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Mechanism of Dissolution of Envelopes of the Extreme Halophile *Halobacterium cutirubrum*[1]

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ABSTRACT

ONISHI, H. (National Research Council, Ottawa, Ontario, Canada), AND D. J. KUSHNER. Mechanism of dissolution of envelopes of the extreme halophile *Halobacterium cutirubrum*. J. Bacteriol. **91**:646–652. 1966.—Envelopes of *Halobacterium cutirubrum* dissolved rapidly in media of low ionic strength. Heating partially inhibited breakdown, probably because of nonspecific protein coagulation rather than inactivation of a lytic enzyme(s). Dissolution of envelopes in water did not involve splitting of peptide bonds or protein-lipid bonds, or any extensive breakdown of carbohydrate polymers. Dissolution was increased by alcohols and urea, even at high salt concentrations, but was not affected by metabolic inhibitors. Thus, no evidence was found for a dilution-activated lytic enzyme that contributes to envelope breakdown. Cells of *H. cutirubrum* were stable in 2 M NaCl, but lysis occurred in 2 M KCl or NH_4Cl. This lysis did not involve an extensive breakdown of the envelope. No evidence for different sites of Na^+, K^+, and NH_4^+ action was obtained from the pattern of release of envelope constituents in different concentrations of these salts. Ultracentrifugation studies showed that adding salts to envelopes that had been dissolved in water led to a nonspecific reaggregation of envelope material. No difference was seen between the effects of KCl and NaCl, except at 3 to 4 M concentrations where KCl caused more aggregation. The preferential effect of Na^+ on intact cells is probably due to its ability specifically to prevent leakage rather than to an overall effect on envelope integrity.

Envelopes of the extremely halophilic bacterium *Halobacterium cutirubrum* are stable in salt solutions of the same high ionic strength as the medium in which this organism grows. When placed in water the envelopes break up very rapidly, most of their protein, lipid, and ultraviolet-absorbing substances being found in particles which are not visible under the phase-contrast microscope, but which can be sedimented by centrifugation for 2 hr at 105,000 × *g* (11, 12). Dissolution of these envelopes and those of *H. halobium* does not involve extensive proteolytic breakdown; with the latter organism, part of the dissolution was found to be unaffected by heating (3).

It has been suggested that the breakdown of envelopes of the extreme halophiles in water involves a nonenzymatic splitting into particulate subunits (3). However, the evidence that dissolution is nonenzymatic did not seem conclusive, and, in addition, we believed that the breakdown of the components other than protein should be studied. Envelopes of *H. cutirubrum* and other extreme halophiles also contain large amounts of lipids and substantial amounts of carbohydrates, including hexosamines, but not muramic acid, as well as smaller amounts of nucleic acids and other phosphorus-containing substances (5, 12). The fate of some of these envelope components during dissolution remained to be investigated.

It was observed earlier that Na^+ preserves the integrity of intact cells of *H. cutirubrum* much more effectively than does K^+ or NH_4^+, but it has no greater effect than these ions on envelopes or on cells made permeable by treatment with

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acid (1, 11). In an attempt to explain this finding, we have now studied the ability of these cations to prevent loss of different envelope components and to reaggregate the particles from dissolved envelopes. We have also compared the lysis of whole cells that takes place in moderate KCl and NH_4Cl concentrations with the lysis that takes place in water.

MATERIALS AND METHODS

The bacteria used, the preparation of envelopes, and many of the analytical methods employed have been described (11, 12). Turbidity was read after 30 min at room temperature in a Hilger Spekker Absorptiometer with a no. 8 (675 mμ) filter. Protein release from envelopes was measured, usually after 30 min at room temperature, by centrifuging for 30 min at 15,000 × *g* and determining the protein content of the supernatant fluid. A 100% turbidity was taken as that found in 4 M NaCl. The per cent protein released was calculated from the total amount present.

Water lysates were prepared by centrifuging envelopes and resuspending in water to a final concentration of 0.5 mg of protein per ml. In the experiments shown in Fig. 1, 2, and 4, the material tested was diluted 25-fold in the appropriate salt solution; in all other experiments, material was diluted 10-fold. All salt solutions tested also contained 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.0).

For measuring cell breakdown in the experiments shown in Table 3, 0.5 ml of a cell suspension in 4 M NaCl was added to 20 ml of each solution tested or to 100 ml of 4 M NaCl; then 80 ml of 5 M NaCl was added to all solutions but the last. After centrifugation, the supernant fluids were poured off, the precipitates were suspended in water, and the lipid phosphorus was measured.

Analytical methods. Lipids were extracted by the method of Bligh and Dyer (2). Carbohydrates were determined by the anthrone reagent (16).

For ultracentrifugation studies, concentrated envelope suspensions in 4.5 M NaCl were first dissolved by dialysis against a large volume of distilled water; this was followed by dialysis against the salt solution to be tested. Each dialysis was carried out for 10 to 16 hr at 25 C. Sedimentation analyses were carried out in a Spinco model E ultracentrifuge at 20 C, with schlieren optics. Sedimentation constants were calculated without correcting for medium viscosity or density.

Dinitrophenylation of intact and dissolved cell envelopes was performed essentially as described by Ingram and Salton (8). For intact envelopes, 10 ml of a suspension (containing about 50 mg of protein) in 4 M NaCl plus 10 mg of $NaHCO_3$ per ml were shaken with 20 ml of a 5% (v/v) ethyl alcoholic solution of fluorodinitrobenzene (saturated with NaCl and containing 0.2 M $MgCl_2$) for 5 hr at room temperature in the dark. For dissolved envelopes, the same weight of envelopes was centrifuged and dissolved in 10 ml of water containing 10 mg of $NaHCO_3$ per ml, and then shaken with 20 ml of a 5% (v/v) ethyl alcoholic solution of fluorodinitrobenzene for 5 hr at room temperature in the dark. Both kinds of envelopes were then dialyzed against 4 M NaCl for 3 days, and equal volumes of 10% trichloroacetic acid were added to the dialyzed solutions. 2,4-Dinitrophenol (DNP)-protein was precipitated by centrifuging at 15,000 × *g* for 10 min, washed four times each with ethyl alcohol and ether, and dried in vacuo. The methods of hydrolysis, extraction, and paper chromatography were the same as used by Ingram and Salton (8).

RESULTS

Search for a lytic enzyme activated by dilution. If envelopes contain a lytic-enzyme system activated by lowering the salt concentration, it might be possible to demonstrate this by adding lysed envelopes to unlysed envelopes at different salt concentrations. Though the action of such an enzyme system might be inhibited at the highest salt concentration, it seemed likely to us that an intermediate concentration could be found at which the addition of a lysate would increase envelope dissolution. However, in NaCl concentrations ranging from 0 to 4 M, addition of dilution-lysed envelopes caused no enhancement of lysis, measured either as turbidity reduction or as protein release (Fig. 1 and 2). A similar result was obtained with envelopes suspended in

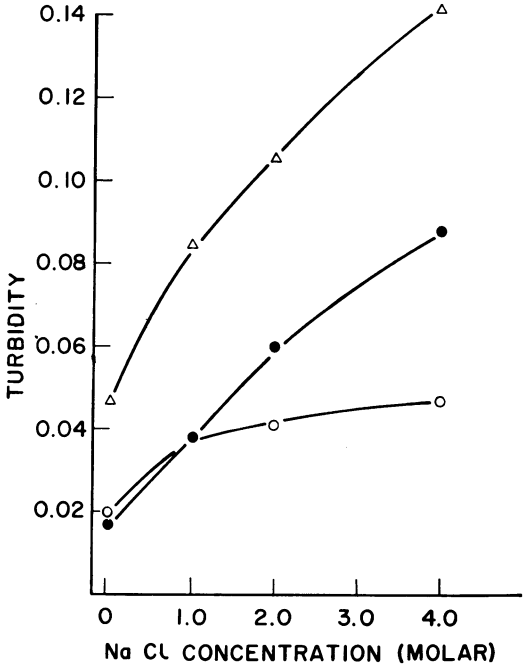

FIG. 1. *Effect of water-lysed envelopes on turbidity of envelopes in different NaCl concentrations. Symbols:* ●, *envelopes;* ○, *lysate;* △, *envelopes plus lysate.*

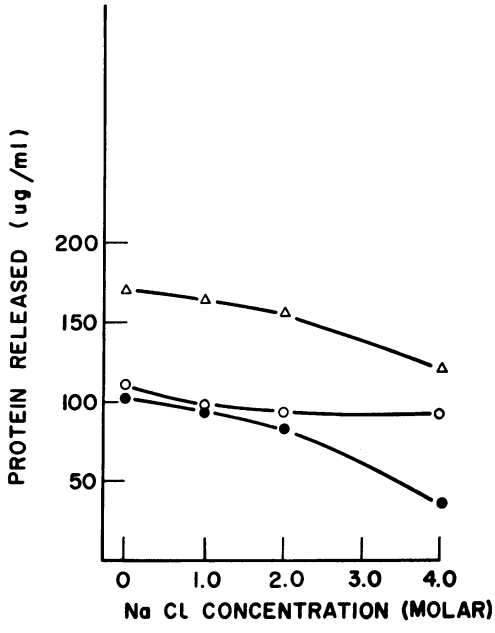

FIG. 2. *Effect of water-lysed envelopes on protein released from envelopes in different salt concentrations. Symbols: ●, envelopes; ○, lysate; △, envelopes plus lysate.*

0 to 0.1 M $MgCl_2$. Thus, these experiments provide no evidence that a dilution-activated lytic-enzyme system exists in envelopes.

Effect of heat on lysis. Abram and Gibbons (1) observed that cells of *H. cutirubrum* were transformed into spheres after 40 min at 60 C, but were as readily lysed by lowering the salt concentration of the suspending medium as unheated cells. Brown (3) found that envelopes of *H. halobium* broke down on dilution in two stages, a very rapid, temperature-independent initial phase and a subsequent slower, temperature-dependent phase. Such a diphasic breakdown was also observed in *H. cutirubrum* envelopes (12). Heating envelopes of *H. halobium* at 100 C for 10 to 80 min did not affect the first stage of breakdown, though it abolished the second (3).

We examined envelopes of *H. cutirubrum* heated either at 100 C for 20 min or at 60 C for 1 hr. Under the former treatment, one-third to one-half of the total material was visibly coagulated and was removed by centrifugation for 10 min at 6,000 × *g*. The supernants fluids contained spherical particles which, in water, broke up into much smaller fragments, but which were still visible under phase contrast. The per cent fall in turbidity in water was almost as great in heated as in unheated cells. However, the per cent protein remaining after centrifugation for 30 min at 15,000 × *g* did not increase on dilution of the heated envelopes in water, in contrast to the large increase found in unheated envelopes (Table 1). The change of shape of heated envelopes may account for their being less readily sedimentable in 4 M NaCl than unheated envelopes. The fragments probably sedimented as well in water as the intact heated envelopes did in 4 M NaCl because of the difference in specific gravity of the two solutions.

TABLE 1. *Dissolution of heated and unheated envelopes in water*

Suspending solution	Per cent turbidity		Per cent protein released	
	Unheated	Heated	Unheated	Heated
NaCl, 4 M	100	100	5	20
Water	14	23	68	21

The failure of these heated envelopes to disintegrate completely could be due either to the nonspecific coagulation of protein or to the inactivation of a lytic enzyme. If the latter were responsible, we would expect that adding a lysate of unheated envelopes to particles remaining after heated envelopes were diluted might dissolve these particles. However, when this experiment was performed, no such digestion, as measured by protein solubilization, was observed after 30 min of incubation at room temperature.

Envelope suspensions heated at 60 C for 1 hr appeared unchanged to the naked eye, but phase-contrast examination revealed many small clumps of coagulated envelopes. Turbidity of these suspensions was less affected by lowering the salt concentration than was that of unheated envelopes (Fig. 3), and many of the heated envelopes remained intact in water. Adding unheated envelopes to heated ones in water did not cause any further decrease in turbidity of the latter (Fig. 4). From these experiments, we conclude that some dissolution occurs in heated envelopes, and that the observed inhibition of dissolution is due to coagulation of envelope protein rather than to the inactivation of a lytic-enzyme system.

Splitting of peptide bonds during envelope dissolution. It is well established that dissolution of envelopes of *H. cutirubrum* and *H. halobium* is not caused by extensive splitting of peptide bonds (3, 11). Brown (3) found that no new amino end groups appeared in the proteins of *H. halobium* after exposure to water, and we observed the same thing with *H. cutirubrum*. Valine, serine, glutamic and aspartic acid, and an unknown

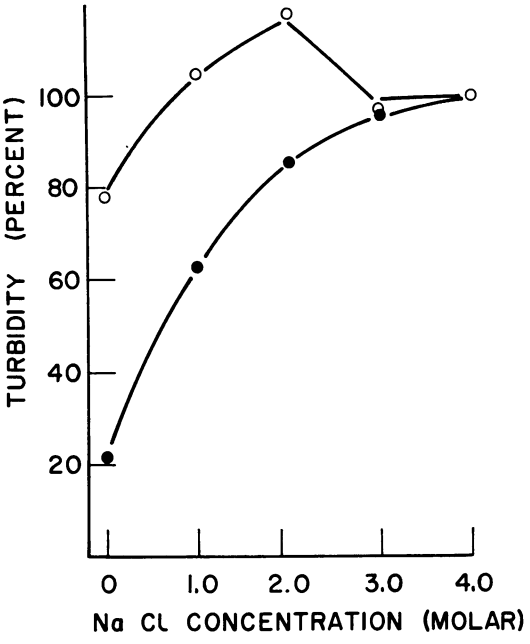

FIG. 3. *Effect of NaCl concentration on turbidity of heated and unheated envelopes. Symbols:* ●, *unheated envelopes;* ○, *heated envelopes.*

component were labeled by treatment with 2,4-dinitrofluorobenzene whether or not envelopes had been exposed to water before treatment. It therefore seems unlikely that any peptide bonds were split during dissolution of *H. cutirubrum* envelopes.

Polysaccharides. The possibility that polysaccharides in the envelopes play a structural role and that these polysaccharides are split on dilution was examined by determining the amounts of total carbohydrate and of hexosamine in lysed envelope preparations that sedimented after 2 hr at 105,000 × *g*. Most of the carbohydrate (90%) and hexosamine (87%) was sedimented, and a subsequent paper shows that no loss of carbohydrate occurred on dialysis (13). Thus, there appears to be no extensive breakdown of polysaccharide on lysis.

Protein-lipid bonds. It has already been shown that lipid as well as protein is sedimented when lysed envelopes are centrifuged at 105,000 × *g* for 2 hr, and this was taken to indicate that the lipoprotein remains intact (11). However, this is not the only possible interpretation, since we found that an aqueous suspension of isolated lipids from *H. cutirubrum* also sediments under these conditions. Protein-bound lipid is not soluble in chloroform, but free lipid is (9). Envelopes contained no free lipid, whether or not they had been exposed to low salt concentrations, and,

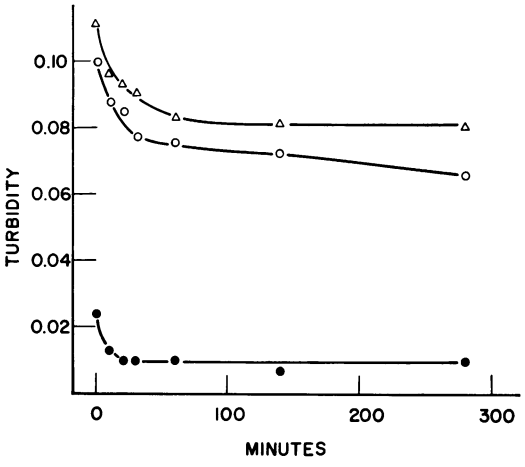

FIG. 4. *Effect of unheated envelopes on the dissolution of heated envelopes in water. Symbols:* ●, *unheated envelopes;* ○, *heated envelopes;* △, *heated plus unheated envelopes.*

again, we conclude that dilution does not break lipid-protein linkages.

Effect of metabolic inhibitors on dissolution of envelopes. The following enzyme inhibitors, all in the concentration 0.01 M, had no effect on the loss of turbidity of envelopes suspended in water: NaN_3, NaCN, and $HgCl_2$. Copper sulfate (0.01 M) increased the turbidity compared with that found in 4 M NaCl, presumably by precipitating the proteins.

Effects of alcohols and urea on envelopes. Abram and Gibbons (1) observed that intact cells of *H. cutirubrum* were lysed by high concentrations of urea, even in the presence of 4 M NaCl, and that surface-active agents also caused lysis. There was considerable dissolution of the envelopes of this organism in ethyl alcohol (10%), *n*-butanol (3%), and urea (5 M), even in the presence of 4 M NaCl (Table 2). The alcohols had no effect on envelopes heated at 100 C for 15 min. Butanol treatment did not increase the free lipid in the envelope, indicating that butanol did not act by breaking lipid-protein bonds, as has been suggested (15). It has also been suggested that butanol acts on hydrogen bonds as urea is thought to do, although urea may also act on hydrophobic bonds (7).

Differential effects of monovalent cations on cells and on envelopes. Earlier work demonstrated that cells did not lyse in 2 M NaCl, but that in 2 M KCl or NH_4Cl lysis (as measured by the fall of turbidity or the release of ultraviolet-absorbing substances) was practically complete, and so rapid (even at 3 C) that its time course could not be measured (1, 11). These experiments did not show, however, whether the loss of cytoplasmic

TABLE 2. *Effect of alcohols and urea on envelopes of Halobacterium cutirubrum**

Treatment	Per cent turbidity	Per cent release of protein
Water	8	83
Butanol, 3%	8	86
NaCl, 4 M	100	20
NaCl, 4 M, + 3% butanol	33	54
Water		83
Ethyl alcohol, 10%		83
NaCl, 4 M		17
NaCl, 4 M + 10% ethyl alcohol		44
Water	28	56
Urea, 5 M	20	72
NaCl, 4 M	100	12
NaCl, 4 M + 5 M urea	43	38

* Figures are taken from three separate experiments.

constituents was accompanied by envelope disintegration or whether lysis was the result of leakage from essentially intact envelopes. This was investigated by determining how much of the cell's lipid phosphorus remained sedimentable after treatment with 2 M KCl or NH_4Cl, with different NaCl concentrations, and with water. All of the cell's lipid phosphorus is found in the envelopes (12), and, hence, as with other bacteria (10), it gives a valid and convenient measure of the amount of envelope present. More lipid phosphorus remained sedimentable after lysis in 2 M KCl or NH_4Cl than after lysis in water (Table 3). The sedimentation after 30 min at 15,000 $\times$ *g* was less than that of mechanically isolated envelopes, which are completely sedimented under these conditions (11, 12), but was virtually complete after 1 hr at 31,000 $\times$ *g*. Hence, the cell envelopes must have broken up, if at all, into fairly large particles. Abram and Gibbons (1) reported that *H. cutirubrum* formed spheres in 2 M KCl or NH_4Cl, a change in shape we also observed. However, since these spheres were dim under phase contrast, it was not possible to determine whether the conversion was quantitative. These experiments do show that lysis in 2 M KCl and NH_4Cl does not involve as extensive envelope breakdown as does lysis in water.

Pattern of release of envelope constituents in different salts. It has been suggested (11) that specific sites on the cell envelope require a high concentration of Na^+ in the surrounding medium for support, and that other sites require a high concentration of K^+ or NH_4^+. If this were so, it seemed possible that Na^+ would specifically prevent the release of certain envelope constituents and that K^+ or NH_4^+ would prevent the release of others; also, a different pattern of release of envelope constituents would be obtained as the concentration of each of these salts was decreased.

TABLE 3. *Sedimentation of lipid phosphorus of Halobacterium cutirubrum after exposure of cells to different salt concentrations*

Salt and concn	Per cent lipid phosphorus* sedimenting after		
	10 min at 6,000 $\times$ *g*	30 min at 15,000 $\times$ *g*	60 min at 31,000 $\times$ *g*
Water	0	14	39
NaCl, 2 M	54	83	89
NaCl, 4 M	94	94	100
KCl, 2 M	11	66	96
NH_4Cl, 2 M	8	21	80

* Sedimentation in 4 M NaCl after 60 min at 31,000 $\times$ *g* is taken as 100%.

After suspending envelopes for 2 hr at room temperature in each solution studied and centrifuging at 15,000 $\times$ *g* for 30 min, the release into the supernatant fluid of protein, total phosphorus, carbohydrate, and ultraviolet-absorbing substances was measured. There was no consistent difference in the pattern of release of these sub-

TABLE 4. *Effect of different salts on release of envelope constituents*

Salt	Concn	Per cent release*			
		Protein	Total phosphorus	Carbohydrate	UV-absorbing (260 mμ)
	M				
None		69	74	63	62
NaCl	1.0	47	40	29	40
	2.0	38	21	17	29
	3.0	14		11	21
	4.0	8	29	7	17
	5.0	8		6	18
KCl	1.0	44	41	26	43
	2.0	39	24	19	38
	3.0	21		11	32
	4.0	13	9	10	26
NH_4Cl	1.0	22	46	11	40
	2.0	30	37	11	33
	3.0	32	39	8	31
	4.0	27	36	6	26
$MgCl_2$	0.01	50	48	30	44
	0.05	35	35	15	29
	0.10	32	35	16	26

* Most figures are the mean values from two or three experiments.

stances in corresponding concentrations of NaCl and KCl (Table 4). NH_4Cl was more effective than NaCl and KCl in preventing the release of carbohydrate and, as found earlier (11), of protein. It was not especially effective in preventing the loss of phosphorus or of ultraviolet-absorbing compounds. The small fragments into which envelopes in 1 and 2 M NH_4Cl break (11) apparently have lost phosphorus and ultraviolet-absorbing substances. As expected (11), $MgCl_2$ was very effective in low concentrations in preventing the release of all constituents studied. It cannot be deduced from these experiments that Na^+ and K^+ act on different sites. They do support the findings that breakdown of envelopes in NH_4Cl is different from that in NaCl and KCl, but this has not been investigated further.

Ultracentrifugation studies. When salt was added to envelopes that had dissociated in water, no reformation of particles was seen under the phase-contrast microscope. However, ultracentrifugation studies, which we carried out with the hope that they would provide some clue to the differential action of NaCl and KCl, have showed that addition of salts can cause an aggregation of smaller particles into larger ones.

In 0.1 M NaCl or KCl, the schlieren pattern showed a "shoulder" of rapidly moving material which pulled away from a slower main peak with an uncorrected sedimentation coefficient, S = 5.2 (Fig. 5). As the salt concentration was increased, the amount of rapidly moving material increased and the size of the residual slower-moving peak decreased. This presumably represents a rather nonspecific aggregation of smaller into larger particles. It is seen most strikingly in concentrations of NaCl and KCl of 2 and 4 M, in which a very large peak appeared immediately but rapidly spread. No difference was seen between the action of NaCl and KCl at concentrations of 2 M or less. A peak remained after 20 min in 3 and 4 M NaCl, but not in 3 and 4 M KCl (3 M not shown in Fig. 5). Thus, at the highest concentrations, KCl, but not NaCl, caused certain envelope particles to aggregate. Because of the obviously random nature of this aggregation, these results are difficult to relate to the effect of monovalent salts on intact cells and envelopes, but they certainly do not show any preferential maintenance of envelope integrity by NaCl.

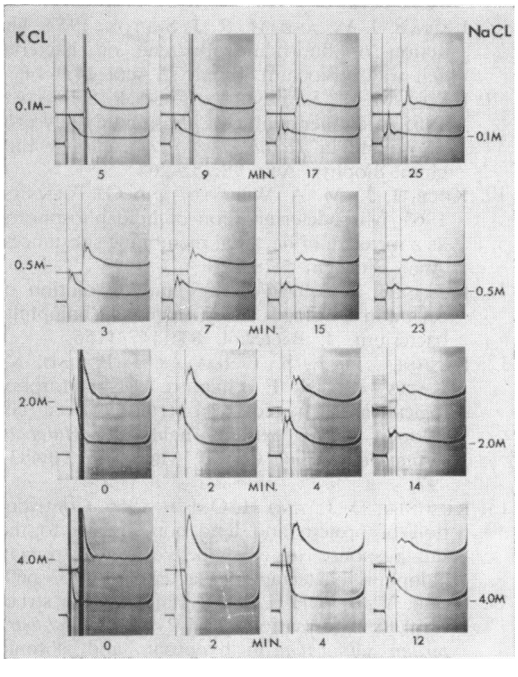

FIG. 5. *Effect of different NaCl and KCl concentrations on the sedimentation behavior of components of dissolved envelopes. Photographs were made at the indicated times after the ultracentrifuge reached its operating speed of 35,600 rev/min.*

DISCUSSION

Mohr and Larsen (14) found that a number of metabolic inhibitors, including cupric and mercuric ions, did not affect the structural transformations or the lysis of *H. salinarium* in varying concentrations of different salts. They concluded that it was very unlikely that such transformations had an enzymatic basis. We have found that cyanide, azide, and mercuric ions do not inhibit envelope dissolution, although cupric ions coagulate envelopes so that their dissolution in water can no longer be measured. Brown (3) concluded from the rapidity of dissolution of envelopes of *H. halobium* and from the fact that peptide bonds were not broken during dissolution that the process was not enzymatic. We have shown here that no extensive digestion of envelope polysaccharide takes place on dissolution. It is, however, obviously harder to prove that no enzyme is involved in envelope dissolution than to show that any specific enzyme is not involved. Supporting, though still equivocal, evidence against enzyme involvement is provided by experiments with heated envelopes. These disintegrate rapidly, but not completely, in water. The residue cannot be digested by a freshly dissolved preparation of unheated envelopes in water, and this probably indicates that the inhibition of disintegration caused by heating is due to a nonspecific protein coagulation rather than to the inactivation of a lytic enzyme. The fact that adding lysed envelopes to originally intact envelopes at different salt concentrations does not affect the lysis of the latter also argues

against the existence of a lytic-enzyme system. Even though it is still possible to argue that a lytic-enzyme system exists and that it has such specialized spatial requirements that it cannot act on any but its immediate surroundings, all the experiments that have been performed on envelopes of these and other extremely halophilic bacteria make the existence of such an enzyme system extremely doubtful.

The possibility that an enzyme(s) from the cytoplasm is involved in the breakdown of envelopes when intact cells are placed in water has not been investigated directly here. However, the results of Mohr and Larsen (14) on enzyme inhibitors and the finding of Abram and Gibbons (1) that heated cells of *H. cutirubrum* lyse in water make such a mechanism appear very unlikely.

The suggestion (11) that Na^+ acts on the outside of envelopes and that K^+ and NH_4^+ act on the inside seemed especially attractive in being practically unsusceptible to experimental proof. On reconsidering the evidence, it seems much more likely to us that Na^+ can act on certain sites on the envelope to prevent leakage, but that the other cations are about as effective in preventing gross envelope breakdown. This is supported by the fact that the lysis which occurs in 2 M KCl and NH_4Cl involves almost as much leakage as lysis in water, but it does not involve as much envelope disintegration. That Na^+ is much more effective than the other cations in preventing leakage of inorganic phosphate under conditions in which cells do not break down was shown earlier in experiments with formalin-treated cells. (11). It seems pertinent that Na^+ has a specific effect on transport in a marine bacterium (6), presumably through an action on some part of the cell membrane.

Ultracentrifugation studies on dissociated envelopes have shown that raising the salt concentration leads to a nonspecific aggregation of the dissolved particles. Brown (4) has recently described a magnesium-dependent dissociation and nonspecific reaggregation of 70*S* particles from sonically treated *Sarcina lutea* protoplasts. Considering the degree of dispersion of halophile envelopes in water and the probability of protein denaturation (13), it is not surprising that the reaggregation in higher salt concentrations is nonspecific and that organized structures are not formed again. However, the fact that aggregation occurs at all is consistent with the supporting role postulated for salts on the envelopes of extreme halophiles.

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LITERATURE CITED

1. ABRAM, D., AND N. E. GIBBONS. 1961. The effect of chlorides of monovalent cations, urea, detergents and heat on morphology and the turbidity of suspensions of red halophilic bacteria. Can. J. Microbiol. **7**:741–750.
2. BLIGH, E. C., AND W. J. DYER. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
3. BROWN, A. D. 1963. The peripheral structure of Gram-negative bacteria. IV. The cation-sensitive dissolution of the cell membrane of the halophilic bacterium, *Halobacterium halobium*. Biochim. Biophys. Acta **75**:425–435.
4. BROWN, J. W. 1965. Evidence for a magnesium-dependent dissociation of bacterial cytoplasmic membrane particles. Biochim. Biophys. Acta **94**:97–101.
5. BROWN, A. D., AND C. D. SHORBY. 1963. The cell envelopes of two extremely halophilic bacteria. J. Cell Biol. **18**:681–689.
6. DRAPEAU, G. R., AND R. A. MACLEOD. 1963. Na^+ dependent active transport of α-aminoisobutyric acid into cells of a marine pseudomonad. Biochem. Biophys. Res. Commun. **12**:111–115.
7. HAUROWITZ, F. 1963. The chemistry and function of proteins, p. 164–165. Academic Press, Inc., New York.
8. INGRAM, J. V., AND M. R. J. SALTON. 1957. The action of fluorodinitrobenzene on bacterial cell walls. Biochim. Biophys. Acta **24**:9–14.
9. KATES, M., L. S. YENGOYAN, AND P. S. SASTRY. 1965. A diether analog of phosphatidyl glycerophosphate in *Halobacterium cutirubrum*. Biochim. Biophys. Acta **98**:252–268.
10. KOLB, J. J., M. A. WEIDNER, AND G. TOENNIES. 1963. Microdetermination of lipid phosphorus as a measure of bacterial membrane substances. Anal. Biochem. **5**:78–82.
11. KUSHNER, D. J. 1964. Lysis and dissolution of cells and envelopes of an extremely halophilic bacterium. J. Bacteriol. **87**:1147–1156.
12. KUSHNER, D. J., S. T. BAYLEY, J. BORING, M. KATES, AND N. E. GIBBONS. 1964. Morphological and chemical properties of cell envelopes of the extreme halophile, *Halobacterium cutirubrum*. Can. J. Microbiol. **10**:483–497.
13. KUSHNER, D. J., AND H. ONISHI. 1966. Contribution of protein and lipid components to the salt response of envelopes of an extremely halophilic bacterium. J. Bacteriol. **91**:653–660.
14. MOHR, V., AND H. LARSEN. 1963. On the structural transformations and lysis of *Halobacterium salinarium* in hypotonic and isotonic solutions. J. Gen. Microbiol. **31**:267–280.
15. PETHICA, B. A. 1958. Lysis by physical and chemical methods. J. Gen. Microbiol. **18**:473–480.
16. SCOTT, T. A., JR., AND E. H. MELVIN. 1953. Determination of dextran with anthrone. Anal. Chem. **25**:1656–1661.