On the Structural Transformations and Lysis of *Halobacterium salinarium* in Hypotonic and Isotonic Solution s

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SUMMARY

When the NaCl concentration of a suspension of *Halobacterium salinarium* is gradually lowered by adding water, the rod-shaped organisms are converted to spheres which lyse. The organisms do not change in size when transformed from rods to spheres. Chemicals which do not possess a strong net charge in aqueous solutions do not protect the structure of the organism when they replace NaCl in iso-osmolar concentrations. The rod shape of the organisms is only maintained by very high concentrations of ions which interact weakly with common proteins; lithium and ammonium ions are exceptions to this rule. Ions which display strong interactions with common proteins, and chemicals which are believed to break secondary bonds between protein molecules, effect a transformation of rods to spheres, and frequently a lysis of the spheres, when brought in contact with organisms suspended in strong NaCl solution. The changes in structure in hypotonic and isotonic solutions are not affected by metabolic inhibitors. Isolated cell-wall fragments disintegrate into smaller units when exposed to conditions under which whole organisms lyse. It is concluded that the structural transformations and lysis of *H. salinarium* in hypotonic solutions are not caused by the action of enzymes and that osmotic phenomena play no, or only a minor, role. The observations support the contention that the globular lipoprotein particles, which constitute the bulk of the material of the cell wall of these bacteria, are bound together mainly by electrostatic forces and secondary bonds. When the cells are exposed to hypotonic solutions, or to ions which bind strongly to proteins, or to chemicals which are believed to break secondary bonds between protein molecules, the linkages binding the lipoprotein particles together are weakened so that the wall structure disintegrates. Only in the presence of high concentrations of sodium and chloride ions, or other ions which bind loosely to proteins, is it possible for the proteinaceous particles of the cell wall to associate in an orderly array.

INTRODUCTION

Bacteria belonging to the genus *Halobacterium* Elazari-Volcani (*Bergey's Manual*, 1957) require at least 2.5 to 3 M-NaCl for growth; most species grow best in a medium containing 4 to 5 M-NaCl. The NaCl requirement for growth is specific; attempts to substitute other chemicals for NaCl have been unsuccessful. When cultivated in a medium giving good growth, the organisms normally are slender uniform rods of varying length. When the salt concentration of a suspension containing normal

rod-shaped organisms is gradually lowered by adding water, the organisms change their shape. At concentrations in the range 3 to 3.5M, most of the rods appear somewhat irregular; at concentrations in the range 2.5 to 3M, bulgy and apiculate structures are formed; at concentrations in the range 1.5 to 2.0M, most organisms appear as spheres. When the salt concentration is further lowered, the individual spheres suddenly lyse. Lysis normally occurs in the salt concentration range 1.0 to 1.5M (Klebahn, 1919; Petter, 1932; Christian, 1956; Abram & Gibbons, 1960). Evidence has been presented that the intracellular salt concentration in *Halobacterium* is very high, possibly as high as in the medium in which the organisms are grown (Christian, 1956; Christian & Ingram, 1959). Therefore, it seemed reasonable to ascribe the deformation and lysis of the organisms in hypotonic solutions to an osmotic effect. When the saline environment was diluted with water, an excess osmotic pressure would build up inside the organisms, resulting in the formation of spherical structures from the rod-shaped forms, and at higher dilutions in a disruption of the cell envelope (Christian, 1956; Ingram, 1957). Abram & Gibbons (1960, 1961) presented data which indicate that the deformation and lysis of *Halobacterium* in hypotonic solutions are not simply due to an osmotic effect. They suggest that *Halobacterium* requires a high sodium chloride content in its environment to maintain a rigid cell-wall structure. The present work was undertaken to examine these phenomena.

METHODS

Organism. *Halobacterium salinarium* strain 1 was isolated in our laboratory from salt fish, and maintained on a yeast-autolysate tryptone salt medium (described below) to which 2% (w/v) agar (Difco) was added.

Growth in liquid cultures. The organisms were usually grown in 500 ml. flasks containing 100 ml. medium of the following composition (% w/v): 25, NaCl; 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.02, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 0.25, tryptone (Oxoid); 5, yeast autolysate; in tap water; pH 7.0. The yeast autolysate was made by incubating a suspension of 500 g. baker's yeast in 500 ml. tap water at 45° for 24 hr. The suspension was then heated to boiling, adjusted to pH 7.0 with NaOH, and filtered. Tryptone and yeast autolysate were autoclaved separately from the salt components. The flasks were inoculated with 1.4 ml. of a 48 hr. culture and incubated at 37° on a reciprocating shaker (84 oscillations/min. of excursion 5 cm.).

For the study on cell walls, organisms were grown in a 25 l. carboy containing 10 l. medium of the composition described above. The inoculum (300 ml.) consisted of organisms grown for 48 hr. on the shaker. The culture was incubated with aeration (20 l./min.) at 37°; 4.0 ml. of autoclaved sperm whale oil were added to prevent foaming.

Chemicals. Guanidine nitrate was synthesized as described in *Organic Syntheses* (1947). The other chemicals were obtained commercially. Commercial monobromoacetic acid was distilled 3 times; commercial monochloroacetic acid and dichloroacetic acid were distilled twice. Commercial monoiodoacetic acid was recrystallized 5 times from light petroleum.

Studies on whole organisms

Preparation of suspensions of organisms. Organisms were harvested after 48 hr. (i.e. close to the end of the exponential growth phase) by centrifugation at 10,000 *g* for 5 min. in the cold. The pellets were washed once in the centrifuge with 4.3 M-NaCl, then resuspended in 4.3 M-NaCl to give a reading of 0.36 in an EEL Portable Colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with 5 ml. standard cuvettes and filter no. 608. Such suspensions will be referred to as 'standard suspensions'. Some difficulty was encountered in suspending the fragile organisms without damage. The following procedure, by which only a small fraction of the organisms was structurally changed, was finally adopted: 5 ml. 4.3 M-NaCl was added to the pellet and the mixture homogenized by being carefully sucked into and blown out from a pipette 3–5 times.

Exposure of organisms to hypotonic solutions. Samples (0.1 ml.) of a standard suspension of organisms was pipetted at room temperature into a series of test tubes, each containing 0.9 ml. of a NaCl solution ranging in concentration from 0 to 4.3M.

Substitution of salts and non-electrolytes for NaCl. The main purpose of these experiments was to obtain information on the effect of various chemicals, substituted in iso-osmolar concentrations for NaCl, upon the shape of the organisms. Because of lack of data in the literature on the dissociation and osmotic pressure of strong salt solutions, osmolarity of the salts in solution was computed from the simple formula $(m+n)M$, in which m and n designate the valencies of the cations and the anions, respectively, and M the molar concentration. The values for osmolarity thus obtained are approximations only, but are considered sufficiently accurate for the present work.

Samples (0.1 ml.) of standard suspension were pipetted at room temperature into a series of test tubes, each containing 0.9 ml. of a mixture of 8.6 osmolar NaCl and 8.6 osmolar solution of test substance in a proportion which varied from tube to tube. The solutions of test substances had previously been adjusted to pH 7.0 with NaOH or HCl; exceptions were the solutions containing $ZnCl_2$, $CdCl_2$ and $MnCl_2$ which were adjusted to pH 6.0–6.5, i.e. the limit at which insoluble hydroxides were formed.

Experiments with metabolic inhibitors. These experiments were designed to test the effect of metabolic inhibitors on the structural transformation patterns which were displayed by *Halobacterium salinarium* when the saline environment was diluted with water, or when other chemicals were substituted for NaCl in iso-osmolar concentrations. To a standard suspension was added metabolic inhibitor in concentration as listed on page 276. After incubation for 20 min. at room temperature 0.1 ml. of the suspension was pipetted into a series of test tubes, each containing 0.9 ml. of a hypotonic or an isotonic salt solution to which had been added inhibitor in the same concentration as in the standard suspension of organisms.

Evaluation of structural changes. A problem was to find a rational way of expressing the degree of structural deformation of the organisms when exposed to unfavourable conditions. In the past, measurements of turbidity or optical density have been used to register lysis (Christian, 1956). For the present work, this method was not satisfactory. We observed, as did Abram & Gibbons (1961), that when lysis of *Halobacterium* was brought about by changes in the concentration of certain salts,

there is no clear correlation between lysis and decrease in optical density. This might, in part, be due to the changes in refractive index of the suspending medium. Furthermore, measurements of optical density did not give satisfactory characterization of the structural changes taking place before lysis.

Table 1. *Numerical expression of different stages in the structural transformation of cells of Halobacterium salinarium*

Structure number	Appearance of cells	Description
1		Mostly uniform rods, some slightly irregular rods
2		Mostly slightly irregular rods, some bulgy and apiculate forms
3		Mostly bulgy and apiculate forms, some spheres
4		Mostly spheres, some bulgy and apiculate forms
5		Spheres lysing rapidly

In most of our experiments, the structural deformations were elicited by exposing non-proliferating organisms to chemicals in high concentrations. During the work, it became clear that when the organisms were deformed as a result of such exposures, the structural changes closely followed the pattern described by earlier workers for *Halobacterium* exposed to a gradual decrease of the salt concentration in the environment. The uniform rods were transformed, through transition forms, into spheres, and the spheres frequently lysed. The degree of structural alteration depended upon the severity, rather than the time, of exposure, e.g. the concentration of a given chemical. When exposed to such unfavourable conditions, the organisms would take on a new shape in less than 30 sec. Transition forms between rods and spheres were stable for several hours. Spheres appeared quite stable under some conditions; under other conditions they lysed more or less rapidly. The two different patterns of conversion from rods to spheres reported by Abram & Gibbons (1961) were not observed in our work. In view of the above considerations, it appeared most useful to express the degree of structural deformation by assigning a number to a certain structural state of the organisms (Table 1). In separate tests, it was shown that the degree of structural alteration resulting from a certain treatment

was reproduced with sufficient accuracy. In all experiments, the structural state of the organisms was observed in a phase contrast microscope 0.5–2 min. after exposure. The 'structure numbers' recorded were used in graphical representations of the results by plotting the numbers along an arbitrary scale.

Studies on cell-wall fragments

Isolation of cell-wall fragments. When the culture in a 25 l. carboy was full-grown (70 hr.), the organisms were harvested with a Sharples centrifuge at room temperature. The following manipulations were then carried out at 0°. The organisms were washed carefully twice on the centrifuge with a standard salt solution containing (% w/v): 25, NaCl; 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.02, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; pH 7.0, and the sediment (17 g. wet weight) was suspended in 30 ml. of the standard salt solution. 50 ml. of glass beads (Ballotini, no. 12) were added and the suspension was treated for 5 min. in a MSE Homogenizer run at maximum speed. The glass beads were separated from the suspension by passage through a coarse sintered glass filter (no. 1). The filtrate was subjected to fractional centrifugation. As demonstrated by electron microscopy, cell-wall fragments were sedimented at 7,000–15,000 *g* (30 min.). The sediment was washed 15 times with 10 ml. of the standard salt solution by re-suspension and centrifugation at 30,000 *g* for 40 min. Purity of the wall preparation was assessed by electron microscope examination of samples. Repeated washing was necessary to obtain a preparation of cell-wall fragments of satisfactory purity. The cell-wall fragments were finally suspended in 0.5 ml. of the standard salt solution and stored at 0°.

Experiments with cell-wall fragments. These experiments were designed to test the effect upon cell-wall fragments of substituting other chemicals for NaCl in iso-osmolar concentrations, and of diluting the saline environment with water. In experiments of the first type, 0.01 ml. samples of the suspension of cell-wall fragments were pipetted into a series of test tubes, each containing 0.09 ml. of an 8.6 osmolar solution prepared by mixing the standard salt solution and an 8.6 osmolar solution of the chemical to be tested. The solutions of test chemicals had previously been adjusted to pH 7.0. Since the standard salt solution was 8.6 osmolar with respect to NaCl, the osmolar concentration of the sum of chemicals was near 8.6 in all the mixtures. In experiments on the effect of diluting the saline environment, 0.01 ml. of the suspension of cell-wall fragments was pipetted into a test tube containing 0.09 ml. water. The resulting suspensions were homogenized by being carefully sucked into and blown out from a Pasteur pipette 3 to 5 times. The experiments were made at room temperature. Samples were removed for examination of the cell-wall fragments in the electron microscope.

Electron microscopy. Carbon replicas of the cell-wall fragments were made by the methods of Dalitz (1953) and Roelofsen, Dalitz & Winjman (1953) for wall constituents of plants. The preparations were shadowed with platinum at 27° before being covered with a film of carbon. As shown in the present work, cell walls of *Halobacterium* dissolve when brought in contact with pure water. It was therefore unnecessary to use chromic acid for removal of the organic material which adhered to the carbon film; keeping the carbon film in distilled water for 2 hr. sufficed. The carbon replicas were examined in a Siemens Elmiskop I electron

microscope at accelerating voltage 80 kV. The salt contained in the suspensions of cell-wall fragments formed numerous crystals in the preparation of specimens for examination in the electron microscope. Fields which showed replicas of these crystals were avoided when photographing the specimens.

RESULTS

Structural changes brought about by dilution of the saline environment with water

The structural changes taking place when normal rod-shaped *Halobacterium salinarium* organisms were exposed to environments of progressively smaller salt content are shown in Fig. 1. The spheres which were formed at concentrations below 5 osmolar NaCl were surprisingly small as compared with the rods from which they originated. The average sizes of spheres and rods were computed on the basis of measurements of many individuals of each type. The results (Table 2) show that the average sphere had a volume about half of that of the average rod. In further experiments, the structural transformations of individual organisms were observed. Such observations could easily be made on a slide preparation of bacteria in 8.6 osmolar NaCl by adding a small drop of water to the edge of the cover slip. It appeared that a short rod normally gave rise to one sphere. Longer rods, however, normally gave rise to 2–4 spheres, depending upon the length of the rod. From observations on many organisms it was concluded that an average of about two spheres was formed from each rod. Within the limits of our simple technique, this figure seemed to be independent of the rate at which the rods were converted to spheres. Considering the average size of the spheres in relation to the average size of the rods (Table 2), it thus appears that the total volume of the spheres was close to the total volume of the rods from which the spheres were formed.

Table 2. *Comparison of size of normal, rod-shaped cells of Halobacterium salinarium and spheres formed from the rods by dilution of the saline environment*

The rod-shaped organisms were suspended in 8.6 osmolar NaCl. The spheres were produced by diluting the suspension with water to 4.0 osmolar NaCl. The figures are arithmetic means of measurements on 40 individuals of each type.

	Rods	Spheres
Length	4.3 μ	—
Width	0.7 μ	1.2 μ
Volume	1.7 μ^3	0.9 μ^3

Structural changes by substitution of various chemicals for Na⁺ and Cl⁻

Replacement of Cl⁻ by other anions. The effects of several Na salts upon the shape of *Halobacterium salinarium* are graphically represented in Figs. 1–4. It appeared that the different anions had strikingly different effects. When chloride was replaced by nitrate or sulphate, the rods took on irregular shapes, the irregularity being greater the higher the degree of replacement (Fig. 1). However, these anions protected the organisms to some extent, since in their presence less deformation occurred than when the NaCl solution was simply diluted with water. On the other hand, when chloride was replaced by perchlorate or thiocyanate, a more extensive deformation of the organisms occurred than when the NaCl solution was simply

diluted with water (Fig. 1). The rod-shaped organisms changed to spheres which lysed rapidly at relatively low degrees of replacement, i.e. at NaCl concentrations still so high that no or only a slow lysis would take place if thiocyanate or perchlorate had not been present. Accordingly, it appeared that these anions enhanced the structural transformation.

The effect of fluoride could not be tested because of the low solubility of NaF. Of the other halides (Fig. 2), only chloride protected the rod shape of the organisms. When chloride was replaced by bromide, the organisms were transformed from rods to spheres as the chloride concentration decreased, at about the same chloride concentrations as if the suspension had been diluted with water. Bromide therefore gave no protection of the rod shape. On the other hand, the spheres that were formed in solutions containing bromide did not lyse even at very small chloride

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Structural transformations of *Halobacterium salinarium* by dilution of saline environment with water and by replacement of NaCl by various Na salts.

Fig. 2. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by Na halides.

Fig. 3. Structural transformations of *Halobacterium salinarium* by substitution of Na chloroacetates for NaCl.

concentrations. Bromide therefore prevented lysis of the spheres. When chloride was replaced by iodide, the rods were transformed to spheres at chloride concentrations which would have protected the rod shape if iodide had not been present. Thus, iodide enhanced the transformation of rods to spheres. The spheres appeared to be relatively stable even at small chloride concentrations, but some lysis occurred upon prolonged incubation. In further experiments, the interesting observation was made that when a comparatively small amount (0.05 mg./ml.) of iodine was present in the iodide solution, the spheres formed would lyse rapidly.

In similar experiments where NaCl was replaced by Na salts of organic acids in iso-osmolar concentrations, the following anions gave good protection of the rod shape: formate, acetate, propionate, lactate, succinate; chlorinated derivatives of acetate, however, did not protect. The structural transformation took place at lower degrees of replacement the higher the number of chlorine atoms in the acetate ion (Fig. 3). The effect of trichloroacetate was especially striking: the organisms changed to spheres which lysed rapidly at comparatively very low degrees of

replacement of chloride by trichloroacetate ion. An analogous picture emerged when the effects of the various monohalogenated acetates were compared. The structural transformations took place at lower degrees of replacement the higher the atomic weight of the halogen (Fig. 4).

Replacement of Na^+ by other cations. The effect of replacing Na^+ by other cations upon the structure of *Halobacterium salinarium* was tested. As in the experiments with anions, strikingly different effects were observed, depending upon the chemical nature of the cation. Some of the results are shown in Fig. 5. The cations could be arranged in a series according to their effect upon the organisms: Na^+ ; K^+ ; Mg^{2+} ; Ca^{2+} ; Mn^{2+} ; Sr^{2+} ; Zn^{2+} ; Li^+ ; NH_4^+ ; Cd^{2+} . When they replaced Na^+ , the cations

Fig. 4

Fig. 4. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by Na halogenated acetates.

Fig. 5

Fig. 5. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by various chlorides.

listed to the left gave a good or fair protection of the rod shape. Decreasing degrees of protection were given by the cations as listed from the left to the right. The cations listed at the right end of the series not only did not protect the rod shape, but appeared to enhance the transformation of rods to spheres. For some of the cations, high degrees of replacement were precluded either by limited solubility of the chloride salts, or by aggregation of the organisms in their presence. In no case was lysis observed. Trimethylamine and guanidine, which in solution at pH 7 have a strong positive charge, were tested in similar replacement experiments. Trimethylamine appeared to give a fair protection of the structure of the organisms; guanidine, however, enhanced the structural transformations.

Replacement of NaCl by some non-electrolytes. When NaCl was replaced by non-electrolytes, none of a number of compounds tested protected the rod shape of the organisms. Some of the compounds appeared to promote a transformation of rods to spheres; a few also caused lysis of the spheres. Some of the results are shown in Fig. 6. The compounds could be arranged in a series according to their effect:

ethyleneglycol, glycerol; acetamide; formamide; formaldehyde, ethanol, urea. When NaCl was replaced by ethylene glycol or glycerol, the structural transformations took place at about the same NaCl concentrations as when diluting with water. The other chemicals rendered the organisms susceptible to the structural changes, and with increasing intensity as listed from the left to the right in the above series.

Effect of chemicals on organisms suspended in strong NaCl solutions

The chemicals used in the replacement experiments reported above were dissolved in 8.6 osmolar NaCl and different amounts were added to organisms suspended in 8.6 osmolar NaCl. The picture which emerged from these experiments was as follows. Those chemicals which showed protection of the rod shape in the replacement experiments did not change the structure of organisms suspended in 8.6 osmolar

Fig. 6

Fig. 7

Fig. 6. Structural transformations of *Halobacterium salinarium* by replacement of NaCl by various non-electrolytes.

Fig. 7. Structural transformations of *Halobacterium salinarium* when suspended in 8.6 osmolar NaCl and exposed to increasing concentrations of various compounds.

NaCl. The same applied to all chemicals which in the replacement experiments gave no protection of the organisms, in the sense that in their presence the organisms changed to spheres which lysed at the same NaCl concentrations as when the NaCl solution was diluted with water. However, all chemicals which enhanced the structural transformations in the replacement experiments did the same to organisms suspended in 8.6 osmolar NaCl, although less drastically, when applied in the same concentrations. Some of the results are given in Fig. 7. The curves should be compared with the corresponding curves in Figs. 2, 3, 5, and 6. It appears that NaCl in high concentrations tended to protect the organisms from chemicals which affected their normal shape.

The non-ionic detergents Tween 20 and Tween 80 only slightly affected the shape of organisms suspended in 8.6 osmolar NaCl when added in concentrations up to 3.5% (w/v). The anionic detergent Na dodecylsulphate is only slightly soluble

in 8.6 osmolar NaCl, and at 0.0035% (w/v) had no immediate effect upon the shape of the organisms. However, after incubation for 24 hr. at room temperature, the rods appeared irregular in shape and the optical density of the suspension had decreased to about one-third of the original value. The cationic detergent Cetavlon (a product of Imperial Chemical Industries Ltd, which consists of a mixture of dodecyl-, tetradecyl- and hexadecyl-trimethyl ammonium bromide) caused rapid lysis of the organisms when added to 0.01% (w/v). In contrast to other chemicals causing lysis, Cetavlon caused the rods to lyse without previous conversion to spheres. These observations with detergents essentially accord with those of Abram & Gibbons (1961) on *Halobacterium cutirubrum*.

The effect on organisms of suspension in solutions of decreasing solute concentration

Halobacterium salinarium organisms were centrifuged down and the sediments suspended in solutions of several chemicals made up in series of dilutions. The amount of NaCl introduced with the sediment gave a concentration of less than 0.02M, which was in itself far too little to protect the organisms from lysis. The results showed that chemicals which had displayed protection of the rod shape at 8.6 osmolar, displayed less protection the less their concentration. In most cases, the transformation of rods to spheres, and lysis, took place at molar concentrations higher than found for NaCl. In no case did a chemical protect the structure of the organisms at concentrations lower than found for NaCl. Na acetate appeared to be about as effective as NaCl, giving about the same degree of protection at any given molar concentration.

Mager (1959) reported that spermine in minute concentrations prevented lysis of *Achromobacter fischeri* in hypotonic solutions. We found that spermine did not affect the lysis pattern of *Halobacterium salinarium*. It is known, however, that *Halobacterium* organisms exposed to acidic conditions become 'fixed' so that they will not greatly change their structure upon dilution of the saline environment (Browne, 1922; Dussault, 1955). We found that acetic and hydrochloric acids were effective as fixing agents.

Effect of metabolic inhibitors

Most of the experiments reported above on structural transformations of *Halobacterium salinarium* by the replacement of NaCl by various chemicals were repeated in the presence of metabolic inhibitors. General and specific metabolic inhibitors were used, and in concentrations known to stop enzymic activities. The inhibitors used were (M): sodium iodoacetate, 0.1; sodium *p*-chloromercuribenzoate, 0.01; sodium arsenite, 0.01; sodium fluoride, 0.1; sodium fluoroacetate, 0.01; sodium arsenate, 0.01; sodium azide, 0.1; potassium cyanide, 0.01; hydroxylamine, 0.01; ethylurethane, 0.1; formaldehyde, 0.1; copper sulphate, 0.01; mercuric chloride, 0.02. None of the inhibitors effected a change in the structural transformation patterns.

Effect of penicillin on proliferating organisms

Na benzylpenicillin inhibited growth of *Halobacterium salinarium* markedly when added at 1000 units/ml. to exponentially growing shake cultures. In concentrations of 3000–4000 units/ml. Na benzylpenicillin effected a transformation of growing organism to spheres which then lysed.

Effect of temperature

Abram & Gibbons (1961) reported in detail on the structural changes which took place when suspensions of *Halobacterium cutirubrum* were exposed to elevated temperatures. Our observations on *H. salinarium* conform with their findings: when a suspension of normal organisms in 4.3 M-NaCl was heated to 65°, the rods were converted to spheres; on prolonged incubation at this temperature, the spheres lysed.

Effect on cell-wall fragments of various chemicals substituted for Na⁺ and Cl⁻

Plate 1, fig. 1, shows an electron micrograph of carbon replicas of cell-wall fragments. The preparation was made directly on material from a suspension of wall fragments in the standard salt solution described under Methods. A hexagonal arrangement of particles in the wall can be seen. A similar pattern was observed by Houwink (1956) in the wall of whole *Halobacterium halobium* organisms, and may possibly be a characteristic feature of all members of the genus. In the many preparations of this kind made, the hexagonal arrangement of particles was observed in every wall fragment examined, indicating that the pattern is expressed on both sides of the wall. The effect upon cell-wall fragments of various chemicals was tested, using concentrations known from the studies reported above to effect lysis of whole organisms. After exposure to 5.2 osmolar NaCl + 3.4 osmolar NaI + 0.05 mg./ml. I₂ (Pl. 1, fig. 2), or 6.9 osmolar NaCl + 1.7 osmolar Na trichloroacetate (Pl. 2, fig. 3), comparatively few cell-wall fragments were found in the suspension by electron microscopy and they had completely lost their hexagonal particle pattern. After exposure to 6.0 osmolar NaCl + 2.6 osmolar urea (Pl. 2, fig. 4), no cell-wall fragments could be seen. Under the conditions used, the chemical obviously effected a disintegration of the wall fragments into smaller units which formed a homogeneous layer on the specimens prepared for electron microscopy. For comparison, similar experiments were carried out on cell-wall fragments of a non-halophilic fluorescent pseudomonad. These fragments were suspended in water and exposed to NaI (+iodine), Na trichloroacetate, and urea of the same concentrations as used for the walls of *H. salinarium*. Examination in the electron microscope showed no alteration in the cell-wall structure.

Effect on cell-wall fragments of dilute saline environments. The suspension of cell-wall fragments in the standard salt solution was diluted ten times with water to a NaCl concentration of 0.86 osmolar. This concentration had been shown previously to cause lysis of whole organisms. Electron microscopy revealed the complete absence of cell-wall fragments in these diluted preparations. The dilution obviously effected a spontaneous disintegration of the wall fragments into smaller units.

DISCUSSION

On the basis of studies of the effect upon *Halobacterium cutirubrum* of cations of the alkaline series, of urea, of detergents, and of heat, Abram & Gibbons (1961) proposed that the extreme fragility of this bacterium might be due to the cell wall being loosely held together by hydrogen bonds and electrostatic forces; only in the presence of high concentrations of NaCl are the electrostatic forces so screened that

the bonds hold the cell wall together in the shape of a rod. As far as the experiments can be compared, our results on *H. salinarium* agree very well with those of Abram & Gibbons. Further, we can offer evidence to strengthen their conclusion about the cause of the extreme fragility of *Halobacterium* cells and the lysis in hypotonic solutions.

If the structural transformations of *Halobacterium* in hypotonic solutions were due primarily to osmotic effects, one might expect an increase in the volume of the organisms upon dilution of the saline environment with water. Furthermore, one might expect that other chemicals, replacing NaCl in iso-osmolar concentrations, might prevent changes in structure. The experiments showed, however, that no measurable increase in the size of the organisms occurred on dilution of the environment, and that only few of the many chemicals used to replace NaCl protected the rod shape of the organisms. The results thus did not support the osmotic theory, which has prevailed in the literature in the past, to account for the structural transformations and lysis of *Halobacterium* in hypotonic solutions (Ingram, 1957). Recent experiments on a marine bacterium suggest that its lysis in hypotonic solutions might be the result of enzymic degradation of a cell-wall component (Brown, 1961). It seems highly unlikely that the structural transformations and lysis of *H. salinarium* in hypotonic and isotonic solutions are due to the action of enzymes, since our experiments showed that these transformations were unaffected by metabolic inhibitors.

From the experiments on the effect of ions upon *Halobacterium salinarium* an interesting relationship emerges. It appears that a high concentration of ions which are generally known to be poorly bound to, or which interact weakly with, common proteins protect the rod shape. Ions which are generally known to display strong interactions with proteins effect a transformation of the rods to spheres, and frequently also lysis of the spheres. These ions also effect a structural transformation of the organisms in strong NaCl solutions, but less drastically than when they replace NaCl. The latter observation shows that NaCl can counteract the detrimental effect of these ions to some extent. Ions known to interact moderately with proteins have moderate effects upon the cell structure. These rules hold strictly for all anions tested. In the case of the cations, however, lithium and ammonium ions are exceptions; they did not protect the rod shape of *H. salinarium* although they are known to interact only weakly with common proteins.

Polyalcohols did not maintain the rod shape when replacing NaCl. On the other hand, chemicals of this type did not promote the structural changes since when added to organisms suspended in strong NaCl solution they caused no change in structure, and when stepwise replacing NaCl, the structural transformations at any given NaCl concentration were about equal to those which occurred when the saline environment was simply diluted with water. Other organic chemicals, which are believed to break secondary bonds between protein molecules when used in high concentrations (e.g. urea, guanidine) did effect a structural transformation in strongly saline environments.

Proof that the structural transformations of these *Halobacterium salinarium* organisms were due to interference with the structure of the cell wall is given by the electron microscope studies of isolated cell-wall fragments. These studies showed that conditions which caused a structural transformation of whole organisms and lysis also caused a disintegration of cell-wall fragments. Smithies, Gibbons &

Bayley (1955) reported that cell walls of *H. salinarium* contain no, or only traces of carbohydrate. Analyses carried out in our laboratory (Lie, Tangen & Larsen, unpublished) did not show any simple sugars in hydrolysates; a small amount of amino sugar was detected. The bulk of the wall material is composed of lipid and protein; the amino acid composition, which includes diaminopimelic acid, closely conforms with that found for the walls of other Gram-negative bacteria. Weidel, Frank & Martin (1960) provided evidence that the cell wall of *Escherichia coli* is composed of three layers: an outer layer of lipoprotein particles; a middle layer of lipopolysaccharide particles of fibrous structure; an inner layer of protein particles tied together by chains of a molecular structure probably identical with the mucopeptide found in such abundant amounts in the walls of Gram-positive bacteria. The lack of simple sugars in hydrolysates of walls of *H. salinarium* can be taken as evidence that there is no polysaccharide fibre, or very little, in the walls of these bacteria. The penicillin-sensitivity of growing *H. salinarium* and the presence of amino sugar and diaminopimelic acid in wall hydrolysates indicate that a mucopeptide structure is present, but the low amino sugar content suggests that this mucopeptide is a minor component which does not contribute as much to the mechanical strength of the wall as is believed for most other bacteria. The dominating components of the wall of *H. salinarium*, proteins and/or lipoproteins, form globular particles of diameter about 130 Å. and are arranged in a hexagonal pattern (Pl. 1, fig. 1). It seems reasonable to attribute the disintegration of the wall by high concentrations of strongly binding ions to disturbances of the electrostatic forces between the proteinaceous particles. The disintegrating effect of guanidine, urea and compounds of similar properties can be interpreted in the light of the alleged tendency of such compounds to disrupt hydrogen and/or hydrophobic bonds between protein particles.

The proteinaceous sub-units of the walls of *Halobacterium salinarium* are held together in an orderly array only in the presence of high concentrations of ions which bind loosely to proteins. It is well known that loosely binding ions tend to decrease the solubility of proteins when applied in high concentrations, allegedly by decreasing the solubilizing interaction between water molecules and the polar groups of proteins (Taylor, 1953). Baxter (1959) suggested that the activating and protective effects of salts upon halophilic enzymes of *Halobacterium* are due to a reduction by the salts of electrostatic repulsions between charged groups in the enzyme proteins. It seems probable that sodium and chloride ions in high concentrations have similar effects upon the proteinaceous sub-units of *Halobacterium* walls, thereby making it possible for the units to associate to form a surface layer which possesses the properties necessary for normal cellular function.

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EXPLANATION OF PLATES

Figs. 1–4 illustrate by electron micrographs of carbon replicas the disintegration of cell-wall fragments of *Halobacterium salinarium* when NaCl in the suspending solution was partially replaced by other chemicals in iso-osmolar concentrations. Magnification throughout $\times 60,000$.

PLATE 1

Fig. 1. Cell-wall fragments as prepared for electron microscopy from a suspension in 8.6 osmolar NaCl.

Fig. 2. After exposure of cell-wall fragments to a mixture containing 5.2 osmolar NaCl, 3.4 osmolar NaI and 0.05 mg. I_2 /ml.

PLATE 2

Fig. 3. After exposure of cell-wall fragments to a mixture containing 6.9 osmolar NaCl and 1.7 osmolar Na trichloroacetate.

Fig. 4. After exposure of cell-wall fragments to a mixture containing 6.0 osmolar NaCl and 2.6 osmolar urea.