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THE EFFECT OF HIGH HYDROSTATIC PRESSURE ON THE PERMEABILITY OF *SACCHAROMYCES CEREVISIAE* TO NEUTRAL RED DYE

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THE EFFECT OF HIGH HYDROSTATIC PRESSURE ON THE PERMEABILITY OF *SACCHAROMYCES CEREVISIAE* TO NEUTRAL RED DYE

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SUMMARY

Little is known of the effect of high hydrostatic pressure on cell permeability. Those studies which have been made vary widely in cell types subjected to pressure, and vary equally widely in their results.

In the present study, the effects of two degrees of pressure application on uptake and adsorption of a vital dye, neutral red, by a microbial cell, Saccharomyces cerevisiae, were examined. In one series of tests, a pressure of 3000 psi was applied to a yeast-dye mixture for a period of ten minutes, the cells were washed, and the pH sensitive dye was extracted by acidification. The extracted dye was measured colorimetrically with a split beam spectrophotometer. The second series was run at a pressure of 6000 psi. All data were compared to controls run at atmospheric pressure.

Results of these experiments indicate a dual effect of pressure on dye uptake. Moderate pressure of 3000 psi stimulates absorption, whereas 6000 psi inhibits the absorption process.

A suggestion is made which may account for the observed results. Future investigations of the effect of hydrostatic pressure on the structural elements of both cell wall and membrane pores are recommended.

CHAPTER I

INTRODUCTION

Controlled investigations of the biological effects of high hydrostatic pressure in the deep oceans began in the last quarter of the nineteenth century. A long standing assumption that the ocean depths were devoid of life was shattered by a dredge returned from a depth of 18,000 feet full of benthic fauna (Regnard, 1891). Since then, investigations of the phenomenon of life-under-pressure have ranged from the study of molecular kinetics to whole body responses, under mild, near-shore conditions or the crushing pressures of deep trenches. Such work has revealed that some biological processes such as plasmolysis are retarded by high pressures and that others like plasmoptysis are accelerated (Murakami, 1963), and that still others are initially accelerated by moderate pressure but finally retarded by higher pressure. Protein synthesis is affected in this dual manner (Landau, 1966). Great differences in pressure sensitivity among species are often reflected in their distribution throughout the water column (Bennet and Elliot, 1969).

Pressure has long been recognized as an important parameter in physical systems through the perfect gas law, PV=nRT. Biological systems, however, differing greatly from the non-molecular-interaction requirements of this law, have no simple dependence upon it. It was not until the impetus of man's invasion into the deep oceans that investigations of the qualitative applications of the gas law came to fruition. The PV=nRT relationship can be modified through the application of reaction kinetics

to yield a biologically representative equation (McElroy and De LaHaba, 1949):

$$\frac{\partial \ln K}{\partial P} = \frac{-\Delta V}{RT}$$

where P is pressure, K is the rate constant of any reaction, and ΔV is the volume change of that reaction. The lower the temperature, the greater the effect of a given change in pressure. Likewise, the greater the volume change occurring in a reaction, the greater the pressure sensitivity of that reaction.

Reactions which involve a volume change and thus may be susceptible to pressure include ionization and unfolding of proteins (Johnson and Eyring, 1970), unimolecular dissociations (Culbertson, Kester, and Pytkowicz, 1967), sol-gel transformation (Marsland and Landau, 1954), emulsification of oil and water (Fenn, 1962), and muscle contraction (Brown, 1957). Many diverse actions have been found susceptible to pressure but nearly all are attributable to a volume change occurring in one of the above ways. Certainly, knowledge of the mechanisms by which pressure interacts with living systems is incomplete. The full potential of thermodynamics (and, to coin a word, volume-dynamics) in the elucidation of hydrostatic pressure effects in biological systems is far from realization.

Since the ocean bottoms at 35,800 feet are environs of near freezing temperature and great pressure, in light of the McElroy equation we can understand oceanographers' surprise in discovering that teleost fish are alive and well at these great depths (Piccard and Dietz, 1961). Some protective, adaptative mechanism or set of mechanisms appears to exist in cellular and sudcellular components of these fish.

Complicating the search for such protective mechanisms is the discovery of Landau and Peabody (1963) that many biochemical processes are instantly reversed in the laboratory upon return to surface pressure (one atmosphere). Much of Landau's work, concerned with just such impermanent alterations, require versatile, sturdy, yet sensitive apparatus, and rapid analytical techniques, without which a response not maintained under pressure during analysis may be undetected. If, however, a passing event were to leave some mark or indication of its passing, imperturbable by a pressure decrease, then we can be confident of detecting the initial response.

Vital dyes can serve as such indicators. There have been numerous studies of plasma membrane permeabilities and binding factors within a cell using vital dyes. For example, Chambers and Chambers (1969) found that under surface pressure the plasma membrane of starfish eggs and amoeba seems to pose no obstacle to the passage of methyl red or neutral red. Whether or not membrane permeabilities to vital dyes are altered under high hydrostatic pressure, however, is a question of some debate with no generalizations having yet been drawn.

A Russian scientist, Golovina (1958), employed neutral red as an agent for investigating permeability of mouse brain cells under pressure. His results agree with the findings of Suzdal'skaya (1955), using mouse digestive glands: under pressures equal to or greater than 6000 psi there is an increase in the cells of their sorptive power, or the tendency to absorb neutral red. Golovina's earlier work with frog muscle (1955) indicates a similar increase in neutral red sorption at 6000 psi -- accompanied by sustained contraction of the muscle. At pressures lower

than that stimulating contraction, however, there was an apparent decrease in sorptive power of the cells. No explanation was offered for these results.

Neutral red, a chloride salt of a basic chromophore, is ideally suited for cellular vital staining. Being basic, it exists in the associated or non-ionized form in an alkaline environment and becomes dissociated in more acid environments such as cell cytoplasm. Such a dye moves predominantly in the direction of maximal dissociation or, under usual conditions, into the cell (Chambers & Chambers, 1961). At physiological pH most cellular proteins are on the alkaline side of their isoelectric range, resulting in affinity for basic rather than acid dyes (Lamana, 1965). Accumulation within the cell is enhanced by the tendency for dye cations to form undissociated complexes with cytoplasmic proteins (Chambers, Cohen, & Pullack, 1931). Diffusion into the cell tends toward equilibrium but the formation of neutral red proteinates prevents the attainment of this equilibrium until all dye is out of solution or until all reactive groups in the cell are occupied -- a condition usually preceded by death of the cell.

A variety of studies of permeability-under-pressure have employed techniques other than staining with variable results. Murakimi (1963) measured the permeability to water of inner epidermal cells of Allium cepa. He reported that the rate of plasmolysis (shrinkage of protoplasm due to loss of water) is depressed by high pressures of 7000 psi in hypertonic solutions of electrolytes (KCl, NaCl, $CaCl_2$, and $MgCl_2$) and in nonelectrolytes (sucrose, glycerin, and urea). The opposite effect, plasmoptysis, is likewise slowed in solutions of divalent cations ($CaCl_2$, $MgCl_2$).

A puzzling acceleration of deplasmolysis, however, is exhibited at 7100 psi in nonelectrolytes and monovalent cations (NaCl, KCl). Zimmerman and Murakami (1968) ran a similar investigation on the swelling of Arbacia eggs in hypotonic salt solutions discovering no great change with pressures up to 8000 psi. A solution of 0.3M Kcl did demonstrate a protective effect compared to an equimolar solution of NaCl at 8000 psi. When exposed to this hypotonic concentration of NaCl, Arbacia eggs swelled and lysed within ten minutes. Only two percent of eggs placed in 0.3M KCl burst under similar pressure conditions.

Indirect studies of permeability such as measurements of resting current and injury potentials of skeletal muscles seem to indicate an increase of permeability at pressures of 6000 psi and lower. (Okada, 1954)

Landau (1966), in examining pressure effects on ^{14}C-amino acid incorporation into protein of E. coli at 37°C, observed a stimulation of protein synthesis at a pressure of 4000 psi, no effect at 6000 psi, and inhibition at 8000 and 10,000 psi. In searching for the cause of this inhibition at 10,000 psi, Landau tested for permeability changes with ^{14}C-leucine. He discovered a ten percent decrease in radioactivity of TCA-soluble material, a change of insufficient magnitude to account for the observed synthesis alterations but definitely indicative of some decrease in permeability.

The purpose of the present study was to examine the sorption of a vital dye under high hydrostatic pressure in a non-marine organism, the yeast, Saccharomyces cerevesiae. This yeast was selected both in order to simplify handling and in order to eliminate the possibility of a priori pressure adaptation. The biological process examined and its indication

of cell permeability is one of importance to an understanding of the cell's contact with its environment.

CHAPTER II

MATERIALS AND METHODS

Organism Used

Sacchromyces cerevisiae was the test organism used throughout this study. It was purchased in the dried form as "Baker's Yeast", suspended in 5% (weight by volume) sucrose and plated on Sabouraud's agar as a stock culture. This culture was maintained by transfer in a 37°C constant temperature room.

Resuspension of Test Culture

Forty-eight hour growths cultured on Sabouraud's medium were removed from the incubator and allowed to cool to room temperature (22°C). The 5% sucrose suspension medium, stored at 5°C, was allowed to warm to room temperature. The purpose of this equilibration of temperature prior to suspension was to avoid thermal shock and possible death of the cells (Knaysi, 1935). Upon scraping, four agar plates thus cooled yielded approximately 80 mg. of yeast, a quantity found suitable for suspension in 80 ml. of sucrose medium. The suspension was constantly stirred both to promote aeration, which is conducive to active synthesis within the cells, and to prevent localized accumulation of end products (Morris, 1958).

The sucrose medium was chosen both because of its isotonicity with yeast cells and because of its nutritive properties. The disaccharide sugar, while providing a carbon source, tends to slow the fermentation process, an advantage where excess accumulation of end products is to be

avoided. (Nord and Weise, 1958). Sucrose, also, aids in the prevention of floculation in S. cerevisiae (Jansen, 1958).

Testing Apparatus

High pressure equipment used in this study consisted of a hand-operated pump with pressure gauge (Blackhawk Manufacturing) equipped for generation of 10,000 psi pressure, a flexible high pressure hose and fittings, and a testing capsule or "bomb" equipped with electrical ports and a screw-on top. Bulk oil was the pressure-transmitting medium. Pressure could be quickly generated (6000 psi in less than ten seconds) and released within one second. Throughout this investigation, maximum pressure was limited to 6000 psi. Heat generated during the act of pressurization was dissipated quickly enough to prevent a spike temperature rise of more than one or two degrees centigrade.

Design of an inner two-compartment chamber was based on extensive modification of design by Landau and Thibodeau (1962). The chamber consisted of two plexiglass cylindrical sections each flanged on one end with provisions for an o-ring seal and cover-slip compartment separator (Fig. 1 and 2). The lower chamber contained a knife and spring for breaking the cover slip when desired. Tension could be applied to the spring by sliding the lower compartment away from the base through a stationary segment while the spring was secured to the base by an arched copper wire. Friction between the lower compartment and base segment held the knife and spring in a cocked position. A high amperage current supplied by an external battery was fed at the closing of a switch through the electrical ports in the bomb to the base of the inner plexiglass chamber. Completion of the circuit was made through the thin copper wire which breaks under

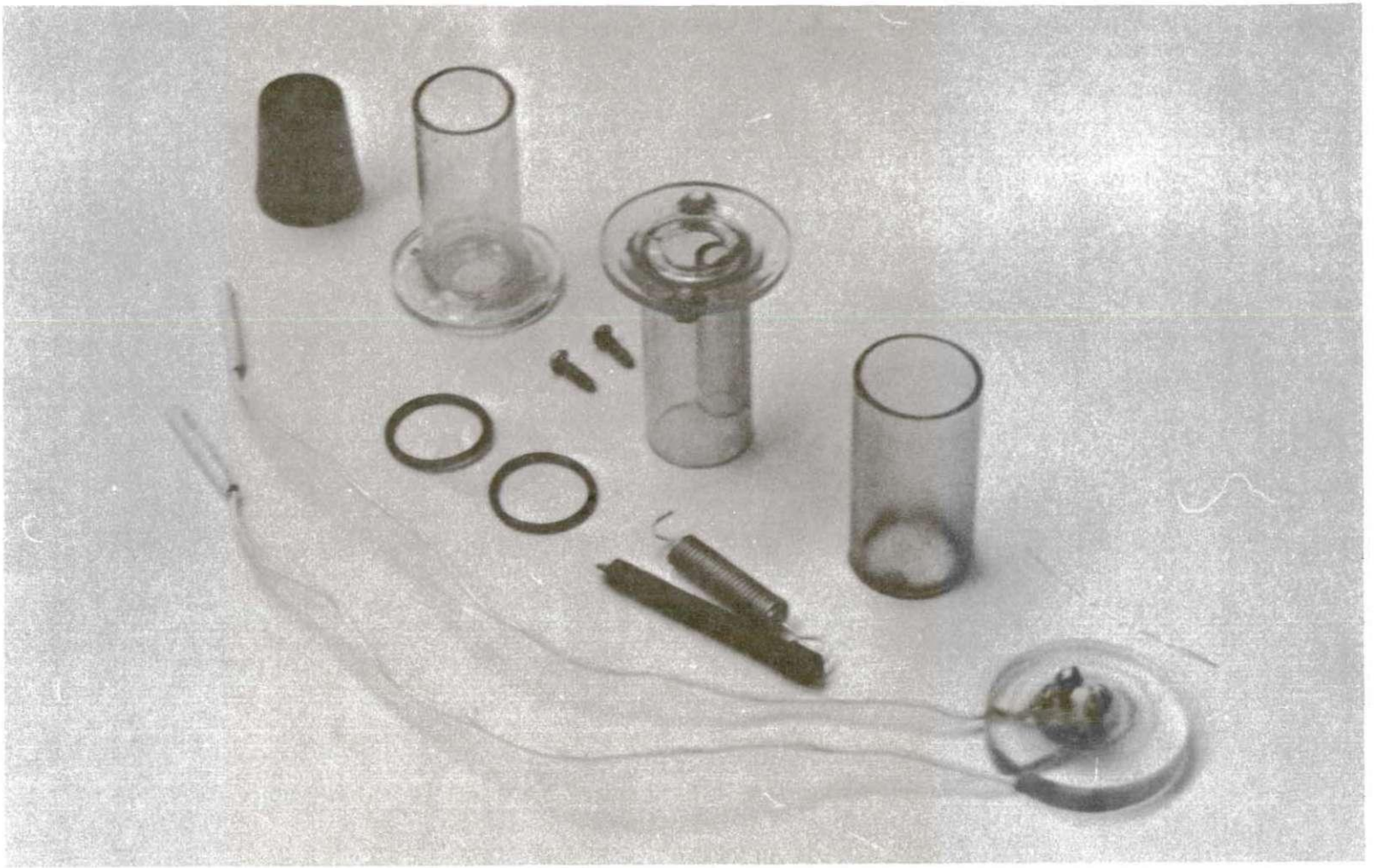


Figure 1a. Disassembled Testing Cell. Assembled in Order from Bottom Right to Top Left.

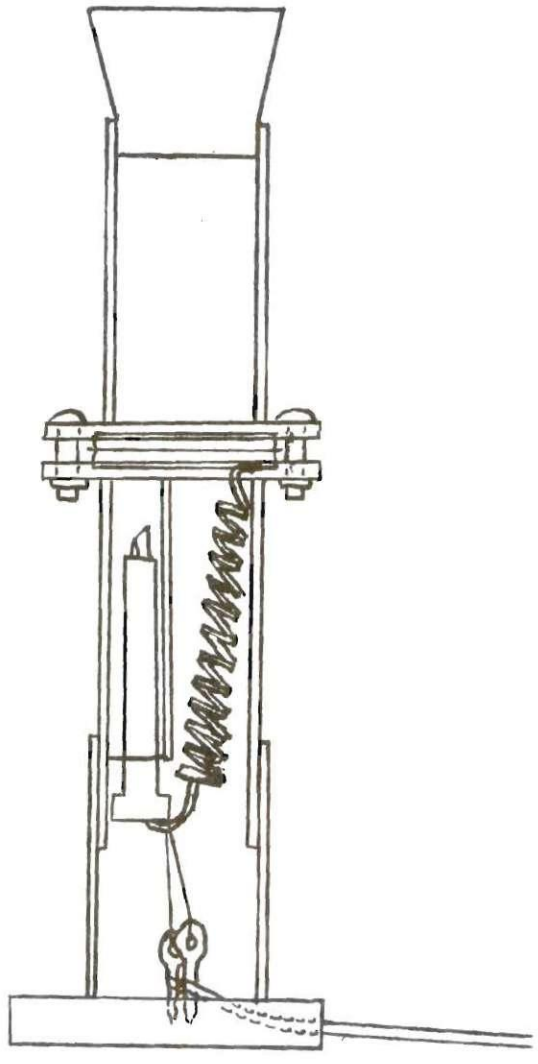


Fig. 1b. Drawing of Assembled Testing Cell.

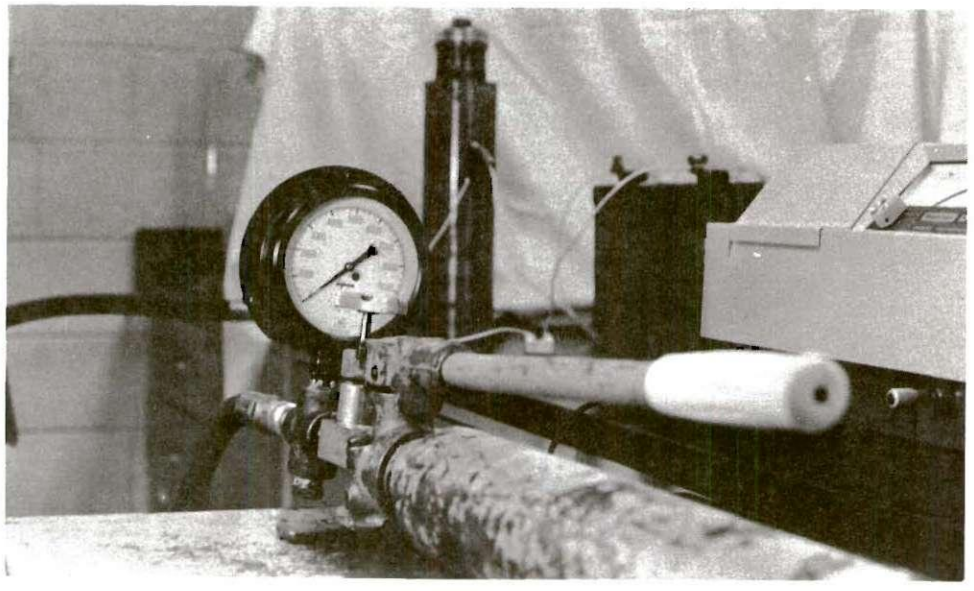
combined heat and tension, releasing the spring-cocked knife. The entire assembly was designed to be disassembled, cleaned, reassembled, and filled between tests.

Assembly and Filling Procedure

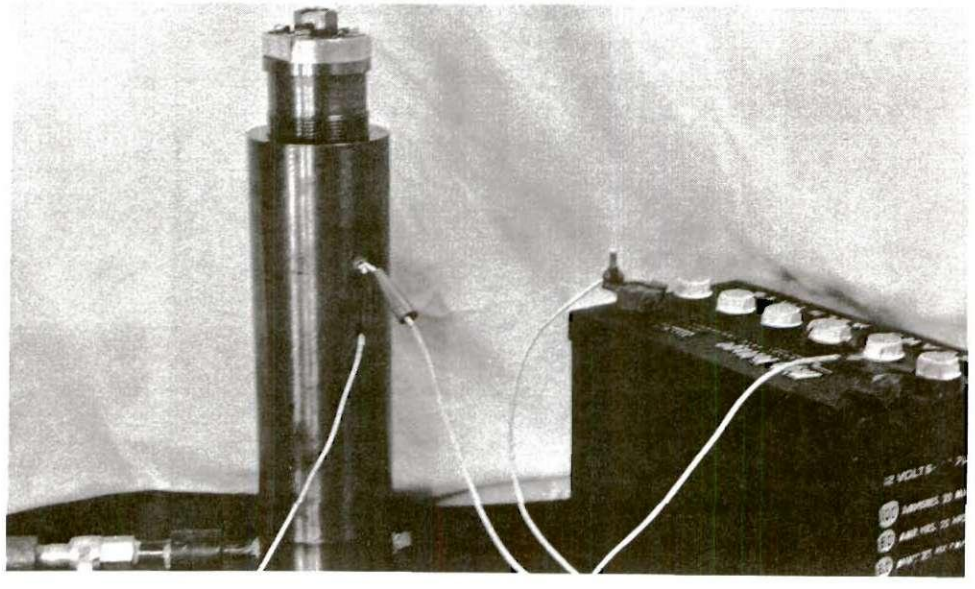
The initial step in assembly began with connection of the copper release wire to the electrical leads in the base plate. The spring-knife unit was hooked through the arch thus formed. The base cylindrical segment was then glued to the base with ethylene dichloride. This seal was easily broken during disassembly with no damage incurred, even with frequent repetition. The lower chamber was then inserted into the base cylinder with the upper point of the spring attached to the chamber flange, and the entire unit cocked.

Into the lower testing chamber was then pipeted 27.5 ml of neutral red dye in a concentration of 0.15 mg/ml. The dye stock contained 1 mg/ml in distilled water and was diluted with 5% sucrose at the beginning of each series of runs resulting in a final sugar concentration of 4.5%.

The bottom chamber was isolated by a 25 mm round cover slip of No. 1 thickness flanked on both sides by o-rings. The seal was completed by securing the top chamber to the bottom by tightening two brass screws along the chamber flanges. Now twelve ml of the yeast suspension was pipetted into the top chamber. A silicon stopper served both as an end seal and as a flexible, pressure-transmitting portion of the assembly. A hypodermic needle temporarily inserted through the stopper served for the relief of pressure and trapped air during sealing. A coat of silicon stopcock grease was placed over all possible points of leakage. Recycling time for the entire operation including testing and analysis was one hour.



(a)



(b)

Figure 2. a) Hand Operated Pump for Generation of High Hydrostatic Pressure.
b) Pressure Chamber. Battery Connected to Electrical Leads in Chamber Wall. Fittings to High Pressure Hose to the Left.

Testing Procedure

The purpose of this series of experiments was to expose S. cerevisiae to neutral red dye under various conditions of hydrostatic pressure. Exposure time was 10 minutes (Landau, 1966) using pressures of 6000 psi, 3000 psi, and controls at atmospheric pressure. Yeast controls maintained at atmospheric pressure were run either immediately before or after each high pressure application, with a total of four tests (2 pressure plus 2 controls) per day.

Following buildup of pressure (or, in the case of controls, sealing of the bomb) mixing of the yeast and dye was triggered by breaking the cover slip. After the lapse of ten minutes the bomb was decompressed, the top cap removed, and the yeast-dye mixture poured into a clear flask for mixing of the settled yeast. Thirty-one ml of this mixture was then poured into a calibrated 40 ml glass centrifuge tube, and the tube placed in a refrigerated (-5°C) International PR-J centrifuge. Elapsed time from decompression to initiation of the centrifugal washing procedure ranged from one minute fifty seconds to two and a half minutes, with an average of two minutes and little variance. It is possible that exposure of the yeast to atmospheric pressure decreased the test sensitivity. Russian scientists have also been annoyed with a similar time lag in their high pressure research (Golovina, 1955).

Yeast cells were washed four times in cold 5% (wt. by vol.) sucrose, being spun each time for ninety seconds at 1800 g. All unbound dye was removed by this procedure. For a measure of dye uptake, 1 ml of 2% (vol. by vol.) HCl was added to the 31 ml of yeast suspension and was allowed to stand for forty seconds; then the suspension was centrifuged

as previously noted. Increased acidity of the external medium from a pH of 6.2 to 2.1 served to extract a portion of the previously bound dye dependent upon binding strength and intracellular dye concentration (Chambers & Chambers, 1961). The extracted dye concentration (in reference to distilled water) was then determined colorimetrically on a Beckman DB-G split-beam scanning spectrophotometer with quartz cells of 1 cm light path. The neutral red absorption peak ranged symetrically from 640 nm to 390 nm with a maximum at 534 nm. This maximum absorbance was utilized in determining absorbance differences between pressure tests and paired controls.

Early in the series of experiments it was determined microscopically that after a ten minute exposure to dye about 85% of the yeast cells showed evidence of staining. Budding and vacuole mobility observed in the majority of the yeast cells seemed to have no relationship to the staining characteristics of the cells. Rupturing of the cells by high pressure was not observed.

CHAPTER III

RESULTS AND DISCUSSION

The results of extracted dye measurements on yeast cells at 6000, 3000, and 0 psi above atmospheric pressure are indicated in Table 1 and Figure 3. Since daily growth yields could not be duplicated, the most meaningful treatment of data was by a percentage difference calculation between a pressure run and the control immediately following or preceding it. In this way, data obtained by test-control pairs would be little affected by variations with time of the tested culture. Size limitations of the equipment did not allow for simultaneous analysis of both tests and controls. Errors introduced by this time delay were minimized by varying the order in which the controls and the tests at both pressures were run during the day.

Five controls were paired with five tests at 6000 psi. Upon subtracting the absorbance value of the control from the absorbance value of its paired test, negative values were obtained for the percent difference of absorbance with an average of -16.7%. This indicates a higher concentration of neutral red in the controls than in the test samples. In Figure 3 this data is represented by "b" on the chart with a range for the values extending to plus and minus two standard errors from the mean.

The "a" in Figure 3 represents the percent difference in absorbance data taken between controls and tests at 3000 psi. The mean for this group of six pairs was +12.7% with a standard error of 3.7%. Under this pressure treatment the test samples tended to absorb more dye than the

Table 1. Results of Pressure Tests at 3000 and 6000 PSI.

	Absorbance 14.7 PSI	3000 PSI	% Difference	
3000 PSI Series				
1.	0.63	0.80	+21.2	
2.	0.60	0.58	- 3.5	
3.	0.44	0.54	+18.5	$\bar{x}$ = +12.7%
4.	0.95	1.05	+ 9.5	S_x = 9.0%
5.	0.93	1.06	+ 8.5	S.E. = 3.7%
6.	0.32	0.41	+22.0	
				Averaged Results: +12.7% ±3.7%
6000 PSI Series		6000 PSI		
1.	0.84	0.72	-14.2	
2.	0.72	0.66	- 8.4	$\bar{x}$ = -16.7%
3.	0.76	0.68	-10.5	S_x = 7.7%
4.	0.85	0.60	-29.5	S.E. = 3.44%
5.	0.71	0.56	-21.0	
				Averaged Results: -16.7% ±3.4%

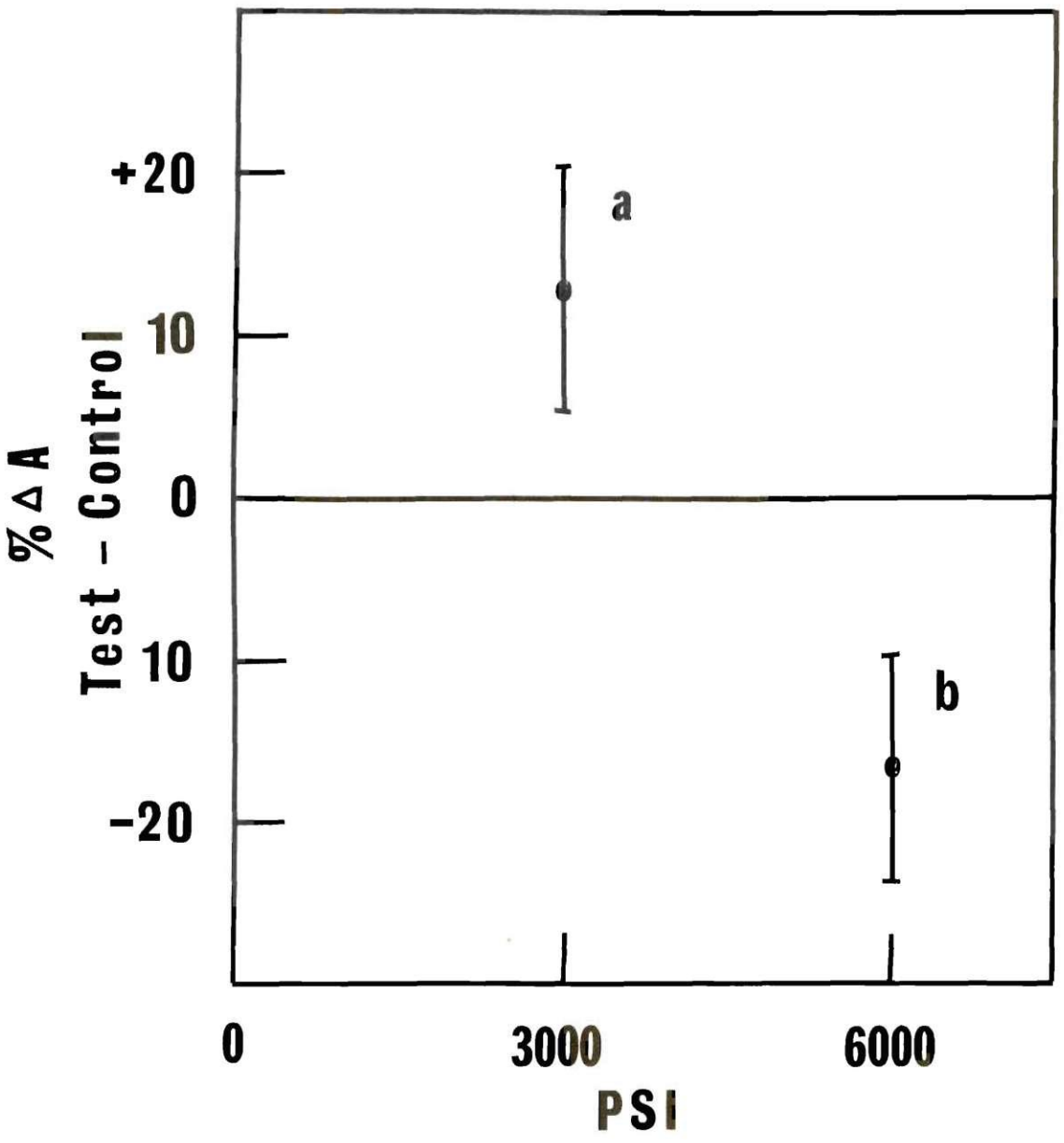


Figure 3. The Per Cent Difference in Absorbance Between Test Samples (Control Subtracted from Test) is Plotted for Tests at 3000 psi (a) and 6000 psi (b). The Range is Expressed Within Two Standard Errors of the Mean.

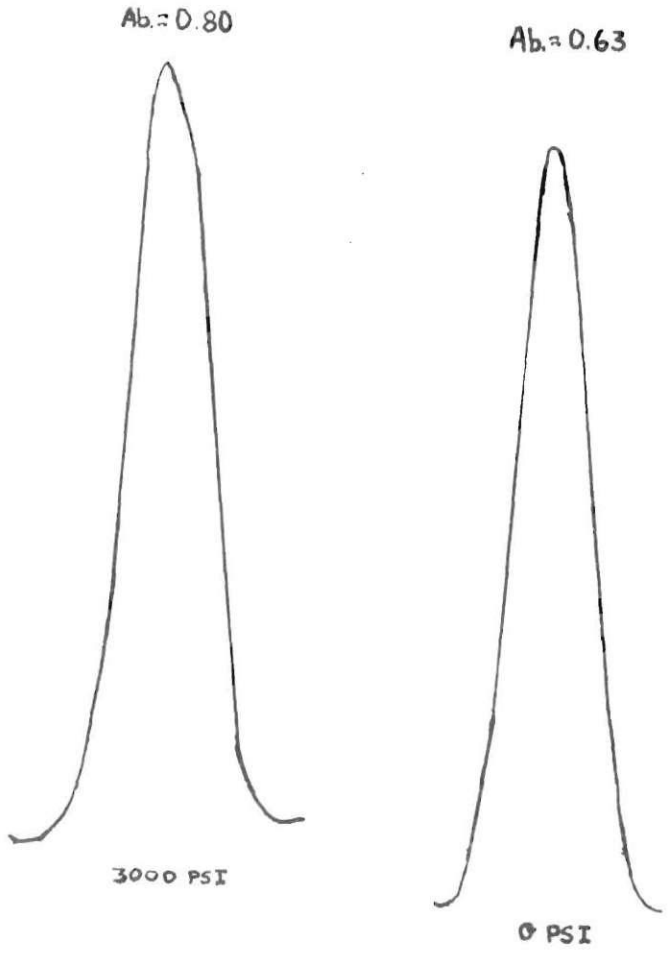


Fig. 4. Sample Absorption Curves.
Wavelength seam of the neutral red peak.

controls. Again, "a" is expressed as a range of plus and minus two standard errors from the mean.

Although dye extraction has been classified as a measure of retention (Lamanna, 1965), one may assume that any pressure effect observed would indicate a change in dye uptake. It is only during this uptake period that pressure is being applied and that the test sample differs from the control. If pressure were to permanently alter some dye binding factor within the cell, then a preferential retention could occur between the altered and non-altered cell during washing -- after the release of pressure -- disguising what might have been equal dye uptake. This possibility is remote, however, as all biochemical pressure alterations thus far identified are instantly reversible upon return to atmospheric pressure from pressures below 10,000 psi (Landau and Peabody, 1963).

A conservative bias was built into the experiment by virtue of unavoidable exposure of the tested samples to atmospheric pressure prior to centrifugal separation of yeast and unbound dye. This served to decrease the identifiable effect of pressure on cell permeability to the dye.

In spite of the conservative bias inherent in this experiment, the absorbance data is clearly separable into two groups related to pressure. It is apparent then that the processes responsible for neutral red uptake are altered by high hydrostatic pressure -- accelerated by moderate pressure (3000 psi) and inhibited by higher pressures (6000 psi). There is a resemblance in this pressure response pattern to the well documented response of bacterial luminescence (Bround, Johnson and Marsland, 1942) and to the ^{14}C-amino acid incorporation work of Landau (1966). Both responses rely upon the dual action of pressure upon activated enzymes.

Pressure will both counteract denaturation of enzymes at supra-optimal temperatures and reduce primary reaction rates by hindering formation of the activated state (perhaps by hindering a volume increase associated with excitation of an orbital electron) (Lamanna, 1965). At a temperature of 37°C, a portion of the rate-controlling enzyme is being denatured, and moderate pressure exhibits its protective effect resulting in an increase of active enzyme and thus higher reaction rate. The effect of higher pressure on the primary reaction rate, however, eventually overcomes this acceleration response and results in a net decrease of reaction rate.

Important differences are to be noted between these results and those of the present study. The first is the magnitude of change. The present results show percent differences only half as great as those encountered in Landau's C^{14} studies. The percentages are comparable, however, to Landau's unexplained permeability decrease (see page 5). This latter point may be significant as the ten percent permeability decrease Landau observed approximates the magnitude of change observed in this experiment.

Secondly, the temperature at which the yeast-dye interaction was maintained is considerably below that used by Landau. In fact, upon Landau's repetition of experiments at 22°C, the acceleration promoted by moderate pressure disappeared, an observation consistent with his theory. At that temperature, little if any enzyme is being denatured. In the bacterial luminescence studies of Brown, Johnson and Marsland, we do find at temperatures of 20°C, and even 15°C, an initial luminescence acceleration at 3000 psi followed by inhibition at 6000 psi. The observation that 6000 psi results in a repression of activity at this temperature is

consistent with the effect of pressure upon primary reaction rates, but the activation hump at lower pressure is unexplained.

One factor which may play a large role in permeability alterations under pressure is change in pore sizes in either or both of the diffusion barriers of the yeast, the cell wall and plasma membrane. These barriers have distinct permeability differences; galactose and arabinose penetrate only as far as the cell wall in S. cerevisiae, with acetic acid filling all polar regions throughout the cell (Conway and Downey, 1950).

The Cell Wall as a Diffusion Barrier

The cell wall in S. cerevisiae is a rather thick meshwork of irregularly arranged macromolecular fibrils, with openings or pores having a maximum diameter of 37 Å (Gerhardt and Judge, 1964). This relatively small size excludes molecules as small as inulin (m.w. 3000) (Conway and Downey, 1950). These observations support the sieve theory of permeability, with pore diameter being dependent on kinetic motion of the fibrils, changes in interfacial forces, and adsorptions (Lamanna, 1965). It should be noted that adsorption to cell wall components, as for example adsorption of lysine in Micrococcus lysodeickticus, does not imply either effects on internal pooling of the lysine or the presence of a real barrier to permeation by the molecule. (Few, Fraser, and Gilby, 1957). However, the fact that the cell wall is notoriously resistant to staining by neutral red should, rule out concern for the latter occurrence.

Interpretation of the results of this experiment in terms of our knowledge of pressure effects on cell walls is not yet possible. Nor are there any obvious hypotheses to guide future experimentation. It is hard to envision a mechanism by which pressure could affect kinetic motion,

interfacial tension, or adsorption in a manner which decreases permeability, but we certainly cannot rule out the possibility that such a mechanism exists, nor can we ignore the possibility that pressure affects a yet unknown feature of cell walls.

The Plasma Membrane as a Diffusion Barrier

Unlike the cell wall, the plasma membrane appears a likely candidate for pressure alteration. Several studies of small ion diffusion have led to calculations for membrane pore sizes in various cell types (assuming cylindrical pores); 4.25 Å radius with a distribution of $1.3 \times 10^{10}/cm^2$ for the plasma membrane of squid giant axons (Villegas and Barnola, 1961), 4Å radius in frog sartorius muscle (Mullins, 1961), and 4.17Å for pores in resting mammalian neurons (Ito et al., 1962). These pore sizes, remarkably similar, are all somewhat larger than the radii of water molecules (1.36Å) or hydrated K+(4.0Å), Na+(3.7Å), or Cl-(3.9Å) ions and quite a bit smaller than the cell wall pores calculated for S. cerevisiae.

The discovery of globular proteins such as ATPases in the plasma membrane has led some authors to picture natural membranes not as a lipid bilayer but as a skeleton of protein monomers assembled into two-dimensional arrays (De Prau, 1968). Globular proteins appear especially sensitive to high pressure alteration (Zimmerman, 1970).

Based on this small amount of information and the results of Brown, Johnson, and Marsland as well as the present results with yeast permeability, the following hypothesis may be proposed: plasma membrane pores may represent spaces in a periphery of at least partially globular proteins. The presence of free or empty space within the framework of these proteins is subject to reduction by pressure, just as in the case of a

balloon by rough analogy. For system operating below the optimum rate of diffusion across the plasma membrane, a slight increase in pore area would accelerate the diffusion-dependent reaction. This is precisely what should happen with the application of moderate pressure -- pore sizes should increase in accordance with the decrease in protein volume. The amount of this area effect is dependent upon the number of peripheral globular units (see Appendix A); the smaller the number of peripheral units, the more sensitive is the pore area to pressure. With radii of 4Å we would expect a minimum number of such units. A strain on bonds between all peripheral units is associated with this pressure-induced increase in area. A further increase in pressure could result in loss of integrity of the pore circumference, or less drastically, a further increase in pore area. Under the latter condition a loss of molecular selectivity could ensue, resulting in competitive inhibition, or alteration of metabolic pathways by the sudden appearance of heretofore excluded inducers. This condition could at least partially explain a decline in reaction or diffusion rate under high pressure (6000 psi) following an increase in the direction of an optimum under lower pressures (3000 psi). (For further discussion see "Implications" in Appendix A.)

Such suggestions, may lead to high pressure research directed toward the role of pores in the plasma membranes and cell walls in reactions of whole cells to hydrostatic pressures above atmospheric.

CHAPTER IV

CONCLUSIONS

Analysis of neutral red adsorption and retention in Sacchromyces cerevisiae under high hydrostatic pressure led to the following observations.

1. A pressure of 3000 psi applied to cells of a forty-eight hour culture for a period of ten minutes stimulates the process of dye uptake and adsorption.

2. A pressure of 6000 psi applied in the above manner depresses dye uptake below that found at atmospheric pressure.

CHAPTER V

RECOMMENDATIONS

From the results of this research the following recommendations for future work are made.

Firstly, it is apparent that experiments utilizing a wide range of controlled temperatures would yield information vital to thermodynamic interpretation. For example, a thermostatically controlled heating and cooling unit could be installed within a high pressure chamber. Or, if rapid temperature changes are not required, a water jacket, surrounding the pressure chamber, may be added.

Another problem encountered is the prolonged exposure of the test sample to atmospheric pressure while it is still in contact with dye. One solution is the rapid cooling of a sample to minimize diffusion following the pressure test. An alternative solution, and prehaps a better one, is washing while the sample is still pressurized. A testing chamber has been designed and partially constructed by this author in which such a washing step can be accomplished. The chamber utilizes a piston to draw first the dye, then later the washing solution, into contact with the sample.

An investigation centering on the influence of pores on permeability may be fruitful. The first step in such a study should be a continuation of extensive electronmicrographic observations. Essentially nothing is known of the pores in plasma membranes if such pores do, in fact, exist.

Pores are known to exist in the cell wall of S. cerevisiae and the effect of pressure on these pores should be investigated, perhaps through

the use of isolated cell walls.

Models such as that in Appendix B should be useful both in planning experiments on globular proteins and in interpreting results of such experiments. The value of the model in the study of pores with globular perimeter units is obvious.

APPENDIX A

Calculation of Pore Area

For a pore formed by a periphery of circular units (being a cross-section of spherical units) we utilize the following method in determining pore area.

Pore area consists of the area of a polygon described by line segments joining the centers of peripheral units minus the summed portions of the circular units included within the polygon.

Area of a regular polygon is $A = \frac{1}{4} Na^2 \operatorname{ctn} (180°/N)$ with "N" being the number of sides (5 in the above case), and "a" the length of each side. The following substitutions are made:

$$R_2 = \frac{a}{2};\ 180°/N = \pi/N = U$$

$$\text{polygon area} = NR_2^2 (\cos u/\sin u)$$

The radius R of a circumscribed circle passing through centers of all circular units is given as:

$$R = \frac{a}{2} \csc (\pi/N)$$

This in turn gives us a relation for R_2, the peripheral circle radii, in terms of R and U.

$$R_2 = R \sin u.$$

Polygon angles are found as: $B = \left(\frac{N-2}{N}\right)\pi$ radians. Accordingly, area of the peripheral circles included in the polygon may be expressed as $\frac{B}{2\pi} (\pi R_2^2)$ or as a sum of all such areas: $\frac{N-2}{2} \pi R_2^2$.

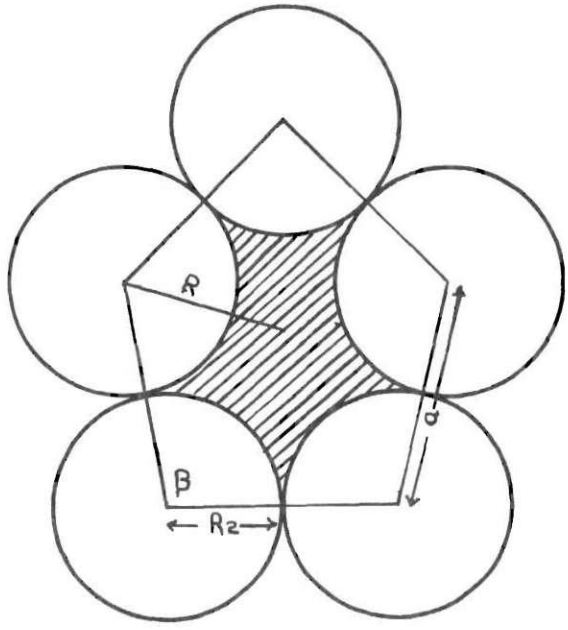


Fig. 5. Model pore. Geometry with five peripheral units (N = 5).

The pore area is therefore expressed as:

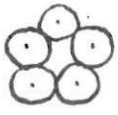
$$Ap = NR_2^2(\cos u/\sin u)\ \frac{N-2}{2}\ \pi R_2^2$$

or simplified to $AP = R_2^2 \left[N\ (\cos u/\sin u) - \pi(\frac{N-2}{2}) \right]$.

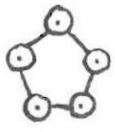
The second column of Table 2 represents pore areas for a value of 1 for R. A rapid increase in pore area is evident as N is initially increased above a minimum of 3, but as N continues to increase the rate of change of pore area decreases, with the pore area limited by the area of a circumscribed circle of radius R.

In a high pressure application, the changing parameter is not N, but the decrease in perimeter unit area. We should envisage the pore under pressure as surrounded by globular units anchored at their center of mass by balanced forces resulting from association with the neighboring matrix. Any pressure-induced decrease in volume will be opposed by bonds between immediate neighbors in the pore perimeter, perhaps with a certain amount of distortion resulting. It is this distortion which can perhaps best be represented as line segments extending between adjacent perimeter unit centers.

Our model pore portrayed at first in this manner:



is altered by pressure to this representation:



The increase in the area of the pore due to compression of its perimeter units is calculated from the per cent change in volume induced by pressure.

If pressure causes a 27% reduction in volume of a globular unit its cross-sectional area will be decreased by 19%. This decrease results in an addition to the pore area expressed in the following manner: $(\frac{N-2}{2})\ P\pi R_2^2$ where P is the per cent reduction in cross-sectional area. The last four columns in Table 2 represent pore areas following, respectively a 14.3% decrease in volume of perimeter units, a 27% volume decrease, and per cent changes in pore area following their respective volume decreases. (N.B. 14.3% and 27% are the volume changes resulting from a 5% and 10% reduction in length of radii.)

Implications

The data of Table 2 illustrates two points.

(a) At a pressure resulting in a 14.3% volume decrease of globular peripheral units, a pore surrounded by 3 units is almost 100 times more sensitive to pressure (capable of being altered) than a pore with fifty perimeter units.

(b) As pressure is further increased, pressure sensitivity of the pore area becomes less dependent upon the number of perimeter units. There is only about a threefold decrease in sensitivity between a pore of three units and one of fifty units.

These considerations not only indicate the possible sensitivity of pores to a high pressure environment, but help explain differential pressure effects encountered in this experiment.

One explanation for a dual response was made on page 23. Another example of this differential action may be found in a modeled reaction

Table 2.

PORE AREA VARIANCE WITH NUMBER OF PERIMETER UNITS
AND PER CENT DECREASES IN UNIT VOLUME UNDER PRESSURE

BASED ON R VALUE OF 1

N	AP	14.3% VOL.	27% VOL.	% DIF.14.3	% DIF.27
3	0.1209	0.2358	0.4595	94.984	279.97
4	0.4292	0.5824	1.1348	35.684	164.41
5	0.7496	0.9083	1.7700	21.178	136.14
6	1.0273	1.1804	2.3003	14.909	123.92
7	1.2579	1.4020	2.7321	11.461	117.20
8	1.4482	1.5828	3.0843	9.2925	112.98
9	1.6063	1.7317	3.3746	7.8073	110.09
10	1.7389	1.8559	3.6167	6.7282	107.98
11	1.8514	1.9608	3.8210	5.9094	106.39
12	1.9478	2.0504	3.9955	5.2672	105.14
13	2.0311	2.1276	4.1460	4.7504	104.13
14	2.1038	2.1948	4.2771	4.3255	103.30
15	2.1678	2.2539	4.3921	3.9702	102.61
16	2.2245	2.3061	4.4939	3.6686	102.02
17	2.2750	2.3526	4.5845	3.4095	101.51
18	2.3203	2.3942	4.6656	3.1845	101.08
19	2.3612	2.4317	4.7387	2.9873	100.69
20	2.3983	2.4657	4.8049	2.8130	100.35
21	2.4320	2.4966	4.8651	2.6579	100.05
22	2.4628	2.5248	4.9201	2.5190	99.779
23	2.4910	2.5507	4.9705	2.3939	99.535
24	2.5171	2.5745	5.0169	2.2806	99.314
25	2.5411	2.5964	5.0597	2.1775	99.113
26	2.5634	2.6168	5.0993	2.0834	98.930
27	2.5841	2.6357	5.1361	1.9970	98.762
28	2.6033	2.6532	5.1703	1.9175	98.607
29	2.6213	2.6696	5.2023	1.8441	98.464
30	2.6381	2.6850	5.2322	1.7761	98.331
31	2.6539	2.6994	5.2602	1.7129	98.208
32	2.6687	2.7129	5.2865	1.6541	98.093
33	2.6827	2.7256	5.3113	1.5991	97.986
34	2.6958	2.7375	5.3346	1.5477	97.886
35	2.7082	2.7488	5.3567	1.4995	97.792
36	2.7200	2.7595	5.3775	1.4542	97.704
37	2.7311	2.7697	5.3972	1.4116	97.621
38	2.7417	2.7793	5.4160	1.3714	97.542
39	2.7517	2.7884	5.4337	1.3334	97.468
40	2.7613	2.7971	5.4507	1.2975	97.398
41	2.7703	2.8053	5.4667	1.2634	97.332
42	2.7790	2.8132	5.4821	1.2311	97.269
43	2.7873	2.8207	5.4967	1.2004	97.209
44	2.7952	2.8279	5.5107	1.1712	97.152
45	2.8027	2.8348	5.5241	1.1434	97.098
46	2.8100	2.8413	5.5369	1.1168	97.046
47	2.8169	2.8476	5.5492	1.0915	96.997
48	2.8235	2.8537	5.5610	1.0673	96.950
49	2.8299	2.8595	5.5723	1.0441	96.905
50	2.8361	2.8650	5.5831	1.0220	96.862

maintained in equilibrium by diffusion of reactants through two pores, one quite small with perhaps three perimeter units (controlling reactant A) and the other composed of 50 perimeter units and therefore relatively large (controlling reactant B).

Upon application of moderate hydrostatic pressure the small pore's area may double in size while the larger pore increases in area only slightly. With the possibility of twice as many A molecules entering the reaction site than can react with B molecules the equilibrium will probably be disrupted.

A further increase of pressure, however, could serve to double not only the previous area of the small pore, but also double the area of the large pore. Hence both reactants, while increasing in quantity, are maintained in a 2 to 1 ratio initiated by the first application of hydrostatic pressure. With the initial effect of disequilibrium thus stabilized at very high pressures, other high pressure effects such as the raising of activation energies may come to the forefront creating the dual response of the system.

It must be remembered that the above considerations apply only for a pore with globular units in the perimeter.

Conclusions

A dual natured response of a system under high hydrostatic pressure could result from the action of membrane pores in each of the following ways:

1. As discussed on page 23, a reaction controlled by pores of similar size may react in one manner to a pressure induced increase in pore area, and in a different manner to a further

increase in pore area under higher pressure. This last response may be due to:

(a) loss of pore integrity

(b) loss of selectivity of reactants.

2. A dual natured response may result from the differential sensitivity of pores of different sizes under varied pressure conditions. Once a diffusion controlled reaction responds to a moderate pressure application by reaching a new equilibrium, a further increase in pressure should have little effect on that equilibrium and instead exert its effects on other processes.

APPENDIX B

Computer Modeling of Globular Molecules

In our discussion of the possible role of membrane pores in explaining the differential action of high hydrostatic pressure in this experiment, the assumption was made that globular molecules or networks of molecules compose the pore perimeter. We are immediately confronted with a lack of knowledge of the mathematical principles involved in the formation of three-dimensional complex networks. This appendix describes one device which may be useful in a modeled description of such formations.

A program for selection of pseudo-random points in a three-dimensional field has been generated for the PDP-8 computer utilizing the FOCAL language. An evaluation and decision for retention or rejection of these randomly generated values is based upon the history of previously retained points. The criterion for retention and storage is the absence of intersection between a line segment defined by the last generated point and the latest retained point, and all other possible line segments when these line segments are joined end to end in order of their generation. Upon endowment of these segments with thickness, a cross-sectional area, the finished product appears similar enough to a globular macromolecule to arouse some interest.

The formation of a complex interfolded protein is yet to be closely observed. The growth of a complex intertwined model may be examined, however, with careful scrutiny. While the processes of formation of the

protein and the model may not be identical, information obtained from the model may be useful in our study of the protein.

We may examine for instance the effect of strand thickness on the compaction of a globular molecule. Furthermore, we can study the probable synergism between strand thickness and high hydrostatic pressure. To the programmed model a thin stranded "molecule" in a volume compacted by pressure is indistinguishable from a thick stranded network at a lower pressure. In fact, the parameter of pressure may be entered into a system by manipulating the computer's control of thickness. Such manipulation, then, is one of the conveniences of this program and constitutes a means of circumventing a previous stumbling block - definition of pressure as a parameter.

There is a mathematical expression borrowed from coincidence probabilities in Coulter counters and redundancy and overlap probabilities in bacterial counts which seems to mimic the rather mechanical decision-making process of the FOCAL program. This expression: $P = A \ln(1-\frac{d}{A})$ may be, in fact, a generalization of the results obtained by the program. For such a generalization we would define the units of the equation thusly: P is the number of attempts at incorporating randomly selected loci into the growing "molecule" without contact or intersection with previously selected loci; d is the number of successfully incorporated loci (for each locus representing an atom of some average atomic weight, d is proportional to the molecular weight of our molecule). A is the volume number field limit, an expression of strand diameter and pressure.

This logarithmic function indicates that, as long as the growth of a molecule is limited to a certain volume, the retention probability of

subsequently generated loci varies inversely with the number of loci retained.

As the volume limits of growth are reduced as an expression of increased pressure or strand diameter, the probability of retention is likewise reduced.

Implications

Through the continued use of computer models in directing high pressure research the following points may be clarified.

A. It is possible that spatial hindrance promoted by high pressure or increases in strand diameter participates in kinetic effects such as enzyme inactivation. This effect may appear either in a pre-formed molecule or during the formation of such a molecule.

B. Variations in hydration of a macromolecule may contribute to changes in strand diameter with possible evolutionary benefits either within or outside a high pressure environment.

C. While a mean decrease in strand diameter may benefit the formation of a macromolecule, an increase may serve to limit pressure sensitivity of a biological process. If, for example, an increase of hydration of globular proteins should decrease the void space available for compaction by pressure, alteration by pressure of a structure composed of these globular units should be diminished. The model pore described in Appendix A may be such an example.

Table 3. PDP-8 Focal program for model of globular molecule.

```
C-FOCAL S 2/71

01.01 T "3 DIMENSIONAL INTERSECTION PROGRAM",!
01.02 C Q=OK/(NO+OK)
01.03 T "PRELIM. INPUT   NINE COORDINATES",!
01.04 S J=FX(1,3165,160)+FX(1,658,3712)
01.06 F PQ=0,2;A XA(PQ),YA(PQ),ZA(PQ);S IB=3
01.09 T "    LOADED",!;T "    N      Q     SLOPE"
01.12 T "       PUNCH RETURN WHEN READY",!;A B;
01.14 C TURN ON PUNCH BEFORE GIVING RETURN
01.16 S N=0;S O=0

02.05 S XA(IB)=FITR(FRAN()*10);S YA(IB)=FITR(FRAN()*10);T*K
            S ZA(IB)=FITR(FRAN()*10);S H=1
02.07 S RP=IB-1;S PQ=IB-2;S CD=IB-3;
02.23 I (XA(IB)-HA(RP))2.25,2.05
02.25 S M1=(YA(IB)-YA(RP))/(XA(IB)-XA(RP))
02.28 S B1=YA(IB)-M1*XA(IB)
02.29 I (XA(PQ)-XA(CD))2.30,2.05
02.30 S M2=(YA(PQ)-YA(CD))/(XA(PQ)-XA(CD))
02.33 S B2=YA(PQ)-M2*XA(PQ)
02.34 I (M1-M2)2.35,2.05
02.35 S XO=(B2-B1)/(M1-M2);S XO=FITR(XO)
02.51 I (XA(IB)-XO)2.55,3.01
02.53 I (XA(RP)-XO)2.56,3.01,2.81
02.55 I (XA(RP)-XO)2.81,3.01
02.56 I (XA(CD)-XO)2.57,3.01,2.58
02.57 I (XA(PQ)-XO)2.81,3.01,3.01
02.58 I (XA(PQ)-XO)3.01,3.01
02.81 S PQ=PQ-1;S CD=CD-1;S H=H+1
02.82 I (PQ)3.01,2.97,2.25
02.97 S IB=IB+1;S O=O+1;G 4.09

03.01 S PQ=IB-H-1;S CD=IB-H-2
03.25 S M1=(ZA(IB)-ZA(RP))/(XA(IB)-XA(RP))
03.28 S B1=ZA(IB)-M1*XA(IB)
03.29 I (XA(PQ)-XA(CD))3.30,2.05
03.30 S M2=(ZA(PQ)-ZA(CD))/(XA(PQ)-XA(CD))
03.33 S B2=ZA(PQ)-M2*XA(PQ)
03.34 I (M1-M2)3.35,2.05
03.35 S XO(1)=(B2-B1)/(M1-M2);S XO(1)=FITR(XO(1))
03.51 I (XA(IB)-XO(1))3.55,3.95
03.53 I (XA(RP)-XO(1))3.56,3.95,2.81
03.55 I (XA(RP)-XO(1))2.81,3.95
03.56 I (XA(CD)-XO(1))3.57,3.95,3.58
03.57 I (XA(PQ)-XO(1))2.81,3.95,3.95
03.58 I (XA(PQ)-XO(1))3.95,3.95,2.81
03.95 I (XO(1)-XO)2.81,3.97,2.81
03.97 S N=N+1

04.09 S G=FDIS(200+5*(N+0),300+500*(O/(N+0)))
04.10 I (O)4.11,2.05
04.11 S Q1=O/(N+0);S Q3=Q1-Q2;S Q2=Q1;S S=100*Q3;T %3,(N+0);T*K
       T %5.05,Q1,S,!
04.12 I (400-(100+20*S))4.16;I (50+(100+20*S))4.16
04.14 S G=FDIS(200+5*(N+0),100+20*S)+FDIS(200+5*(N+0),100)
04.16 I (N+0-100)2.05
04.18 T %2,"   END OF RUN    POINTS ACCEPTED OUT OF 100 =",O,!
04.20 T "*****************************************************"
04.21 T "*****************************************************"
04.22 T "*****************************************************",!
04.25 S N=0;S O=0;S IB=3;G 2.05
*
```

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