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THE PERIPHERAL STRUCTURES OF GRAM-NEGATIVE BACTERIA

IV. THE CATION-SENSITIVE DISSOLUTION OF THE CELL MEMBRANE OF THE HALOPHILIC BACTERIUM, *HALOBACTERIUM HALOBIUM*

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SUMMARY

The cell envelope of *Halobacterium halobium*, when isolated in 2.2 M NaCl, consists of a single unit membrane which dissolves rapidly if the suspending salt solution is diluted. The extent of dissolution is approximately inversely proportional to salt concentration and is complete at sufficiently high dilution. From the extreme rapidity of the dissolution, even at 0°, the occurrence of the phenomenon in heated membranes, the separation of several distinct macromolecular components in the ultracentrifuge and evidence for no peptide bond breakage, it was concluded that the dissolution process consisted of non-enzymic disaggregation into macromolecular sub-units of the membrane. The effects of salts and pH on the process were consistent with the belief that disaggregation is caused by an excess of negative charges on membrane proteins. The relatively high content of aspartic and glutamic acids in membrane proteins together with effects of pH provide evidence that the negative charges arise predominantly from the β- and γ-carboxyl groups of these two amino acids. Stabilization of the membranes is discussed and a mechanism is proposed whereby high salt concentrations can increase mechanical rigidity of the membrane.

INTRODUCTION

The outermost permeability barrier of a unicellular organism must have effectively direct contact with the cell's external environment. Permeability barriers are usually lipoprotein membranes. It follows that the structural integrity and biological function of the membrane must be maintained when in contact with a solution which does not necessarily have the same ionic compositon as the intracellular contents. Although knowledge of membrane structure is limited, it is a reasonable assumption that maintainance of biological function will require the membrane proteins to be in their native conformation.

Bacteria of various kinds thrive in environments which range in ionic strength from very close to zero up to that of saturated aqueous NaCl. Properties of membrane proteins and the manner of association of sub-units into a membrane are likely to vary with the ionic strength of the natural habitat. The sea, with an ionic strength in

the region of 0.68, lies close to the lower end of the ionic strength range of natural environments. The external membrane of marine pseudomonad NCMB 845 autolyses proteolytically at reduced salt concentration[1,2]. The autolysis, which is inhibited by H^+ and other cations, was attributed to conformational changes induced in membrane proteins by the appearance of negative charges on the proteins[2]. The extreme halophil, *H. halobium* grows optimally in 4–5 M NaCl and quite well in saturated aqueous solutions of NaCl. The envelope of this organism can therefore be subjected to an "ion deficit" (defined below) considerably greater than that obtainable with the marine organism or other organisms of moderate salt requirements. It is to be expected, therefore, that effects of salt dilution, which are evident in the membrane of the marine organism and which are proportional to the ion deficit, will be more pronounced in the corresponding structures of an extreme halophil. The cell envelope of *H. halobium* consists of a single structure which, in the whole organism, is possibly a compound (5-layered) membrane; on isolation in 12.5 % (w/v) NaCl, however, the envelope is obtained as a simple unit membrane[3]. When the suspending salt solution is diluted the isolated membrane of *H. halobium* dissolves very rapidly.

The present study was undertaken to examine the dissolution process, the forces immediately responsible for it and to consider what information the dissolution mechanism might provide about the structure of the membrane. Some of the findings have been discussed elsewhere in a preliminary form[4].

MATERIALS AND METHODS

Organism and growth medium

H. halobium was grown for 6 days at 30° with aeration on Oxoid peptone (1 % w/v) in the basal salts solution described by SEHGAL AND GIBBONS[5] (containing 25 % w/v NaCl).

Isolation of cell membranes

The organisms were harvested and resuspended in cold (4°) half-strength basal salts solution. The yield from 1.5 l culture medium was resuspended in 80 ml and the organisms were disrupted in a Mickle disintegrator for 18–20 min[6]. Membranes were isolated by a series of fast and slow centrifugations[6] in cold half-strength basal salts solution. The final suspension in the salts solution was then either freeze-dried or stored in cold half-strength basal salts solution until treated in some other way as described below.

Measurement of membrane dissolution

Membrane dissolution was followed turbidimetrically at 700 mμ (in 1-cm cylindrical tubes). All readings were referred to a distilled water blank and, where necessary, correction was applied for differences in the refractive indices of the suspending salt solutions.

Examination of soluble breakdown products

Membrane solutions were prepared for ultracentrifugal analysis by dialysis against cold distilled water, concentration in the cold to a final protein content of

about 1 % (w/v) and finally dialysis against cold 0.1 M NaCl. Initially the protein content of the solution was determined by the method of LOWRY *et al.*[7] but subsequently the absorption of the membrane solution at 475–480 mμ (caused by the pink, presumably carotenoid, pigment of the organism), after calibration against protein concentration, was found to be a sufficiently reliable index for this purpose. Sedimentation behaviour was observed at 59780 rev./min in a Spinco Model E analytical ultracentrifuge at about 5° and 20°.

Cell membranes and their breakdown products were dinitrophenylated under the conditions used by INGRAM AND SALTON[8]. At the end of the reaction period suspensions of DNP-membranes and dissolved membrane were acidified with HCl and extracted with ether to remove excess FDNB. The ether extract was re-extracted with aqueous $NaHCO_3$ (1 % w/v); the $NaHCO_3$ solution was then acidified with HCl and extracted with ether. This ether extract was examined for free DNP-amino acids. DNP-dialysates were extracted with ether directly from the alkaline reaction mixture at the end of the period of reaction with FDNB. DNP-membranes, DNP-membrane solutes (non-dialysable fraction) and DNP-dialysates were hydrolysed for 15 h at 100° in constant boiling HCl in sealed glass ampoules. Ether-soluble DNP-amino acids were identified by paper chromatography in the "toluene"–1.5 M phosphate 2-dimensional system of LEVY *et al.*[9] and estimated by measuring absorbancy at 360 mμ after elution from the chromatogram with aqueous $NaHCO_3$ (4 ml, 1 % w/v). Standard mixtures containing 0.1–0.4 μmoles of DNP-DL-alanine, DNP-L-aspartic acid, DNP-L-glutamine and DNP-DL-serine were hydrolysed, chromatographed and estimated under conditions identical to those just described. These four compounds were prepared by SANGER's method[9,10] and recrystallized twice from appropriate solvents. The standard error of the estimation using crystalline DNP-amino acids (0.2 μmole) was ±2 % of the mean. DNP-amino acids other than these four were estimated with reference to DNP-alanine with corrections where necessary for differences in molar extinction coefficients[9]. An aliquot of the aqueous phase of the hydrolysate was chromatographed in *tert.*-amyl alcohol plus phthalate (pH 6.0)[11]; ε-DNP-lysine was estimated after elution from such chromatograms with aqueous $NaHCO_3$.

Complete (but approximate) amino acid analyses were done by dinitrophenylating the aqueous phase of the above hydrolysates according to LEVY *et al.*[9] and, after removal of excess FDNB, separating the reaction products into ether-soluble and water-soluble fractions. Aliquots of the former were chromatographed in the 2-dimensional system and estimated as above. The water-soluble fraction was chromatographed in the 1-dimensional system from which chromatograms DNP-arginine and occasionally small amounts of some other DNP-amino acids were estimated.

Definition

The term "ion deficit" is proposed and defined as the reduction in ionic strength or molar salt concentration of a solvent (or suspending solution) below that at which a biologically active macromolecule such as a protein is in its native conformation. Native conformation is assumed in this context to be associated with optimal biological activity; in many cases, of course, this will be found within a range of ionic strengths rather than at a single value.

EXPERIMENTAL AND RESULTS

Effects of salt concentration and temperature on dissolution of the membrane

The first turbidity reading (zero time) was taken soon after adding the membrane suspension to the other constituents in the spectrophotometer tube. For equal amounts of membrane suspension this initial reading varied with the final salt concentration of the diluted suspension suggesting that a very rapid breakdown of the membranes was occurring. For this reason all constituents of the reaction mixture were maintained at 0° and the first turbidity reading was taken at that temperature. The time lapse between diluting the membrane suspension and taking the first reading was reduced as much as possible consistent with adequate mixing; readings were obtained after an interval of 10–15 sec. Subsequent turbidity readings were taken after incubating the diluted suspension at either 0° or 35°. Results are given in Fig. 1 from which it is evident that the greater part of the dissolution occurred during an initial very rapid phase of breakdown. Fig. 1 also shows that, apart from its effect on the rate of initial rapid breakdown (inferred from the first reading), the principal effect of

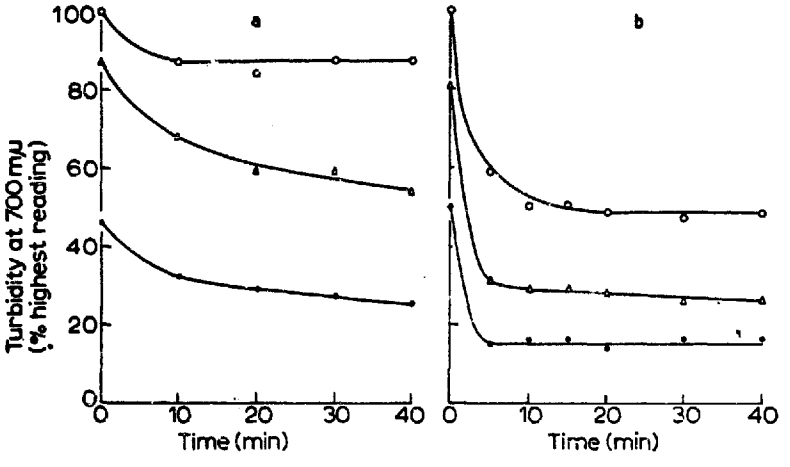

Fig. 1. Effects of temperature and salt concentration on dissolution of isolated membranes of *H. halobium* in 0.0125 M Tris buffer (pH 8.0). a. Membrane suspension diluted and incubated at 0°. b. Membrane suspension diluted at 0° and the first reading taken at that temperature (see text); subsequently incubated at 35°. O—O, 1.25 M NaCl; △—△ 0.62 M NaCl; ●—●, 0.31 M NaCl. At 2.2 M NaCl there was no dissolution; at 0.22 M NaCl the turbidity–time curves were only slightly below those obtained at 0.31 M NaCl.

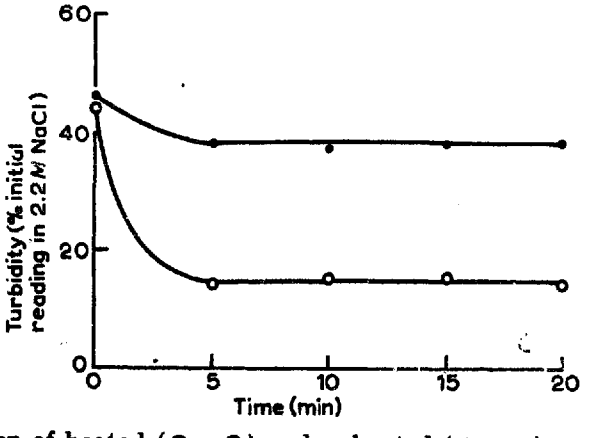

Fig. 2. The dissolution of heated (●—●) and unheated (O—O) membranes of *H. halobium* in a solution containing 0.0125 M Tris buffer (pH 8.0) and 0.22 M NaCl. Similar results were obtained with membranes heated at 100° for 10, 20, 40 and 80 (illustrated) min.

NaCl concentration was on the extent of dissolution. The extent of dissolution for each salt concentration below 2.5 M NaCl was different at each of the two temperatures.

The effect of Ca^{2+} on dissolution was measured over the range 0.001–0.2 M $CaCl_2$ in the presence of 0.22 M NaCl and 0.0125 M Tris buffer (pH 8.0). At 0°, 0.001 M $CaCl_2$ had no effect, 0.005–0.1 M completely suppressed dissolution and 0.2 M caused some aggregation of the suspension. Ca^{2+} ions had less effect at 35° at which temperature 0.005 M $CaCl_2$ caused only about 23 % inhibition and 0.01 M about 70 % inhibition of membrane dissolution. The absolute effect of both NaCl and $CaCl_2$ varied slightly from one membrane preparation to another.

Membrane suspensions (in half-strength basal salts solution) were heated at 100° for periods up to 80 min and their dissolution measured at a final concentration of 0.22 M NaCl. Results are given in Fig. 2 from which it is seen that the initial rapid breakdown was similar in both heated and unheated membranes but the final extent of dissolution was less with the heated membranes.

Effect of pH on dissolution of the membrane

Membrane dissolution was measured in acetate and phosphate buffers over the range of pH 2–7. Results are given in Figs. 3 and 4. In Fig. 3 selected curves show the turbidities of membrane suspensions at several values of pH. Both the initial turbidity

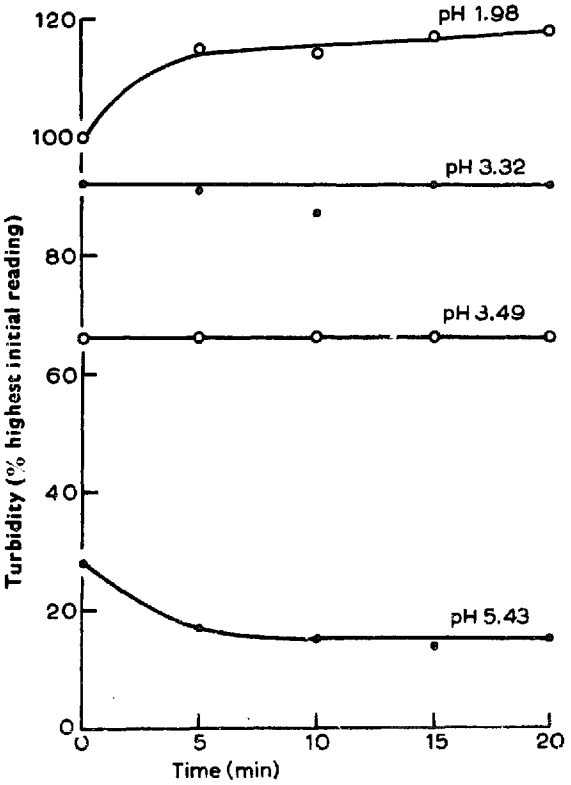

Fig. 3. Selected curves illustrating changes in turbidity of membrane suspensions at different values of pH in 0.02 M acetate buffer at 35° in the presence of 0.33 M NaCl. It is unlikely that the initial readings measured simply different degrees of aggregation of the suspension because the reading corresponding to 100 % was approximately the same as that obtained in unbuffered 2.2 M NaCl.

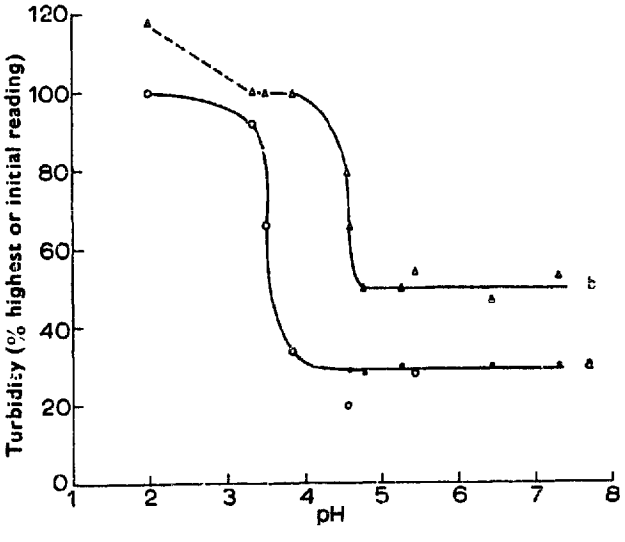

Fig. 4. The effect of pH on initial and final turbidities of membrane suspensions. The values plotted are derived from curves of the type shown in Fig. 3. Curve a shows the change with pH in initial turbidity (expressed as per cent of highest initial reading of the series). Curve b shows the change with pH of the turbidity after 20 min at 35° (expressed as per cent of initial reading at the same pH). O—O and Δ—Δ, 0.02 M acetate buffer; ●—● and ▲—▲, 0.02 M phosphate buffer. Values of pH are those of the diluted membrane suspension in the presence of 0.33 M NaCl.

of the diluted suspension and subsequent breakdown during incubation at 35° were affected by pH but the effect was different in the two cases. The results are summarized in Fig. 4 from which it is evident that dependence of initial turbidity on pH was maximal near pH 3.5 while dependence of the second phase was maximal at about pH 4.5.

Properties of membrane solutions

For preparative purposes membrane solutions were obtained by centrifuging the membrane suspension in half-strength basal salts solution and resuspending the pellet in distilled water (at 4° or at room temperature) or in buffer solution as indicated. The solution was then centrifuged in the cold at 14500–17000 × *g* for 45 min. A very small pellet was usually obtained from this centrifugation; in early preparations this was again resuspended and centrifuged in distilled water. After the second resuspension no residue remained beyond an occasional trace of material which was obviously an impurity. In later preparations the second resuspension was omitted. The residue lost by this omission was very small and avoidance of excessive dilution (and subsequently prolonged concentration) of the membrane solution was considered advantageous. Membrane solutions were concentrated in the cold from dialysis bags with or without prior dialysis against cold distilled water.

Membrane preparations (either dry or in suspension) were pink and this colour went into aqueous solution with the membrane. The pigment was retained during dialysis. The absorption spectrum of an unbuffered aqueous solution showed a non-specific rise in absorbancy at wavelengths below about 650 mμ with slight shoulders near 540, 450–480 and 420 mμ. The colour could be extracted from an aqueous solution or suspension of membranes by butanol or diethyl ether and from dried membrane preparations with butanol or ethanol. The pigment in aqueous butanol had high absorption below about 550 mμ with peaks of about equal height at 400, 465 and 495 mμ. The absorption spectra of methanolic solutions of bacteriopurpurins from *Bacterium halobium* are reported to have maxima at 460, 495 and 528 mμ for α-bacteriopurpurin and 452, 482 and 502 mμ for β-bacteriopurpurin[12]. Most of the membrane components were precipitated from solution by half saturation with $(NH_4)_2SO_4$; the precipitate included the pink pigment.

The ultracentrifuge separated a major component from others with higher sedimentation rates (Fig. 5). In one preparation the sedimentation coefficient of the major component (measured near 20°) was $s_{20,w} = 3.6$ S. In a second preparation the sedimentation coefficient of the major component was $s_{20,w} = 3.9$ S when run near 20° and $s_{20,w} = 4.0$ S when run near 5°. Sedimentation coefficients of the faster components were not determined because their behaviour in the centrifuge was slightly anomalous. An inverted peak which was assumed to be caused by lipoprotein was observed in all preparations (see Fig. 5).

Membrane solutions were subjected to paper electrophoresis in buffers of *I* 0.1 at pH 7.0, 5.3 and 2.5, for various times at various potentials. Separation was poor with considerable streaking but some information was obtained. At pH 7.0 a relatively large amount of material, including the pigment, migrated towards the anode. At pH 5.3 the pigment remained at the origin but unpigmented protein moved towards the anode. At pH 2.5 there was no migration. At no pH did any component migrate towards the cathode.

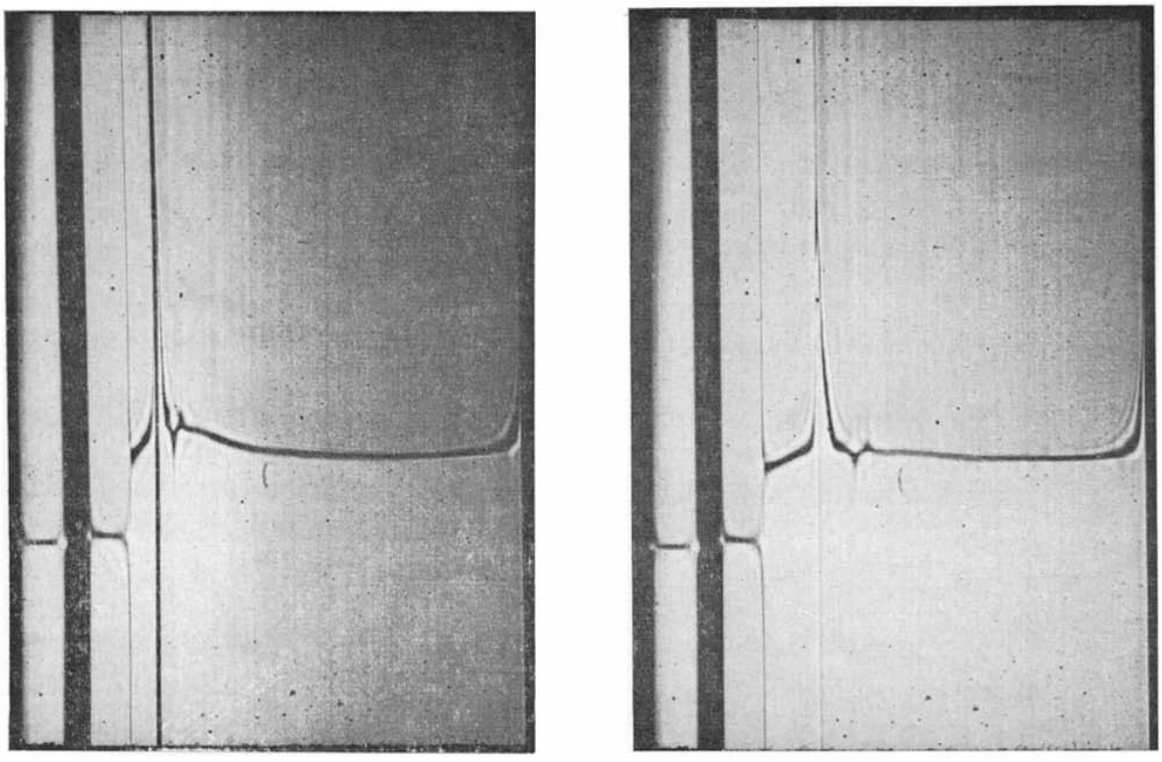

Fig. 5. Sedimentation patterns in 0.1 M NaCl at 18.8°, (a) 12 min and (b) 28 min after reaching maximum speed. The dark zone to the right of each picture is caused by a component containing the pink pigment of the membrane. The sedimentation coefficient of the main peak in this preparation is $s_{20,w} = 3.9$ S.

Membrane solutions which had been freeze-dried could be redissolved in distilled water leaving little or no residue. Membrane solutions were not precipitated by salt solutions which had been found adequate to stabilize intact membranes.

Dinitrophenylation of whole and dissolved membranes

Half of each membrane preparation was freeze-dried from suspension in half-strength basal salts solution and described as "whole" membrane. Membrane solutions were obtained by dissolving the other half of the relevant preparations in (i) distilled water, (ii) 0.01 M phosphate buffer (pH 7.0) or (iii) distilled water after heating the membrane suspension for 30 min at 100°. Whole membranes and membrane solutes (with or without prior dialysis) were reacted with FDNB as described above.

The same DNP-amino acids were obtained from whole membranes, the material from undialysed membrane solutions and the non-dialysable material from membrane solutions. No DNP-amino acids were obtained from the dialysate.

Quantitative expression of the end-group analyses in terms of mass of starting material was difficult and unreliable for whole membranes because these preparations contained large amounts of salt. The results for dialysed membrane solutions could be treated in this way, however, and since there was no evidence of loss of material during dialysis, the assumption was made that the original mass of membrane was equal to that of the dialysed soluble material. End-group analyses expressed on this assumption are given in Table I which also contains comparable figures for the marine organism NCMB 845. Table II gives end-groups as mole percentages of the total amino acid content; results for whole membranes are amenable to this treatment. The results

TABLE I

NH_2-TERMINAL ACIDS OF *H. halobium* MEMBRANES AND DISSOLVED MEMBRANES

Yields are calculated, for two preparations, on the mass of dissolved membrane following removal of salt by dialysis (see text). Comparative results are included for three envelope preparations from the marine organism NCMB 845. Aspartic and glutamic acids are combined because they were not always completely separated on the chromatograms. The high DNP-serine content of NCMB 845 envelope was not caused by lipid-bound serine (*cf* MACFARLANE[13]). DNP-ethanolamine but no DNP-amino acid was obtained from hydrolysed membrane DNP-lipid of this organism[14].

NH_2-terminal acid	*μmoles DNP-amino acid per g of*		
	H. halobium membrane	*H. halobium dissolved membrane*	*NCMB 845 envelope*
Asp + Glu	1.0, 1.9	1.1, 1.3	2.2, 1.9, 2.6
Ala	3.3, 3.5	3.5, 4.0	1.4, 2.2, 2.6
Ser	2.0, 2.1	2.3, 2.6	7.1, 4.8, 6.4
Thr	0.3, 1.3	0.4, 0.7	0, 0, 0

TABLE II

MOLE PERCENTAGES OF NH_2-TERMINAL ACIDS OF *H. halobium* MEMBRANES AND DISSOLVED MEMBRANES

Results are given for the same two preparations as listed in Table I. No difference in mole percentages of end groups could be demonstrated between whole membranes and membrane solutes obtained by the other dissolution procedures described in the text. Comparative results for two preparations of NCMB 845 are included. The average number of amino acids in the polypeptide chains of the membranes was calculated with the assumptions that there was no branching and all free α-NH_2 groups were accessible and reactive.

NH_2-terminal acid	*Mole per cent end group in*		
	H. halobium membrane	*H. halobium dissolved membrane*	*NCMB 845 envelope*
Asp + Glu	0.12, 0.07	0.05, 0.07	0.06, 0.08
Ala	0.27, 0.27	0.20, 0.28	0.07, 0.05
Ser	0.15, 0.17	0.11, 0.19	0.14, 0.24
Thr	0.04, 0.02	0.03, 0.03	0, 0
Total	0.58, 0.53	0.39, 0.57	0.27, 0.37
Average chain length	177 189	256 176	370 270

showed that no qualitative or significant quantitative difference in the NH_2-termini was evident between whole and dissolved membranes.

Free DNP-serine, DNP-aspartic acid and DNP-glutamic acid were recovered together or separately from some, but not all, of the dinitrophenylation reaction mixtures. The greatest amounts of DNP-serine and DNP-aspartic plus DNP-glutamic acids recovered corresponded respectively to 29 and 11 μmoles per g membrane solute. There was no correlation between the occurrence or proportion of free DNP-amino acids in the reaction mixture and the proportion of these same DNP-amino acids released on acid hydrolysis of the DNP-membrane. It is not known at present whether the free amino acids were derived from contaminating material or released from alkali-labile attachment to a membrane constituent.

It has been suggested that the cation-sensitive autolysis of the envelope of

marine pseudomonad NCMB 845 is initiated by the appearance of negative charges on the membrane[2]. Because dissolution of the halophil membranes was under consideration as possibly an extreme example of this phenomenon, attention was directed to the content in the membranes of the major acidic and basic amino acids. The mean mole percentages of these amino acids were: aspartic plus glutamic acids, 24.4 (9 preparations); arginine, 5.4 (7 preparations); lysine, 4.3 (3 preparations). Comparable figures for two envelope preparations of NCMB 845 were: aspartic plus glutamic acids, 15.9; arginine, 5.6; lysine 5.4 Some asparagine was present in the envelope of each organism but no correction has been made for amide. Thus, if amide is ignored, the halophil membrane contains an excess of acidic over basic amino acids of about 15 mole per cent compared with an excess of about 5 mole per cent in envelopes of the marine organism.

DISCUSSION

The extreme rapidity of dissolution, even at 0°, the occurrence of dissolution in heated membranes, the separation of several distinct macromolecular components in the ultracentrifuge and the evidence for no breakage of peptide bonds during dissolution all lead to the conclusion that the process of dissolution is a non-enzymic disaggregation into the macromolecular components of the membrane. It is relevant to an understanding of membrane structure that the products of disaggregation appeared to consist of protein plus lipoprotein (broadly speaking) rather than protein plus lipid. (There is, at present, no evidence related to the possible breakage of non-peptidic covalent bonds. This is not considered likely but, should it occur, such breakage is not sufficiently extensive to produce significant quantities of low molecular weight products.) The disaggregation of membranes of this kind presents an unusual, perhaps even a unique, opportunity of obtaining all the components of a lipoprotein membrane in aqueous solution and hence in a form which is potentially susceptible to fractionation by chromatographic and related methods.

The effects of salt concentration, bivalent cations and pH on disaggregation are all consistent with a mechanism which operates principally through exposure, on the membrane, of a net negative charge. This type of mechanism was proposed to account for the changes leading to autolysis in the cell envelope of the marine organism NCMB 845 (ref. 2). In the envelope of the marine organism a reduction in ionic strength led to proteolysis, presumably as a consequence of conformational changes in membrane proteins[2]. In the halophil membrane, however, the overall effect was more drastic; the forces induced on lowering salt concentration were sufficiently great to separate components rapidly from each other without invoking proteolysis. That this difference in repulsive forces involved a difference in magnitude of the net negative charge is supported partly by the different iso-electric points of the two types of envelope. The envelope of NCMB 845 had an iso-electric point near pH 4.5; judging from aggregation of membrane suspensions, that of the halophil membrane is less than pH 3 (Fig. 4).

The two curves of Fig. 4 have the shape of titration curves with pK values of about 3.5 and 4.5 respectively. From a study of acid-dissociation constants (see ref. 15), it can be seen that, of the major acid groups occurring in lipoprotein membranes, the acid dissociation constants of (lipid) phosphate are sufficiently different from

pK 3.5 and 4.5 to exclude it as a likely factor in disaggregation. On the other hand the dissociation constants of the β-and γ-carboxyl groups of peptide-bound aspartic and glutamic acid are close enough to pK 3.5 and 4.5 to implicate these two amino acids as a probable source of the negative charges responsible for membrane disaggregation. This evidence is supported by the relatively high proportion of these two amino acids in the halophil membranes. It would be surprising however, if the simple molar proportions of aspartic and glutamic acids were the only factor causing membrane disaggregation. The effectiveness of the total net charge developed on lowering the salt concentration is likely to be affected also by the proximity of $-COO^-$ groups to each other and to the basic side chains of arginine and lysine.

If the foregoing mechanism proposed for disaggregation is valid then stabilization of membranes by NaCl is in agreement with the predictions of the Debye–Hückel theory, according to which each negative charge will be surrounded by an "atmosphere" of Na^+ ions. Bivalent cations will act similarly with the added likelihood that salt bridges will be formed between neighbouring $-COO^-$ groups. The much greater effectiveness of Ca^{2+} than Na^+ is probably explained in this way (as it was in stabilizing the envelope of NCMB 845 against autolysis[2]).

Under favourable physiological conditions *H. halobium* seems to be quite rigid. In addition to stabilizing the membranes against disaggregation, cations probably also make an important contribution to the rigidity of the cell envelope which, in this organism, contains no peptidoaminopolysaccharide (mucopeptide)[3]. Rigidity in a membrane is a manifestation of resistance to flexing. If the charged groups on a membrane are surrounded by an ion atmosphere of opposite sign, movement of any of these groups through the solution will be resisted by a mechanism similar to the relaxation or asymmetry effect which opposes migration of an ion in solution. The membrane has a number of negative charges distributed along a surface, however, and some of these charges are no doubt close enough to one another to cause interaction of their respective ion atmospheres. When this happens orientation of solvent and solute ions will occur along the membrane surface and disturbance of this orientation, as in flexing the membrane, should require work to be done. Again bivalent cations will act similarly but, for a given ionic strength, should be more effective in stiffening if they are able to form bridges between two negative charges.

The effectiveness of an ion atmosphere in stiffening a membrane in accordance with this hypothesis should increase with the number of available charges on the membrane and the ionic strength of the suspending solution. Thus halophilic bacteria should be not only more dependent on high salt concentration but more able to benefit from it morphologically than bacteria of moderate salt requirements, such as NCMB 845, which have a lower content of aspartic and glutamic acids in the cell envelope. From these considerations it is evidently desirable, when assessing properties of the cell envelope of halophilic bacteria, to regard the ion atmosphere as an essential component of that structure.

ABRAM AND GIBBONS[16] examined the effects of salt and other factors on the morphology of halophilic bacteria and suggested that "the cell walls of these organisms are held together rather loosely, as by hydrogen bonds, coulomb forces or "salt" linkages". Present evidence is that under normal conditions the membranes of *H. halobium* are no weaker and, in the light of the foregoing discussion, might even be stronger than many other types of lipoprotein membranes. Because they can be

exposed to a greater ion deficit, however, they can be subjected to correspondingly greater disruptive forces than are possible to develop in membranes of other organisms.

It is to be expected from the evidence of this work and the interpretation which has been placed upon it that there will be a broad relation between the content of excess aspartic and glutamic acid in membrane proteins and the ionic conditions required to stabilize that membrane. The validity of this expectation should be readily established by a sufficient number of observations.

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