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A basis of the specific sodium requirement for morphological integrity of *Halobacterium halobium*

Halophilic bacteria require high concentrations of NaCl for growth and for their morphological integrity[1,2]. Extensive substitution of sodium by other monovalent cations will not allow growth and, in certain cases, will not preserve the morphology of the organisms. Explanations of this specific requirement have been proposed both in terms of osmotic pressure[3] and electrostatic screening[4,5]. More recently, KUSHNER[6], ONISHI AND KUSHNER[7] and KUSHNER AND ONISHI[8] made a new suggestion which was that halophils are stabilized better by Na^+ than K^+ or NH_4^+ because there are specific binding sites for Na^+ on the (morphological) outside surface of the membrane and specific sites for K^+ and NH_4^+ on the (morphological) inside surface. The reasons for this proposal are equivocal but it was justified as being attractive "because it is practically unsusceptible to experimental proof"[7]. ONISHI AND KUSHNER[7] modified the suggestion and proposed that the specific contribution of Na^+ is to "act on certain sites on the membrane to prevent leakage whereas other monovalent cations are about as effective as Na^+ in preventing gross disintegration of the membrane". The present work describes effects of various salts on the morphological integrity of *Halobacterium halobium* and explains the results in terms of simple physicochemical processes.

Halobacterium halobium was grown with aeration for 5 days at 37°. The growth medium contained Oxoid peptone (1%, w/v) in the salts solution described by SEGHAL AND GIBBONS[9]. For experiments involving suspensions of whole organisms in various salt solutions the organisms were washed twice in cold 5 M NaCl and resuspended at as high a concentration as practicable in 5 M NaCl. Membranes were isolated as described previously[10] except that in the present work cold 5 M NaCl was used throughout as a suspending solution. When sufficiently purified, the membranes were suspended at as high a concentration as practicable in 5 M NaCl. Turbidity of suspensions of organisms and of membranes was measured at 700 mμ in 1-cm cuvettes. Measurements were made on suspensions incubated for 20 min in 0.025 M Tris buffer (pH 7.0) at 35°. Suspensions were prepared by diluting the concentrated suspension (in 5 M NaCl) 25–50-fold with the appropriate solution. The resulting minimal NaCl concentration (0.1–0.2 M) was ignored in the quantitative expression of the results.

Fig. 1 shows turbidities of suspensions of *H. halobium* in solutions of various chlorides. $MgCl_2$ (0.02 M) was added to all solutions of the monovalent cations. Qualitatively similar results (with some variation in the relative positions of the K^+ and NH_4^+ curves) were obtained without added $MgCl_2$; such results are similar to those already reported for *H. cutirubrum*[5]. The relative levels of effectiveness of K^+ and NH_4^+ were affected by temperature, K^+ being more effective relative to NH_4^+ at 20° than at 40°. When suspended in NaCl and LiCl the organisms were normal rods at 4 M, distorted at 3 M, spherical at 2 M and completely lysed at lower concentrations of salt. In KCl the same sequence of morphological changes was sometimes encountered but it occurred at higher concentrations of the salt. In most experiments the organisms lysed in 4 M NH_4Cl and in all experiments they lysed at lower concentrations of this salt. The organisms retained their normal rod-shape in concentrations of $CaCl_2$ and $MgCl_2$ above 0.5 M.

Fig. 2 shows the stability of isolated membranes in chlorides of the four mono-

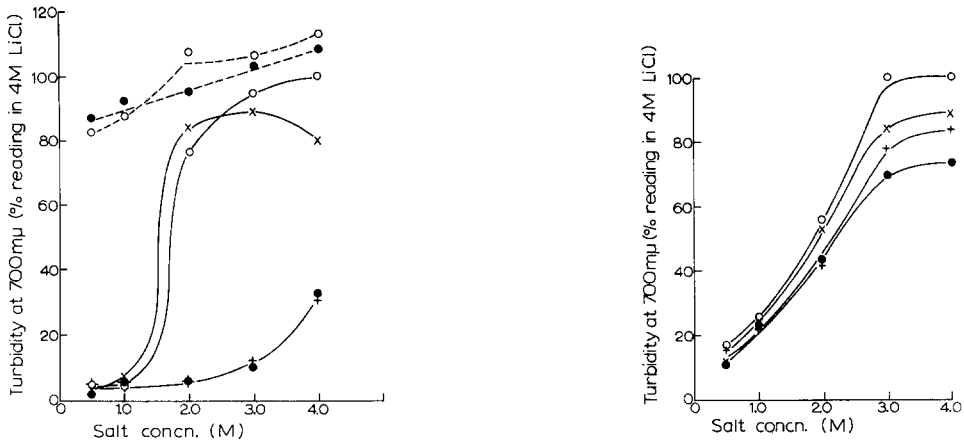

Fig. 1. Turbidities of suspensions of whole cells of *H. halobium* in five concentrations of various salts, buffered as described in the text. Continuous lines, monovalent cations; broken lines, bivalent cations. ○, LiCl and $MgCl_2$; ●, KCl and $CaCl_2$; ×, NaCl; +, NH_4Cl. $MgCl_2$ was added to the solutions of the four monovalent chlorides to give a final concentration of 0.02 M. NH_4Cl was adjusted to pH 7.0 with NH_4OH before use.

Fig. 2. Turbidities of suspensions of membranes of *H. halobium* in five concentrations of various salts. ○—○, LiCl; ×—×, NaCl; ●—●, KCl; +—+, NH_4Cl. NH_4Cl was adjusted to pH 7.0 with NH_4OH before use.

valent cations. At 4 M concentration there were slight differences between the salts, differences which were in a similar direction to those encountered with whole organisms. The differences were smaller than obtained with whole organisms, however, and were negligible at lower salt concentrations.

There are two factors which are necessarily involved in the effect of salts on the morphological stability of halophilic bacteria. The first is prevention by cations of electrostatic disaggregation of the membrane and the second is osmotic pressure. Although the magnitude of the cellular osmotic pressure is unknown when the organisms are at equilibrium in any salt solution, a transient increase in pressure must occur when, as in experiments of this kind, the suspending solution is diluted[2] or when the suspending solute is changed to one which penetrates more readily. The present simple experiments enable the two effects of osmosis and electrostatic disaggregation to be separated. Thus Fig. 1 shows different abilities of several salts to stabilize the whole organisms but Fig. 2 shows the four monovalent cations to be about equally effective in preventing electrostatic disaggregation of the membrane. This suggests that the differences between cations observed with whole organisms are osmotic, an interpretation which is confirmed virtually unequivocally when whole organisms are suspended in various salts *plus* 0.02 M $MgCl_2$. At this concentration Mg^{2+} completely prevents the electrostatic disaggregation but offers negligible osmotic protection; use has been made of this fact to isolate the membrane of *H. halobium*[11]. Thus the differences between the monovalent cations depicted in Fig. 1 can be explained entirely by differences in degree of penetration with correspondingly different levels of osmotic protection. The four monovalent cations protected the organisms in the order, $Li^+ > Na^+ > K^+ \geqslant NH_4^+$ (with occasional reversal of K^+ and NH_4^+) which is the same order as their hydrated volumes, the reverse order of the ionic radii[12] and, it must be assumed, the reverse order of the

amount of penetration by the ions. In addition, different ammonium salts varied in their ability to protect the whole organisms; the determining factor in this case was presumably the penetration by the anion. For example, in the presence of Mg^{2+}, 4 M $(NH_4)_2SO_4$ was up to 6 times more effective than NH_4NO_3 at stabilizing whole organisms. Various other ammonium salts, including NH_4Cl, gave intermediate levels of protection.

Therefore, in the absence of Mg^{2+}, the effect of salt concentration on stability of the organisms is the sum of the electrostatic and osmotic effects. The differences between monovalent cations at any one concentration are largely osmotic with, perhaps, a minor supplementary effect caused by slight differences in the electrostatic screening efficiencies of the four monovalent cations at high concentrations. There is no need to explain any of these phenomena by "specific sites" on the membrane and there is no evidence for such sites. Indeed, since the measurements were made at equilibrium, the contribution of the cell membrane to the final result is doubtful (the significance of this statement will be elaborated elsewhere). The selection of ions and the reversal of the relative amounts of penetration by K^+ and NH_4^+ with a change in temperature are compatible with a mechanism based on intracellular charge distribution such as proposed by LING[13].

The results given by Ca^{2+} and Mg^{2+} require a slightly different interpretation. The ability of bivalent cations to prevent disaggregation, presumably by bridging neighbouring negative charges, is so great that the additional bonds which are formed in this way at salt concentrations above 0.5 M are evidently strong enough to protect the organism during the transient increase in osmotic pressure. It is also possible that bivalent cations cause some disorganisation of the cell and thereby increase permeability to water so that the duration of the increase in osmotic pressure is shortened.

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