the floating plug, the check valve will immediately close preventing entry of water into the sampler. Eventually, i.e. when the sampling is completed, the floating plug will again return to its extreme upwards position, pushed by the piston head.

As the filling of the sampler begins, the distilled water behind the piston head is forced out of the sampler through the hole in the piston rod, the ball check valve (28) and into an expandable rubber bulb (3) avoiding the mixing externally of escaping distilled water with sample seawater entering through the filling system.

So that the surface ship will know that the sampling has been completed, an electrical signal is provided in the form of a shipboard light or alarm, activated by a mercury switch (18). This mercury switch must tilt 90° to function. This action is accomplished as follows: A latch trip rod (6) is provided and is attached via the piston rod end plate (5) to the piston rod (7). Thus the latch trip rod moves up with the piston rod, carrying with it a latch trip pin (not visible in Fig. 142). This pin also prevents the piston rod from rotating and tangling the electric conductors. The same pin also engages a latch bar (not visible in Fig. 142) which is secured via several parts to the mercury switch. This engagement rotates the mercury switch into a horizontal position. The same latch trip pin and bar also serve to release a pair of reversing thermometers held in a protecting rack (15). The 180° swing of the thermometer assembly is powered by a torsion spring and the thermometers are locked in the downward position by a plunger lock pin.

The pressure differential between the internal and the external water will automatically close the check valve (21) and thus preserve the *in situ* hydrostatic pressure of the sample once filling is complete and the sampler is raised.

The sampler is provided with an attached pressure tranducer (20) which will indicate the internal hydrostatic pressure of the sampler with a precision of approximately ± 2%. A needle valve (22) serves as a connector to the transfer system (see below) or as means to release the pressure of the sampler at any desired time.

To prevent a change of temperature in the water sample, an insulation blanket (14) surrounds the cylinder housing (9).

The sampler and all parts are made of high quality non-corrosive stainless steel. All internal parts, which come in contact with the water sample, are lined with non-toxic, inert materials such as glass, gold, or they are silicone coated. The total internal volume of the HIPS prototype is about 1000 cc and will withstand a pressure differential of 1000 atm with a safety factor of 2.

TRANSFER SYSTEM (See Figure 143)

For experimental purposes, aliquots of the deep-water sample can, with preservation of the original hydrostatic pressure, be transferred to a series of secondary pressure vessels, hereafter referred to as *transfer units*. The set-up and the principal connecting tubes, etc., used in the water transfer, are semi-schematically shown in Fig. 143. Each transfer unit (B in Fig. 143) is

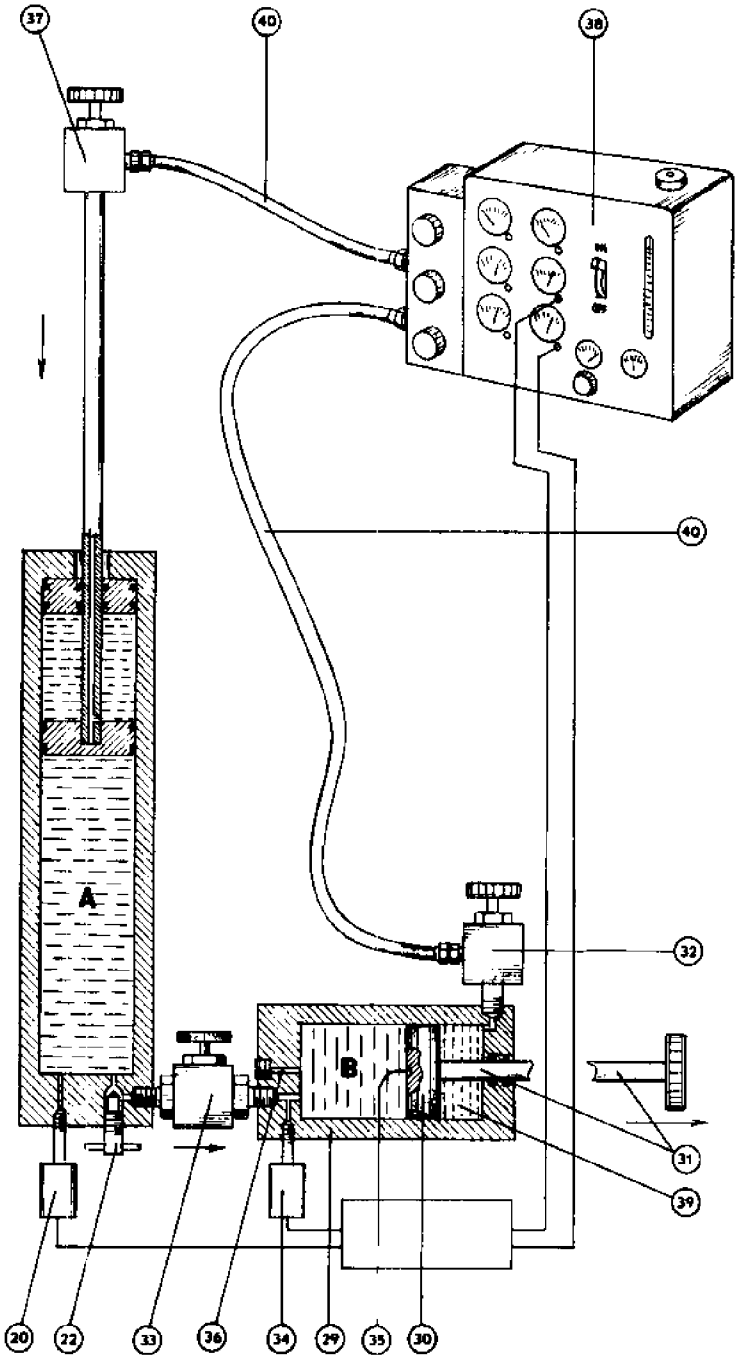

Figure 143 Transfer of seawater from HIPS (A) to transfer unit (B). The figure shows the transfer nearly completed. Further explanation in the text.

constructed essentially as HIPS (A in Fig. 143), i.e. consisting of a housing (29) with a piston head (30) and a piston rod (31). It is further provided with a upper (32) and a lower needle valve (33) and a pressure transducer (34) but lacks the ball check valves and the floating plug of HIPS. The internal diameter of the transfer units and the area of the rear side of the piston head are exactly the same as in HIPS but the volume capacity may vary from 1/10 (for approximately 8 subsamples) to 1/3 (2 subsamples) in different transfer units. A nutrient groove (35), situated in the front end of the piston head, permits the automatic addition to the water of any experimental substance which will dissolve or become suspended in the water as it enters. The nutrient(s) is added to the groove through the plugable port (36) in advance of transfer and may be sterilized together with the unit. The internal surfaces are, as in HIPS, lined with biologically and chemically inert materials.

Water transfer is prepared as follows: The needle valve on HIPS (22) is tightly closed and then the ball check valve (21) and attached filling system are removed. The needle valve (33) of the transfer unit, which at this point has the piston head in a fully downward position, is thereafter aseptically connected to the fitting on the HIPS needle valve (22). The cavities of the connecting needle valve and tubing must be as small as possible to minimize the unavoidable slight pressure drop at the moment when the bridge becomes filled with water from HIPS. The ball check valve, previously present at the distal end of the HIPS piston rod (28, Fig. 142), is replaced by a needle valve (37). This valve and the upper valve of the transfer unit (32) are now connected with flexible pressure tubing (40) to a hydraulic pump (38). This entire rear system, including the transfer unit cavity (39), is then filled with sterile hydraulic fluid and a pressure, equal to or slightly exceeding the internal pressure of HIPS, is produced with the pump (38).

When the HIPS needle valve (22), and the transfer unit needle valve (33) are opened, water will immediately fill the connecting tubing up to the front end of the piston head of the transfer unit. Any minor pressure changes produced during this operation can be monitored on the pump panel (38) which is electrically connected with the transducers (20) and (34). Pressure corrections are provided by the hydraulic system.

The transfer of water from HIPS to the transfer unit can now be accomplished by a simple mechanical downward push on the HIPS piston or by pulling the piston rod (31) of the transfer unit. This action will also force the hydraulic fluid to flow into the cavity created behind the piston head of HIPS; exactly equal volumes of water and hydraulic fluid, both under the same pressure, will simply deplace each other in the closed circular system. After completion of the transfer, all needle valves are closed and the transfer unit is disconnected for incubation. Subsequent transfers are performed in the same manner until HIPS has been drained. The entire operation will take place in a room set at the ambient temperature of the original water sample.

It is obvious that the design of this transfer system will permit any desired numbers of subsequent transfers. The possibility also exists that subsamples may be transferred to specially constructed pressure vessels, etc. Partial transfers from HIPS or any transfer unit are also possible without the need for sacrificing the left-over water or culture in the donor vessel.

The conception of this pressure device and its subsequent development was inspired by the pioneering work of Claude E. ZoBell.

Discussion Following Berger Paper

(Question): Has this been used?
BERGER: This particular one has not. The prototype does not incorporate the various electrical gadgetry, and was messenger-actuated, instead of a transducer or a pressure gauge-actuated one. The initial problem was in working out the ball valves as check valves. The check valves are teflon-coated steel or just straight teflon spheres and they seem to seat themselves quite nicely.
(Question): How's the piston locked out?
BERGER: By the very pressure of the water - unless you get a lot of stuff coming in with the water.
BRAUER: How well is the pressure maintained?
BERGER: As I recall, based on the runs that were done, I would say, off the top of my head, within 90% of what you would compute. The original, very first, models were made out of aircraft hydraulic landing gear cylinders. They were aluminum and they couldn't go quite as far.
SAUNDERS: We used floating pistons in some of our systems, and we had trouble with them cocking on us and rolling up the O-ring or just jamming. We found we had to lengthen the bodies more than the diameter of the floating pistons. I notice these are fairly narrow.
BERGER: No, the major problem was actually binding of the shaft at the top. In the original prototype there were two of these held in parallel and sometimes this gave trouble. This new model has not been built, but the opening is much wider, and I would guess that this would tend to solve the problem. I also think that the type of O-ring used might be better.
VIDAVER: It seems to me that using that electromechanical cable, primarily just to trip this thing, it's not necessary. I'm sure you can devise a mechanical device that would trip it.
BERGER: I rather feel that way myself.
VIDAVER: Another thing, also, I'd like to suggest that to have a cable running to operate the transducer, when all you want to know is when you get it up to the top is: Is it full or not? Is it under pressure? Why not just have the transducer all hooked up and then plug into?
BERGER: The purpose of the transducer is really in order to maintain checks on what the pressure is when you want to transfer.

VIDAVER: Well, that can all be done up at the top. You don't need to know what it's doing when it's down at the bottom.
HESSLER: It's fairly easy to do those things without wires anyway. There's a tremendous advantage to knowing if your thing's working when it's down in the ocean because it may take you three or four hours to lower it down there. But you could use an electrical pinger, or you can have acoustically operated devices, too. Benthos makes a 4-channel pinger that could be for this purpose almost immediately - it's a shelf item.

SUBJECT INDEX

AUTHOR INDEX