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Microbial Water Stress

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INTRODUCTION

A fellow of my acquaintance, on seeing a colleague drink undiluted water (55.5 molal), has been known to comment in disapproval that water at such a concentration should not be used for that purpose and that its main function is for putting around the outside of boats. He conceded that dilution with a little salt is acceptable for boats but for no other purpose. The proponent of this philosophy is not a biologist and it is unlikely that many biologists would accept his generalization without some qualification. Nevertheless, it is a point of view.

Another point of view with which all biologists might not agree, at least initially, is one which I wish to advance in this review. It is that, notwithstanding the indispensability of water in living systems and the unique properties of solvent water, quantitative variations in the amount of water available are of less *direct* microbiological significance than is generally conceded.

Discussions of microbial water relations usually emphasize the stresses associated with limiting water availability rather than with an excess of water. A major reason why this is so, as already pointed out (19), is that any distribution curve of microbial biomass or number of microbial species against salt concentration would be asymmetrical, with the peak or peaks in the freshwater-seawater region. Similarly, plant and animal physiologists usually treat water stress as a stress of deficiency rather than of abundance. This review is no exception,

and emphasis is placed on those microorganisms that tolerate and sometimes require an environment in which the amount of thermodynamically available water is greatly reduced. Tolerance, in this context, refers to the ability to thrive, not merely survive.

In very general terms, there is probably not much difference in the range of habitats, as defined by water availability, tolerated by appropriate representatives of higher plants, animals, and microorganisms. The mechanistic details of adjustment to water stress are quite distinct in the case of microorganisms, however, although microbial adaptive mechanisms are sometimes encountered to a limited extent in higher organisms.

The nutritional physiology of the protista is itself sufficient to cause a distinctive type of adaptation mechanism. Since prokaryotes, and many unicellular eukaryotes, can acquire their nutrients only from solution, they can be grown only in direct contact with liquid water (there are some marginal situations associated with ice). A desiccated environment for a *growing* prokaryote is therefore a concentrated solution with which the microbial cell must come directly to thermodynamic terms. Although it is true that plants also obtain their nutrients from solution, land plants, at least, have a large aerial component. Their water relations are affected profoundly by intermittent wetting and the problem of reducing water loss by evaporation. This is achieved partly by control of gas exchange by mechanical means such as closing stomata. The same kind of generalization is applicable to animals that, in many examples of adaptation to a desert situation, conserve water by excreting concentrated urine, coprophagy, and by various dodges that reduce evaporation.

As the following quantitative treatment explains, however, no plant or animal is known whose cells can tolerate solute concentrations even remotely approaching the extreme levels at which some groups of microorganisms can thrive and which some exceptional microorganisms even require. This review is concerned primarily with such organisms.

PHYSICOCHEMICAL PARAMETERS

Biological water relations have been traditionally and, to a large extent still are, discussed in terms of osmotic pressure. This parameter is useful when turgor and related phenomena are under consideration, but it is much less useful in relation to the rigid-walled prokaryotes in which turgor pressure cannot be reliably measured by current techniques and for which attempts to derive them indirectly have been handicapped by many assumptions and oversimplifications (19). Nevertheless, osmotic pressure, as a colligative property of solutions, can be validly employed if its limitations are recognized.

By strict definition, osmotic pressure is not a pressure that can be measured directly. If a solution is placed in an osmometer, however, a hydrostatic pressure will develop, and this pressure can be measured. With a pure ideal solvent on one side of the osmometer membrane, the hydrostatic pressure necessary to prevent a flow of solvent is, by definition, numerically equal to the osmotic pressure of the solution.

Notwithstanding textbook definitions, there is confusion and some disagreement about the physical implications of osmotic pressure. Two schools of thought can be identified among biologists. The first (group 1) considers, in essence, that osmotic pressure is an intrinsic property of a solution that exists independently of any osmometer; as already stated, the osmometer reveals a derived hydrostatic pressure. The other school (group 2) considers that osmotic pressure is not an intrinsic property and is manifest only with the involvement of an osmometer.

Statements such as "the osmotic pressure of the suspending solution was increased," which are common in microbiological publications, will mean different things to the two groups. To group 1, the cell is subjected to an increased external pressure; to group 2, the cell is subjected to a decreased internal pressure. Furthermore, the expression "osmotically active solute" should logically have a different meaning to the two groups. In fact, it seems to mean much the same thing to everyone, suggesting a breakdown in the logic of group 1 and, once again, confusion between osmotic and hydrostatic pressures. To group 1, all solutes must, by definition, be osmotically active, but in practice they are so described only if they are effectively retained by a membrane and can contribute thereby to the generation of a hydrostatic pressure in an osmometer. Considerations of this type have led some cell physiologists to use the term "osmotic potential" to denote a capacity to develop a specified hydrostatic pressure under appropriate conditions (97).

Osmotic pressure is related positively to the concentration of solutes. For a dilute ideal solution, the Van't Hoff relation states:

$$\Pi_s = RT \sum_j \frac{n_j}{\bar{V}_w \cdot n_w} \qquad (1)$$

in which Π_s is osmotic pressure (the subscript s

being used to denote an ideal solvent), R is the universal gas constant, T the absolute temperature, n_j the number of moles of species j, $\bar{V}_w$ the partial molal volume of the solvent (water), and n_w is the number of moles of water. The summation, $\sum_j$, denotes that all solutes in the system are considered. The expression $n_j/\bar{V}_w n_w$ is a statement of concentration and can be substituted by a symbol for concentration, c_j. Thus, equation 1 becomes

$$\Pi_s = RT \sum_j c_j \quad (2)$$

(The derivation of this equation and those that follow is given in a number of books on the physical chemistry of solutions. Those by Robinson and Stokes [115] and Nobel [97] are of particular relevance, the latter being oriented throughout to biological systems.)

In a solution of "real" (that is, nonideal) solutes in an ideal solvent, equation 2 becomes:

$$\Pi_s = RT \sum_j \gamma_j c_j = RT \sum_j a_j \quad (3)$$

where γ_j is the activity coefficient (1 in an ideal solution, <1 in nearly all other cases), and a_j is the activity of the solute.

There is no evidence that osmotically generated hydrostatic pressure is a factor of any significance in distinguishing the physiology of, say, a halophilic bacterium from one thriving in a dilute environment. Although the natural environment of halophils would develop an enormous pressure in an osmometer, their internal contents are also very concentrated (see below) and, as a consequence, these organisms are subjected to a net hydrostatic pressure that is probably less than that developed in freshwater microorganisms. The evidence generally is that the only serious requirement of hydrostatic pressure is that it be kept within those limits sufficient to maintain a cell in a satisfactory state of turgor. There is no evidence, of which I am aware, that variations in hydrostatic pressure within the limits operable in a functioning cell, or within the limits encountered between healthy cells of different habitats, have any measurable effect on enzyme function. Of course, hydrostatic pressure can be a factor of great significance in a transient situation, such as a change in solute concentration of the environment of a microorganism. Under such conditions hydrostatic pressure sufficient to burst susceptible cells can be generated, but this is not the kind of phenomenon with which we are concerned in this review.

Nevertheless, between dilute and concentrated environments there are fundamental physicochemical differences that require quantitative description. The other colligative properties of a solution, namely, elevation of boiling point, depression of freezing point, and the lowering of vapor pressure of the solvent, are no less useful than osmotic pressure in providing a numerical expression related to a thermodynamic property of the solution. These measures are rarely used in biology, however, presumably because they lack the apparent direct physiological significance that is commonly attributed to osmotic pressure. This independence could be advantageous.

There are two fundamental properties of solutions directly related to all four colligative properties. These are the chemical potential of a solvent and the thermodynamic solvent activity. The chemical potential, μ, of substance j is defined as the partial molal Gibbs free energy and is denoted thus,

$$\mu_j = \left(\frac{\partial F}{\partial n_j}\right) n_i, T, P, E, h \quad (4)$$

where F is the Gibbs free energy, n_j the number of moles of substance j, n_i the number of moles of all other substances present, and T, P, E and h being temperature, pressure electrical potential, and height, respectively.

Chemical potential is calculated from the equation,

$$\mu_j = \mu_j^\circ + RT \ln a_j + \bar{V}_j P + Z_j F E + m_j g h \quad (5)$$

where μ_j° is the chemical potential of substance j in a standard state that can be arbitrarily chosen, a_j is the thermodynamic activity of substance j, $\bar{V}_j$ is the partial molal volume of substance j, $Z_j FE$ is a term representing the effect of electrical potential on chemical potential, and $m_j gh$ is a gravitational term that is of importance, for example, in relation to sap movement in trees but of negligible significance for microorganisms. The electrical term can be omitted for nonelectrolytes and for water, and thus the chemical potential of water in a situation where gravitational effects are ignored can be written:

$$\mu_w = \mu_w^\circ + RT \ln a_w + \bar{V}_w P \quad (6)$$

where a_w is the thermodynamic activity of water. The dimensions of chemical potential are expressed as energy per mole, the units commonly used being calories per mole or joules per mole.

Equation 6 shows that assignment of an ab-

solute value to μ requires an absolute value for μ^0 that is not obtainable. This is no problem in biological situations, however, in which the significance of a chemical potential, like that of other potential notations, lies in potential difference and the value for the standard state is constant.

The potential difference, $(\mu_w - \mu_w^0)/(\bar{V}_w)$, is sometimes called the *water potential* and is given the symbol ψ. Thus, from equation 6:

$$\psi = \frac{RTlna_w}{\bar{V}_w} + P \qquad (7)$$

The parameter, ψ, has received little attention from microbiologists but is frequently used by plant physiologists, although not always correctly. Thus Hsiao (61) equated ψ with $lna_w(RT/\bar{V})$, which is, in fact, an expression for the determination of osmotic pressure (see below).

Over recent years, microbiologists have tended to discuss microbial water relations in terms of water activity, a_w, as used in the preceding equations. This practice received a major impetus in food microbiology, largely from the work of W. J. Scott (see, for example, reference 121). As the previous equations show, lna_w is related directly to water potential and there are some practical advantages in its use. Among these advantages are its relative ease of experimental determination, its simple mathematical manipulation, and its direct relation to some other easily recognized and measured properties of solutions.

In the foregoing discussion, a_j, the activity of substance j, can be defined on a molar or molal concentration scale or as a mole fraction. The activity of a solvent is usually based on its mole fraction. Water activity, a_w, is defined as $\gamma_w N_w$ where γ_w is the activity coefficient and N_w is the mole fraction of water = $n_w/(n_w + n_i)$ in which n_w is the number of moles of water and n_i is the number of moles of all solutes. This definition, of course, has the same form for any other solvent. Therefore, in a pure ideal solvent, $n_i = 0$ and $a_w = 1$.

One form of expressing Raoult's law is shown in equation 8:

$$\frac{P}{P_0} = \frac{n_w}{n_w + N_i} \qquad (8)$$

where P and P_0 are the vapor pressures of solution and solvent, respectively. Thus, water (or solvent) activity is numerically equal to the vapor pressure of the solution relative to that of the pure solvent.

A convenient numerical example sometimes used to illustrate equation 8 is that of a 1 molal ideal aqueous solution. Since the molecular weight of water is 18.016, 1 kg of water contains 55.51 mol; in other words, pure water is 55.51 molal. For a 1 molal solution, the right-hand side of equation 8 is 55.51/56.51 = 0.9823. Furthermore, it is apparent that such a solution will equilibrate with an atmosphere of 98.23% relative humidity. The water activity of a solution is therefore numerically equal to $10^{-2}\times$ the equilibrium relative humidity of a solution when the latter is expressed as a percentage.

In an ideal solution, a_w is independent of temperature. The effect of temperature is small for dilute nonideal solutions and, for concentrated nonideal solutions, is significant to the extent that it affects the activity coefficient of solvent or solute.

Finally, the fundamental relation between osmotic pressure, Π, and a_w is shown in equation 9:

$$\Pi = \frac{-RT \cdot lna_w}{\bar{V}_w} \qquad (9)$$

The negative sign demonstrates that osmotic pressure responds oppositely to changes in a_w.

Table 1 shows a relation between water activity and the ability of microorganisms to grow. Some other biological and biophysical phenomena, as well as representative foodstuffs, are included in the table as a series of reference points. Conspicuous among these reference points is the high a_w at which plants wilt. No vascular plant is known whose cells can tolerate levels of water activity as low as those of a wide range of common bacteria, let alone yeasts or molds.

This observation not only exemplifies a fundamental physiological difference in the water relations of plants and microorganisms, but it also illustrates an ecological aspect of microbial water relations mentioned in the introductory comments of this review. When soils dry out, the water that remains eventually becomes discontinuous, the zones most resistant to evaporation being occluded within soil particles. Plants can obtain this occluded water via their root hairs. Since water obtained in this fashion will prevent wilt, it follows that the occluded soil water must be more dilute than the wilt point of plants; in other words, it must have a water activity greater than about 0.98 a_w. These and other aspects of plant water relations have been discussed extensively by plant physiologists (for example, Slatyer [127]). In turn, this emphasizes that the water relations of soil bacteria are not commonly a manifestation of growth at low a_w but are,

TABLE 1. *Approximate limiting water activities for microbial growth*[a]

Water activity (a_w)	Reference points	Foods	Bacteria	Yeasts	Fungi
1.00	Blood Plant wilt Seawater	Vegetables Meat, fruit	*Caulobacter* *Spirillum* spp.		
0.95		Bread	Most gram rods	Basidiomycetous yeasts	Basidiomycetes
0.90		Ham	Most cocci *Lactobacillus* *Bacillus*	Ascomycetous yeasts	*Fusarium* *Mucorales*
0.85		Salami	*Staphylococcus*	*Saccharomyces rouxii*, (in salt) *Debaryomyces* (in salt)	
0.80		Fruit cake Conserves		*S. bailii* (in sugars)	*Penicillium*
0.75	Salt lake	Salt fish	Halophils		*Wallemia* *Aspergillus* *Chrysosporum*
0.70		Cereals Confectionary Dried fruit			
0.65					*Eurotium*
0.60				*S. rouxii* (in sugars)	*Xeromyces bisporus*
0.55	DNA disordered				

[a] This table was obtained in its essentials from J. I. Pitt. With the exception of the halophilic bacteria, the organisms listed had a tolerance range from a_w 1.00 (approximately) down to their tabulated level. The halophilic bacteria, together with halophilic species of the alga *Dunaliella* and the halophilic actinomycete *Actinospora halophila*, have an upper limit as well (see text). The growth characteristics of halophilic bacteria suggest that they are limited at 0.75 a_w by the solubility of salt rather than by their physiology.

instead, a consequence of physical discontinuities of liquid water. Under such circumstances, the problem confronting a soil bacterium is one of surviving equilibration with air, or whatever the gas phase might be, in a soil microenvironment. Survival under these circumstances is a very complex phenomenon and involves more than peculiarities of microbial physiology. Thus, for example, the clay, montmorillonite, enhances the survival during desiccation of fast-growing strains of *Rhizobium* but not the slow-growing strains (91).

SOME ECOLOGICAL ASPECTS OF MICROBIAL WATER RELATIONS

Microbial Water Relations and Foods

Human foods, which are usually reasonably nutritious for heterotrophic microorganisms, even if not always for humans, are preserved essentially by eliminating viable microorganisms and preventing readmission (as in canning) or by adjusting physicochemical conditions so that microbial growth is retarded or prevented. Lowering water availability is one of the major processes in this second category. It can be achieved by freezing, by the physical removal of water, or by the addition of solutes, as in curing.

As already indicated, the use of the parameter, a_w, in microbial physiology and ecology received a major impetus from food microbiologists. Notwithstanding the comments later in this review in which the direct physiological significance of a_w is questioned, there can be no doubt that a_w is a very useful, perhaps the most useful, parameter so far employed to describe microbial water relations in a complex environment, such as a foodstuff. This is reflected in Table 1, which, among other things, puts salmonellosis and staphylococcal food poisoning, as well as outright food spoilage, in perspective on a water activity scale and in relation to specific types of foods. The table also shows that some of the extreme types of microorganisms, such as the halophilic bacteria and xerotolerant (osmophilic) yeasts, are potential spoilage organisms for various types of relatively dry foods.

A detailed discussion of this topic, however, is beyond the scope of the present review. Additional information is contained in articles by Ingram (62), Scott (121), Christian and Waltho (40), Pitt and Christian (109), and Pitt (108).

The Saline Environment

Table 1 states, in essence, that the prokaryotes that can tolerate the lowest a_w are the extremely halophilic bacteria that grow well at 0.75 a_w, the value for a saturated solution of sodium chloride. The physiology of these bacteria is dominated by an absolute requirement for sodium chloride at a high concentration; they will not grow at less than about 2.9 M sodium chloride. Although some other salts such as calcium chloride are tolerated at moderate con-

centration, sodium chloride, as Brown (19) has pointed out, is the only readily soluble salt, which is known to be able to support some form of life over its entire concentration range. No other salt of any monovalent cation is known even to approximate that capability.

A requirement for salt at concentrations above 3 M is, of course, associated with a very distinctive ecology. Highly concentrated and often saturated solutions of salt occur naturally in marine pools concentrated by solar evaporation, the Dead Sea (total salt concentration, 23 to 33%, wt/vol) and various inland lakes, for example in Australia and the United States, in which salt concentration reaches saturation. Halophilic microorganisms have been isolated from all of these sources. Some soils are quite saline but, as already pointed out, uneven distribution of water, both temporally and spatially, presents special problems of microbial survival in soils over and above ability to thrive in an environment of specified composition. There is little reliable information about the occurrence of halophilic microorganisms in soils. Halophils are well known in some industries. The preparation of salt by solar evaporation of seawater is sometimes accompanied by the appearance of a red coloration attributed to the growth of halophilic bacteria. The development of this color has been recognized since ancient times and frequently used as a guide to the degree of concentration of the brine (7). Brines used for curing meat and fish products by modern techniques usually contain about 12% (wt/vol) sodium chloride, together with some other salts such as sodium nitrate. There is also a significant amount of organic nutrient extracted from the food being processed. Such brines provide excellent growth media for moderately halophilic and salt-tolerant microorganisms; according to Ingram, counts of 10^7 to 10^8/ml are common (49). Some of these bacteria have a role in the curing process by reducing nitrate and contributing to the color and flavor of the foodstuff. An account of the microbiology of curing brines is contained in a symposium edited by Eddy (49). A salt-tolerant strain of *Desulfovibrio* has been implicated in the spoilage of brines used for pickling olives (110).

Since high salt concentrations constitute a powerful selective influence, the ecology of salt lakes is inevitably simpler (that is, there are fewer species) than that of marine or freshwater environments. Early reports (7) that brine shrimps, worms, etc., die out as salt concentration approaches saturation do not seem to have been disproved. I am not aware of any reports of animals or vascular plants in saturated-salt lakes. Aspects of salt tolerance by some animals have been discussed by Bayly (10). Nevertheless, such lakes commonly harbor microbial communities, which implies either chemotrophs sustained entirely by elution of nutrient from the surrounding land or else an ecosystem that contains both photosynthetic and heterotrophic microorganisms. The latter condition is the general one, although there has been one report (94) of an Antarctic pond with a freezing point of about −48°C, which apparently contained bacteria and yeasts but no viable algae. (One diatom frustule was observed.) The predominant cation in the pond was Ca^{2+}. The authors reported culturing several bacteria and yeasts, but the significance of this is uncertain, since they apparently used nonsaline growth media. On the other hand, K. Kerry (personal communication) observed bacteria, yeasts, blue-green algae, and eukaryotic algae in another Antarctic lake which contained salts in the same proportion as in seawater but which remained unfrozen at −18°C.

In warmer climates, evaporation from salt lakes can proceed to the point of saturation with sodium chloride. The concentration process is accompanied by sequential changes in the viable population of the lakes; in the later stages, a red or pink color is sometimes developed in the water or on the salt crystals that are deposited on the foreshores. Apparently, such a color can be caused by ferric oxides or hydroxides (7), but it can also indicate a microbial bloom. There seem to be differences of opinion as to whether the blooms are predominantly algal (such as *Dunaliella salina*) or bacterial, and it can reasonably be assumed that both types occur. The bloom expresses a selection by salt concentration of extremely halophilic or halotolerant microorganisms. It also implies, of course, an unusually high nutrient concentration. Evaporation would increase nutrient concentration in at least the same proportion as it increased salt concentration. In fact, the proportion is likely to be greater, since the increasing salt concentration would normally kill off some organisms present in the "dilute" lake and convert them, thereby, from biomass to nonviable organic matter available to the surviving heterotrophs. There is also evidence (see below) that some extremely halophilic bacteria (species of *Halobacterium*) are capable of photophosphorylation, in which case they might be expected to make very efficient use of organic nutrients for biosynthesis under appropriate conditions.

Few types of halophilic microorganisms have been identified. Those that have been are so ubiquitous in highly saline environments that there can be little doubt that they are indeed

the major representatives of the halophils. On the other hand, it is unlikely that they are the only types. Among the algae, the genus *Dunaliella* is outstanding and, indeed, is commonly the only algal species encountered in saturated-salt lakes (17). This genus has marine and halophilic representatives. The three best known halophilic species are *Dunaliella salina*, *Dunaliella viridis*, and *Dunaliella parva*; their salt relations are a little different from each other and substantially different from those of the extremely halophilic bacteria (see below). Nonetheless, at least the first two can grow in saturated solutions of sodium chloride. A halophilic photosynthetic bacterium, *Ectothiorhodospira halophila*, has been isolated (113); its salt relations are closer to those of the halophilic *Dunaliella* than to the extremely halophilic bacteria. Recently, an extremely halophilic actinomycete, *Actinopolyspora halophila*, has been isolated and described (54).

The extremely halophilic bacteria are heterotrophic, aerobic, and usually highly pigmented—either red or pink. There are two major types. One is rod shaped and lacks a "conventional" bacterial wall (see below) and should therefore be classified as gram negative. (Direct Gram stains on halophils are of doubtful value unless adequate precautions are taken against disruption of the bacteria on the slide.) This genus is *Halobacterium*, the major species being *Halobacterium halobium*, *Halobacterium salinarium*, and *Halobacterium cutirubrum*. The validity of the species differentiation is questionable. The other major type is a thick-walled (i.e., gram-positive) coccus for which the nomenclature is less clear than for the halobacteria. The generic names *Sarcina*, *Micrococcus*, and *Halococcus* have been used (70). The name *Halococcus* will be used henceforth in this review. In addition there are apparently fragile (gram-negative) cocci that have not been investigated systematically. We have encountered such organisms in water samples from Australian salt lakes.

I am not aware of reports of extremely halophilic chemotrophic anaerobes, nor reliable reports of yeasts or molds in saturated salt lakes. Table 2 lists the approximate salt relations of a number of microorganisms.

A curious aspect of the ecology of the extreme halophils is their ubiquity. The natural habitats of these organisms are discrete and widely separated, yet, so far as we know, all salt lakes that are not otherwise inhibitory contain halophilic microorganisms. Presumably, this ubiquity is the result of dissemination rather than independent evolution in each lake. Halophils, especially the bacteria, are extremely sensitive to dilution (the halobacteria are killed at salt concentrations less than about 2.8 M) and to virtual dissolution in a dilute environment. They would seem, therefore, to be highly vulnerable to rain, especially during transport by wind, birds, etc.

The Nonionic Environment

There are few natural habitats in which a low water activity occurs predominantly because of a high concentration of a nonelectrolyte. To some extent, of course, this generalization is modified by one's definition of a natural habitat. When such environments do occur, their major microbial inhabitants are the sugar-tolerant yeasts and fungi. Sugar-tolerant yeasts are normally found in nectar and are transmitted by bees among nectaries and to honeycomb (62). When honey is spoiled by fermentation, these yeasts are usually responsible.

The circumstances under which sugar- or salt-tolerant yeasts are most commonly encountered by microbiologists are associated with the food industry, in which they frequently appear as spoilage organisms and the agents of undesirable fermentations. Some indication of their spoilage potential, as a function of water activity, is given in Table 1. Since most known sugar-tolerant yeasts and molds are tolerant of, but do not require, high solute concentrations (there are some exceptions; see below), they are able to grow over a very wide range of water activity. Thus, they have been associated with the spoilage of honey, wine must, maple syrup, fruit juices, dessert wines, dried fruits, molasses, malt extract, conserves, etc. Salt-tolerant yeasts frequently inhabitat pickling brines and are needed in the preparation of some oriental fermented foods such as soy sauce and miso paste. The biology, distribution, and nomenclature of these yeasts are described by Scott (121), Ingram (49, 62), Onishi (106), and Pitt (108). Scott's and Pitt's reviews include two of the few accounts of aspects of the biology of xerophilic molds.

Regardless of their ecological and industrial significance, however, these organisms warrant physiological study because, as Table 1 implies, they are able to thrive at water activities far lower than any other known organism or, more specifically, the cells of any other type of organism.

HYPOTHETICAL EXPLANATIONS OF THE TOLERANCE OF LOW WATER ACTIVITY

Microbiologists with even a passing acquaintance with the extreme types of microor-

TABLE 2. *Approximate salt (sodium chloride) relations of some representative microorganisms*[a]

Organism	Salt tolerance (M)	Salt optimum (M)
Non-halophilic bacteria		
Gram-negative		
Spirillum serpens	0–0.2	
S. undula		
Enterobacteriaceae	0–0.7	0.2–0.3
	0–1.4	
Pseudomonas, *Achromobacter*, *Serratia*	0–0.7	0.2–0.3
Vibrio parahaemolyticus	0.2–1.5	
Marine bacteria	0.2–0.7	
	0.2–>1.4	
	0.3–0.7	
	0.7	
	0.7–>1.4	
Gram-positive		
Anaerobic sporers	0–0.9	0.2
	0–1.6	
Aerobic sporers	0–2.6	0.2
	0–3.4	
Lactobacillus plantarum	0–1.6	Low
Streptococcus faecalis	0–1.7	Low
Cocci	0–2	Low
Staphylococcus aureus	0–>3	0.2
Moderately halophilic bacteria		
Vibrio costicolus		
Micrococcus halodenitrificans	0.2–4.0	1.0
Halophilic and extremely halophilic bacteria		
Ectothiorhodospira halophila	1.5–5.1	1.9–3.8
Halococci	2–saturated	3.4–5.0
Halobacteria	2.6–saturated	3.4–5.0
Marine and halophilic algae		
Dunaliella tertiolecta	0.17–1.5	0.17
D. viridis	1.7–saturated	1.7
Halophilic actinomycete		
Actinospora halophila	2–saturated	

[a] The salt relations listed in this table are simplifications and are intended primarily as a general guide. Salt tolerances vary with other environmental factors such as temperature, pH, and nutrition. The listing of more than one tolerance range for a type of organism implies that subgroups have been recognized. When given as a single concentration, salt optima should be treated even more cautiously than the tolerances, but the optima do indicate which end of the tolerance range supports better growth. A tolerance limit of 0 indicates that growth can occur, at least in some media, without the specific addition of sodium chloride. The information listed in this table was compiled from various sources, including my observations and references 15, 38, 39, 54, 75, 92, and 113.

ganisms under discussion have generally been intrigued by the physiological basis of their unusual environmental tolerances. The extremely halophilic bacteria have attracted the most attention. It is of some interest that the U.S. National Aeronautics and Space Administration (NASA) supports a research program into these bacteria, presumably because that they are among the most extraordinary microorganisms on earth, at least in their environmental requirements, and therefore more likely than any others to resemble any extraterrestrial organisms which might be encountered in NASA's exploratory activities.

For many years biologists were reluctant to accept the idea that any living system could operate in a saturated salt solution and, for this reason, sought explanations of halophil physiology in a supposedly dilute interior. It is now well known that the interior is not dilute and, moreover, that there is no mechanism available to bacteria that could make it so (see Brown, reference 19).

A microorganism responds to a new physicochemical situation essentially in two stages. The first occurs when the organism, which might or might not be growing, is transferred to a new environment. The changes that occur

in stage 1 are associated with the cell's thermodynamic adjustment to the new conditions and are comparatively rapid. When, as in the circumstances under discussion, the environmental change involves a change in water activity, the thermodynamic adjustment always involves a transient osmotic stress. The adjustment process can be modified if the organism has a source of energy.

The second stage of adaptation occurs when the organisms have adjusted thermodynamically and then grow. Stage 2 involves alterations in the organism's metabolism, levels of enzyme activity, etc., by the new environmental conditions. The processes are more complex and much slower than those of stage 1. Initially they involve changes in enzyme activities and in the details of enzyme regulation (each of which can be relatively fast) and subsequently modification of biosyntheses and changes in the details of control of enzyme formation. These latter processes can be expected to have a time scale similar to that of a generation of the organism.

When an organism is transferred to a new environment, it faces, in stage 1, a simple, binary choice, death or survival. If it survives, the limits of its environmental tolerance are then determined by the nature and extent of the adaptive processes that occur in stage 2. Once an organism is through stage 2 and into a physiological steady state, or something like a steady state, there are two basic types of explanation that are theoretically possible for a tolerance of or requirement for a low a_w. These are: mechanism I, the proteins of a tolerant organism are fundamentally and generally different from those of a nontolerant organism and are intrinsically better able to function under the extreme environmental conditions; or mechanism II, the proteins of tolerant and nontolerant organisms are essentially similar but, in the tolerant species, enzymes can function because intracellular conditions are modified so that the inhibitory effect of the environment is diminished. As discussed below, halophilic bacteria fit into class I; a functional halophil enzyme can be selected at random and recognized by its response to salt. On the other hand, although bacterial endospores do not represent the type of biological phenomenon under discussion, they do provide a good example of a type II situation, that is, enzyme protection by a highly specialized set of intrasporal (intracellular) conditions. The water relations of "osmophilic" yeasts, "xerophilic" molds, and halophilic algae can also largely be explained on the basis of mechanism II (see below). Of course, it is an oversimplification to suggest that only two mechanisms are possible or that they are mutually exclusive. For example, notwithstanding the salt tolerance of halophil enzymes, it will be shown that halophilic bacteria depend also on mechanism II.

A variation or combination of both mechanisms can be invoked if, for example, tolerant organisms use distinctive metabolic pathways. Metabolic peculiarities might be relevant either because they are catalyzed by enzymes that, in any organism, have intrinsically favorable water (or solute) relations (mechanism IA) or because the end product of the distinctive pathways can modify intracellular conditions so as to diminish environmental inhibition (mechanism IIA). We are not aware of any examples of IA but, as will be shown, IIA is used by all known xerotolerant eukaryotic microorganisms.

HALOPHILIC BACTERIA

The extremely halophilic bacteria grow in sodium chloride at concentrations between about 2.8 M and saturated (6.2 molal). The lower limit varies slightly with genus (*Halobacterium* and *Halococcus*), with nutritional conditions, and with temperature. The growth curves suggest that these bacteria would grow at sodium chloride concentrations above 6.2 molal were it possible to achieve them. The general biology of the extreme halophils is well documented (19, 70, 75, 76) and does not warrant extensive repetition here. There are several distinctive and intrinsically interesting characteristics of these organisms, however, which are presumably a result of environmental selection but which, on present evidence, do not appear to have a deterministic role in halophil salt relations.

Cell Envelope and Lipid Biochemistry

One such characteristic is the absence of bacterial peptidoglycan, not only in the envelopes of the halobacteria (19, 27) but also in the walls of the gram-positive halococci (24). The latter bacteria, as already mentioned, have very thick walls of the gram-positive type, the polymeric details of which have not yet been determined. Acid hydrolysates of halococcal walls contain sugars and amino sugars, together with substantial amounts of glycine, but no muramic acid (24). Presumably it is an inability to synthesize this compound that is responsible for the lack of peptidoglycan in halophilic bacteria. Recent evidence suggests that the major halococcal wall polymer is a complex sulfated het-

eroglycan (131). On the other hand, the halophilic actinomycete *Actinopolyspora halophila* does contain diaminopimelic acid and is lysozyme sensitive (54).

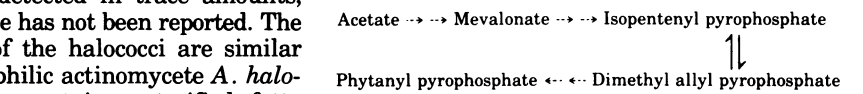
The extreme halophils are also noteworthy for their membrane lipids, which do not contain esterified fatty acids in any more than trace amounts, but which have, instead, hydrocarbon side chains bound to glycerol by an ether bridge. The definitive work on halophilic bacterial lipids has come from Kates and his associates and has been collated and reviewed by Kates (66). The major phosphatides of halobacterial lipids are derivatives of 2,3-di-*O*-phytanyl-*sn*-glycerol. The predominant halobacterial phospholipid is the diether analogue of phosphatidyl glycerol phosphate, which can account for more than 70% of lipid phosphorus in *H. cutirubrum* (122) and as much as 80% in *H. halobium* (89). The next most abundant phosphatide in both these species is the ether analogue of phosphatidyl glycerol, which accounts for 6.0 to 7.5% of lipid phosphorus in each species. Phosphatidyl choline (lecithin, diether analogue) has been detected in trace amounts; phosphatidyl choline has not been reported. The membrane lipids of the halococci are similar (149), but the halophilic actinomycete *A. halophila*, in contrast, contains esterified fatty acids, not phytanyl ether-linked lipids (54).

Ether lipids are not unique to halophils (129), but they are unusual in bacteria. They have been reported, however, in some other bacteria with extreme environmental tolerances. These are the acidophilic thermophilic organisms, *Thermoplasma acidophilum*, which requires a growth medium at pH 2.00 or less and 59°C or more, and the sulfur oxidizer, *Sulfolobus acidocaldarius*, which requires a pH of 3.0 or less and a temperature in the range 65 to 90°C. Neither of these species produces fatty acids to any significant extent, and both contain as their major lipid component isopranol ($C_{40}H_{50}$) glycerol diethers (71, 72, 128). *Sulfolobus acidocaldarius* is also distinguished by some unusual glyco- and phosphoglycolipids (71).

Halophil lipids are very acidic, a property that is of direct relevance to the salt relations of these organisms (see below). The synthesis of their ether lipids, however, seems to be almost a chance result of a selection process rather than an essential mechanism of adaptation to a highly saline environment. The synthesis of these lipids provides an interesting biochemical example of "where there's a will there's a way."

H. cutirubrum does, in fact, have the genetic specification of a fatty acid-synthesizing system and does, in fact, produce enzymes that can synthesize fatty acids from malonyl-coenzyme A. The enzymes are very salt sensitive, however, and indeed, are inhibited by salt to a greater extent than is the corresponding fatty acid-synthesizing system from *Escherichia coli* (III). Apart from any of its other implications, this curious set of circumstances argues that halophils evolved from organisms with much lower salt requirements rather than by any other process. From the point of view of fatty acid synthesis, the adaptive process might be said to be incomplete but, since the bacteria manage well enough without fatty acids, there is no apparent evolutionary disadvantage in this.

Most extreme halophils are highly pigmented with carotenoids which, indeed, are the major nonpolar lipid constituents. The biochemical ingenuity of halophil lipid metabolism lies in the adaptation of a pathway used for the biosynthesis of the isoprenoid chain of carotenoids to the production of the phytanyl chain of the lipid. This pathway includes the following steps (66):



The metabolic peculiarities of the extreme halophils which culminate in their lack of muramic acid and fatty acids are perhaps reflected in an aspect of the antibiotic sensitivity pattern of these bacteria. Neither the halobacteria nor the halococci are affected by the so-called wall antibiotics (i.e., those, like penicillin, that specifically inhibit peptidoglycan synthesis) with the notable exception of bacitracin which, at a concentration of 20 μg/ml, completely inhibits the growth of *H. halobium* and *H. salinarium* (unpublished observation from S. Cumming in our laboratory). Recently, bacitracin has also been reported to cause sphere formation in a *Halobacterium* species (93).

The biosynthesis of peptidoglycan and some other polysaccharides associated with bacterial cell envelopes involves the transfer of a sugar or sugar complex from anhydride combination with a nucleotide (as, for example, in a uridine diphosphate sugar) to the growing polymer through its intermediate attachment to a lipid-soluble compound known as glycosyl carrier lipid (GCL) (117). According to the type of polymer being synthesized, attachment of the sugar to the GCL is either through a pyrophosphate (sugar-P-P-GCL) or a monophosphate (sugar-P-GCL) group. The sugar or sugar complex is then transferred to the growing polymer with-

out the pyrophosphate (or phosphate), which remains bound to the GCL as GCL-P-P (or GCL-P). The function of the GCL in this system, as its name suggests, is the catalytic one of a carrier that is used repeatedly in a cycle. To accept another molecule of sugar from the nucleotide, the GCL must be available as the monophosphate (GCL-P). In those pathways that involve a GCL-P-P-sugar, therefore, a dephosphorylation is required after the GCL has released its sugar:

$$\text{GCL-P-P} \rightarrow \text{GCL-P} + P_i$$

It is this reaction that is inhibited by bacitracin.

The GCL has been identified in other bacterial systems as a C_{55}-polyisoprenoid alcohol. This might be fortunate for the halophils that, as stated, do not have an effective means of making fatty acids but do synthesize other polyisoprenoid compounds. Both the membrane and outer layer of the halobacterial cell envelope yield sugars and amino sugars on acid hydrolysis. It can reasonably be assumed that these sugars are incorporated into envelope polymers, whether glycoproteins or polysaccharides, with the mediation of a GCL of the type similar to that found in nonhalophilic bacteria.

Nutrition

Extreme environmental conditions should be expected, a priori, to select against some metabolic pathways. Such a selection is reflected in the cell envelope and lipid composition already discussed and also, presumably, in halophil nutritional requirements. The halobacteria and halococci are somewhat fastidious nutritionally. They make little use of sugars as an energy source and do not usually produce, from carbohydrates, carboxylic acids in quantities large enough to be measured by conventional diagnostic bacteriological methods. *Halobacterium salinarium* produces a glucose dehydrogenase and a glucose-6-phosphate dehydrogenase only when induced, and then the enzymes do not have a high specific activity (1).

Synthetic media have been devised for halophilic bacteria. Dundas et al. (48) used a medium containing 10 amino acids and cytidylic acid which supported limited growth. Onishi et al. (107) used a medium containing 15 amino acids, 2 nucleotides, glycerol, and either asparagine or NH_4^+, which gave better results, although Gochnauer and Kushner have pointed out that the concentration of K^+ in this medium is suboptimal and, under some conditions, limiting for both growth rate and yield of the bacteria (53). The two sets of authors differed as to which amino acids were essential for growth. Dundas et al. concluded that valine, methionine, isoleucine, and leucine were essential for the growth of *H. salinarium*. Their results showed, among other things, that lysine was not essential. On the other hand, Onishi et al. (107) found the amino acids essential to *H. cutirubrum* to be arginine, leucine, lysine, and valine. A. Markides, in our laboratory, using the medium of Onishi et al., has confirmed that lysine is essential for all the halophils in our collection, which includes species of *Halobacterium* and *Halococcus*. Dundas et al. (48) diluted but did not wash their inocula; the discrepancy can possibly be attributed to this.

Miscellaneous Characteristics

Gas vacuoles. An unusual feature of some strains of *Halobacterium* is the presence of gas vacuoles. They were first reported by Houwink (60); they are unusual among microorganisms but are not unique to halophils. Their formation requires the presence of a specific type of thin, lipid-free protein membrane. Microbial gas vacuoles have been discussed at length by Walsby (145).

"Satellite" DNA. Extreme halophils have another peculiarity that is not related in any obviously directly deterministic manner to their salt requirements but which might have important indirect implications. This is a "satellite" deoxyribonucleic acid (DNA) originally described by Joshi et al. (64) in preparations of DNA from *H. salinarium* and *H. cutirubrum* and later by Moore and McCarthy (95) for a number of other extreme halophils, including the halococci. The "satellite" component accounted for 11 to 36% of the DNA in the various preparations. DNA prepared by Moore and McCarthy from two moderate halophils and from *E. halophila* was homogeneous.

The satellite DNA was, in all cases, less dense than the major component and had a different base composition from it. For reasons outlined previously (19), there is a possibility that the satellite DNA is an artifact of preparation. If it is not an artifact, then it is not unique. Satellite DNAs have been discussed in the context of information theory with interesting evolutionary implications (51).

Halophil bacteriophages. Until recently no virus of halophilic bacteria had been reported. Even the halophils have been overtaken by inevitability, however, and two reports now attest to the existence of salt-dependent bacterio-

phages able to infect species of *Halobacterium* (138, 143).

Light Reactions of the Red Halophils

Most extremely halophilic bacteria are heavily pigmented with carotenoids that might reasonably be expected to modify halophil physiology, especially in a brightly illuminated environment such as constitutes the common natural habitat of these bacteria. Larsen (75) has discussed a possible role of carotenoids in protecting the organisms against damage by visible radiation and, in collaboration with Dundas (47), has shown a colorless mutant of *H. salinarium* to be more vulnerable to visible light than the pigmented parent strain. More recently, however, Hescox and Carlberg (58) questioned this simple interpretation and produced evidence that the carotenoids are involved in an energy transfer capacity that facilitates photoreactivation by visible light after exposure to damaging doses of ultraviolet.

The evidence is now clear that radiant energy trapped by membrane pigments is used by halophilic bacteria. First, at a simple level, *H. halobium* produces more pigment when grown in the light than in the dark (A. D. Brown, unpublished observation). In this respect it resembles other carotenoid-pigmented bacteria (136).

The membrane of *H. halobium* contains a pigmented protein complex that in many respects resembles rhodopsin (101). This protein has been named "bacteriorhodopsin"; it occurs in the membrane bound to vitamin A aldehyde. The production of bacteriorhodopsin is enhanced by growing the organism at reduced oxygen tensions. The pigment forms patches of "purple membrane" that can account for as much as 50% of the membrane surface. If the bacteria are incubated anaerobically in the dark, their adenosine 5'-triphosphate (ATP) content decreases sharply but it can be restored by O_2 or by anaerobic incubation in the light. Respiratory-chain inhibitors abolish the response to oxygen but not to light. Uncouplers of phosphorylation that function as proton translocators abolish the light response (44).

Unlike rhodopsin, bacteriorhodopsin is not bleached by light. Instead, it responds to a light flash by a reversible change in its absorption peak from 560 to 415 nm and a concomitant release of protons. Illumination of whole bacteria leads to proton excretion, inhibition of respiration, and an increase in intracellular ATP. These effects can be observed to best advantage in starved bacteria that are heavily pigmented.

If bacteriorhodopsin is illuminated continuously, the pigment apparently oscillates between the two forms identified by their absorption peaks. When it is incorporated into a membrane, the oscillation is accompanied by a vectorial release and uptake of H^+, which results in a net outward flow of protons from the bacterium (44). The direction of the proton flux (outwards) is the same as that which occurs during respiration of the bacteria or of mitochondria. It is the opposite of the light-induced proton flux of chloroplasts. The proton flux causes an electrochemical gradient which, according to Danon and Stoeckenius (44), can be used in phosphorylation within the framework of Mitchell's chemiosmotic mechanism.

Racker and Stoeckenius (112) reported the incorporation of bacteriorhodopsin into phospholipid vesicles which then transported protons in the opposite direction from that which occurred in whole bacteria. In other words, they accumulated protons in the light and released them in the dark. Uncouplers of oxidative photophosphorylation abolished the proton uptake. Inclusion of a mitochondrial adenosine triphosphatase in the vesicles, however, enabled them to catalyze phosphorylation. Furthermore, illuminated cell envelope vesicles of *H. halobium* actively accumulate leucine. The leucine transport system is not dependent on ATP hydrolysis nor is it a function of the proton gradient. It does respond to membrane potential, however, and there is some evidence that leucine transport is facilitated by the associated transport of Na^+ (85). Cyclic electron flow also energizes amino acid transport in vesicles from *Rhodopseudomonas spheroides* (56).

The overall process of photophosphorylation in halobacteria is not yet well understood, but it apparently involves a cyclic electron flow inasmuch as there seems to be no need for an electron donor (in isolated vesicles), nor are there reports of the production of reduced pyridine nucleotides. Recently, Oesterhelt (100) described the whole phenomenon in some detail. Illumination also elicits a motor response in *H. halobium*; the response is apparently mediated by the purple membrane (59). A three-dimensional model at 0.7-nm resolution of the purple membrane has been obtained by electron microscopy (57).

There is no evidence yet that their capacity for photophosphorylation has any determining role in their salt requirements or water relations, but presumably it gives the bacteria an ecological advantage in their peculiar environment. It should enable them to function anaerobically in the day time, and it would also lessen their demand for organic energy sources. This

last factor is likely to be of importance in the halophil blooms that are reported from time to time (see above). Their capacity for photophosphorylation might also reflect a phylogenetic relationship with photosynthetic bacteria, although there is a substantial difference in DNA base composition between the red halophils and two photosynthetic bacteria, *R. spheroides* and *Ectothiorhodospira halophila* (95).

Halophilic Characteristics

Up to this point the properties that have been discussed, although generally characteristic of halophilic bacteria and of intrinsic interest, do not have an obvious directly deterministic role in the salt or water relations of the organisms. One exception is the acidic nature of the membrane phospholipids (see below). Indeed, the halobacterial and, by inference, the halococcal membranes in toto do have a major function in determining the environmental requirements of these bacteria. This is to be expected, of course, when a microorganism has an absolute specific requirement for a high concentration of a solute and where, as is usually the case, there is effective exclusion of that solute from the cell.

The halobacterial envelope comprises a lipoprotein membrane with an outer glycoprotein layer (19, 27, 32, 90, 133). If the halobacterial environment is diluted, the bacteria, predictably, will burst. Osmosis is a major factor in their bursting but, in addition, there is an equally significant change in the cell envelope itself; this change is not a result of osmotic factors. If isolated envelopes are exposed to progressively more dilute suspending solutions, they soon lose the outer glycoprotein layer and later disaggregate to give what, for most practical purposes, is a lipoprotein solution (18, 25, 26, 73, 90).

Although the halococcal membrane has not yet been examined in these terms, it probably behaves similarly to the halobacterial membrane. Halococcal lipids are similar to halobacterial lipids (149) and, although the halococci do not burst in dilute solutions, they are disorganized internally.

The osmotic disruption of halobacteria has its counterpart in any sufficiently fragile microorganism that equilibrates with a concentrated solution and is then transferred to a dilute one. But for the inherent instability of the envelope it would be reasonable to assume that (in morphological terms) halobacteria could be "trained" to accept the simple osmotic consequences of a dilute suspending medium or the replacement of salt by sucrose. In this sense, part of the halophilic salt relations can be seen as a tolerance, rather than a requirement.

This aspect of halophil physiology falls within the province of microbial water relations in the sense in which that expression is commonly used. On the other hand, the instability of the cell envelope reflects a very specific solute requirement that can be described as "water relations" only by severely stretching definitions and concepts. The role of salt in maintaining the structural integrity of halophil envelopes has been discussed by Brown (19) in terms of the possible contributions of the salt to water structure, hydrophobic bonding, and electrostatic forces; it was attributed largely to the electrostatic effects (18–21) of salts in neutralizing excess charges on the membrane. A similar explanation had been invoked earlier by Baxter (9) to explain the inactivation in dilute solution of a halophil lactate dehydrogenase. The electrostatic events in the membrane were attributed by Brown to a net negative charge caused by an excess of aspartic and glutamic acids in the membrane protein. More recently, Lanyi (74) has argued persuasively that hydrophobic interactions are a major factor in the salt relations of halophil proteins generally.

Lanyi's argument, however, is not that electrostatic effects do not occur but, rather, that they do not need *very* high concentrations of salt to overcome them. He points out (validly) that, as a general rule, the net charge on polyanions is overcome by salt concentrations of 0.5 M or less. He also points out that the α-helical structure of polyglutamic acid, which is highly charged, becomes unstable at very high salt concentrations. He suggests that the electrostatic phenomena attributed to halophil proteins do, in fact, occur at relatively low salt concentrations (0.5 M or less) and that the demand for very high concentrations is probably a reflection of other factors such as hydrophobic interactions. His evidence includes specific differences of various anions, an effect which he points out is difficult to reconcile with a simple charge-shielding hypothesis. For example, the order of effectiveness of various sodium salts in stabilizing the envelope of *H. cutirubrum* was $NaCl > NaNO_3 > NaClO_4$ (73). Those components that remained particulate at less than about 0.7 M salt, however, had little specificity in their salt requirements. The order of effectiveness of the salts in stabilizing the envelope is also the order of increasing "salting in" capability. Lanyi's results have an experimental error large enough to cast some doubt on the differences attributed to sodium chloride and

sodium nitrate, but there was no doubt about the difference of sodium chlorate from the other two.

Other evidence relevant to the involvement of electrostatic forces and hydrophobic bonds in determining the characteristic properties of halophil proteins, and especially the halophil envelope, is (briefly) as follows.

First, there is a theoretical basis, in the Debye-Hückel theory, for stabilization of polyions and polyionic aggregates by salts although, strictly, this theory is quantitatively applicable only to dilute solutions. A more recent and mathematically more versatile theory of ionic solutions has been advanced by Olivares and McQuarrie (104). Second, the disintegration of halophil envelopes responds quantitatively to pH to give titration curves with pK values very close to those of the β- and γ-carboxyl groups of aspartic and glutamic acids (18). Furthermore, there is a substantial excess of these amino acids (over the basic amino acids) in the membrane proteins (18, 90). Structural changes in the envelopes of halobacteria occur in response to changes in salt concentration within any range, but the response is steep at low concentrations (21). The outer layer, which accounts for about 15% of the envelope, is lost competely in 1 M sodium chloride (90). The sequence of events in envelope disintegration has been described in some detail by Lanyi (73).

Magnesium chloride, at a concentration of 0.02 M, however, will substitute for 4 M sodium chloride in stabilizing the membrane, but it does not prevent loss of the outer layer (28, 90). Stabilization of the membrane by 0.02 M magnesium chloride is effective even to the extent of keeping the respiratory chain intact (31). Bi- and multivalent cations can form bridges between neighboring negative charges and, because of this, they are much more effective than Debye-Hückel atmospheres of monovalent counterions in neutralizing charges on the surface of a polymer or aggregate. When a phenomenon of this kind, that is, effective replacement of a high concentration of a monovalent ion by a low concentration of a divalent one, is encountered, it provides strong evidence that electrostatic forces are involved in the structural changes under investigation. Bivalent cations are also able to form coordination complexes. Since the magnesium in a halophil envelope can be displaced by suitably high concentrations of sodium chloride, however, coordination complexes are not likely to be implicated to a major extent in membrane stabilization by magnesium chloride. It is thus very difficult to avoid the conclusion that unneutralized charges provide the forces that disintegrate the halophil envelope. Of course, this conclusion does not exclude a role of hydrophobic interactions as one of the types of bond that stabilizes or tends to stabilize the envelope against the disruptive forces generated by removing neutralizing counterions. Indeed, in a lipoprotein membrane, hydrophobic interactions are axiomatic. The question is whether these bonds assume abnormal significance in a halophil and whether their strength is substantially affected by changes of salt concentration above about 0.5 M.

The relative ease with which the outer glycoprotein layer of *H. halobium* is lost from the envelope on lowering salt concentration might reflect a particular involvement of hydrophobic bonds in its attachment. The outer layer is marginally more acidic than the membrane proteins, but it contains no lipid and it has a slightly lower content of amino acids with nonpolar side chains (90). Furthermore, such access as it might have to the membrane lipid and to the nonpolar side chain of the membrane proteins is likely to be restricted. Its attachment to the envelope is easier to reconcile with polar bonds of some kind (hydrogen bonds, ion pairs, or salt bridges) than it is with hydrophobic bonding.

Other evidence for a role of surface charges in membrane disaggregation was given by succinylating the envelope of a marine pseudomonad (20). This treatment, which substituted $-COO^-$ for $-NH_3^+$ groups, increased the surplus of carboxyls in the envelope to a level only slightly less than that occurring naturally in the membrane of *H. halobium*. Succinylation eliminated the autolytic properties that characterized the untreated pseudomonad envelope and endowed it with an ability to disaggregate in a similar manner to the halophil envelope. Salt inhibited disaggregation, but at lower concentrations than required by halophil envelopes. Furthermore, acetylation, which, by merely masking $-NH_3^+$ groups, caused a smaller surplus of excess carboxyls than did succinylation, produced an intermediate level of salt dependence.

The obvious rigidity of whole halobacteria and isolated envelopes in high salt concentrations requires, in the absence of peptidoglycan, an explanation that is almost certain to involve environmental salt. A plausible explanation, based on "relaxation effects" is available (19); relaxation effects are electrostatic phenomena. It can also be assumed that any increase in the strength of hydrophobic interactions that occurs at high salt concentrations would also

stiffen an aggregated structure such as a membrane and thus supplement the electrostatic stiffening.

A factor of perhaps crucial importance in the membrane, however, is the effect of lipid phosphate on the charge density of protein carboxyls. This factor is frequently ignored in those arguments based on the simple numerical excess of acidic over basic amino acids in the membrane proteins. It has already been pointed out that halophil membrane lipids are very acidic and that there is almost a complete absence of neutral phospholipids. Brown (21) showed that essentially all the carboxyl groups on the membrane proteins could be titrated with hydrogen ions (in fact, more carboxyls titrated on the envelope than in the dispersed lipoproteins, a phenomenon attributed to conformational stabilization on the membrane). The majority of basic groups, namely, the ϵ-amino groups of lysine, the phenolic hydroxyls of tyrosine and, by inference, the guanidinium ion of arginine, did not titrate in the envelope. This apparent "burial" of the basic groups was attributed to ion pairing with lipid phosphate. Such an association is not, in itself, sufficient to prevent titration, but it is a condition necessary for the group to remain "buried" and inaccessible to the titrant; burial of an unmasked charge requires an expenditure of 40 to 400 kJ of free energy/mol (137). In the membrane, the groups most likely to be associated with the basic groups on the protein are lipid phosphate. Neither the titer of phosphate nor the guanidinium ion of arginine could be determined accurately in these experiments (21), but some calculations suggest that all the lipid phosphate groups are likely to be involved in ion pairs in the intact membrane. Under the relevant experimental conditions, the membrane of *H. halobium* contains about 0.8% P (89). This is equivalent to four to five atoms of P/100 amino acid residues, assuming an average molecular weight of 150 for the amino acids. One hundred moles of amino acids include about 6.5 mol of arginine + lysine (90). In each molecule of phosphatidyl glycerophosphate, there are two P atoms, with a total of three dissociable groups. If all the lipid phosphorus were present in this form, four atoms of P would be equivalent to six dissociable groups, which is approximately the number needed to neutralize the basic amino acids. In fact, about 80% of the lipid P occurs as phosphatidyl glycerol phosphate, with another 7.5% as phosphatidyl glycerol, which has one dissociable group/P atom. It is thus evident that the membrane contains phosphate and basic amino acids in about the proportions needed for neutralization.

The immediate effect of this arrangement is to increase the effective net surplus of acidic groups in the membrane protein from about 19 to about 26 mol/100 mol. Of probably greater significance, however, is the effect the ion pairing has on protein orientation. The carboxyl groups, which are not compensated by basic groups, are held firmly on the membrane surface to give a charge density substantially greater than they could possibly achieve in the dispersed lipoprotein.

Furthermore, there are at least two types of bonds involved in stabilizing the intact membrane. One of these is ionic, which disrupts when the membrane disintegrates; this type of bond was detected only because it broke on disaggregation of the membrane. The other is a noncovalent and probably nonpolar association of lipid with protein, which is highly stable and remains intact when the membrane disaggregates into dispersed lipoprotein (21).

The conclusion seems inescapable that the forces which are directly responsible for the gross disaggregation of halophil membranes originate predominantly in protein carboxyl groups on the membrane surface and that the exposure of their charges is effected by the removal of neutralizing cations. It is equally inescapable that, since there are demonstrably hydrophobic interactions in a lipoprotein membrane, such interactions must supply part of the total bond strength that holds the membrane together. There is a body of indirect evidence and some good argument that, in halophils, hydrophobic bonds are intrinsically weaker than in nonhalophils and that they require the "salting out" effect of high concentrations of sodium chloride to bring them up to "normal" strength. It is therefore reasonable to assume that the structural changes that occur in halophil membranes in response to a slight lowering of salt concentration are primarily the result of weakening cohesive bonds, whereas the structural disruption that occurs on lowering the salt concentration further is primarily the result of generating disruptive forces. Unfortunately, there is insufficient evidence to enable this statement to be rephrased quantitatively, nor is it easy to distinguish between conformational changes of membrane proteins and the separation of lipoprotein complexes from one another.

Quantitative information could probably be obtained by exploiting the different relative effects of temperature and pressure on hydrophobic bonds, hydrogen bonds, and electrostatic bonds (ion pairs); increasing temperature

strengthens hydrophobic bonds but weakens the other two; increasing pressure has the opposite effect (99). To my knowledge, there is no information about effects of hydrostatic pressure on halophil membrane stability and only limited data about temperature. Such evidence as there is does not, in fact, support a significant quantitative role of hydrophobic bonds in determining the response of halophil membranes to changes of sodium chloride concentration within the range 0.31 to 1.25 M. Within that range, the *extent* of membrane disaggregation was much greater at 30°C than at 0°C (18), but it is of interest and relevance to Lanyi's argument that the proportional difference between the extent of disaggregation at the two temperatures was greatest at the intermediate salt concentration of 0.62 M.

There is clearly room for additional measurements of the type suggested but, inasmuch as it is relevant to the main theme of this review, the disaggregating properties of the halophil membrane give no support whatever to any supposition that they are a response to a_w rather than the type and concentration of salt in the suspending solution.

Intracellular Physiology

The intracellular physiology of extreme halophils is dominated by the massive accumulation of K^+ and Cl^- and by the effective exclusion of Na^+. This was first demonstrated by Christian and Waltho (37), who found that *H. salinarium*, when grown to stationary phase in a medium containing 4 M sodium chloride and 0.03 M potassium chloride, accumulated potassium to a concentration of about 4.5 molal and had apparent sodium and chloride contents of about 1.4 and 3.6 molal, respectively. They also reported that *Sarcina* (*Halococcus*) *morrhuae* contained about 2 molal potassium and 3 molal sodium after similar growth conditions. It is possible that the sodium content reported for *H. salinarium* was too high because of leakage during centrifugation and technical difficulties of accounting accurately for Na^+ in interstitial water. It is virtually certain that the sodium and potassium contents reported for *H. morrhuae* were substantially too high and low, respectively, because of errors associated with occlusion of "extracellular" salts in the enormously thick cell walls of these bacteria (see reference 24). Nevertheless, the basic observation of massive potassium accumulation and effective sodium exclusion by halophilic bacteria has been amply confirmed in many laboratories. For example, C. E. Armstrong (Ph.D. thesis, University of New South Wales, Sydney, 1975) reported potassium concentrations of 4.5, 5.5, and 7.5 molal in mid-exponential-phase *H. salinarium* grown in media containing 3.4, 4.3, and 5.1 M sodium chloride, respectively. Sodium content was apparently 0.4 to 0.8 molal in all cases. It is noteworthy that 7.5 molal exceeds the solubility of KCl. Conditions of this sort naturally raise many questions about the details of enzyme function within the cell.

The discussion that follows is confined to those features of the salt relations of halophil enzymes that are of immediate relevance to the main theme of this review. A broad spectrum of halophil enzymes has been discussed in some detail by Lanyi (74). From the outset, however, a clear distinction should be made between effects of salt on the activity, on one hand and, on the other, the stability of an enzyme. Conditions that allow an enzyme to function vigorously might also allow a significant rate of inactivation. Conversely, some enzymes are stable under conditions that severely inhibit activity. Thus, a halophil isocitrate dehydrogenase is quite stable in but severely inhibited by 4 to 5 M sodium chloride (3).

This section is confined almost entirely to enzyme activity, since it is reasonable to assume that, in the viable organism, enzyme stability is not a major variable in determining the response of halophils to the environment. Nevertheless, the different effects that salts can have on stability and activity of an enzyme are relevant to the interpretation of enzymological results and to arguments about cell physiology that might arise from such interpretation. Thus, a question that is seldom answered is to what extent the history of an enzyme preparation might affect its properties as measured in the ensuing experiments.

A special case of an interaction of stability with measured activity is to be found in the interesting work of Louis and Fitt (79–83) on a DNA-dependent ribonucleic acid (RNA) polymerase of *H. cutirubrum*. According to Louis and Fitt, this halophil enzyme comprises two subunits, each of about 18,000 molecular weight. The subunits, designated α and β, have very different degrees of stability on removal of salt by dialysis. The α subunit retains virtually full activity for up to 24 h after dialysis against a salt-free buffer and 50% activity after 20 days of dialysis. The β subunit, on the other hand, is completely and irreversibly inactivated after dialysis against a salt-free buffer. In a "high-salt buffer" (2.5 M potassium chloride plus 1.0 M sodium chloride) the subunits are stable, but they do not aggregate, nor do they catalyze the synthesis of RNA unless bivalent cations are

present. The addition of manganous chloride (10 mM) to the high-salt buffer causes the subunits to dimerize to a complex of molecular weight 36,000, which is the active form of the enzyme. Magnesium will not substitute for manganese in the dimerization but, even when dimerized, the enzyme will not function without the addition of Mg^{2+} (100 mM), which is needed for attachment of the enzyme to the DNA template.

The effect of salt concentration on template specificity is of special interest. At a high salt concentration (1.5 M potassium chloride + 0.6 M sodium chloride), halobacterial DNA is transcribed, whereas calf thymus DNA is not. In the absence of added salt the converse is true: calf thymus DNA promotes RNA synthesis, whereas halobacterial DNA is inactive.

The effects of salt on substrate specificity are not correlated with the guanine + cytosine content of the DNA. The assay used by Louis and Fitt lasted 1 h at 37°C, during which time the β-protein would presumably have been extensively inactivated under conditions of low salt concentration. In fact, this is apparently the cause of the changed template specificity that occurs at low salt concentration. The α-protein alone can synthesize RNA from a DNA template provided the reaction is primed with a suitable dinucleoside phosphate that supplies the first diester bond of the new polymer. The α-protein alone will not initiate polymerization without a primer and, in this respect, it resembles the much larger core enzyme of *Escherichia coli* RNA polymerase. Furthermore, in a primed reaction, the α-protein alone shows no template specificity with any of four different types of DNA, nor does it show any salt dependence. The β-protein, which is unstable in the absence of salt, cannot catalyze RNA polymerization, but it is entirely responsible for chain initiation and template specificity in the complete enzyme. In these respects, it resembles the σ factor of the *E. coli* RNA polymerase.

Thus, the halophil RNA polymerase has some properties that distinguish it sharply from other halophil enzymes on one hand, and from the corresponding polymerase from *E. coli* on the other.

Halophil enzymes have now been sufficiently well studied to enable a working generalization of their salt requirements to be advanced; of course, there are exceptions. In general, enzymes associated with the cell membrane have an optimum in the region of 4 M NaCl or KCl; ribosomal enzymes have a specific requirement for KCl at a sharp optimum of about 4 M. Soluble enzymes of intermediary metabolism collectively have a wider range of salt optima, but there are many, such as the nicotinamide adenine dinucleotide phosphate (NADP)-specific isocitrate dehydrogenase, which have an optimum in the vicinity of 0.5 to 1.0 M salt (NaCl or KCl).

Thus, from the outset, halophilic bacteria can be recognized as having a physiology that invites explanation by both the hypothetical mechanisms already advanced. The high salt optima must reflect proteins intrinsically different from those of other organisms, whereas the accumulation of K^+ and virtual exclusion of Na^+ denotes an intracellular environment with enzymological implications that are likely to be very different from those of the bacterium's habitat. An apparent anomaly lies in the relatively low salt optimum of some soluble cytoplasmic enzymes, with the implication that those enzymes function under conditions of inhibition in the intact cell.

A simple explanation of this is shown for isocitrate dehydrogenase in Fig. 1. At the optimal salt concentration, enzyme activity was higher in either lithium chloride or sodium chloride than in potassium chloride, but the activity fell off very sharply in the first two salts at higher concentrations. On the other hand, the inhibition caused by potassium chloride at concentrations above optimal was rela-

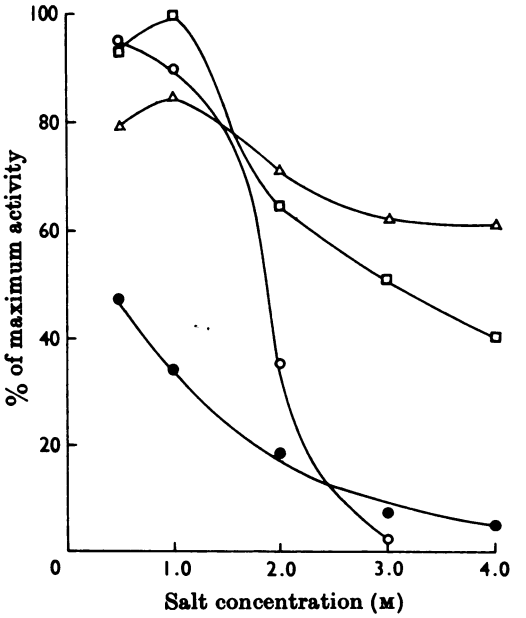


FIG. 1. *Effects of salts on the activity of a halophil NADP-specific isocitrate dehydrogenase. Symbols: ○, LiCl; □, NaCl; △, KCl; ●, NH_4Cl. Results of Aitken et al. (3) are reprinted with permission.*

tively slight. Thus, although potassium chloride was less effective than either sodium chloride or lithium chloride in activating the enzyme, it was also less effective as an inhibitor at concentrations approaching physiological conditions for the organism. Figure 1 allows two other points to be made. First, there is no evidence whatever that a_w is a determinant of enzyme activity under the experimental conditions, although the range of salt concentrations used was great enough to cause substantial changes in a_w. This is scarcely surprising. With most biological systems and, especially with cell-free enzyme preparations, we are accustomed to thinking in terms of direct interactions between electrolytes and proteins rather than indirect interactions in which the electrolyte has a nonspecific role in merely changing a_w. This does not exclude a possible indirect effect of the salt on water *structure* and hence, presumably, on hydrophobic bonding within the protein molecule(s); indeed, there are reasons already discussed for supposing that, at high concentrations, salts act partly in this way.

Second, generalizations about the relative effects of salts on enzymes are of little value unless the comparison makes due allowance for salt concentration. Clearly, a comparison within Fig. 1 would lead to a set of conclusions which were completely different at, say, 3.0 M salt on the one hand, and 1.0 M salt on the other. For this reason comparisons like that given, for example, in Lanyi's (74) Table 2 are of doubtful value.

A similar but more sophisticated comparison is shown in Fig. 2: a series of values of apparent V_{max} are plotted against salt concentration. The comparison between the effects of potassium chloride and sodium chloride is qualitatively similar to that of Fig. 1, but it differs quantitatively and introduces a new parameter, inasmuch as the concentration of fixed substrate affects the comparison. This is another reason why simple comparisons of salt effects are difficult to defend. Figure 1 shows that it is the effect of sodium chloride on enzyme activity which is conspicuously susceptible to fixed substrate concentration. In potassium chloride the substrate effect was minor.

Thus, the apparent anomaly of an enzyme's having a salt optimum well below the salt concentration within the cell can, at this stage, be given a simple explanation. The organism virtually excludes Na^+ and accumulates such massive concentrations of KCl that its intracellular contents are dominated by it. Although these concentrations are well above the optimum for some enzymes, KCl is a poor inhibitor and, even at those concentrations, produces only a minor degree of inhibition. Both from the point of view of its accumulation and its effective "protection" of enzymes at high salt concentrations, potassium chloride is a physiologically compatible substance for halophilic bacteria.

The use, by halophilic bacteria, of both the physiological mechanisms proposed above is now evident. The proteins are intrinsically distinctive and nearly all require a salt concentration that is very high by nonhalophilic stan-

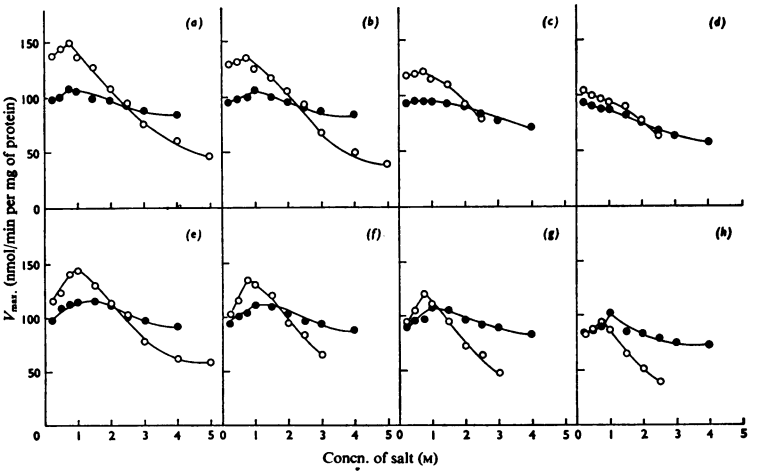


FIG. 2. *Effects of salt concentration on apparent V_{max} of a halophil NADP-specific isocitrate dehydrogenase. The results were obtained at several concentrations of each fixed substrate. In the top panels (a–d) $NADP^+$ was used at fixed concentrations of (left to right) 0.80, 0.60, 0.40, and 0.20 mM. In the lower panels (e–h) sodium isocitrate was used at fixed concentrations of (left to right) 1.0, 0.50, 0.25, and 0.125 mM. Symbols: ○, Sodium chloride; ●, potassium chloride. Results of Aitken and Brown (2) are reprinted with permission.*

dards. This is true even of enzymes which, like isocitrate dehydrogenase, have a salt optimum at 1.0 M or less. (It it not true of the fatty acid synthetase system, which is not functional in the viable organism.) On the other hand, those cytoplasmic enzymes that are inhibited by high salt concentrations can function because the intracellular composition is such that the impact of the organism's environment is softened. If potassium chloride were not accumulated, the intracellular a_w and, hence, total solute concentration would still be about the same. Its thermodynamic adjustment to the environment would be achieved either by loss of water, in which case the major intracellular solutes would be "pool" intermediary metabolites, miscellaneous salts, etc., which, for reasons discussed below would be severely inhibitory at such concentrations, or else the bacteria would accumulate sodium chloride which, as shown above, is also inhibitory.

Potassium chloride thus has a physiological function in halophilic bacteria which is very similar to that of the polyhydric alcohols in xerotolerant yeasts and halophilic algae (see sections, Xerotolerant Yeasts and Halophilic Algae). We have called such substances "compatible solutes" (29). Their essential properties and mode of action are discussed in the section, Compatible Solutes.

XEROTOLERANT YEASTS

General Biology

The adjective "osmophilic" was first used by Richter in 1912 (106). The word has been useful, but it is misleading in both of its components. For reasons already outlined, osmotic pressure is not a major factor in the peculiar physiology of these yeasts, and most known species (not all, however) are tolerant, not "-philic" of high solute concentrations. The term "sugar-tolerant" is used and has been advocated (5), but there is some evidence for the existence of a group of yeasts distinguished by their salt tolerance. The dominant sugar-tolerant genus is *Saccharomyces*. Two species and one variety have been clearly identified; they are *Saccharomyces rouxii* (Table 1), *Saccharomyces rouxii* var. *polymorphus* and *Saccharomyces mellis* (Lodder and Kreger-van Rij). Yeasts associated with moderate salt concentrations include several genera among which *Saccharomyces (rouxii), Debaryomyces, Hansenula,* and *Pichia* are prominent. The nomenclature and distribution of the tolerant yeasts have been discussed by Onishi (106).

A term that involves minimal bending of descriptions already in use and is generally applicable to the group as a whole is "xerotolerant"; it will be used henceforth in this review. The subdivisions of sugar- and salt-tolerant will also be used where appropriate.

A definition of xerotolerance will not be attempted at this stage since any definition based on growth characteristics will be either vague or inaccurate. The major physiological characteristics of the group should become clearer in the discussion which follows. Scarr and Rose (119) defined "osmophilic" yeasts as those that can grow in sugar solutions at concentrations above "65° Brix" (65%, wt/wt) at 20°C; this definition can be used as a useful working description of the sugar-tolerant group.

A reliable definition based on tolerance at low a_w cannot be advanced because the water relations of these organisms vary greatly with solute used to adjust a_w. This is revealed in Table 1, for example, which attributed widely different water relations to *S. rouxii* in sugar and salt solutions. There are many other examples of this kind of phenomenon, some of which are cited by Onishi (106). The differences, however, are not confined to those between salts and nonelectrolytes. One interesting example reported by Anand (Ph.D. thesis, University of New South Wales, 1969) occurred with a sugar-tolerant strain that could not grow in a medium adjusted with sucrose to 0.85 a_w but did grow in the same medium when the water activity was lowered even further (to 0.80) by the supplementary addition of glycerol. Furthermore, growth in media adjusted to specified levels of water activity with polyethylene glycol (molecular weight, 200) generally diminished the difference in minimal levels of water activity tolerated by sugar-tolerant and nontolerant yeasts (Fig. 3 and 4).

Results of this type illustrate very clearly, at least for a simple environment, that any direct deterministic effect of a_w on the yeast is overwhelmed by the type and concentration of solute used to adjust a_w. Figures 3 and 4 also show a fairly consistent difference in maximal growth rates between tolerant and nontolerant strains; the tolerant strains have exponential growth rates which, on average, are about half those of their nontolerant counterparts. (The paper by Anand and Brown [5] should be consulted for comments on the "water relations" of some of the intermediate strains.)

There is, moreover, a complication introduced by temperature. Opposing effects on biological processes of increasing temperature, on the one hand, and increasing solute concentration, on the other, are common (19). With some strains of xerotolerant yeast these effects can become large enough to cause a change from a

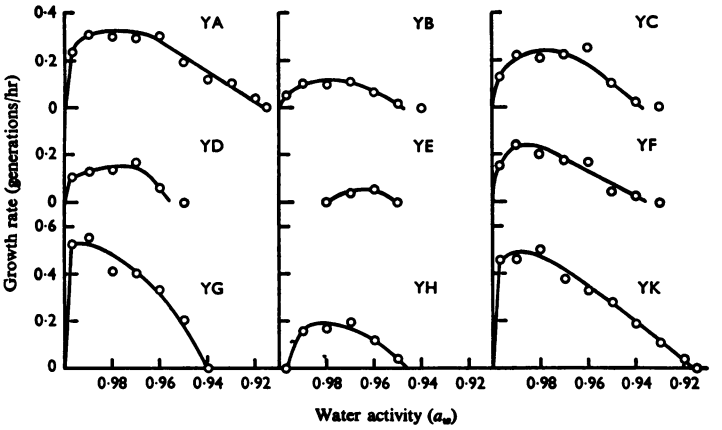


FIG. 3. *Response of exponential growth rate of nine xerotolerant yeasts to water activity in media adjusted with polyethylene glycol (molecular weight, 200). Results of Anand and Brown (5) are reprinted with permission. The original paper should be consulted for the identity of the yeasts.*

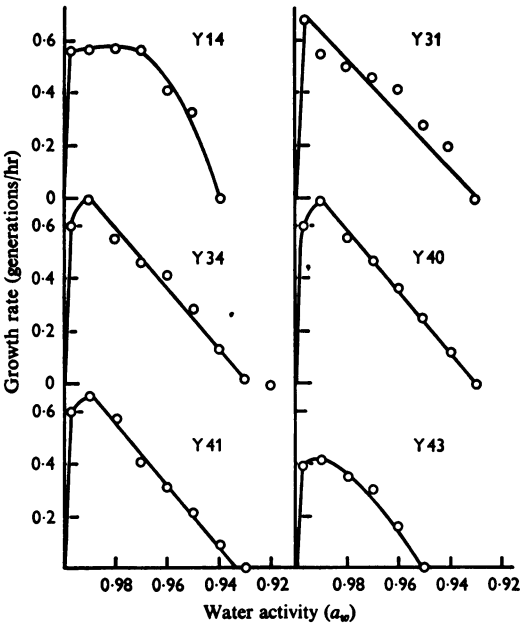


FIG. 4. *Response of exponential growth rate of six nonxerotolerant yeasts to water activity in media adjusted with polyethylene glycol (molecular weight, 200). Results of Anand and Brown (5) are reprinted with permission. The original paper should be consulted for the identity of the yeasts.*

tolerance of to a requirement for a high solute concentration. For example, Onishi (105) has reported that, at 30°C, the yeast *Torulopsis halonitratophila* would not grow in a dilute medium but did grow in the presence of 6% (wt/vol?) sodium chloride. At 20°C, however, it grew in dilute medium. Thus at the higher temperature the organism was halo- or xerophilic, whereas at 20°C it was xerotolerant. Onishi (106) has cited several other examples of this type of phenomenon. In our own experience, the yeast *Zygosaccharomyces nectarophilus* (5), which will not grow in a dilute basal medium at 30°C (Fig. 3), will do so in the temperature range of 16 to 23°C (M. Edgley, unpublished data).

Physiology

The available evidence will not support many generalizations about the nutritional requirements of xerotolerant yeasts. One of the few supplementary growth factors that appears to be generally required is biotin (130). The strain of *S. rouxii* used extensively in this laboratory requires biotin and pantothenate (A. J. Markides, unpublished data).

Some effects of solute concentration on the ability of yeasts to assimilate sugar have been reported and summarized by Onishi (106). For example, a strain of *S. rouxii* assimilated glucose readily in a dilute medium or in the presence of 18% sodium chloride. It also grew readily in basal medium with galactose or maltose as sole carbon source but very poorly on either of those sugars in 18% sodium chloride. Onishi has added the comment that very few strains would assimilate or ferment these sugars in highly saline media. Observations of this kind are to be expected. They need reflect no more than differential effects of a_w (or solute concentration) on the kinetics of the relevant reactions. Similar effects occur with other physicochemical factors such as temperature.

Although the xerotolerant yeasts can thrive at levels of water activity even lower than those tolerated by the extremely halophilic bacteria,

the two types of organism are fundamentally different, inasmuch as the yeasts have no absolute requirement for a specific solute. Neither do they generally require a low a_w although, as stated, there are some exceptional strains that do appear to have such a requirement, which is temperature dependent. Partly for this reason and partly because of the great strength of their thick walls, xerotolerant yeasts do not have the problems of structural integrity which beset the halophilic bacteria in dilute solutions.

There was, therefore, no a priori reason to look initially to the cell membrane of the yeasts, as was done with the halophils, for direct explanations of their environmental peculiarities. Of course, the yeast membrane is involved in the overall deterministic physiology (see below) but, on present evidence, not in any specific sense. Instead, it seemed probable that the primary explanation of their water relations would lie inside the cell within the broad framework of mechanism I or II as outlined above.

Of immediate relevance to these mechanisms was a comparison of the kinetics, water relations, and electrophoretic properties of an NADP-specific isocitrate dehydrogenase from the tolerant *S. rouxii* and the nontolerant *S. cerevisiae*. This comparison, which was made by Anand (Ph.D. thesis, University of New South Wales, 1969) and extended by Brown (unpublished data), failed to show any significant difference, by the criteria used, between the enzyme preparations from the two yeasts. Although the systematic comparison was confined to one enzyme it was, in our view, sufficient to discount mechanism I (above) as an explanation of the sugar tolerance of *S. rouxii*. As already noted, any functional halophilic bacterial enzyme has distinctive salt requirements.

It followed, therefore, that mechanism II, or some modification of it, should operate and that some intracellular property such as composition should be substantially and consistently different in the two types of yeasts. Broadly speaking, intracellular composition will be determined by the uptake of solutes from the extracellular fluid and by the retention of metabolites. Since the different water relations of the tolerant and nontolerant strains show up clearly in high sugar concentrations, any differences in composition arising from solute uptake might reasonably be expected to show up with sugars and other nonelectrolytes. Evidence for significant penetration of some solutes lies in the effects of different solutes on the growth responses of the yeasts to a_w (see above). It is difficult, for example, to explain the effects of supplementary addition of glycerol to a medium containing sucrose (above) without invoking significant penetration of at least one of the solutes.

Brown (22) found consistent differences between *S. cerevisiae* and *S. rouxii* in the amount of solute taken up as a function of the extracellular solute concentration when the yeasts were incubated in buffered solutions of any of several sugars or glycerol. *S. rouxii* had a lower capacity for the nonelectrolytes than *S. cerevisiae*. The way in which the two species responded to sucrose is of some practical significance, since this sugar is often encountered at high concentrations in foods and because the differences were consistent between all sugar-tolerant and -nontolerant strains examined. *Saccharomyces cerevisiae* produces an invertase; *S. rouxii* does not. As a consequence, solute uptake by *S. cerevisiae* was an active process dominated by metabolism to the extent that no free sucrose was recovered from the yeast. In *S. rouxii*, however, sucrose uptake occurred by diffusion and, under the experimental conditions, the strain used (YA) equilibrated with the suspending solution in such a way that the intracellular concentration was about 40% of that of the extracellular fluid (22). In this sugar, then, there are major qualitative differences in the intracellular composition of the two species, differences attributed directly to metabolism in one type but not the other.

Quantitative differences between the species attributable simply to uptake of nonmetabolized sugars can be illustrated by lactose which, in *S. cerevisiae*, equilibrated at about 70% of the extracellular concentration and, in *S. rouxii*, at about 10% (which probably means exclusion from the protoplast, since the walls can take up about as much as this). Glycerol equilibrated in *S. cerevisiae* at about the same concentration as in the suspending solution with an upper limit to the intracellular accumulation of about 1 M. *S. rouxii*, on the other hand, approximated an equilibrated condition at 35 to 40% of the extracellular concentration (22).

These differences were minor, however, and doubtless themselves partly determined by another major difference in intracellular composition between the two types of yeast. All our xerotolerant strains contained high intracellular concentrations of a polyhydric alcohol; the nontolerant strains did not. With one exception, the major polyol accumulated was arabitol (presumably the D-isomer) when the yeasts were grown in a basal medium. The exception was a small unidentified yeast, YO (29), which accumulated mannitol. Another variant was YE (5), the temperature-dependent xerophilic

yeast (see above). In addition to arabitol, this yeast accumulated significant quantities of glycerol and a hexitol, presumably mannitol.

The concentration of polyol within *S. rouxii* (strain A, reference 5) when growing exponentially in a basal nutrient medium containing (initially) 0.2% glucose is normally within the range 0.6 to 0.9 molal (22). At mid-exponential phase, the growth medium is likely to contain about 0.002 M polyol, although polyol yields are variable in response to nutritional and other environmental factors (106, 130). Nevertheless, the quantities cited indicate a concentration factor within the yeast of several hundred.

One of the environmental parameters that affects both polyol production and intracellular polyol accumulation is the water activity (or the solute concentration) of the growth medium. Onishi (106) and Spencer (130) have discussed effects of salt on (extracellular) polyol production by various species and strains of salt-tolerant yeasts. Onishi reported a range of responses to sodium chloride (18%, wt/vol) by various strains from a doubled yield of polyol with *S. rouxii* strain N28 to a halved yield with *S. acidifaciens* var. *halomembranis*. The responses were also affected by aeration.

Spencer (130) has summarized related results from various sources, among which is a report of a shift in the type of polyol produced by *Pichia miso* in response to potassium chloride within the range 0 to 3 M. Increasing concentrations of this salt shifted polyol production from predominantly erythritol to predominantly glycerol. M. Edgley (unpublished data) has observed an increase in the proportion of intracellular glycerol (relative to arabitol) on growing *S. rouxii* in elevated concentrations of polyethylene glycol (molecular weight, 200) or especially sodium chloride. The reviews of Onishi (106) and Spencer (130) should be consulted for their extensive tabulation of polyol yields under various growth conditions.

As already intimated, however, the published information refers only to extracellular polyol. The intracellular polyol content is more difficult to determine accurately, especially after growth of the yeast in a high concentration of sugar.

There are, nevertheless, some clear trends in the response of intracellular polyol content of *S. rouxii* to the water activity of the growth medium. The basic trend is that a decrease in a_w causes an increase in the intracellular polyol concentration. This happens not only when a sugar is the solute, under which conditions such an effect might be expected as a direct consequence of extensive metabolism of the sugar, but also with nonmetabolites such as polyethylene glycol. Polyol can reach a concentration of at least 5 molal within *S. rouxii* in media adjusted to 0.95 a_w or lower (22). The amount of polyol that leaks out on washing is also affected by the growth conditions, by polyol content, and by the solute concentration in a washing fluid. Thus yeast grown at a low a_w and containing polyol at a high concentration leaks more when washed in water in 0°C than yeast grown in a dilute medium and containing less polyol. The result of this is that, at least under the experimental conditions used by Brown (22), *S. rouxii* grown in several media at 30°C and then washed with water at 0°C contained, after the washing, polyol in the range approximately 9 to 15% of the dry mass of the washed yeast.

As already stated, there is no evidence that mechanism I (above) explains the water relations of the xerotolerant yeasts, but their intracellular composition, which is consistently and substantially different from that of their nontolerant counterparts, provides the basic requirement for mechanism II. Since the difference in composition obviously arises from a metabolic peculiarity, mechanism IIA is implied.

What remains to be demonstrated is a role of the polyol in yeast water relations. Working with nonelectrolytes, Anand (Ph.D. thesis, University of New South Wales, 1969) showed that the extent of enzyme inhibition at reduced water activity was dependent on the solute used to adjust a_w. Specifically, he showed differences in the rates of the reaction catalyzed by a yeast isocitrate dehydrogenase in solutions of polyethylene glycol (molecular weight, 200) and of sucrose. He also obtained preliminary evidence that glycerol was far less inhibitory than the other two solutes. This second observation was confirmed by Brown and Simpson (29) and is illustrated in Fig. 5. Glycerol and sucrose functioned solely as inhibitors; there was no evidence of their activating the enzyme as salt, at low concentrations, activated the corresponding halophil enzyme.

Thus, in solutions of nonelectrolytes as well as electrolytes, water activity (a_w) is neither a simple nor a direct determinant of enzyme activity. Simpson (Ph.D. thesis, University of New South Wales, Sydney, 1976) has since extended these comparisons to a wide range of nonelectrolytes; some aspects of her findings are discussed in the section, Compatible Solutes.

Briefly, then, polyols make two major physicochemical contributions to the physiology of xerotolerant yeasts; they function (i) as an "osmoregulator" and (ii) as a compatible solute, a

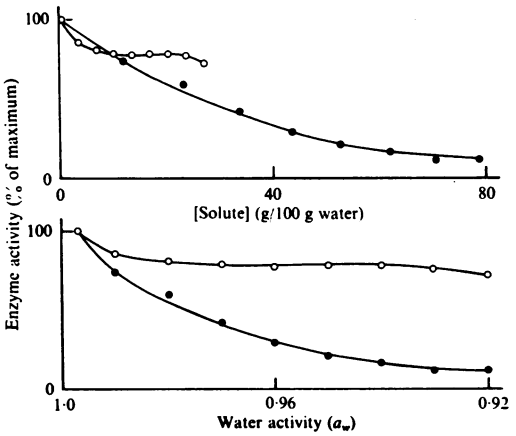


FIG. 5. *Activity of an NADP-specific isocitrate dehydrogenase from the xerotolerant yeast, Saccharomyces rouxii, measured in various concentrations of glycerol (○) and sucrose (●). The upper and lower panels show the same results with solution properties represented in two different ways. Curves showing enzyme activity as a function of molal solute concentration were basically similar to those in the lower panel. Results of Brown and Simpson (29) are reprinted with permission.*

substance that at high concentration protects enzymes against inhibition or inactivation. In addition, polyols can be expected to serve as a reserve food supply, but there are some constraints on this role as discussed for halophilic algae (below). The physiological role is thus very similar to that of K^+ (and KCl) in halophilic bacteria; it is discussed in greater detail in the section, Compatible Solutes. Fungal water relations have recently been discussed by Pitt (108). The physiological basis of xerotolerance by fungi imperfecti has not been studied in any detail, but there is reason to assume a general similarity with that of the xerotolerant yeasts. Polyol accumulation by fungi is common (78).

HALOPHILIC ALGAE

The terms "halophyte," as applied to higher plants, and "halophil," as applied to unicellular algae, are commonly used loosely to denote some degree of salt tolerance without necessarily giving a very clear indication of the range of concentrations tolerated. This interpretation is too broad for present purposes and the noun, halophil, will be used to apply, somewhat arbitrarily, to those algae with a requirement for salt, usually at concentrations of about 1.5 M or more. In fact its use, even in this way, is questionable. As will be shown, there are no eukaryotes known to have absolute and clearly defined salt requirements similar to those of the extremely halophilic bacteria. There are indeed halophilic algae with a salt requirement of the order of 1.5 M and a tolerance of up to saturated sodium chloride but, for reasons which will become apparent, the designation of even these organisms as halophilic is open to challenge.

Halophilic algae are found within the phylum *Chlorophyta*, order *Volvocales*. The principle genera with halophilic species are *Dunaliella* and *Chlamydomonas*. Both are flagellates; the major morphological distinction between them is the lack of a cell wall in *Dunaliella*. The halophilic algae that have been subjected to the closest scrutiny are species of *Dunaliella*. Little detail is known of the salt relations of *Chlamydomonas*, but ecological evidence (17) suggests that the genus is much less halotolerant than *Dunaliella*. The best known halophilic species of *Dunaliella* are *Dunaliella parva, Dunaliella viridis*, and *Dunaliella salina* which, in that order, appear to have increasing tolerance of high salt concentrations. In addition, the marine species *Dunaliella tertiolecta* has also been the subject of a number of investigations and has been used for comparison with the halophilic algae.

The species named above are characterized collectively and individually by a remarkably wide range of salt tolerance, probably the widest known for any unicellular organisms. *D. salina* has been reported (L. A. Loeblich, Ph.D thesis, University of California, San Diego, 1972) to grow throughout the range 0.3 M to saturated sodium chloride and to be able to withstand sudden substantial changes in salinity (87, 88, 139). Ben-Amotz and Avron (14) have reported that *D. parva* can withstand a change in salt concentration from 1.5 to 0.6 M sodium chloride without apparent leakage of cell contents.

The halophilic *D. viridis* grew over the range of approximately 1.6 M to saturated sodium chloride when the culture medium was inoculated directly with a suspension grown in 3.4 M sodium chloride, with the maximum growth rate occurring close to the bottom of the concentration range (15). Similarly, the optimum salt concentration for the marine species *Dunaliella tertiolecta* was at the bottom of its effective tolerance range (approximately 0.17 to 1.5 M sodium chloride) when the inoculum was grown in 0.17 M sodium chloride (15). The range of salt concentrations tolerated and the location of the optimum each differentiate *Dunaliella* clearly from the halophilic bacteria.

A more fundamental distinction from halo-

philic bacteria lies in the ability of the algae to be "trained" to extend their limits of salt tolerance, both up and down, with the implication that these limits are determined in part by the history of the organism. Thus, McLachlan (86) reported that *D. tertiolecta* grew within the range approximately 0.06 to 2 M sodium chloride. Craigie and McLachlan (43) extended this to about 2.6 M by serial subculturing through intermediate salt concentrations. Latorella and Vadas (77) adapted this species to 3.6 M salt. Conversely, D. S. Kessly (personal communication) has trained the halophil, *D. viridis*, again by serial subculture, down to 0.3 M sodium chloride.

Another biological difference from halophilic bacteria lies in the ability of *D. viridis* to accept replacement of a major part of its salt requirement by a nonelectrolyte such as sucrose. Successful transfer to a sucrose medium can be made in one step, but growth is much slower than in purely saline media of the same water activity (D. S. Kessly, personal communication). In its ability to accept substitution of salt by a nonelectrolyte, *D. viridis* has, at least qualitatively, a property in common with the xerotolerant yeasts. There is, nevertheless, an apparent minimal requirement by the species for sodium chloride. In the presence of 1.0 M sucrose this is about 0.05 M sodium chloride (D. S. Kessley, personal communication).

At yet another level, the fundamental difference between halophilic algae and bacteria is reflected in the complete absence of any evidence of halophilic characteristics in algal proteins. Thus, although the *Dunaliella* surface carries a net negative charge (A. C. T. Jokela, Ph.D thesis, University of California, San Diego, 1969), as indeed do most microbial surfaces, they show none of the instability at reduced salt concentrations which is so characteristic of halobacterial membranes. Again, although halophilic algal enzymes have not been studied nearly as extensively as their halophilic bacterial counterparts, of those that have been studied, there is no evidence whatever of any unusual salt requirements. Halophilic algal enzymes are sharply inhibited by salt concentrations well below that encountered in the growth medium (63, 15). Furthermore, the salt relations of two enzymes, glucose-6-phosphate dehydrogenase and glycerol dehydrogenase, are functionally identical in preparations from the marine species *D. tertiolecta* and the halophil *D. viridis* (15). Admittedly, these comparisons are limited but, as pointed out elsewhere in this review, a random selection of a functional halobacterial enzyme will reveal its peculiar salt requirements.

Intracellular Composition

The inevitable debate over the internal composition of halophilic algae has resembled in some respects the early speculation about halophilic bacteria, although there does not seem to have been explicit advocacy of a "dilute interior." The debate has centered predominantly on whether or not salt of any kind is accumulated to a concentration to match that outside the cell. Technical problems of estimating salt concentrations within the cells have facilitated the expression of opposite opinions. These problems are substantial with *Dunaliella*, which is a mechanically delicate organism and susceptible to leakage and salt exchange during centrifugation.

The evidence for and against high concentrations of salt within halophilic species of *Dunaliella* has not, in general, been based on direct analyses. Thus, Trezzi et al. (139) drew inferences from volume changes in *D. salina* in response to changes in environmental salinity. They concluded that the plasma membrane is freely permeable to salt and that the algae should therefore contain salt at a concentration close to that of the growth medium. Similar conclusions were drawn by Marrè and Servettaz (88) and Ginzburg (52). On the other hand, Johnson et al. (63) argued from the salt sensitivity of a number of cell-free enzyme preparations from *D. viridis* that this species excludes salt. Ben-Amotz and Avron (12), however, interpreted similar findings, together with the salt sensitivity of isolated chloroplasts, as indicative of compartments of low salt concentration within the cell. Borowitzka and Brown (15) confirmed the salt sensitivity of two enzymes from *D. viridis* and *D. tertiolecta* (and, as already stated, demonstrated the functional identity of the corresponding enzymes from the two species).

Of more immediate relevance to the question of intracellular composition was the demonstration that species of *Dunaliella* accumulate glycerol. Craigie and McLachlan (43) had demonstrated glycerol production by *D. tertiolecta* in 1964. Later, Ben-Amotz and Avron (14) showed that glycerol accumulated to a concentration of about 2 M in *D. parva* when the alga was adapted to 1.5 M sodium chloride. Ben-Amotz and Avron recognized glycerol as an "osmoregulator," as had Wegmann (147), inasmuch as its concentration responded positively to environmental salinity changes. Similarly, Borowitzka and Brown (15) demonstrated glycerol accumulation in direct proportion to extracellular salt concentration in *D. tertiolecta* and *D. viridis*. In the latter species, glycerol reached a

concentration of about 4.4 molal when the organism was grown in 4.25 M sodium chloride. This value is similar to the potassium concentration in halophilic bacteria and to total polyol in xerotolerant yeasts when grown at comparable levels of a_w.

In simple terms, the physiological implication of glycerol accumulation is that it does not leave room for much salt in the algal cell. The experimental results suggest that glycerol concentration is sufficient to bring cellular water potential to about the same level as that outside the cell. Moreover, if salt did enter the cell to a concentration similar to its external concentration, the delicate membrane of *Dunaliella* would not be able to withstand the osmotic/hydrostatic consequences of the considerable reduction in internal water potential caused by the additive effect of the two solutes. Thus, with glycerol at the stated concentrations, salt uptake should be no more than a minor event sufficient to make up any discrepancy between internal and external water potentials (with allowance, of course, for the slightly lower internal water potential required for turgor). This limited uptake is likely to be met by potassium chloride rather than sodium chloride; experimental results with *D. tertiolecta* and *D. viridis* support this assumption (15).

Polyol production is common in plants generally, including algae, and has been reviewed extensively by Lewis and Smith (78), although those authors specifically omitted glycerol from consideration. Mannitol is a common algal polyol, particularly among the brown seaweeds. Lewis and Smith have also pointed out the ubiquity of polyols at high concentrations in lichens and noted the possible function of these compounds as osmoregulators in marine algae and seaweeds.

Physiological Role of Glycerol

Glycerol accumulation can be assumed to have three major functions in the physiology of marine and halophilic species of *Dunaliella*. The first and most obvious function is that of an osmoregulator, that is, a substance whose concentration responds positively to extracellular solute concentration (negatively to a_w), which maintains thereby approximate parity between internal and external a_w (or water potential) and which therefore minimizes osmotic stresses and dehydration to which the cell would otherwise be subjected. Although this is a vital function, it is not particularly specific. Any solute retained within a cell will contribute to its osmotic status and, in the absence of a dominant osmoregulatory solute, thermodynamic adjustment to a low water potential will be achieved by a water flux.

The second role is that of a compatible solute, or protector of enzyme activity. Glycerol has not been compared with other nonelectrolytes in its effect on algal enzymes, as it has for a yeast enzyme, and as potassium chloride has been compared with sodium chloride for halophilic bacterial enzymes (see sections, Halophilic Bacteria and Xerotolerant Yeasts). Some simple comparisons of glycerol and salt have been made using algal enzymes (15, 55). Nevertheless, its protective action on yeast isocitrate dehydrogenase (sections Xerotolerant Yeasts and Halophilic Algae), its failure to inhibit *Dunaliella* glucose-6-phosphate dehydrogenase at concentrations below about 4 M (15) and the simple proposition that the algae would not grow if the glycerol were not protective make its compatible nature in algae a virtual certainty.

Third, it can act as a food reserve under some conditions. Lewis and Smith (78) have discussed polyols as food reserves, but it should be noted that extensive consumption of the polyol would deplete an algal cell of its compatible solute and leave it with diminished protection against environmental salinity. It is likely, therefore, that glycerol becomes available as a carbon source in significant quantities only as a result of a drop in salt concentration.

The biosynthetic pathway of glycerol in *Dunaliella* has not yet been studied in any detail. Since glycerol is an early product of photosynthesis (43, 147) and can also be formed in the dark from accumulated starch (D. S. Kessly, personal communication), it is possibly produced in the chloroplasts. Furthermore, the presence of an active NADP-linked dehydrogenase (11, 13, 15) suggests that the final steps of the sequence are:

$$\text{Triose phosphate} \xrightarrow[P_i]{} \text{Dihydroxyacetone} \rightleftharpoons \text{Glycerol}$$

Glycerol could be expected to diffuse readily from the chloroplast into the cytosol. Normally, dihydroxyacetone phosphate is the major product of photosynthesis which passes from chloroplast to cytosol (144). If glycerol were produced in the cytosol from triose phosphate, we could expect glycolytic enzymes to catalyze the process, in which case the sequence would probably be:

$$\text{Dihydroxyacetone phosphate} \rightleftharpoons \text{Glycerol-3-phosphate} \xrightarrow[P_i]{} \text{Glycerol}$$

In this case the reduction step would require NADH, not NADPH. This is essentially the mechanism suggested by Wegmann (146, 147), but it does not seem to happen that way.

Regulation of Glycerol Production

The regulation by extracellular solute concentration of production and accumulation of compatible solutes is a complex process that is poorly understood. It is apparent, however, that glycerol content varies as a result of synthesis or degradation, not simply by concentration or dilution caused by water fluxes (11, 15).

The NADP-specific glycerol dehydrogenase of *Dunaliella* has a high apparent Michaelis constant for glycerol (1.5 to 3.5 M) and a low apparent Michaelis constant for dihydroxyacetone (0.8 to 2.2 mM) (13, 15; L. J. Borowitzka, Ph.D. thesis, University of New South Wales, 1974). In spite of this big difference between the two constants, the enzyme can be assumed to function as a freely reversible dehydrogenase in the cell, since the glycerol concentrations are of the same order as the Michaelis constants; this point has already been emphasized (15). Moreover, the enzyme does not have simple Michaelis-Menten kinetics. Under some circumstances, notably with glycerol as variable substrate, it gives parabolic double reciprocal plots (15). These, and related results described by Borowitzka (Ph.D thesis, University of New South Wales, 1974), suggest that glycerol adds twice in the reaction sequence, once as a substrate and once as an effector. The enzyme is thus likely to be crucial in the overall process of regulating glycerol production.

Borowitzka (Ph.D. thesis, University of New South Wales, 1974) has argued that there should be two levels at which glycerol concentration is regulated. One of these is the homeostatic process by which glycerol is maintained more or less at a constant concentration in any one set of environmental conditions. The second level is that of the direct response of glycerol content to environmental salinity.

Borowitzka has suggested that the nonlinear kinetics are important at the first level since, at low glycerol concentrations corresponding to those normally encountered in *D. tertiolecta*, reaction velocity changes sharply in response to glycerol concentration. On the other hand, at high glycerol concentrations, such as occur in *D. viridis*, reaction velocity responds relatively slightly to changes in glycerol concentration. In other words, the enzyme kinetics suggest a fairly coarse control of glycerol oxidation by glycerol concentration in the marine species and a fine control in the halophil. In turn, this suggests that glycerol concentration should oscillate to a greater extent in *D. tertiolecta* than in *D. viridis*. It is not yet known if, in fact, this happens. The problem of regulating glycerol concentration in response to environmental salinity has many features in common with the regulation of polyol production in xerotolerant yeasts and, so far, is largely unexplored. Some additional comments on regulation are made in the section, Compatible Solutes.

Physiological Basis of Algal Halophilism

We have argued elsewhere (15, 29), and in this review, that cells that thrive at biological extremes of low water activity must accumulate a compatible solute. Glycerol accumulation can explain in large measure the *tolerance* by *Dunaliella* of high salt concentrations but, on present evidence, it is insufficient to explain the apparent *requirement* of *D. viridis* for 1.5 M sodium chloride. Nor does it explain the different salt relations of *D. tertiolecta* and *D. viridis*, since each can be trained to grow in the other's domain and, when it does, it contains glycerol at a concentration appropriate to that domain (D. S. Kessly, personal communication).

The essential function of a compatible solute is to confer a *tolerance*, not a *requirement*. It is true that a mechanically delicate alga such as *Dunaliella* might, for purely osmotic reasons, require a certain minimal salt concentration to maintain cellular integrity, but this is a "chicken-and-egg" argument. The presence of glycerol might explain why *D. viridis* needs training to grow at a lowered salt concentration, but it does not explain why *D. tertiolecta* needs training to grow at higher salt concentrations.

In fact, there is no significant information of which I am aware that can explain the basically different salt relations of *D. tertiolecta* and *D. viridis*. Certainly the differences between these species do not appear to lie in any generalized intrinsic properties of their enzymes. As already stated, there is no apparent difference in their ability to adjust their absolute glycerol content to a specific salinity.

A notable difference between the two species, however, is observed when *D. tertiolecta* is trained to grow in the salt concentration range of *D. viridis*. Under those conditions it (*D. tertiolecta*) still has a substantially higher growth rate than the halophil (D. S. Kessly, personal communication). Kessly's experiments were done under conditions of continuous illumination, and it is relevant, therefore, that *D. tertiolecta* is reported (77) to require continuous illumination when, after training, it grows at salt concentrations greater than 2.5 M.

The difference in growth rate between the two species is reminiscent of the generally low growth rates of the xerotolerant yeasts (5) and

suggests that a fundamental difference between the two algal types might be found in their energy metabolism. Thus, if the halophil were to divert a substantially greater proportion of its carbon to glycerol production it would have less carbon and NADPH available for other biosynthetic processes. The greater amount of glycerol so produced would impose a need for a higher salinity for the osmotic reasons already mentioned, but this is unlikely to be the whole explanation. Inasmuch as the solute requirement of *D. viridis* is not absolutely specific for salt, the alga resembles those xerotolerant yeasts such as *Torulopsis halonitratophila* (105) and *Zygosaccharomyces nectarophilus* (strain YE, reference 5) which have an apparent requirement for a lowered a_w. Furthermore, both these yeasts lose this requirement with a reduction in temperature (105; M. Edgley, personal communication). The effect of temperature has not yet been satisfactorily explained nor, to my knowledge, have the effects of temperature on halophilic algal salt requirements been investigated.

Algae Other than *Dunaliella*

The genus *Chlamydomonas* has species with a minimal requirement of about 0.34 M sodium chloride, an ability to grow in 1.7 M sodium chloride, but an uncertain upper limit of concentration (102, 140). Blue-green algae have been isolated from the Dead Sea and, of these, a species of *Aphanocapsa* was reported to have a minimum salt requirement of 1.0 M and to grow best between 1.5 and 3 M sodium chloride (142). The Dead Sea, like concentrated marine pools and the saline Arctic pools mentioned earlier, contains substantial concentrations of Mg^{2+}, which is itself a significant factor in a definition of salinity tolerance.

Little is known of the physiological basis of salt tolerance in any of these algae. *Chlamydomonas* is reported to contain about 0.2 M Na^+ and 0.05 M K^+ when grown in 1.7 M sodium chloride (103). If these estimations are reasonably accurate, then there is an obvious need for an additional osmoregulatory solute(s). One might fairly assume that such a solute would be a polyol or something chemically similar. A compatible solute of some kind can be assumed to accumulate in all algae with significant salt tolerance. This is discussed further in the sections, Compatible Solutes and Loose Ends.

COMPATIBLE SOLUTES

Electrolytes

The results discussed so far have shown that all microorganisms capable of growth at biologically extremely low levels of a_w and appropriately studied accumulate compatible solutes to a concentration of a similar order to that of the extracellular solute(s). The solutes are K^+, Cl^-, and KCl in halophilic bacteria and one or more polyhydric alcohols in the other organisms we have studied. It is likely that all other protista with a conspicuously xerotolerant physiology accumulate either a polyol or a closely related hydroxylated organic metabolite. Compatible solutes function partly as osmotically active substances and partly as protectors of enzyme activity. The mechanism of osmotic adjustment is obvious and relatively nonspecific; it does not warrant detailed discussion.

Enzyme protection, however, is less obvious. A semantic distinction might be made between an intracellular solute such as potassium chloride when it functions as an activator as, for example, with halophil ribosomal enzyme systems and as an inhibitor as, for example, with the isocitrate dehydrogenase under halophilic physiological conditions. The compatible nature of potassium chloride is apparent under the latter conditions because it is a very poor enzyme inhibitor. On the other hand, our limited experimental evidence supplemented with a generous helping of intuition suggests that the polyols function under inhibitory conditions (i.e., at or above any optimum that might exist) with nearly all enzymes other than those involved directy in their metabolism. Like potassium chloride, they are very poor enzyme inhibitors and, at high concentrations, display their compatible properties because of this.

Potassium chloride inhibits halophil isocitrate dehydrogenase not only less severely than does sodium chloride, but it does so in a much simpler fashion. Figures 6 and 7 illustrate this statement in kinetic terms. Secondary plots of slope and intercept show potassium chloride to be a simple linear noncompetitive inhibitor, whereas sodium chloride gives a complex nonlinear inhibition pattern. The nonlinearity can be overcome by increasing the concentration of $NADP^+$ (3).

Nonlinear kinetics of this kind imply a cooperative action of the inhibitor which, in turn, means multiple addition of the inhibitor in the reaction sequence and/or a change in state of the enzyme caused by the inhibitor. It is likely that a change in state is involved since (i) the effect can be modified by high substrate concentrations and (ii) the enzyme has a higher apparent molecular weight in sodium chloride than in potassium chloride. Apparent molecular weights are: in a low ionic strength buffer without added potassium chloride or sodium chloride, 70,900; in 1.0 M KCl, 122,000; in 4.0 M

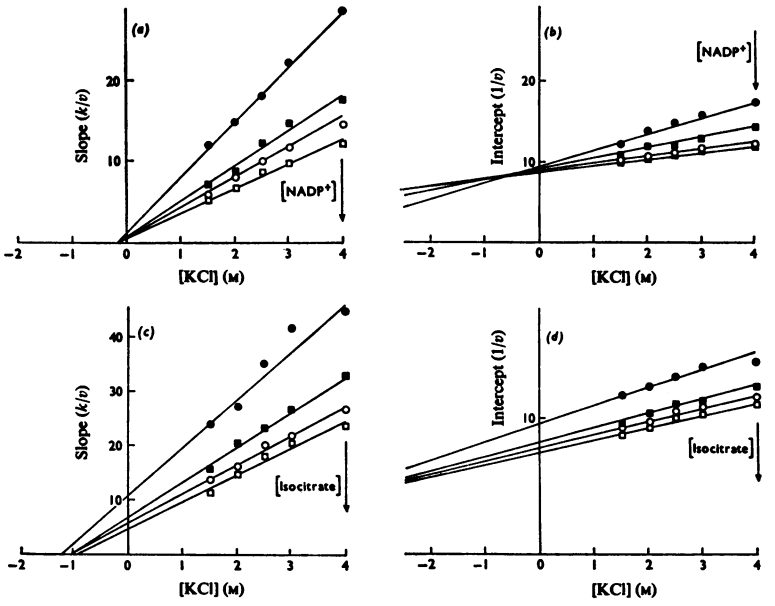


FIG. 6. *Halophil NADP-specific isocitrate dehydrogenase. Secondary plots of slopes and intercepts against concentration of potassium chloride. Top panels (a and b): NADP+ used at fixed concentrations of (top to bottom) 0.20, 0.40, 0.60, and 0.80 mM. Bottom panels (c and d): sodium isocitrate used at fixed concentrations of (top to bottom) 0.125, 0.25, 0.50, and 1.0 mM. Results of Aitken and Brown (2) are reprinted with permission.*

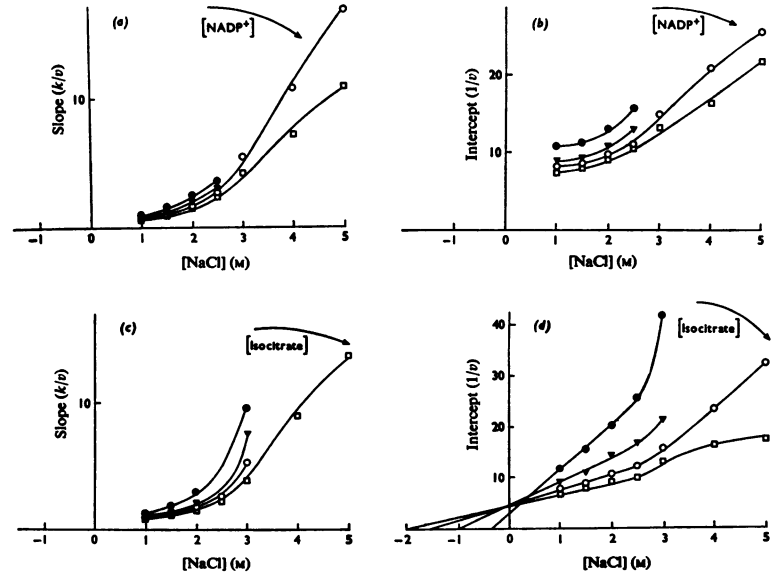
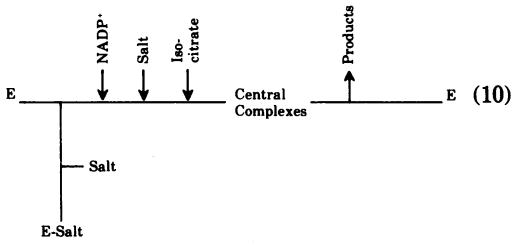


FIG. 7. *Halophil NADP-specific isocitrate dehydrogenase. Secondary plots of slopes and intercepts against concentration of sodium chloride. Top panels (a and b): NADP+ used at fixed concentrations of (top to bottom) 0.20, 0.40, 0.60, and 0.80 mM. Bottom panels (c and d): sodium isocitrate used at fixed concentrations of (top to bottom) 0.125, 0.25, 0.50, and 1.0 mM. (At high concentrations of sodium chloride and low fixed substrate concentration, the kinetics were not Michaelis-Menten.) Results of Aitken and Brown (2) are reprinted with permission.*

KCl, 135,000; in 1.0 M NaCl, 224,000; in 4.0 M NaCl, 251,000 (2). Thus, the enzyme probably has a monomeric molecular weight of about 71,000, dimerizes in 1 to 4 M potassium chloride (the physiological condition), and forms a trimer or tetramer in 1 to 4 M sodium chloride. The higher apparent molecular weight in sodium chloride is consistent with the "salting out" characteristics of this salt, as discussed by Lanyi (74).

There is evidence that salt concentration affects the reaction mechanism of the halophil isocitrate dehydrogenase, but under physiological conditions it has a sequential mechanism, normal for pyridine nucleotide-linked dehydrogenases, in which $NADP^+$ is the first substrate added and NADPH is the last product released. The evidence also shows, however, that the effects of salt in activating the enzyme, and at high concentrations inhibiting it, can be interpreted in a kinetic sense as if salt were a third substrate, causing substrate inhibition at high concentrations. The reaction mechanism can be represented as in reaction (10):



(10)

Thus, salt adds in a substrate-like manner between $NADP^+$ and isocitrate and also, in an inhibitory manner, to form a dead-end complex before addition of $NADP^+$. Whatever the physicochemical explanation of this association might be, there are extensive implications for the physiology of halophilic bacteria in the fact that a relatively nonspecific substance such as a common inorganic salt can modify an enzyme in such a way that the kinetics suggest a fairly precise interaction with the active site of the enzyme.

The kinetics also allow a calculation of the dissociation constants of the constants of the enzyme-salt complexes. The reaction mechanism shown above implies two salt-enzyme dissociation constants. One constant, that of the enzyme-"substrate salt" complex is formally similar to a Michaelis constant (K_m). The other, that of the dead-end complex, is a normal inhibitor constant (K_i). The values of these constants for both salts are shown in Table 3.

The table shows that the two salts give identical "Michaelis constants," and there is nothing at this level to distinguish between them as enzyme activators. The magnitude of the "Michaelis constants" is normal for a substrate. On the other hand, the inhibitor constants show some conspicuous and important differences. First, for both salts they are orders of magnitude greater than the "Michaelis constants." A difference in this direction is predictable from the simple fact that the salts do activate at low concentrations and do inhibit at high concentrations. The difference in magnitude is not necessarily so obvious. The outstanding difference, however, is between the two salts, the K_i (KCl) being about 10 times as great as the K_i (NaCl) and well in excess of the solubility of either salt.

In simple terms, therefore, the "compatible" nature of potassium/chloride for a halophil enzyme is a direct consequence of its very low affinity for the enzyme at the "inhibition site"; sodium chloride is a much more powerful inhibitor because of its tighter binding. The aggregation of the enzyme is associated with the tighter binding of sodium chloride.

The physicochemical processes underlying this kinetic explanation are undoubtedly complex. From the outset, the assumption can be made that in the low (activating) range of salt concentration the salts are extensively ionized and the quantitative differences between the salts under these conditions are attributable to differences between K^+ and Na^+. Thus, there is essentially no difference between the two cations as reflected by the "K_m" of the enzyme-salt complex. There are differences, however, which are shown in the relations between the enzyme and its substrates. In the low (activating) range of salt concentration, K_m (isocitrate) was consistently higher in sodium chloride than in potassium chloride, but the opposite was true of K_m (NADP). Furthermore, at low salt concentrations apparent V_{max} was higher in sodium chloride than in potassium chloride except with

TABLE 3. *"Dissociation constants" of salt (or cation) complexes of halobacterial isocitrate dehydrogenase*[a]

Constant	NaCl	KCl
"K_m"	31–32 mM	30–31 mM
K_i	0.9 M	9.5 M

[a] The values were calculated from data of Aitken et al. (3). In its role as an activator, expressed in the "K_m," the salt probably exerted its effect largely through the cation. On the other hand, the inhibitor constant (K_i) must be assumed to reflect an interaction with a substantial proportion of undissociated salt (see text).

very low fixed concentrations of isocitrate (2).

The differences between the two cations as well as Li^+ and NH_4^+ (at low concentrations, Fig. 1) can be interpreted superficially in terms of radii or, inversely, of hydrated volumes of the ions. Not only are explanations of this type superficial, but they are also serious oversimplifications that are likely to be wrong on several counts. Although it is true that the ionic radii, or hydrated volumes, do correlate with and can predict the behavior of ions under some circumstances, these circumstances are usually restricted by a number of factors including a relatively narrow concentration range of the salts.

Of far greater predictive value is the thermodynamic explanation developed largely by Eisenman. A description of this theory is beyond the scope of the present review, but it has been discussed in detail in Diamond and Wright (45). Some comment is appropriate, however. In an aqueous environment the relative affinities of a site on a protein, a membrane, etc., for two different cations will be a function of the free energies of hydration and of binding to the site;

$$\Delta F_{Na^+}(\text{site}) - \Delta F_{K^+}(\text{site}) - (\Delta F_{Na^+}h - \Delta F_{K^+}h) \quad (11)$$

where ΔF_x (site) is the free energy of interaction between the cation, X, and the site; $\Delta F_x h$ is the free energy of hydration of the cation, X.

Thus, the "K_m" values for the two salts, which we assume actually reflect cation binding to the enzyme, suggest that the sum of expression (11) is about the same for Na^+ and K^+. Perhaps this is coincidental, since the free energies of hydration are certainly not the same (116). A complication, of course, is that the "K_m" is not an equilibrium constant but, as with any conventional K_m, it reflects a steady-state phenomenon. Even though the cation does not change in the reaction, it cannot dissociate (after the reaction) from the same form of the enzyme as that to which it initially attached. This is evident from reaction 10. There can be no recurrence of the enzyme-$NADP^+$ complex from which the cation would dissociate to give a true equilibrium. Nevertheless, it is inconceivable that the thermodynamic considerations discussed by Diamond and Wright (45) do not apply in some measure to an enzymic situation such as this. Clearly, it is an area for detailed investigation.

In the high, inhibitory range of salt concentration the situation is more complex because, among other things, the salts become proportionately less dissociated with increasing concentration. Furthermore, the activity coefficients of the two salts respond differently to concentration: that for sodium chloride increases over the relevant concentration range, whereas that of potassium chloride remains relatively constant (Fig. 8). This basic difference in solution properties of the two salts possibly contributes to the complex inhibition patterns caused by sodium chloride, but it is unlikely to provide the whole explanation. For one thing, it cannot explain the differences in apparent molecular weight determined in the two salts nor, for another, does it overcome the differences in apparent V_{max} encountered at high salt concentration, although a correction for activity does reduce those differences substantially (Fig. 9).

A physicochemical explanation of the special properties of compatible solutes should also take into account, wherever possible, the relevant properties of solvent water. Solvent properties and the thermodynamics of solvation assume greater relative importance when a solute has an affinity for an enzyme that is not very different from the affinity of water for the enzyme. In the extreme case in which solute and water are bound to a polymer in the same proportion as they occur in solution, the solute effectively has zero affinity for the polymer and, by definition, is not adsorbed (30).

There is now very good experimental evidence, based largely on nuclear magnetic resonance and dielectric dispersion measure-

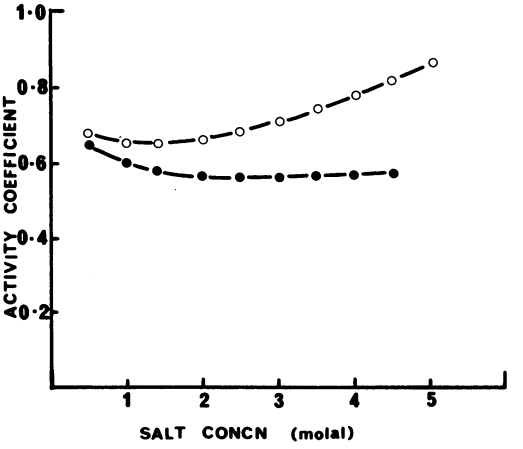


FIG. 8. *Activity coefficients of sodium chloride (○) and potassium chloride (●) as a function of concentration. Plotted from data in Robinson and Stokes (115).*

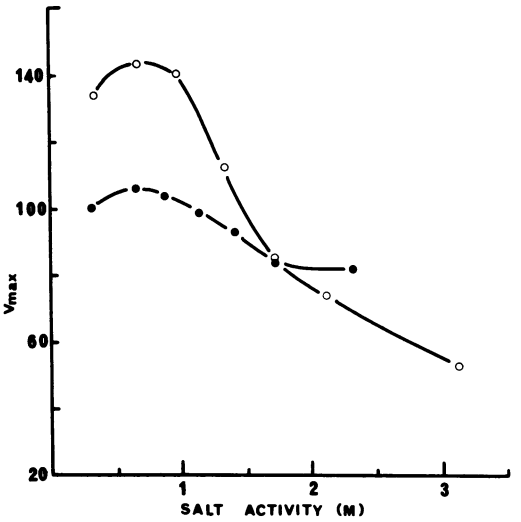


FIG. 9. *Halophil NADP-specific isocitrate dehydrogenase. V_{max} as a function of salt activity in sodium chloride* (○) *and potassium chloride* (●). *Replotted from results of Aitken et al. (3).*

ments, for three "types" of water in an aqueous solution of a protein. Cooke and Kuntz (42) have described these three types in the following way. Type I—"bulk water," the rotational and translational properties of which do not appear to be appreciably altered by binding to macromolecules. A protein solution (20%, wt/vol) has about 90% of its water in this form. Type II—"bound water." The rotational motions and freezing point of this water are substantially modified by interaction with the surface of macromolecules. This water seems to exist as one to two monolayers and is present in the proportion of about 0.3 to 0.6 g of water/g of protein. A 20% protein solution has about 10% of its water in this form. Type III—"irrotationally bound water," in which water molecules are essentially bound to specific sites on the polymer for periods of microseconds. A 20% protein solution has about 0.1% of is water in this form.

It can be assumed that the dissolution of salt to a high concentration will nonspecifically affect the amount or activity of type I water, will specifically affect type II water in some way, and probably will not affect type III. Physicochemical information about salt-protein interactions at high salt concentrations is limited, but what information there is seems to be consistent with the preceding comments and with the properties of compatible solutes. For example, Bull and Breese (30) have studied water and solute binding to egg albumin over a salt concentration range of approximately 1 to 3 molal. Their results suggest that, under these conditions, the chloride of the alkali cations are only slightly bound to the protein and that their major effect is on the amount of bound water. The values cited by Bull and Breese suggest that their "bound water" is the type II water as defined by Cooke and Kuntz (above). For example, they quote 740 mol of water bound/mol of egg albumin at 97% relative humidity. This is equivalent to 0.3 g of water/g of protein. In solution with no binding of salt, about 1,350 mol of water is bound per mol of protein (0.5 g/g). These values are within the range of type II water.

Water bound to the protein over a salt concentration range of 1 to 3 molal was constant in both potassium chloride and sodium chloride but different for each salt: 211 mol/mol (0.08 g/g) for sodium chloride and 360 mol/mol (0.14 g/g) for potassium chloride.

It thus seems likely that the compatible nature of potassium chloride is expressed in part through the limited disturbance that it causes to the type II bound water. It would clearly be desirable to examine the inhibition of a halophil enzyme caused by rubidium chloride and cesium chloride since, according to Bull and Breese, they cause even less dehydration of egg albumin than does potassium chloride. Furthermore, sodium sulfate actually enhances the degree of solvation; kinetic studies of its effects should also be informative.

It is also likely that salts modify enzymic reactions through their effect on "activation volume," that is, the change in volume that occurs when an enzyme-substrate complex changes from the ground state to the transition state. Effects of salt on this process, however, are generally restricted to salt concentrations below about 300 mM (84).

Nonelectrolytes

The compatible solute role of polyhydric alcohols was established in the first instance by comparative studies of the effects of sucrose, on the one hand, and glycerol, on the other, on the kinetics of the NADP-specific isocitrate dehydrogenase of *S. rouxii*. In a number of respects this investigation was complementary to that of Aitken and Brown (2) on the effects of salts on the halophil isocitrate dehydrogenase. At the time of writing, the greater part of the nonelectrolyte studies is unpublished but can be found in the Ph.D. thesis of J. R. Simpson (University of New South Wales, 1976).

Interpretation of the kinetics of this enzyme was complicated by nonlinear double-recipro-

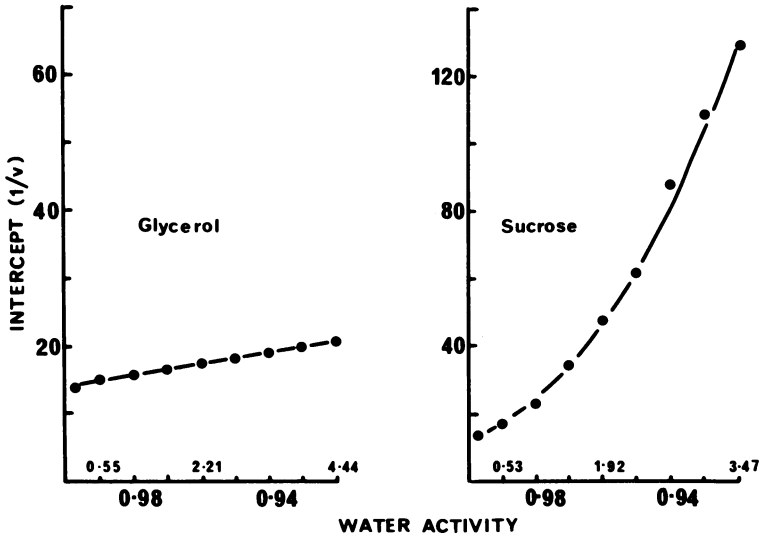


FIG. 10. *Yeast NADP-specific isocitrate dehydrogenase. Secondary plots of intercept against water activity in solutions adjusted with glycerol (left panel) and sucrose (right panel). The small numerals above the abscissa indicate the concentration (molal) of the solutes at selected points. Previously unpublished data of J. R. Simpson and A. D. Brown.*

cal plots (concave down). Associated with the nonlinearity was gel-electrophoretic evidence that the enzyme occurred in several forms, probably signifying different states of aggregation. Multiple forms of isocitrate dehydrogenases have been reported from other sources (114, 118, 123). Because of the nonlinearity of the double-reciprocal plots, secondary plots of slope were not possible, although intercept could be so treated. Figure 10 illustrates such plots and gives a striking comparison with the corresponding analysis of the halophil isocitrate dehydrogenase in potassium chloride and sodium chloride (Fig. 6 and 7). Briefly, the comparison shows intercept replots for glycerol to be linear with a small slope (i.e., inhibition changed only slightly with inhibitor concentration), whereas the corresponding plot for sucrose is steep and nonlinear (inhibition increased sharply with concentration and the rate of increase was concentration dependent).

In this respect glycerol, a compatible solute in halophilic algae and some xerotolerant yeasts, resembles potassium chloride, the compatible solute for halophilic bacteria. There is an equally striking similarity between the nonelectrolyte sucrose, which is severely inhibitory and partly excluded by xerotolerant yeasts, and the electrolyte sodium chloride, also severely inhibitory and largely excluded by halophilic bacteria. Furthermore, by extrapolating secondary plots of the type shown in Fig. 10, inhibitor constants can be derived for the two nonelectrolytes. Obviously, any constant derived in this way for sucrose can be, at best, an approximation. Nevertheless, even a casual inspection of Fig. 10 reveals a major difference in the K_i of the two inhibitors, that for sucrose being by far the smaller. The numerical values derived for these constants are: K_i (glycerol), 13 molal; K_i (sucrose), 1.5 molal.

Thus, there is again a striking resemblance to the values obtained for the halophil enzyme (Table 3). It is evident that, with nonelectrolytes as well as electrolytes, compatible solutes are distinguished as inhibitors with a very low affinity for an enzyme.

To some extent, however, the selection of sucrose for these comparisons was fortuitous. Simpson has extended her investigation to cover a range of nonelectrolytes to include the fully hydroxylated polyols up to hexitols, incompletely hydroxylated di- and triols, and a range of sugars.

In all cases, inhibition was assessed from the slope of plots of reciprocal velocity (1/v) against inhibitor concentration; the steeper the slopes, the greater the response of inhibition to inhibitor concentration.

When measured in this way, the inhibition caused by the fully hydroxylated alcohols was positively correlated with chain length (molecular weight), but the relation was sigmoidal (Fig. 11). Thus, although chain length dominated the inhibitory properties of the polyols,

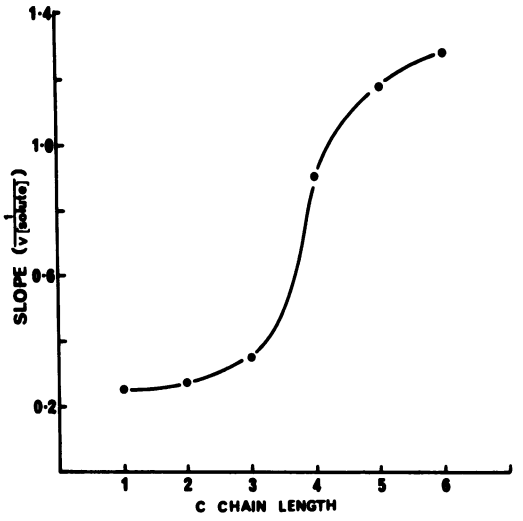


FIG. 11. *Yeast NADP-specific isocitrate dehydrogenase. Inhibition, expressed as the slope of double-reciprocal plots, as a function of the chain length of acyclic fully hydroxylated polyhydric alcohols. The C_1 alcohol was methanol. Previously unpublished data of J. R. Simpson and A. D. Brown.*

there was another determining factor(s) as well. Some indication of the level at which this additional factor operates was obtained by plotting slope against the chromatographic R_f of the polyol. Values of R_f were obtained for the solvent butan-1-ol + acetic acid + water (6:1:2, by volume) although, since the polyols are nonelectrolytes, virtually any solvent mixture capable of reflecting an "oil"-water partition coefficient might be expected to illustrate the point. When this was done an essentially linear relation was obtained between slope and R_f, the highest R_f correlating with the lowest slope. The probable implications of this are discussed below.

The situation with sugars is far more complex in that there was no correlation between slope (as used above) and molecular weight. Stereochemical factors apparently exert a major influence and several sugars, including sucrose, gave nonlinear plots of 1/v versus inhibitor concentration. The generalizations that can be made about inhibition by sugars are briefly: (i) inhibition caused by straight-chain aldoses, glyceraldehyde and erythrose, is substantially irreversible, implying complexing between the aldehyde group and the enzyme; (ii) hexoses were generally less inhibitory than other sugars, the least inhibitory being fructose which gave about the same slope as glycerol (it is probably no coincidence that some of the lowest values of a_w supporting growth of microorganisms have been obtained in fructose); (iii) some sugars, notably sucrose and ribose, gave nonlinear plots of 1/v versus inhibitor concentration, the slope increasing with inhibitor concentration. Moreover, there was no correlation of slope with the chromatographic R_f of the sugars.

The incompletely hydroxylated diols and triols (propane and butane diols and butane triol) all gave biphasic plots of 1/v versus polyol concentration, the plots being divisible into two straight lines, with the greater slope occurring at the higher polyol concentration. The order of increasing inhibition caused by these di- and triols was: propane-1,2-diol; propane-1,3-diol; butane-1,2,4-triol; butane-2,3-diol; butane-1,4-diol; and butane-1,3-diol.

Physicochemical Mechanism of Nonelectrolyte Action

To this point the evidence is clear that at a series of specified levels of a_w an enzyme behaves differently in solutions of different nonelectrolytes. The conclusion is inescapable that a_w cannot account for the differences shown by the various solutes and is thus unlikely to be the major determinant in the total quantitative effect produced by the nonelectrolyte solutions. This should scarcely be surprising for solutions which might contain 10 or 15% less thermodynamically available water but several orders of magnitude more solute than "conventional" dilute biological solutions. It is equally evident that nonelectrolytes exert a direct action on the enzyme. There is insufficient information to enable any firm conclusions to be drawn about the action mechanism, although there are some useful pointers available from various sources.

Nonelectrolytes might affect enzyme action by changing the viscosity, pH, ionic strength, or dielectric constant of a solution, by changing water "structure" or by directly reacting with the protein molecule in any of several possible ways. A partial correlation of enzyme inhibition with viscosity suggests that this might, at most, be a supplementary factor in determining inhibition (Brown, unpublished data). The minor effects of the solutes on pH do not explain differences in inhibition (J. R. Simpson, Ph.D. thesis, University of New South Wales, 1976). Although ionic strength is increased in direct proportion to the lowering of a_w and thus might contribute to the total inhibition encountered in nonelectrolyte solutions, the increase should be the same for all solutes. Dielectric constant (4) correlates with inhibition caused by at least some polyols and one sugar (glucose), but not with sucrose (J. R. Simpson and A. D. Brown, unpublished data). The limited information available for dielectric constant, together with

that given by the correlation with R_f (see above), suggests a possible involvement of hydrophobic interactions in the inhibition caused by polyols and some sugars.

Another possible mechanism is the modification of water "structure" by the solute. In this case, it can be assumed from the outset that any relevant changes in water structure would occur in the type II bound water described above.

There is evidence that the solutes can modify the aggregation state of a protein. Thus, Simpson has shown by gel electrophoresis that ammonium sulfate fractionation appears to depolymerize the yeast isocitrate dehydrogenase, and she has obtained some evidence that glycerol (2 molal) has a similar effect. If this is so, it agrees with earlier findings of Contaxis and Reithel (41) that glycerol depolymerizes an enzyme. These authors made a series of comparisons of propane-1,2-diol, ethylene glycol, and glycerol on the physical chemistry and enzyme activity of jack bean urease. They observed that, on exposure to aqueous solutions (90% vol/vol) of each of the alcohols, urease dissociated to a molecular weight of 240,000, half the normal value. The rate of dissociation increased in the order: glycerol, propane diol, ethylene glycol. There was no evidence of any significant conformational change in the 240,000-molecular-weight monomers during the dissociation, and enzyme activity remained at 80 to 82% of the untreated enzyme. Moreover, the "monomer" reassociated to the dimer on removal of the alcohol. On prolonged exposure (2 days) to ethylene glycol or propane diol, the enzyme reassociated in a manner different from that described above to give an enzymically inactive polymer. This did not happen in glycerol, in which the enzyme remained active on prolonged exposure. β-Lactoglobulin also dissociates to a "monomer" in about 10% ethylene glycol and reassociates (dimerizes) in about 20% glycol (69).

Since the dissociation of urease did not apparently cause a conformational change in the protein, Contaxis and Reithel assumed that the association was confined to a restricted zone on the surface of the 240,000-molecular-weight subunit. They also assumed that the alcohols promoted dissociation by modifying water structure and by "competing with water for hydrogen bonding." A change in hydration was presumed to occur near hydrophobic areas on the protein and, by virtue of the entropy change that accompanied it, to contribute to the negative free energy of dissociation in the polyol solution.

Douzou, who, for some years, has studied enzyme activity at subzero temperatures, has recently reviewed the theory and methodology of this field of investigation (46). High solute concentrations are needed to prevent freezing at the temperatures of Douzou's experiments, and it is scarcely a coincidence that polyols, notably, ethylene glycol, propane diol, and glycerol, were the solutes used for this purpose. Douzou comments that, at normal ambient temperatures, these polyols are far less effective "denaturing" agents than monohydric alcohols, including ethanol, and that many proteins are stable in 1:1 (by volume) aqueous solutions of the polyols. At first sight this observation conflicts with Simpson's finding (above) that the inhibitory action of methanol on isocitrate dehydrogenase was (marginally) less than that of ethylene glycol or glycerol. This apparent discrepancy might be explained by the distinction between inhibition and denaturation; Simpson's measurements of enzyme activity were limited to periods of a few minutes.

If nonelectrolytes do affect enzyme activity by disturbing water structure, it is probably type II water, as suggested already, which is so affected. It is possible, however, the nonelectrolytes can act by more than one mechanism. The negative correlation of inhibition by polyols with chromatographic R_f (R_f being used as a convenient measure of a hydrophobic/hydrophilic partition coefficient), together with the kinetic evidence of tight binding by inhibitors (weak binding by compatible solutes), suggest that the greater the affinity for a hydrophobic region on a protein the more effective the inhibition. Conversely, compatible solutes apparently have a lower affinity for the hydrophobic regions and a correspondingly greater affinity for water (a low free energy of hydration). Although it is phrased somewhat differently, this interpretation is in general accord with the assumptions made by Contaxis and Reithel. It is also consistent with the interpretation of Nozaki and Tanford (98) of thermodynamic aspects of protein denaturation in which ethylene glycol was considered to be much less effective than urea in reducing the free energy of hydration of nonpolar groups on the protein. Moreover, their interpretation agrees with the action KCl proposed for as a compatible solute for halophil enzymes (see above).

This reasoning cannot be easily extended to the sugars, however, whose stereochemistry appears to dominate their inhibitory efficiency and for which there is no correlation with a hydrophobic/hydrophilic distribution coefficient. Generalizations about the significance of sugar configuration or conformation are difficult, partly because of inadequate understand-

ing of effects of concentration on the equilibrium conformer composition of sugars in aqueous solutions.

As already stated, the severe inhibition caused by glyceraldehyde and erythrose is partly irreversible, from which a direct, possibly covalent reaction between the carbonyl group and a reactive group on the protein might be assumed. In dilute aqueous solution, glyceraldehyde equilibrates to a mixture that contains about 20% free aldehyde. Glyceraldehyde is substantially more inhibitory than erythrose which, at equilibrium, contains about 1% free aldehyde. Ribose contains about 0.01% free aldehyde, whereas the other sugars used have negligible proportions (S. J. Angyal, personal communication).

Sucrose was the most inhibitory of the sugars giving strictly reversible inhibition. The severity of its inhibition cannot be explained simply on the basis of its molecular weight since another disaccharide, maltose, was much less inhibitory and, in fact, was less inhibitory than the pentose, arabinose. The next most inhibitory sugar was ribose, which shared with sucrose the property of causing "nonlinear" inhibition, the plot of 1/v versus inhibitor concentration being concave up in both cases. The nonlinearity of ribose inhibition might be attributed to variations in anomeric and isomeric composition, which is known to change with concentration, but the same explanation cannot be applied to sucrose. This sugar is apparently conformationally stable over a wide range of concentration in aqueous solution (S. Angyal, personal communication).

The fructose moiety of sucrose is in the furanose form; ribose, in dilute aqueous solutions, contains about 24% furanose ($\alpha + \beta$) (6). On the other hand, free fructose contains about 31% β-furanose (50). Fructose was the least inhibitory sugar being roughly equivalent to glycerol. There is no less difficulty in attempting to correlate inhibitory properties with the proportions of the 1C and C1 forms of the sugars. Other aspects of sugar conformation in relation to inhibition are discussed by Simpson (Ph.D. thesis, University of New South Wales, 1976).

Finally, it cannot be assumed that all enzymes will respond quantitatively to nonelectrolytes exactly as described for the yeast isocitrate dehydrogenase. For example, Heimer (55) reported substantial differences in glycerol inhibition of a nitrate reductase from *D. parva*, *Chlorella pyrenoidosa*, and XD cells of tobacco. In every case, however, glycerol was far less inhibitory than sodium chloride, the only solute with which it was systematically compared. On the other hand, generalizations about nonelectrolyte action will probably be broadly applicable to virtually any enzyme that does not have a specific interaction with any of the solutes.

Regulation of Compatible Solute Accumulation

The direct proportionality between potassium concentration in halophilic bacteria and sodium chloride concentration in their growth medium can probably be explained physicochemically on the basis of maintaining approximate parity between internal and external water potential, coupled with the relatively impermeant nature of Na^+. A detailed discussion of this mechanism is beyond the scope of this review.

The eukaryotic polyol content also varies directly with external solute concentration (inversely with water activity). In this case, although water movement must contribute to the final internal polyol concentration, the major variable is the actual proportion of polyol of the dry cell mass. This is shown clearly in the response of the glycerol content of *Dunaliella* to external salt concentration (15). (The polyol status of the eukaryotic organelles is not known. Like bacteria in a concentrated medium, the nucleus, mitochondria, and chloroplasts have no mechanism for maintaining a dilute interior against a concentrated cytoplasm. We must assume that they contain a compatible solute that might or might not be the same as in the cytoplasm.)

It is thus evident that a control mechanism of some kind operates across the plasma membrane: the concentration of an intracellular solute regulates the biosynthesis of an intracellular solute. Such a regulatory process has extensive implications for cell physiology generally but, at present, little if anything is known of its mechanism.

The following observations are relevant to a study and ultimate explanation of the control of compatible solute production.

(i) A similar response is shown by glycerol in algae and by polyols in xerotolerant yeasts.

(ii) Internal polyol content varies broadly in response to a_w with little effect of the chemical nature of the external solute on the total polyol accumulation. Thus, in *Dunaliella*, glycerol responds to sucrose or sodium chloride (Kessly, personal communication); in *S. rouxii*, polyol content responds to glucose, polyethylene glycol or salt (22; M. Edgley, personal communication).

There is, however, a specific aspect of the effect of salt on polyol accumulation by *S. rouxii*. It has long been recognized that *S.*

rouxii gives increased yields of (extracellular) glycerol in response to increased salt concentration of the growth medium (106). The earlier work did not distinguish clearly between the types of polyols produced; polyol yields were commonly expressed as "glycerol." Nevertheless, the basic observation that more glycerol is formed in response to increased salinity is true and conspicuously includes intracellular polyol. When grown in basal medium, *S. rouxii* accumulates arabitol plus traces of glycerol (22, 29). Increasing the salt concentration of the growth medium causes an increase of total intracellular polyol, but the increase is entirely attributable to glycerol and is, moreover, accompanied by a slight diminution in arabitol content (M. Edgley, personal communication).

(iii) *Dunaliella* contains an NADP-linked glycerol dehydrogenase (13, 15). *S. rouxii* has an NADP-dependent ability to dehydrogenate D-arabitol and xylitol as well as an NAD-dependent ability to dehydrogenate xylitol, ribitol, sorbitol, and mannitol. It is apparently unable to dehydrogenate glycerol, at least via a pyridine nucleotide dehydrogenase, but it can dehydrogenate butane-1,2,4-triol and butane-2,3-diol, both with NAD^+ (J. R. Simpson, Ph.D. thesis, University of New South Wales, 1976).

(iv) *Dunaliella* responds to increased salt concentration in the dark, producing more glycerol from accumulated starch (Kessly, personal communication).

(v) *Dunaliella* produces extra glycerol in response to increased salt concentration under conditions of nitrogen starvation, suggesting that the regulatory mechanism might not require the induction or repression of an enzyme (Kessly, personal communication).

S. rouxii requires aerobic conditions for arabitol production and reverts to an ethanolic fermentation under anaerobic conditions (106). Unlike *S. cerevisiae*, *S. rouxii* is not subject to catabolite repression of its respiratory capability by high concentrations of glucose in the growth medium (23). It is not known whether this reflects a fundamental difference in the control of gene expression in the two species or whether it represents another manifestation of protection by the cell's compatible solute. It has been shown, however, that glycerol, at concentrations up to about 2 M, will substitute for cyclic adenosine 5'-monophosphate and cyclic adenosine 5'-monophosphate receptor by stimulating transcription of the *gal* operon in *E. coli* (96).

LOOSE ENDS

There are many microorganisms with a solute tolerance substantially less than those of the extreme types already discussed. Marine microorganisms tolerate about 3.5% (0.6 M) sodium chloride, plus smaller quantities of other salts, and there are some bacteria, commonly described as moderately halophilic, which require or tolerate salt at a concentration of 1 to 1.5 M or sometimes higher. Furthermore, the genus *Staphylococcus* is well known for strains with a wide range of salt tolerance, although in no sense are these bacteria halophilic.

The physiological basis of intermediate solute (or a_w) tolerance is not understood, although there are some pointers. Moderately tolerant cells, like their extremely tolerant counterparts, must adjust thermodynamically to reduced a_w by sustaining a reduced internal a_w. This means that those solutes which are retained within the cell will increase in concentration and, by definition, be osmotically active. Furthermore, since enzymes continued to function under these conditions, it also follows that the solutes which are present at such increased concentrations are not excessively inhibitory. If the *content* (per dry mass of cell, as distinct from the *concentration*) of any solute increases under these conditions it should be suspected as a possible intermediate type of compatible solute.

In fact, there are solutes that accumulate in various organisms under these conditions. These substances lie within a limited range of chemical types but occur within quite a wide range of organisms. They accumulate in direct response to a lowering of external a_w and, although they have not yet been studied generally as enzyme inhibitors, their occurrence under these conditions suggests that they do, in fact, act as compatible solutes at moderate levels of water stress.

Polyols or their derivatives accumulate in eukaryotic protista. Thus, as already described, the marine alga *D. tertiolecta* accumulates glycerol, but to a lower concentration than its halophilic relative, *D. viridis*. The freshwater alga, *Ochromonas malhamensis*, produces α-galactosyl glycerol in response to the relatively small changes in solute concentration which it can tolerate (67, 68).

Bacteria, however, do not apparently accumulate polyols or carbohydrate derivatives in this manner. A positive correlation between K^+ accumulation from a standard low-salt growth medium and salt tolerance of some 32 bacterial strains was demonstrated by Christian and Waltho (36). Their experimental conditions included values for a_w down to 0.88 (plus one salt-tolerant coccus that grew at a_w 0.84); potassium contents ranged between about 200 and 1,050 μmol/g (dry mass). The water content of bacte-

ria growing at 0.90 a_w is about 0.83 g/g (dry mass) (39). Assuming no additional uptake, relative to cell mass, at the low levels of a_w, dehydration would result in a K^+ concentration within the tolerant species of about 1.3 molal which, for KCl, is roughly equivalent to 0.95 to 0.96 a_w (see reference 115). It is not known whether any supplementary active uptake of K^+ occurs in nonhalophils in response to the lowering of external a_w, but Christian and Waltho (39) have reported that the increased K^+ concentration in *Staphylococcus aureus* is largely the result of dehydration. If that is so, the value of 1.3 molal should be reasonably accurate for bacteria grown at the low levels of a_w, and a substantial concentration of additional solute(s) would be needed to meet the thermodynamic requirements of the situation. Furthermore, although the accumulation of K^+ is reminiscent of the situation in halophilic bacteria, we do not know whether K^+ acts to any appreciable extent as a compatible solute for enzymes of salt-tolerant but nonhalophilic bacteria.

Some bacteria accumulate specific metabolites in response to water stress and it is likely that at least one such compound, proline, might function as a compatible solute. In fact, three amino acids correlate, like K^+, in two ways with a tolerance of reduced a_w. The first correlation is between "intrinsic" amino acid content and inherent or potential tolerance of water stress. The second correlation is the response of intracellular amino acid content to changes in a_w. There are actually few reports of intrinsically high levels of these amino acids in tolerant bacteria. I have, however, observed a much higher concentration of "pool" aspartate, glutamate, and proline in salt-tolerant strains of *Staphylococcus* than in nontolerant strains (unpublished data). On the other hand, there is ample documentation of responses of these amino acids to changes in a_w. Tempest et al. (135) report that, in continuous culture, sudden increases in the salinity of the growth medium cause extensive and rapid increases in the "pool" free glutamate concentration in gram-negative bacteria and similar, but slower, responses in gram-positive bacteria. Whether or not glutamate functions as a compatible solute is uncertain. Tempest et al. (135) reported a glutamate concentration of about 0.1 M in *Aerobacter aerogenes* growing at a dilution rate of 0.3 h^{-1} in 4% sodium chloride. This was the highest concentration obtained for any amino acid in *A. aerogenes*, although slightly higher concentrations were achieved in some other bacteria. In fact, their reported concentrations are almost certainly low by a factor of about 3. A water content of 4 g/g (dry mass) had been assumed, whereas under their growth conditions a value of about 1.5 g/g is to be expected (see, for example, reference 39; my experience agrees with Christian and Waltho in this respect). Thus, a glutamate concentration of about 0.3 M was apparently achieved in bacteria growing in 4% (about 0.6 M) sodium chloride. Glutamate could also serve as a counterion for K^+ and would presumably promote the accumulation of that ion. Glutamate thus made a significant contribution to the overall osmotic balance of *A. aerogenes* and, at a concentration of 0.3 M, it is unlikely to be a very effective general enzyme inhibitor.

There are also reports of enhanced proline accumulation by bacteria in response to lowered a_w (16, 35). Christian and Hall (35) showed that proline increased linearly in *Salmonella oranienburg* with decreasing a_w to reach a value of 1.5 mmol/g (dry mass) at about 0.95 a_w. This is approximately equivalent to a concentration of 1.36 molal.

Measures (92) has also demonstrated an accumulation of glutamate, γ-aminobutyrate, or proline in bacteria in response to increased salt concentration. There was a qualitative trend in the type of amino acid accumulation; glutamate predominated in the least salt-tolerant, whereas proline predominated in the most tolerant bacteria of the group studied.

Prima facie evidence that proline might function as a compatible solute was provided in 1955 by Christian (33, 34) who showed that, in a defined medium, this amino acid was essential for the growth of *S. oranienburg* at values of a_w less than 0.97. Cristian and Waltho (40) also showed that proline stimulated the respiration of this and other bacteria at reduced a_w. It is relevant to this interpretation that the use of glycerol to adjust the a_w of the suspending solution did not appreciably inhibit respiration down to about 0.96 a_w (38).

Proline also accumulates in plants as a part of their response to water stress. Contents of up to 3.9 mg of proline/g of dry tissue have been reported for barley plants subjected to −10 to −20 bars of osmotic potential, compared with about 0.25 mg/g in watered plants (125). Up to 5 mg/g was detected in excised leaf laminae exposed to polyethylene glycol (molecular weight, 4,000), producing an "osmotic potential of −20 bars" (126). This is equivalent to a proline concentration of about 10 mmolal (assuming 4 g of water/g of dry tissue), which is negligible by microbial standards.

Proline concentration in halophytes can be a little higher. Stewart and Lee (132) have reported that proline in the halophyte *Triglochin*

maritima is equivalent to 113 μmol/g of wet tissue or about 10% of the dry mass of shoot tissues of plants collected in the field. Together, these figures imply a water content of about 6.7 g/g of dry tissue which, if true, suggests a substantial contribution by vascular tissue, with the implication that the proline was unlikely to have been distributed uniformly in the material that was analyzed. Taken at their face value, however, the figures imply a mean proline concentration of about 17 mmolal, again negligible by microbial standards. The general trend of these results is entirely consistent with the high a_w at which plants, in general, wilt (Table 1).

Nevertheless, Stewart and Lee have provided some enzymological evidence of a possible function of proline as a compatible solute. Up to a concentration of 0.7 M it did not inhibit glutamate dehydrogenase, acetolactate synthase, nitrate reductase, or glutamine synthetase from *T. maritima*. Sodium chloride (0.6 M), on the other hand, caused more than 50% inhibition of the enzymes.

There are many reports of osmoregulation by metabolites, frequently amino acids, in a wide range of plants, animals, and microorganisms (e.g., 8, 65, 120, 134, 141, 148). Most, if not all, of the multicellular organisms so considered have a very limited range of *cellular* tolerance of a_w. Even under the relatively mild conditions that prevail for the cells of multicellular organisms, however, thermodynamic adjustment ("osmoregulation") must occur and solute(s) must concentrate to achieve that adjustment to reduced a_w. If salts are inhibitory, as they frequently are even at relatively low concentrations, then the continued activity of the organism will demand that osmotic balance be achieved by a noninhibitory solute. At concentrations up to about 0.5 M, few common metabolites are likely to be *general* enzyme inhibitors, although some would obviously inhibit specific enzymes very effectively.

Thus, amino acids might well act as osmoregulators under mild conditions and function as low-grade compatible solutes. It is not surprising that aspartate and glutamate accumulate in some organisms, since these amino acids are at the beginning of several biosynthetic pathways and are thus unlikely to have any widespread function as feedback inhibitors. Proline seems to be in a somewhat different class, however, and warrants a reasonably detailed enzymological study.

Finally, it is to be expected that there will be occasional circumstances in which a specific substance at low concentration can relieve some of the major effects of water stress in a way reminiscent of the modification by a substrate of effects of high salt concentrations on an enzyme (see section, Halophilic Bacteria). A substance acting in this way should not be regarded as a compatible solute. A possible example involving whole bacteria lies in some interesting work of Avi-Dor and associates. In a recent report (124), it was shown that betaine (0.5 mM) relieved the salt inhibition of growth and respiration of a halotolerant bacterium. Betaine was slowly accumulated (to an intracellular concentration of about 800 mM when the bacteria were suspended in 2.0 M sodium chloride), but only extracellular betaine was effective in relieving salt inhibition. Apparently, the action of betaine is on the cell membrane.

Survival

Although this review is concerned with microbial activity, not survival, some brief comments on the latter situation might not be out of order. It is a long- and well-established practice to assist the preservation of freeze-dried microorganisms by the inclusion of a nonelectrolyte such as glycerol or a sugar. This practice apparently reduces mortality during dehydration, storage, and rehydration. It is virtually certain that, in this kind of circumstance, nonelectrolytes function by directly substituting as a "solvating" molecule for the water that is removed on dehydration. Hydration of the polar sites on organic molecules commonly involves hydrogen bonds; removal of the solvent can, and frequently does, lead to the substitution of site-solvent H bonds by site-site H bonds, which can be intra- or intermolecular. This is exactly what happens in the manufacture of a sheet of paper and, presumably, is the significance of the use of glucose for obtaining the three-dimensional 0.07-nm structure of the purple membrane of *H. halobium* in the anhydrous environment of an electron microscope (57).

The formation of intra- or intermolecular H bonds under conditions of excessive dehydration, however, causes irreversible changes in some proteins which lead to enzyme inactivation. The addition of hydroxylated compounds provides an alternative source of H bonds that can prevent the formation of the intersite bonds and thereby prevent the protein inactivation which accompanies them.

Thus, although there are some superficial similarities between the function of glycerol, etc., in this context and the role we have proposed for compatible solutes, there is an important basic difference in the mechanism of action in the two situations. As discussed, our evi-

dence suggests that in aqueous solutions compatible solutes have an unusually low affinity for enzyme proteins, or at least for those regions of the enzyme molecules where binding would be inhibitory. The affinity of compatible solutes is for solvent water.

SUMMARY

Microbial water stress has been discussed primarily in relation to three distinctive groups of microorganisms, namely, the extremely halophilic bacteria, the halophilic algae, and the xerotolerant ("osmophilic") yeasts. Brief reference was also made to other xerotolerant fungi.

The halophilic bacteria are distinguished by an absolute, specific requirement for high concentrations of sodium chloride. The halophilic algae (notably *Dunaliella*), notwithstanding a certain minimal salt requirement, have a physiology that is generally much more characteristic of a tolerance than of an absolute requirement for salt. Moreover, the halophilic algae can be trained to grow outside their normal salt tolerance ranges and they can accept replacement of a substantial part of the salt requirement by a nonelectrolyte. In both respects, the halophilic algae differ fundamentally from the halophilic bacteria. In the second respect, they are reminiscent of the xerotolerant yeasts. The xerotolerant yeasts are generally distinguished by a remarkable tolerance of low water activity (a_w), although the tolerance range is dependent on the solute used to adjust a_w. Occasionally, a requirement for a reduced water activity is encountered among the yeasts; such a requirement is usually temperature dependent.

The salt requirements of the extremely halophilic bacteria are determined by a need for salt partly to maintain membrane integrity and the function of those enzyme systems associated with the cell membrane and with ribosomes. Their overall salt relations are also affected by the ability of some cytoplasmic enzymes to *tolerate* salt at intracellular concentrations. These enzymes have an optimum in the region of 0.5 to 1 M salt. Their function is possible because the bacteria accumulate potassium chloride and effectively exclude sodium chloride. In marked contrast to sodium chloride, potassium chloride is a poor enzyme inhibitor at high concentrations. Because of this, it protects the relevant enzymes against the inhibition that would occur at the same water activity in its absence (that is, if a_w were determined by sodium chloride or by common intermediary metabolites). Salt inhibition is discussed in some detail in relation to an isocitrate dehydrogenase.

Halophilic algae and xerotolerant yeasts do not apparently produce enzymes with a distinctive tolerance of low a_w (or increased solute concentration). The water relations of these organisms are largely determined by the accumulation of polyhydric alcohols to a concentration commensurate with extracellular a_w. As potassium chloride does in halophilic bacteria, the polyols function, not only as osmoregulators, but also as protectors of enzyme activity. Substances that function in this way have been called compatible solutes.

The enzymological and physiological significance of compatible solutes was discussed. It is evident that enzyme function responds, not to changes in a_w per se, but to the nature and concentration of the solute used to adjust a_w. Nevertheless, a_w remains a valuable parameter to describe microbial water relations in a complex medium.

The role of "intermediate" or "low-grade" compatible solutes in cells with intermediate levels of xerotolerance was considered briefly. Perhaps the most notable solute of this type is proline, which accumulates in response to water stress in some higher plants as well as in some bacteria.

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ADDENDUM

P. S. Low and G. N. Somero (Proc. Natl. Acad. Sci. U.S.A. 72:3305–3309, 1975) have extended their evidence and argue that salts affect the velocities of enzyme-catalyzed reactions by changing the activation volume of an enzyme-substrate complex (See section, Compatible Solutes and reference 84). They demonstrated a linear relation between activation volume and reaction rate, but pointed out that the relation is not causal. Their interpretation supplements the comments made in Compatible Solutes on the role of water. It is important to note, however, that the effects reported by Low and Somero are confined to salt concentrations less than about 300 mM.

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