

FIGURE 4. Photographs, in polarized light, of spherulites formed in thin layers of a 5M glycerol solution, illustrating the increasing number and decreasing size of the crystallization units at increasing cooling rates and decreasing temperatures.—Temp. of freezing bath: (a) -60°; (b) -70°; (c) -75°. $\times 83$.

When a layer 75μ thick of a 50 per cent gelatin gel is cooled at a rate of a few thousand degrees per second by immersion in an isopentane bath at -150° , the x-ray diffraction diagram shows no reflection peak (FIGURE 5, Tracing 1). When a similar preparation is immersed in a bath at -60° or -30° , the cooling rate being of the order of hundreds of degrees per second, three peaks emerge in succession: first, Peak No. 2, which one would normally expect to be produced by the basal planes 002 of hexagonal crystals, then Peaks 5 and 7, from Planes 110 and 112 (Tracings 2 and 3). Peak No. 1 (from Planes 100) also begins to emerge. When the temperature of the coolant bath is -20° , -15° or -10° , which reduces the cooling rates to tens of degrees per second, Peak No. 1 completes its rise, and is followed by Peak No. 3, from Planes 101, and finally by Peaks 4 and 6, from Planes 102 and 103 (Tracings 4 to 6).

The facts that: (a) the most rapidly cooled specimens have no peaks (Tracing 1), (b) the specimens cooled at intermediate rates show three peaks which occupy the same position in the diagram as the three principal peaks of cubic ice (Tracings 2 and 3), and (c) the most slowly cooled specimens exhibit the full set of peaks of hexagonal ice (Tracings 4, 5 and 6) gave rise to the idea that the three cooling rates result in the formation of, respectively, amorphous, cubic and hexagonal ice.

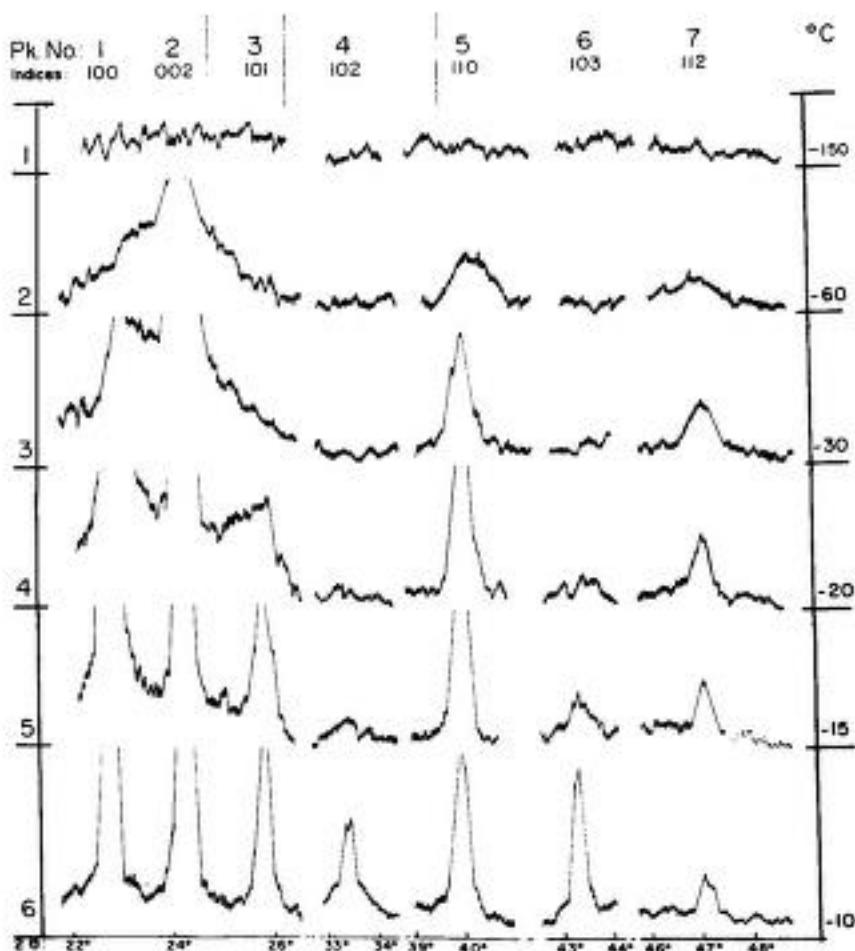
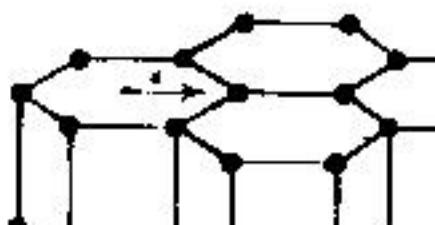
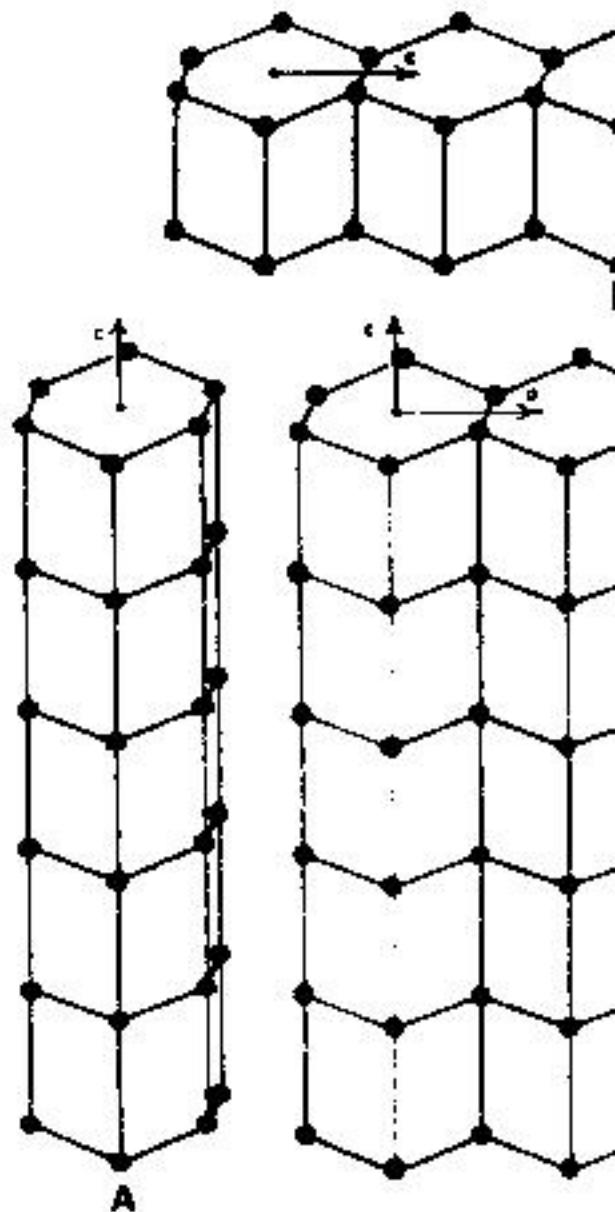


FIGURE 5. X-ray diffraction diagrams of ice in gelatin gels of 50% concentration frozen in thin layers by immersion in baths at the temperatures indicated on the right side of each diagram. — The order numbers of the angles, or peaks, are given in the top line, the indices on the second line, the order numbers of the tracings in the column at left, the freezing temperatures in the column at right, and the scale of two-theta angles underneath the tracings. — Tracing 1: No evidence of any rise. Tracing 2: Inverted V at Angle 2 and humps at Angles 5 and 7. Tracing 3: Inverted V at Angle 1, spikes at 2 and 5, inverted V at 7. Tracing 4: High spikes at Angles 2, 5 and 1, and inverted V at 3. Tracing 5: Peak at Angle 3, and elevation or inverted V at 4 and 6. Tracing 6: Tall peaks at Angles 2, 5, 1, 3 and 6. (Reproduced from Luyet, Tanner & Rapatz. 1962, with permission of *Biodynamics*.)

Luyet: Phase



The authors note that the *gradual emergence of Peak 1*, which does not bely favor of the theory of cubic ice. (original paper.)

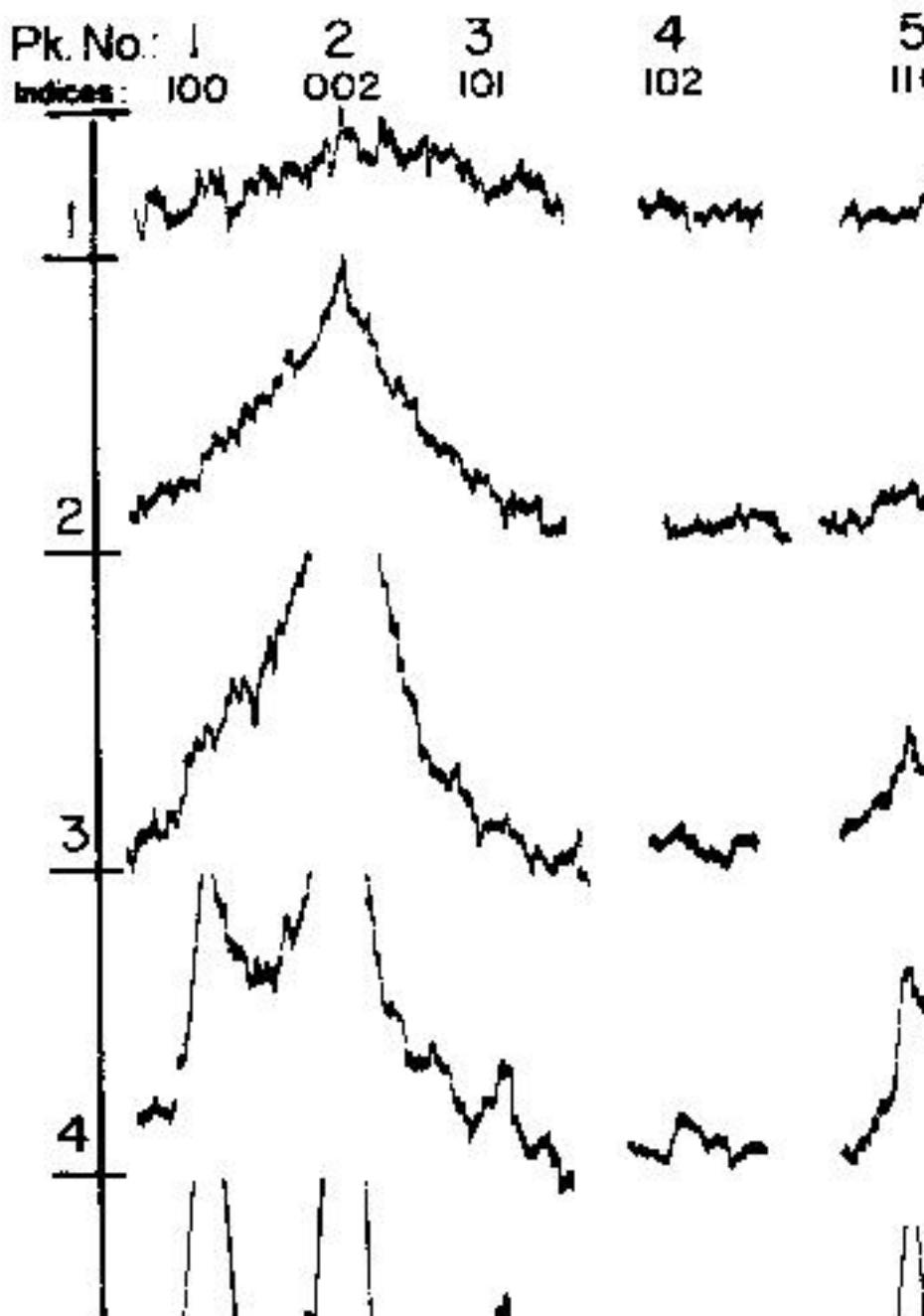
On the whole, if the interpretation is correct, rapid cooling would him ment in some preferred directions.

Effect of rapid cooling in limiting titative determination of the degree by rapid cooling has been attempte A microdilatometer method (in whic ters) gave the amounts of ice form pyrrolidone (PVP) and, by differen to freeze, when the cooling rate (degrees per second.

The results, plotted in the curves the freezable water which remain creases from small values at PVP 50 at a concentration of 35 per cent is maximum, and decreases to small increases to 45 per cent.

The question arises then of the c

Luyet: Phase



interrupted or inhibited crystalline category designated by the physicists of opacity is rather abrupt, when range, we called the phenomenon "

This type of recrystallization is c illustrates well the process; but one from other forms of recrystallizatio

(b) General form of recrystalliza attention from the physicists consider in a population at the expense of s them as "migratory recrystallizati molecules from the small to the large caused by the difference in surface particles. The rate of the migration the process is particularly noticeable point, where one can actually see masses grow larger.

The same principle controls the the size below which crystals are higher rate than that at which the

The principal characteristic of rate of crystalline growth increase

Luyet: Phase

TABLE
TEMPERATURES OF "IRRUPTIVE RECRYSTALLIZATION"
AQUEOUS SOLUTIONS

Solute:	Temp. °C.
Hemoglobin	- 3.5
Soluble starch	- 5.0
Bovine albumin	- 5.3
Dextrin	- 9.9
Gelatin	-11.5
Polyvinyl pyrrolidone	-14.5

The temperatures recorded are those at which the solution becomes intensely opaque in one minute. With the exception of gelatin, the temperature-time curve is practically as follows:

Nature of the phase transitions to the older concept, as was already a devitrification. I mentioned obse it is a recrystallization. But the exclusive. There is the possibility a devitrification, followed by a ch other. This is what would happen Dowell, Moline and Rinfret (1960) they obtained during the *rewarm* diagrams, which are quite similar very rapidly frozen water, were in during rewarming, from vitreous cubic to hexagonal ice.

The x-ray diagrams that we obt gelatin gel was rewarmed gradu as those obtained by Dowell, Mol evidence for a *gradual* rise in the peratures and for the *intrusion* of which they do not belong. The stri

upon rewarming and of those obtained upon rapid freezing to the same temperatures suggests that they result from the same process. So, it appears that here again, rewarming merely permits the completion of the crystallization which has been hindered and interrupted in the course of rapid freezing. One should note that the greatest change takes place in the recrystallization range, at -10° in the case of a gelatin solution.

Recrystallization during rapid freezing (spontaneous recrystallization). Since rapid cooling results in the formation of spherulites, that is, in a crystallization, it involves a release of latent heat during the course of cooling. The heat released is sufficient, in some places, to raise the temperature locally, and so, to bring about recrystallization. This we call spontaneous recrystallization during cooling, and distinguish it from recrystallization induced by rewarming. The phenomenon is illustrated in FIGURE 9, in which one sees (Photo. 1) areas that became opaque as a result of a recrystallization brought about by the heat released at the meeting points of several neighbor spherulites. When the freezing temperature is closer to the recrystallization temperature, the opaque borderlines between spherulites are broader (Photo. 2). Electron micrographs of specimens cooled very rapidly and freeze-dried show clouds of spontaneous recrystallization along the border lines of the evanescent spherulites (white specks

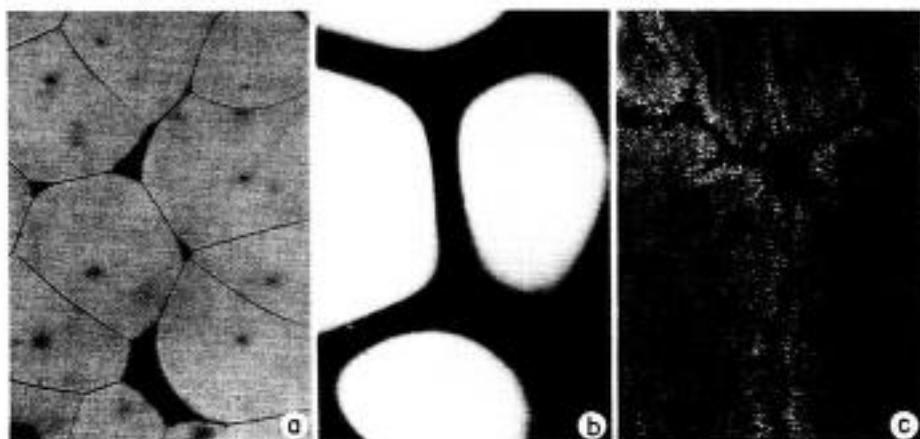


FIGURE 9. Spontaneous recrystallization. (a) Recrystallization clouds (dark areas) formed at the meeting of several adjacent spherulites. $\times 51$. (b) Recrystallization clouds covering larger areas when the temperature of freezing is closer to the recrystallization temperature. (The white areas are evanescent spherulites.) $\times 51$. (c) Electron micrograph showing recrystallization clouds (white specks) formed on the borderlines between adjacent spherulites. $\times 4,860$. a: reproduced from Luyet & Rapatz, 1958, with permission of *Biodynamica*; c: reproduced from paper in press by MacKenzie & Luyet, with permission of *Biodynamica*.)

in electron micrograph 3 of FIGURE 3, the probable universality of spontaneous

(C) *Overall Picture of the Process*

General considerations. The precise nature of the phase transitions encountered during the process of slow cooling and subsequent rewarming are fundamental to an understanding of the process. It is believed that they take place in slow freezing and rewarming processes, the former being merely to hinder some of the latter, and the latter being to permit the completion of the desired stages.

The transitions involve three main steps: (1) the formation of nuclei, (2) the growth of nuclei to a certain size, and (3) the growth of nuclei to larger sizes and, if that growth has been completed, the final stage. The first two steps constitute crystallization, and the third step, according to the principle, one or two of the three steps may be omitted. In the case of recrystallization, all three steps may be omitted.

Variations in cooling rates involve variations in the rate of nucleation, which in turn, controls the rate of growth of nuclei. It is of interest to note that the above brief summary of the process of crystallization and recrystallization briefly some of the problems involved in the process. The situations in which these two notions are related to each other, and the overall relationships.

Luyet: Phase

amorphous or semicrystalline state. This is due to hindering the motion of the water molecules by the presence of molecules of solutes which

One of the manifestations of the concentration effect is the delay in the appearance of crystalline structures when concentrated solutions are cooled to very low temperatures. The magnitude of that delay when the temperature is raised from -10° to -100° at a concentration higher than 50 per cent (Luyet, 1959) causes the crystallization to take place over a period of several days) unless the temperature is lowered again to -10°. This (Luyet, 1959) causes the crystallization to take place over a period of several days) unless the temperature is lowered again to -10°. This is due to hindering the motion of the water molecules by the presence of molecules of solutes which

Equilibrium versus nonequilibrium transitions encountered in the rapid freezing of aqueous systems may be considered as a particular case in the study of the relationships between the two factors, concentration and the occurrence of phase

substance. (Studies of such overall solutions of three other substances

From the point of view of the extinctions in gelatin gels, there are four ranges to consider: C₁: up to 20 per cent; C₂: 20 to 50 per cent; C₃: 50 to 65 per cent; C₄: above 65 per cent.

Most of what has been said in the literature has referred to Range C₁, which is encountered during rapid cooling or during slow cooling in which one obtains evanescent structures which disappear on warming to -10°. What has been said applies to all ranges, including, in particular, the limitations of the extinction method which applies typically to this range.

In Range C₂, the same general picture is observed as was reported above, instead of rapidly growing crystalline balls which disappear on warming until the temperature is raised to a value at which they attain microscopic or macroscopic dimensions, these crystalline balls undergo recrystallization.

In Range C₃, there is no evidence of extinction, no matter what the cooling rate is, provided that it is slow enough on warming. Apparently the material is completely amorphous.

In Range C₄ (dilute solutions), complete extinction is observed on cooling, but disappears on warming.

Luyet: Phase T

- LUYET, B. & G. RAPATZ. 1957. Devitrification of ice in glycerol-water mixtures. *Biodynamica* 2: 342.
- LUYET, B. & G. RAPATZ. 1958. Patterns of ice formation in rapidly frozen gelatin solutions. *Biodynamica* 8: 1-68.
- LUYET, B., C. KROENER & G. RAPATZ. 1958. X-ray analysis of rapidly frozen gelatin solutions. *Biodynamica* 8: 69-72.
- MACKENZIE, A. P. & B. J. LUYET. 1962. X-ray analysis of rapidly frozen gelatin solutions. *Biodynamica* 12: 1-12.
- MERYMAN, H. T. 1958. X-ray analysis of rapidly frozen gelatin solutions. *Biodynamica* 8: 69-72.
- MORAN, T. 1926. The freezing of gelatine gels. *J. Phys. Chem.* 30: 101-106.
- PERSIDSKY, M. & B. LUYET. 1959. Low-temperature X-ray analysis of rapidly frozen gelatin gels and its relationship to concentration. *Biodynamica* 9: 1-12.
- RANDALL, J. T. 1934. The Diffraction of Light by Liquids. *Trans. Faraday Soc.* 30: 101-106.
- SCHULZ, R. E. 1955. The Structure of Gels. *Advances in Colloid Science* 1: 1-12.
- RAPATZ, G. & B. LUYET. 1959. Recrystallization of ice in gelatin gels subjected to various temperatures. *Biodynamica* 9: 85-106.
- TAMMANN, G. 1898. Ueber die Abhaengigkeit der Viscositat verschiedener untermuehlten Fluessigkeiten. *Physikalische Chemie* 25: 441-479.
- ZACHARIASEN, W. H. 1932. The atomic arrangement in ice. *J. Phys. Chem.* 54: 3841-3851.

FACTORS AFFECTING THE FORMATION OF ICE IN THE FREEZER

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American Foundation for E

INTRODUCTION

During the past 25 years, freezing has assumed increasing importance as means for preserving foodstuffs and pharmaceutical systems. Early developments, summarized by us,¹ were followed by steady progress reported and discussed at international meetings²⁻⁵ and in several texts.⁶⁻¹⁰ There has been a marked increase in the number of successful applications of freezing, both in the theoretical aspects of the process and in the advances in practical procedure to date.

It is at once apparent, however, that the design of apparatus have resulted in different types of results in terms of vapor flow from specimen to the system,⁷⁻¹⁰ analysis has not been made of the various stages in the formation of the processes taking place, and it has been argued that it is the latter process

MacKenzie: Freeze

from the specimen, have thus supposed mation of ice, followed by passage of necting directly with the sample surface unfrozen water into channels left empty enon discussed in considerable detail [1].

The inadequacy, *in certain cases*, of drying was first apparent in results of even more strongly apparent to the p various experiments which are best su order. *Firstly*, it was observed, with the microbalance,¹¹ that the rate at which a given size freeze-dries, at a given temperature, depends on the amount of the solute present. This point is, perhaps, its significance appears often to have been again with the aid of the microbalance, frequently not determined by the resistance to narrowness of channels vacated by the sublimation. The velocity of sublimation is not always primarily determined by the size of the channels; for example, 30 per cent polyvinyl pyrrolidone and thus, presumably, channels with diameters about 100 microns, respectively, freez

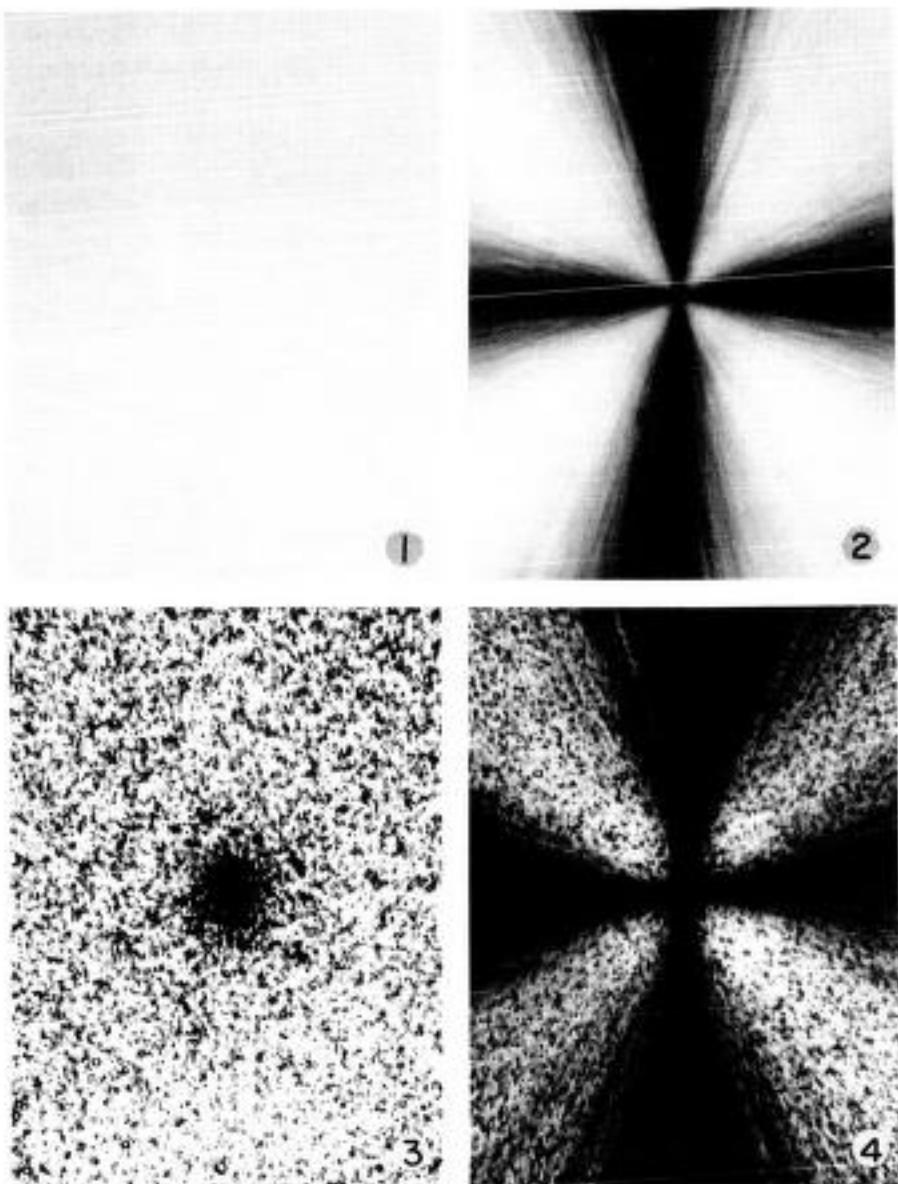


FIGURE 1. Effect of rewarming on a gelatin solution frozen very rapidly by abrupt immersion in a cooling bath at -50°C . Concentration: 30%. Sample thickness: ca. 20 microns. Photos. 1 and 2: before rewarming (sample transparent to naked eye); photos 3 and 4: after rewarming to -10°C . for 10 minutes (sample white and almost opaque to naked eye). Photos 1 and 3: ordinary light; photos 2 and 4: polarized light, $\times 75$.

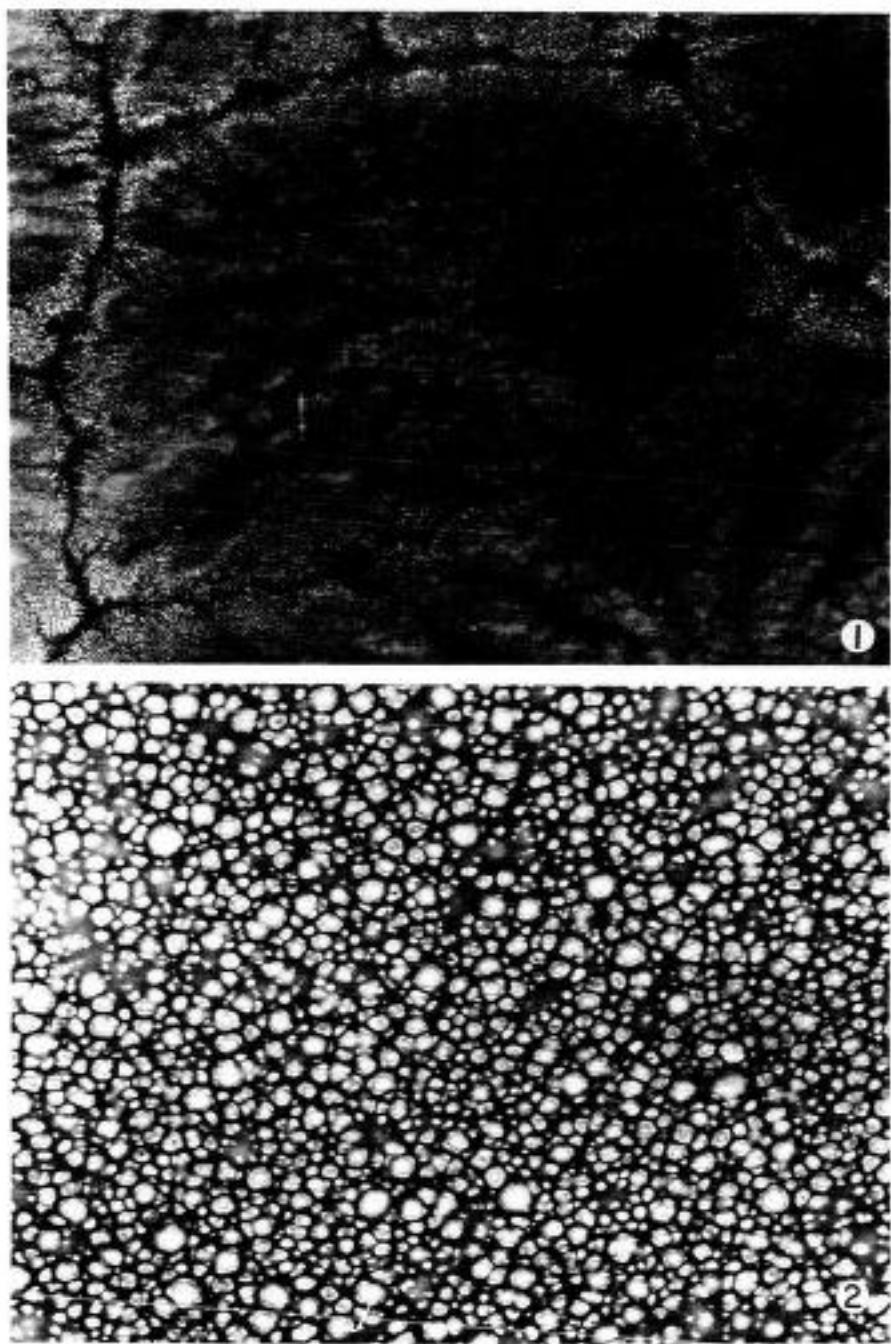


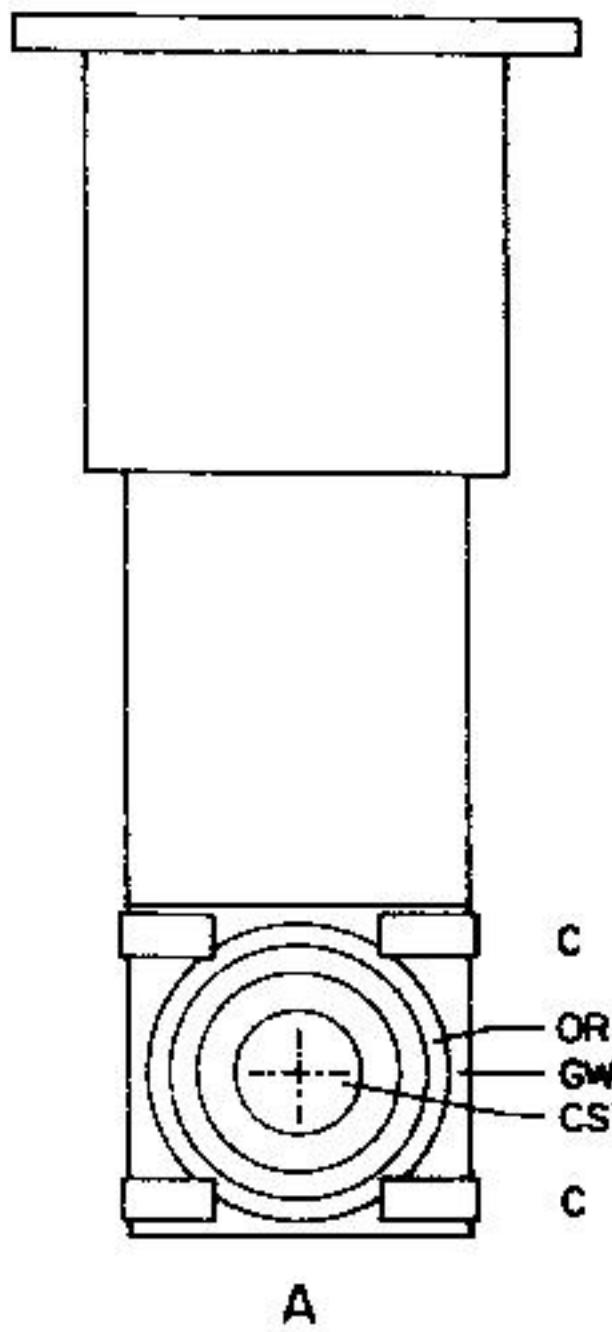
FIGURE 2. Electron micrographs illustrating effect of rewarming on very thin layers of gelatin solution frozen by sudden immersion in isopentane at -78°C . Concentration: ca. 25%. Sample thickness: ca. 1/4 micron (collodion sandwich film technique). Photo. 1: before rewarming; photo 2: after rewarming to -6°C , for 30 minutes. Note that recrystallization upon rewarming has occurred to an extent sufficient to eliminate all evidence of initial freezing pattern. Approx. $\times 3,300$.

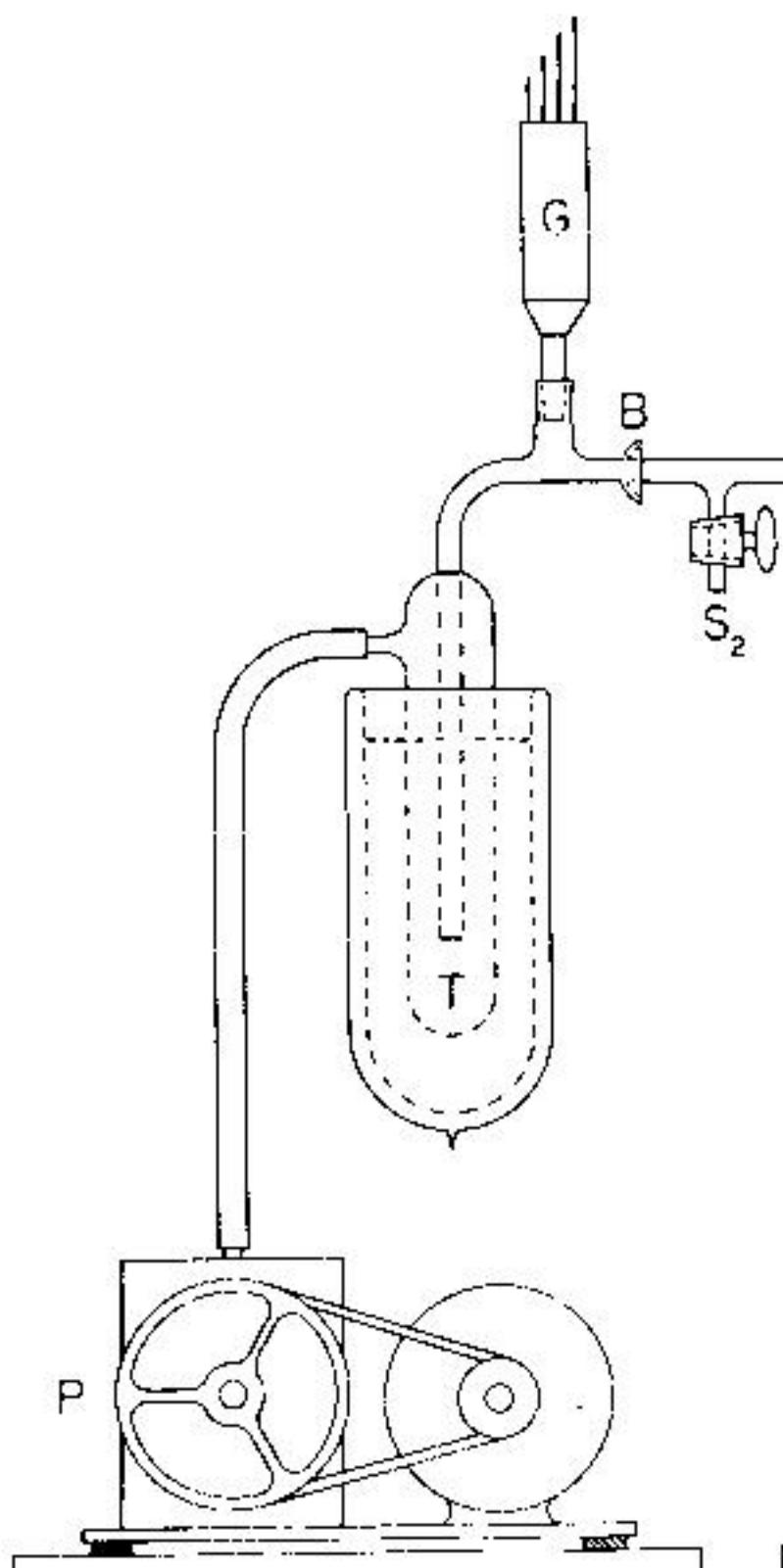
are commonly encountered in materials distinguished from cracks formed by freezing. The variation in the extent of secondary formation of cracks does not appear, however, to have considerable bearing on a discussion of the behavior of a freeze-drying material.

Lastly, several sugar solutions undergo complete loss of structure, at -30°C . (the temperature at which ice crystals begin to melt during warming). That is, freeze-drying, preserves the solute framework and the final product devoid of cavities. Such results are in agreement with those of other experiments in which sugar was dried at lower temperatures, when compared with the results obtained by preservation by freeze-drying.

These results, diverse in nature, are in accordance with the evidence of variations of the accepted theory of diffusion. In particular, to demonstrate in some materials homogeneous barriers to movement of molecules, it was found that the narrowest real channels were not necessarily the most effective. The factual evidence was most apparent in the case of sucrose, where a new method of analysis was now under way. A versatile form of sucrose, which could be dried without loss of structure, was obtained by

MacKenzie: Freeze-





MacKenzie: Freeze-

of freeze-drying of the specimen can chamber (FIGURE 3) takes the form of a dows on its two main faces and a flange in position in the apparatus) for connec mal contact between the specimen and of which is regulated to $\pm 0.1^{\circ}\text{C}.$, or be glass windows.

Samples may be mounted between coverslip and one of the chamber windows by the rate at which the specimen is required — and the method of assembly where slow freezing is to be employed, t assembled at room temperature. The lowered and freezing is initiated, if nec rod. Specimens may be further cooled or drying commenced by opening the stop drying chamber/condenser assembly and the condenser with dry ice or liquid nitro

Freeze-drying takes place in directio of the microscope and is most convenie tography or by cinematography. One c perature during the course of freeze-d

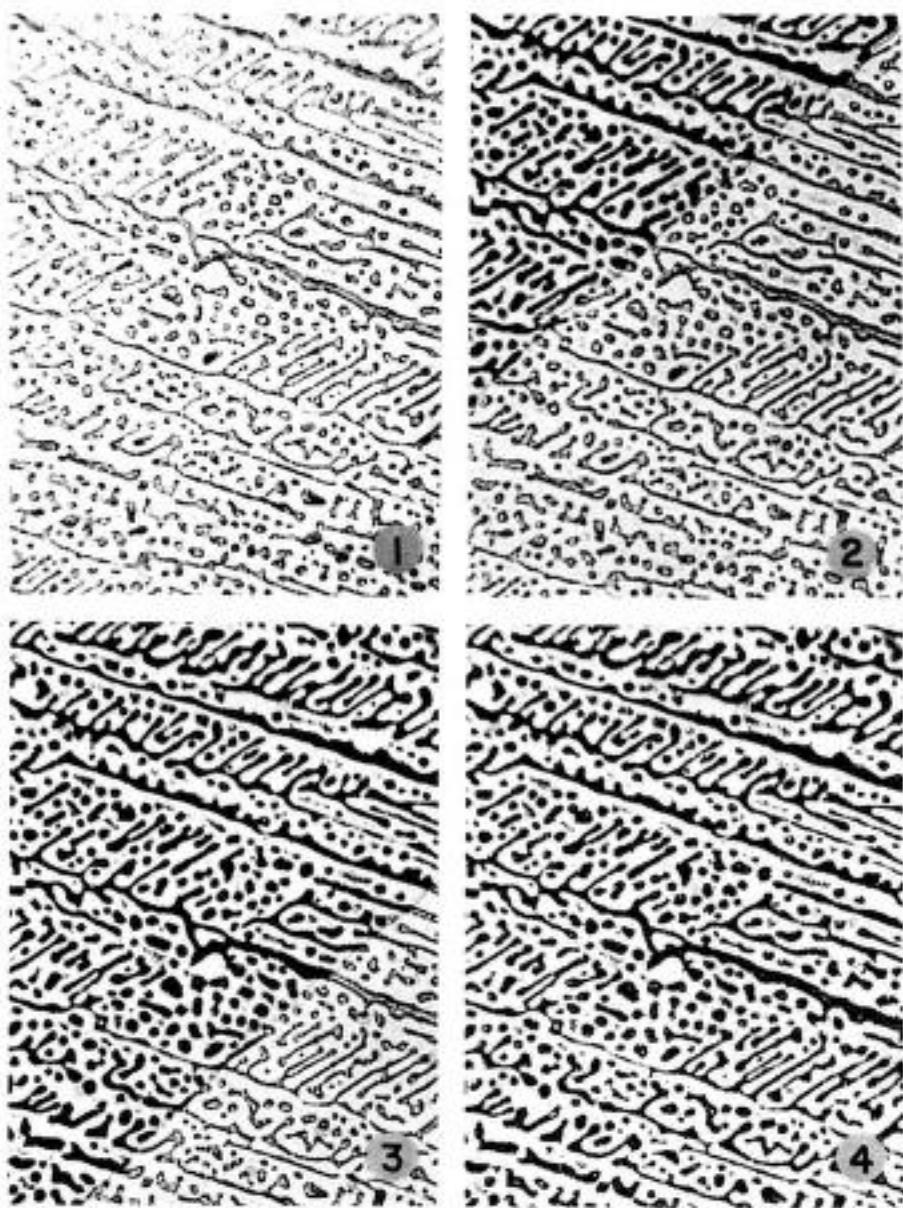


FIGURE 5. Sequence showing freeze-drying by direct sublimation of ice from a slowly frozen KCl solution. Water vapor travels via spaces between salt crystals. Concentration: 10%. Sample thickness: ca. 10 microns. Freeze-drying temperature: -30°C. Photo. 1: before freeze-drying; photos. 2 and 3: during freeze-drying; photo. 4: after completion of freeze-drying. Magnification: approx. $\times 300$.

MacKenzie: Freeze-Drying

apparently unchanged, in the pattern it follows during freezing. There is no contraction of size or cracking — frozen solutions of hydrate-free solutes can be freeze-dried, however, by sublimation without loss of water or efflorescence of the hydrated crystals.

Dilute solutions (up to several per cent) of non-ionizing solutes, of low and of high molecular weight, can be freeze-dry by this mechanism but only if the solutes are completely soluble in the ice (conditions of very little or no supercooling). It is noteworthy that such conditions of complete solubility are frequently encountered in the practice of freeze-drying. However, another mechanism for the removal of water from solutions freeze-dry is described in the third section.

2. Water vapor escaping through the boundary layer. The physical structure of many frozen solutions is altered by rewarming treatments in such a way that the ice crystals undergo complete disintegration, leaving small, irregular particles, each distinctly separate from the others^{12,13} (see FIGURES 1 and 2). Solutions containing gelatin, so treated, subjected to freeze-



FIGURE 6. Successive stages in freeze-drying by transport of water molecules through the concentrated solute phase present in a frozen polyvinylpyrrolidone solution. The sample was frozen at -40°C and recrystallized by rewarming to -10°C , for 4 hours prior to freeze-drying. Concentration: 50%. Sample thickness: ca. 20 microns. Freeze-drying temperature: -30°C . Freeze-drying occurs from upper left to lower right in each case. Note the progression of the ice/void interface through the cavities marked by arrows. Approx. $\times 300$.

try of the sample. With the flat samples of the type necessary in the freeze-drying microscope, cracking during freeze-drying has been recorded in the cases of several different solutes, the most marked fissures being seen in materials of high molecular weight. FIGURE 7 shows a sequence representing development of cracks in the freeze-drying at -30°C . of a salt-free solution of recrystallized ovalbumin, 30 per cent w/w, rapidly frozen (note how cracks are formed close behind the freeze-drying front). Similar cracking is observed in freeze-drying of this solution at -40° and at -20°C . Dextran solutions behave in some cases in a similar way and one deduces that very little secondary drying is necessary in those cases to cause crack formation. Solutions of sucrose and of polyvinyl pyrrolidone, on the other hand, were observed to crack upon prolonged freeze-drying at low temperatures but cracks extended only a small fraction of the way from the edge of the preparation to the interface where sublimation of ice was occurring.

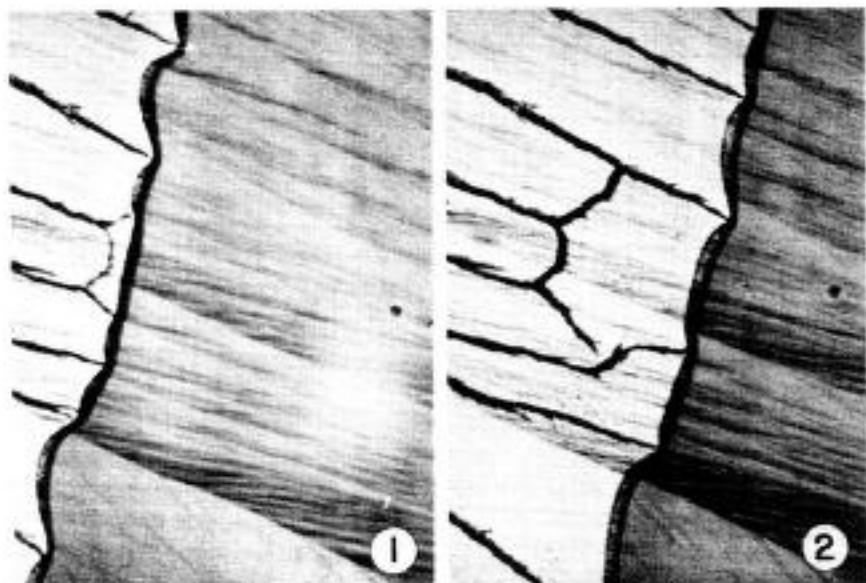


FIGURE 7. Photographs illustrating the formation of cracks in the freeze-dried portion of a rapidly frozen salt-free albumin solution. Concentration: 30%. Sample thickness: ca. 20 microns. Freeze-drying temperature: -30°C . Note the deeper penetration of the front at points ahead of the fissures. Note also the increase in crack width at any point with progression of secondary drying. Magnification: approx. $\times 270$.

It is evident from the distribution both via channels left by sublimation resolved in FIGURE 7) and by fissures of the freeze-drying front it is resistance to vapor flow than do the

One may also note, in passing, that if one cracks a specimen; Stephenson has shown this diagrammatic form in his well known paper.²⁰ The further possibility exists that if one allows the first parts to freeze-dry, then the cracks formed by freezing greatly facilitate the formation of conduits as drying proceeds.

4. *Water vapor escaping via cracks*
ice is present only as distinct and
other process. FIGURE 8 shows a
of 50 per cent salt-free, crystallin-
lized by rewarming. In this case,
extremely readily, causes cracks
ice containing cavity from another
cavity, secondary drying of the
place; cracks form in the wall, ice
and water vapor passes through

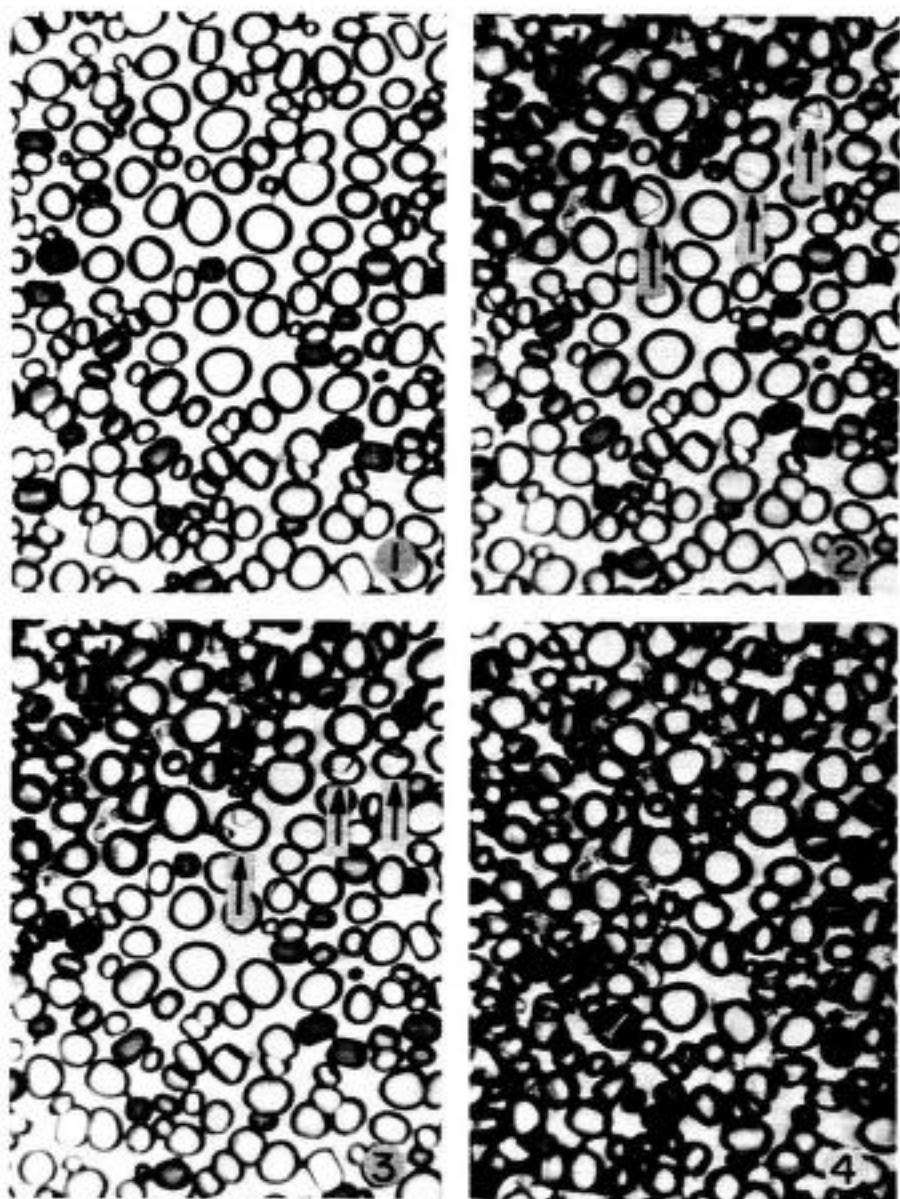


FIGURE 8. Sequence showing how crack formation may facilitate freeze-drying of a system containing many discrete ice particles isolated one from another by concentrated solute. Sample: salt-free ovalbumin frozen rapidly, rewarmed to -1°C . for 1 hour, and cooled slowly thereafter to -30°C . Concentration: 50%. Sample thickness: ca. 20 microns. Freeze-drying temperature: -30°C . Photo. 1: before freeze-drying; photos. 2 and 3: during freeze-drying; photo. 4: after completion of freeze-drying. Photographed in polarized light. Note the cracks (dark lines) which interconnect cavities in the freeze-dried material. Note also the ice/void interface on each subliming ice crystal (see arrows). Approx. $\times 210$.



FIGURE 9. Micrographs illustrating "freeze-drying" resulting in total collapse of the solute matrix. Sample: polyvinylpyrrolidone solution, frozen at -40° and recrystallized by rewarming to $-10^{\circ}\text{ C}.$ for 4 hours. Concentration: 50%. Sample thickness: ca. 20 microns. Freeze-drying temperature: -25° (photo 1), $-20^{\circ}\text{ C}.$ (photos 2, 3, and 4). Freeze-drying progresses in each photograph from lower left to upper right. The area under the dotted line in photo 1 represents the portion freeze-dried at -25° . Note how freeze-drying at -25° leads to preservation of frozen structure while freeze-drying at -20° causes its total disappearance. Note also that warming to -20° following a period of freeze-drying at -25° does not result in disappearance of structure in the portion freeze-dried prior to warming. Approx. $\times 183$.

on the nature of the solute but not on its concentration (within the range 10 to 50 per cent w/w) and only to vary with freezing rates when the latter are extremely high. Similar changes in mechanism have been observed at about -30° and -40°C., more than 20 and 30 degrees lower than the freezing points of the solutions, in the cases of sucrose and glucose respectively. Note also that in every case salts were absent.

6. Behavior of salts close to eutectic temperature. FIGURE 10 shows the freeze-drying of a 15 per cent KCl solution maintained at -30°, -20°, -15°, -13° and -20°C. in succession, the bands in the freeze-dried portion denoting freeze-drying at the temperatures recorded and reproduced in FIGURE 11. One sees that sublimation of ice from a mixture of ice and salt crystals takes place at -30° and at -20°C. according to the first of the mechanisms just described. At higher temperatures, however (-15° and -13°), a growth of salt crystals accompanies the sublimation of ice (despite the slight and very localized evaporative cooling which must occur in the interface). That it is freeze-drying, and not rewarming of the frozen material, which brings about the growth of salt crystals is seen by reference to the appearance of the portion remaining to be freeze-dried in FIGURE 10, which resembles very closely the structure of the portions freeze-dried at -30° and -20°C. Further evidence for this conclusion is provided by the appearance of the band freeze-dried at -20° following exposure to -13°. One may also note that an increase in temperature of the entire preparation from -11.5° to -11°C. caused eutectic melting of the frozen portions. The matter of the behavior of salts at temperatures close to their respective eutectic temperatures is perhaps of some importance in relation to their probable sub-microscopic behavior upon freeze-drying at considerably lower temperatures.

DISCUSSION

This section will consist of the following parts: (1) An examination, in terms of water forms and the properties of solute molecules, of the basic processes likely to occur in freeze-drying, depending on conditions. (2) An evaluation of the effect of experimental factors in determining the observed mechanisms. Some points of practical significance arising from the findings will then be discussed separately in an Appendix.

1. Basic Processes

The following list is based on the fact that water must exist in one of two forms in frozen biological materials, that is, as ice or as water which, for one reason or another, does not freeze (water in the latter form is often described as "bound water" but it is most important to remember that the freezing and post-freezing treatments frequently determine the quantity of water which does not freeze and that a distribution or "spectrum" of

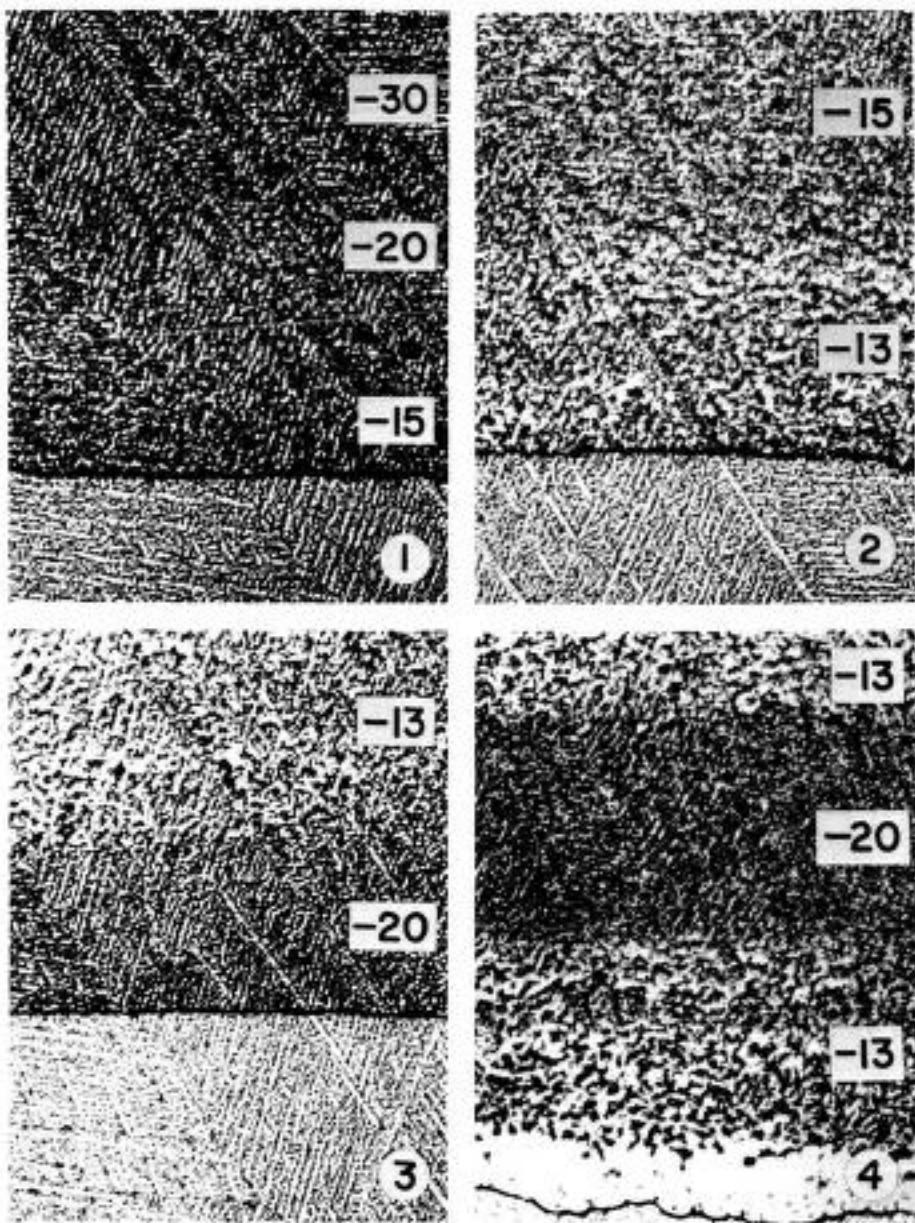


FIGURE 10. Sequence illustrating behavior of a KCl solution freeze-dried at temperatures close to the eutectic melting point. Concentration: 15%. Sample thickness: ca. 20 microns. Freeze-drying temperature: -30, -20, -15, -13, -20, and -13; in succession (see FIGURE 11). Photo. 1: freeze-drying at -30, -20, and -15; photo. 2: freeze-drying at -15 and -13; photo. 3: freeze-drying at -13 and -20; photo. 4: freeze-drying at -13, -20, and -13, followed by warming to -9°C. The portion not freeze-dried underwent eutectic melting at -11°C. Approx. $\times 200$.

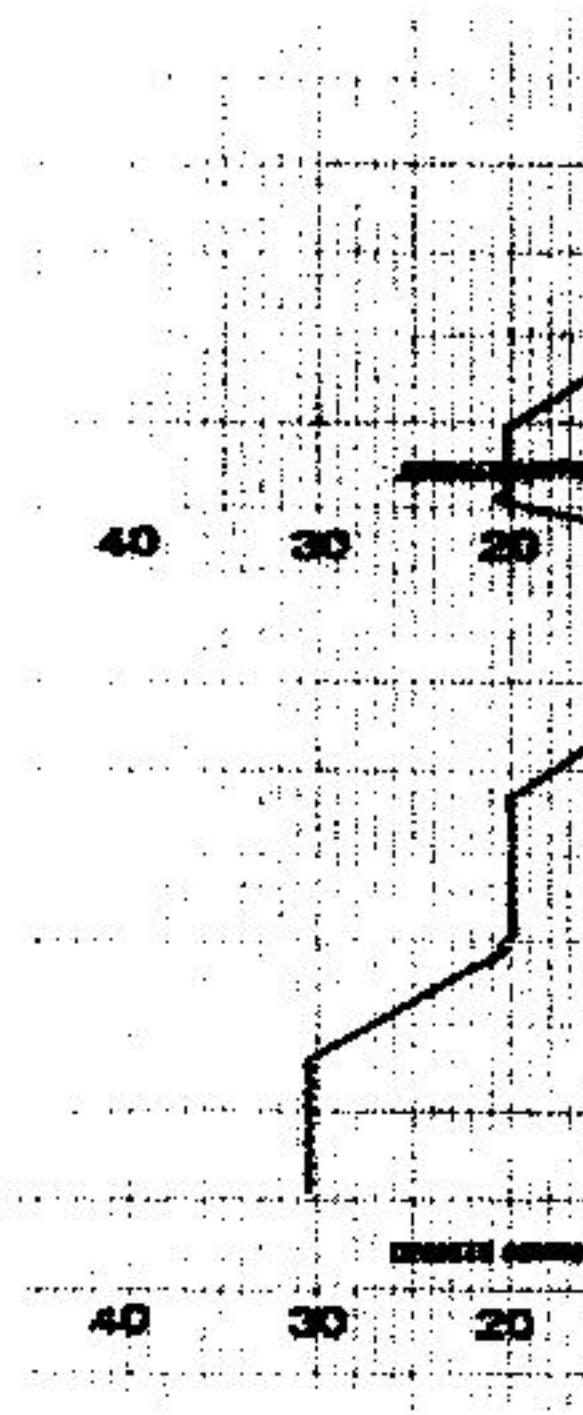


FIGURE 11. Temperature/time record illustrating freeze drying experiments conducted to determine optimum conditions.

observations and those reported earlier. These facts will suffice to show the way in which the two may interact to produce a given behavior.

First among the six mechanisms
which, according to the convention,
the one in which the sublimation of
Observations of various solutions
first place through channels vacat
surface and, in the later stages, al
Such a description will be but sl
recourse to the list of basic proces
to consist of the three simultaneou
water, conversion of internally to
of the latter; formation of crack
shrinkage of the solute and will
also the formation of extra solute
mechanism of initiation of the cr
of each basic process is essential to
form of freeze-drying, the most imp
perhaps, that sublimation of ice
begins.

The second mechanism. By con-

MacKenzie: Freeze-Drying

is, on the one hand, the direct transport through spaces from which ice has just desorbed; externally bound water and internally bound water lead, via molecular solute bonding and shrinkage that grossly alter the size of the solute phase. Cracks are then initiated at the surface of the system. In rapidly frozen ovalbumin, for example, during freeze-drying, cracks easily and shrinks considerably. The cracks formed may be much wider than the ice crystals themselves. Liquid water will thus flow much more easily through the channels vacated by ice and freeze-drying. This is due, in part, to the presence of the cracks, a fact which is important in determining the final dimensions of the shape of the freeze-dried product. The process extends inwards into the sample. Freeze-drying is a process which is dependent, but only for its increased velocity, on the rate of secondary drying.

The fourth of the reported mechanism of freeze-drying is that the sublimation of ice can take place only if the water molecules can escape before the sublimation of ice can take place. If this were true, freeze-drying would have to proceed at a much slower rate than it does. The ready formation of cracks in some samples, however, where none exist, suggests that this mechanism is not the dominant factor in freeze-drying.

through the latter, etc. Since ice crystals on the surface before freeze-drying are quite large, they diminish in size, direct sublimation, for example, is eliminated. In such conditions it would be more appropriate to speak of the "dissolution" of ice rather than of its sublimation and it may even be called "freeze-drying." Alternately,

Sixth, and last, of the reported cases of salt solutions freeze-dried at temperatures below their eutectic points (electron microscopy) the phenomenon may occur at temperatures above the eutectic point. Sublimation of ice is undoubtedly the mechanism responsible for the formation of ice crystals upon freeze-drying (FIGURE 1). It is also likely that there is a tendency towards a reduction in the size of the ice crystals. While the most likely mechanism is dissolution of the solute from the solution via a thin surface film on the ice, it is also possible that the solute remains below the eutectic temperature resulting in a reduction in the size of the ice crystals.

2. Dependence of Mechanism

It is now logical to ask what factors influence the geometric distribution of ice crystals and the tendency to crack upon dehydration, and whether or not the mechanism of

MacKenzie: Freeze-

of the solute to flow is determined principally by the temperature of the bath and the freeze-drying temperature though other factors controlling the distribution of ice may also have an effect. In particular, a recrystallization of the solute may be used to raise the threshold temperature, probably by about 10° C., for the solute. (4) Recrystallization of salts may depend upon freeze-drying temperature but the mechanism is almost uninvestigated. It is only possible to say that the simultaneous presence of non-crystallizing solutes in small amounts tends to inhibit the phenomenon.

Much further fundamental research is required to catalogue the behavior of solutions. It is particularly important to study the behavior of solutes, some of which have already been studied by infrared analysis and by conductivity measurements. The results obtained by these methods and also from the more sophisticated microscopic, electron microscopic and X-ray methods have, however, suggested that studies of simplified systems will not be sufficient. It will be necessary to study the more elaborate cellular structures. The freeze-drying of the latter for various purposes, particularly the drying of organized structures will be greatly simplified by the use of the technique of *in vitro* experiments.

1. Chances of attaining specific conditions.

It is apparent from the foregoing that, given a system containing solutes, certain ranges of water content, concentrations of solutes, temperatures, the number of possible conditions is limited. It is therefore of interest to consider the relative likelihood that experimental objects will attain specific conditions.

For instance, the freeze-drying process can be considered in terms of preservation of ultrastructural and functional integrity. In such a system, an extremely rapid initial freezing will result in many very small isolated ice crystals. The rate of crystallization will be limited by the rate of diffusion of water molecules into the matrix. If the rate of diffusion is slow enough, crystallization upon cooling will be rapid. In such a system, freeze-dried at the very low temperature of -70°C., it will be necessary for preventing recrystallization to proceed either by transport of water molecules through the matrix, which will be a very slow process, or by formation of fissures, if they are formed. The latter will be a source of artifacts. A series of artifacts will have been introduced into the system by the freezing process. The temperatures where solute flow occurs must obviously be avoided on account of the resulting artifacts.

2. Some seemingly desirable features of freeze-drying.

Further and most important questions are:

MacKenzie: Freeze-

those in which one aims for exhaustive

(A) Greaves¹ has attributed the survival of bacteria to the ability of the suspending agent to protect the system during experimental conditions were supposed, by secondary drying to preserve the system from loss of essential water. Nei,² more recently, has shown that the survival of a suspension of *E. coli* in distilled water can be increased by 10 times if the experimental approach led to a limitation of secondary drying. This was achieved with success. The notion of limited drying must be considered from both theoretical and technical viewpoints. It is well known that complete hydration is essential to prevent enzymatic actions upon rearming freeze-dried products. It is also known, however, that over-drying may lead (a) to increased oxidative damage in particular, which may, for example, increase the rate of reactions, and (b) to irreversible destruction of membrane systems.^{30,31}

Different cell systems, however, depend on different protective agents, if any, used for freezing at different temperatures without loss of viability. For example, yeast cells can be freeze-dried above these temperatures by maintaining the sample at a given low temperature, e.g., -20°C., and for removing all the

sidered as a means for reducing species, even up to complete dryness that substances which protect against of little use in protecting cell suspensions, the need for a new search for effective protective agents.

The further possibility arises to reduce the rate of drying in the absence of protective agent by increasing the freeze-drying temperature when carrying out primary and secondary drying at extra low temperatures. This is such that solutes are most readily removed from the liquid phase (in contrast to present practice where the rate of removal is slow during secondary drying). Such an approach would be particularly valuable if capable of controlled, limited dehydration. It is not known whether the process and the doubt remains that the rate of removal of water during the dehydration will not disrupt membranes or other components of the system at the low drying temperature. Experiments are in progress and it is hoped to report the results shortly.

In summary, two freeze-drying processes have been developed and require careful examination.

1. Limited dehydration, based on the use of protective agents.
2. Complete dehydration at low temperatures, based on the use of protective agents and drying at very low temperatures.

MacKenzie: Freeze-

12. MENZ, L. J. & B. J. LUYET, 1965. Some observations on the effect of temperature on the freeze-drying of aqueous solutions. *Biodynamica*. In press.
13. NEI, T. 1964. Formation of cracks in biological preparations. *Biodynamics* 9: 214-220.
14. FERNANDEZ-MORAN, H. 1960. Low-temperature electron microscopy of biological specimens at temperatures near liquid helium II. *Ann. N. Y. Acad. Sci.* 92: 101-113.
15. NEI, T. 1962. Electron microscopic studies on freezing and drying: cinematographic observations. *Exptl. Cell Res.* 28: 560-575.
16. COLEMAN, J. W. 1962. A freezing technique giving self supporting specimens. Ed. by S. S. Breese Jr., Ed. Academic Press.
17. MACKENZIE, A. P. 1964. Apparatus for freeze-drying. *Biodynamica* 9: 214-220.
18. LUYET, B. J. & G. L. RAPATZ. 1957. Unpublished.
19. MACKENZIE, A. P. & B. J. LUYET. 1965. The recrystallization of ice in rapidly frozen aqueous solutions. *Biodynamica* 9: 221-232. In press.
20. STEPHENSON, J. L. 1953. Theory of ice formation. *Bull. Math. Biophys.* 15: 411-429.
21. LUYET, B. J. 1939. The devitrification of aqueous solutions. I. The hydrate series. *J. Phys. Chem.* 43: 88-94.
22. LUYET, B. J. 1957. On the growth of ice crystals. *Proc. Roy. Soc. B.* 147: 434-451.
23. MACKENZIE, A. P. & B. J. LUYET. 1965. The effect of temperature on the rate of ice formation in aqueous solutions. *Biodynamica* 9: 1-68.
24. LUYET, B. J. & G. L. RAPATZ. 1958. Patterns of ice formation in aqueous solutions. *Biodynamica* 8: 1-68.

SEPARATE EFFECTS OF DRYING L

R. I. N. Greav

The University Department of

Intr

Freezing, thawing and drying a
ing cells. Yet, if water is immobil
the cell metabolism ceases and, if i
be preserved indefinitely.

Organisms vary considerably in resistance to drying, but even with freeze drying is considered they are dried.

The discovery that glycerol and protected cells against injury by freezing. It was found to be preserved by freezing. Unfortunately it did not succeed in freeze-drying. However, it was found that other substances, such as glucose, lactose and sucrose, and

Greaves & Davies: I

A Kipp Micrograph Recorder was used to record the analysis of a system as a function of temperature, resistance and different reaction rates.

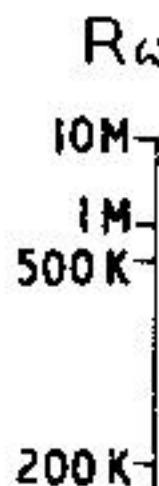
Results

FIGURE 1 records the analysis of a eutectic mixture. The arrows indicate the direction of change.

On cooling an exothermic reaction starts at 500°K. This is followed by crystallization of the eutectic mixture. This continues until the resistance rapidly rises to over 1 M ω .

On warming, this resistance does not return to its original value. It has reached when it suddenly falls to a very low value.

NaCl



perature shows an endothermic rise of the eutectic mixture.

The main points to observe from marked degree of supercooling than ment of the eutectic temperature on cooling and thawing of the eutectic mixture by differential thermal measurement.

FIGURE 2 shows a similar experiment with 0.9 per cent NaCl. On cooling, the resistance continues to rise slowly not reaching a maximum until the temperature is below -60° C. On warming, the resistance approximates the cooling curve, indicating no hysteresis on cooling. Differential thermal analysis shows no evidence of melting or freezing of a eutectic mixture.

Ten per cent DMSO in 0.9 per cent NaCl shows a similar behavior except that the resistance did not reach a maximum until the temperature was about -10° C. and did not reach 1 M_o, until the temperature was about -60° C. The water and the NaCl eutectic were also at the same temperature.

FIGURE 3 records an unique experiment with 0.9 per cent NaCl. On cooling the resistance

Greaves & Davies:

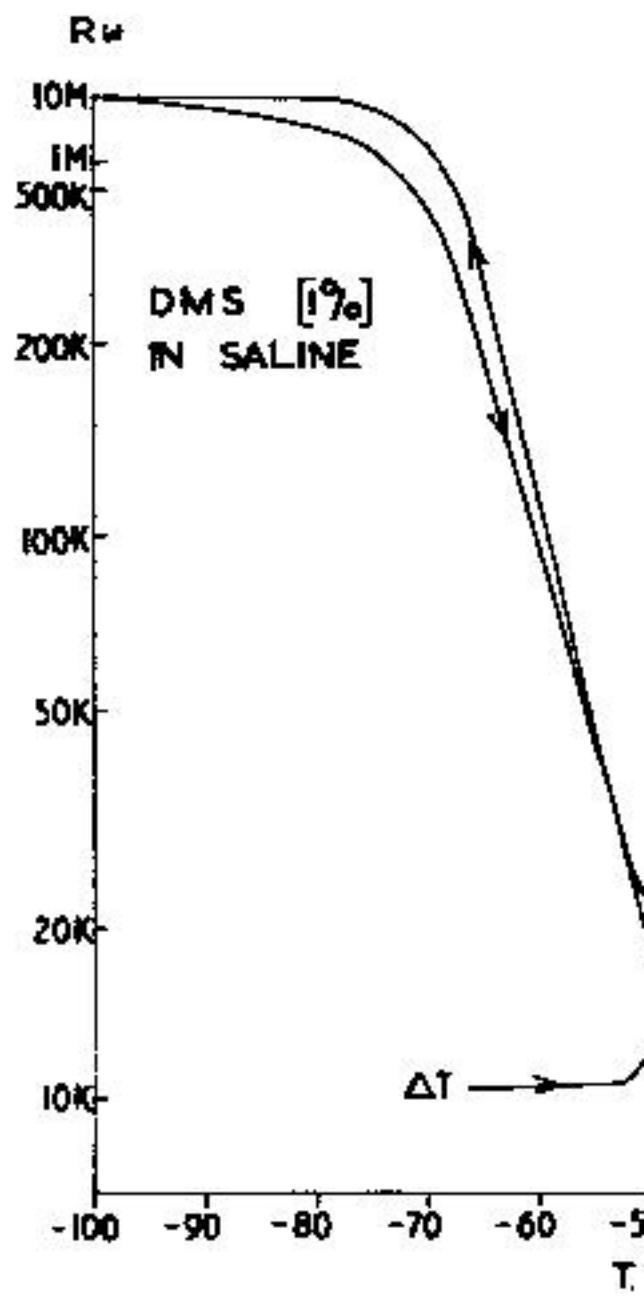


FIGURE 3. Thermal analysis of 1% DMS in saline. The graph shows the increase in resistance on thawing which occurs

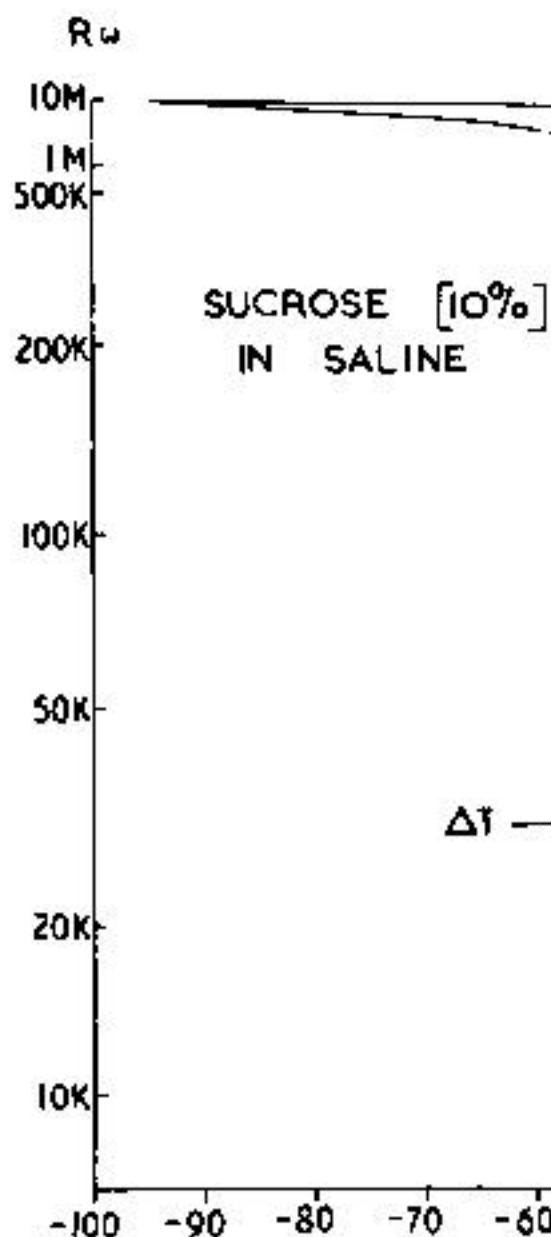


FIGURE 4. Thermal analysis of 10% sucrose in saline. The graph shows the relationship between the change in temperature (Δt) and the relaxation rate (R_ω). The y-axis is logarithmic, ranging from 10K to 10M. The x-axis ranges from -100 to -60. The curve shows a decrease in R_ω as Δt increases, indicating a change in the direction of change.

Greaves & Davies:

first instance and metastable in the second. The addition of the extract, however, failed to s

Conclusion

All the additives tested which are effective in preventing freezing injury prevent the formation of glasses. They appear to do this by producing a glass which becomes increasingly hardens as the temperature falls.

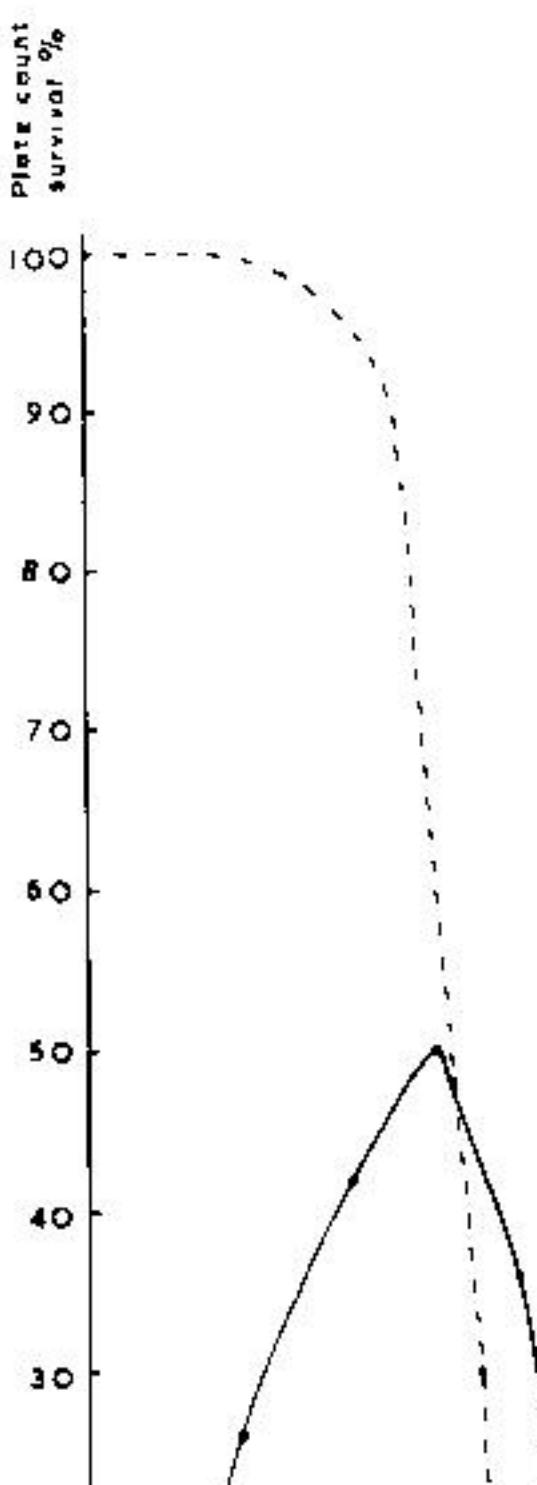
Glasses formed on slow freezing temperatures are metastable, the metastability being recorded.

The additives tested could be placed in the following order to form glasses and prevent eutectic coagulation: sucrose the most efficient, followed by glycerol, glucose, and in order.

FREEZE-DRIED GLASS

Preliminary experiments were carried out by Greaves & Davies on nylon gauze in an apparatus similar to that used by Hargreaves (1959). This apparatus relies on the use of a bell jar connected by a large air-ballasted pump. A calibrated stopcock and a manometer line enables various freezing rates and

FIGURE 5 presents the results of dry ice freezing of suspensions in one per cent bovine albumin. The



Greaves & Davies:

Description of

Thermoelectric refrigeration seemed to offer a simple solution to the problems and a two-stage 'Frigistor' refrigerator was mounted on a thick brass base plate which was bolted to the side. A recessed "O" ring in this plate gave a good seal to the top of the desiccator (FIGURE 6).

The desiccator chamber was connected to a vacuum system by a valve and a phosphorus pentoxide trap.

At maximum current the first stage reached -40°C., the second stage -55 to -60°C. Ampules of dry ice were placed in blocks on either stage.

There are several ways in which the apparatus can be used. For experimental purposes the ampules are placed in the trap and the dry ice placed in the trap is used as the drying agent. If the trap is placed between the drying chamber and the trap, it can be used to control the rate of the drying rate and consequently the temperature of the ampules. For drying the ampules are placed on the base plate and the trap is used as a refrigerated condenser at -60°C.

The temperature can be held constant by varying the current in the refrigerator or by reverting to the heating coil when the temperature has been heated to ambient temperature. In this way the temperature can be varied over a wide range.



FIGURE 6. Experimental drying apparatus. The two stage thermoelectric Frigistor refrigerator can be seen mounted on a solid brass base plate. The DC power supply is mounted below the desiccator chamber. The different types of aluminum blocks for holding tubes and ampules can also be seen.

Greaves & Davies:

TABLE
PERCENTAGE SURVIVAL IMMEDIATELY
DIFFERENT DRY

Suspending medium	Perce
	-12.5°C.
Distilled water	1
Glucose 7-1/2 %	71
Sucrose 12-1/2 %	63
Peptone 10 %	59
Na glutamate 5 %	31
PVP 10 %	4
Glucose 7-1/2 % /	

organism. The prefreezing rate and
and the temperature was not allow

These experiments would sugge
avoided the lower the drying tempe
gest that the effectiveness of a dry
tective effect it might have agains

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1. MAZUR, P. 1960. Physical factors
at subzero temperatures. *Ann. N. Y. Acad.* 92: 293-318.
2. MERYMAN, H. T. 1959. Survival
of insects at low temperatures. *J. Insect
Physiol.* 184: 470.
3. REY, L. R. 1960. Thermal analysis
of biological materials. *Proc. N. Y. Acad.
Sci.* 85: 510-534.

TRANSPORT PROPER

Alexander

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The introduction of isotopes in the study of membrane permeability has afforded information unobtainable by other measurement. At the same time the interpretation of the results was made more complicated. This complication is particularly great in the case of water. Without isotopes it was not possible to measure net water movement across a membrane permeability barrier, one could measure only the net movement of solutes. In the case of water movement experiments in which a hydrostatic pressure gradient was used for net transfers of water or from one compartment to another, a pressure gradient of a nonpenetrating or poorly penetrating solute across a membrane permeability barrier provided an equivalent driving force.

For a simple binary aqueous solution in which one component is permeable to the solute, these relations hold:

$$\frac{J_v}{\Delta p - \Delta \pi} = \frac{P_v}{P_v + P_s}$$

in a membrane in which the interaction of water is great, the presence of large channels between water and membrane that may arise in the barrier will be that arising with water in such large channels.

The friction term, f_{ws} , is dependent upon the number of water molecules that move. Since it also is defined per unit area and unit thickness of the membrane, its value if individual molecules move through the membrane during the process of diffusion - *vide infra*, can be expected to be small. If, however, flow through large channels when hydrophilic groups occurs. Within these limits the frictional interactions in the direction of flow will range from zero to the maximal value which is dependent upon the number of water molecules that move. If it is assumed that laminar flow is established in a membrane through which the water moves at a constant velocity, the frictional forces characterize the frictional interactions in the limiting models, however, it seems

It seems probable that if aqueous membranes, hydrophilic groups within

Leaf: Transport Pr

the barrier if interactions of individual water molecules approximate those in bulk water. If the solute and membrane are either much greater or much smaller materials, much less, the barrier in all likelihood will be similar to water. If water becomes organized at the surface of the channel or at nonpolar regions, the increased ice-like structure of the water may affect the penetrability of the barrier. This organization creates the marked difference in the viscosity of an increased structure of water which may not obstruct the passage of water but possibly may exist. The increased organization of water molecules with charged groups arises as a result of the interaction of water molecules with charged groups. An attraction between neighboring water molecules would be increased by the presence of water in the membrane in this situation. The increased structure of water results, on the other hand, from the interaction of water with the nonpolar groups of the membrane. Such attractions may result in a more ice-like water structure. The resistance between water and membranes may be high in the former and may be low in the latter. The latter is due to the slippage at the interface between liquid water and the membrane.

the molecular friction between it and molecules.

In general²

$$\omega = \frac{D}{\Delta x}$$

the permeability coefficient, ω , is inversely proportional to the sum of the frictional coefficients of water and water vapor, f_{wv} , and directly proportional to the fraction of membrane, a_m . In a very finely porous membrane, a_m is unity. In less porous membranes the importance of the diffusion coefficient, D , becomes a determining factor, as in free diffusion processes. In the case of THO, penetrating the membrane

$$J_{THO} =$$

with J_{THO} the quantity of labeled water vapor per unit time; $\Delta c / \Delta x$ the gradient of carrier, A is the fraction of membrane available to the isotopic water, and D is the diffusion coefficient of water in the membrane. The frictional forces that the isotopic water experiences are largely those of water-water interaction. The familiar free diffusion constant, D , is

Leaf: Transport Pro

in which r is the mean radius of the of water and $\Delta p/\Delta x$ is the gradient which provides the driving force for the flow, J_v , to the diffusional flow, J_1 , in the absence of bulk flow, is

$$\frac{J_v}{J_D V_w} = \frac{8}{3}$$

From this relationship the equivalent calculated.^{5, 6} As emphasized by several is even as large as 10 Å, net transfer may exceed that by diffusion.

For a diffusional process the flux ratio of the activity of the diffusing species or if J_{12} is the flux of the diffusing species 1 and J_{21} is the flux in the reverse direc

$$\frac{J_{12}}{J_{21}} =$$

with a_1 and a_2 the activity of the uncharged 1 and 2, respectively. If however, the across a homogeneous semipermeable

To summarize, we may state that encounter during either diffusional (acting singly) or bulk transfer (associated with a permeability barrier are the same. The rate of water movement per molecule may be markedly different. The relative importance and dominance of this type of movement over the other is due to the strong tendency of water to associate through hydrogen bonding.

Next, this author would like to comment on the results reported above in regard to water movement across the urinary bladder which we have been studying because of its similarity in function and in its activities to those of the port system. It has been shown by Ewer¹⁰ that in the toad the urinary bladder is a reabsorbing organ which may be reabsorbed during periods of water loss and that in response to neurohypophyseal hormones it may increase its water content.

The toad urinary bladder is a large, thin-walled sac which occupies one or more of the entire abdominal cavity. It can easily be removed and used as either a whole organ or a segment. Its wall is transparently thin consisting of a single layer of epithelial cells supported on a thin connecting tissue layer. It is supplied by blood vessels and muscle and capillaries. A serosa lining is present on the outer surface.

As very dilute urine may remain in the bladder for long periods of time, it is of interest to know whether or not the

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TABLE
EFFECT OF VASOPRESSIN ON DIFFUSION
WATER FLUX) OF ISOLATED TOAD BL.
 D_2O OR T_2O IN ABSENCE

Preparation*	Periods (30 minutes) ($\mu l/cm.^2/hr.$)		
	1	2	3
Control	343	338	339
With hormone	338	543	599

*Control includes 10 experiments; seven in one direction, three in reverse direction. Hormone added at end of experiment. Vasopressin to medium bathing serosal surface; 3.14 cm.² area. *J. Physiol.* 1962, 160, 103-113. & A. Leaf. 1962. Studies on the movement of water across the isolated toad bladder and its modification by vasopres-

upper line shows the large net transfe

passively across the bladder. The active reabsorption of sodium and reabsorption which requires energy.

Thus water moves passively across the bladder. The high unidirectional diffusion permeability occurs in the absence of the ability to induce large net transfers. The increase in unidirectional diffusion can modify the bladder to produce the effect. Koefoed-Johnsen and Ussing⁶ have given at the present time on the basis of their hypothesis, net water movement in aqueous pores through the bladder. Vasopressin increases the net transfer in individual pores. As discussed earlier, the radius (or pore radius squared) available for flow while laminar flow, according to the theory, the fourth power of the radius of the pore, Δr , will affect diffusion on the order of $(r + \Delta)^4 - r^4$. The hormones as altering the responsive small pores to one containing few

Einstein College of Medicine and the author have obtained information¹² perhaps pertinent to this discussion. Both theory and experiments support the view that viscosity and self-diffusion in bulk water are largely determined by intermolecular hydrogen-bonding. Deductions regarding the degree of hydrogen-bonding and, therefore, the state of water can be made from the activation energies for diffusion and viscous flow in water. The temperature dependence of both processes has been shown by Wang and associates¹³ to be essentially identical, yielding activation energies of 4.6 and 4.59 kilocalories per mole at 25°C., respectively. We have therefore examined the temperature dependence for both diffusion of tritiated water (THO) and net transfers of water through the toad bladder. FIGURE 2

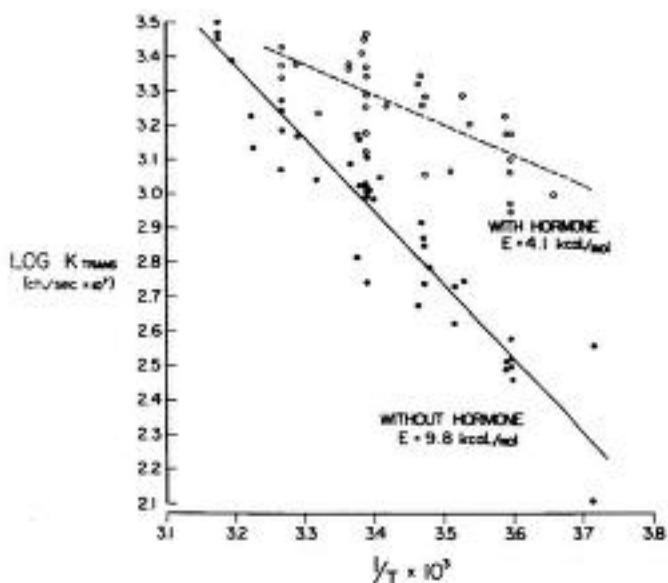


FIGURE 2. Temperature dependence of diffusion of THO through isolated toad bladder. K_{trans} is the transepithelial permeability coefficient for diffusion of THO and $1/T$ is the reciprocal of the absolute temperature. (From Hays, R. M. & A. Leaf. 1962. The state of water in the isolated toad bladder in the presence and absence of vasopressin. *J. Gen. Physiol.* 45: 933).

shows Hays' findings on the temperature dependence of THO through the toad bladder in the presence and absence of vasopressin.¹² The results are presented as a familiar Arrhenius plot of the reciprocal of absolute temperature on the abscissa and the logarithm of the rate constant, in this case the transepithelial permeability coefficient for THO, on the ordinate. From the slopes of the experimental lines obtained the activation energies are readily calculated. In the absence of vasopressin a high value of 9.8

kilocalories per mole was obtained which dropped to 4.1 kilocalories in value is sufficiently close to the frictional resistance that the diffusing bulk water. However, the value of hormone means that the frictional molecule meets in the membrane countered during diffusion in bulk w

This finding in the absence of possible interpretations regarding a barrier in the bladder. A continuous molecular lipid layers studied by *A* such high activation energies for other hand, if continuous aqueous diffusion barrier in the absence of water molecules in these channels other or to the membrane than is the resistance is determined by intermolecular friction energy obtained for bulk transpressin was found to be 4.6 kilocalories per mole, the value of Wang and associates

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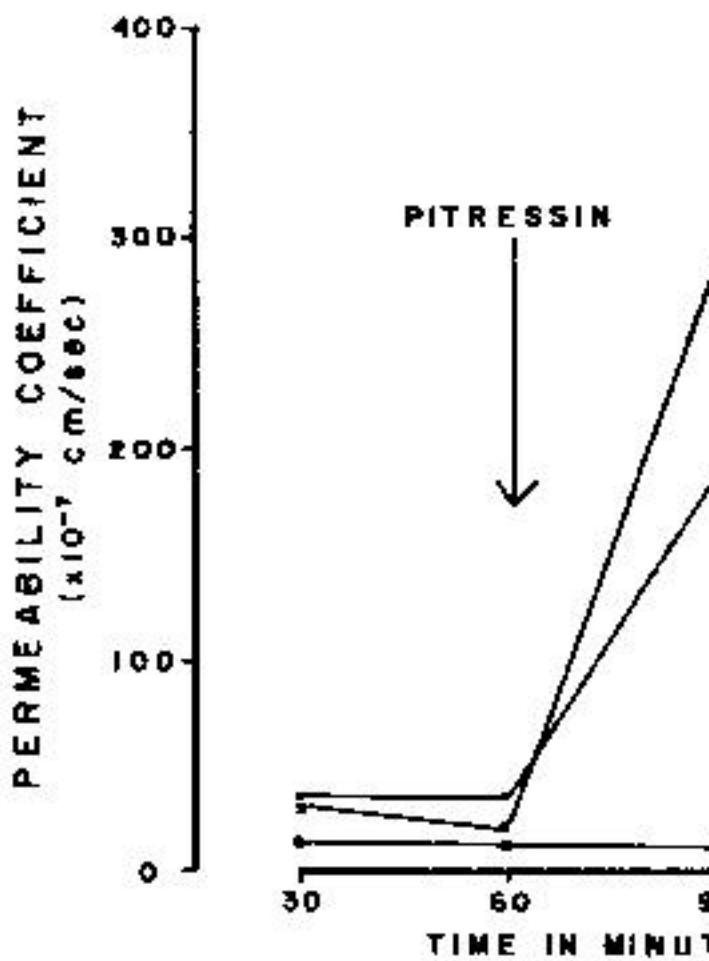


FIGURE 3. Effect of vasopressin on the permeability coefficient of the toad skin to urea, acetamide and tiourea. After two hours of equilibration, vasopressin was added to the medium bathing the serosal side. (Courtesy of R. H., R. M. Hays, E. Lamdin & A. Leaf. The effect of various hormones on the permeability of the toad skin. *J. Physiol.* 163: 629-630.)

The evidence from the effect of solutes that seems so irreconcilable action of vasopressin is the specific to solutes, just considered, and the bladder retains to small solutes even of water. The reflection coefficient obtained¹⁶ in the presence of large $\mu\text{l}/\text{cm}^2 \text{ hr}$. A value of 1.0 for the reflectability of the membrane for the sodium ion in a completely nonselective membrane was 0.995 and for chloride 0.993, with its rate of penetration of some 80 $\mu\text{l}/\text{cm}^2 \text{ hr}$.

We have good reasons to believe with respect to all three of its effects, and sodium ions, is in or near the urinary surface of the single layer. In order to circumvent the apparent difficulty to accommodate the large net movement of impermeability to small solute molecules, various hypotheses have been proposed. A fine diffusion barrier, selective for water and sodium, but effectively blocking the passage of other solutes, has been

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Referen

1. STAVERMAN, A. J. 1951. The theory Recueil Travaux Chem. Pays-Pas. 70: 1.
2. KEDEM, O. & A. KATCHALSKY. 1961. nomenclological coefficients of membe 143.
3. FRANK, H. S. 1958. Covalency in the water and ice. Proc. Roy. Soc. Londo
4. DEBYE, P. & R. L. CLELAND. 1959. F vycor. J. Appl. Phys. 30: 843.
5. PAPPENHEIMER, J. R., E. M. RENKIN diffusion and molecular sieving thr Am. J. Physiol. 167: 13.
6. KOEFOED-JOHNSEN, V. & H. H. USSIN and flow to the passage of D₂O thr Scand. 28: 60.
7. SOLOMON, A. K. 1960. Pores in the cel
8. ROBBINS, E. & A. MAURO. 1960. Expe diffusion and hydrodynamic perme branes. J. Gen. Physiol. 43: 523.
9. SIDEL, V. W. & J. F. HOFFMAN. 196 analogues. Fed. Proc. 20: 137.
10. EWER, R. F. 1952. The effect of pi regularis Reuss. J. Exptl. Biol. 29: 17.
11. HAYS, R. M. & A. LEAF. 1962. Studie the isolated toad bladder and its Physiol. 45: 905.
12. HAYS, R. M. & A. LEAF. 1962. The stat

DIFFUSION AND THE NONELECTRO

Samuel B. Horowi

*Laboratory of
Albert Einstein Medi*

The observation was made by firmed¹⁻⁶ that the passive transpo commonly related to the chemical studies have shown that a corre rates and the equilibrium distribu and a nonpolar solvent: roughly, t the solute in the nonpolar phase. These observations occupy a centr on cell permeability. It has become port as involving diffusional and by a membrane at the surface of of lipid composition. Modification has kept step with the experimental transport, including, for example, pores as a primary route of penet

Horowitz & Fenichel:

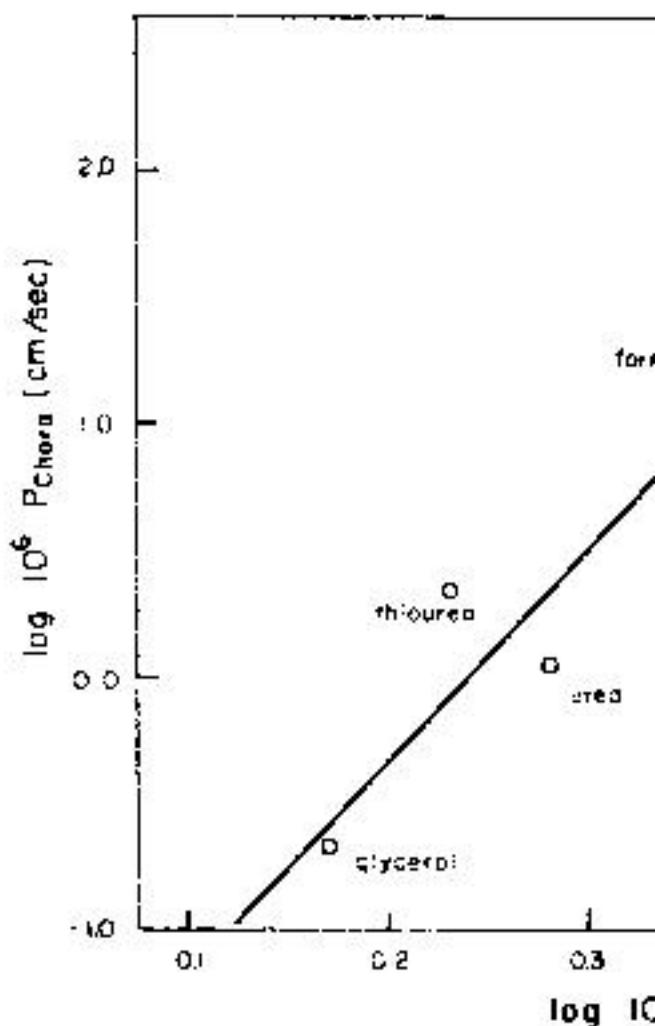


FIGURE 1. Correlation of permeability of *Chara* at 18-23°C.³ with diffusion coefficient of solutes in polyacrylate gel at 15.3°C.¹¹ Only the first members of the series are included. This correlation should be compared with the solvent/water equilibrium distribution coefficient.

III. Barrier, as a first approximation, the cell and owing its properties to the cell. Specificity primarily

From the point of view of theoretical models, there is no significant difference among the models based on the same mechanism, but their composition. A model barrier may be considered as a membrane because of the small quantity of lipid molecules which it contains, and the barriers. While the specificity which is characteristic of the barrier is mainly adequate to account for the main features of cellular transport, the operation of this mechanism is limited by the physical requirements on the structure of the barrier. It must contain a lipid phase which is continuous throughout the barrier, a number of pores of molecular dimensions, and a sufficiently strong intermolecular interaction to prevent penetration of even highly lipid soluble substances.

On the other hand, a diffusion-controlled barrier, as a first approximation, requires that the barrier be composed of a polymer having H-bonding. A high density of H-bonds is not sufficient in itself, as may be shown by the example of the barrier in strongly H-bonding liquids.^{11,12} The specificity of cellular transport a polymer, and the specificity of the barrier, whereby the effect on specificity is

whereas that for 1-butanol transport is of this difference is not that to be expected as has been shown by Barrer.¹⁵ Little application of this type of physical chemistry to lipid membrane permeation models.

In view of the imperfect theoretical of the possibilities of multiple barriers mechanism, EM evidence does not consider solubility mechanism. The hypothesis are transport barriers, though reasonable if one accepts this hypothesis, the possibility may impose specificity by a diffusional ordered protein phase. From this point component of membranes seem disorganized. It has demonstrated the intimate involvement of the Schwann cell in the synthesis of the membrane. Morán and Finean¹⁷ showed that extraction of the membrane leads to marked dimensional changes. The general universal model of the cell membrane is based on the fluid mosaic theory. Were there no alternative to the fluid mosaic model it would be logical and simplifying, but the existence of some other models, as the par-

of lipids and cellular membranes. The surface membrane structure, and the lipid content is just that required to form a layer (cf. Ponder²¹). Nevertheless, it is to be expected from a lipid transpor-

The Distribution

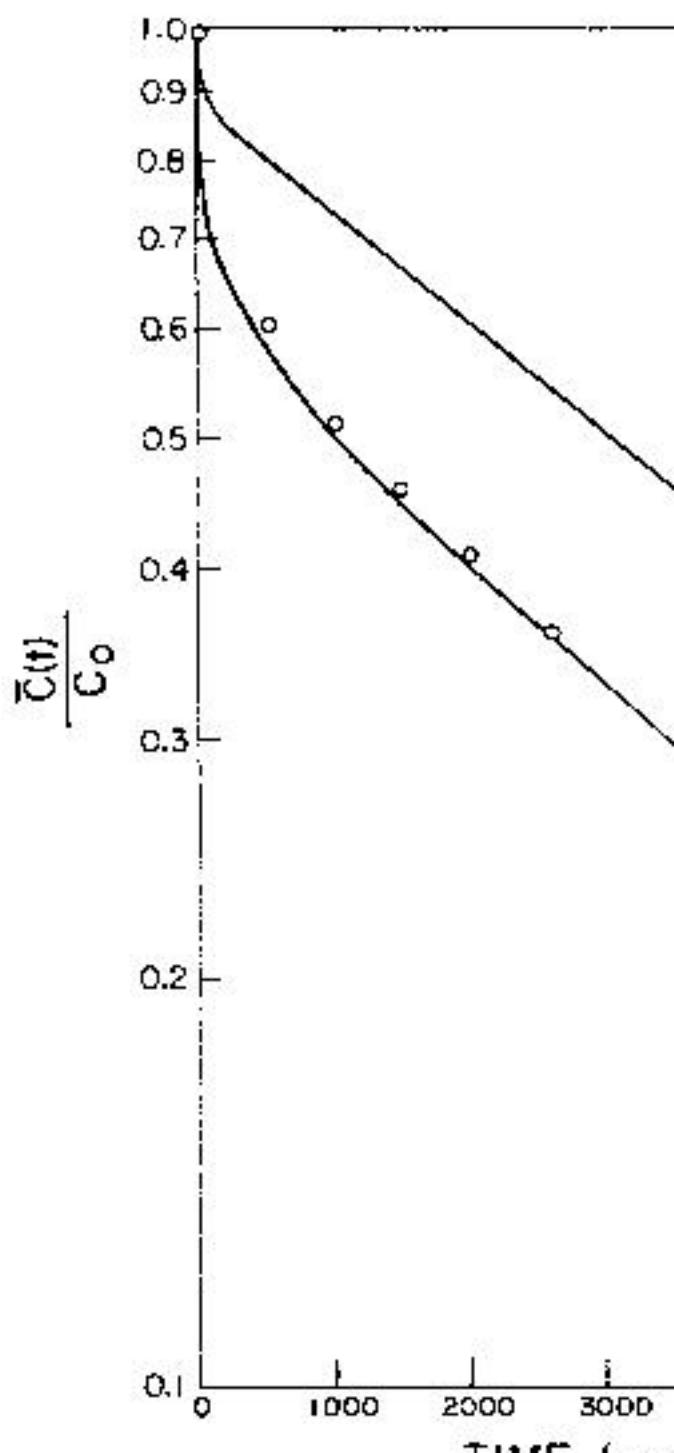
The form of the kinetics of flux will depend on the form of the distribution through the cell or tissue. Three extreme models may be considered above. The extreme models, those of the discontinuous and of the continuously distributed, give the extreme forms of the kinetics.

Consider tracer efflux from a large cell containing a uniform initial concentration of tracer. In the single surface barrier model, the thickness of the membrane is usually taken as 75-100 Å; this is small enough to neglect diffusion across the membrane thickness, so that the kinetics, to the linear approximation, are given by

$$\frac{\bar{C}(t)}{C_0} = e^{-kt}$$

where C_0 is the uniform initial concentration, $\bar{C}(t)$ is the mean concentration at time t . The rate constant k is a constant; for a membrane of permeability P and area A , $k = PA/P_m$.

Horowitz & Fenichel:



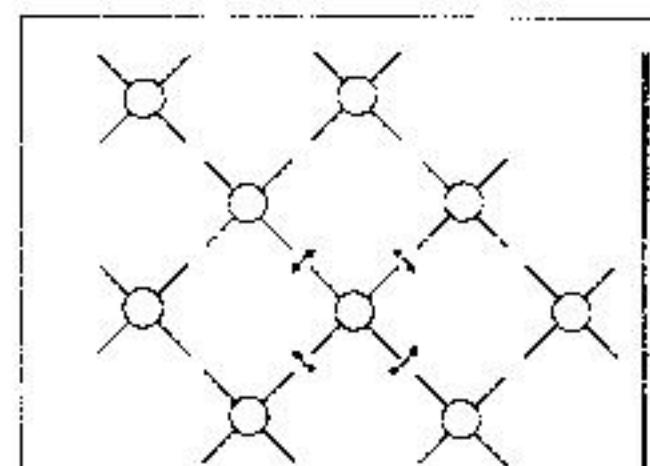
a multimembrane barrier. The latter distribution existed either a number of barriers are involved, of such geometrically appropriate set of first order curvature of frog skeletal muscle provides an alternative. The longitudinally oriented tubules of the sarcoplasmic reticulum; this is to be continuous with the extracellular space radially from within the muscle fiber. Successively through these sheaths, if one accepts a single transport barrier, one would expect kind of series membranes. However, even though the sheaths does not strengthen the sheaths are not continuous as is the case in lacunar, so that extensive free pathways exist.

Justification for considering a multimembrane barrier at the ultrastructure level, from the high resolution electron microscopy, X-ray diffraction²⁶ and nuclear magnetic resonance, extends to the molecular level as well as in other cells and macromolecular systems at the present symposium. These molecules in apposition to protein

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ular diffusion path. For example, in solution is not extensive, the major diffusional bulk water, and diffusional coefficient of water.³¹ The applicability to the cell of pure water is therefore contingent on the assumption that is such as to determine the overall transparency that this assumption is reasonable.

Pure water systems. The properties of a system is dependent upon the modified H-bonding dynamics. To elucidate these dynamics we shall consider a perfect tetrahedrally H-bonded network schematically in FIGURE 3a. The water molecules are represented by a central circle with four lines extending from it to the centers of four surrounding circles. The molecules are arranged in a square lattice. Arrows indicate the movement of one molecule along a path consisting of two adjacent molecules. The path starts at the bottom center molecule, moves up-right to the top center molecule, then down-right to the right center molecule, then down-left to the bottom center molecule, and finally up-left to the left center molecule.



The consequences of this become such as a Bjerrum rotational defect H-bond. A water molecule at the bottom of a fairly deep potential well in the di-torsional oscillation about this direction and the molecule will therefore extend. Since the orientations of the other molecules are relatively rigidly fixed they also oscillate with greater amplitude, "smeared out". This is schematized by the potential wells seen by neighboring molecules being directional,* so that these too oscillate.

*This effect may be reinforced by the presence of a defect molecule in the 4-bonded and non-4-bonded positions which is transmitted to successive neighbors so that the defect increases.

The overall effect of the defects is to produce partial lattice disorder. In ice, the density of Bjerrum defects, on the order of one per 10^3 water molecules, the number of defects in the system increases with disorder overlap. In the overlap region the probability of breaking H-bonds will be small. We

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where a is the jump distance, N_1 is the number of defects per unit volume, v is the frequency of jumps, ΔG_1^{\ddagger} is the free energy of activation, and g is a factor near unity which includes the effect of finite size of the defect. Taking, as usual, $v = 10^{12} - 10^{13}$ sec⁻¹, $a = 10^{-8}$ cm, and $\Delta G_1^{\ddagger} = 10$ kcal./mole, we find that in the intermolecular spacing in a water molecule, equal to about 10^{-8} cm in ice and 10^{-9} cm in liquid water, the activation energy for diffusion in the region around a defect will be smaller than the corresponding activation energy for diffusion in the bulk. This is supported for the diffusion of water molecules by calculations of Jaccard.³⁵ In liquid water, the activation energy for diffusion is about 4.4–4.6 kcal.,³⁶ which suggests that $\Delta H_1^{\ddagger} \approx 10$ kcal./mole (cf.) Zener).³⁷ The difference of $N_1 v g$ between the bulk and the region around a defect, then, probably represents largely a difference in activation energy.

Protein-water systems. Let us introduce the concept of a protein molecule with the characteristic of having H-bonds to some small multiple of a characteristic number of water molecules. Consider the positions of a tetrahedral water structure in the neighborhood of a protein molecule subject to the increased oscillational and rotational motion of the water molecules when a defect is nearby. This motion is linked to a molecule of immense inertia, such as a globular protein molecule, and may be

Let us assume that the diffusion mechanism is larger, which must overlap one or more mechanisms. The rate will be determined by the probability of finding one or more vacancies (empty lattice sites) next to the molecule; and by the probability of a subsequent place change. Both of these depend upon the chemical nature of the solute. For the case of simple solute diffusion we may write

$$D_2 = g a^2$$

which differs from Equation 3 in that it contains the factor g and the activation energy, ΔG_2^\ddagger , for the specific diffusing solute.

Size specificity. Let us focus attention upon the factor g . We find that g and ΔG_2^\ddagger depend only upon the number of lattice sites required for diffusion, n . In an idealized rigid lattice, we will find that

and

$$\Delta G_2^\ddagger = \frac{1}{2} k T \ln n$$

where n is the number of lattice sites required for diffusion. For a spherical molecule, $n = 6$.

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when the solute molecule is too small, a to fit an integral number of lattice sites the diffusion coefficient.

Chemical specificity. The chemical nature found effect on diffusion in the protein both N_2 and ΔG^\ddagger . The reason for this effect of the introduction of a non-H-bonded group of the unfilled H-bonds adjacent to the vacancy is similar to those around the vacancy in the lattice will be different in the protein water. In the liquid the absence of lattice neighbor permits water molecules to reorient these defects, while still forming molecules, which are free to accommodate "cage" molecules. On the other hand, if such reorientation is only possible if two orientated water molecules with the bar in a preordered system; as a result, they ("bound") with the solute.*

The presence of bound defects, together reduces the need for additional defects reducing the magnitude of ΔG^\ddagger . Diffusion

solute, and estimate their strength. The solute to the water lattice will depend on the nature of the ordering groups in the lattice. A number of different lattices are possible; for some of them H-bonding has been obtained.³⁹⁻⁴⁰ In systems in which water is ordered by the presence of ordering groups, a simple configuration of ordering groups. A simple relationship has been made.^{41,42} In the absence of ordering groups, other points can be offered as probable. A site which has the greatest number of H-bonding sites of greatest number of bonds to the lattice, have as a result the slowest diffusion. The site which is misaligned with lattice sites will have the greatest degree of lattice disorder increasing with the depth of minima in the potential function.

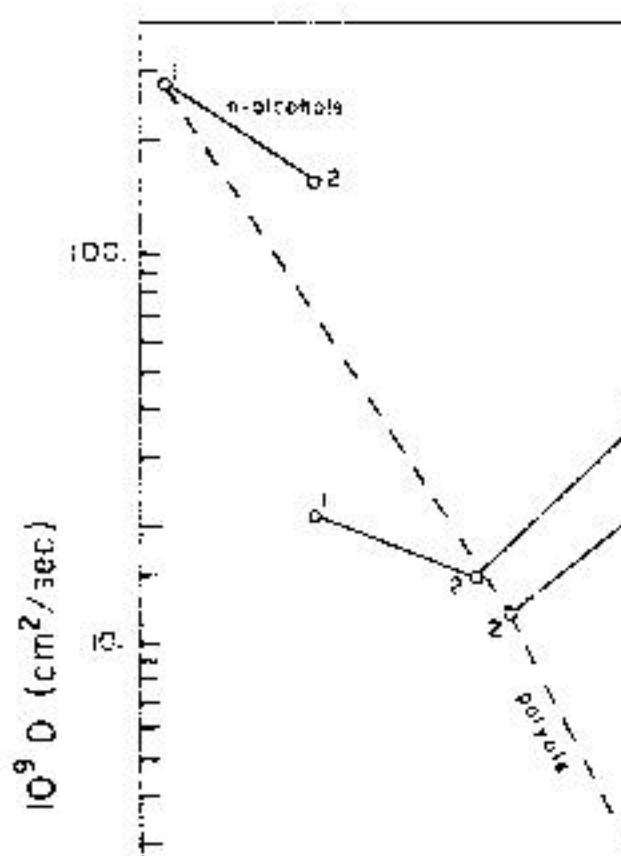
The quantitative predictions which have been made are still yet limited. Using data on water diffusion, $D_1 \sim 10^{-7}$ to $10^{-8} \text{ cm}^2/\text{sec}$, and $D_2 \sim 10^{-10} \text{ cm}^2/\text{sec}$, one would expect the following:

- (1) For solutes which are non-H-bonding, but have large nonpolar

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where D'_n and D_n are, respectively, in nonpolar; C_n is the concentration of n in the nonpolar. Potentially, the size of from k .

Comparison with experimental data. in three different biological systems, distance throughout the cell are presented :



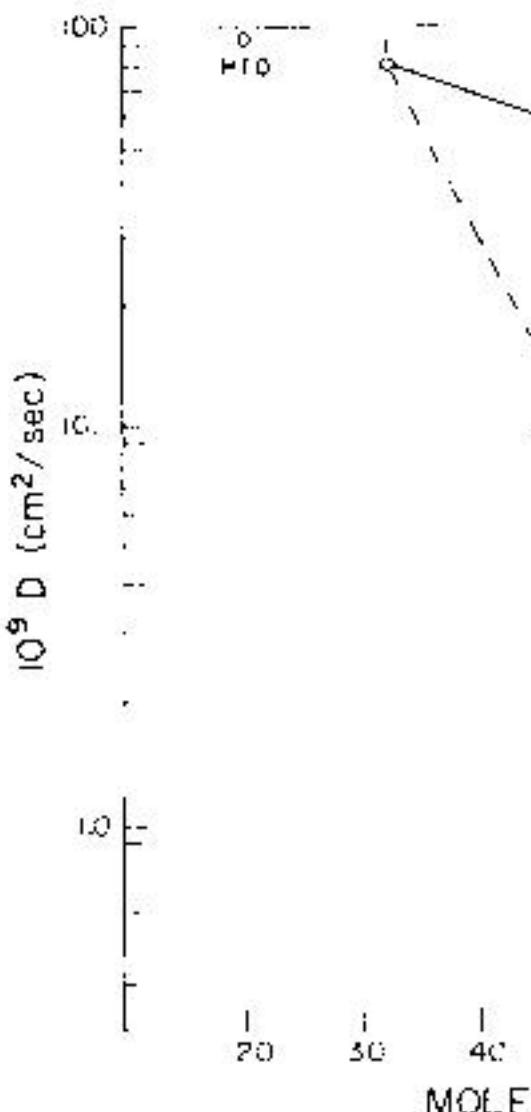


FIGURE 5. Intracellular diffusion epithelium of the toad bladder, as a connect members of homologous series. The average thickness of the bladder mucosal epithelium, an essentially co taken as the diffusional barrier, and meability coefficients,² K_{muc} , in the ness, d , by the equation $D = K_{muc} \cdot d$

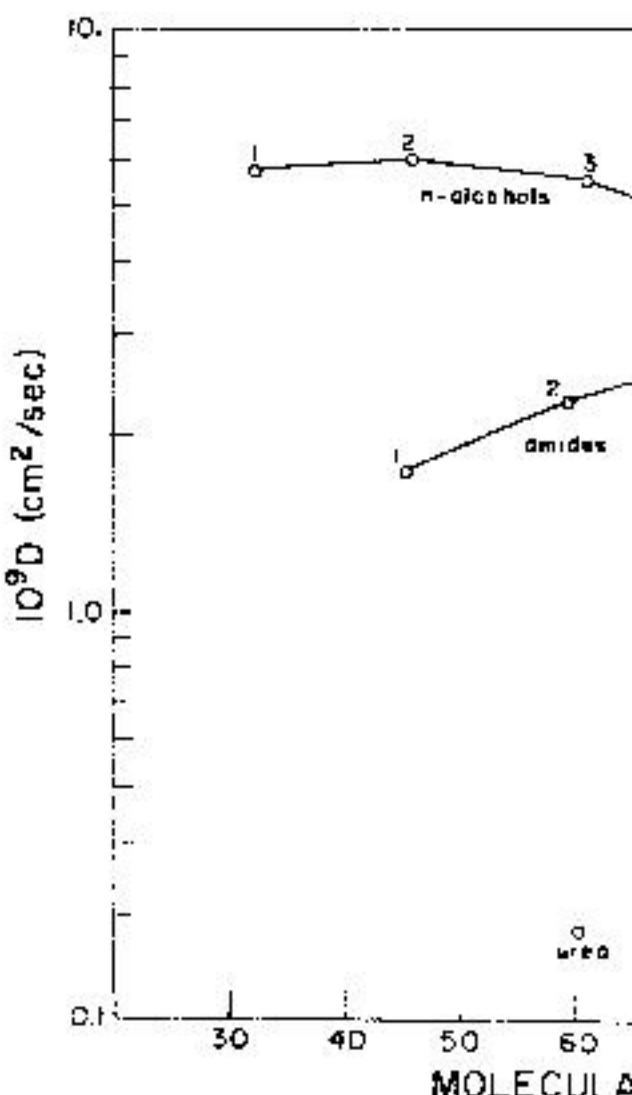
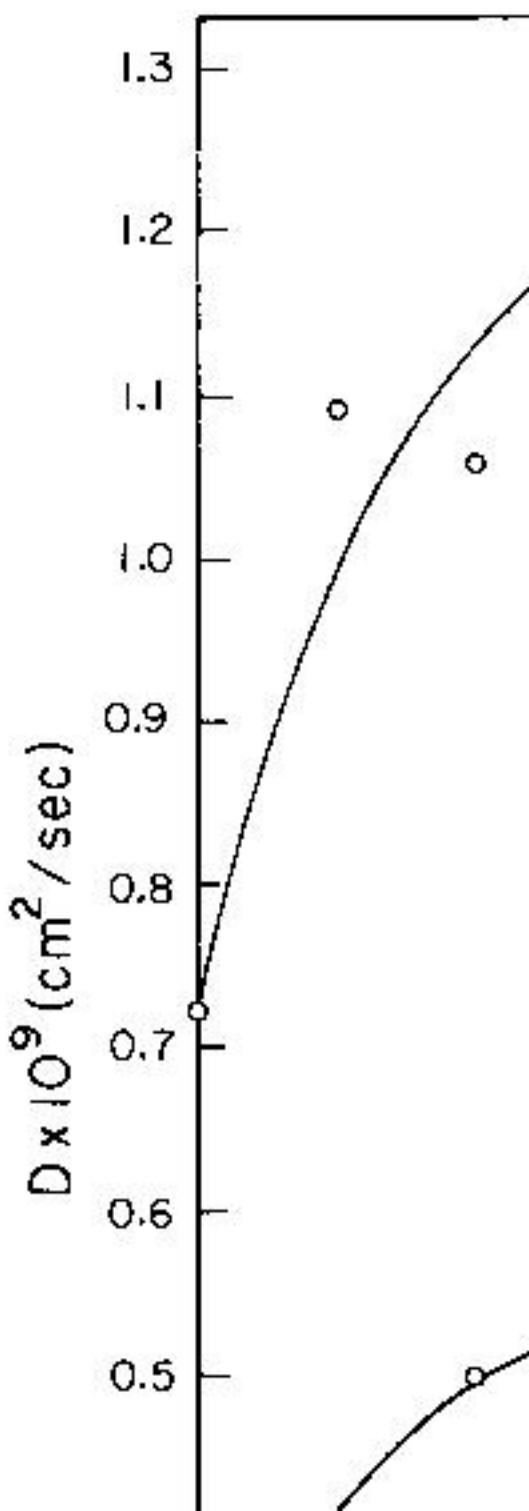


FIGURE 6. Intracellular diffusion coefficient of *Aedes aegypti* and *Anopheles* *punctipennis*, as a function of molecular weight for three homologous series. D was calculated from the equation $D = r_n^2 / 4t$; r_n was measured to be 34.9μ in **FIGURES 4-6**, the sartorius has the largest diffusion coefficient. Comparison of the sartorius data with that of *Anopheles* *digitii* IV⁶ shows that the values of D here for slowly diffusing solutes, whereas for rapidly diffusing solutes, the molecular weight strongly affects the calculated D .



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by three homologous alcohols was studied at a urea concentration of 60mM. The ratios D'_2/D_2 of urea diffusion to that of water in the presence of 60mM of each of the alcohols were 1.45, 1.64, and 2.00 for 1-pentanol, 1-butanol, and 1-propanol, respectively, at 5.6°C. The increase in the ratio of diffusion rates of water to urea with increasing size is in accord with the expectation of a hydrodynamic model. Concentrations of urea and glycerol used were such as to give equal diffusion of urea.

An enhancement of the transport rate of water by vasopressin, retaining solutes, as well as water and urea, in the presence of nonpolar material such as 1-pentanol, has been suggested. A simple suggestion can be offered whereby the effect of vasopressin on the transport will, nevertheless, be the same. The water lattice and stability of the water lattice is dependent on the configuration of H-bonding sites of the water molecule. The configuration, resulting from vasopressin, is such as to increase the disorder of the water lattice. The incompleteness of this simple picture is made evident by the lack of effect of vasopressin: no effect is seen in the presence of thiourea. No model has been offered to account for the lack of effect of the present model in the direction of the effect of vasopressin.

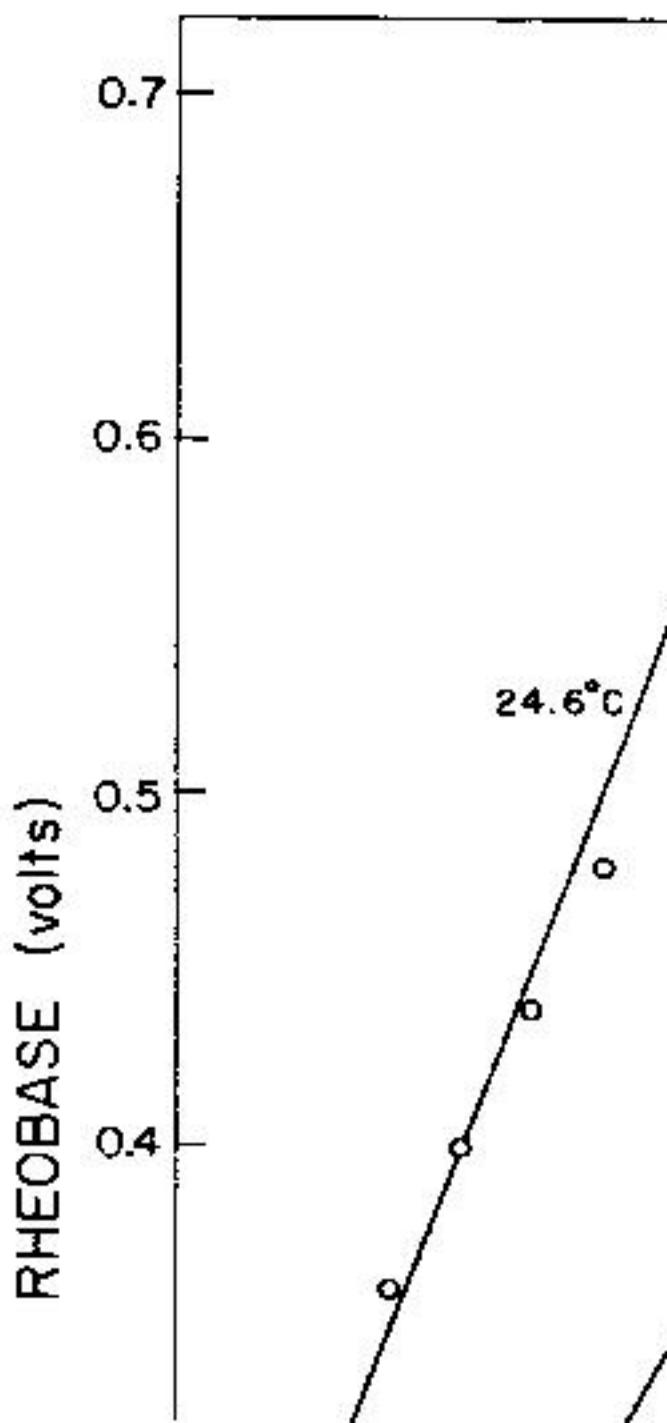
cooperative lattice disordering ("runaway narcotic concentration leads ex-

A number of other theories of primary importance to a lipid-pore model include the possibility of these agents interacting with the membrane. Recently, two versions of a hypothesis concerning the interaction of cellular water have appeared which may be relevant to the formation, in the presence of a narcotic, of either hydrate microcrystals or icebergs, structures which have been examined by several workers in this paper. However, because the hypothesis of the present "disordering" hypothesis has been discussed in detail in another paper, a brief comparison is included here.

The basic assumption of the two theories of cellular water is that the microcrystal hypothesis postulates that the lipid-pore system is able to stabilize specific polar solutes. The disordered hypothesis postulates that the preexisting protein-water system is able to stabilize a specific type of water structure which would be disrupted by the addition of nonpolar solutes. In this section, these two theories differ.

(1) The presence of microcrystals

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formation of crystalline cages from the vapor in the cage. Using the narcotic effect in terms of the concentration and temperature coefficient at constant concentration, we have

$$\frac{\partial \ln \frac{P}{P_0}}{\partial T} = -\frac{\Delta H_2}{RT^2} + \frac{\Delta S_2}{R}$$

where ΔH_2 is the enthalpy of solution of the solute in the water, and ΔS_2 is the enthalpy of formation of the cage. The Frank and Evans "icebergs," which are negative, since it is determined principally by the interaction between the crystalline cage and the solute, are about 12 to 20 kcal. mole of solute; the size of the cage molecule is of the size of 1-pentanol. Thus the temperature coefficient of narcosis is about 1.5°. The enthalpy of a hypothetical 1-pentanol hydrate is about 100 kcal. mole. Two factors could reduce this discrepancy: (1) It is conceivable that the entry of 1-pentanol into the water system requires breakage of hydrogen bonds, the energy for this is about 8 kcal. (2) The water molecule may be "pre-stabilized" relative to the solute. From data, the ΔH of this stabilization would be a value greater than the ΔH of formation of the cage.

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The model, furthermore, leads to an which some support has been obtained.

Acknowledgments

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References

1. OVERTON, E. 1902. *Pfluegers Arch.* Ge
2. COLLANDER, R. & H. BÄRLUND. 1933. Ac
3. DAVSON, H. & J. F. DANIELLI. 1943. branes. Cambridge Univ. Press. Ca
4. HÖBER, R. 1950. *Physical Chemistry of* York, N. Y.
5. LEAF, A. & R. M. HAYS. 1962. *J. Gen. Ph*
6. FENICHEL, I. R. & S. B. HOROWITZ. 1 221) : 1.
7. PORTER, K. 1961. *In The Cell.* J. Br Academic Press, New York, N. Y.
8. VOELLER, B. R. 1964. *In The Cell.* J. Br Academic Press, New York, N. Y.
9. HUXLEY, H. E. 1960. *In The Cell.* J. Br Academic Press, New York, N. Y.
10. HODGE, A. J. 1959. *Rev. Mod. Phys.* 31:
11. HOROWITZ, S. B. & I. R. FENICHEL. 1964
12. FENICHEL, I. R. & S. B. HOROWITZ. 1965
- 13.AITKEN, A. & R. M. BARRER. 1955. *Trans*

33. GRÄNICHER, H. 1963. Phys. kondens. Materie 3: 1.
34. SHEWMON, P. G. 1963. Diffusion in Crystalline Solids. Academic Press, New York, N. Y.
35. JACCARD, C. 1959. Helv. Phys. Acta 32: 1.
36. WANG, J. H., C. V. ROBINSON & R. D. KENNEDY. 1963. J. Phys. Chem. 67: 466.
37. ZENER, C. 1952. In Imperfections in Metals. Eds. J. H. Hollomon, R. Maurer, McGraw-Hill, New York, N. Y.
38. DICK, D. A. T. 1964. J. Theoret. Phys. Chem. Solids 25: 1.
39. BERTIE, J. E. & E. WHALLEY. 1964. J. Phys. Chem. 68: 1.
40. TAYLOR, M. J. & E. WHALLEY. 1964. J. Phys. Chem. 68: 1.
41. BERENDSEN, H. J. C. & C. MICCHELE. 1964. J. Phys. Chem. 68: 1.
42. WARNER, D. T. 1965. This Annal.
43. COLLANDER, R. 1930. Acta Botanica Fennica 1: 1.
44. LEAF, A., J. ANDERSON & L. B. PALEY. 1964. J. Phys. Chem. 68: 1.
45. PEACHEY, L. D. & H. RASMUSSEN. 1964. J. Phys. Chem. 68: 1.
46. FENICHEL, I. R. & S. B. HOROWITZ. 1964. J. Phys. Chem. 68: 1.
47. MAFFLEY, R. H., R. M. HAYS, E. WHALLEY. 1964. J. Phys. Chem. 68: 1.
48. FERGUSON, J. 1939. Proc. Roy. Soc. (London) A 170: 1.
49. BRINK, F. & J. M. POSTERNAK. 1964. J. Phys. Chem. 68: 1.
50. MULLINS, L. J. 1954. Chem. Rev. 54: 1.
51. PAULING, L. 1961. Science 134: 1.
52. MILLER, S. L. 1961. Proc. Nat. Acad. Sci. U.S. 48: 1.
53. BAIRD, S. L., JR., G. KARREMAN & R. D. KENNEDY. 1964. Proc. Nat. Acad. Sci. U.S. 51: 7.
54. SZENT-GYÖRGYI. 1957. Bioenergetics. Academic Press, New York, N. Y.
55. ELEY, D. D. 1962. In Horizons in Physics. Eds. S. S. Adler, J. A. Wheeler, and B. DeWitt. Academic Press, New York, N. Y.

THE HYDRATE MICROCL ANESTHESIA

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Linus Pauling in his paper entitled "The Nature of Anesthesia,"¹ first published in the journal *Science*, has shown that this theory was forced on us by the following facts:

The phenomenon of anesthesia can be explained as follows: When the application of a physical agent to an organism produces a reversible depression of sensory activity, starting with the most sensitive nerve cells in the highest centers and gradually spreading until the person decides that the depression of activity is sufficient to inflict the trauma contemplated without causing pain, or causing the patient to feel pain but not sensations of pain. Thus the degree of depression of activity is proportional to the intensity of the stimulus.

whole series of lectures on the subposals of Meyer and Overton⁶ that upon a suitable partition coefficient of the brain; they "proved" their anesthetic potency with solubility lations fail to explain the anesthetic gas xenon,⁷ or the anesthetic action sium ion, or the fact that many ex are devoid of anesthetic action. Mo bility of anesthetic agents in solut their solubility in water, and sug solubility served to transport the a leave the lipoid to combine with the lipoid theories were held to be nea they seem still to constitute the ad anesthesia.

So much progress has been made lar structure of biological materia possible to make a new attack on the cal agents produce these extraordin that the answers will not be comple structure of water being asked at t

modynamic activity, forces one to conclude that anesthesia is being produced in similar ways by dissimilar anesthetic agents.

The list of general anesthetics includes such gases as xenon, argon and even nitrogen, which can produce anesthesia under pressure (as anyone familiar with the dangers of scuba diving knows as "raptures of the deep") and it is impossible to believe that these gases produce anesthesia by taking part in ordinary chemical reactions. Butler¹⁰ in 1950 wrote, "the action of these spherically symmetrical atoms without any permanent dipoles furnishes the most conclusive demonstration that anesthesia need not depend on the specific effects of any structural grouping."

The most surprising group of anesthetic agents are the noble gases such as xenon.¹¹ Xenon has no ability to form ordinary chemical compounds by covalent or ionic bonding; its only known chemical property, in biological systems, is its ability to form clathrate compounds with water. Xenon, like most of the general physical anesthetics, does not form the hydrogen bonds which are known to play an important part in many physiological processes.

Crystals of xenon hydrate have been shown by x-ray diffraction studies to have the same structure as the hydrates of other small molecules such as chlorine and methane.¹² In 1952, Pauling and Marsh¹³ made a thorough study of chlorine hydrate and found it to consist of cages formed by 20 water molecules joined tetrahedrally by hydrogen bonds so as to form twelve sided polygons, each facet being pentagonal. These pentagonal dodecahedra

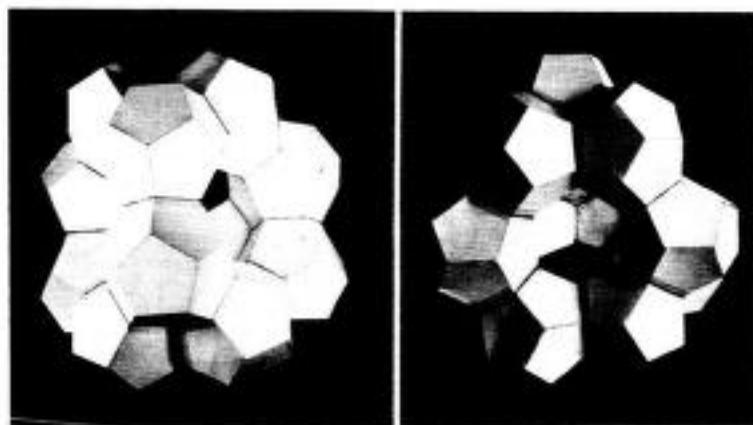


FIGURE 1. Two views of a paper model of the hexakaidecahedron cavity of the 17 Angstrom (Type II) hydrate structure. The cavity is formed by 12 dodecahedra, each contributing one pentagonal face to the cavity. The centers of each hexagonal face would lie at the corners of a regular tetrahedron. Each of the four hexagonal faces of a single hexakaidecahedron will be shared by other hexakaidecahedra.

fill space in such a way that there are dodecahedra, each with 14 sides (faces.) The chlorine molecules were in tetrakaidecahedra. The smaller dodecahedra contained single water molecules. The repeating unit of the hydrate has an edge of 11.8 Angstroms, each surrounded tetrahedrally by four water molecules at a distance of 2.75 Angstroms, reported by Sir Humphrey Davy.² However, a different arrangement of dodecahedra forms a cubic unit of structure of the chloride hydrates on an edge and contains 16 water molecules filling space in such a way that it has 16 sides, 4 of which are hexagonal and shared with the dodecahedra. Ordinary ice contains 12 water molecules at a distance of 2.76 Angstroms per water molecule of the cubic unit. This is approximately 16 per cent greater than ordinary ice. The 17 Angstrom chloride hydrates contain 16 water molecules at a distance of 2.76 Angstroms per water molecule 18 per cent greater than ordinary ice. The difference in volume of the chloride hydrates is partly due to the V-shaped arrangement of the water molecules and partly to the presence of the anesthetic molecules.

Catchpool: Hydrate M

per mole, from which must be subtracted the Van der Waals repulsion of the xenon atoms and the water molecules. His calculations show how nearly the stability of the hydrate can be understood in terms of the Van der Waals theory.

Since the energy of the Van der Waals interaction between a molecule and its cage of water molecules depends on the refraction of the trapped molecules, the equilibrium between the crystals and water and ice at 0° and the melting temperature of the hydrate should bear a relation to the interactions affect the free energy of formation. The logarithm of the equilibrium pressure of the gas at the melting point of the hydrate, the melting point of the hydrate, and the mole refractions of the water and the gas on a slightly curved line and the mole refractions of the water and the gas on another slightly curved line. Approximate prediction is shown in FIGURE 2, which is reproduced from *Science*.¹ The deviations from a smooth curve are in the direction to be expected, since the large size of the cages and the stability of the hydrate depend on the repulsions at close range between the xenon atoms.

The temperature at which chloroform dissolves in water is 20°, but this can be raised by forcing xenon into the cages of the hydrate crystal, where it occupies about one-third of the

similarly stabilized by filling more he formulated his theory of genera G. A. Jeffrey of the University of ture of a crystalline hydrate of an have a clathratelike structure res 25 . He then speculated that if filled, the hydrate crystal would These alkylammonium salts close teins. If hydrate crystals were to brain by incorporating molecules also interfere with the motion of chains that normally contribute to These electric oscillations are invol ory, and interference or damping enough to cause loss of consciousness the microcrystals might be maskin

It is obvious that anesthesia is hydrates of the chlorine and chlor be necessary to stabilize these hydride is anesthetic for mammals at a at a temperature of 37°, but the h pressure of 40 atmospheres is ma

Catchpool: Hydrate M

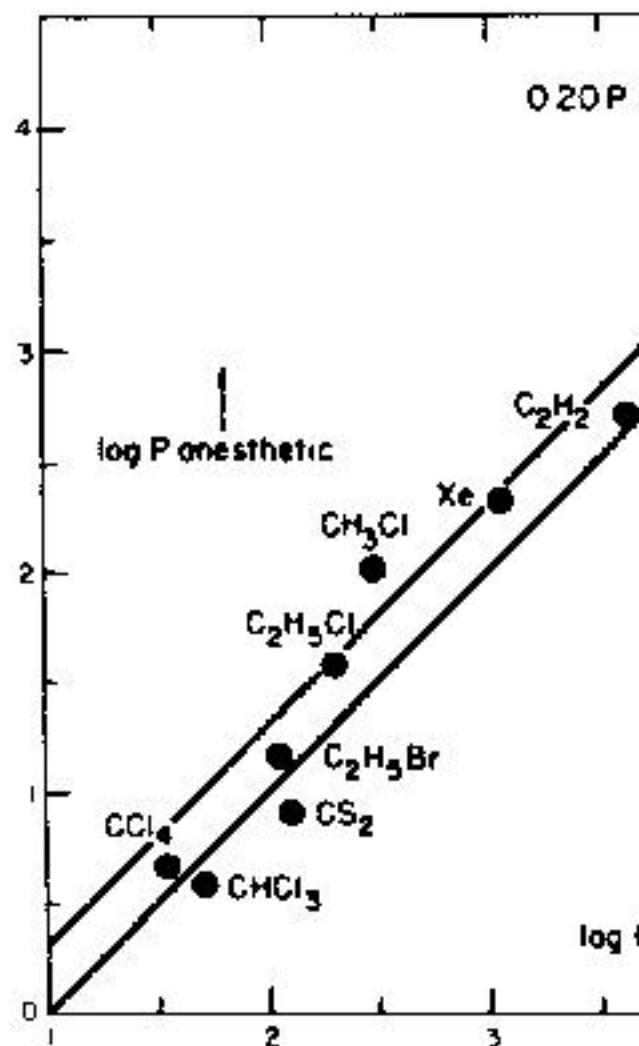
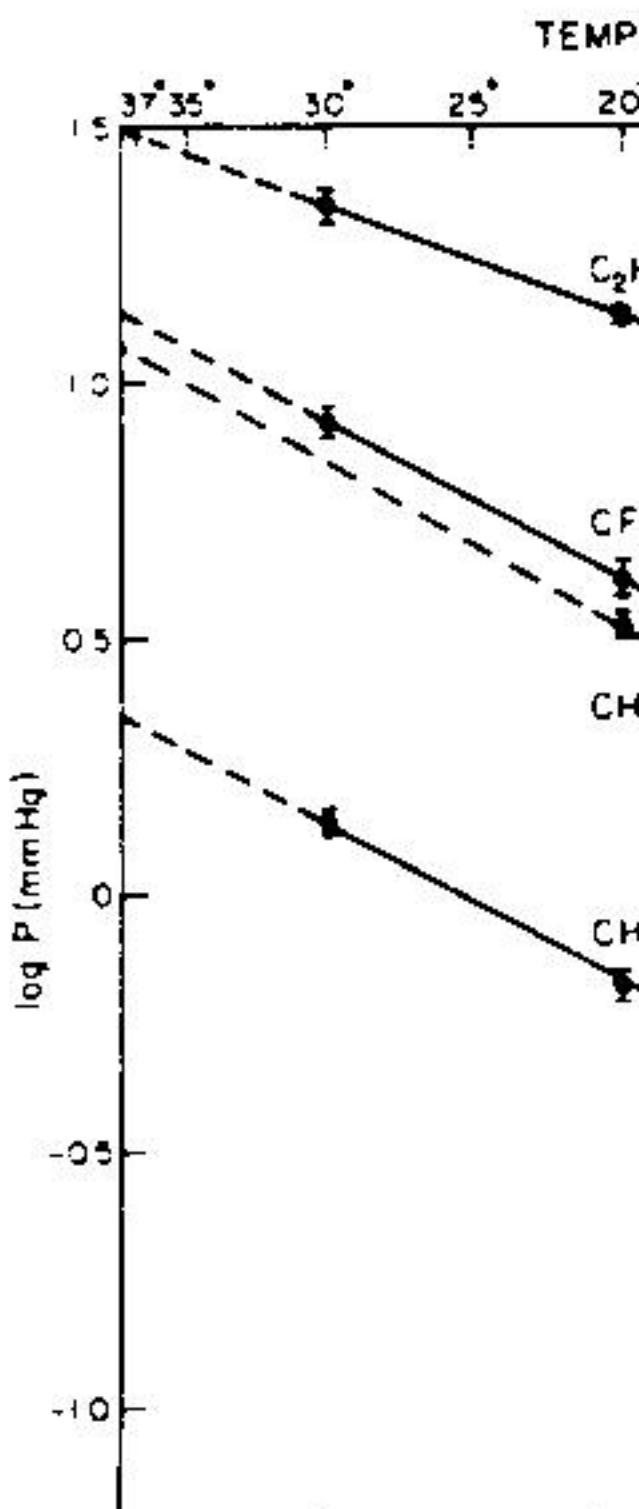


FIGURE 3. The logarithm of the anesthetic bonding anesthetic agents plotted against their hydrate crystals. (From Pauling, Sci

mised that hydrate formation, or "icebergs," it would have the same effect with and w



Catchpool: Hydrate M

drate formation by alcohols.¹⁸ It is likely that the interaction of diethyl ether and other ethers is to be a combination reactions with other molecules (water) involving hydrogen bond formation.¹⁹ Dielectric measurements have shown that an increase in the amount of irroration of hemoglobin containing dissolved xenon, which apparently does appear to increase the amount of steric hindrance around the protein molecule.²⁰

The hydrate microcrystal theory of anesthesia asserts what was pointed out nearly 100 years ago that ether is not merely a special poison to the nervous system, but acts on all tissues by numbing them, temporarily.²¹ That this is true has been confirmed by the action of anesthetics on nearly every tissue of the body, including the nervous system. The action of the nerve membrane, or stiffening of the nerve membrane, or stiffening of the nerve membrane." He also suggests that anesthesia is produced by induction along a nerve by increasing the viscosity of the water at its interface with the protein molecule, microcrystals are formed in the water surrounding the protein molecule.

16. CHERKIN, A. & J. F. CATCHPOOL. 1964. *Science* **144**: 1460.
17. FRANK, H. S. & A. S. QUIST. 1961. *J. Chem. Phys.* **65**: 560.
18. GLEW, D. N. 1962. *Nature* **195**: 698.
19. PAULING, L. 1964. *Anes. Analg.* **43**: 1.
20. SCHOENHORN, B. P., P. O. VOGELHUT & R. M. FEATHERSTONE. 1964. *Nature* **202**: 695.

A PROPOSED WATER-PROT ITS APPLICATION TO THE TOBACCO MOSAIC V

Donald T.

The Upjohn Company

The elucidation of the primary structure of occurring polypeptides and proteins has studies have brought to light many structures such as disulfide-stabilized rings, closed decapeptides, and cyclic peptides. In addition, structures have frequently been identified which such as insulin, vasopressin and gramicidin, protein, ribonuclease,¹ the presence of disulfide linkage has been indicated.

These special structural features have been interpreted in the light of existing concepts of tertiary structure, the alpha helix or the pleated sheet.² Thus Scheraga's model³ postulates the disulfide-stabilized octapeptide loop connecting two alpha helical segments. The cyclic de-

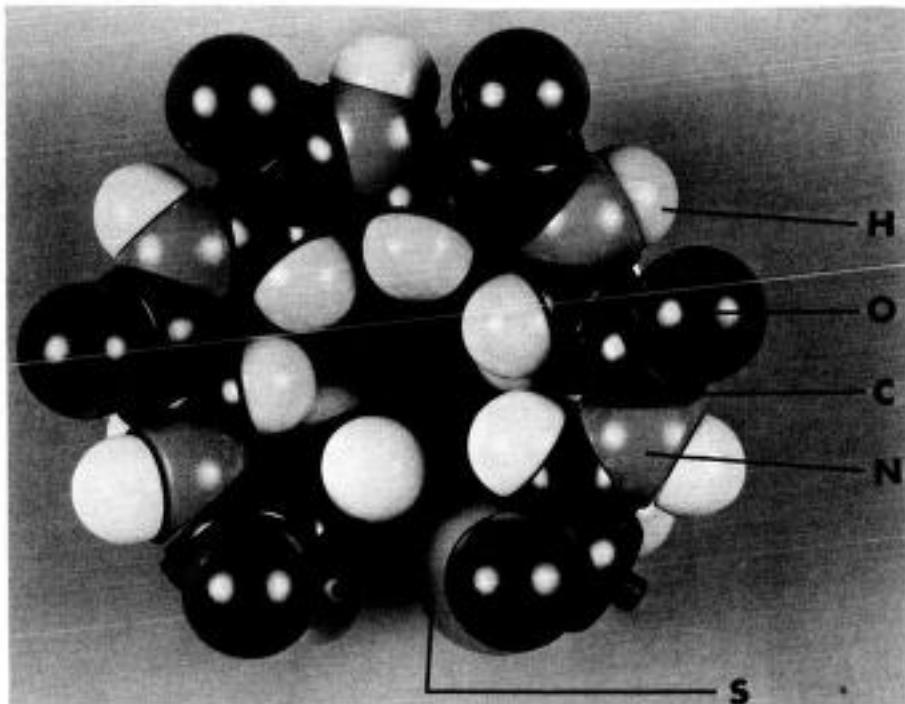


FIGURE 1. Cyclic hexapeptide ring of insulin.

A chain, it could be shown that this peptide ring can be laid out so that the peptide oxygens form a uniform hexagon.⁵ In this arrangement the carbonyl oxygens lie in a common plane as indicated in FIGURE 1, and the model of the ring shows all of the peptide linkages and the α -carbon atoms on one surface, while the other surface of the ring (not illustrated) contains mainly the side chains. The peptide surface is for convenience designated as the "hydrophilic surface" to distinguish it from the side chain or "hydrophobic surface."

The uniform hexagonal arrangement of peptide carbonyl oxygens may be readily extended to the cyclic decapeptide case by laying out the 10 peptide oxygens in the form of two fused hexagons as in FIGURE 2, a result which can be readily achieved without straining the normal bond angles. Although FIGURES 1 and 2 are simplified models with some side chains omitted to emphasize the carbonyl oxygen arrangements in print, several models of known sequences with all the side chains attached were also constructed and studied in detail.^{5,6} In the complete models incorporating these hexagonal carbonyl oxygen features, it was readily seen that the available side chain positions for the various amino acid residues also favored a consider-

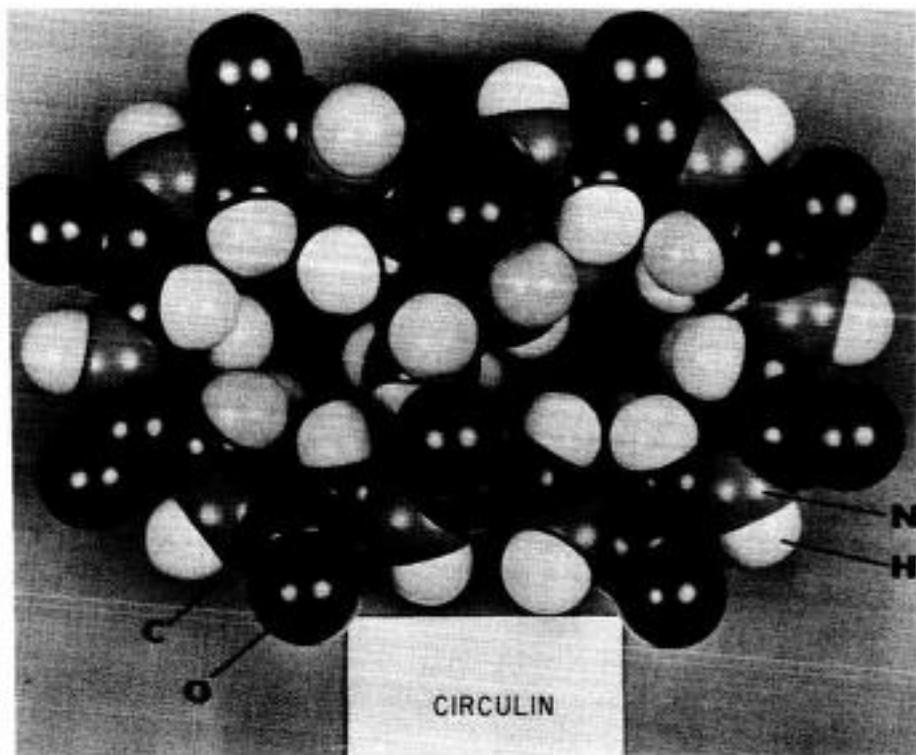


FIGURE 2. Cyclic decapeptide ring.

able number of hydrophobic and polar interactions among the side chains. Particular attention was given to the case of gramicidin S for the sake of comparison with the modified pleated sheet conformation suggested by other workers.² In the alternate hexagonal conformation for gramicidin S, this cyclic decapeptide presents a "hydrophilic surface" which is similar to FIGURE 2 except that the open hexagonal centers may be occupied by the δ -amino groups of the two ornithine side chains and two of the $-\text{NH}$ groups are replaced by the $-\text{NCH}_2-$ of proline residues. By way of contrast the "hydrophobic surface" of this molecule from the model in FIGURE 3 is seen to consist almost entirely of paraffinic or aromatic groups in very orderly and compact contact. The degree of hydrophobic contact is appreciably greater in the fused hexagon model shown here than in the pleated sheet array, and the permissible placement of the aromatic D-phenylalanine rings allows better aromatic interaction in such a way that the hexagonal model suggests a structural preference for the D-isomer.

Further details of other antibiotic and open chain peptide models in the hexagonal conformation are also included in the previous papers.^{3,6} I would

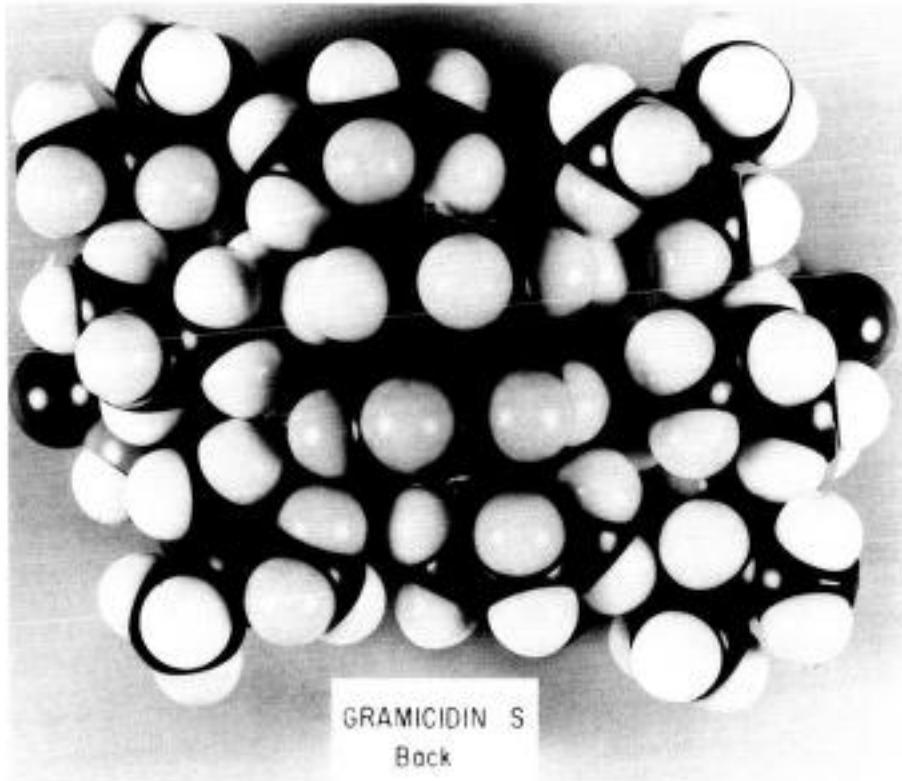


FIGURE 3. Hydrophobic surface of gramicidin S in fused hexagon conformation.

like to emphasize that within the limits of the hexagonal concept as it is applied to the "hydrophilic surface" various placements of the side chains on the "hydrophobic surface" are usually possible because of the free rotations of the various β -carbon atoms around the $\alpha\text{-}\beta$ carbon-carbon linkage; and I certainly would not care to imply that the side chain arrangements there presented are the only possible ones or necessarily the most favored ones. The model studies do indicate unmistakably that the hexagonal conformation applied to known sequences allows excellent side chain interactions on the suggested "hydrophobic surface." A most interesting and perhaps more significant observation about the hexagonal pattern of the "hydrophilic surface" was made, however, while comparing its oxygen pattern with the oxygen pattern in an ordered water structure. It was clearly seen that the second neighbor oxygens of the ice lattice also form a honeycomb pattern of regular hexagons which are also coplanar, and even more striking was the realization that the hexagonal water pattern and the hexag-

Warner: Water-Pro

onal peptide oxygen pattern are almost gen-oxygen distances of about 4.8 Å. The tide layer in our suggested hexagonal peptide layer by exactly positioned col bond strength for each bond and a maxi presented itself as an exciting way of b picture in a useful structural sense, w mutual water-protein stabilization by oxygen patterns. Therefore, we immed hexagonal principle of chain conformat

In our initial efforts with open chain tained perhaps one or two hexagons; bu was of interest not only to study the sid phobic" side but also to see whether the on the "hydrophilic" surface could be sequence which I have thus far construct is the B chain of insulin. Starting with chain, the respective carbonyl oxygens hexagon (positions 1 to 6), then an adj using carbonyl oxygen positions 7 throu tions 1 and 6 of the first hexagon, proc

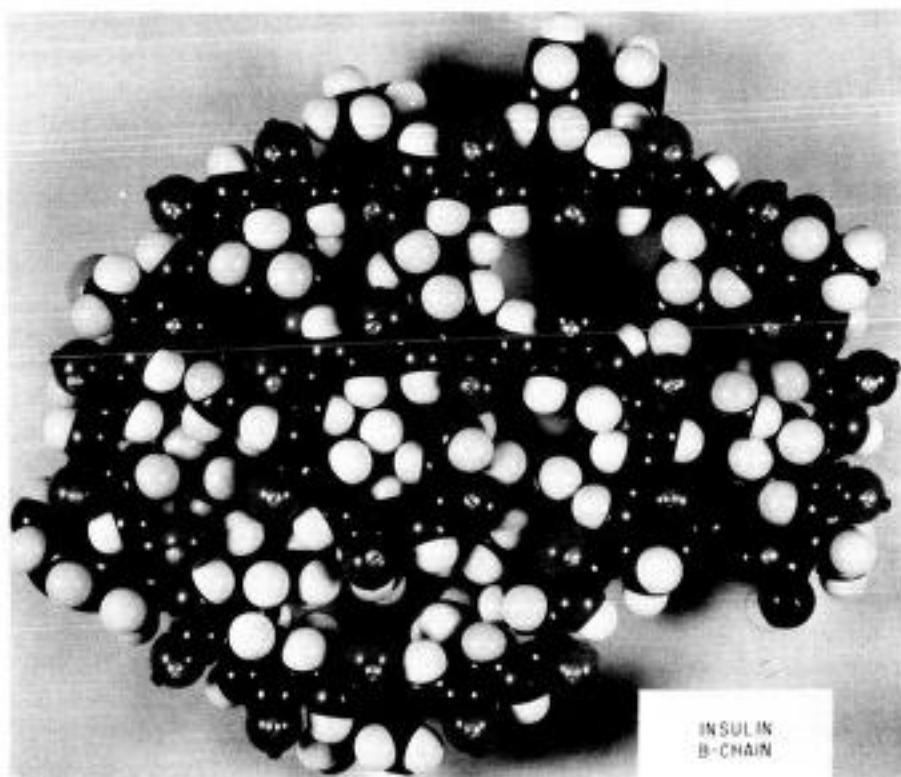


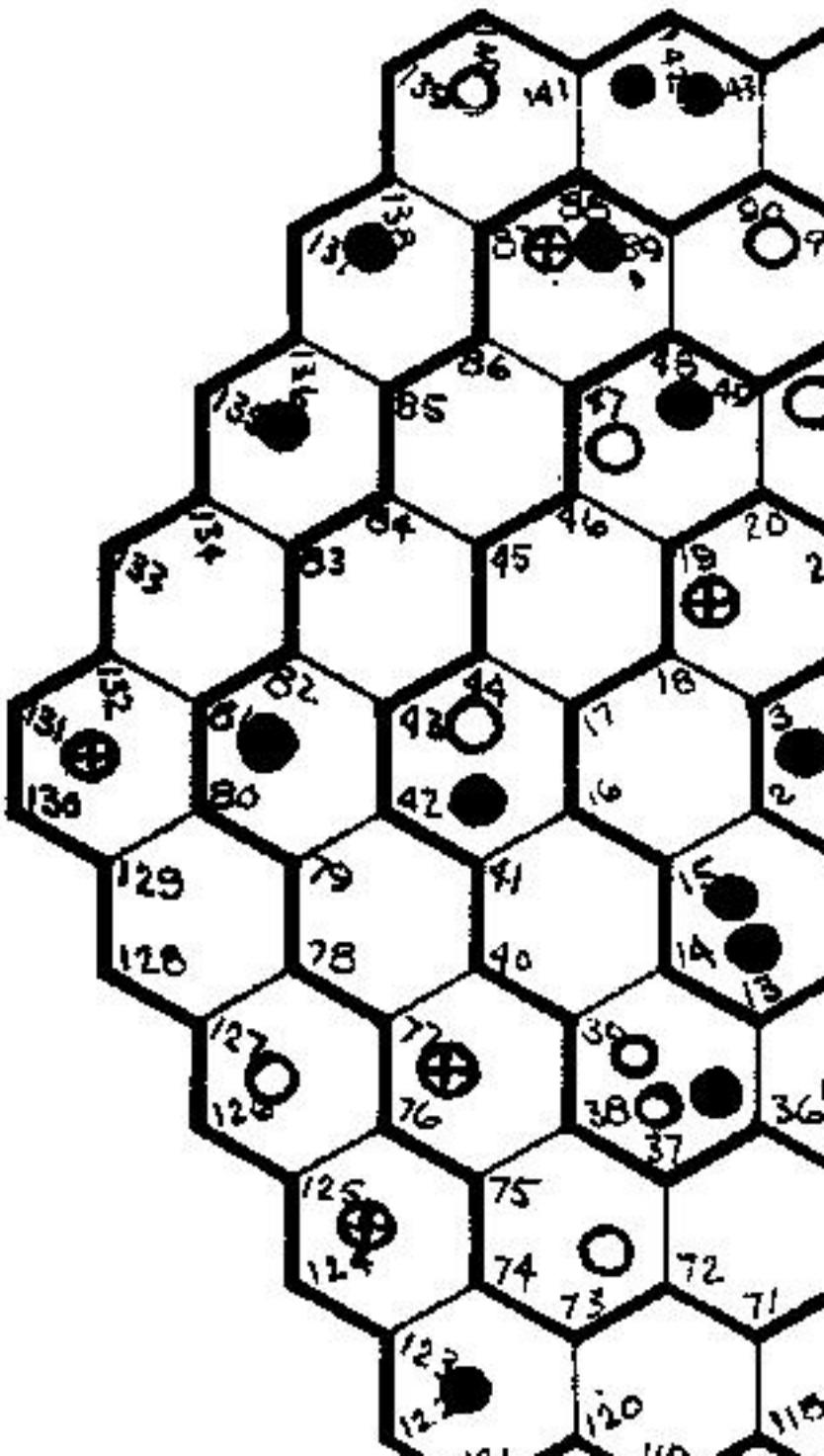
FIGURE 5. Catalin model of B chain of insulin (hydrophilic surface).

hexagonal conformation serve to form the corner positions of nine contiguous regular hexagons of a honeycomb pattern (letters A-I). In FIGURE 5 an actual model of the B chain of insulin as constructed with Catalin models ("hydrophilic surface") is shown in the same relative position as in FIGURE 4. In spite of small discrepancies in the bond angles of some of the model atoms, it is quite feasible to arrange the model chain to conform to the pattern of FIGURE 4. The actual model (FIGURE 5) suggested an interesting possibility about the -NH groups of the various peptide bonds. It is readily appreciated that in the second neighbor oxygen pattern of water there will be an oxygen position above the center of each hexagon which will not be contacting a peptide carbonyl oxygen. Although in some instances this water oxygen could be contacting polar side chain groups projecting into the hexagonal center, as I have already suggested,⁵ the peptide -NH groups offer another possible bonding pair. Each of the nine hexagons except A and B has two peptide -NH groups pointing into the hexagonal central area to allow the bonding of an additional oxygen of

Warner: Water-Pro

water there. Hexagon B could employ one or both of the two NH groups for water bonding in a similar manner. Hexagon A has one NH group which, while not quite so favorable. In any event the hexagonal principle of assembly of the subunits of the B chain has inherent possibilities for satisfying the requirement for each and every -CO-NH- bond by a hydrogen bond to a water molecule. Another item of potential importance is the position of the -CH₂- groups of the B chain. Judging from their position in a Dreiding stereomodels, α -hydrogens are in proper distance to hydrogen bond to water molecules within each hexagon. Sutor⁷ has recently suggested the possibility of C-H---O hydrogen bonds, especially between a hydrocarbon chain and a hetero atom, and has suggested the importance of such systems.

The favorable results with the B chain, the hexagonal principle of assembly of the subunits of the B chain, and the possible water stabilization provide the basis for applying the hexagonal principle to an actual problem. The problem is the stabilization of the subunit by water molecules during the study of subunit assembly to form the virus particle in the medium of water cementing as a lamellar structure. In the case of tobacco mosaic virus (TMV)



Warner: Water-Pro

of carbonyl oxygens, forming exact equivalent layer of second neighborhood, yields a sort of laminated structure of the two interacting partners, perhaps members of a geodesic dome but in any case of the proposed subunit contains nonpolar surface capable of promoting side chain attachment, maintaining the proposed subunit in the correct orientation. Of equal importance, this surface also provides a plane studded with interacting groups for complementary zones of side chain attachment of other protein subunits. Various models are elucidated in the complete manuscript.

We may now ask whether it is feasible to use this shape and size in constructing a model. The dimensions of the subunit clearly allow for a central core, three of the models are shown with the same point of each subunit oriented between subunits in this FIGURE is the bridging of the peptide oxygens of the otherwise repel each other upon close approach. "combination of three" has its own

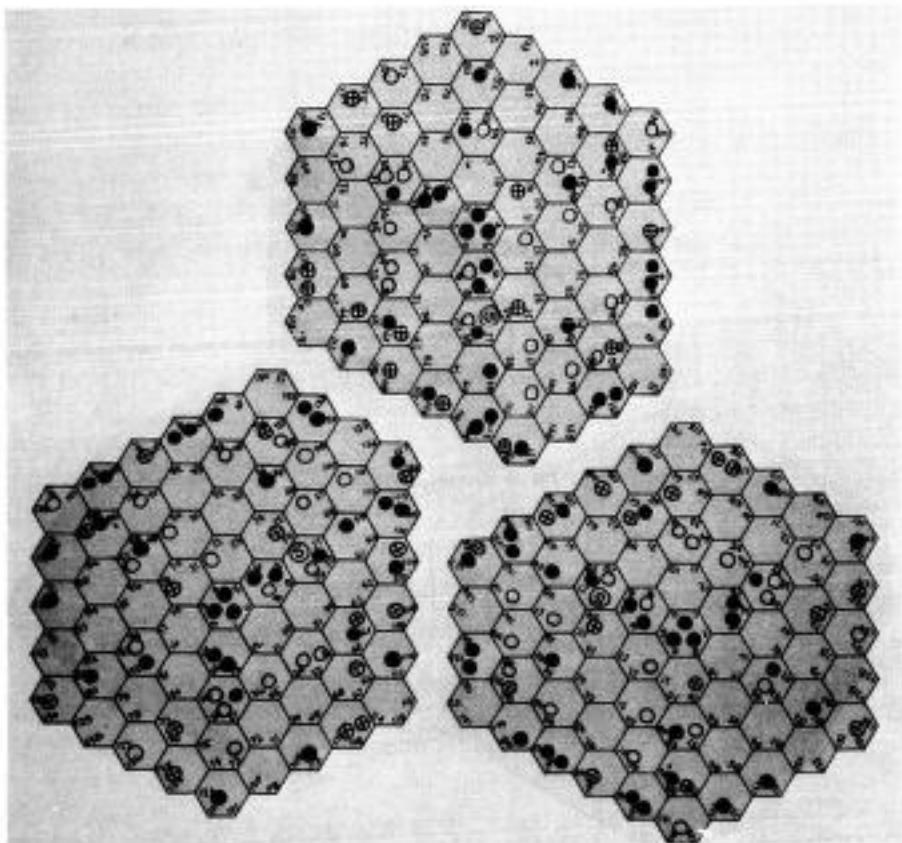


FIGURE 7. Radial combination of three TMV subunits.

- (c) The postulated side chain combinations (amide-amide, amide-carboxyl, etc.) suggested by Buzzell¹² are frequently encountered as indicated by the code symbols in the overlap zones with the hexagonal paper models.¹¹
- (d) The total of six subunits in the assembly is consistent with the value for the so-called "A-protein" of Schramm¹³ although the possibility that "A-protein" may not be a single entity has recently been presented by Caspar.¹⁷

If we now take the assembly of six subunits indicated in FIGURE 8 and picture it schematically as in FIGURE 9 to give a slightly exaggerated feeling of the thickness of the individual subunit layers, we arrive at a staggered sandwich model where the hydrophobic surface forces (polar, nonpolar, perhaps some ionic) cement the two slices together, and the upper and lower surfaces are pictured as hydrophilic hexagonal arrays of peptide

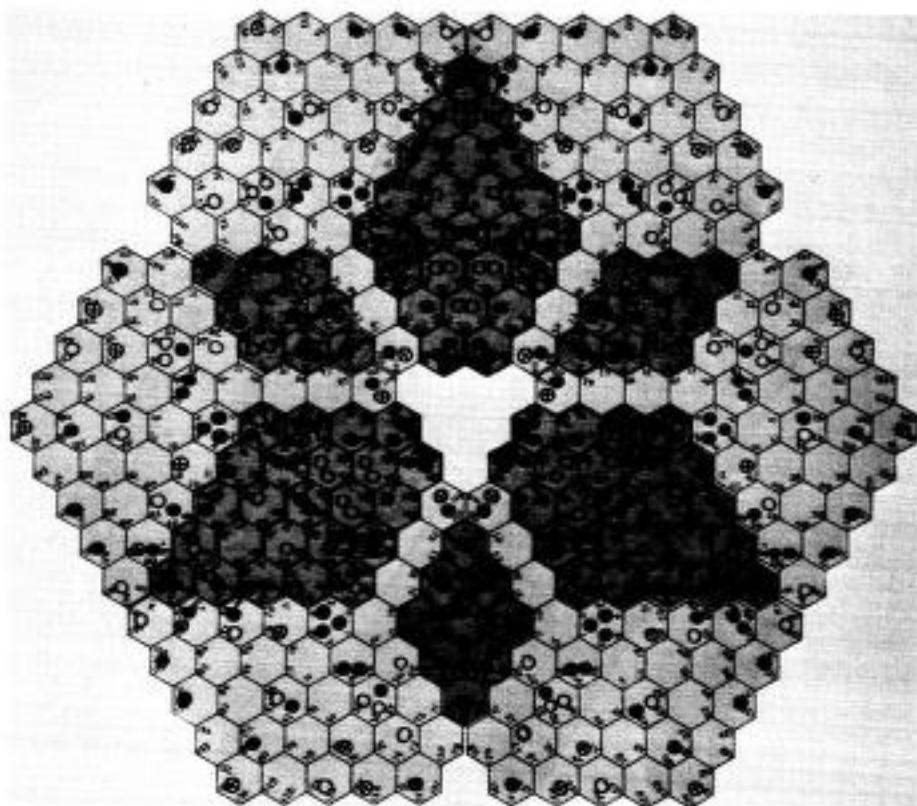


FIGURE 8. Hydrophobic overlap of two radial combinations in schematic "A-Protein" model.

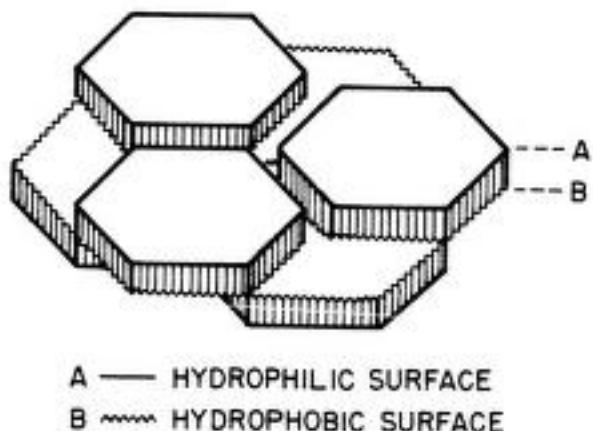
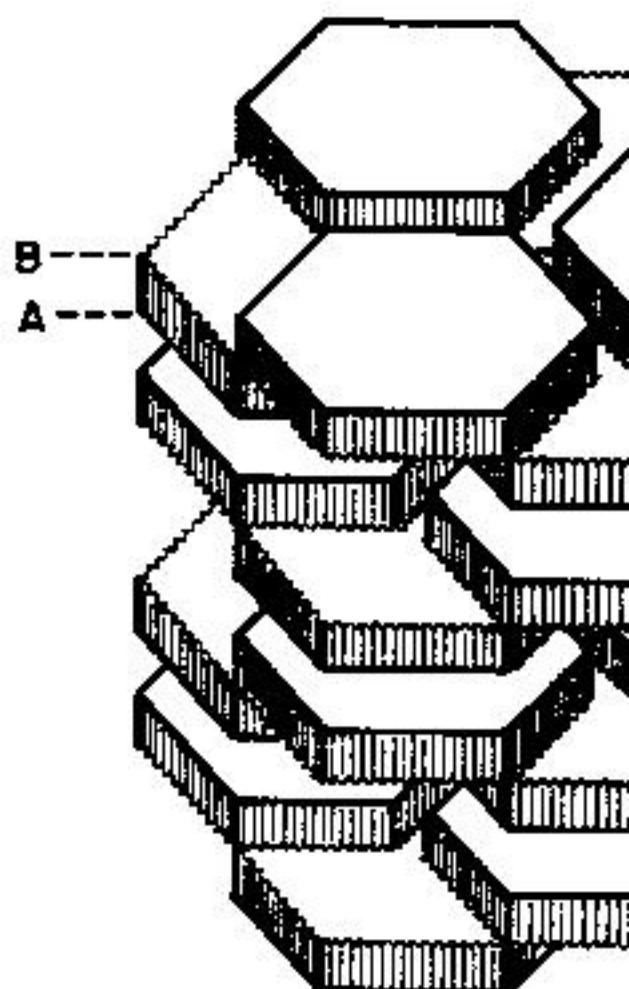


FIGURE 9. Three-dimensional schematic "A-Protein" model.

oxygens which may be water-coated in an aqueous environment. Presuming FIGURE 9 is a working model for "A-protein", how can we postulate the formation of the rod-like particles from such building units? This author has already indicated that the respective peptide carbonyl oxygens are about 4.8 Å. apart, and as a refinement of this distance has stipulated the possibility that this distance may vary between about 4.76 Å. and 4.90 Å. for the *cis* and *trans* forms of the peptide bonds respectively. Since the second neighbor oxygen distance in water should also vary with temperature, water must be warmed to about 25-30°C. to achieve an average second neighbor distance of about 4.76 Å., based on the first neighbor oxygen value of about 2.94 Å. at 30°C. determined by Brady and Krause.¹² Therefore, the hexagonal peptide array of the TMV subunit (FIGURE 6) and the hexagonal second neighbor oxygen pattern of water should begin to be dimensionally similar at about 25-30°. Although water undoubtedly also bonds to proteins at lower temperatures; if the respective hexagonal patterns are not dimensionally similar (with the water pattern theoretically smaller below about 25°) so that the bonds are not exactly collinear, the net effect based on a mechanical analogy would be a tendency to produce a concave surface for the protein subunit. Such a water-layered concave surface would have only a small contact area with other similar concave surfaces, just as the concave surfaces of two water-wet watch glasses make contact only at the edges. However, at temperatures where the water and peptide patterns are dimensionally similar and the cooperative forces might theoretically be expected to yield a planar water-layered peptide surface, one protein subunit can be expected to have a large intimate contact with another similar planar subunit while requiring only a monolayer of ordered surface water between them. A crude analogy would be two glass plates held in close and firm contact by a few drops of water spread in a thin layer between their contacting surfaces. In the case of the protein layers, the analogy would perhaps be further refined to include electrostatic attractions capable of being effective between two planar surfaces in close contact which would not be allowable when the two surfaces were concave and consequently separated by a considerable distance at certain points. These probable electrostatic interactions would also be influenced by the pH of the medium and the isoelectric point in the protein situation.

Going back now to the probable model of the "A-protein" of TMV in FIGURE 9, if this material in aqueous solution at the proper pH and at a relatively low temperature (*ca.* 10°C.) is gradually warmed to about 30°, the average second neighbor water distances will gradually approach the hexagonal peptide distances of about 4.8 Å. At the optimum water temperature the hydrophilic surfaces of the upper (or lower) TMV subunits of the proposed "A-protein" model would be presumably exactly planar and

Warner: Water-Pr



A — HYDROPHILIC
B ~~~ HYDROPHOBIC

FIGURE 10. TMV protein rod.

is incompatible with the idea of a rod, a discrepancy which suggests structure which still need to be re-evaluating the possibilities of any model.

In addition to the axial array of subunits shown in FIGURE 10, it is also possible to examine the edges of the subunits by overlapping electron micrographs of TMV preparations showing interlocking of particles^{13,14} although this was not carried out by Markham, Hitchborn and colleagues. The penetration of adjacent virus rods is a factor which varies with concentration and dilution and this factor can be applied to the "A-particle" of TMV in our study. The schematic diagram of the various proposed models is a rather complex one and we refer to our recent publication¹ for the benefit of the reader. For the present discussion it is adequate to say that the spacings which appear between the subunits in the overlap occur at definite easily measurable distances in the respective rods. These spacings are due to zones of lower electron density in the subunits and the ends of these lower electron density zones

Warner: Water-Pro

oxygens.²¹ Similarly, the oxygens on "second neighbor" trio in a different flexibility of the water oxygen pattern stable arrangement of the oxygen net the inositol oxygen trios may be thou and quite definitely fixed arrangement oxygens at only one particular water In this sense the inhibiting effect of life forms observed by Webb²² and the on freezing-thawing damage²³ are conc their ability to replace segments of the protein or nucleic acids under condit hydroxyl patterns of the carbohydrates less favorable ambient water oxygen stru. It is likely that the "inositol" use but the more readily available *myo*-inse tive thing about *myo*-inositol which ha in another connection. This particular c but in this instance five of the hydro hydroxyl is axial. In biological system dized at the axial hydroxyl only. The oxidized at 11.²⁴ We can conclude that

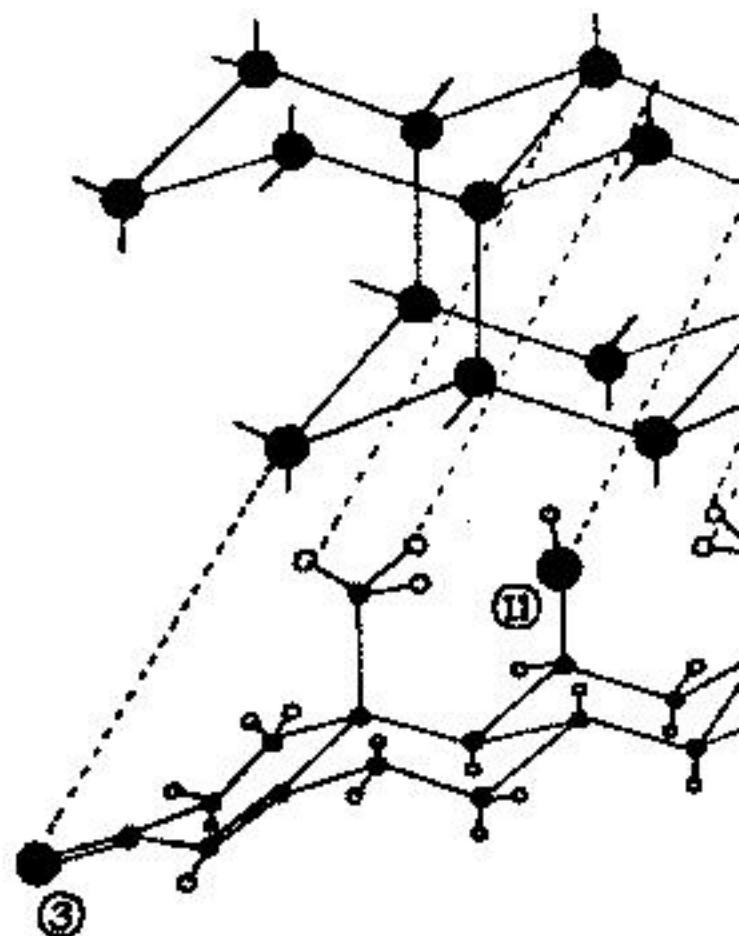


FIGURE 11. Stere

Warner: Water-Precipitated

at best only three degrees of resonance freedom with these remaining resonance freedom of the 18-CH₃ and 19-CH₃ groups. These interfere with the two near water oxygens. The dotted lines of FIGURE 11, the hydroxyl groups insert themselves by their fixed covale- tions of what had been two resonating ties in the water, thus drastically altering the atom of the 11-hydroxyl group will result in resonance pathways. Instead we find two or more former resonance outlets. (Note: These pathways are shown in the diagram as the steroid is inserted into the diamine.) In this comparison the physical models show that the degree of allowable resonance freedom of the steroid with a single water position, and the resulting resonating possibilities does result in two or more resonance pathways, then the biological activity may be inherent in the environment. It is undoubtedly true that any reactive molecule depends on its immediate surroundings, and that the ability of these surroundings to undergo short-term perturbations to more distant no-

TA

WATER-SUBSTRATE

Water model*

Substrate

a. Biotin

b. 1, 4-Quinone

stability of water at a specific location and in a particular way. The one example of a nonring compound in TABLE 1, the triglyceride of Example e, is interesting in view of current concepts of membrane structure involving protein-lipid bilayers with water layers between. If triglyceride in this conformation can interact favorably with structured water, and the protein in the hexagonal conformation can also interact favorably with this same structured water, then water should of course be theoretically capable of serving as a structural cement at protein-lipid interfaces as well as at protein-protein interfaces. The fact that the ester carbonyl is perhaps less capable of existing in the enol form than the amide carbonyl of the peptide is worth keeping in mind on a theoretical basis, since the enol form of a carbonyl is potentially capable of interaction with water in a different spacing relation than the keto form.²¹ O. Hechter and I are considering the application of these concepts to peptide hormone interactions with specific receptors in the cell membrane and their relation to the molecular organization of the cell membrane. Some of these concepts are discussed by Hechter in his paper.

Summary

The importance of water in life processes is a factor that is widely recognized but little understood. Although it is well known that protein contains a considerable quantity of bound water, neither the α -helix nor the pleated sheet models make provision for the incorporation of this water into the model structures. However, the "hexagonal" conformations of polypeptides and proteins presented for discussion in this paper contain structural features which permit the incorporation of water in a uniform manner, implying that the incorporated water is present in an ice-like lattice in hydrogen-bonded contact with each peptide oxygen and probably each -NH group as well. The hexagonal structure is capable of incorporating small cyclic rings and decapeptide structures which the α -helix cannot assimilate without distortion. In the case of the TMV protein, the hexagonal principle can be consistently applied to yield a subunit model capable of serving as a "building block" for the TMV rod.

Other biological components such as sugars, steroids and triglycerides have reactive groups which in many instances are also capable of accommodating themselves to the oxygen lattice of the water structure. Some of the possible implications of these close "fittings" are discussed in the light of their biological consequences and importance. The value of models in picturing the relationships has been stressed. It is hoped that these preliminary studies will encourage others to utilize model building in attempting to understand structural relationships, especially of known compounds in their relationship to the water structure.

Acknow

I would like to thank the editor of FIGURES 1-3 and the publishers of the use of FIGURES 6-10.

Ref

1. HIRS, C. H. W. 1960. N. Y. Acad. Sci. 93: 103.
2. PAULING, L. & R. B. COREY. 1952. Proc. Roy. Soc. (London) **141**: 11.
3. SCHMIDT, G. M. J., D. C. HODGKIN, E. M. STANLEY, & W. M. STANLEY. 1959. Proc. Roy. Soc. (London) **151**: 744; 752.
4. LINDLEY, H. & J. S. ROLLETT. 1959. Nature (London) **183**: 1030.
5. WARNER, D. T. 1961. Nature (London) **191**: 1075.
6. WARNER, D. T. 1961. J. Theoret. Biol. **3**: 111.
7. SUTTOR, D. J. 1963. J. Chem. Soc.: 1133.
8. KLUG, A. & D. L. D. CASPAR. 1960. J. Mol. Biol. **3**: 155.
9. TSUGITA, A., D. T. GISH, J. YOUNG, & W. M. STANLEY. 1960. Proc. Natl. Acad. Sci. U.S.A. **47**: 1035.
10. ANDERER, F. A., E. WEBER, H. UHLIG, & W. M. STANLEY. 1960. Proc. Roy. Soc. (London) **186**: 922.
11. WARNER, D. T. 1964. J. Theoret. Biol. **4**: 111.
12. BUZZELL, A. 1962. Biophys. J. **2**: 223.
13. WILLIAMS, R. C. 1952. Biochim. Biophys. Acta **46**: 103.
14. MATTHEWS, R. E. F., R. W. HORN, & W. M. STANLEY. 1960. Proc. Roy. Soc. (London) **178**: 635.
15. MATTERN, C. F. T. 1962. Virology **17**: 103.
16. SCHRAMM, G. 1947. Z. Naturf. **2b**: 103.
17. CASPAR, D. L. D. 1963. In: Advances in Protein Chemistry, Vol. 17, Jr. M. L. Anson, J. T. Edsall, & J. H. Fraenkel-Conrat, Eds., Academic Press, New York, p. 1.

INTRACELLULAR WATER MECHANISMS OF TRANSPORT

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Preface

The history of ideas shows that whenever a new concept is proposed which connects previously unconnected facts in a meaningful unifying configuration, another model is immediately proposed which is its antithesis. The coexistence of these two models is tolerable; the partisans on both sides, using Occum's razor, attack with data. In the course of time, the data accumulate so that one concept is victorious and the other is rejected. As time passes and more data are collected, the overall system is achieved, it then becomes apparent that the system as it has been based upon an overly simple model. In the context of new information, some ideas which were previously valid are now found to be invalid, some invalid ideas are revaluated, and others are added.

These concepts differ fundamentally with respect to the importance of the cell interior in control of the transport of substances into and out of the cell. Each concept uses a set of different assumptions concerning the state and structure of water, ions, solutes and macromolecules in the cell interior; they also differ as to how metabolic energy is utilized for transport processes. One of these concepts has been almost universally accepted, the other rejected. The concept which is generally accepted postulates that the selection and exclusion of solutes by cells is determined exclusively, or primarily, by the properties of a thin lipoprotein permeability barrier at the cell surface, first designated as the cell membrane, later as the plasma membrane. The generally rejected alternative is a holistic concept which holds that structural relationships in the cell as a whole, not just in the membrane, determine what substances enter or leave and which substances are excluded or accumulated in the cell.

The Two Concepts of Transport

Membrane thesis. The widely accepted thesis (for partial listing of reviews cf.¹⁻¹²) assumes that the interior of the cell may be considered as one (or more) relatively simple "well stirred" compartment(s) containing ions, small molecules and the polymers of the cell bounded by a cell membrane regarded as the primary permeability barrier limiting the entry and exit of substances. The bulk of the water and monovalent cations within such a cell is assumed to be "free" and in essentially the same state inside and outside of the cell. A small fraction (10-20 per cent) of the total intracellular monovalent cations may be "bound" to structural components; a fraction of cell water corresponding to "bound" ion is likewise "bound" and unavailable as solvent for permeant electrolytes or nonelectrolytes. The intracellular nondiffusible biopolymers, primarily proteins, possess sites potentially available for "binding" permeant solutes (via electrostatic forces, hydrogen-bonding, etc.), but it is categorically assumed that such binding sites are not *selective* in the sense that they are not able to discriminate between closely related chemical species, for example as between K⁺ and Na⁺, or stereoisomers in the sugar or amino acid series.

Selective binding sites are required to account for the observed selectivity of the transport of various substances in cells; these sites are classically assumed to be localized exclusively in the plasma membrane. The binding sites in the plasma membrane—few in number—serve a catalytic role in two types of transport processes, differentiated thermodynamically in terms of whether the transmembrane flow of solute is *uphill* (active) or *downhill* (passive). The transmembrane flow of specific solutes *with* the gradient is assumed to involve membrane "pores" or "channels" possessing binding sites for appropriate selectivity. Passive processes of this type do not require the obligatory coupling of metabolic

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energy with solute flow; metabolic energy is used in maintaining the membrane or "pores" in equilibrium with solute flow. The inflow of Na^+ and outflow of K^+ during the propagation of nerve serves as an example. In a depolarized nerve membrane with the gradients of substances in the cell represents translocation of Na^+ across an apparent thermodynamic gradient, which in this type are attributed to metabolically coupled Na^+-K^+ pump was the first membrane pump to be postulated; this pump moves Na^+ from an aqueous intracellular region to the external aqueous medium, while it moves K^+ from the external aqueous medium to the cell interior. Membrane pumps for amino acids were later to be postulated and were shown to be specifically accumulated by the cell. These membrane pumps represent specialized use of metabolic energy into specific osmotic work for the energetic reaction of permeant translocation, so arranged so as to permit translocation of specific solute through the membrane against an apparent thermodynamic gradient.

certain fixed negative sites in the ordered intracellular system, the sodium ion being excluded from the ordered phase. This thesis demands that selective binding sites postulated be present in stoichiometric number to account for the solute accumulated. Thus there must be enough fixed negative sites (carboxyl, phosphate, etc.) present within the cell to interact with the K⁺ accumulated within the cell. The idea utilized to account for Na⁺ exclusion can be generalized; thus all permeant substances which distribute in only a small fraction of the total water of the cell are assumed to be excluded from a greater or lesser volume of the aqueous regions which comprise the ordered unitized intracellular system.

The holistic proposals envisage a requirement for metabolic energy — primarily for the formation of the ordered selective intracellular system; once formed, maintenance of the ordered system requires only minimal energy expenditure.^{13,17} Energy is thus required for *organization* and is not directly coupled to solute flow. The ordered selective intracellular system may be disturbed, so that it becomes disordered (in part or *in toto*) and in this case selectivity of the affected region is altered. Thus, excitation (as in nerve or muscle) can be considered to alter the ordered system, so that certain negative sites lose their selectivity for potassium ions; in this case K⁺ would leave and Na⁺ enter the cell with the gradient. Reformation of the ordered lattice system requires metabolic energy and K⁺; upon reformation of the ordered system, Na⁺ would leave and K⁺ enter the cell. In effect holistic proposals envisage an energized *intracellular* mechanism coupling Na⁺ and K⁺ translocation as an alternative to a cation pump in the plasma membrane.

Present Status of the Concepts

There is insufficient space to attempt here a critical review of the data *pro* and *con*, which has been advanced to support one theory as against the other. A voluminous literature of review articles, monographs and proceedings of symposia¹⁻¹² present the record of cogent formidable arguments advanced historically in favor of the membrane concept, and against the data and theoretical proposals of the holists. The monographs of Ling¹³ and Troschin¹⁴ provide literature summaries from the minority point of view.

Perusal of the writings bearing on these opposing views reveals that, with a few notable exceptions, discussions of transport resemble the monologues of political or theological controversies, rather than scientific dialogues between the protagonists. Views are stated in monistic "either-or" form together with supporting evidence on a few cell types, extrapolated to cells generally; serious attention has rarely been given to arguments of opponents, or to the possibility of penetrating deeply behind the differ-

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ences of interpretation within the community, and a profound understanding of the problem.

It is the thesis of this presentation that a clear understanding of the principles of structural biology and biochemistry of the cell provide a basis for the pluralistic view of the cell. The conflict between these opposing concepts of the cell can be resolved only by the concurrent development of methods for isolation and biochemical analysis of the characteristic organelles of the cell — the nucleus, the cytoplasm, and the membrane. The principles of the structural and functional organization of the cell are characterized not only by uniformity but diversity. The cell biologists have developed their different models of the cell, based on the results of light microscopy; these models have been modified and refined in the course of electron microscopy despite the revolutionary changes in our knowledge of the cell, produced by electron microscopy. The models of the cell, which are employed, and diversity as well as uniformity, must be taken into account, certain ideas advanced by both schools of thought are found to be valid. In the context of the present discussion, the models of transport across membranes are seen to be complementary rather than antagonists.

As the first step in this attempted
several of the cardinal predictions of

membranes of cells generally. Indeed, it has been utilized as a marker for the presence of membrane fragments in broken cell preparations.

A second cardinal prediction of the theory is that the primary importance of the surface membrane lies in its role as a source of energy in the generation of bioelectric potentials. This prediction can now be regarded as established. Perfusion of the peripheral nerve fiber with a solution where the bulk of axoplasm have been replaced by an aqueous solution of electrolytes, e.g., sucrose, results in action potentials²³ which are in essential respects similar to those predicted by the membrane theory of Hodgkin and Huxley. The membrane theory, however, does not predict that the origin of membrane potentials is to be found in the membrane itself (cf. ²⁴), than originally envisaged in the theory of the "leaky membrane."

Concurrent with these developments, there has been considerable progress in the study of the membrane as a barrier for transport phenomenon — long believed to be of little practical interest. It has now been unequivocally demonstrated that the membrane is a highly active barrier, capable of selectively controlling the movement of substances across it. Studies of the membrane properties of intact cells have led to discovery of a particulate membrane fraction, the "microsomal" vesicular membrane fraction, which is a membrane system of the sarcoplasmic

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interior side of the inner membranes.² other divalent cations) together with p to calcium (or Mg^{++}) precipitates with chondrion. The system involved in energ in mitochondria from all cell types stud flow through the electron transfer chain considered to be localized in the inner m

Energized processes leading to the amino acids in isolated thymic nuclei ganelle is likewise membrane bounded. systems in the cell exhibit so-called a to the view³⁵ that cellular membrane pendent of whether they are at the sur possess specialized macromolecular ass energy into osmotic work.

Nature of Membrane

Let us therefore reexamine the function in the light of present information about organization principles of the cell (cf. 2

Electron microscopy has revealed that exhibit a profusion of membrane syste

of cells, was revealed by electron microscopic morphological features which the lipoprotein membrane of a general Danielli model of the plasma membrane preparations a characteristic trilaminar pattern of two narrow dense lines separated by a wide, the thickness of the trilaminar layer revealed that most, if not all, of the membranes exhibited this characteristic trilaminar formity of cellular membranes finding support for the concept of the *unit-membrane* view. This concept holds that all of the membranes are organized on a single fundamental design, the organization involves two monolayers of fully spread monolayers of non-lipoproteins — differing somehow in structure — of the membrane. The similarities and differences between the Davison-Danielli model have been discussed.

We have already seen that the morphological uniformity is expressed in the units associated with the various membranes, the diversity is expressed in profound

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in the membrane; an encouraging developmental problem has been achieved partly where periodically arranged membrane Fernández-Mórán²² and then by others²³ by the chemistry of all of the essential components of the parts of these membrane subunits. A fundamental attempt of H. Fernández-Mórán²² with their associates,²³ to correlate some properties of mitochondrial membrane systems has attracted considerable interest. Several groups are now actively working on this problem. The mechanism of active translocation of solutes through a membrane "pump" (independent of other mechanisms) is still to be determined. The progress already achieved in this field is remarkable. The mechanism of the pump for divalent cations may well be the first to be dissected and fully described.

Functional diversity in membranes may be due to more than differences in transducing units. There are also functional differences as between membranes containing mucopolysaccharide or glycoproteins which are located at the outermost membrane layers (cilia). Coatings of this type have been widely studied. The asymmetrical nature of the unit mem-

of membranes or highly ordered structures (eg. the contractile system in muscle, the chromosomes of the nuclei; (c) water within unit membranes, as in the aqueous membranes of the mitochondria, of the endoplasmic reticulum; (d) water within intermembrane spaces of the mitochondrion or between the structural components of the nucleus; (e) water in the hyaloplasm, the extracellular fluid, and throughout the cell.

The point to be made is that a single "typical" cell is intimately associated with water at the surface or *within* the membranes, and is also associated with water unassociated with membranes or other "solid" structures. Water is sharply differentiated from the water in the cytoplasm, where most of the water is mobile and may differ in some properties from the water structure of water soluble proteins. Part of the water is associated with "mobile" solutes, some of which are enzymes and certain RNA's) and part is associated with another in the hyaloplasm, eventually reaching the molecular surface (eg. a membrane). The water in the extracellular fluid, however, would resemble the types of water found in the cytoplasm.

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in various functional processes in the cell, such as those associated with the membrane potential or with the transport of ions across the membrane. In addition to the cell as a whole, Fernández-Morales' concept serves as a powerful mechanism for understanding the regulation of essential processes in the membrane. The ability of the membrane to undergo changes in ordered water structure can provide a conceptual basis for conformational changes in proteins that modify the arrangement of the polar head groups in the lipid leaflet to a less tightly packed micelle. Such changes could pass through the various phases of the membrane structure changes reversibly, and provide a local perturbation. Selective permeability of membranes can be explained by the presence of molecular sieves lined with ordered water structures. Changes induced by excitation could be due to the presence of water structures in special regions of the membrane in a hydrogen bonding medium, ordered water lattices. A fast protonic charge transport mechanism has been proposed by Eigen and DeMaeyer,⁵⁵ or in electron transfer by the hydride ion as suggested by Klotz.⁵⁶ The presence of ordered water lattices as an integral part of the membrane, as promulgated by Fernández-Morales, provides a conceptual basis for understanding a variety of mechanisms associated with membrane function.

provide substantial support for the structural link for the protein and structures.

Elsewhere I have described⁵⁰⁻⁶¹ a
trate the fundamental molecular con-
in effect a variety of ideas dealing with
ture and function, developed by us
a unifying configuration. The new re-
details of protein and water structure
concept. Starting with Warner's hexa-
tein structure (discussed by Warner⁵²)
to conceptually envisage a system
arranged in a hexameric goedesic.
of these layers being "cemented" into
a hexagonal ice-like arrangement of
unit-membrane. The hexagonal ar-
rangement which is visualized by Warner provides an ordered system of hydro-
gens of the amide bonds of polypeptides
ment which corresponds to a second
of the "ice lattice" (4.8 Å). This pos-
sive ordered system of hydrogen atoms
ranged hexagonally as in ice. War-

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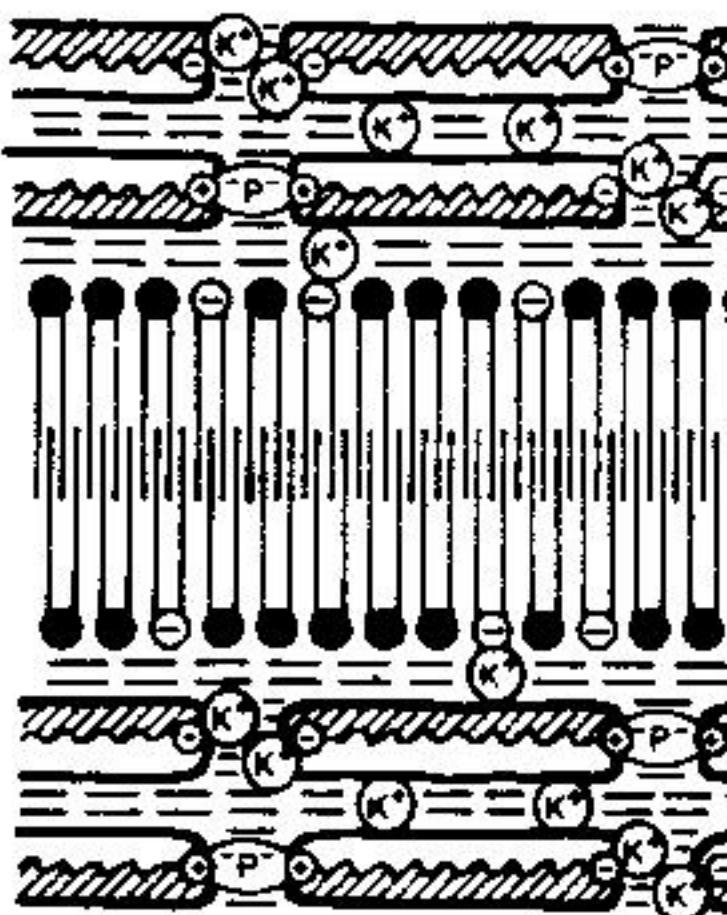


FIGURE 1. A schematic representation of the basic features of the unit-membrane concept. The subunits are represented as a system of interlocked hexagonal units surrounded by water layers in an ice-like arrangement. The individual hexagonal subunits have their hydrophobic surfaces turned inward to form "disc units" in an ice-like state, this type of water being channels in the protein layer of the membrane and are shown in a "staggered" relation-

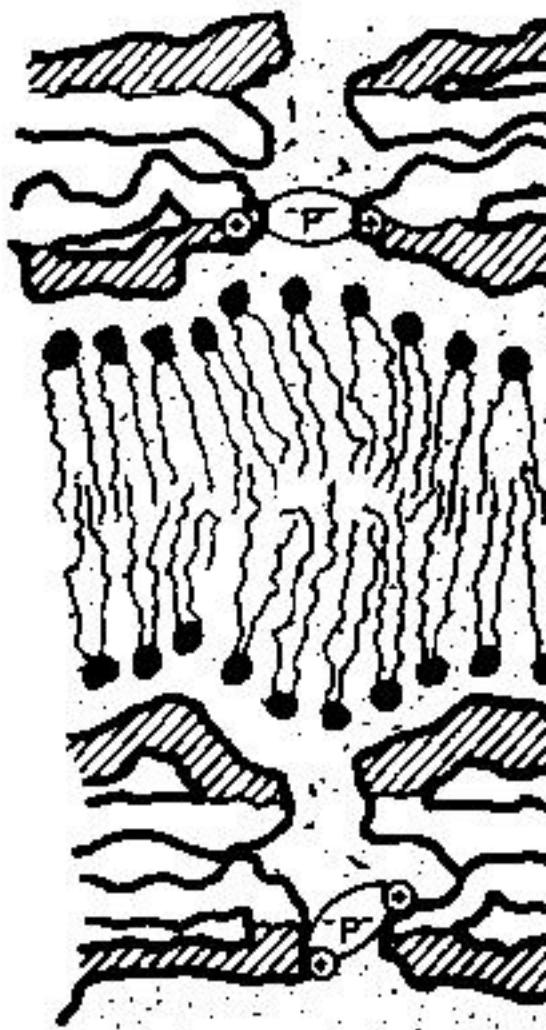


FIGURE 2. A schematic representation of the arrangement of protein subunits in a precisely ordered lattice. For purposes in the various structural components. The protein subunits have changed from helical form, the lipid bilayer to a more

Hechter: Intracellular

hyaloplasm, the intracellular water which is water assumed in the plasma membrane. Likewise, if a particular cell type is "loaded" with water, the relative volume of hyaloplasm is small. It is foundly different in structure and properties from the water envisaged in the extracellular fluid.

Given profound diversity in cell structure and in relation to membrane systems, it is apparent that the proportions of water types may be very different. It has been estimated that in some cell types, where the endoplasmic reticulum and associated membrane systems of the cell plus attached components account for as much as 80-90 per cent of the total mass, the proportion of membrane plus componentry in the cytoplasm is about 50-60 per cent. In bacterial protoplasts, where nucleoli are not present, the cell membrane accounts for 70 per cent of the mass;¹⁶ the value for the membrane in plant cells is much less than 10 per cent. These rough figures illustrate the variations in the proportions of hyaloplasm in different cell types. They illustrate the hazard of extrapolating to the water of one cell type (say erythrocytes) the properties of water in another (say, muscle). Water is an important, but largely neglected factor in cell physiology.

a more "typical" cell (where the $\frac{1}{2}$ cent of the cell volume) is more like binding to selective sites on membrane and to energized translocation devices. There are two types of selective binding sites involved in solution, neither catalytic nor stoichiometric, which would vary from one cell type to another. The first type is the extent of the intracellular membrane. The two types of selective binding sites are production devices serving a catalytic function in the membrane; the other type serves a structural sense as envisaged by the holistic view of the membrane phase.

Both types of selective binding sites are involved in calcium accumulation by the isolated vesicles of skeletal muscle. If the vesicles are energized by the addition of ATP, with Ca^{++} and a calcium precipitating agent (e.g., citrate or phate), Ca^{++} accumulation involves the membrane phase as demonstrated by the isolation of the membrane vesicle (cf.²⁷). This transport is specific for Ca^{++} since Mg^{++} , though it is required for the reaction, is not substituted. However, if vesicles are isolated under

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oxalate (or phosphate) forming egg shells and structure of intervesicular water at pH. One wonders, therefore, how activity can be estimated in an undefined aqueous medium.

The difficulty in determining whether the tubular vesicles actually represents active transport reappears in the mitochondrial system. Energized translation of Ca^{++} and phosphate leading to the precipitation of calcium within the mitochondrion (cf.^{31,60-71}). In addition to the variation of activity coefficients of ions in the interstitium, another complicating factor arises from the fact that it must be taken into account. Mitchell⁷² has indicated the participation of pyridine nucleotides, which utilize the asymmetrical discharge of protons of OH^- to the interior chamber of the membrane to move hydroxyl ions to facilitate calcium precipitation. Phosphate (or oxalate) is well known to facilitate the energized translocation of divalent cations. This is termed the energized production and accumulation of OH^- across a membrane. If the H^+ and OH^- are moved by the ATPase in sarcotubular vesicles, the

lation associated with Na^+ exclusion binding sites for K^+ , unavailable to mitochondrial membranes; the selectivity (cf. Discussion of Gamble's study) that during the energized translocation of K^+ into the cell by the membrane of liver, kidney, heart, and brain, while external Na^+ enters;¹⁵ thus the process is widely distributed in the body. It is specifically required for protein, since other cations are ineffective, suggesting that it is selective for K^+ in this system as well as in the translocation of Na^+ and K^+ against a concentration gradient in isolated nuclei,¹⁷ where fixed negative charges are available.

Thus intracellular membrane systems have binding sites for K^+ . Depending upon the membrane systems of a particular cell (and the molecular structural components) the fraction of the K^+ accumulated by the cell will depend upon the relative number of selective intracellular binding sites and the ratio of "hyaloplasm" to "membrane". The relative binding of K^+ for accumulation by the cell will depend upon the relative number of selective intracellular binding sites and the ratio of "hyaloplasm" to "membrane".

membrane but may be present in the endoplasmic reticulum as well. The recent finding of an ATPase in the sarcoplasmic reticulum of skeletal muscle¹¹ activated by either K⁺ or Na⁺, and which is not ouabain-sensitive, serves to remind us that more than one kind of membrane and more than a single type of enzymatic mechanism may be involved in the old problem of K⁺ accumulation and Na⁺ exclusion. Conway's¹² findings suggesting that the Na pump in frog skeletal muscle appears to involve a redox system pump, where lactic dehydrogenase and not ATPase participates, would be consistent with the above idea.

It must be emphasized that in specialized differentiated cells, the various membrane systems of the cell are likewise differentiated, both in structure and function. We have seen that specialized endoplasmic reticulum of skeletal and cardiac muscle fibers possesses a sequestering system for Ca⁺⁺ which is highly selective. The endoplasmic reticulum in other cell types, such as liver¹³ does not possess this system. In cells other than muscle fibers, the mitochondria appear to be the primary devices utilized to sequester Ca⁺⁺, but other divalent cations (such as Mg⁺⁺, Mn⁺⁺) are similarly sequestered. Thus, the mitochondrial system is not as selective for Ca⁺⁺ (or as fast) as the sarcotubular system in muscle. Given functionally specialized cells, exhibiting uniformity and diversity at all levels, it may safely be predicted that when the molecular mechanisms of maintaining an unequal distribution of Na⁺ and K⁺ in cells are discovered, once again a pattern of uniformity associated with diversity will emerge.

Epilogue

We have discussed the two opposing classical concepts of transport. Upon analysis, both ideas are shown to be right in part; and both partially wrong. The proponents of the plasma membrane thesis of transport were wrong in that they neglected the role of the cell interior; the holists were wrong in their deemphasis of the plasma membrane and of intracellular membranes generally. If one considers that membrane systems throughout the cell are involved in transport, and that cells exhibit diversity as well as uniformity, a pluralistic resolution is achieved. This conclusion, so obvious to me today, was first presented to me in 1959 by a friend of many years, Theodore Sheldovsky of the Rockefeller Institute. We had been discussing the nature and structure of water in the cell, the polyelectrolyte character of intracellular macromolecules and certain findings in my laboratory, all of which seemed to me to be wholly inconsistent with the plasma membrane thesis. Sheldovsky agreed. However, I also knew of other data which almost forced one, as in chess, to the plasma membrane thesis. Sheldovsky agreed. And then he said: "What if what we call transport involves a membrane system *throughout the cell*, so that what we call the cell membrane is really packed inside the cell as well as at the surface?"

I heard then, but did not really listen. Monistic configurations which are all encompassing are so powerful in attractiveness that it was only after several years that I came to understand what Sheldovsky had said.

References*

- HARRIS, E. J. 1960. In *Transport and Accumulation in Biological Systems*. 2nd Ed. Butterworth Scientific Publications. London, England.
- CHRISTENSON, H. N. 1962. In *Biological Transport*. W. A. Benjamin. New York, N. Y.
- HODGKIN, A. L. 1958. Proc. Royal Soc. (B) 148: 1.
- LEAF, A. 1959. Ann. N. Y. Acad. Sci. 72: 396.
- GLYNN, I. M. 1959. Internat. Rev. Cytology 8: 449.
- WILBRANDT, W. 1961. Internat. Rev. Cytology 13: 203.
- SHANES, A. M. 1959. Pharm. Rev. 10: 59.
- WHITTEM, R. W. 1959. Ann. Reports Chem. Soc. 72: 396.
- EDELMAN, I. S. 1961. Ann. Rev. Physiol. 23: 37.
- WILBRANDT, W. 1959. J. Pharm. Pharmacol. 11: 65.
- PARK, C. R., D. REINWEIN, M. J. HENDERSON, E. CADENAS & H. E. MORGAN. 1959. Amer. J. Med. 26: 674.
- RANDLE, P. J. & F. G. YOUNG. 1960. Brit. Med. Bull. 16: 224.
- LING, G. N. 1962. In *A Physical Theory of the Ling State: The Association-Induction Hypothesis*. Blaisdell Publishing Co. New York, N. Y.
- TROSCHEIN, A. S. 1958. In *Das Problem der Zellpermeabilität*. Fischer. Jena, Germany.
- ERNST, E. 1958. In *Die Muskeltätigkeit; Versuch einer Biophysik des querge streiften Muskels*. :355. Verlag Ungarischen Akad. Wissenschaften. Budapest, Hungary.
- SIMON, S. E., F. H. SHAW, S. BENNETT & M. MULLER. 1957. J. Gen. Physiol. 40: 753.
- HECHTER, O. & G. LESTER. 1960. Recent Progr. Hormone Res. 16: 139.
- HOFFMAN, F. 1962. Circulation 26: 1201.
- STRAUB, F. B. 1954. Acta Physiol. Hung. 4: 235.
- POST, R. L. & P. C. JOLLY. 1957. Biochem. Biophys. Acta 25: 118.
- SKOU, J. C. 1961. In *Membrane Transport and Metabolism*. A. Kleinzeller & A. Kotyk, Eds.: 228. Academic Press, Inc. New York, N. Y.
- KINSOLVING, C. R., R. L. POST & D. L. BEAVER. 1963. J. Cell Comp. Physiol. 62: 85.
- BAKER, P. F., A. L. HODGKIN & T. I. SHAW. 1961. Nature (Lond.) 190: 885; 1962. J. Physiol. 164: 355.
- HODGKIN, A. L. & A. F. HUXLEY. 1952. J. Physiol. 117: 500.
- TASAKI, I. & T. SHIMAMURA. 1962. Proc. Nat. Acad. Sci. (U.S.) 48: 1571.
- FRANZINI-ARMSTRONG, C. 1964. Federation Proc. 23: 887.
- HASSELBACH, W. 1964. Federation Proc. 23: 909.
- WEBER, A., R. HERZ & I. REISS. 1964. Federation Proc. 23: 896.
- DAVIES, R. E. 1963. Nature (Lond.) 199: 1068.
- PODOLSKY, R. J. & I. L. COSTANTIN. 1964. Federation Proc. 23: 933.
- LEHNINGER, A. L. 1964. In *The Mitochondrion*. : 157. W. A. Benjamin, Inc. New York, N. Y.
- PALADE, G. 1964. Proc. Nat. Acad. Sci. (U. S.) 52: 613.
- FERNANDEZ-MORAN, H., T. ODA, P. V. BLAIR & D. E. GREEN. 1964. J. Cell Biol. 22: 63.

*The references listed in this conceptual survey of transport and accumulation represent a small selection of the voluminous literature bearing on this field. Many important papers and reviews necessarily have had to be omitted in this type of treatment.

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34. ALLFREY, V. G., R. MENDT, J. W. Hoschek & J. D. Robertson. 1960. Proc. Natl. Acad. Sci. (U.S.) **47**: 907.
35. LEHNINGER, A. L. 1964. Neuroscience and Behavior. Academic Press, Inc., New York.
36. DAVSON, H. & J. DANIELLI. 1952. In *Handbook of Physiology*. Vol. I. Ed.: 2nd Ed.: 111. Cambridge University Press, London.
37. ROBERTSON, J. D. 1957. J. Physiol. **140**: 297.
38. ROBERTSON, J. D. 1959. Biochem. Soc. Trans. **7**: 103.
39. ROBERTSON, J. D. 1960. Progr. Biophys. **5**: 131.
40. ROBERTSON, J. D. 1962. Res. Pub. Assoc. Amer. **1**: 101.
41. ROBERTSON, J. D. 1964. In *Cellular Biology*. Ed.: 24. Academic Press, Inc. New York.
42. SIEKOWITZ, P. 1963. Ann. Rev. Physiol. **24**: 101.
43. FERNÁNDEZ-MÓRAN, H. 1957. In *Metabolic Pathways in the Cell*. Ed.: 1. Pergamon Press, London, England.
44. FERNÁNDEZ-MÓRAN, H. 1959. Rev. Mod. Phys. **31**: 101.
45. FERNÁNDEZ-MÓRAN, H. 1959. In *Biochemistry of the Cell*. Ed.: L. Onley, Ed.: 319. John Wiley & Sons, New York.
46. FERNÁNDEZ-MÓRAN, H. 1962. Circulation **25**: 101.
47. FERNÁNDEZ-MÓRAN, H. 1964. J. Royal Microscopical Society **83**: 101.
48. PARSONS, D. F. 1963. Science **140**: 985.
49. SMITH, D. S. 1963. J. Cell Biol. **19**: 115.
50. STOECKENIUS, W. 1963. J. Cell Biol. **16**: 101.
51. FAWCETT, D. W. 1962. Circulation **26**: 101.
52. SCHMITT, F. O., R. L. BEAR & K. J. HARRIS. 1964. J. Cell Biol. **18**: 31.
53. FINEAN, J. B. 1957. J. Biochem. Biophys. **42**: 101.
54. SZENT-GYÖRGYI, A. 1957. In *Bioenergetics*. Ed.: 1. Academic Press, Inc., New York, N. Y.
55. EIGEN, M. & L. DE MAEYER. 1959. In *Bioenergetics*. Ed.: 1. Academic Press, Inc., New York, N. Y.

73. BONTING, S. L. & L. L. CARAVAGLIO. 1964. *Science* 144: 125.
74. GAMBLE, J. J. JR. 1962. Am. J. Physiol. 202: 1030.
75. LEHNINGER, A. L. 1964. Abst. 6th Intern. Congr. Biochem. 1964. Federation Proc. 23: 623.
- 75a. LURIN, M. 1964. Federation Proc. 23: 623.
76. TANAKA, R. & L. G. ABOOD. 1964. *Science* 144: 125.
77. ENGEL, A. G. & L. W. TICE. 1964. *Science* 144: 125.
78. CONWAY, E. J. 1963. Nature (London) 200: 125.
79. SCHUEL, H., R. SCHUEL & L. LORBER. 1964. *Science* 144: 125.

RAPID FREEZING AND

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Techniques for the preservation of blood for long periods of time are needed today, more than ever before, in the event of major disasters and fluctuations in blood supply. With increasing medical specificity of blood and the ever-increasing cost of blood banking, the banking of sufficient supplies of universal blood is of great importance. Since presently accepted methods of preservation may be used within three weeks after collection, it is difficult to maintain blood for much longer than this period.

For long-term preservation blood must be maintained at temperatures below -100°C . In this temperature range the chemical and biological activity becomes negligible. It has been shown that temperatures below -100°C . are suitable for the long-term storage of blood without evidence of damage.

Luyet¹⁰ and Meryman and Kafig and thawing of blood in thin films form using liquid nitrogen as a red blood cells with and without firmed in our laboratory.¹¹ Subse rates of cooling (several degrees second) with small amounts of e torily inhibited cell hemolysis.

Experiments primarily with th than 10 cc. of blood were used to freezing process. When transfusio of mean molecular weights between vide protection at concentrations erties of the red cells, research w of blood sufficient for *in vivo* red

Processing, minimally, 50 cc. of producible results, involved extensive biophysical, engineering and medical studies of containers for freezing for sterile transfer and freezing a with respect to the biochemistry, erythrocytes. These studies have

quantity of blood to be frozen.¹² For processing 50 cc. of blood a flat-walled container measuring approximately 7 x 8 cm. is used. For half and one pint units of blood the walls are corrugated in order to increase the surface area available for the transport of heat.

Freezing

It was determined early in the study with PVP-protected blood that changes in cooling rates only at the interface between the container surface and the liquid nitrogen were not sufficient for optimal recovery of undamaged cells and that agitation of the blood during phase change was a necessity. FIGURE 1 shows the effect of agitation rate on cell recovery. Fifty cc. samples of blood were frozen in rectangular aluminum containers of 110 cc. capacity. Identical thawing conditions were utilized for all samples. This FIGURE shows that direct recovery of the red cells was not significantly affected. Saline resuspension recovery, however, increased

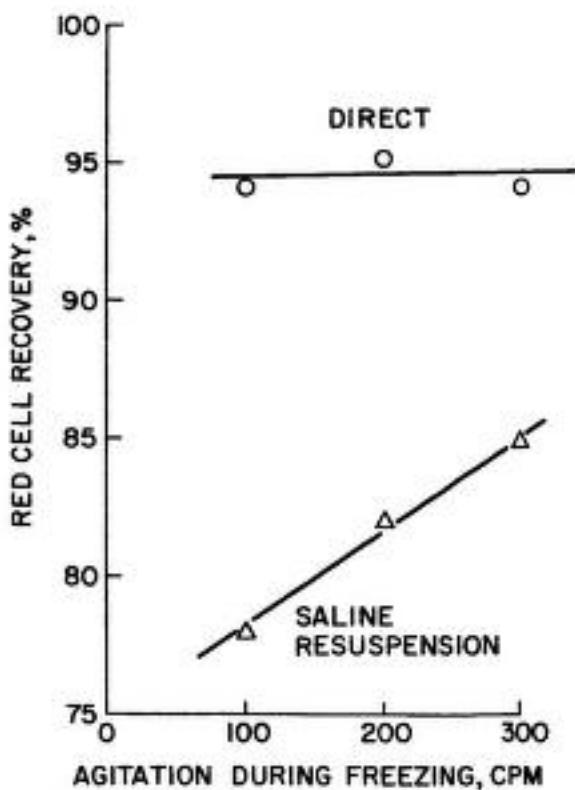


FIGURE 1. Effect of agitation rate of the "Linde" Blood Processing Unit on recovery of 50 cc. samples of blood frozen in a rectangular aluminum container of 110 cc. capacity.

significantly with increase in agitation is a measure of the cells which remaining and resuspension in an excess volume of saline solution. These data suggest process is essential when volumes of milliliters are frozen in containers of saline resuspension recovery is not except as a limiting value.

Container surface modifications to container to boiling liquid nitrogen can experiments. With proper control of cell recoveries can be attained by agitation. This is very important in terms of life of the processing equipment by the higher frequency of agitation.

Changes can be made in the rate of temperature of a boiling cold liquid by material to be cooled.^{15,16} TABLE 1 shows films on the cooling rate of a metal probe in liquid nitrogen. The coatings are a liquid solution then vaporizing the prior to immersion in the liquid n

At this point, let us digress and consider how to increase cooling rates by actually increasing the use of insulating films. Several situations exist where heat transfer occurs from a warm surface to a boiling liquid. In one case, the temperature difference between the surface and liquid is increased and boiling occurs heat transfer is increased and boiling occurs heat transfer due to the agitation of the liquid. At the same time, nucleate boiling provides maximum heat transfer for a given temperature difference. A highly insulating film results in lowered rates of heat transfer. In blood freezing, therefore, it is to lower the temperature of the liquid to the nucleate boiling range. Cooling rates are higher allowable rates of heat transfer.

To obtain the recovery data shown in Figure 1, we used aluminum containers using an agitator. As shown there exists an optimum rate of agitation without a coating and the maximum rate of agitation with a coating. Available techniques do not allow accurate measurement of the heating and cooling rates in the various regions of the graph under different conditions of agitation. Hence such data as heating and cooling rates are critical, these data should be obtained under controlled conditions.

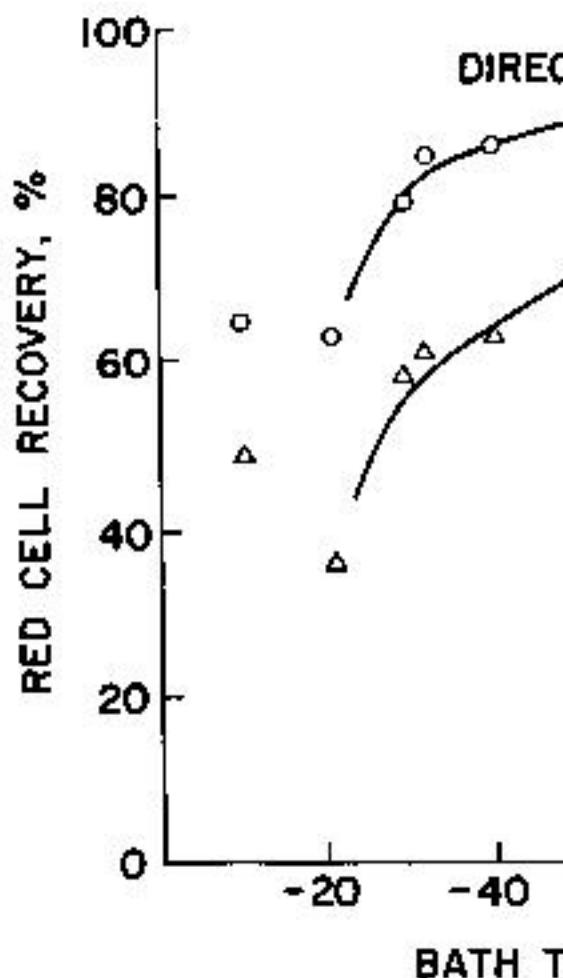


FIGURE 2. Recovery of red cells frozen in a liquid, nonboiling refrigerant manner. Thawing conditions were all identical.

conventional procedure in transparency of half mil (0.0005 inch).

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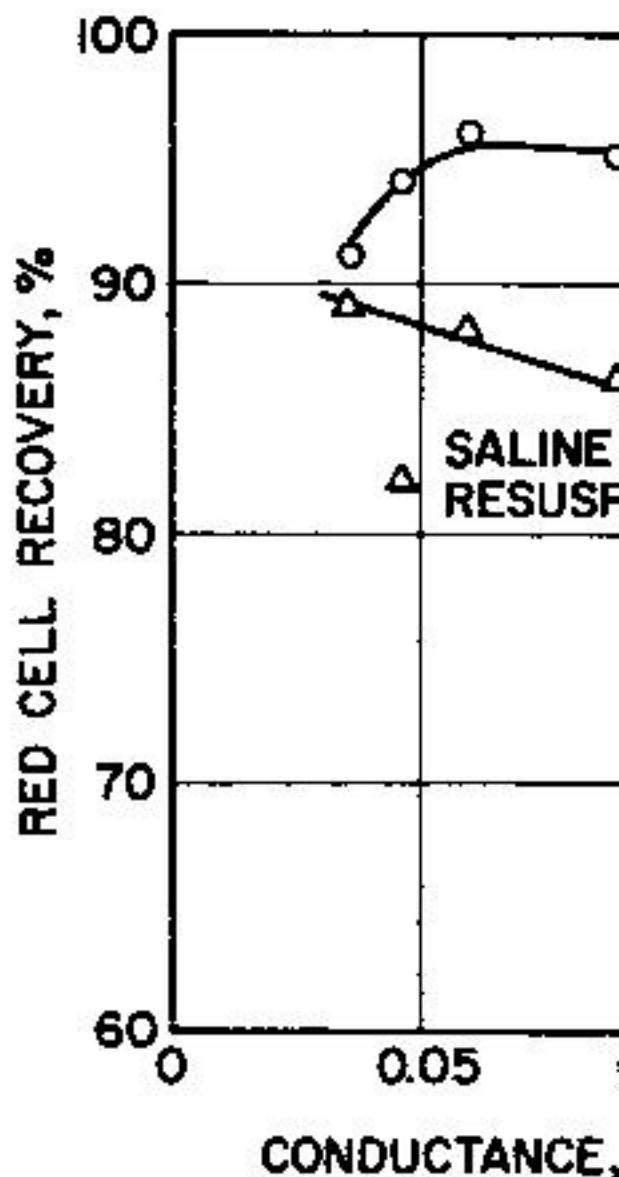


FIGURE 3. Recovery of red cells frozen at different conductances. The recovery of red cells depends on the freezing rate, thickness and resistance to heat transfer during freezing and thawing thus reducing the resistance of

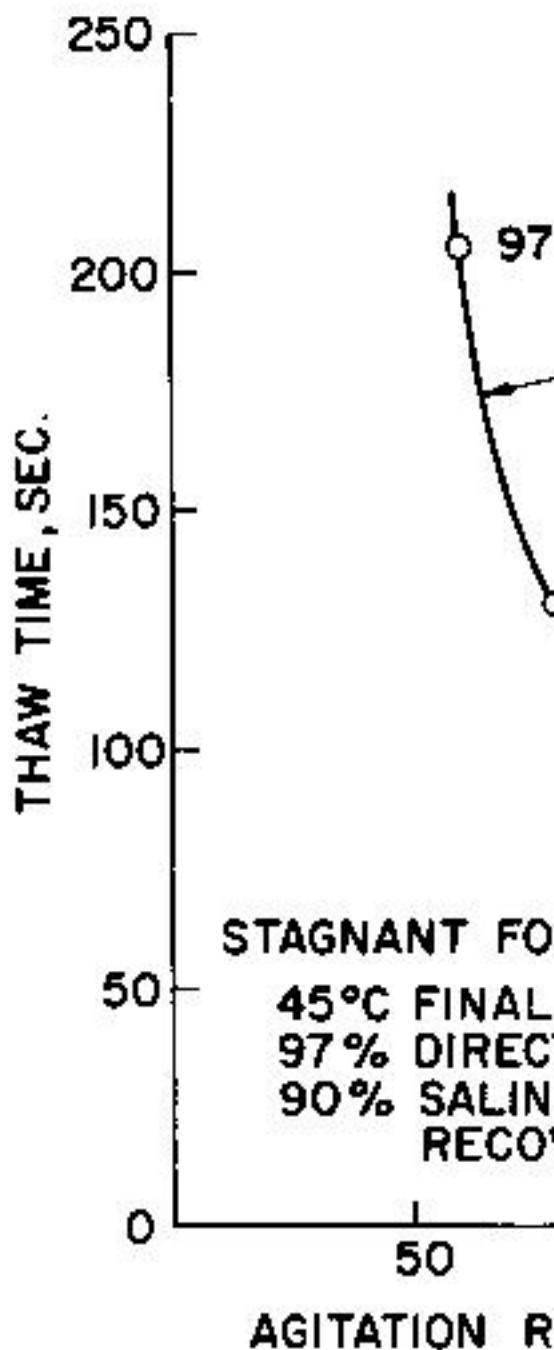


TABLE II
EFFECT OF THAWING RATE

Thawing conditions	No. tests	
machine—150 cpm.	6	
machine—75 cpm.	2	
manual—gentle	3	

Specimens thawed in 45° C. water.

blood have resulted in survival data with five per cent for the testing of a given

TABLE 2 presents data showing the *in vivo* red cell survival. Fifty cc. quantities in 110 cc. rectangular aluminum containers were used for all specimens. The effect of the use of different agitation conditions was determined by varying the decreasing rates of heat transfer. All experiments were conducted at 45° C. However, *in vitro* survival of the thrombocytes was measured at 37° C. The average value for the 24-hour survival

plastic, commercially available containers were used. These were rectangular with variable volumes. In our study of processing parameters we found *in vitro* results equivalent to the best values reproducibly obtained. Of course the freezing rates were lower in the plastic than the metal containers, but the cell recovery values were identical. Cell viability was much impaired with the slower thawing rates.

Discussion

The long-term preservation of blood is a problem which has been studied in detail if useful yields of viable cells can be obtained. At low temperatures, volumes of blood which approach the volume of a single cell can be frozen. We find that simple scale-up of experiments in fractions of a ml. up to 50 ml. is feasible and results in intact red cells. At the same time it is difficult to obtain information concerning the distribution of freezing damage in the different regions of a sample. Empirical methods were thus used to determine the best freezing and thawing procedures.

We have presented here a few aspects of one facet in the study of cryobiology.

Sakaida et al.: Rapid Freezing of Human Blood

References

1. BLOOM, M. L., A. P. RINFRET, E. WILSON & T. M. BOW. 1962. Frozen and thawed blood. Proc. 8th Congr. Int. Blood Transf. (Mexico), 1962.
2. HAYNES, L. L., J. L. TULLIS, H. M. PEARCE & C. TURVILLE. 1960. Clinical use of frozen plasma. *Transfusion* 3(12): 1657-1663.
3. HAYNES, L. L., W. C. TURVILLE, M. T. BLOOM & A. P. RINFRET. 1962. Long term blood preservation. *Transfusion* 3(12): 1664-1670.
4. HUGGINS, C. E. 1963. Prevention of haemolysis of erythrocytes when rapidly frozen and thawed in polyvinyl chloride bags. *Transfusion* 3(6): 483-493.
5. HUGGINS, C. E. 1963. Preservation of human erythrocytes in dimethyl sulfoxide and a novel way of freezing. *Transfusion* 3(6): 193-194.
6. DOEBBLER, G. F., R. G. BUCHHEIT & A. P. RINFRET. 1963. Rapid freezing and *in vivo* survival of rabbit erythrocytes. *Transfusion* 3(6): 494-498.
7. RINFRET, A. P., C. W. COWLEY, G. F. DOEBBLER & R. G. BUCHHEIT. 1963. The preservation of blood by rapid freezing. Proc. 8th Congr. Int. Blood Transf. (Mexico, 1962), 80-88.
8. SLOVITER, H. A. & R. G. RAVDIN. 1962. Survival of human erythrocytes after freezing in polyvinyl chloride bags. *Transfusion* 3(6): 899-900.
9. LUYET, B. J. 1949. Effects of ultra-rapid freezing on mammalian erythrocytes. *Biodynamics* 1(1): 1-10.
10. MERYMAN, H. T. & E. KAFIG. 1955. Freezing of human blood. Proc. Soc. Exptl. Biol. Med. 90(1): 10-13.
11. RINFRET, A. P. & G. F. DOEBBLER. 1963. Rapid freezing of human blood. *Transfusion* 3(6): 499-503.

THE ROLE OF CELL MEMBRANE OF YEAST AND OTHER MICROORGANISMS

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Much of the damage in cells that appears ascribable to two physical processes that accompanies ice formation, and that occurs within the cell (Mazur, 1965). However, it is no means invariably lethal, for a cell can survive exposure to -190°C . and below under appropriate conditions. It is known that freezing occurs under a particular set of conditions, among the most important of which is the presence of the plasma membrane. The presence of the membrane influences the likelihood of intracellular damage. Cells killed by freezing and thawing, therefore, are variably disrupted. There are substances that are released as consequence of freezing, and not

The Cell Membrane

The freezing point of most nonliving

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TABLE
SUPERCOOLING OF CELLS IN THE PRESENCE

Cell, tissue, or organism	Extent of supercooling of water (°C)	
	External ice present	External ice absent
(1) Guinea pig testis	- 6 to - 10	- 18
(2) Rabbit corneal tissue	- 5 to - 10	- 5
(3) <i>Amoeba</i>	- 5 to - 8	
(4) Sea urchin eggs	- 8	< - 10
(5) Various plant cells	- 7	- 20
(6) Insect larvae	- 10 to - 15	- 20
(7) Cabbage leaf	- 13	
(8) Yeast	- 10†	< - 10
(9) Vesicles of lemon	< - 7	- 12
(10) Muscle fibers (frog)	10	

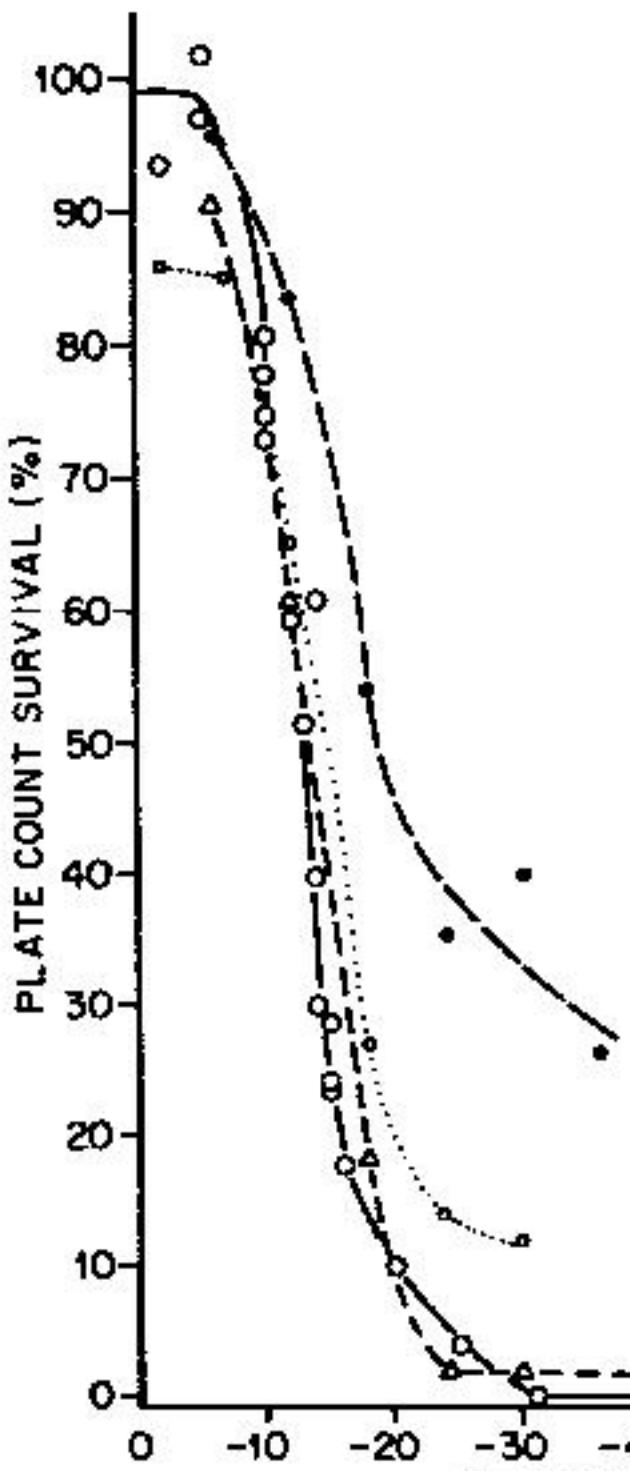
pension of 4×10^9 yeast cells/ml. Thus, since the external medium is liquid water during freezing at all. The proportion of liquid (q) will

$$q \approx$$

where m_i is the concentration of number of species into which the (Mazur, 1963a, 1965); 1.86 is the In the example cited $vm_1 = 0.003$ and 0.06 per cent at -10°C .

If intracellular freezing occurs with ice, one would expect that the greater would be the likelihood probability of intracellular freezing evidence is strong that death in rapid freezing (Mazur, 1961a; 1963a, 1965) but death is related to temperature frozen. As shown in FIGURE 1, survival at -20°C . in solutions ranging in concentration from 0.01 to 6 per cent. Furthermore,

Mazur: Membranes and



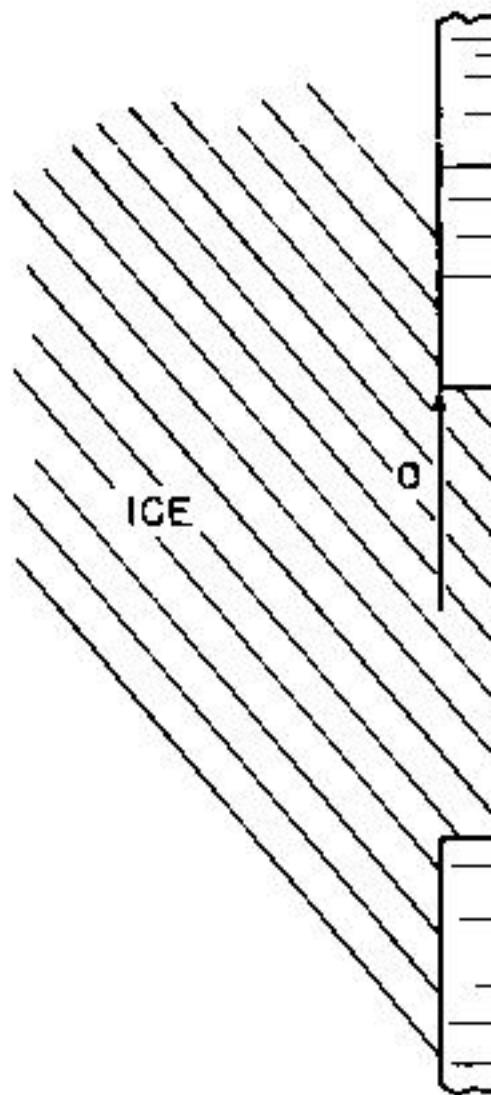


FIGURE 2. Schematic diagram of the a cell membrane. See text for details.

expression for the change in free

Mazur: Membranes and

it would melt, but that it could pass through. Although the lower melting point of ice may partially account for the barrier properties, there is a large quantitative discrepancy. The membrane ceases to be a barrier at approximately -10°C. According to Equation 4, the critical pore radius at -10°C. is 300 Å. The measured critical pore size from permeability studies is 30 Å. What is the source of this discrepancy? One possibility is that the membrane does not completely block nucleation above certain temperatures, leaving small pores. Another is that the value of 300 Å. is erroneous. It is known that the values of σ_{SL} and L_f obtained by the capillary rise method are often erroneous in micro systems, and therefore unreliable (Van Hook, 1961, p. 76). But there is another reason. If Θ is not 0° . If, for example, Θ were 80° , the critical pore radius at -10°C. would become

$$\Delta T \approx \frac{5}{\sin \Theta}$$

and the pore radius at $-10^\circ C.$ would be 100 Å. These values derived from permeability studies are in better agreement with the values of σ_{SL} and L_f .

Although there is no evidence whatsoever to support this, there are reasons to believe that Θ is greater than 0° . Plasma membranes are known to have a

ACTIVATION ENERGIES FOR THE PERMEATION OF WATER

Cell or tissue	Activation energy (kcal./mole)
<i>Arbacia</i> eggs (unfertilized)	13-17
<i>Ascites</i> tumor	9.6
Toad bladder:	
(- vasopressin)	9.8
(+ vasopressin)	4.1
Human red cells	5.7
Self-diffusion and viscous flow of water	4.5
	6.4

of caution is in order. Interfacial curvature apply to assemblages of molecules which are blurred in microscopic systems of all sizes (17). A single layer of

Mazur: Membranes and Freezing

There is a question whether these observations are rapid enough to account for the nuclear changes which rapidly elapse between the onset of cooling and the onset of cellular freezing. The evidence is meager. Mazur (1962) suggests that intracellular freezing in *Saccharomyces cerevisiae* and *Centrotus* is due to alterations of the cell membrane, either by precipitation of salts or by the completion of ice formation in the cytoplasm. On the other hand, studies on yeast and other microorganisms show that the changes in the ability of the plasma membrane to intracellularly exclude solutes (Mazur, 1965, and below).

One might think that cell membranes are ruptured by the mechanical forces produced by the formation of ice. This is generally not to be the case. This point has been discussed elsewhere (Mazur, 1965), so let me just mention one example. Cells of yeast can survive external ice formation if the temperature remains above -10°C. The rate of cooling is either slow ($1^{\circ}\text{C./minute}$) or very

The Plasma Membrane and Freezing

As long as the plasma membrane is permeable to solutes, including cellular supercooled water, the higher the

b and Q_{10} is

$$\ln Q_{10} = 10b \quad (7)$$

Investigators usually give the temperature characteristics of the permeability constant in terms of an Arrhenius equation of the form

$$k = k_0 e^{-E/R(1/T - 1/T_0)} \quad (6a)$$

when E is the activation energy in cal./mole.

Over a restricted temperature range, Equations 6 and 6a are nearly equivalent. Between +20 and -30°C., the relation between b and E is closely described by

$$E \approx 1.5 \times 10^6 b \quad (8)$$

The driving force for the loss in water is the ratio of the internal to external vapor pressures. As shown previously (Mazur, 1963b), this ratio increases with decreasing temperature, according to the relation

$$\frac{d \ln(p_i/p_e)}{dT} = \frac{nv_1}{V(V + nv_1)} \frac{dV}{dT} - \frac{L_v}{RT^2}, \quad (9)$$

where n is the number of osmoles of solute in the cell.

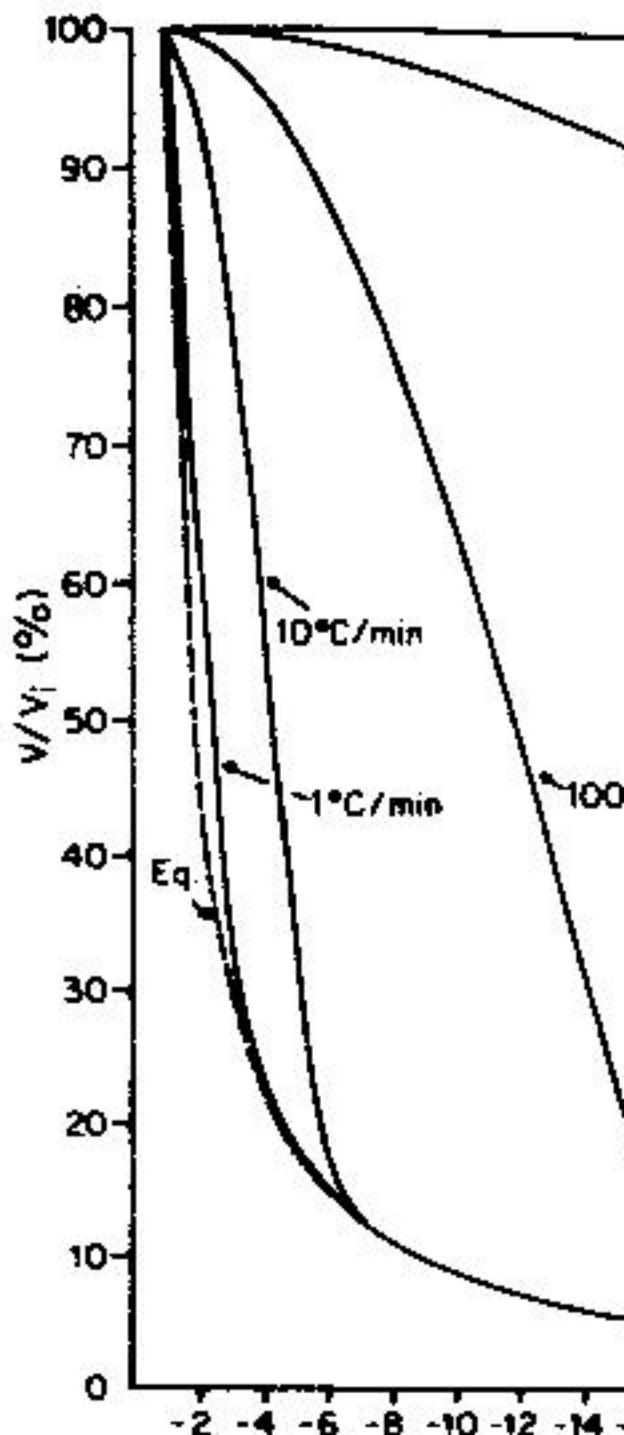
If T and t are equated by means of the cooling velocity ($dT/dt = B$), Equations 5, 6, and 9 can be combined to give an equation that relates the amount of supercooled water in a cell to temperature and the several parameters (Mazur, 1963b), namely,

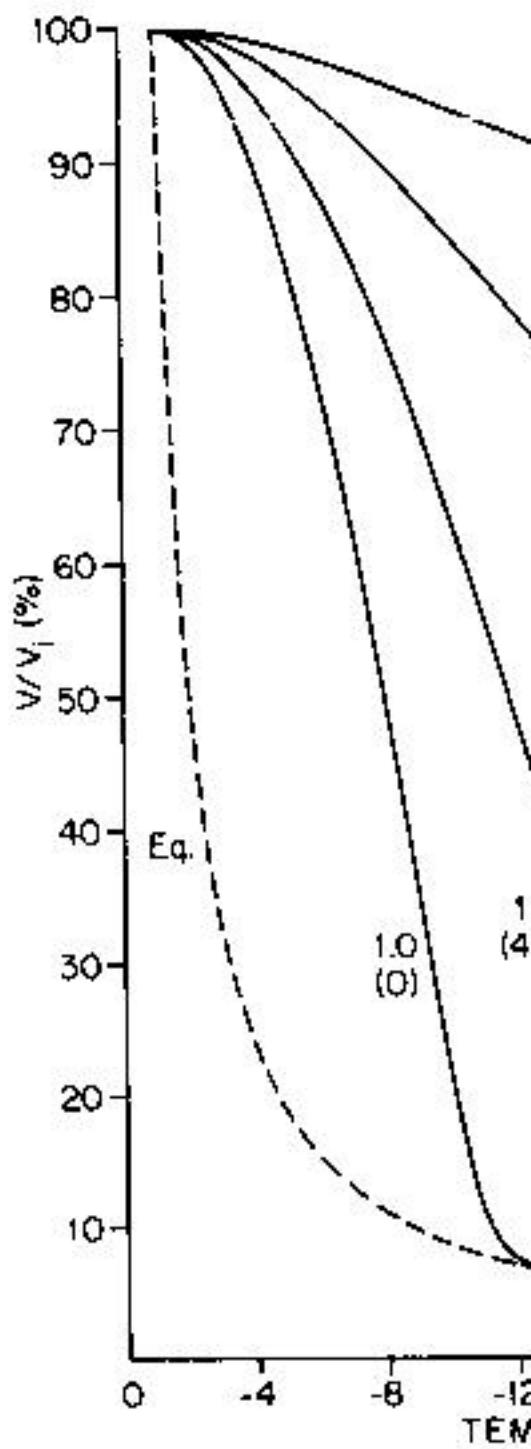
$$Te^{b(T_e - T)} \frac{d^2V}{dT^2} = \left[(bT + 1)e^{b(T_e - T)} - \frac{ARk_0 n}{B(V + nv_1)} \cdot \frac{T^2}{V} \right] \frac{dV}{dT} - \frac{L_v A k_0}{B v_1} \quad (10)$$

Numerical solutions to this equation give an estimate of the effect of various parameters on the likelihood of intracellular freezing. FIGURE 3, for example, shows the calculated effect of cooling velocity on the water content of a 6μ diameter cell. The curve Eq. shows the equilibrium water content as a function of temperature; i.e., it shows the water content the cell would have to maintain in order to avoid supercooling. It can be seen that cells cooled at $1^\circ\text{C}/\text{minute}$ are no longer supercooled at -10°C . or below; therefore, they should not freeze internally. On the other hand, cells cooled at $100^\circ\text{C}/\text{minute}$ are supercooled below -10°C . and should, therefore, undergo intracellular freezing.

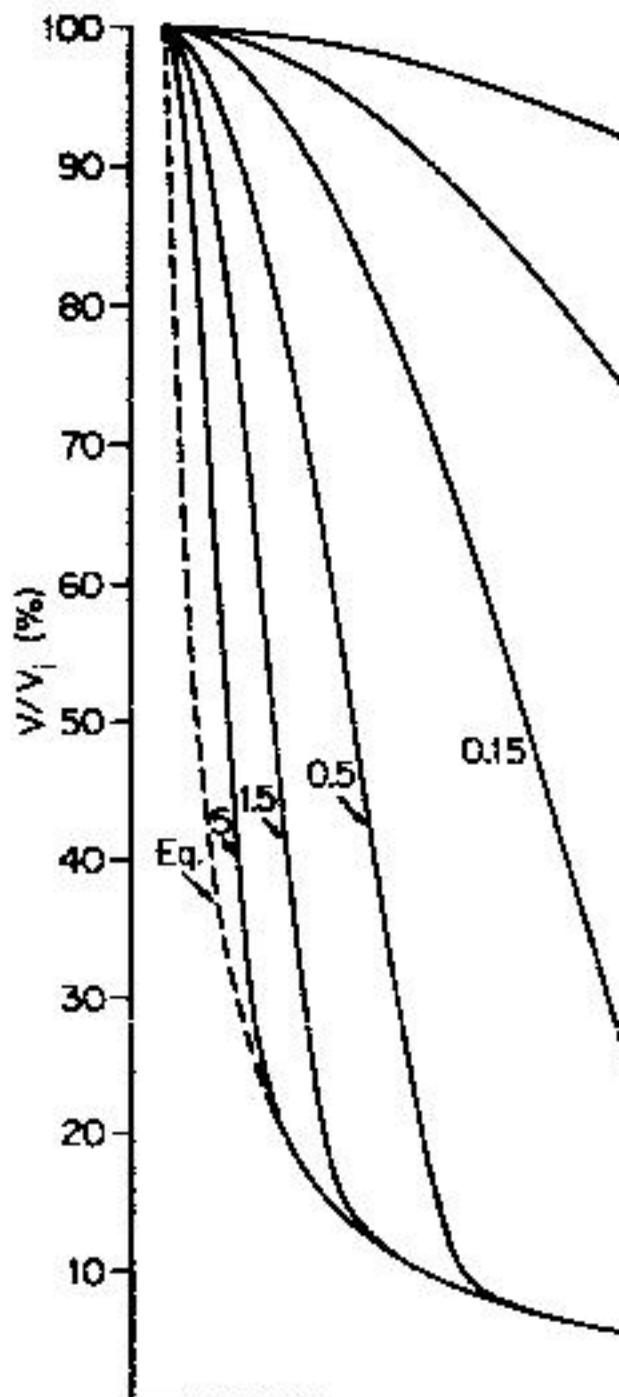
The permeability of the cell profoundly affects the water content and hence the likelihood of freezing. The solutions for the curves in FIGURE 3 used a permeability constant of $0.15 \mu \text{ minute}^{-1} \text{ atm}^{-1}$ and a temperature coefficient b of the permeability constant of 0.0325 ($Q_{10} = 1.4$; activation energy = 4900 cal./mole). FIGURE 4 shows the large effect of varying the temperature coefficient and activation energy. The value of 4900 cal./mole is about that found for blood cells, while the value of 15,000 cal./mole is that characteristic of sea urchin eggs (cf. TABLE 2).

Mazur: Membranes and





Mazur: Membranes and



TA
MINIMAL COOLING VELOCITIES FOR T

Organism	Cooling Calculated (°C./min.)
<i>Amoeba</i>	<1
Sea urchin egg (<i>Strongylocentrotus</i> <i>nudus</i> , unfertilized)	1
Yeast (<i>S. cerevisiae</i>)	20
<i>Escherichia coli</i>	~ 500
Red cells (mammalian)	5000

*The calculated cooling rate was still contained 20% of its intracellular to Equation 10. Known values for the parameters were taken from (1963b). Assumed values were used for $b = 0.1034$ (the measured value for b in NaCl at $T = 30^\circ\text{C}$), $k = 0.0001$ (the measured value for k in NaCl at $T = 30^\circ\text{C}$), and $\rho = 1000$ (the density of water at $T = 30^\circ\text{C}$).

Mazur: Membranes a

per cent of their total solids suggests low molecular weight, for low molecular weight solutes are responsible for most of the molarity. The measurements of the rate at which the solutes escape through the membrane are supported by dialysis experiments (see references in Mazur, 1965).

The quantity of escaping solutes is related to the percentage of cells that are killed (TABLE 5). The fact that all frozen-thawed cells escape suggests that the membrane damage is all-or-none; that is, it suggests that a relatively small amount of damage results in a greater loss from all cells, but to markedly different degrees in different cells. Normal viable yeast cells are killed by freezing. It can be seen from TABLE 5 that the percent of cells killed is approximately equal to the percent of cells that escape, which parallels the percentage of cells that are killed. Other evidence comes from examining the diameters of unfrozen and frozen-thawed yeast cells. The diameter of unfrozen cells appears to be normal while the diameter of frozen-thawed cells (those that are dead) also appears to be normal while the diameter of unfrozen cells appears to be normal.

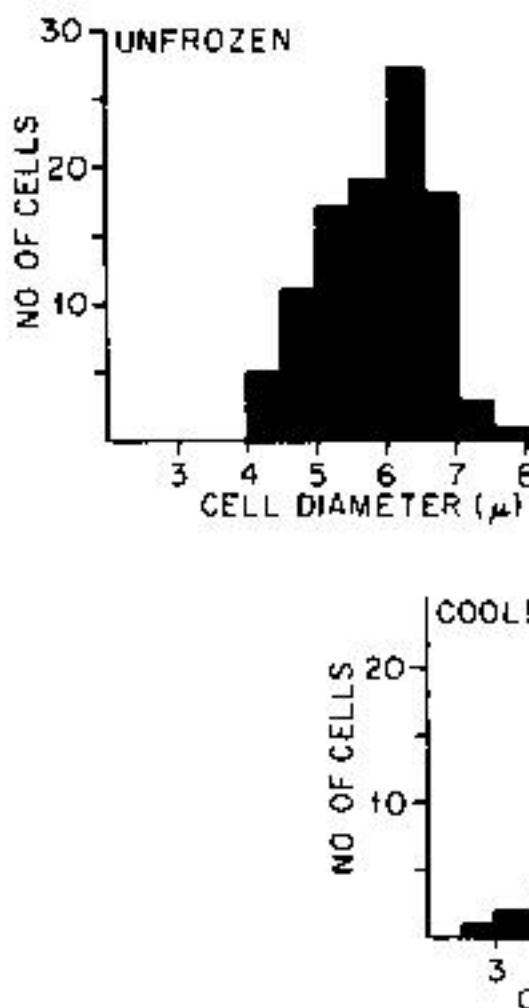
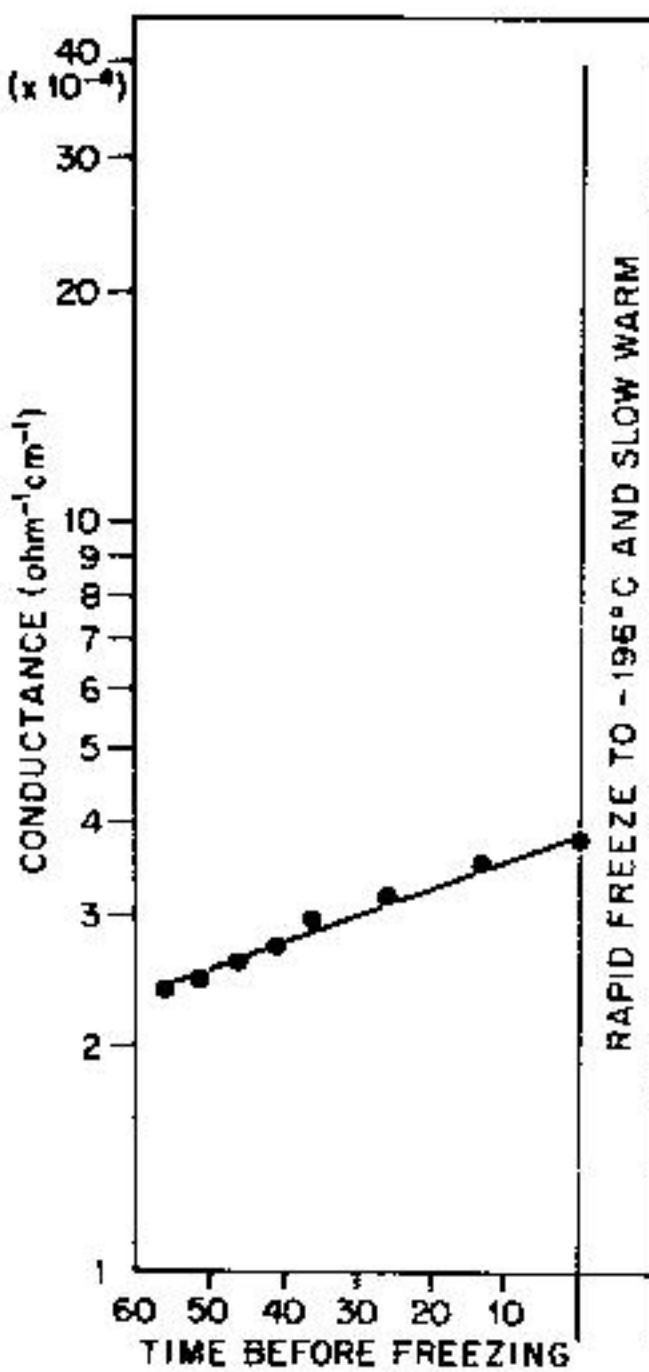


FIGURE 6. Frequency distribution of *Saccharomyces cerevisiae* subjected to indicated treatments. Cells were killed by rapid cooling; about 10% survived. Treated cells were warmed at 1° C./min after freezing, and measuring the cells by microphotometry (see Fig. 1, 1961b).

Mazur: Membranes and



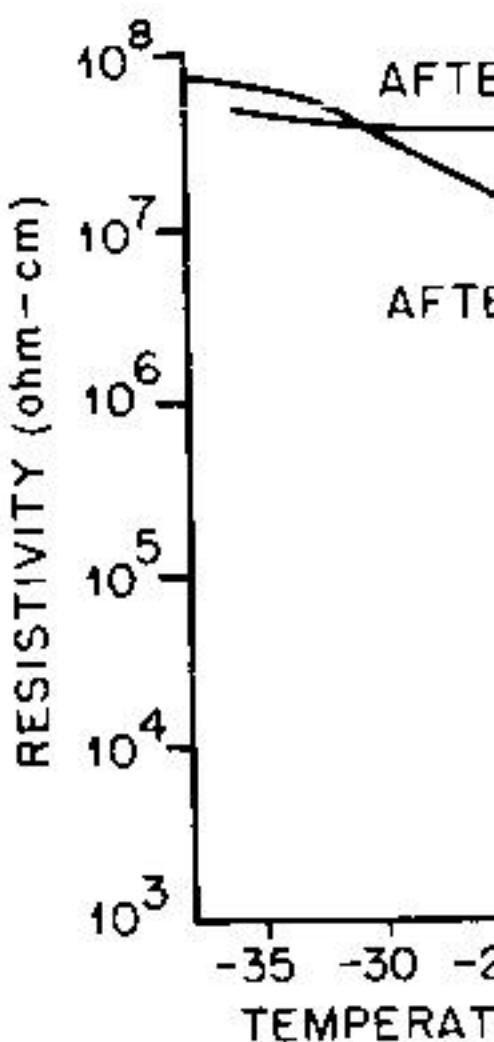


FIGURE 8. Resistivity of frozen samples plotted against temperature during warming and thawing. The samples were frozen in liquid nitrogen at 300° C./minute, and thawed at 1° C./minute (upper curve). The samples were frozen and the resistance measured at -35° C. The frequency was 1000 cps. From M. J. Fawcett, *J. Biophys. J.* 3: 674 (1963).

Mazur: Membranes an

TABLE
RESISTIVITIES OF *Saccharomyces cerevisiae*
VARIOUS LOW TEMPERATURES

Suspension cooled to minimum of -2°C.*	
Treatment	Resistivity at indicated temperatures† (ohm-cm.)
Untreated at 25°C.	4600
Supercooled to -1.0°C.	7500
Frozen at -2°C.; equilibrated at -1°C.	250,000
Thawed; supercooled again to -1°C.	6870

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- ARAKI, T. & T. NEI. 1962. Low Temp. Effect on Enzymes. *Enzyme* 10: 83-126.
- ASAHINA, E. 1956. Contributions from the Department of Botany, Faculty of Agriculture, No. 10 : 83-126. Hokkaido University.
- ASAHINA, E. 1961. Nature 191: 1263-1264.
- ASAHINA, E. 1962. Nature 196: 445-446.
- CHAMBERS, R. & H. P. HALE. 1932. *Plant Physiology* 17: 352.
- DAVSON, H. & J. F. DANIELLI. 1952. *The Structure of the Cell Surface*. 2nd Ed. Cambridge University Press.
- DICK, D. A. T. 1959. Intern. Rev. Cytol. 10: 1-100.
- FLETCHER, N. H. 1962. The Physics of Plant Cells. Cambridge, England.
- FRANK, H. S. & M. W. EVANS. 1945. J. Gen. Physiol. 32: 353-368.
- HAYS, R. M. & A. LEAF. 1962. J. Gen. Physiol. 38: 101-112.
- HEMLING, H. G. 1960. J. Gen. Physiol. 37: 101-112.
- JACOBS, M. H., H. N. GLASSMAN & R. L. HEDGES. 1958. *Plant Physiol.* 33: 197-225.
- KAUZMANN, W. 1959. In *Advances in Protein Chemistry*, Vol. 14, Eds. J. M. Danielli et al., Eds. 14: 1-63. Academic Press.
- LEITCH, G. J. & J. M. TORIAS. 1964. *Plant Physiol.* 39: 101-112.
- LOELOCK, J. E. 1954. Nature 173: 65-67.
- LUCAS, J. W. 1954. *Plant Physiol.* 29: 101-112.
- LUYET, B. J., G. L. RAPATZ & P. M. MAZUR. 1960. Ann. N. Y. Acad. Sci. 92: 101-112.
- MAZUR, P. 1961a. *Biophys. J.* 1: 247-254.
- MAZUR, P. 1961b. *J. Bacteriol.* 82: 669-676.
- MAZUR, P. 1961c. *J. Bacteriol.* 82: 677-684.

NEW APPROACHES IN MEASUREMENT OF ICE CRYSTALLIZATION IN AQUEOUS SOLUTI

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The problem of measuring the crystallization rate of aqueous solutes has occupied the interest of many scientists since the 19th century, in particular, Tammann and his school. The main importance of this problem lies in the fact that the more recent studies in the field of low-temperature biology have added additional interest¹⁻⁶ and opened another aspect of the problem. It is well accepted now in cryobiology that the damage to living systems at low temperatures occurs during the process of crystallization. The rate of crystallization of aqueous systems at low temperatures is of profound importance in cryobiology. The information available on the rate of crystallization of aqueous systems at low temperatures is very limited, and a systematic study of the problem over a wide temperature range is lacking. The only method used so far to measure the rate of crystallization of aqueous systems is the measurement of the linear crystallization velocity. This method is based on the assumption that the ice crystals grow within a demarcated distance from the

MATERIAL

Ins

The main limiting factor of precision of the measurements was spontaneous crystallization of the liquid during the brief period of time in a refrigerating bath. A much greater degree of supercooling can be obtained by freezing in a thin-walled glass capillary tube and growing ice into a cooling bath. The ice is initially immersed in the tube and grows in an upward direction, the velocity with which the tube is being immersed being proportional to the rate of ice growth, so that the ice remains at the level of the fluid in the cooling bath. This level at any given bath temperature depends on the cooling of the liquid in the tube, and measurements of the immersion velocity give the linear crystallization rate of the liquid under the conditions. More detailed discussion of this method will follow later.

The velocity with which the crystallization front moves in a cooling bath can be controlled either by varying the

Persidsky & Richards: New

Light will pass through the unfrozen
the frozen portion of the solution.

Based on light intensity measurements to provide the sensing signal to the persulfide cell, RCA type 7163, was chosen. The needed, good fast time constant, and calculations indicated the output voltage needed by the amplifier of the Honeywell system. The sensitivity that would be required was designed, using the photocell as one photocell indicated it should have a resistance at the light levels to be encountered. It was found to have an impedance of 100 K ohms. The bridge was a 1- 1½ volt dry cell. Due to the battery life will be essentially its sensitivity it was possible to connect the cell bridge. The divider consisted of a one megohm resistor. The output fed to the voltage across the 100 ohm resistor had a low loading factor on the photocell. The linear voltage output with respect to the ohm source of signal voltage provided

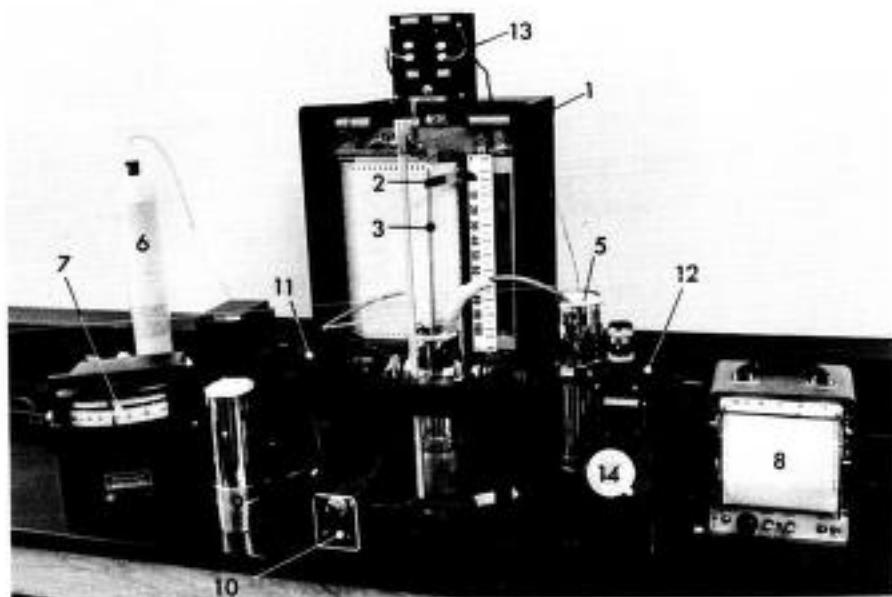


FIGURE 1. The instrument for measuring the rate of crystallization velocity in aqueous solutions. (See text for details.)

which opens and closes a solenoid valve on the aspirating line. In addition, the temperature of the bath is also recorded with a Varian chart recorder, (8) type G-11A, by which it can be read with an accuracy of $\pm 0.2^\circ$. The accuracy of these readings is improved by periodically adjusting the 0°C . reading by means of an ice water bath in a Dewar flask (9) using a selective switch (10) for the various thermocouples. A Bausch and Lomb horizontal microscope (11) with a $2 \times$ magnification objective was used to project the image of the moving ice front onto the photocell (12). The fine and coarse adjustments controlling the movement of the capillary tube are located on the Wheatstone Bridge (13).

A record of the tube travel versus time is made either directly on the chart of the Honeywell recorder (1) or by using an electrical timer (14). The timer is activated automatically by a microswitch which is attached to the pen mechanism and is turned on during the 10 cm. travel distance of the glass tube. The temperature inside the capillary tube was measured during freezing by means of a 0.003 inch thick thermocouple, and was recorded with the Varian recorder (8). The capillary tubes used for measurements with this instrument had an O.D. of 2.0 mm., an I.D. of 1.5 mm. and an overall length of 30 cm.

Persidsky & Richards: New

The instrument with manual control of the linear crystallization velocities of the polymer was designed to obtain the required response from polarized light was not practical with the present method of determining the crystalline orientation in the instrument. The linear crystallization velocity was determined by immersing vertically into a cooling bath. The tube was attached to a carriage which moved on a horizontal plane. The carriage was connected by a belt to a motor which gave manual movements. The capillary tube had an outer diameter of 1.0, an inner diameter of 0.5 mm. and a length of one-half foot long. It was immersed in a Dewar flask, the upper portion of which was used for the cooling bath. The bath was cooled by passing liquid nitrogen through a copper coil in the flask. The bath was agitated by bubbling dry air through it. The temperature was controlled and measured the same way as in the previous instrument to maintain the ice front at a constant position. The intensity of polarized light through a binocular microscope was measured by a photometer. The tube was driven downward by hand until the ice front was determined by measuring the time required for the light intensity to drop to 55% of its initial value.

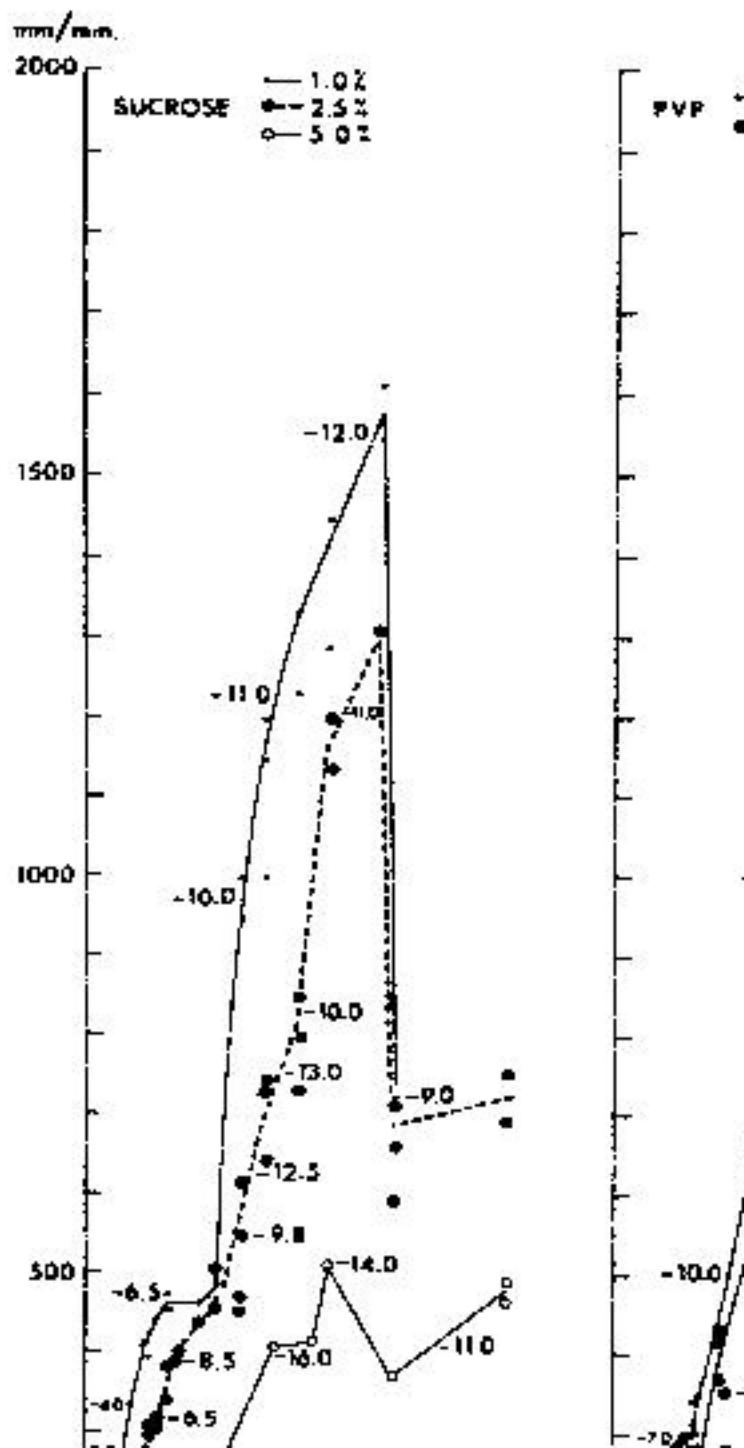
glass distilled water. The capillary tube was then filled with a solution and then, by the third method, had one end sealed.

OBSE

The performance of the first instrument was studied by making a record of the temperature of its immersion into a cooling bath. Figure 1 shows a graph which gave a wide range of freezing points. A graph of an original record made with this instrument shows degrees of line slope indicates different temperatures which are characteristic of given solutions at a given bath temperature. The straight lines with which the tubes were immersed in the bath in these chart records was, however, for a number of measurements, therefore an estimate was made it possible to make these measurements.

Measurements were made with this instrument and some of the results obtained are shown in figure 2. The curves represent the changes of the water

Persidsky & Richards: New



temperature changes impossible. (See determining the temperature in the ca

It is important to point out that when bath below -20°C., it was necessary to of the bath and the advancing ice front crystallization.

mm/sec.

350

300

250

200

Persidsky & Richards: New

VELOCITY mm/sec.

160

140

120

100

80

60

40



form. While in water, the velocity of the ice front increased at approximately -8°C . In measurements, the velocities with NaCl solution which persists for several degrees. compare well with the results obtained with automatic controls. The liquid inside the tube was supercooled to the temperature of the bath for a period of 30 to 60 seconds.

DISCUSSION

In evaluating the validity of the first method of linear crystallization in aqueous solutions, it must be noted first, the thermal condition produced by the apparatus. The automatic adjustment of the velocity of the ice front creates a state of dynamic equilibrium between several parameters:

1. The distance of the ice front from the bottom of the tube, or the depth of the immersion at which the tube is held, having been determined experimentally.
2. The length of time the tube is exposed to the bath, which is constant at any given distance from the bottom of the tube, determined by the immersion velocity of the tube.
3. The thermal condition of the bath, which is controlled by the rate of heat removal.

Persidsky & Richards: New

the advancing ice front. This is made
mocouple wire to a holder above the c
drawn from the tube during its imr
junction with respect to the ice-liqui
its distance from the ice front made
will remain for a sufficient period of
thermal equilibrium with it, and ma
ture possible. This study is now in pr
elsewhere.

The second method using manually
producible results than the first meth
better reproducibility can be achieved

The preliminary data obtained in
the evaluation of a new method for
A few comments, however, can be m
curves shown in FIGURES 3, 4 and 5. T
velocity, occurring between -8° and $-$
in this temperature region very rapi
small decrease in velocity was obser
affect the velocity of crystallization a
change in orientation of crystalline a

propagation. The authors also wish
nical assistance.

REFE

1. TAMMANN, G. 1897. Ueber die Ers
23: 326-328.
2. FRIEDLÄNDER, J. & G. TAMMANN
schwindigkeit. Z. Phys. Chem. 24:
3. TAMMANN, G. 1898. Ueber die Kry
Chem. 26: 307-316.
4. BOGOJAWLENSKY, A. 1898. Ueber
Phys. Chem. 27: 585-600.
5. TAMMANN, G. 1899. Herrn F. W. B
sationsgeschwindigkeit. Z. Phys. Chem.
6. TAMMANN, G. 1899. Ueber die Kry
Chem. 29: 51-76.
7. WALTON, J. H., JR. & R. C. JUDD.
of undercooled water. J. Phys. Chem.
8. HARTMANN, R. 1914. Über die sp
serigen Lösungen. Z. Anorg. Chem.
9. FREUNDLICH, H. & F. OPPENHEIM
schwindigkeit unterkühlter wäs
10. VOLMER, M. & M. MARBER. 1931. Z
geschwindigkeit unterkühlter Se
kationen. Z. Phys. Chem. 154: 97-
11. KAISCHEW, R. & I. N. STRANSKI.
sationsgeschwindigkeit. Z. Phys.
12. FÖRSTER, T. 1936. Ueber die ex
Kinetik Wasserstoffionen bei Kälte.

ENZYME PATTERNS OF TUMORS HISTOCHEMICALLY IN

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Introduction

This report concerns a histochemical study of enzyme patterns in about 195 human malignant tumors, including fresh autopsy material, as well as normal human tissues and human tissues as controls. It began as a pilot study of a group of about 25 breast carcinomas, since the enzyme patterns might give some hint as to whether they were autonomous, since the histological differentiation.¹ TABLES 1a and 1b summarize the results. Differentiated breast cancers generally show enzyme patterns characteristic of their normal tissue cells of origin, whereas undifferentiated carcinomas frequently encountered in the undifferentiated.

This study was extended to include a

TA
ENZYME PATTERNS OF
(1958-1960)

	Esterase	Cholinest.
Adenoca. 8 Cases	8	5
Scirr. Ca. 9 Cases	9	2
Medull. Ca. 4 Cases	2	0
Mucin Ca. 2 Cases	2	0
Fibroad. 2 Cases	2	2

E 16
 CARCINOMAS OF THE LUNG
 (25 CASES)

Dehydrogenases

	Malic Dehy.	Isocit. Dehy.	Glut. Dehy.	B-Hyd. But. D.	Lact. Dehy.	Cyto. Oxid.
7	8	8	8	7	8	8
6	8	7	9	9	9	9
6	8	7	9	9	9	9
6	8	7	9	9	9	9

Melnick: Enzyme P

TA

ENZYME PATTERNS OF
(1958-196

	Esterase	Cholinest.
Diff. squ. cell ca. 6 Cases	6	3
Undiff. squ. cell ca. 9 Cases	8	4
Undiff. adenoca. 5 Cases	5	2
Undiff. small cell & oat cell ca. 5 Cases	1	0

LE 2b
 CARCINOMAS OF THE LUNG
 (1, 31 CASES)

Dehydrogenases

		Malic Dehy.	Isoct. Dehy.	Glut. Dehy.	B-Hyd But. D.	Lact. Dehy.	Cyto Oxid.	
4	5			6	6	6	6	6
								0
								7
								8
								6

Melnick: Enzyme Pa

tivity of any given enzyme was very variable in different types of prostatic carcinomas; in such cases it is better to compare all freezing rates.

The findings of this cryogenic study of prostatic carcinomas during the past three years are summarized in Tables 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 6*a* and *b*. Without giving the details of the methods and the purposes of simplification the tables show the degree of activity of each group in which activity was found. It is not possible to indicate their degree of activity, since even the most active enzymes are not used for chemotherapy. The number of different types of cancers in which freezing rates were encountered, that is, in which the freezing rates were determined, are thereby more clearly indicated. The results of a comparative study of freezing rates between prostatic carcinomas and other tumors are given in the reference cited.*

The significant findings were again the same as those observed previously in the series of breast and lung carcinomas. In almost all of the enzymes there were marked differences in the freezing profiles in the different types of carcinomas. For example, in more of the enzymes of their tissue origin, the undifferentiated cancers often had many enzyme activities which were not found in the differentiated cancers (Table 1).

Melnick: Enzyme Pa

TABLE 3a
CARCINOMAS OF THE LUNG
(1, 31 CASES)

Hydrolases

	5'- Nuc.	G-6 Ph. ase	ATP ase	Beta Gluc.	Leuc. Amin.	Ala. Amin.	Sulfat.
	5	6	6	6	6	5	6
	7	8	8	6	9	6	10

TABLE 3b
CARCINOMAS OF THE LUNG
(64, 31 CASES)

Dehydrogenases							Cyto Oxid.		
Nic al y.	Glut. Dehy.	B-Hyd. But. D.	Lact. Dehy.	Glyc- Ph. D.	Eth. Deh.	G6P Deh.	6PG	Cyto Oxid.	
5	4	6	6	0	6	6	6	6	
3	3	4	4	3	4	5	6	11	

Melnick: Enzyme P

TABLE 4a
OF MALIGNANT TUMORS
(2-1964)

Hydrolases						
	5'- Nuc.	G-6 Ph.	ATP ase	Beta- Gluc.	Leuc. Amin.	Ala. Amin.
	18	14	20	20	22	13
						25
	6	6	6	6	5	4
						6

TABLE 4b
S OF MALIGNANT TUMORS
(1962-1964)

Melnick: Enzyme P

TABLE 5d
OF MALIGNANT TUMORS
(1962-1964)

Hydrolases

	5'- Nuc. Ph.	G-6 ase	ATP ase	Beta- Gluc.	Leuc. Amin.	Ala. Amin.	Sulfat.
	2	2	2	2	1	1	4
	3	1	3	1	1	0	3
	2	3	3	3	1	0	3

TABLE 5b
OF MALIGNANT TUMORS
(1962-1964)

Dehydrogenases

	Glut. Dehy.	B-Hyd. But. D.	Lact. Dehy.	Glyc. Ph. D.	Eth. Deh.	G6P Deh.	6PG Deh.	Cyto. Oxid.
2	2	1	5	2	3	4	3	5
1	3	0	3	2	0	3	3	3

TABLE 6a
HYDROLASES IN MALIGNANT TUMORS
(1964)

Hydrolases						
	Nuc. O.S.	5'- Nuc.	G-6 Ph.	ATP ase	Beta- Gluc.	Leuc. Amin.
-	4	3	5	5	4	2
-	1	1	1	1	1	1
-	2	2	2	2	1	0
-	2	2	2	2	1	0
-	0	2	2	2	2	2

Melnick: Enzyme P

TABLE 6b
OF MALIGNANT TUMORS
(1962-1964)

Dehydrogenases

	Malic Dehy.	Glut. Dehy.	B-Hyd. But. D.	Lact. Dehy.	Glyc.- Ph. D.	Eth. Deh.	C6P Deh.	6PG Deh.	Cyto. Ox.d.
	5	5	5	5	2	0	5	5	5
1	1	1	1	1	1	0	1	1	1
2	2	0	0	2	0	0	2	2	2
3	2	0	0	2	0	0	2	2	2

Melnick: Enzyme Pathology

Latent enzymes. Unmasking of histoenzymes by freezing suggests that other mechanisms of enzyme activity should be investigated. Lawrence,⁷ application of histochemical methods in immunoelectrophoretic preparations showed that the carrier for all of the serum enzymes showed feeble or no enzyme reactivity after being heated or treated with ether or detergents, or in other well-known examples is the unmasking of hydrolases and cathepsins in degenerating matters as the availability and concentration of competitive activities of closely related enzymes release enzyme activity under physiologic conditions about which so little knowledge has until recently been present a central problem for biochemical come.

Abnormal enzymes. The often greater understanding of the nature of the abnormal DNA and RNA templates are abnormality considered by some is binding by toxic substances to the nucleic acids, or perhaps they become eliminated in daughter cells.

Enzyme

The purpose of the present study was to determine what enzymes tumor cells contain and how they are affected by freezing. Slow freezing preserves the integrity of cells, and rapid freezing is generally considered to be deleterious to cells. *et al.*⁸ and others have demonstrated the presence of various enzymes in rapidly frozen tissues. It has been shown that cell organelles in the course of rapid freezing are destroyed by the formation of ice crystals which damage active sites of enzymes which in turn affect the integrity of the cell until needed. As mentioned, an example of this is the presence of hydrolases in degenerating lysosomes. The presence of these enzymes in slowly frozen tissues is due to the fact that the tissue even only a few hours postmortem had been separated from their blood supply, had been processed, and which therefore had suffered more cellular injury than fresh specimens. Levitation of the tissue during freezing in slowly frozen tissues compresses the tissue and causes the reduction of sulphhydryls to form disulfide bonds between adjacent proteins at the active sites. Such a mechanism may explain the preservation of enzymes in slowly frozen tissues, and it can be demonstrated further with histochemical methods. Shikama¹⁰ believes that freezing

Melnick: Enzyme Pa

developed on normal fresh animal tissue and therefore understandably requires to observe the different electrophoretic animal species.¹¹ Furthermore, animal tissue before hypoxic and other degenerative changes is not usually possible in human surgery, perhaps of prime importance, in human tissue produced by disease will require close attention in histochemistry. Discussion of the first four enzymes studied as recorded and summarized is conveniently grouped under the following heading.

Hydrolases. (1) Among the hydrolases tested with two substrates each, were found, and unpredictably acid phosphatase and alkaline phosphatase. Examination of the slowly frozen sections alone would have resulted in the false conclusions for these enzymes, or at least only rapidly frozen tissue quenched at -160° F. of enzyme activity was observed. These enzymes studied (with the exception of cytochrome oxidase detailed below) are the following:

of these two enzymes is often observed correlations in material such as the

(6) Leucine aminopeptidase studies in most tumors, but was also unpaired various kinds. Alanine aminopeptidase tumors examined, chiefly in carcinoma tract. Aside from the hormone-producing and keratinizing tumors, the subjects such as those that contain peptide examine. It may be that histochemical aid in probing problems of function from the opportunities they furnish synthetic pathways.

Dehydrogenases. Bioenergetic metabolism has been one of the most active fields of research in recent years. The best efforts and intense interest have been directed toward the problem of the controlled release of chemical energy within the cell. For this reason the enzymatic reactions involved in metabolic and oxidative pathways have attracted considerable attention. From the TABLES, these are especially prominent in carcinomas, but also occur unpredictably in normal tissues. Possible hypotheses for this phenomenon include the marked variability of tumors, to the inactivation

Melnick: Enzyme Patterns

been widely accepted. At the same time, the chemotherapy of cancer and those who do have always been profoundly impressed among tumors. This is indicated by the words "cancer" or "neoplastic" is entirely (1956) recognized this diversity in the apparent biochemical similarities among cells. While it is difficult to reconcile the unity and diversity in cancer biology, the hypothesis of carcinogenesis. Such a comparison of cells from normal cells appears to have other theories of carcinogenesis and problems of cancer...."

The results of the present study taken in the light of the deletion hypothesis of LePage and Henderson, and others; and the oxidative enzyme groups in these tumors, variable enzyme patterns seen in these tumors, but are seen in an endless variety of animals. Yeasts prefer to ferment carbohydrates; bacteria contain no Krebs cycle but prefer glycolysis with lactic acid; a host of microorganisms acquire or lose many different enzymes.

resembling in this way their normal undifferentiated cancers many were

Glycolytic pathway. The enzymes soluble and to dissolve rapidly into ful histochemical technics have shown them. However, lactic dehydrogenase, the TABLES indicate, it was very active in such cancers. The Pasteur, Warburg and co-workers found that in many of them succinic, malic and ethanol dehydrogenase was completely absent. Apparently the human organism can utilize ethanol as does yeast. It would be interesting to know what it could do with this enzyme. Alpha-ketoglutarate dehydrogenase is active in a number of tumors, so that perhaps the tumor metabolism, and perhaps the Pasteur effect.

Tricarboxylic acid cycle. One of the most important findings was the impairment of the Krebs cycle in tumors. Malic and isocitric (DPN) dehydrogenases were found to be active at the same time lactic, beta hydroxybutyrate and alpha ketoglutarate dehydrogenases, which bring materials to the Krebs cycle, respectively, were frequently active in tumors.

Melnick: Enzyme Pa

which transfer electrons from so many tral importance as cytochrome C oxidase tumors in which glycolysis is so prominent is seen.

It may also be possible that tumors nisms under different circumstances. T often greatly impaired (i.e., bulky tu in their centers), and such impaired when oxygen tension is severely diminution, enzymes concerned with glucose n to become synthesized, so tumors that oxidative metabolism although they ma Histochemical methods may help to elu by the range of enzyme activity in v various distances from its blood supply

Cytochrome C oxidase. The experience of the tumors and normal tissues examined showed that observed for the hydrolases and de tissues quenched in isopentane at -100 totally inactive or at most showed only tissues and in the standard cryostat feebly to moderately active in many tu

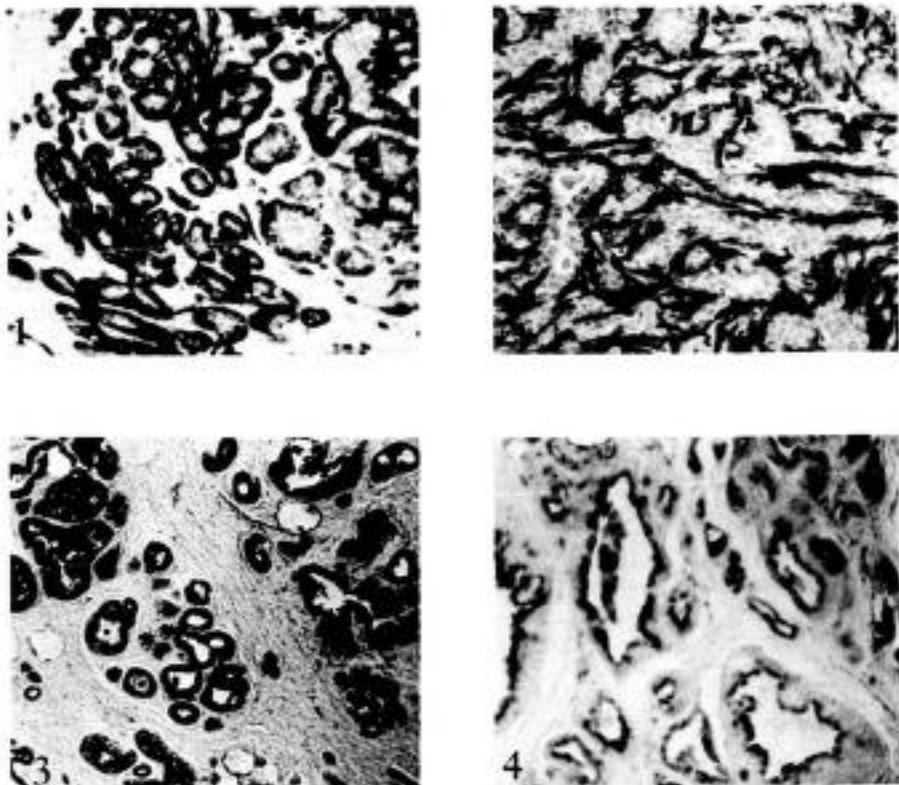


FIGURE 1. Differentiated adenocarcinoma of the breast (H-3-59; $\times 225$). Alkaline phosphatase, Gomori method. Enzyme reaction product is seen throughout the cytoplasm of the tumor cells.

FIGURE 2. Differentiated adenocarcinoma of the breast (H-10-59; $\times 225$). Alkaline phosphatase, Gomori method. Enzyme activity is present only in the myoepithelial layer.

FIGURE 3. Differentiated adenocarcinoma of the breast (H-10-59; $\times 225$). Esterase, azo dye method. Enzyme reaction product is seen in this example throughout the cytoplasm of the tumor cells.

FIGURE 4. Differentiated adenocarcinoma of the breast (H-3-59; $\times 350$). Esterase, azo dye method. Enzyme reaction product is seen only in the luminal border of the cells of this tumor.

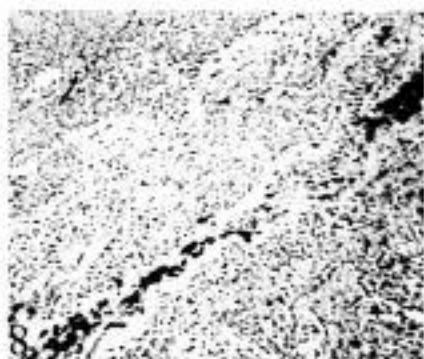
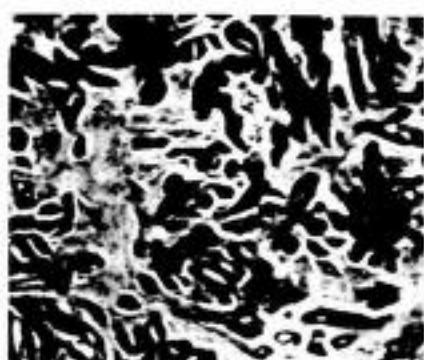
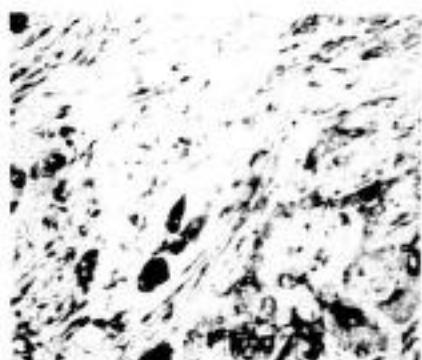


FIGURE 5. Scirrhous carcinoma of the breast (H-12-59; $\times 250$). Alkaline phosphatase, Gomori method. Enzyme reaction product is seen in an occasional ductule, but the cords of tumor cells contain no enzyme activity.

FIGURE 6. Scirrhous carcinoma of the breast (H-12-59; $\times 400$). Esterase, azo dye method. Enzyme activity is seen throughout the cytoplasm of the tumor cells.

FIGURE 7. Undifferentiated medullary carcinoma of the breast (H-4-59; $\times 175$). Alkaline phosphatase, Gomori method. No enzyme activity is present in the tumor cells, but it is seen in the stromal fibroblasts.

FIGURE 8. Undifferentiated medullary carcinoma of the breast (H-4-59; $\times 225$). Esterase, azo dye method. No enzyme activity is seen in the tumor cells, but it is seen in clumps of histiocytes in the stroma.

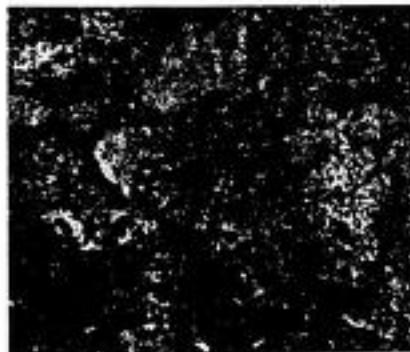
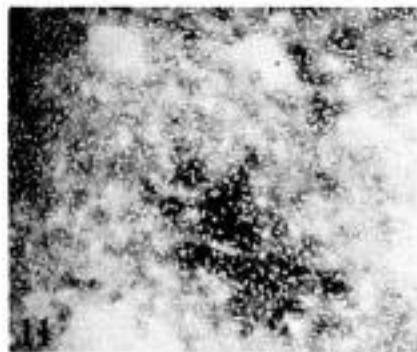
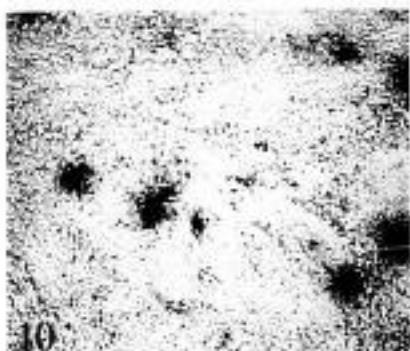
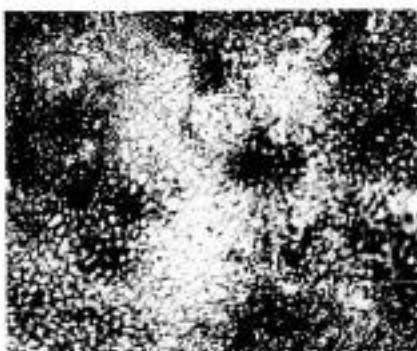


FIGURE 9. Undifferentiated squamous cell carcinoma of the larynx (H-59D-64; $\times 275$). Cytochrome C oxidase, Burstone method. Slowly frozen section. Enzyme activity is seen in some tumor cells, but not in others where, instead, clumps of reaction product are seen unrelated to formed elements.

FIGURE 10. Undifferentiated squamous cell carcinoma of the larynx (H-59E-64; $\times 250$). Cytochrome C oxidase, Burstone method. This section is from the rapidly frozen block and demonstrates that freezing, especially rapid freezing, crystallizes and thus sequesters intrinsic cytochrome C, rendering it unavailable as the substrate for the reaction except in the localized areas where it is present. By adding cytochrome C to the incubating mixture the reaction is uniform throughout, in both the rapidly frozen and slowly frozen blocks (not illustrated). This demonstrates that this enzyme is apparently not affected by freezing, in contrast to all the other enzymes examined.

FIGURE 11. Glioma of the brain (H-18D; $\times 300$). Cholinesterase, Gomori method. Slowly frozen block. Moderate amounts of enzyme reaction product is seen in the cytoplasm of some of the tumor cells, but in the majority there is only feeble enzyme activity in this slowly frozen block.

FIGURE 12. Glioma of the brain (H-18E-64; $\times 250$). Cholinesterase, Gomori method. In this section from the rapidly frozen block enzyme activity is intense.



13

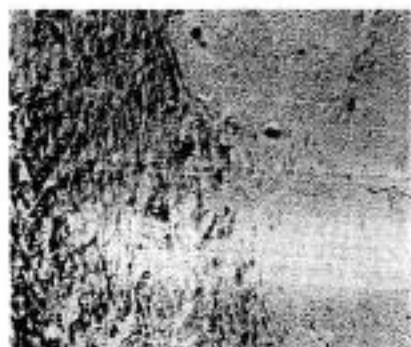
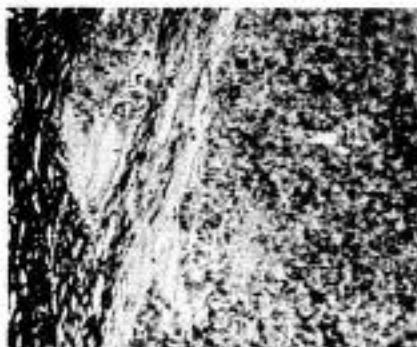


FIGURE 13. Metastasis of an undifferentiated carcinoma of the lung to the kidney (H-65D-64; $\times 150$). Succinic dehydrogenase. Slowly frozen block. Histochemical enzyme activity is not present in the tumor, and is very feeble in the kidney in this section from the slowly frozen block.

FIGURE 14. Metastasis of an undifferentiated carcinoma of the lung to the kidney (H-65E-64; $\times 200$). Succinic dehydrogenase. Rapidly frozen block. Enzyme activity appears in the tumor, and is greatly enhanced in the kidney. (FIGURES 15 and 16 illustrate an example of what is probably a true enzyme deletion in a tumor.)

FIGURE 15. Metastasis of an undifferentiated carcinoma of the lung to the liver (H-52D-64; $\times 175$). Beta-hydroxy-butyric dehydrogenase. Slowly frozen block. Histochemical enzyme is absent in the tumor, and is feeble in the adjacent liver parenchyma.

FIGURE 16. Metastasis of an undifferentiated carcinoma of the lung to the liver (H-52E-64; $\times 225$). Beta-hydroxy-butyric dehydrogenase. Rapidly frozen block. In this section the enzyme activity in the liver can be seen greatly intensified, but is absent in the tumor.

per ml. This is immediately reduced in incubating system so that ample time was available for the procedure, that had not been done previously. The result was that the enzyme was found in all normal tissues. Even so, in the rapidly freezing technique, an intense concentration of cytochrome C oxidase product often persisted in addition to the normal enzyme throughout the section.

Cytochrome C oxidase is an unusual enzyme in that it has two forms. Its active site has an identical structure in all organisms from yeast which is 600 million years old to man. In fact, in the apo-enzyme varies in different species. It is interesting to note that this enzyme was found active in all normal tissues. It is the key enzyme that receives electrons from the cytochrome C system and passes them on to molecular oxygen. It is therefore important that the activity of this enzyme were found.

A number of troubling questions remain concerning the function of cytochrome C oxidase. One question is whether it is involved in oxidative metabolism. One possibility is that it may be involved in the production of energy in tumors. In fact, in some types of tumors it has been found that the enzyme is defective. Another possibility is that it may act as a substrate because of the presence of a large amount of the enzyme in the tumor tissue. This may be found in those tumors in which the enzyme is present in large amounts.

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4. MELNICK, P. J. 1965. Effectiveness of various methods for identification of enzyme activity. *Cryobiology* 8: S259-S268.
5. MELNICK, P. J. Histochemical identification of enzymes in normal tissues and tumors. *Cryobiology* 1: 1-10.
6. STOWELL, R. E. 1964. (Chairman). Fixation methods and their significance. In: *Proceedings of the Int. Congress of Histo- and Cytochemistry*, Vol. 1, Eds.: H. H. Wolff, & H. H. Wolff, Eds. : 111-112. Berlin.
7. LAWRENCE, S. H. & P. J. MELNICK. 1964. Lipoprotein in immunoelectrophoresis. *Cancer Research* 24: 998-1001.
8. WARAVDEKAR, V. S., P. J. GOLDBLATT, & R. E. STOWELL. 1964. Effect of freezing on mitochondrial enzymes of mouse liver. *Cancer Research* 24: 501-503.
9. LEVITT, J. 1964. Cryobiology as viewed by a physiologist. *Cancer Research* 24: 504-508.
10. SHIKAMA, K. 1963. Denaturation of proteins during freezing and thawing. *Science Rep. Tohoku Univ.* 10: 1-10.
11. LAWRENCE, S. H., P. J. MELNICK, & R. E. STOWELL. 1964. Comparison of serum proteins and lipoproteins in normal and tumor tissue. *Proc. Soc. Exptl. Biol. Med.* 115: 57-61.
12. LEPAGE, G. A. & J. F. HENDERSON. 1964. *Principles and Practice of Tissue Freezing in Experimental Tumor Research*. Lippincott Co. Philadelphia, Pa.
13. GREENBERG, D. M., Ed. 1960. *Metabolic Pathways*. Academic Press. New York, N. Y.
14. THANNHAUSER'S TEXTBOOK OF METABOLIC PATHWAYS. 2nd Ed. N. G. Lloyd, Ed. T. C. Thompson, Ed. Academic Press. New York, N. Y.

CHEMICAL AND MORPHOLOGIC CHANGES IN THE PROSTATE FOLLOWING PROSTATEctomy

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The prostate in man is an accessory gland of the male reproductive system, situated immediately below the bladder. It forms the first portion of the male urethra. Normally, during micturition, the prostate is intravesicalized as the detrusor muscle contracts, markedly reducing urethral resistance. There are anatomical changes which prevent this reduction of resistance to void and ultimately ending in bladder neck obstruction and intolerable symptoms. These pathologic changes are due to inflammation of the prostate. All operations designed to relieve obstruction create the intravesical prostate by removing portions of the prostate.

Thirty years ago, the operative mortality rate was as high as 15 per cent. Improved surgical technique, blood replacement, and antibiotic therapy have reduced the mortality rate to two per cent or less. However, mortality is still a factor in the procedure with the heritage of a much higher

dropped to -150° and freezing continued for four minutes. The animals were then sacrificed at intervals, the prostate serially sectioned, and the pathologic changes studied.

Pathology

Gross. After thawing, there was immediate swelling of the prostate gland with hemorrhage (FIGURE 1). By the 14th day, the prostate was



FIGURE 1. Swelling of the gland with hemorrhage.

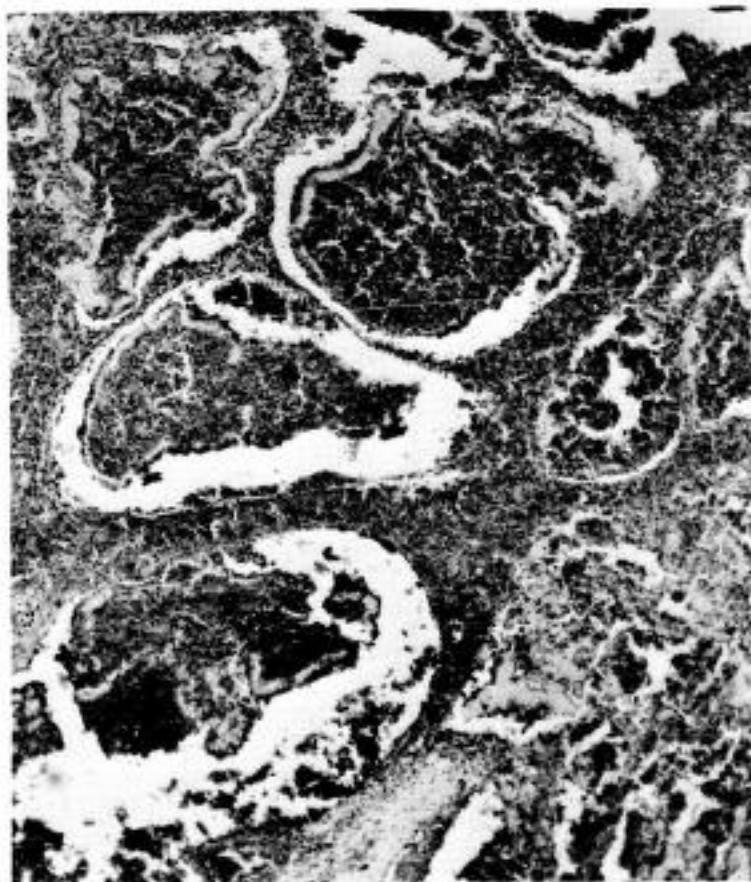


FIGURE 2. Necrosis and hemorrhage noted at five days. Nuclear detritus in lumen of glands. Ghost outline of glands remain but cellular membrane destroyed.

definitely decreased in volume. By the 22nd day, there was gross diminution in size but no other visible abnormality.

Microscopic. Microscopically, there was immediate necrosis (FIGURE 2) which progressed with ghost cells apparent and breakdown in cellular membranes and collections of nuclear detritus. Hemorrhage into the tissue is marked. This is from the area which was cooled to the lowest temperature. As we progressed towards the periphery of the injury, glandular structures are damaged (FIGURE 3), but noticeably spared to a degree. By nine days there is distinct fibroblastic activity around the periphery of the injury. There is still hemorrhage noted and only ghost outlines of glandular and stromal substance. There was marked histiocytic activity seen and hemosiderosis demonstrated. By the 14th day, microscopic ne-

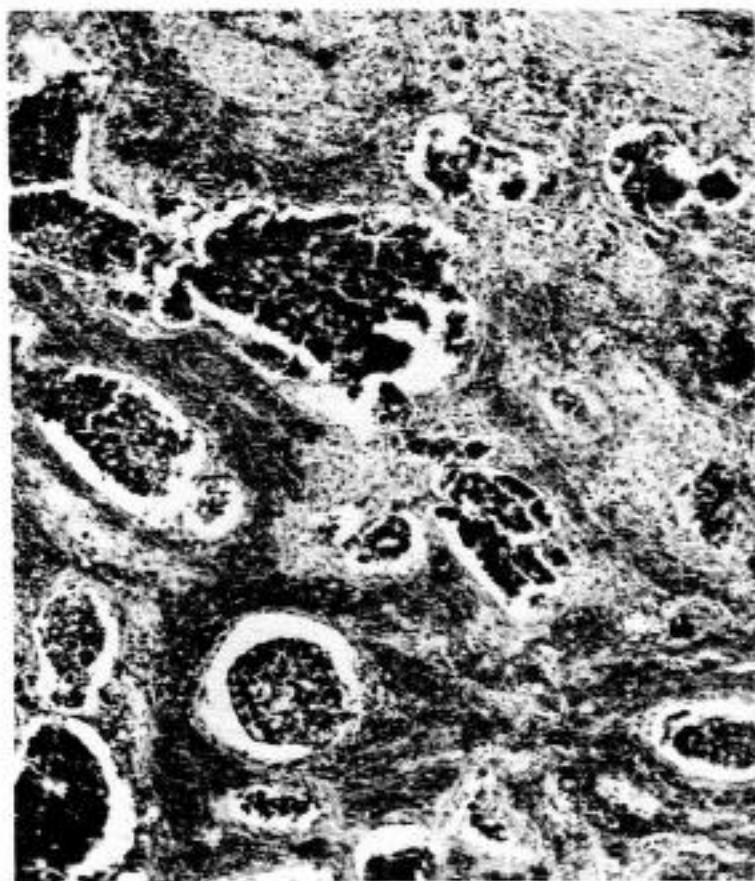


FIGURE 3. Periphery of damaged area at five days. Some cells at basement membrane of gland survive.

rosis was still evident. There is still evidence of some nuclear detritus. Hemorrhage is less evident and collections of hemosiderin are prominent. It is noted at this point, that there is beginning regrowth of tubules in an immature fashion, at the periphery of the injury (FIGURE 4). The periphery of the injury, likewise, shows well-advanced fibrosis with some remaining hemorrhage and hemosiderosis. In several specimens at this stage, there was evidence of squamous metaplasia in the glands surrounding the necrotic area. Inflammation *per se* was still quite minimal. At 22 days, the microscopic picture is that of maturation of the glandular regeneration. There is modest scar formation and minimal inflammatory reaction. Histiocytic activity and hemosiderosis continue. At 28, 35 and 45 days, the regrowth of tissue continues with distinct budding and branch-

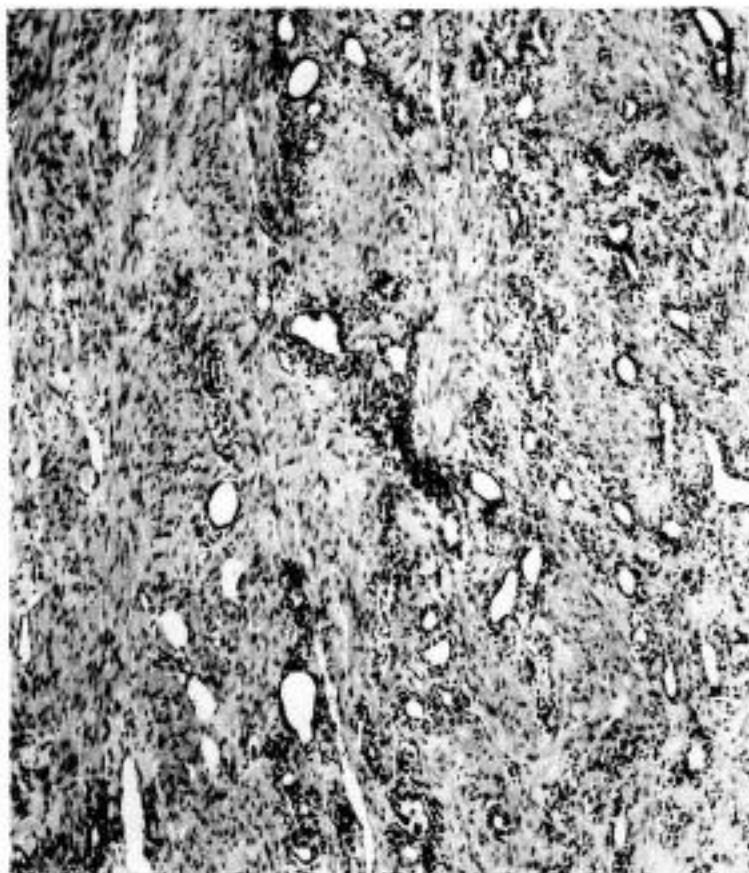


FIGURE 4. Periphery of lesion at 14 days. Demonstrating beginning regrowth of tubules.

ing noted (FIGURE 5). The epithelial cell is more typical. FIGURE 6 demonstrates the reduction in size and obvious alteration of the histologic pattern grossly apparent at 13 days.

We feel that these experiments demonstrated that freezing causes cellular breakdown which was ultimately resorbed with minimal inflammatory reaction. Cellular destruction was most complete when the temperature was the lowest and where freezing occurred the fastest. In those areas at the periphery of the lesion where freezing was slower and the temperature less cold, there was considerable fibrosis seen. There was minimal inflammatory reaction early and late, and this suggested to us that there was a minimal amount of denaturation of protein. Grossly, the prostate was definitely smaller where frozen, by the second week. Due to the fact the



FIGURE 5. Histology at 45 days; glands are beginning to branch.

degree of cold could be carefully controlled and simple freezing did not cause total necrosis, we felt we could begin to study the effects on human prostate with safety.

Before and during this study, we had anticipated certain problems which needed resolution before and while the treatment of prostatic urethral obstructions in man was carried out by this method:

1. Toxicity of the patient post-treatment due to tissue necrosis. Denaturation of protein might cause a foreign protein-type of reaction.
2. Initially, the equipment available for freezing tissues, particularly in organs with a large blood supply, presented a technical problem.
3. We thought that bleeding might be a problem due to the obvious histologic hemorrhage.

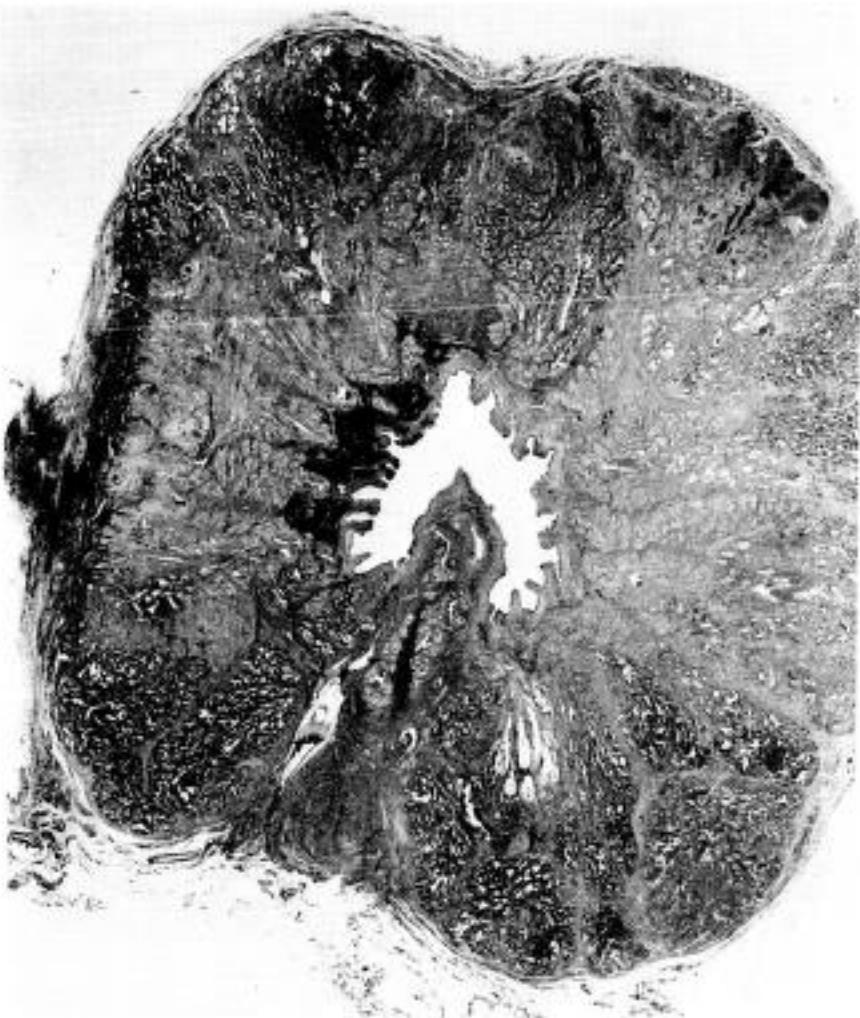


FIGURE 6. Reduction in size apparent by 13 days.

4. The length of time required for relief of the obstruction after treatment. There was considerable swelling secondary to the freezing injury, and the patient might require catheter drainage for an inordinate period of time.
5. There is a critical relationship between bladder function and urethral resistance, and there was a danger of damaging either the external urethral sphincter, bladder or rectum due to the anatomic relationships of these structures to the enlarged prostate.

6. The fibrosis occurring at the periphery of the injury could result in a marked narrowing of the prostatic urethra during the healing phase.
7. Almost all enlargements of the prostate are associated with infection and the necrotic tissue would be vulnerable to this infection.
8. It appears that some cells might resist freezing more than others, and inadequate destruction of the mixture of stromal and glandular cells making up the prostate, might be a possibility.
9. It was essential that we have a simple method to facilitate a wider application of this procedure.

With these considerations in mind, we began to create cold lesions, study pathologic changes and to develop the technical equipment required. It was apparent that *that* tissue cooled the fastest resulted in the most complete destruction, whereas the slower freezing at the periphery noted the survival of certain cells. The injured but surviving tissue was stimulated to produce granulation tissue. This encouraged us from several aspects; primarily, it indicated that if the majority of the freezing was applied to the urethral surface, then the possibility of fistula and damage to adjacent structures would be minimized.

The *enlarged* prostate in man is fitted snugly in a firm, fibrous capsule which has a separate blood supply from the adjacent membranous urethra and bladder. As freezing continues, the prostate enlarges, increasing the pressure between the frozen area and this firm, fibrous capsule, thus de-

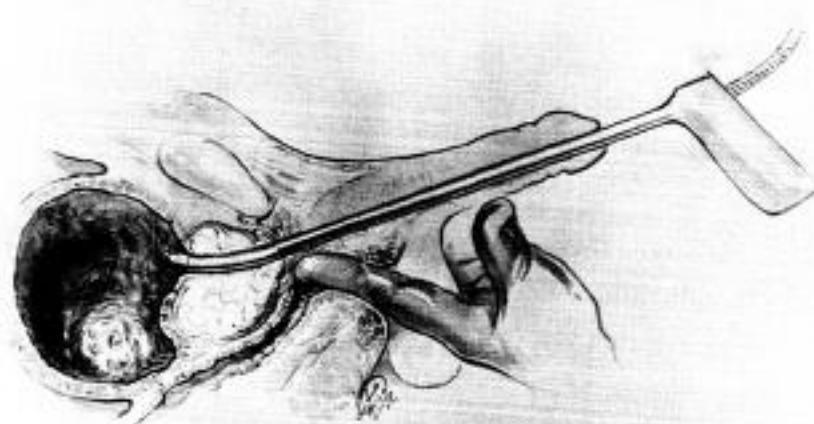


FIGURE 7. Freezing probe in place in urethra.

creasing the blood supply and accelerating the freezing process. Those adjacent structures with separate blood supply were cooled but not frozen. Definitely within the survival range.

We ascertained that we could advance from a rather complicated procedure, using relatively small probes, to a larger instrument allowing increased freezing capabilities. This enables us to freeze larger amounts of prostatic tissue colder with little damage to adjacent structures.

Method

The bladder is filled with air, holding the bladder walls away from the prostatic urethra (FIGURE 7). The freezing surface is $2\frac{3}{8}''$ long, located at the end of the probe. One cm. from that freezing surface, there is a

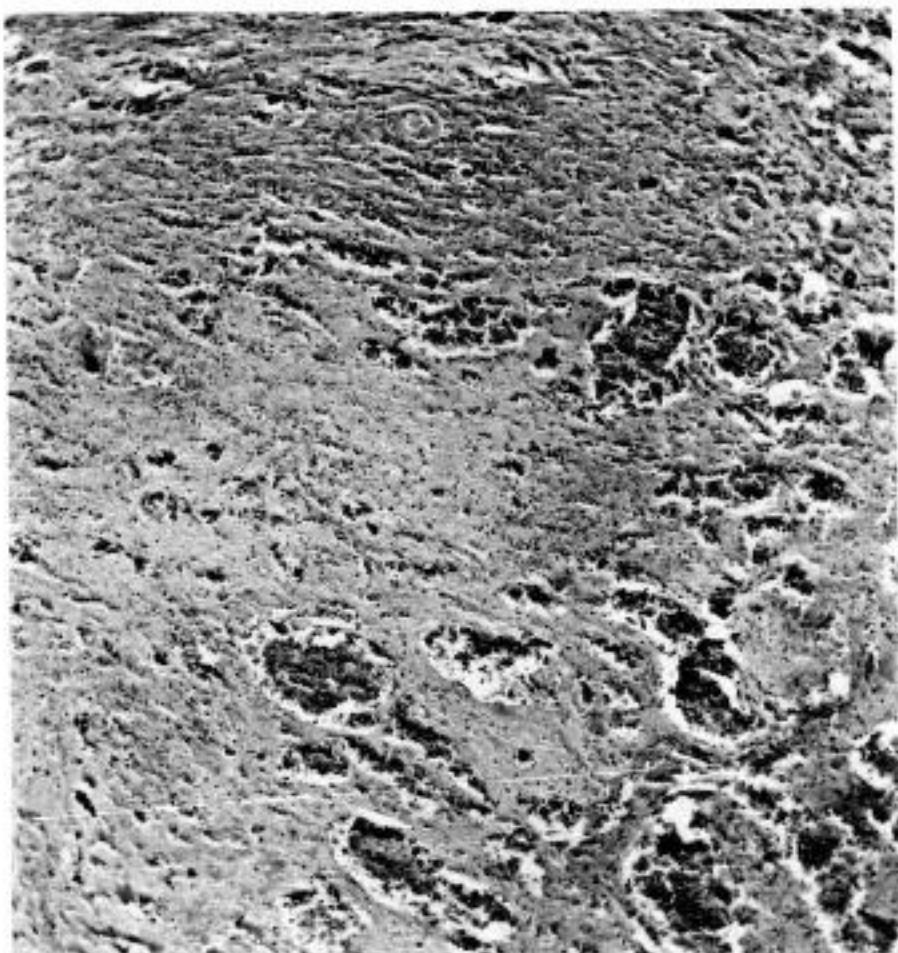


FIGURE 8. Necrosis at five days.

button which is palpable rectally and this is placed at the apex of the prostate gland. Any extra length projects into the bladder, and the bladder is insulated by the air. The prostate is palpably and visibly frozen within 30 seconds. The temperature of the probe tip is reduced to -190° and the freezing process continued for five minutes. As cooling starts, the prostate becomes adherent to freezing surface. As the temperature is reduced, there is obvious expansion within the frozen prostate. The lesions produced resemble those noted in the animal experimentation. The necrosis apparent (FIGURE 8) at five days in that area adjacent to the urethra was complete. Towards the periphery, there was partial survival. At the very edge of the prostate, there was tissue that seemed histologically near-normal.

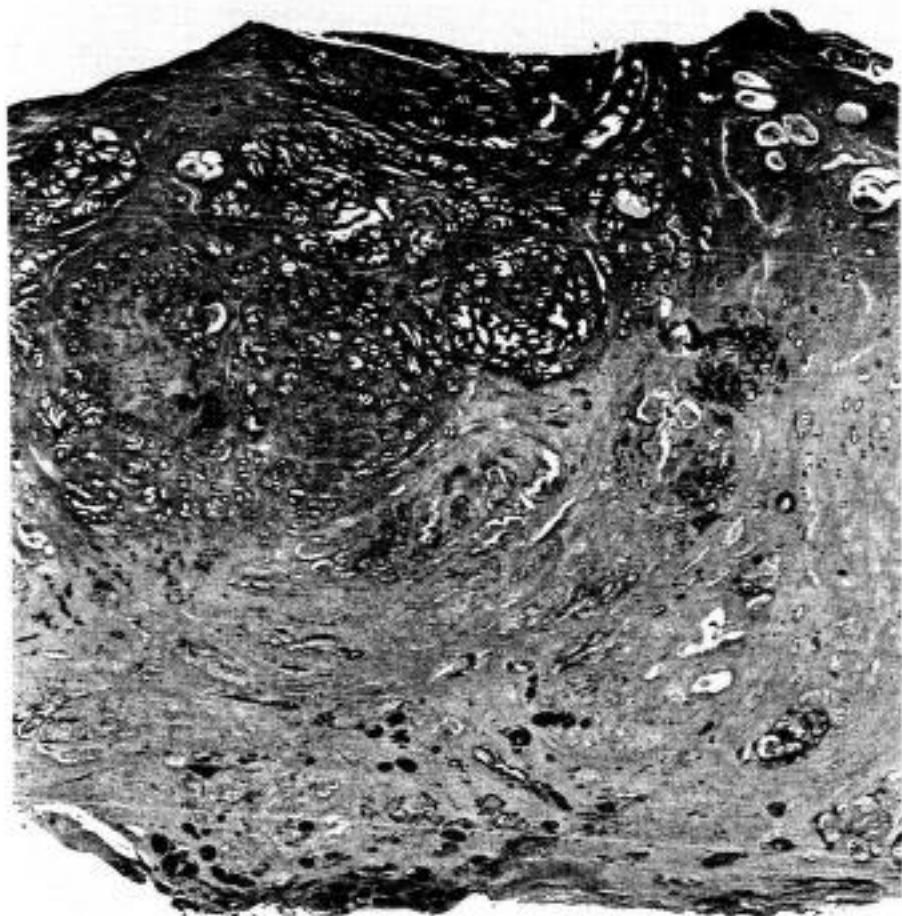


FIGURE 9. Full thickness prostate gland.

Utilizing a Gomori histologic technique, there is, histologically, no acid phosphatase activity in this tissue. Apparently, the rather sophisticated function of the prostate epithelium to produce enzymes is suspended, at least temporarily by the freezing process. The whole thickness of the prostate gland is shown (FIGURE 9). Much of the necrotic portion has been discharged in the urine. Some remains, and the more normal hypertrophied prostate remaining is demonstrated. This elimination of necrotic prostate within five days is illustrated (FIGURE 10) by these x-ray pictures on the left showing the air-filled bladder with the probe in place, and the hypertrophied prostatic impinging on the floor of the bladder, and on the right upper, a preoperative urethrogram, and on the lower picture, the urethrogram taken at five days, clearly demonstrating rather marked widening of the bladder and intravesicalization of the bladder neck.

Several prostatic carcinomas have been frozen rather thoroughly. FIGURE 11 demonstrates tissue removed at 14 days for biopsy purposes. Extensive necrosis is seen. Squamous metaplasia is present in a malignant gland.



FIGURE 10. Left, air-filled bladder with probe in place, hypertrophied prostate impinging on floor of bladder. Right upper, preoperative urethrogram. Lower, urethrogram taken at five days, demonstrating rather marked widening of bladder and intravesicalization of bladder neck.

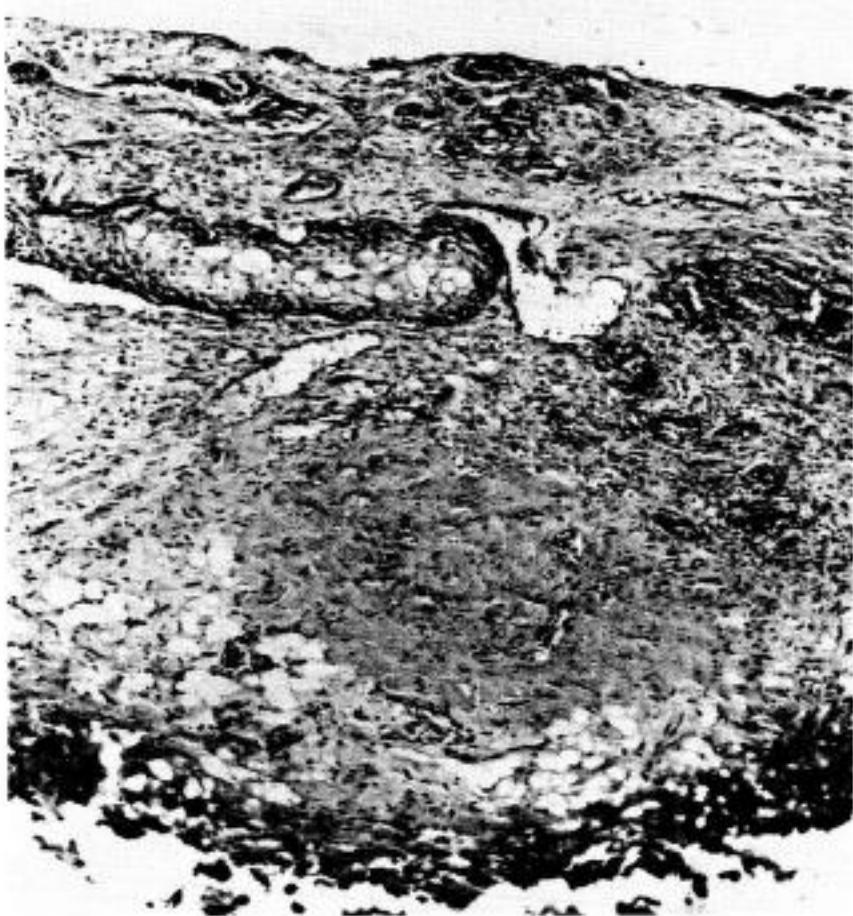


FIGURE 11. Necrosis and squamous metaplasia in prostatic carcinoma, 14 days.

There are scattered cells which may represent nuclear detritus or may represent remaining malignancy. Other sections of the biopsy specimen revealed only empty spaces where that glandular epithelium had been with stromal fibrosis and little resemblance to the earlier picture. FIGURE 12 demonstrates the reaction produced within the prostate when the gland is frozen to -20° and no colder. There is necrosis to a less marked degree. There is severe fibrosis and squamous metaplasia seen. This is a typical reaction of the prostate to injury of any sort.

We have now treated 15 patients with prostatism entirely by this method. To recapitulate our parameters:

1. None of the patients have run a fever or been otherwise ill post-treatment.



FIGURE 12. Squamous metaplasia and little necrosis seen when temperature only $-20^{\circ}\text{C}.$; 14 days.

2. We now feel that we have adequate equipment to destroy the prostate by freezing.
3. There has been transient hematuria. In every case, this has cleared within 12 hours, thus far presenting no clinical problem.
4. In most cases, catheter drainage was not required after five days. In all cases, the catheter had been removed in 14 days.
5. There has been no damage to the bladder function or to the external urethral sphincter or rectum. This is apparently due to the fact that these structures have a separate blood supply which is unaffected by the freezing of the prostate by this technique.

Gonder *et al.*: Chemical and

6. Although fibrosis in the remaining tissue may be a problem, we are dealing with a structure of such width that contracture of the urethra occurred, it did not become clinically apparent.
7. Bacterial infections of the necrotic tissue were not clinically apparent.
8. Adequate destruction of the prostate was consistently achieved and occurred in all cases.
9. The method is simple and safe, requires no special equipment and caused no significant blood loss.

We are now proceeding in an ordered manner to extend the use of this treatment to all patients presenting with symptoms of prostatitis.

Summary

We have presented a description of procedures for transurethral resection of the prostate using a chemical agent. We have described the equipment adapted for clinical use. It is our opinion that the technique is safe and effective. We have commented on those currently available and effective treatments for prostatitis. We believe that the use of this technique in patients with mild and incipient difficult cases of prostatitis should be deferred until the patient's condition is more advanced.

SUMMATION AND

H. S. FRANK (*University of P*)
posed procedure was that the disc
with the discussion of the other p
of this morning's program. The
and I will make some preliminary
cussion, perhaps giving some furt
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looking forward all week. Fernández
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As the next item I have an exc
Steering Committee had tried to se
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havior of water as a solvent, computed in solution, discussing the influence giving the first quantum-mechanical treatment of the hydrogen ion in acid solutions. His effort and in this regard is still a me

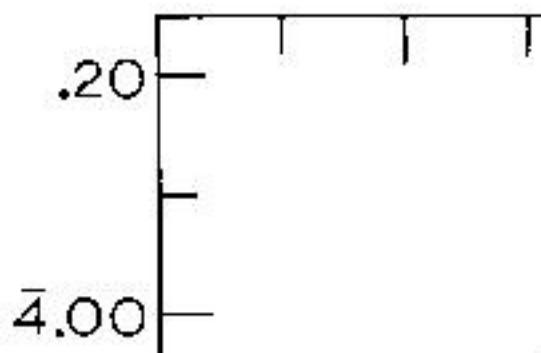
Again, for those who may wish to know more about him, he is now Professor of Crystallography at the University of London. He is a Fellow of the Royal Society and number of other distinguished societies. He was a student of Fowler, of Cambridge University, one of the great physicists of the last generation. He did not write the formal parts of the joint paper, but carried through in respect of water.

Another respect in which a dedication to Bernal is particularly appropriate is that the field in which he has been active is the discussion of the physical properties of water. In his Presidential Address to the Meetings of the Physical Society (1948), he said: "I am sure you will all agree with me that it would have been a source of much pleasure to me if I could have spoken here, but that the meetings were more interesting than they have been for some time. We are grateful to

As a starting point for some intri-

structure are drawn from light scattering data added to this list.

Weillaufer and his associates in the University of Minnesota, interested in denaturating agents in protein systems, have studied model systems (1964, J. Am. Chem. Soc., 86, 1000) in which the hydrocarbons chosen in this work were the simple aliphatic hydrocarbons which are found on the amino acids of proteins. They measured the solubility of these hydrocarbons in water and in urea by the same method, in seven-molar urea at 25°C. Methane is less soluble in the urea than it is in water, ethane is more soluble, propane is more so at high temperatures (FIGURE 1). The data in TABLE 1, namely, that the transition from water to the seven-molar urea solution is spontaneous, ΔH° is positive, but ΔS° is negative, indicates that the hydrocarbons, so to speak, swim up to the surface of the urea solution.



Frank:

TA
THERMODYNAMIC FUNCTIONS FOR

Solute	ΔH°
	kcal./mole
Methane	1.3
Ethane	1.9
Propane	1.7
Butane	1.9
Isobutane	1.5
Neopentane	1.7

pound formation, because that would be the most stable form. The hydrocarbons tend to go where they must do so for reasons connected with entropy.

Clearly, then, there is something more than an entropy effect. It has presumably to do with the size of the molecule. Franks and I have worked during the last few years on this problem, and our attempt has required enlarging some

Another of the things that makes abundantly obvious in the earlier sessions does different things in different concentrations of behavior which it imposes upon it. To make dilute solutions, you get a little structure in this direction or that direction; more concentrated the modifications but can change in kind, and when they are strong and you have the water against a membrane structure, then what the water does we know but under different influences. But, in order to want to what water does in any one case, we know as much as we can about what it does is something else which should be kept in mind.

Except for this interrelationship, I am the interloper at these sessions, grateful, however, for the opportunity of learning things during the week which I have not learned as a physical chemist.

If the present state of the study of water is what we look for now? What should we at present be trying to make a contribution to?

Frank: Di

there is no cooperativeness between what a neighboring water molecule does.

The opposite explicit assumption is that if there is cooperativeness, then what do in patches. There will, instantaneously structured patches and these patches will grow and so on. They will also be forming at a very high frequency. We speak of this at a time to go into a lengthy discussion of the hypothesis on theoretical grounds, but to me certainly is. It is also attractive on experimental grounds because you can account for the maximum of different properties of water. As you know, as water its viscosity goes down, and a whole lot of other things, including some recent spectroscopic ones, which I will not go into now. You will have to refer to in any detail. At any rate, this is a model. But, do not ever forget that some people give you quite convincing arguments why mixtures are "for the birds." This is a question of taste, and you takes your choice."

I, as you see, have plunked my money down that I think water consists of flickering fluctuations. Now, it is true that water is not a

We assume, then, that water is made up of flickering clusters, and that a thermal fluctuation will cause some molecules to jump to attention in a structured chunk here, which will then melt down, and a structure chunk there that will melt down. This is happening all over the place very rapidly. In that case we must try to see what these species are like. How do you describe the bulky species? One species has to be bulky, otherwise you do not get the maximum of density. Well, in the case of the bulky species we have an embarrassment of riches, because there are so many bulky structures of water molecules which are known. There is hexagonal Ice I and cubic Ice I and there are the structures of the clathrates, which we saw yesterday. Solid water can do all of these things, and this produces an embarrassment of riches in speculating about liquid water, which I think may have something to do with Drost-Hansen's embarrassment with the liquid seeming to behave differently in different temperature ranges. This would be because there are so many different kinds of structures which could exist, and the fact that there is no reason why the one which is most favored in one set of circumstances (temperature and pressure) should also be most favored in another.

When we come to the dense species the embarrassment is of a different sort, because it is the existence of the dense species which is denied by the physicists who do not like mixture models, and one must not minimize the difficulties. What is this dense species like? How can water be close-packed when its molecule has so large a dipole moment? Well, a quantum mechanical argument can be advanced which I think is pretty good, making use of the fact that when a water molecule is rotating in certain states its dipole moment gets washed out. Quantum mechanics indicates that this does indeed happen. In such states you might be able to have rotating molecules which could be close packed, and then you would be able to have one of the dense species that has been proposed by a number of people. Or the dense species may be one in which hydrogen bonds are bent, and Scheraga has essentially taken the Lennard Jones-Pople water model and allowed the LJP water to be the dense species that surrounds, and changes places with, the structured clusters. At any rate, this is something which is on the agenda for further elucidation, what can be the nature of the dense species.

Then, what kind of an equilibrium can there be between the dense and bulky species? This might be a simple mass-action equilibrium, as a lot of people have written, or it could be a more complicated mass action equilibrium in which an exponent is put in for the polymer-number of the clusters. There could also be an entirely different equilibrium constant such as is obtained when you do the statistical thermodynamics for an interstitial model, in which the dense species consists of separate monomers inside of clathrate cages. In this model liquid water could be called a "water hydrate"

Frank:

in the same way that we have xenon these possible equilibrium constants to a change in temperature, a change etc. This is therefore also on the ag there are probably several simultaneous species with three equilibria among offer an interpretation of the phenomena about.

Again, whatever we say that water of the behavior of aqueous solutions, kinds of solutes: ionic solutes all have of them, if the ions are small and have lining-up of the water molecules, but structure-breaking. This is easy to understand: an ion tries to orient the water one way to orient another way, and there is a region around it where this will produce a region of breaking.

Then there are the hydrogen bonds: water structure reasonably well, so water, for instance, is not very nonideal. It like water pretty well, and behave nonideal. However, NH_3 does not do

Then we have polyfunctional solvents. An alcohol molecule has an OH group and it is a good solvent for alcohols the other morning. Amino acids are polyfunctional solutes. They have a zwitter-ion structure on the zwitter-ion end and a hydrocarbon tail on the other end. This making of structure dominates the behavior of amino acids in aqueous solutions (but not the dielectric properties). As the concentration of the solutes get more concentrated, the interactions between the solutes are not simply additive and we get some quite interesting effects. Of course, what this meeting is all about is what water does in rather complex systems containing polyfunctional solutes.

There are several outstanding puzzles in this field. One of them is telling us about the change in the NMR when hydrocarbon tails are packed closely together. It was thought that hydrogen bonds were being broken. However, it was not clear exactly what was happening. In inventing a euphemism for this, we said that hydrogen bonds were becoming more "ordered". We did not know exactly what happens there. On the first hand, it was thought that hydrogen bonds were being broken, because the temperature were being raised, but our thermodynamic information tells us that the entropy information obtained from the relaxation times is not consistent with this.

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model. Here, I would suggest that an It happens that in Pittsburgh my co studying the equilibrium in TMV pro units which make up the rods. At a pH at the ice point there are no rods, but w rods are formed. You can see this by electron microscopy, and it is a reversible up and down as often as one wants. Th ture has caused agglomeration of the s posed to mean a loss in entropy, because the subunits. On the other hand, if it g this means that there has been a gain happened, therefore, is that when you units come together, they release some w subunits, and the release of water prod then drives the reaction forward. The been determined within modest limits, a meetings, has done some measurement amount of water released when the ro tobacco mosaic virus protein which pu up of subunits is going to have to be a

instantaneously distinguishable species, operational value to the biologist, stimulating and challenging ideas who with remarkable foresight may not only the *mater*, mother, it is tually paraphrase the title of this "Formed by Water." We, as biologists, that all biological systems are com nated and is maintained in a water began to consider water as more than a structured matrix, that as Szent-Györgyi, a fantastic and fascinating world years and particularly during these this concept of "structured-water" by the biologist. This is in large measure presented by physicists and physi a number of unique properties which of a filling agent.

Even though it is evident from a disciplinary approach that the study is in a difficult stage, one cannot help evidence in support for a mixture

we detect "ordered" water in biologic approaches are feasible?

Obviously, these questions have been stimulating and original papers that have reminded us, water does many differentiations. If we are to conceive of ordered highly differentiated mosaic of organization at the macromolecular and molecular levels in living systems, it is only natural to ask specifically tailored to its molecular example opportunity for good use to be made of possibilities of the bulky species in constituting a living cell: from the crystalline hydrates to possibilities of frameworks of approximation, and the particularly appealing case of Fortuitously, today we have at our disposal tools, such as nuclear magnetic resonance, x-ray diffraction, x-ray scattering and diffraction, the state of water in biological systems. For example, the beautiful studies on the hydrogen molecules by H. J. Berendsen¹⁵ and C. M. Grana's magnetic resonance are already yielding very

for accurate determination of the crystal structure and growth by low-temperature methods. Recent technical limitations set hitherto by vacuum contamination, have made it possible to record electron micrographs and extend the results of previous studies. Thus, electron micrographs and recorded from minute ice crystals and protected from contamination and extending the results of previous studies. However, much more a critical investigation is needed of the amorphous or vitreous ice. At the present stage of our knowledge, the diffuse ring patterns obtained may be due to "vitreous" ice. This is of the greatest importance for the structure of ice is intimately linked with the presence of impurities at temperatures.

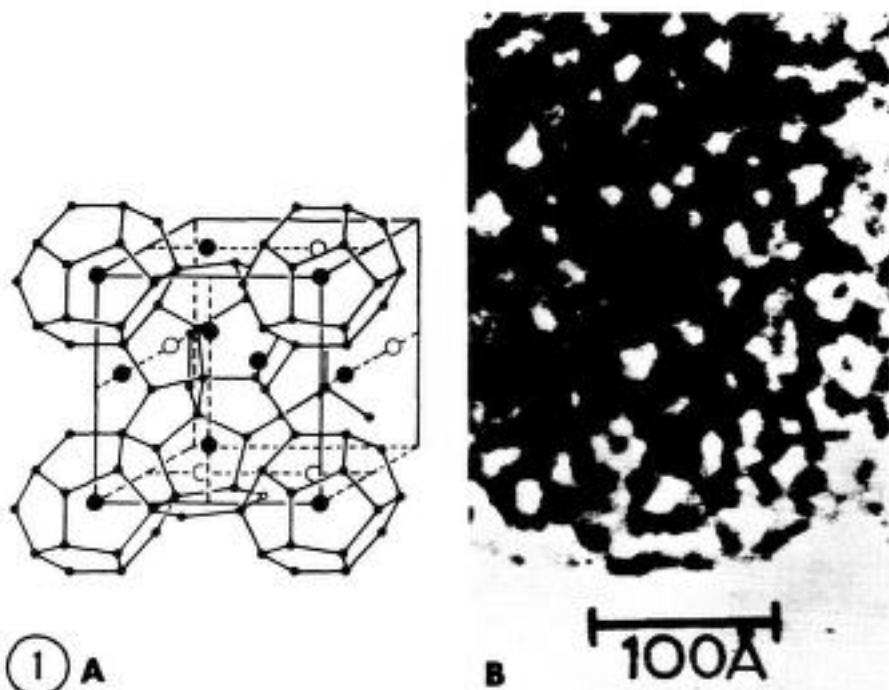


FIGURE 1a. Model of arrangement of water molecules in crystalline nonpolar hydrates according to von Stackelberg *et al.*

FIGURE 1b. High resolution electron micrograph of microcrystalline hydrates of tetra-n-butyl ammonium tungstate enclosed in vacuum-tight microchamber. $\times 2,300,000$.

Using the described experimental approach of low temperature electron microscopy, we have been examining the hydrates of tetra-n-butyl ammonium tungstate (which was kindly supplied by Richard K. McMullan). Ions instead of molecules occupy the cavities within the icelike lattice of the quaternary ammonium salt hydrates, and the n-butyl compounds have tetragonal crystal structures with a unit cell, $a = 23.6 \text{ \AA}$; $c = 12.4 \text{ \AA}$.²⁷ As shown in FIGURE 1b electron microscopy of these compounds, suitably prepared in special vacuum-tight microchambers and examined at low temperature, reveals typical cuboidal-shaped microcrystals giving characteristic single-crystal electron diffraction patterns. At higher magnifications, the microcrystals exhibit an exceptionally regular polyhedral structure, with periodic subunits of 10 to 20 \AA resembling the general arrangement in the postulated models of crystalline hydrates, in a form not unlike so-called negative staining, due to the built-in heavy tungstate ions (FIGURE 1b).

Continuing earlier work, we have been engaged in attempts to provide direct experimental verification of organized water structures of the postulated crystalline hydrate type through local formation of microcrystalline, noble gas hydrates in selected lipid, lipoprotein complexes and cell membranes. By application of argon and xenon under controlled high pressure (approximately 1500 pounds per square inch) and temperature in special specimen chambers, one can produce characteristic electron-dense microcrystalline hydrates (about 10 to 20 Å) in which the chemically completely unreactive noble gas atoms occupy polyhedral cavities in a hydrogen-bonded framework of water molecules.^{2,10} This either contributes further to stabilize and make electron-optically visible any pre-existing organized water structures of the hydrate type in lipoprotein systems, or alternatively, to bind available free water molecules in the process of entrapping the noble gas atoms. This technique has been successfully employed in combination with negative embedding procedures, to visualize argon or xenon hydrate microcrystals of 10 to 20 Å, localized mainly in the hydrophilic regions of isolated lipoprotein layers (FIGURES 3, 4) and cell membranes. When this

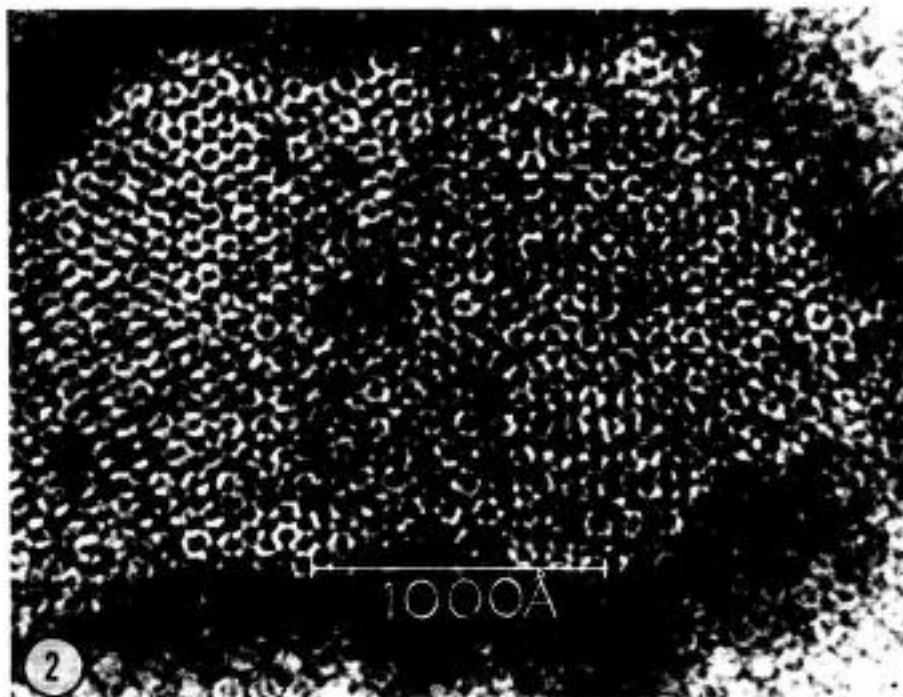


FIGURE 2. Electron micrograph of Ferritin/PTA microdroplet exposed to argon gas under pressure showing ordered ferritin aggregates. $\times 360,000$.

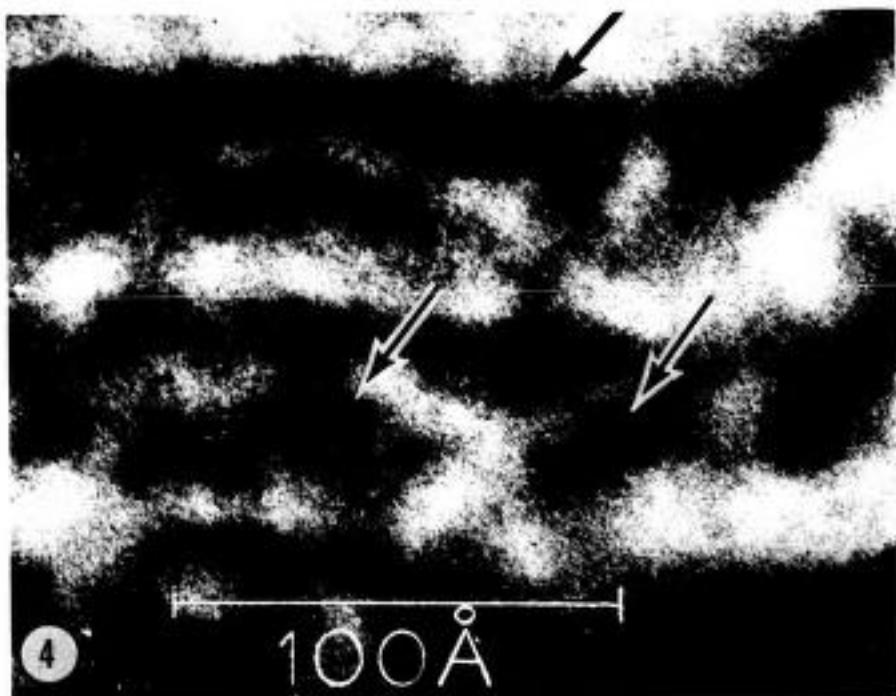


FIGURE 4. Low-temperature electron micrograph of lecithin micelles embedded in thin phosphotungstate film exposed to argon gas under pressure showing dense aggregate (arrows) presumably induced by the formation of argon hydrate microcrystals. $\times 5,600,000$.

These experiments are of relevance to the molecular theory of general anesthesia, postulated by Pauling and his associates. Pauling²⁸ and subsequently Miller, postulated that formation by anesthetic agents, including noble gases, of clathrate-like structures in the aqueous portion of nervous tissues could cause anesthesia by modifying the electrical activity through entrapment of ions and electrically charged side chains of protein molecules, due to increased amounts of structured water in these regions. The described techniques for direct electron microscopic visualization of noble gas hydrates, particularly of xenon which has high electron density, could provide direct experimental proof of the existence of these hydrate microcrystals forming reversibly in membranes. In fact, one might even go beyond this theory of Pauling, which has certain features in common with the hypothesis of anesthesia as propounded by Claude Bernard.²⁹ According to Claude Bernard's theory of anesthesia, anesthetics would produce a reversible coagulation of the constituents of the nerve cell and of other less sensitive tissues. If, indeed, such reversible "coagulations" or

phase changes can occur in the nerve would be the synaptic membranes. A suppose that these transformations are but perhaps with certain rhythmic c In any event, reversible modifications highly complex structures in the cen take into account the structure and ticularly in biological lamellar system transfer, hydrate structures of this tein layers would provide an interco protonic charged-transport mechanism

We come next to the problem of att systems in the native hydrated state considered to be insuperable. This wa tron microscopy has to be carried out sidered impossible to get sharp pic suspensions of particulate components tion. Actually it has proven to be le Recently, it has been possible in our lab *et al.*, and, in a more thorough fashion to build special vacuum-tight microch liquid or an aqueous suspension of

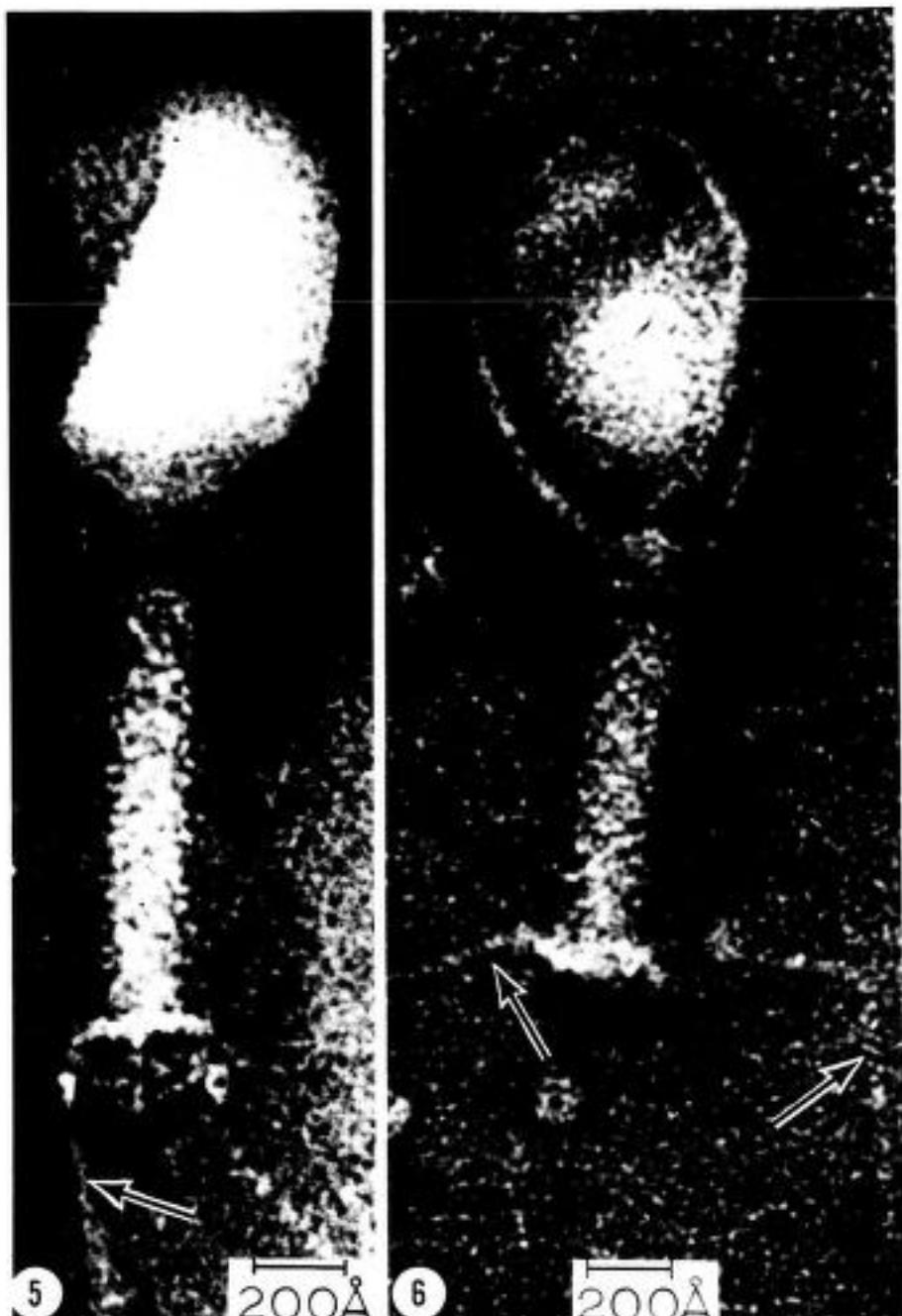


FIGURE 5. Electron micrograph of T2 bacteriophage negatively stained with PTA. $\times 575,000$.

FIGURE 6. Low-temperature electron micrograph of partially hydrated T2 bacteriophage enclosed in vacuum-tight microchamber. $\times 525,000$.

microscopy, proving by subsequent culturing that the bacteria have not been killed and are still viable.

Modern electron microscopes are capable of resolving directly the array of atoms in crystalline lattices of the order of a few angstroms (FIGURE 8). Beyond this there are certain characteristic electron-optical phenomena, such as moiré patterns which are particularly suitable to the study of organized water and of ice crystals. Thus, when two ice crystals overlap the electron microscope images display regular fringe patterns, that may be interpreted as a moiré pattern arising from the coincidence of the projected planes of atoms in the overlapping lattices. By means of such moiré patterns, indirect resolution of the atomic array in crystalline lattices has been achieved and the lattice imperfections can also be studied.¹⁵ When ice crystals are grown at -90°C. on mica or graphite single crystals, (FIGURE 7), it is possible to detect, in suitably oriented ice crystals, a series of dense fringes with regular spacings from 15 Å upward. The corresponding electron diffraction pattern show the typical arrangement of double

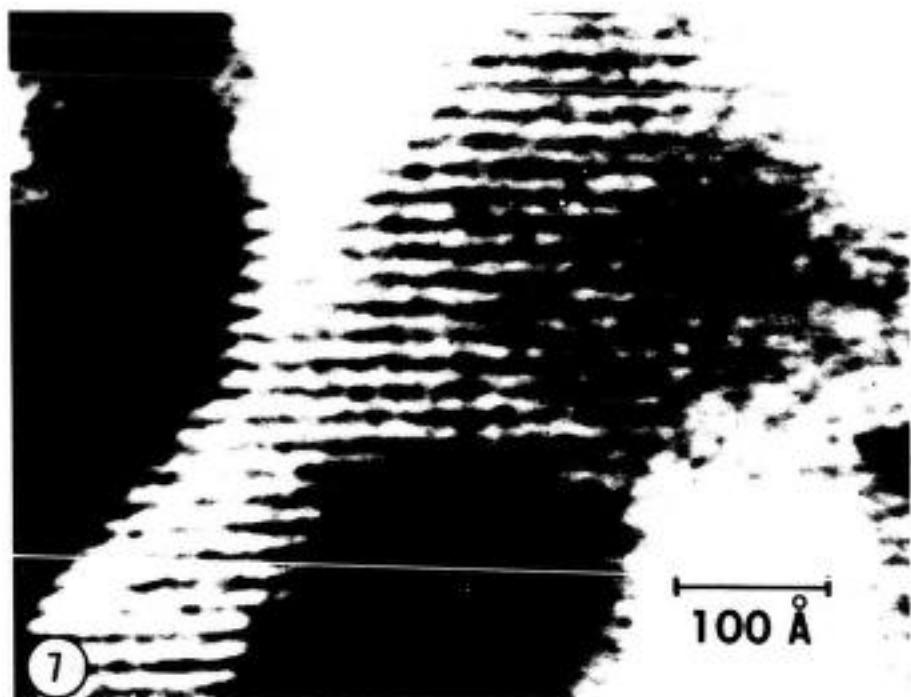


FIGURE 7. Low-temperature electron micrograph of moiré pattern exhibited by ice crystals grown on a single-crystal mica film at -80°C., with a regular period of 18 Å. $\times 2,000,000$.

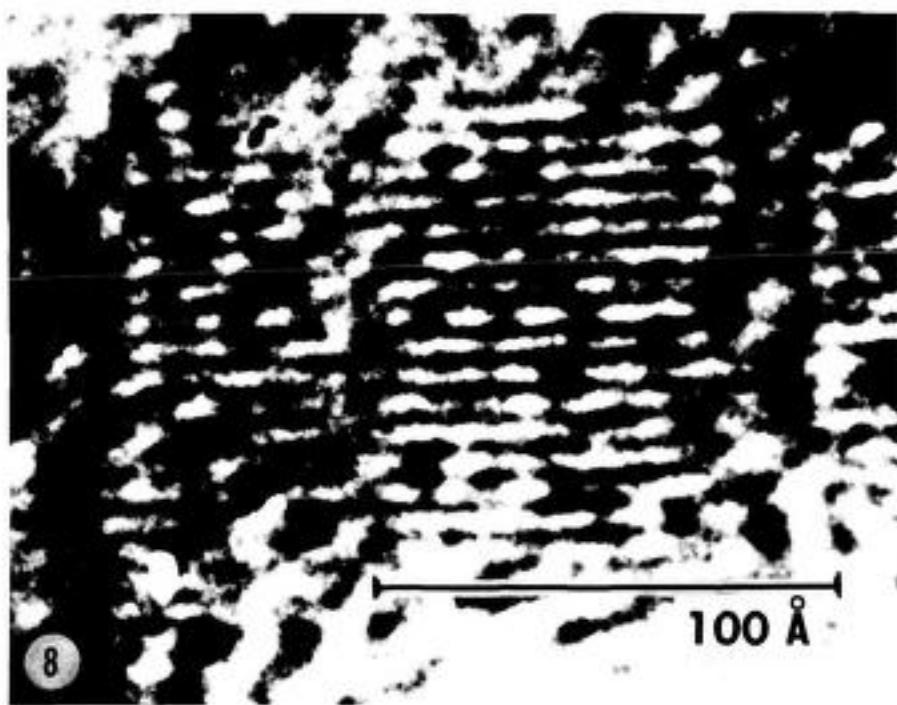


FIGURE 8. Low-temperature electron micrograph of K_2PtCl_4 microcrystal showing lattice spacing of (100) planes. Spacing resolved by direct illumination method is 6.99 Å. $\times 5,900,000$.

diffraction spots around the primary diffraction spots. Although interpretation of these moiré patterns is still at a preliminary stage, it is hoped eventually to observe patterns that are related more directly to the lattice structure of the ice crystals. It may also be possible to recognize lattice dislocations and other modifications by combining high resolution electron diffraction patterns with the corresponding electron micrographs. Rapid changes in the moiré patterns and associated modifications of the diffraction patterns of ice crystals subjected to irradiation at low temperatures have also been recorded in motion picture sequences. Direct observation of ultra-rapid processes occurring in microseconds or even shorter intervals may eventually be possible by using stroboscopic illumination obtained through pulsed-T-F emission from pointed filaments in combination with high speed photography.^{16,21,22} By virtue of this inherent capacity for achieving both high spatial and high temporal resolution, improved electron optical methods can be expected to play a key role in the elucidation of the structure of ice and other phases of water, including organized water in biological systems.

Beyond its fundamental role as a structured matrix of life, water may be endowed with an equally important, and yet more subtle role, namely as the mediator of information and energy transfer at the molecular level in biological systems. I am reminded here of the fascinating simile made by Frank in explaining the flickering clusters. Frank, in referring to the flips which are always taking place whether we are looking at them or not, points out that the Atlantic Ocean has waves in it whether we are there to see the waves or not. One wonders whether these waves of incredibly short duration (10^{-11} seconds) and involving clusters of only 100 molecules of water might not be playing an essential role in sustaining the incessant movement that we all witness when we examine living cells. Could it not be that one of the unique attributes of living protoplasm is that its molecular constituents are able to respond, to "resonate" harmoniously with the flickering clusters in water, "riding" in synchrony with these molecular waves during life, and bobbing around at random like an inert cork of dead matter once they have lost this capability? Moreover, we can ask ourselves, what role does water play in the basic functions of living organisms, which according to Schrödinger,²² maintain their organization by extracting 'order' from the environment? Is water the entropy drainage system par excellence? These and many other questions will be more meaningfully posed in the years to come.

We come back full cycle to Szent-Györgyi's definition that "research is to see what everybody has seen and to think what nobody has thought before." Thanks to present advances in concepts and methodology, our generation of biologists is now able to start thinking about water and its cardinal role as no other generation has been able to do before. Ultimately, we will probably have far deeper reason than Thales of Miletus in acknowledging the full truth of his cryptic saying that "Water is best." I feel that the problem of water is not only of critical importance to an understanding of terrestrial systems, but water or equivalent compounds may very well play a great role in understanding extraterrestrial phenomena, as Miller,²³ has already suggested.

To conclude with a humorous note, some years ago *The New Yorker* had a cartoon depicting an obviously very far off planet with a wrecked space ship, from which a weird, alien creature emerged, with drooping antennae, straggling away, gasping for "ammonia, ammonia!"

References

1. BERNAL, J. D. & R. H. FOWLER. 1933. Theory of water and ionic solution with particular reference to hydrogen and hydroxyl ions. *J. Chem. Phys.* 1: 515.
2. FRANK, H. S. & WEN, W. 1957. Ion-solvent interaction: structural aspects of ion-solvent interaction in aqueous solutions, a suggested picture of water structure. *Disc. Farad. Soc.* 24: 133.

3. FRANK, H. S. 1958. Covalency in the hydrogen bond and the properties of water and ice. *Proc. Roy. Soc. A247*: 481-492.
4. SZENT-GYÖRGYI, A. 1957. *Bioenergetics*. Academic Press, New York, N. Y.
5. EGELOSTAFF, P. A. 1962. *Advances Phys.* 11: 203.
6. FERNÁNDEZ-MORÁN, H. 1959. Fine structure of biological lamellar systems. *Revs. Modern Phys.* 31: 5.
7. HECHTER, O. 1964. On the role of water structures in the molecular organization of nerve cell membranes. Presented at American Cancer Society Conference on Cryobiology. Rye, New York, October 9, 10, 1964. In press.
8. FERNÁNDEZ-MORÁN, H. 1960. Improved pointed filaments of tungsten, rhenium and tantalum for high resolution electron microscopy and electron diffraction. *J. Appl. Phys.* 31: 1840.
- 9a. FERNÁNDEZ-MORÁN, H. & J. B. FINEAN. 1957. Electron microscope and low-angle x-ray diffraction studies of the nerve myelin sheath. *J. Biophys. Biochem. Cytol.* 3: 725.
- 9b. FERNÁNDEZ-MORÁN, H. 1962. New approaches in the study of biological ultrastructure by high-resolution electron microscopy. Paper presented at Symposium of the International Society for Cell Biology, Berne (September, 1961). *In Symposia of the International Society for Cell Biology*: 1: 411-427. R. J. C. Harris, Ed. Academic Press, Ltd. London, England.
10. FERNÁNDEZ-MORÁN, H. 1962. Cell membrane ultrastructure. *Circulation* 26: 1039.
11. FERNÁNDEZ-MORÁN, H., T. ODA, P. V. BLAIR & D. E. GREEN. 1964. A macromolecular repeating unit of mitochondrial structure and function. *J. Cell Biol.* 22: 63.
12. FERNÁNDEZ-MORÁN, H. 1961. The fine structure of vertebrate and invertebrate photoreceptors as revealed by low temperature electron microscopy. *In The Structure of the Eye*: 521-556. G. K. Smeles, Ed. Academic Press. New York, N. Y.
13. SCHMIDT, F. O. 1959. Molecular biology and the physical basis of life processes. *Revs. Modern Phys.* 31: 5.
14. NEMETHY, G. & H. A. SCHERAGA. 1962. *J. Chem. Phys.* 36: 3382, 3401.
15. BERENDSEN, H. J. C. 1960. The structure of water in tissue, as studied by nuclear magnetic resonance. *Biol. Bull.* 119: 287.
16. FERNÁNDEZ-MORÁN, H. 1964. New approaches in correlative studies of biological ultrastructure by high-resolution electron microscopy. *J. Roy. Microsc. Soc.* 83: 183-195.
17. HAINE, M. E. & V. E. COSSLITT. 1961. *The electron microscope*. Interscience. New York, N. Y.
18. FERNÁNDEZ-MORÁN, H. 1960. Low temperature preparation techniques for electron microscopy of biological specimens based on rapid freezing with Helium II. *Ann. New York Acad. Sci.* 85: 689.
19. FERNÁNDEZ-MORÁN, H. 1961. High resolution electron microscopy of hydrated biological systems. *Proc. Internat. Biophys. Congr.* :324 Stockholm, Sweden.
20. FERNÁNDEZ-MORÁN, H. 1959. Cryofixation and supplementary low-temperature preparation techniques applied to the study of tissue ultra-structure. *J. Appl. Phys.* 30: 2038.
21. FERNÁNDEZ-MORÁN, H. 1960. Direct study of ice crystals and of hydrated systems by low-temperature electron microscopy. *J. Appl. Phys.* 31: 1841.
22. FERNÁNDEZ-MORÁN, H. 1960. Low temperature electron microscopy of hydrated systems, in fast fundamental transfer processes in aqueous biomolecular systems. :33. Massachusetts Institute of Technology, Department of Biology. Cambridge, Massachusetts.
23. CLAUSSSEN, W. F. 1951. Suggested structures of water in inert gas hydrates. *J. Chem. Phys.* 19: 259, 1425.

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24. PAULING, L. & R. E. MARSH. 1952. THE HYDROPHOBIC NATURE OF PROTEINS. *Nat. Acad. Sci.* 38: 112.
25. STACKELBERG, M. v. & H. R. MÜLLER. 1953. Die Anwendung der Elektrolyttheorie auf die Raumchemie. *Ztschr. Elektrochem.* 57: 101.
26. KLOTZ, I. M. 1960. Protein molecules in solution. *J. Phys. Chem.* 64: 1025.
27. McMULLAN, R. & G. A. JEFFREY. 1959. Interaction of tetra i-amyl quaternary ammonium salts with proteins. *J. Phys. Chem.* 63: 1550.
28. PAULING, L. 1961. A molecular theory of the hydrophobic effect. *J. Phys. Chem.* 65: 15.
29. BERNARD, C. 1875. *Leçons sur les anémies*. Baillière, France.
30. EIGEN, M. & L. DEMAYER. 1959. Hydrogen bonding, ionization, and proton transfer in aqueous and non-aqueous electrolytic Solutions: 64-85. W. J. H. D. H. S. New York, N. Y.
31. DUPUOY, G. & F. PERRIER. 1962. J. M. 1962. *J. Phys. Chem.* 66: 1025.
32. SCHRÖDINGER, E. 1948. What is life? Cambridge University Press, Cambridge.
33. MILLER, S. L. 1961. The occurrence of hydrophobic groups in proteins. *Nat. Acad. Sci.* 47: 1798.

GENERAL

FINNEMA (*University of Wisconsin*)
make a few comments on the clathrate
some work in the area of clathrate co-
that I have observed with the charac-
with the theory as we have seen it.

I would like to cite several exa-
consider the formation of the clathrate
consisting solely of water plus the
find it is not particularly easy to fo-
sort, at least in the systems with
have the clathrate former present
be saturated or nearly saturated, w-

Second, the system must be und-
tion temperature of the clathrate w-
several degrees undercooling. The
rate will form.

Another condition which genera-
that of agitation. If we do not agi-
tate a solution of gas with a li-

General Discussion

By adding gases such as hydrogen, carbon dioxide, and other ingredients of this sort, we can improve the properties of these compounds slightly. It has been my observation that it is necessary to exert severe pressures on the system to improve its properties.

It is suggested then that if there are certain substances such as proteins or other colloidal constituents which are present in the clathrate compounds and allow them to decompose.

We have formed clathrates in aqueous systems containing mixtures of protein, lipid, and carbohydrate. There is very little difference between the decomposition temperatures of clathrates as compared to systems in which no protein is present.

If we look at the whole pattern of decomposition of sodium chloride or sugar for example, at different temperatures, we find that the critical decomposition temperature of a clathrate is the same as that of the salt. If we add salt to a clathrate, we find that it loses its water of crystallization to a magnitude which is nearly identical with that which it would have if it were a pure salt. That is, the water of crystallization of the salt is lost to the same extent as that of the clathrate.

If you try to take a clathrate like sucrose and decompose it at a low temperature, again formation of a true clathrate is unlikely to be due to the temperature.

clathrates, like half hydrogens in water structure. I do not know, however, they do seem to be quite different.

H. FERNANDEZ-MORAN (*University of Chicago, Chicago, Ill.*): Listening to what we just heard, we are quite amazed at the ease with which Faraday over 100 years ago made clathrates and with the ease with which any student really can produce clathrates. It is true that many of the variables you have enumerated hold, but it is by no means necessary to fulfill all of them.

You have to define very rigorous criteria for establishing whether you have clathrates or not. What you are talking about are crystals of clathrates for x-ray diffraction study. This is quite a different order of problem than forming microcrystals, which you can only detect with polarization optics, electron microscopy and electron diffraction.

H. A. SCHERAGA (*Cornell University, Ithaca, N. Y.*): I just want to speak to only one aspect of the many that the speaker raised, that is, the stability of the clathrate. He referred to the concentrations. In the theory of hydrocarbon solutions, which Nemethy and I developed, we did not use the concept of a complete clathrate cage around the hydrocarbon, but rather a partial cage. The origin of this partial cage was a shift in energy levels of those occupied by the water molecules. One of these levels, namely the lowest one which corresponded to tetrabonded water, was shifted downward because the hydrocarbon could interact with the tetrabonded water species via Van der Waals type interaction so that the water in effect became pentacoordinated. In hydrocarbons you have a very dilute solution so that a particular tetrabonded water species is going to have only one hydrocarbon molecule. If you are in a concentrated system such as in the crystalline hydrates, you have the possibility of a cooperation such that a water molecule in one cage may see a hydrocarbon inside that cage and there may be a hydrocarbon in the next cage. In other words, the hydrocarbons are filling neighboring polyhedral cages and you are getting greater stability in that more water molecules can interact with hydrocarbons and thereby stabilize complete cages as you have in acrystalline hydrates. In aqueous solution where these are dilute and, of course, in anesthesia, you do not have the hydrocarbons near enough to each other because the solubilities are too low to provide this cooperation. Thus we cannot stabilize complete cages.

I would agree with the speaker in the sense that in a microcrystalline range, I do not think you would expect to find complete polyhedral cages encapsulating or enclosing hydrocarbons as, I believe, was postulated in the anesthesia case. If you want to say that the partial cages which we have talked about can function in the anesthesia case, that is another possibility. But, I do not think that you can have complete cages in dilute

General Discussion

aqueous solution as you do have in the crystalline hydrate.

H. S. FRANK (*University of Pittsburgh*): I don't think it would be too literal in talking about a theory of anesthesia to say that Scheraga has given us a theory if you say microcrystals. The statistician has shown that the probability of increased and this would produce the effect of anesthesia is increased if the discussant mentioned at the very beginning of his paper was repeating the words of the theory, but he did not repeat them. Then we have something really good.

O. HECHTER (*The Worcester Foundation for Experimental Biology, Shrewsbury, Mass.*): My understanding of the molecular theory of anesthesia is that it arises from the action of agents, which are chemically nonreactive, on membranes. This unexpected biologic effect. I do not believe that it is part of Pauling or Miller that other agents, which might effect metabolic reactions in the body, in other ways influence ionic fluxes. This is the basis for an unexpected biologic finding. In this conceptual base with which to operate, the membranes assume great importance in determining the excitability properties that are being discussed.

force. Unless you are going to say differently from the way in which I then I do not believe what you have

A. LEAF (*Massachusetts General*) very important to point out again Fenichel was referring, these poor membranes are highly lipid and all thus permitting the urea to leak off his evidence has anything to do with

I. R. FENICHEL: I was certainly not referring to the effect of the action of a nonpolar solvent is on the membrane. I was simply referring to the Pauling's work which he reported on the aqueous phase of the

H. A. SCHERAGA: You have a very good point. I am sure there are many effects involved. I think it has raised a very fundamental question. I would like to ask him if he has some picture as to what concentrated solutions is doing to water.

H. S. FRANK: The idea is that if you have a structured and unstructured species, then urea will dissolve one way in the structured and another way in the unstructured. In the structured species, the water molecules will be held

O. HECHTER: It is important to remember that when you translate simple aqueous solutions to the cell, this is an extrapolation which has no meaning for a biologist. You know that urea, for example, has an effect on some proteins. It might be breaking hydrophobic bonds, as well as having effects on hydrogen bonding systems. In the context of the cell interior where you have an agent that acts both on proteins as well as on water, the effects in some cases are much more mysterious than simple aqueous solutions.

G. N. LING (*Pennsylvania Hospital, Philadelphia, Pa.*): I have heard so much about structure breaking and structure forming. For my own information I would very much like to hear to what structure are we referring? It seems to me that there are all kinds of structures. One kind is the clathrate and the other kind is what is referred to as the bound water. Warner has produced another kind of water which seems to be related to the water lattice in ice. We have been learning about these in this Conference. When we begin to talk about structure breaking, I would like to see if it is a single and unidirectional process. Does this mean that it applies to all. I would like to hear further comments, because it seems to me that an agent which may be structure breaking in one case might be doing just the opposite when the structure is different.

S. B. HOROWITZ (*Albert Einstein Medical Center, Philadelphia, Pa.*): Ling made an extremely important point and the last comment of Frank cast some light on some of the confusion that may be existing here. Frank, in summarizing what we had previously said, said it is either aqueous solution inside the cell or it is acting in a way which is very different from aqueous solution, as we understand it, on the outside. That is precisely the point we are making here. The point again ties into Ling's observation. Inside the cell we are maintaining that a nonpolar solute acts to disorder, just as in my original talk we showed the inverse, namely, that a nonpolar solute in aqueous solution must be producing order to explain the diffusional properties. So the very point here is the question of the states of water in these two phases and whether we can really so blithely extrapolate from the properties of urea in aqueous solution to the properties of urea intracellularly.

H. S. FRANK: We cannot discuss problems of this sort without postulating models. What you do is specify the properties of the model, account for those properties, and then show how those properties account for the phenomena under discussion. If you are making a model in which urea inside the cell does something very different from what it does outside, then it is incumbent upon you to specify how this can be expected to come about. In the absence of such specification I would suggest that the model is very incomplete.

LUCK: Drost-Hansen has shown that we have four anomalous temperatures in water. These were 15, 30, 45 and 60 degrees and it may be possible

to say something about the point of 60 degrees. We know from the technique by Fonteil and Pimentel that the cyclic hydrogen bonding has its own infrared band and it is possible, I think, in the infrared band of pure water to show that the optical densities in the region of this band will have a maximum near 65 degrees.

The optical density in pure water in the region of the band of cyclic hydrogen bonding is maximal in the region of 65 degrees. The infrared analysis has some difficulties because our assumption was that during melting you have clusters — it may be 100 or 300 molecules — and these clusters have some subunits which are combined with cyclic hydrogen bonding. It may be that the cyclic hydrogen bonding is increasing the temperatures and with higher temperatures the clusters become smaller and smaller. In this case we have more linear hydrogen bonding and it may be that the data shown by Drost-Hansen make this point of view more probable. It is necessary, however, to make the infrared analysis more exacting.

W. DROST-HANSEN (*Jersey Production Research Co., Tulsa, Okla.*): There is other evidence from infrared and raman spectroscopy for the reality of this.

H. FERNANDEZ-MORAN: These are new data to me. The thermophilic bacteria and other organisms exist at temperatures above 65 degrees. I wonder whether this might have any biologic significance.

W. DROST-HANSEN: There is a group of bacteria which we refer to as the thermophilic bacteria which ordinarily have maxima in the vicinity of 55 degrees and even temperatures ranging from 60 to 62 degrees. There are also a few strains of bacteria and indeed some algae which may tolerate considerably higher temperatures. I am not attempting to explain these, nor to explain them away. They are there and I do not know what they mean. They certainly do not seem to fit into the scheme that I am proposing.

H. A. SCHERAGA: The thermophilic bacteria may also involve another phenomenon which may have nothing to do with the kinks that Drost-Hansen mentioned. The enthalpy of formation of a hydrogen bond is positive at low temperatures, but it is temperature dependent. As you raise the temperature to the neighborhood of 50 to 60 degrees, the magnitude of ΔH becomes zero and at higher temperatures becomes negative. This is perfectly rationalized in the terms of properties of aqueous hydrocarbon solutions on which the whole theory is based. It is the phenomena which are involved in hydrocarbon solubility which may be playing the role in the thermophilic bacteria and not these structural transitions.

O. HECHTER: Regarding Frank's comment pointing out that the water in the cell might be different, I believe it is quite fair to recognize that Berendsen presented evidence that a typical macromolecule, collagen, had water that was highly structured. The structured water in that system was clearly different than those obtained in simple aqueous solutions. Per-

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haps Berendsen could comment a few words. I think it is important to consider very clearly that the extrapolation from solution to cell is not without danger.

H. S. FRANK: I could not agree with you more. I think that the structure ideas now are something that we have been led to believe for years ago. Whenever anything showed some catalytic effect, they said "catalysis" and everyone was so eager to believe in it that they were willing to use the word structure in the same way. I think that anyone who says he can explain anything cannot explain everything. I do not know how one can try to specify what the differences are between aqueous solution and the cell, if the different behavior is alleged. I agree with you.

H. A. SCHERAGA: I believe, however, that you have made a good argument. I would like to add a few comments you made before Dr. Frank. If you take the behavior in the aqueous solution versus behavior in the cell, there are certain phenomena which must be built into the model which have not as yet put in aqueous solution.

O. HECHTER: We are not talking about the behavior of collagen.

H. A. SCHERAGA: Whether it is a complex system or a simple system, you have to have a model. That is the basic idea that you must have a model for whatever system you are interested in, which will incorporate all the phenomena.

This is a kind of cage that is different before. It contains two pentagonal planes. The pentagonal planes are behind each other and if you build a space filling model of it, you will find a room inside for one water molecule. This is the same orientation pattern that one would get from a water molecule. When this cage is filled, this agrees with what is found at 2.9 Å and certainly not at 2.74 Å, although there is a peak at 2.74 Å, either due to two peaks at something like 2.74 and 2.9 Å or something else. It may be due to a lengthening of the hydrogen bond distance, which is likely because 2.9 Å is the average hydrogen bond energy of the water itself. I believe that this is probably the case. You could give an explanation, because if you have two distances and you will get two peaks, they will emerge to a peak at an average of 2.85 Å.

You can build normal ice structures with these cages. In fact, this will fit very nicely. It also fits a

From these cages you can build structures that are found in collagen. Such structures have a 10-12 Å repeating unit, and it has the repeating distance of 28.6 Å, which corresponds to this 28.6 Å of the collagen fiber.

D. T. WARNER (*The Upjohn Co., Kalamazoo, Mich.*) : We have found the stereo models of the water permit very free rotation around the various bonds. As we look at Berendsen's model we see that it has really five inter-fused boats of near neighborhood oxygens. In the stereo models, if you make the near neighbors oxygens, (six of them), in the boat form you find there is a lot of flexibility in this structure and this free rotation around each one of the bonds. On the other hand, if you take these six near neighbor oxygens and convert them over into the chair arrangement, then we have a completely rigid and stable mechanical form, no longer with a free rotation. Whether this has any significance in our thoughts about protein structure in the water relation, I am not sure.

One of the things that we can see in the ice structure is that in ordinary ice you have planes running in one direction all of which are chairs. When you look at the planes which run in the opposite direction, they are all boats. Ordinary ice is a mixture apparently of these two types, whereas in stable low temperature ice, each one of the arrangements of the six near neighbors is in the chair form exclusively. This will require a little thinking in terms of stability of chair and boats as applied to the cyclohexane system.

I have one other comment concerning our general thinking about the relation of centrally located oxygen which has four oxygens around it in tetrahedral configuration. Perhaps I tend to look at these things in too simplified a form, but let us just take that central oxygen which in the water structure is associated with two free hydrogens and two hydrogen-bonded hydrogens. When we replace that particular oxygen with a nitrogen we would find a situation in which the nitrogen furnishes no hydrogens to that arrangement of oxygens around it. In the case of HF it would furnish one hydrogen; in the case of water, two; in the case of ammonia, three, and in the case of methane, four. It seems to me that from our study of the interaction of these various components and the inserting of them in the water structure, some of the things that we see may be important. When we have water in contact with a biologic system, the possibility of draining off this resonant disturbance is always there. When we are dealing simply with a component in water, there may be no possibility of draining off this resonant disturbance thereby compelling us to do tremendous things like cooling it down or putting a tremendous pressure on it in order to achieve some sort of a crystalline form.

P. MAZUR (*Oak Ridge National Laboratory, Oak Ridge, Tenn.*) : If one takes precautions to eliminate external ice, it is possible to supercool cells, many to minus 15 degrees and some to minus 30. This seems to me to indicate that cells do not contain effective nucleating agents since the cloud physicists and others who have studied the subject find that silver iodide will nucleate at -2°; in many compounds it will nucleate above -10°. If the cell does not contain effective nucleating agents, does this not mean

that if ordered water exists, the lumps or patches in which it exists must be very small because the nucleating efficiency depends on the size of the particle and only large particles are effective nucleating agents at higher temperatures? If one does not have effective nucleating agents that are present, it means that they are either small, with radial curvatures, or else it means that the material has little crystallographic affinity to ice. Apparently there is also correlation between the nucleating ability of a substance and its crystallographic affinity to ice. One question I have in regard to this is, can clathrates nucleate supercooled water?

H. FERNANDEZ-MORAN: The point that Mazur raises is a very important one and we have puzzled about it a great deal. Could it not be that the moment you cool slowly, you do get instead of the diffused rings some sharpening of the diffraction rings. Could it not be that instead of getting ice, we still would get organized water, but not in domains but rather in sheets? This is a problem that I would like to pose to the physical chemists. Is it that supercooling enhances the organization of the water, but not necessarily in the direction of ice crystal formation?

P. MAZUR: All I can say about supercooled water is that it is supercooled in the sense that it gives up the full length of heat of fusion when it finally does freeze.

O. HECHTER: In the model of the cell membrane that we proposed previously, you notice that the ice was sandwiched between layers of macromolecules. This kind of ice might not readily be available for nucleating agents.

Recently, we have been looking at the water in mitochondrial membranes furnished by David Green. In effect we are trying to get at this problem with higher dilution NMR spectroscopy. We have made two preliminary findings that are interesting. We know the amount of water in the system by weight and when we do high resolution NMR spectroscopy, part of the water that should be registering as a proton signal just does not do so. Maybe it is highly structured. Quantitatively we cannot put a number on this, but there seems to be part of that.

Secondly, when we take mitochondrial membranes in sucrose and then begin to wash out the sucrose, using 98 per cent D_2O - 2 per cent H_2O and 0.1 M tetramethylammonium chloride, we are able to see two proton signals, one from the methyl group and one from the H_2O proton. We noticed at first that this is at least a three compartment system. There is water outside. There is water in the interior chamber and in the membrane phase. We noticed at first that most of the interstitial water is instantaneously replaced with D_2O . Despite seven washings of these mitochondria membranes with this salt mixture over a four-hour period, we still have a sizable fraction of water represented by H_2O protons that are still registering. It is very difficult to wash out all of the proton water from this

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system. Despite this our evidence for protons enter into the interior chamber membrane phase that just does not seem reaction would be almost instantaneous. I am very hopeful that by pursuing this approach some aspects of this water in-

H. FERNANDEZ-MORAN: May I call attention to the problem of residual water that seems to be associated with the water system?

H. T. MERYMAN (*Naval Medical Research Institute*): What is residual water? I would consider it residual unless you would consider speculation that by making a general observation about the discussions that have been made here, we are accustomed to measuring simple systems and are accustomed to describing complex systems. This has become very popular and the assumption is that two groups, assemble them, and they will work. The trouble is that it is not possible to measure a system once it gets too complex, because of the assay. We have all seen examples of mea-

it would die or survive and not necessarily been killed, but that we have been circumstances of the experiment system that involves healing as well.

The word which I believe is of 'systems.' The purpose of research is function. We are not going to be shape of things because in the long to understand.

H. FERNANDEZ-MORAN: Thank you marks, but you still have not answered

FELIX FRANKS (*Bradford Institute*)
I should like to come back to the question Luck discussed. This brings out one point. There have been some measurements made by the Raman Spectra Laboratories on the Raman spectrum conclusion that at about 60° there is water. That mainly poses the question "What is there on?" Luck has presented information to the cyclic hydrogen bond. This theory of hydrogen bonding at the critical temperature

of solutes in which the yeast cells are frozen. He also mentioned that the concentrated suspensions of bacterial cells are more resistant to freezing and thawing than are dilute cell suspensions.

Previous investigators have found that the physiologic state of bacterial cells, for example *Escherichia coli* which grow in a complex, undefined nutrient broth in the experiments, affected the sensitivity to repeated freezing and thawing. Exponentially grown *E. coli* were approximately five times more sensitive than were resting stationary cells. Anaerobically regrown cells were 5 to 10 times more sensitive than the aerobically grown cultures. Ingraham and I have just shown that under strictly controlled conditions the rate of repeated freezing and thawing is not a function of initial concentration of cells frozen, nor is it a function of variation of the physiological state of the cells. These controls include, as Mazur indicated, freezing and thawing at a constant rate, keeping the time before plating constant, freezing and thawing in a medium, and thawing at a temperature that precludes rapid growth or increases in cell numbers. This is most critical and it is not usually observed.

We grew *E. coli* in buffered basal salts medium with glucose and carbon compounds under anaerobic conditions. Samples were obtained during exponential growth and during the stationary phase.

Cells were frozen to -78° and thawed to 11° . There was no statistically significant differences in the slopes of the growth curves. Death by complete freezing and thawing is not due to lysis alone.

Unless one controls the ionic composition of the freezing medium you can get all kinds of killing rates and cannot quantitatively study this effect. One way is by washing. Another way is by nonionic composition, but you have to know the extent of washing.

O. HECHTER: Previously we talked about the possible significance of water structures in the cell and their implications for active transport. I wonder, Dr. Ling, what in your opinion has evolved from this Conference concerning active transport, and whether there has been any reconciliation anywhere along the line with regard to the subject?

G. N. LING: As to the physical state of water in the living cell, I believe the great weight of discussion has centered around the cell membrane, which is usually considered about 100 Angstrom units thick. For example, a muscle cell which is $60 \text{ } \mu$ across, constitutes 1000th of the total protoplasm. Consequently the question still remains, what is the state of water in the living cell? I mean, the remainder of the cell.

The theses which we have presented very rapidly have not reached very many people. It is asking too much. But, basic issues can be discussed somewhat more clearly and without a great deal of controversy. If one assumes the water inside the cell to be free water, then one has to provide energy for all the parts of the cell and so far the question of the demand

for energy of total metabolism of the question has been raised since 1951 has answered this question. Therefore people who do not want to accept an idea come out and answer it. Have we done "holes" in our argument? Perhaps we have in every sense. In fact, I have never

The other side of the picture shows tains proteins with ionic groups following can be entirely duplicated by the ion exchange resin, of sheep's wool, and dried out course, living processes. Yet, kinetically identical. In fact, from these kinetic studies the association of these ions and no behavior of these surface sites quantitatively equilibrium distribution patterns of yesterday. Quantitatively as well as qualitatively the behavior of the cell membrane

If we accept a picture which Heschl's ionic side chains would have something to do with the cell surface. The ionic side chains belong to the beta and gamma carboxy-

more is really at issue than just the active transport in the membrane. We have merely made some preliminary excursions in association with David Green and his associates who really have done the biochemistry.

Essentially what it boils down to is this. We have evidence both from the biochemical and ultrastructural side that in the membranes, at least in one type of membrane that we have studied extensively, namely the mitochondrial membrane, there are multienzyme complexes which in the process of playing a major role in the production of ATP, electron transport and oxidative phosphorylation, also mediate and in effect act as ion pumps. This means that the membrane is much more active than we thought, and I am sure that this will shed light on many problems.

This is a fragment of a much greater problem, one that is at the center of research and controversy in nerve physiology and in so many other fields. Personally, I am sorry that we do not have either the time or the participants to bring this out. My own view is that like everything it will be neither black nor white, but gray. In fact, it will be watery gray. Water will play a very significant role in this whole theory of energy transduction.

D. T. WARNER: I would just like to mention one aspect about our model that is delaying water between protein layers. It has to do with the possible mechanics, shall we say, on the molecular level. I believe the way we would like to look at this is that the carbonyl oxygen in the peptide linkage is not a completely nonionic situation. It has the possibilities of going from, shall we say, keto to an enol form. We have pointed out these possibilities in the paper dealing with carbohydrates and they may have been overlooked by some of the people in the protein field.

If you take a layer of water and attach it to a peptide surface, with carbonyl oxygens in the enol form, you have the possibility of having an upper peptide layer and a lower peptide layer with two layers of water between. The distance is about 6.8 Angstrom units. If you convert this carbonyl oxygen into the keto form, then the water will rearrange itself. You will still have two layers between the two peptide layers, but the distance between the peptide layers now becomes about 4.8–4.9 Å. In our model we have the possibility for a sort of a contraction phenomena, which would simply be brought about by pH changes, which might be at local sites due to local acidity such as the release of phosphate groups.

I believe when we are talking about membrane openings and things that might produce membrane openings, we have to begin to think about the mechanics on the molecular level. Similarly, we must do this in the case of some of the sugars where we have these rigid cyclohexane rings with hydroxyls in either the equatorial or the axial position. In the case, for example of myoinositol, which has only one axial hydroxyl not being capable of making contact with one of the oxygens of the water structure in the water model, the axial hydroxyl is preferentially oxidized in the

system. As a result it becomes a keto-oxygen which comes much closer to fulfilling a favorable interaction with the water structure. By the consequence of having oxidized it, we have introduced not only an energy into that system, but also a different ordering of the water molecules around it. I believe some of these things should be begun to be thought of in the terms of the mechanics at the molecular level, if we are going to be molecular biologists.

H. FERNANDEZ-MORAN: Thank you, Dr. Warner. I could not agree with you more. In fact, the work I alluded to previously on the multienzyme complexes that we are working on with Green, and on a newly isolated contractual protein, go precisely in this direction.

COLACICCO (*Albert Einstein College of Medicine, Bronx, N.Y.*): I should like to ask Henry Frank with regard to the concept of structure meeting and structure breaking of urea. We know that if we go from water to ice, the dielectric constant increases. We also know that if we take a urea solution, urea increases the dielectric constant of water. Have you any comment on or any explanation for this increase in the dielectric constant of water by urea with respect to the structure making and structure breaking?

H. S. FRANK: I do not think they are directly connected. When you have a mixture in an electric field, there are contributions from all of the species which are present. In pure liquid water some of the dielectric constants come from the reorientation of the unstructured material and some come from the flickering of Bjerrum salts in the structured material. When you have urea present, some will come from urea orientation of water. Urea happens to have a pretty big dipole moment. If I am right, it is in the midst of a region where there is no structure. It would be very free to rotate and this would fit in nicely enough with the high dielectric constant.

DR. COLACICCO: The importance of dipole-dipole and charge-charge interactions in water has strongly been overlooked by the students of the structure of water who have put tremendous emphasis on hydrophobic bond theory.

I have mentioned to H. A. Scheraga that I have landed on the other extreme end of the molecule that is on the head charge or on the head group. In that respect, I incline to visualize the iceberg structure of water not so much around the nonpolar site of the molecules, but around the polar site of the molecule. Phenomenal electrostriction, I believe, is worthy of investigation. Kauzmann has initiated some studies in that direction but we have not heard much more in the sense that probably work in that direction will bring some conflict between the hydrophobic bond theory and the possibility of a hydrofluoric bond theory. I wonder if H. Frank would be generous enough to comment on this possibility?

H. S. FRANK: You have these polyfunctional solutes. It is quite true that what one end of the molecule does with water may be different from what

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the other end of the molecule does. I think we have not reached the place of being able to predict what all these interactions, in particular,

On the molecular level, I am very familiar with what it means, but it obviously takes place.

R. STOWELL (*Armed Forces Institute of Pathology*)
several questions of P. J. Melnick for presentation.

First, it was not clear to me to what extent variability between different tumors in their known phenomena, and to what extent variability alone with control of other variables.

I would like to know whether he can compare his histochemical results are well known to him compared with biochemical results. Does he use free substitution, freeze drying, chemical fixation or some other method? What is the explanation in his work? This would give us an idea of the variation might be due to the technique.

Along a similar line, what is his estimate of the amount of error which might be introduced into his work?

What I have told you is true for both the hydrolases and the dehydrogenases, but exactly the opposite is true for cytochrome oxidase. Freezing crystallized and sequestered cytochrome C so that it was unavailable as the substrate for the enzyme.

By adding cytochrome C to the incubating system the full activity resulted and this is, of course, understandable with such a key enzyme. May I suggest that although water is the universal medium in which all life exists, we would also be justified to turn out attention to other substances in living cells that are also effected by cryogenic modalities.

H. FERNANDEZ-MORAN: Thank you very much. Unless there is a very pressing point that remains to be discussed I thank the field. Much as I would like to continue, I understand that the cryobiologists will have a meeting tomorrow and they will have a chance to continue their discussion.

May I now turn the meeting over to J. Flynn.

JOHN FLYNN: (*Office of Naval Research Branch Office, New York, N. Y.*): Now that the moment of adjournment is finally reached, I would like on behalf of the conveners of this Conference to express to all of you a very warm appreciation for all that you have accomplished in the last three and a half days. It has more than met our expectations. It has confirmed amply our belief that a Conference on the Forms of Water in Biological Systems, dealing as this has done with fundamental issues of physics and physical chemistry and biological sciences, would be instructive and profitable.

I would like also to say a word of thanks to all of those whose support and generosity has made this Conference possible, to The New York Academy of Sciences which carried the major financial load, to its supremely effective, efficient Executive Director, Mrs. Miner, and to Admiral Schantze, who for a number of years has been doing an admirable job in managing conferences, for their many courtesies and for the fine and efficient manner in which they handled all the innumerable details, to the National Aeronautics and Space Administration which contributed heavily. I must say, also, a word of appreciation and thanks to my colleagues in the Office of Naval Research, who have responded generously to my request for participation; to the Session Chairmen, who have done a marvelous job; to the speakers, and I wish there had been more time and more of them; and to all participants.

In a moderately long and largely misspent life, filled with too many conferences, I think I have never in my life attended a conference in which the general level of interest remained as high as in this. The ball was kept bouncing at all times. There was always something of interest to discuss.

I leave it with the profound conviction that the influence of this Conference will be felt in biological sciences for years to come. To all of you, I thank you, and auf Wiedersehen.