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Contribution of Protein and Lipid Components to the Salt Response of Envelopes of an Extremely Halophilic Bacterium[1]

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ABSTRACT

KUSHNER, D. J. (National Research Council, Ottawa, Ontario, Canada), AND H. ONISHI. Contribution of protein and lipid components to the salt response of envelopes of an extremely halophilic bacterium. J. Bacteriol. **91**:653–660. 1966.—Removal of protein from envelopes of *Halobacterium cutirubrum* by peptic digestion left residues that required little or no salt for stability. The salt requirement of envelopes was also lowered by incubation in 0.1 M $MgCl_2$, and could be lowered even further by digestion with trypsin or chymotrypsin in 0.1 M $MgCl_2$. Dissolution of envelopes in low salt concentrations made their protein more susceptible to attack by these and other proteolytic enzymes. Removal of lipids raised the requirement for divalent cations, particularly for Mg^{++}; it slightly increased the Na^+ requirement and did not affect the requirement for K^+. It was concluded that the requirement for high salt concentrations in extreme halophiles is due to mutual repulsion between negatively charged groups on proteins rather than to repulsion between negatively charged phosphate groups on the lipids. The latter act primarily as sites on which divalent cations, especially Mg^{++} which is required in high concentrations by growing cells, are bound. In this manner, the phosphate groups support envelope structure.

Work in this laboratory and elsewhere (1, 3, 10, 13) has shown that the breakdown in the absence of salts of envelopes of the extreme halophiles is a rapid and probably nonenzymatic process, and that most of the envelope substance can be recovered in the form of sedimentable particles. This breakdown is thought to be due to electrostatic repulsion between negatively charged groups in the envelope. The most important of these groups are the dicarboxylic acids of the acidic protein and the phospholipid phosphate groups, all of which have *p*K values of about 3.5 (3, 6, 10). However, little is known of the structural relations of such groups, or of their relative importance in producing electrostatic repulsion in the absence of shielding cations. This question has been investigated by using envelopes from which most of the lipid or protein has been removed.

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As another indication of the structural changes accompanying envelope dissolution, we have investigated the effect of dilution on the susceptibility of envelope proteins to proteolytic digestion.

MATERIALS AND METHODS

The preparation of envelopes of *Halobacterium cutirubrum* and most of the methods used have been described (10, 13). Envelopes were stored at 5 C in "buffered NaCl," i.e., 4.5 M NaCl containing 0.1 M $MgCl_2$ and 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.0). Before use, envelopes were centrifuged and resuspended in the appropriate salt solution. For turbidity measurements, envelopes were resuspended in 4.0 or 4.5 M NaCl, diluted 10 to 20 times (as specified) in the salt solution to be tested, and turbidity was measured after 30 min at room temperature with a Hilger Spekker Absorptiometer at 675 mμ. Acid-treated cells, whose salt response is similar to that of mechanically isolated envelopes (8), were prepared as before (9) with variations as shown in the text. In one experiment (Fig. 3) with acid-treated cells, turbidity was measured at 660 mμ in a Coleman Junior spectrophotometer.

Envelope concentration is usually expressed as milligrams of protein per milliliter of suspension,

since it is difficult to determine the dry weight of envelopes suspended in strong salt solutions; envelopes contain about 50% protein on a dry-weight basis (10).

Analytical methods. Protein was determined with the Folin phenol reagent by the method of Lowry et al. (11). Proteolytic activity was measured in some experiments as the release of trichloroacetic acid-soluble material reacting with the phenol reagent. This reagent, which measures tyrosine residues, gives different color values per residue with different proteins (11). However, we have observed that in experiments with different proteolytic enzymes the sum of the trichloroacetic acid-insoluble and -soluble "protein" was equal to the amount of protein originally present, and we feel justified in expressing trichloroacetic acid-soluble material reacting with the phenol reagent in terms of a percentage of the total protein (*see* Fig. 1 and 2).

For amino acid determinations, envelopes were hydrolyzed anaerobically in 6 N HCl for 20 and 70 hr at 110 C as recommended by Moore and Stein (12). After removal of HCl by repeated drying, amino acids were determined in a Beckman-Spinco amino acid analyzer. The values obtained after 70 hr of hydrolysis are reported for all amino acids except serine, threonine, and tyrosine; figures for these amino acids are corrected for destruction during hydrolysis (12), and the 70-hr figure for NH_3 is corrected for the contribution made by these amino acids.

Proteolytic enzymes. The following proteolytic enzymes were used in these experiments: pepsin (three times crystallized) and chymotrypsin (salt-free from ethyl alcohol), Nutritional Biochemicals Corp., Cleveland, Ohio; trypsin (two times crystallized), Worthington Biochemical Corp., Freehold, N.J.; pronase P from *Streptomyces griseus*, Kaken Chemical Co., Tokyo, Japan; and nagarse from *Bacillus subtilis* var. *bioticus* A, Teikoku Chemical Industry Co., Osaka, Japan. Both pronase and nagarse are relatively nonspecific proteinases (4).

RESULTS

Effect of envelope dissolution on susceptibility of envelopes to proteolytic digestion. Most of the protein of envelopes in 0.1 M $MgCl_2$ was converted into a trichloroacetic acid-soluble form in 2 hr at 37 C by trypsin, chymotrypsin, pronase, or nagarse at *p*H 7.0 or 8.0, or by pepsin in 0.06 M HCl. Both the rate of proteolytic attack and the total amount of protein solubilized was increased when envelopes were dissolved in water before $MgCl_2$ was added. Activity of all the enzymes in 4 M NaCl was low, although there was an increased initial proteolytic activity after a 30-min exposure to water. In the absence of added proteolytic enzymes, acid-soluble material did not increase with time in the presence of 4 M NaCl, but increased slowly in envelopes or dissolved envelopes suspended in 0.1 M $MgCl_2$. These results are illustrated in Fig. 1 (trypsin, no enzyme added) and Fig. 2 (pronase). The other enzymes gave similar patterns of response; the protein converted into a trichloroacetic acid-soluble form after 2 hr in 0.1 M $MgCl_2$ was: with chymotrypsin, 72%; pepsin, 83%; and with nagarse, 58%.

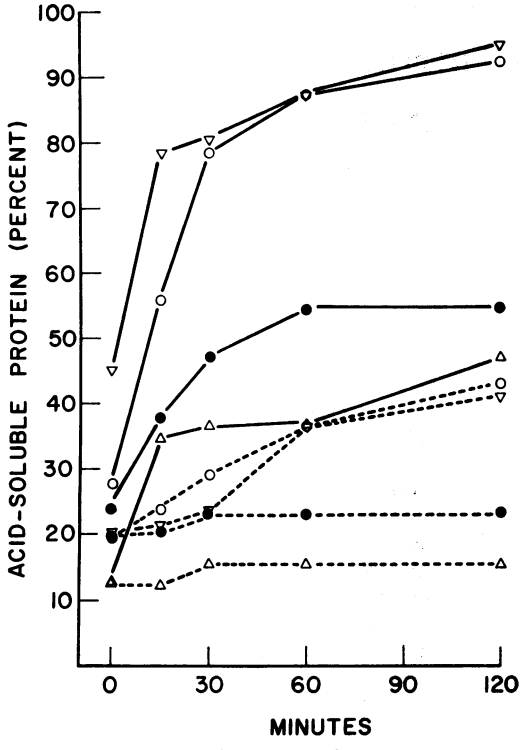

FIG. 1. *Effect of salts and of dilution on the tryptic digestion of envelopes of Halobacterium cutirubrum. An envelope suspension (0.25 ml), in 4.0 M NaCl, containing 6.6 mg/ml of protein was added in duplicate to the solutions shown below, all of which contained 0.01 M Tris buffer (pH 8.0). To one set of tubes, 0.5 ml of trypsin (1 mg/ml dissolved in 0.001 M HCl) was added. Envelopes were added to the following:* ○, *4.75 ml of 0.1 M MgCl ;* ▽, *4.63 ml of water, followed after 30 min at room temperature by the addition of 0.12 ml of 4 M $MgCl_2$ (final MgCl concentration, 0.1 M);* △, *4.75 ml of 4 M NaCl;* ●, *4.42 ml of water, followed after 30 min at room temperature by the addition of 1.11 g of solid NaCl (final NaCl concentration, 4 M). Solid lines, with enzyme; dotted lines, without. After incubation at 36 C for the times shown, 0.5 ml of each digest was added to 1.5 ml of 5% trichloroacetic acid; after standing overnight at 5 C the precipitate was centrifuged at 15,000 × g for 15 min, and protein (expressed as per cent of the total) was determined in the supernatant fluid.*

These results did not show whether the liberation of material from envelopes by trypsin treatment represented a true solubilization or simply

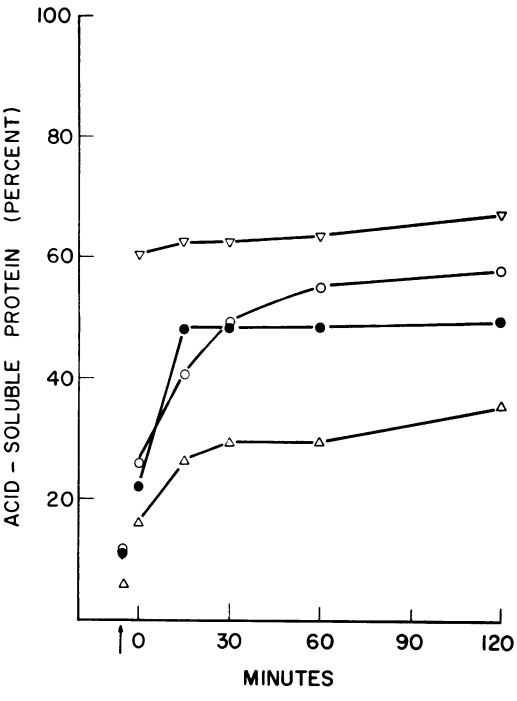

FIG. 2. *Effect of salts and of dilution on the digestion of envelopes of Halobacterium cutirubrum by pronase. Conditions and symbols as in Fig. 1, except that 0.01 M Tris buffer, pH 7.0, was used; the enzyme (1 mg/ml) was dissolved in water; and 0.005 M $CaCl_2$ was added to all samples before adding the enzyme. Arrow indicates the amount of acid-soluble protein found before the enzyme was added.*

TABLE 1. *Release and trichloroacetic acid extractability of protein-reacting substances from envelopes of Halobacterium cutirubrum in the presence and absence of trypsin**

Experimental conditions	Time	Per cent protein in supernatant fluid	
		Without trypsin	Trypsin added
	min		
Trichloroacetic acid added to total digest	0	15	15
	30	30	62
	120	49	84
Trichloroacetic acid added to digest supernatant fluid	0	19	53
	30	49	66
	120	54	81

* Two 0.3-ml samples of an envelope suspension containing 10.6 mg of protein per ml were added to tubes containing 5.7 ml of 0.1 M $MgCl_2$ and 0.01 M Tris buffer (*p*H 8.0). To one tube, 0.5 mg of trypsin was added and both tubes were incubated at 36 C. At the time intervals shown 1.0-ml samples of each digest were centrifuged at 0 C for 30 min at 15,000 × *g*, and 0.5 ml of the supernatant fluid was added to 1.5 ml of 5% trichloroacetic acid; at the same time intervals 0.5-ml samples of each total digest were added to 1.5 ml of 5% trichloroacetic acid. After standing overnight at 5 C, all samples were centrifuged at 15,000 × *g* for 15 min and protein was determined in the supernatant fluid.

an increased extractability by trichloroacetic acid. However, no more material was released when trichloroacetic acid was added to the trypsin-treated envelope suspension before centrifuging than when it was added to the supernatant fluid after centrifuging; the same thing was true for the slow proteolysis that took place in the absence of trypsin (Table 1). In the early stages of proteolytic digestion, more protein was released from the samples centrifuged before the addition of trichloroacetic acid, probably because of the digestion that took place during centrifugation. A very rough estimate of the molecular weight of the material released by trypsin was obtained by measuring the amount of such material in the trichloroacetic acid supernatant fluid that passed through a dialysis membrane on overnight incubation against 5% trichloroacetic acid. About half of the material released at each interval studied, up to 3 hr, was dialyzable (Table 2); hence, its molecular weight was less than 10,000.

Proteolytic digestion of envelopes was also studied by measuring the loss of protein from a dialysis sac after incubation with each enzyme for 6 and 24 hr (Table 3). In 0.1 M $MgCl_2$, 77% or more of the protein was made dialyzable in 24 hr by each enzyme studied. With chymotrypsin, pronase, and nagarse, the loss after 6 hr was greater in envelopes that had been first exposed to water. In 4 M NaCl, proteolytic digestion was slower and less extensive than in 0.1 M $MgCl_2$, but the rate and extent of digestion was increased in envelopes that had been exposed to water before the salt was added. In these experiments, none of the enzymes caused any loss of carbohydrate. After 24 hr at *p*H 1.9, about 30% of the carbohydrate was lost in 0.1 M $MgCl_2$ and 10% in 4 M NaCl. This loss was not increased by pepsin, and it may have been due to the hydrolysis of of polysaccharides.

Other envelope constituents were not studied in any detail, but, after pronase digestion under the various ionic conditions described above, there was no release of trichloroacetic acid-soluble phosphorus and no loss of phosphorus through a dialysis membrane.

TABLE 2. *Dialyzability of trichloroacetic acid-soluble material released from envelopes of Halobacterium cutirubrum by trypsin**

Time	Per cent of total protein in	
	Trichloroacetic acid supernatant fluid	Trichloroacetic and supernatant fluid after dialysis
min		
0	33	21
30	59	28
60	66	34
120	73	35
180	77	38

* To 11.1 ml of 0.01 M Tris buffer (*p*H 8.0) was added 0.6 ml of a suspension of envelopes in 4 M NaCl containing 7.84 mg/ml of protein. After 30 min, 0.3 ml of 4 M $MgCl_2$ was added to give a final $MgCl_2$ concentration of 0.1 M. To 10.0 ml of this suspension, 1 ml of trypsin (1 mg/ml) was added. After incubation at 35 C for the intervals shown, 1.0-ml samples were added to 3.0 ml of 5% trichloroacetic acid, and supernatant fluids were prepared as in Table 1. Protein was determined in supernatant fluids immediately or after overnight dialysis at 5 C against 3.75% trichloroacetic acid.

In the absence of added proteolytic enzymes, there was a 25% loss of protein in 24 hr from envelopes suspended in 0.1 M $MgCl_2$ (Table 3), and a similar loss was noted from envelopes dissolved in water. An earlier report (8) that envelopes in water did not lose protein through a dialysis sac was based on 3 hr of incubation. Though some autolysis does occur, it is much too slow to play a part in the extremely rapid dissolution of envelopes in water.

Effect of treatment with proteolytic enzymes on the salt response of envelopes. We next examined the salt response of envelopes from which most of the protein had been removed by enzymatic digestion. The most clear-cut results were obtained with envelopes digested with pepsin. These had lost most of their protein, and there was a large increase in the carbohydrate-protein ratio. Phase-contrast examination showed that a few rod-shaped envelopes remained, more were twisted or otherwise distorted, and most had broken up into smaller fragments. The integrity of digested envelopes was maintained by lower concentrations of NaCl and $MgCl_2$ than were required by undigested envelopes (Table 4).

To explore the salt response at concentrations lower than 0.45 M NaCl, greater dilutions than the 10- or 20-fold dilutions used for mechanically prepared envelopes (Table 4) were required; for this, thick suspensions of acid-treated cells were prepared. These, when treated with pepsin, were more stable in low concentrations of NaCl, KCl, and $MgCl_2$ than were cells treated only with acid (Fig. 3). Measurement of solubilization of protein or of carbohydrate showed the same lowered salt dependence of digested envelopes as did turbidity.

Incubation with 0.06 M HCl alone made the envelopes less sensitive to the absence of salts than were untreated envelopes; this is probably

TABLE 3. *Effect of salts and water on release of dialyzable material from envelopes by proteolytic enzymes**

Treatment of envelopes before adding enzyme	Per cent protein lost from dialysis sac											
	No enzyme (*p*H 7.0)	Trypsin		Chymotrypsin		Pronase		Nagarse		No enzyme (*p*H 1.9)	Pepsin	
	24 hr†	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr	24 hr	6 hr	24 hr
0.1 M $MgCl_2$	25	52	81	43	77	68	79	64	79	18	66	94
Water, then 0.1 M $MgCl_2$	12	49	88	56	88	83	88	78	86	37	65	89
4.0 M NaCl	17	9	41	25	43	32	41	34	45	12	19	60
Water, then 4.0 M NaCl	16	28	62	37	69	52	64	53	71	26	43	84

* Enzymes and envelopes were prepared as in Fig. 1 and 2. To 5.0 ml of each envelope suspension or solution (containing 0.6 to 0.9 mg/ml of envelope protein) 0.5 ml of enzyme (1 mg/ml) was added, and 4.0 ml of the mixture was dialyzed against the same salt solution containing 0.01 M Tris buffer (*p*H 7.0) for trypsin, chymotrypsin, pronase, and nagarse, or 0.06 N HCl adjusted to *p*H 1.9 for pepsin. For chymotrypsin, pronase, and nagarse, 0.005 M $CaCl_2$ was added to all samples before adding the enzyme. Samples from inside the dialysis sac were analyzed for protein at the time intervals shown. Figures are corrected for the contribution made by the enzyme protein.

† Time of incubation.

TABLE 4. *Effect of acid treatment and pepsin digestion on the salt response of envelopes of Halobacterium cutirubrum**

Expt	Treatment of envelopes†	NaCl concn (M)						$MgCl_2$ concn (M)					
		0	0.5	1.0	2.0	3.0	4.0	0	0.01	0.03	0.05	0.1	0.5
1	None	27					100	27					
	Acid	49	70	95	95	112	100	49	96	117	116	131	
	Acid + pepsin (0.2 mg)	66	89	102	105	109	100	66	101	130	125	144	
2	Acid	43		70	101	96	100	43	52	83	82	99	97
	Acid + pepsin (0.2 mg)	83		98	112	101	100	83	102	141	113	152	110
3	Acid	17	54	68	92		100						
	Acid + pepsin (3 mg)	40	86	96	103		100						
4	None	25					100						
	Acid	35					100						
	Acid + pepsin (3 mg)	100					100						

* Results expressed as per cent turbidity. Turbidity in 4.0 M NaCl was taken as 100%. About 60 mg of envelopes was suspended in 20 to 30 ml of 0.06 M HCl, with additions as shown, for 6 to 22 hr at 36 C. Treated envelopes were washed twice and suspended in 4.5 M NaCl plus 0.1 M Tris buffer (*p*H 8.1), then diluted 20-fold or 10-fold (experiment 4) in the salt solution to be tested.

† Recovery of envelope-protein after acid treatment and washing was 25 and 44% in experiments 3 and 4, respectively. In experiments 3 and 4 the envelopes treated with acid plus pepsin contained, respectively, 15 and 7% protein as compared with the acid-treated envelopes. In experiment 3 the carbohydrate-protein ratio (w/w) was 0.089 for acid-treated envelopes and 0.260 for envelopes treated with acid plus pepsin.

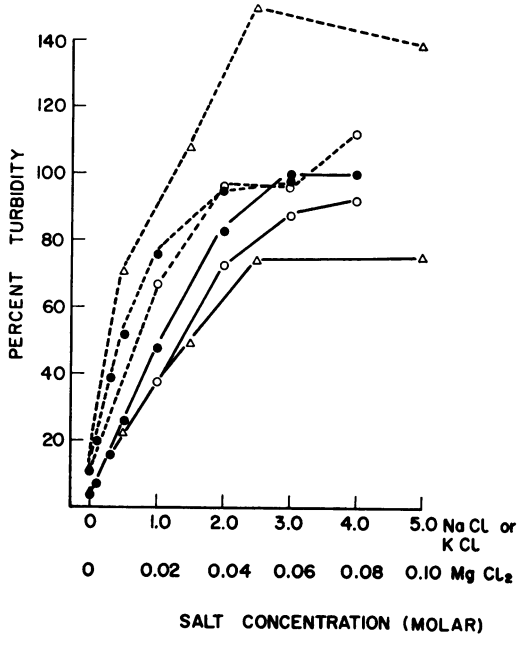

FIG. 3. *Effect of pepsin treatment on the salt response of acid-treated cells of Halobacterium cutirubrum. Cells (900 mg, dry weight) were suspended in 60 ml of 4.5 M NaCl containing 0.06 M HCl, centrifuged and resuspended in 60 ml of 0.06 M HCl. To half the suspension was added 0.5 mg of pepsin. After incubation for 22 hr at 36 C, the cells were centrifuged, washed twice, and then suspended in 2.0 ml of 4.5 M NaCl containing 0.1 M Tris (pH 8.1). For turbidity measurements, the cell suspensions were diluted 50-fold with the salt solutions shown. Pepsin treatment had caused a 77% loss of protein; carbohydrate-protein ratio (w/w) in the acid-treated cells was 0.028, and in those also treated with pepsin, 0.252. Symbols: ●, NaCl; ○, KCl; △, $MgCl_2$; solid line, acid-treated cells; broken line, cells treated with acid plus pepsin.*

connected with the loss of protein under acid conditions noted in Table 3.

Envelope protein contains a surplus of dicarboxylic amino acids (10), and, although most of the protein was lost on pepsin treatment, it seemed barely possible that the residual protein might contain most or all of the acidic amino acids, so that there would be no reduction in net negative charge. Amino acid analyses (Table 5) showed that there was little change in composition after acid treatment. After treatment with acid and pepsin there were few striking changes, and the overall result was a decrease rather than an increase of net negative charge per milligram of protein. Thus, pepsin, by removing most of the protein, greatly reduced the net negative charges on the envelopes.

The amino acid analyses of untreated en-

TABLE 5. *Effect of acid treatment and peptic digestion on the amino acid content of envelopes of Halobacterium cutirubrum**

Amino acid	Content		
	Untreated envelopes	Acid-treated envelopes	Acid + pepsin-treated envelopes
Lysine	0.13	0.15	0.087
Histidine	0.093	0.082	0.077
Arginine	0.21	0.24	0.16
Aspartic acid	0.85	0.97	0.57
Threonine	0.54	0.54	0.45
Serine	0.50	0.51	0.42
Glutamic acid	0.68	0.74	0.53
Proline	0.28	0.23	trace
Glycine	0.78	0.64	0.64
Alanine	0.71	0.58	0.47
Valine	0.71	0.55	0.41
Methionine	0.088	0.082	0.00
Isoleucine	0.31	0.30	0.26
Leucine	0.45	0.47	0.43
Tyrosine	0.19	0.22	0.093
Phenylalanine	0.21	0.21	0.15
Ammonia	0.55	0.65	1.85

* Samples analyzed were from experiment 4 of Table 4. Results are expressed as micromoles of amino acid per milligram of protein.

velopes are similar to those reported earlier (10), but may be considered more accurate (*see* Materials and Methods). In contrast to the earlier findings, methionine appears in these envelopes but not methionine sulphone, presumably because of the steps taken to exclude air before hydrolysis.

The effects of other proteolytic enzymes on the salt response of envelopes were less clear-cut than those of pepsin. Adding trypsin or chymotrypsin (up to 0.3 mg/ml final concentration) to envelopes (1 to 2 mg of protein per ml) incubated in 0.1 M $MgCl_2$ at *p*H 7.0 for 2 hr at 35 C decreased the sensitivity to dilution more than that of control cells in two out of three experiments. Incubation in 0.1 M $MgCl_2$ alone also reduced the sensitivity of envelopes to dilution. Sensitivity decreased after 2 hr at 37 C, but not at 0 C. After overnight incubation at 37 C, the sensitivity of envelopes usually completely disappeared. The loss of sensitivity on incubation with $MgCl_2$ was not increased by increasing the $MgCl_2$ concentration, nor was sensitivity to water restored to $MgCl_2$-treated envelopes by treatment with 10^{-6} to 10^{-2} M ethylenediaminetetraacetic acid (EDTA). The loss of sensitivity is probably not due to binding of Mg^{++} ions to the envelope, since such binding would take place in the cold and might be susceptible to reversal by EDTA treatment. The loss of sensitivity may be, instead, the result of an enzymatic process, perhaps the slow proteolysis described above (Fig. 1 and Table 3), but this has not been investigated further.

Effect of removal of lipids on the salt response of envelopes. Direct extraction with neutral solutions containing high concentrations of ethyl alcohol led to dissolution of envelopes, but this could be prevented by the addition of acid. Envelopes in 4.5 M NaCl plus 1% acetic acid were therefore extracted twice at room temperature with 75% ethyl alcohol containing 1% acetic acid, washed in and resuspended in 4.5 M NaCl. This procedure removed all the pigment and 80% of the lipid phosphorus from envelopes, leaving colorless residues which still had the shapes of intact envelopes. In contrast to the deproteinized envelopes, these had not lost any of their sensitivity to dilution. The requirement of defatted envelopes for NaCl was somewhat greater than that of untreated envelopes, whereas the KCl requirement was only slightly changed (Fig. 4). In the experiment shown, higher turbidities were obtained in 3 and 4 M KCl in lipid-extracted envelopes than in control envelopes, but this was

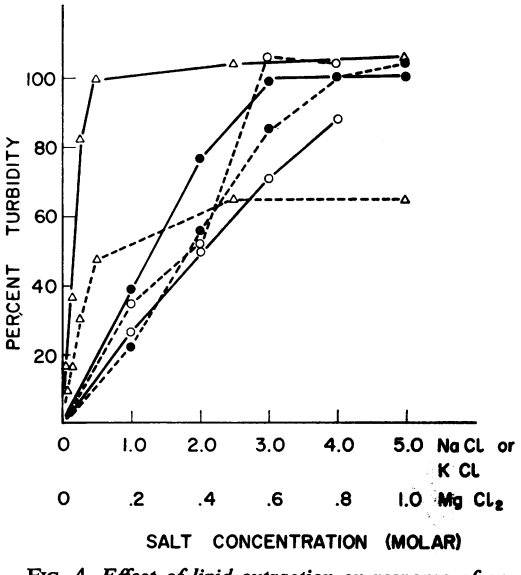

FIG. 4. *Effect of lipid extraction on response of envelopes of Halobacterium cutirubrum to NaCl, KCl, and $MgCl_2$. Suspensions of envelopes in 4.5 M NaCl were added to 10 volumes of the salt solutions shown or 10 volumes of water, the latter being used as blanks. Control and lipid-extracted cells had similar turbidity values in water (100% turbidity = that found in 4.0 M NaCl). Symbols: ●, NaCl; ○, KCl; △, $MgCl_2$; solid lines, untreated envelopes; broken lines, lipid-extracted envelopes.*

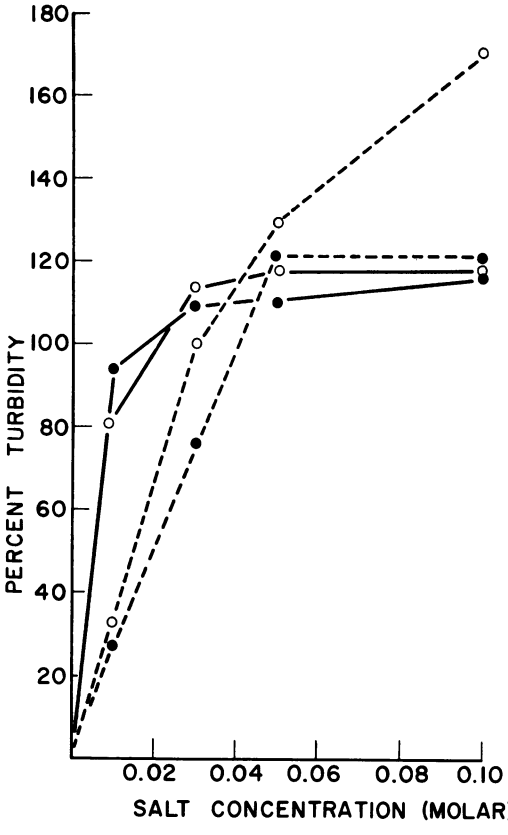

FIG. 5. *Effect of lipid extraction on response of envelopes of Halobacterium cutirubrum to $CaCl_2$ and $MnCl_2$. Conditions as in Fig. 4, except that suspensions were added to 20 volumes of each salt solution. Symbols: ●, $CaCl_2$; ○, $MnCl_2$; solid lines, untreated envelopes; broken lines, lipid-extracted envelopes.*

not usually observed. The most striking difference between lipid-extracted and control envelopes was their response to Mg^{++} ions. The requirement for this ion had greatly increased, and in the concentration range studied it was no longer possible to obtain maximal turbidity of lipid-extracted envelopes. Below 0.05 M, higher concentrations of Ca^{++} and Mn^{++} were also required to maintain turbidity in extracted envelopes. At and above this concentration of $CaCl_2$ there was little difference between extracted and control envelopes. There was an increase in turbidity of extracted, relative to control, envelopes in higher $MnCl_2$ concentrations (Fig. 5). Microscopic examination showed that the former were easily coagulated by low $MnCl_2$ concentrations, but this has not been further investigated.

DISCUSSION

The susceptibility of envelopes to proteolytic enzymes was used in these studies to indicate the degree of disorganization caused by exposure to water. The decrease in the orderly arrangement of the peptide chains within the protein molecule that constitutes denaturation is known to increase the susceptibility of many proteins to attack by proteolytic enzymes (7). Several enzymes of extremely halophilic bacteria require salts and are inactivated by exposure to water (2); such inactivation may or may not be irreversible (5). After studying the effects of salts on the lactic dehydrogenase of *H. salinarium*, Baxter (2) suggested that "this enzyme differs from enzymes of nonhalophilic organisms in being less firmly held in its native catalytically active conformation. At low salt concentrations the electrostatic repulsion between ionized groups on the enzyme expands it to a form which is not catalytically active and which can readily expand further to an irreversibly denatured form." It seems likely that an exposure to water leads to a denaturation of envelope protein as well as to a breaking up of the envelope into particulate subunits (13), although we cannot at present distinguish the contribution each process makes to susceptibility to proteolytic attack.

Our results also show that intact envelopes, in 0.1 M $MgCl_2$ and even in 4.0 M NaCl, can be attacked by proteolytic enzymes, trypsin, and pepsin, causing the removal of about 90% of the protein. The nonspecificity of pronase and nagarse did not make them more effective in degrading envelopes in these experiments, which, however, were more concerned with measuring the removal of protein from particulate or trichloroacetic acid-precipitable envelope material than with the subsequent degradation of the liberated polypeptides.

The negatively charged groups on proteins are primarily responsible for envelope dissolution in the absence of salt, but the negatively charged lipid phosphate groups play quite another role. Thus, removing most of the protein lowers the net negative charge on envelopes, and lowers or abolishes the dependence of envelopes on salts for stability. Removal of lipids also lowers the net negative charge but does not lower the salt requirement. The sodium requirement is slightly increased, and the requirement for potassium ions remains unchanged. The most striking effect of lipid extraction is on the response to divalent cations. The requirement for Mg^{++} is greatly increased, and, in fact, it is not possible to obtain maximal turbidity with concentrations of $MgCl_2$ 10 times as high as those that

suffice with untreated envelopes. Lipid extraction also increases the requirement for Ca^{++} and Mn^{++}, but low concentrations of these ions are able to maintain maximal turbidity. Recent studies (Kates and Palameta, *unpublished data*) have shown that the phosphatide from *H. cutirubrum* can bind both Na^{+} and K^{+} and has an especially strong attraction for Mg^{++}.

These studies give the following picture of the role of different envelope constituents in envelope stability. Envelopes are unstable at neutral *p*H values in the absence of salts because of repulsion between negatively charged ionized dicarboxylic acids in their protein. Removal of most of the protein leaves a carbohydrate-rich residue, stable on dilution, which may be important in determining the shape of these bacteria. Phosphate groups on envelope lipids, though negatively charged, do not contribute to envelope dissolution in the absence of salts. On the contrary, such groups serve as linkage sites by means of which divalent cations can hold the envelope together, and in the absence of such groups the divalent ion requirement is increased. Mg^{++} seems to form bridges primarily between lipid phosphate groups, since quite high concentrations of these ions cannot maintain maximal turbidity in envelopes lacking such groups. The ability of low concentrations of Ca^{++} and Mn^{++} to maintain maximal turbidity may indicate a greater affinity of these ions than of Mg^{++} for envelope protein.

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LITERATURE CITED

1. ABRAM, D., AND N. E. GIBBONS. 1960. Turbidity of suspensions and morphology of red halophilic bacteria as influenced by sodium chloride concentration. Can. J. Microbiol. **6**:535–543.
2. BAXTER, R. M. 1959. An interpretation of the effects of salts on the lactic dehydrogenase of *Halobacterium salinarium*. Can. J. Microbiol. **5**:47–57.
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. HILL, R. L. 1965. Hydrolysis of proteins. Advan. Protein Chem. **20**:37–107.
5. HOLMES, P. K., AND H. O. HALVORSON. 1963. The inactivation and reactivation of salt-requiring enzymes from an extreme obligate halophile. Can. J. Microbiol. **9**:904–906.
6. 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.
7. KAUZMANN, W. 1959. Some factors in the interpretation of protein denaturation. Advan. Protein Chem. **14**:1–63.
8. KUSHNER, D. J. 1964. Lysis and dissolution of cells and envelopes of an extremely halophilic bacterium. J. Bacteriol. **87**:1147–1156.
9. KUSHNER, D. J., AND S. T. BAYLEY. 1963. The effect of pH on surface structure and morphology of the extreme halophile, *Halobacterium cutirubrum*. Can. J. Microbiol. **9**:53–63.
10. 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.
11. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. MOORE, S., AND W. H. STEIN. 1963. Chromatographic determination of amino acids by the use of automatic recording equipment, p. 819–831. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 6. Academic Press, Inc., New York.
13. ONISHI, H., AND D. J. KUSHNER. 1966. Mechanism of dissolution of envelopes of the extreme halophile *Halobacterium cutirubrum*. J. Bacteriol. **91**:646–652.