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Regulation of Cell Volume and Ion Concentrations in a *Halobacterium*

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Summary. Changes in cell volume and ion content of a *Halobacterium* species are described in terms of the NaCl concentration (0.5–3.5 M) and pH(4–8) of the suspending medium. Cell volume, per unit content of protein of bacteria in stationary phase cultures, rose as the [NaCl] of the growth medium was increased. Logarithmic-phase bacteria shrank as the pH fell from 7 to 5.5. These changes are characteristic of bacteria with a moderate or rapid rate of O_2 consumption. Starving (i.e. nonmetabolizing) bacteria, on the other hand, did not change in size within the above ranges of [NaCl] and pH. At lower values, however, such bacteria swelled and eventually lysed. Effects of low pH on cell ions are compared in metabolizing and starving bacteria, and it is shown that changes in the state of the cell K are correlated with movements of cell Na. It appears that the cell K is used to maintain cell [Na] below the NaCl concentration of the medium. The results are explained in terms of a model involving interactions between polyelectrolytes, salts and water in the concentrated cytoplasm of these halophilic organisms.

The extremely halophilic bacteria grow in media containing NaCl at concentrations approaching saturation. The cells are characterized by similarly high concentrations of potassium even though the outside medium may contain less than 1 mM K^+ (Christian & Waltho, 1962; Ginzburg, Sachs & Ginzburg, 1970; Gochnauer & Kushner, 1971; Lanyi & Silverman, 1972). In a species of *Halobacterium* isolated from the Dead Sea the high potassium content was found to be maintained at all phases of the life-cycle of the bacterial cultures; the concentration of cell sodium – at all times lower than outside sodium – fell with the metabolic activity of the bacteria (Ginzburg, Sachs & Ginzburg, 1970, 1971). Thus, the behavior of the cell ions is quite different from that of mesophilic tissues examined up to now, in which the prolonged maintenance of ionic concentration gradients across the cell membrane is dependent upon the metabolic activity of the tissue.

A further peculiarity of these halophilic bacteria, described in this paper, is that the cell volume is very variable; each bacterium is enclosed within an elastic cell-envelope (Larsen, 1967) and the cell volume varies with the pH and NaCl concentration of the medium.

The work described here was undertaken to elucidate the mechanisms of control of the cell volume and of the concentrations of the major cell ions (K^+, Na^+, Cl^-). In the present paper changes in cell volume with changes in pH and NaCl concentration of the ambient medium are described. These changes could not be explained by any known osmotic mechanism; they appear difficult to understand except by invoking interactions in which the cellular polyelectrolytes are involved. The same changes in pH and NaCl concentration which affect cell volume have also been found to affect the content of cell potassium although no causal correlation between these two parameters has been detected. A certain fraction of the cell potassium does, however, play a role in the regulation of the cell sodium content.

Materials and Methods

Methods of culture of the *Halobacterium* sp. used have already been described (Ginzburg *et al.*, 1970). The methods of analysis and calculation were described in the same paper. For the preparation of starved bacteria *see* Ginzburg *et al.* (1971).

Growth of Halobacterium sp. at Different NaCl Concentrations in the Medium

A series of culture media was prepared, of which the members were identical in all respects except for the concentration of NaCl which varied from 2 to 5 M. The different media were inoculated at one and the same time, with equal quantities of the same mother-culture. Measurements were made after a 48-hr incubation period, by which time growth (as measured by increase in protein content) had stopped in all the cultures.

Measurements of Cell Mass

Volume. The method for the measurement of packed cell pellets has been described in an earlier paper (Ginzburg *et al.*, 1970).

Fresh Weight and Dry Weight of Bacterial Pellets and Cells. Aliquots of bacterial suspension were centrifuged in tared glass Sorvall tubes for 20 min at $13{,}000 \times g$. The supernatant was removed, leaving the packed bacteria as a tight red mass adhering strongly to the bottom of the tube. The tube sides were quickly rinsed with water and acetone and dried with paper tissue. Tubes were then weighed, yielding the fresh weight of pellets, which were of the order of 20–40 mg. After drying for 48 hr at 105 °C, the tubes were cooled and reweighed to determine the dry weight of the pellets.

This method gave similar results to those obtained by the more laborious methods described earlier (Ginzburg *et al.*, 1970); the value of $35.7 \pm 0.5\%$ for the dry weight of pellets from 48-hr cultures and given in Table 1 of this paper is not very different from that of $38.3 \pm 0.8\%$ for 72-hr cultures quoted in the earlier paper. The dry weights of the cells are,

Table 1. Variation of dry weight content of *Halobacterium sp.* pellets and cells with NaCl concentration of medium

State of bacteria	NaCl (M)	% dry wt		No. of determinations
		Pellets	Cells	
Growing[a]	1.7	38.7±0.5	43.7±1.5	5
	2	34.5±0.25	38.9±1.25	5
	3.5	35.7±0.46	39.2±1.46	5
	5.0	37.2±0.38	39.6±1.38	4
Starving[b]	1	30.3±0.6	36.1±1.6	7
	2	34.5±0.7	40.1±1.7	7
	3	39.2±0.8	44.7±1.8	7
	4	40.3±0.7	45.1±1.7	8

[a] Cultures had been growing for 48 hr at 37 °C.
[b] After equilibration for 2 hr at 20 °C.

however, lower than those of the earlier paper which contained an error in the weight of the dried residue of the supernatant (quoted value: 0.24 mg/ml; actual value 0.24 g/ml).

Experimental Methods

Effect of Nature and Concentration of Salt Solution on Volume of Starved Bacteria. Four-ml portions of a suspension of starving bacteria were centrifuged at 13,000 × g in a Sorvall RC 2-B centrifuge at room temperature for 10 min. The supernatant was poured off and all remaining droplets removed by suction. Four ml of the experimental salt solution were poured onto the pellet which was detached from the tube wall by scraping with a spatula. The experimental salt solution contained the major salt – NaCl or LiCl – at the desired concentration together with 2 mM/liter KCl and 10 mM/liter N,N-bis-2-hydroxyethyl-2-aminoethane sulfonic acid buffer (BES) at pH 7.0. The tube contents were transferred to a 25-ml Erlenmeyer flask which was shaken at 20 °C for 2 hr.

At the end of the incubation period samples were taken for the measurement of bacterial volume and protein in measured samples of suspension.

pH Experiments with Growing Bacteria. Logarithmic-phase cultures at an optical density of 0.2 were used (400–465 nm). About 70 ml of bacterial suspension were poured from the culture flask into a jacketed cylindrical vessel with an inner diameter of 8 cm, forming a liquid layer of about 1.5 cm. This was stirred by means of a magnetic bar. The vessel was covered with Parafilm. A combined electrode (Radiometer GK 264 B, largely insensitive to high salt concentrations below pH 10) connected to a Radiometer pH meter (Type 22 S) dipped into the suspension: the pH was controlled manually by addition of small volumes of 0.01 N HCl or NaOH in 3.5 M NaCl. The temperature of the bacterial suspension was maintained constant by means of a current of water at 37 °C passing through the jacket of the vessel. Samples for the measurement of ions, protein or cell volume were removed from the bacterial suspension as required; 300 µl aliquots were centrifuged in a Beckman Microfuge and the resultant cell mass analyzed for K, Na, Cl or protein, according to methods which have been described elsewhere.

The pellets of bacteria spun down in glass Sorvall tubes under the conditions described consist partly of bacteria and partly of medium trapped between the bacteria. This latter component forms $28 \pm 3\%$ of the total fresh weight of the pellets (mean of five determinations using Dextran blue, a Sephadex product with a molecular weight of 2,000,000 and which does not penetrate through the bacterial membrane). By using the figure of $28 \pm 3\%$ as an estimate of the trapped medium, it is possible to calculate the dry/fresh weight ratio of the bacteria from the measured dry/fresh weight ratio of the pellets.

pH Experiments with Starving Bacteria. The same vessel and experimental techniques were used as for growing bacteria. Other methods of sampling had to be used, however, owing to the large amount of fluid taken up by the bacteria which made them too fragile to be analyzed by the usual means. Eight 10-ml aliquots were centrifuged in weighed glass Sorvall tubes; the supernatants were discarded and the inner walls of the tubes cleaned as described for the fresh weight measurements. All the tubes were weighed. Four were put to dry at 105 °C for 48 hr and then reweighed. These served to determine the water content of the pellets and hence of the cells, the volume of trapped medium being known. The inner walls of the remaining tubes were cleaned particularly carefully to rid them of any contaminating NaCl from the medium. Ten ml of distilled water was added to the tube, the pellet dispersed and the concentrations of K, Na and Cl measured. The values obtained by these methods for five separate suspensions of bacteria at pH 7 were: K: 2.8 M; Na: 1.3 M; Cl: 2.9 M and are close to the values of K: 3.0 M; Na: 1.1 M; Cl: 2.5 M obtained by the methods described formerly (Ginzburg *et al.*, 1971).

Analysis of Ions and Protein

Methods are described in Ginzburg *et al.* (1970). Ions were measured by means of an Eppendorf flame photometer. Protein was measured by use of the Lowry test (Lowry *et al.*, 1951).

Calculation of Results

The volume of bacterial pellets is expressed relative to the bacterial protein content of the same suspensions, in mm^3 per 100 µg bacterial protein. It is assumed that although the volume of a unit of bacterial mass may change, its protein content should remain constant.

In many experiments the bacterial ion content is also expressed relative to protein. In dealing with cell K, a distinction is made in certain experiments between K_{Cl}, that portion of the cell potassium which is balanced by Cl, and K_x, the remainder. K_{Cl} is calculated as the difference between the *measured* Cl and Na; K_x equals total K minus K_{Cl}.

Results

Effect of NaCl Concentration in Medium on Cell Volume

In cultures at the stationary phase of growth the volume of packed pellets of bacteria, per unit of protein, increased with the NaCl concentration of the medium in which the bacteria had grown. These observations are summarized in Fig. 1, curve *A*, from which it can be inferred that there

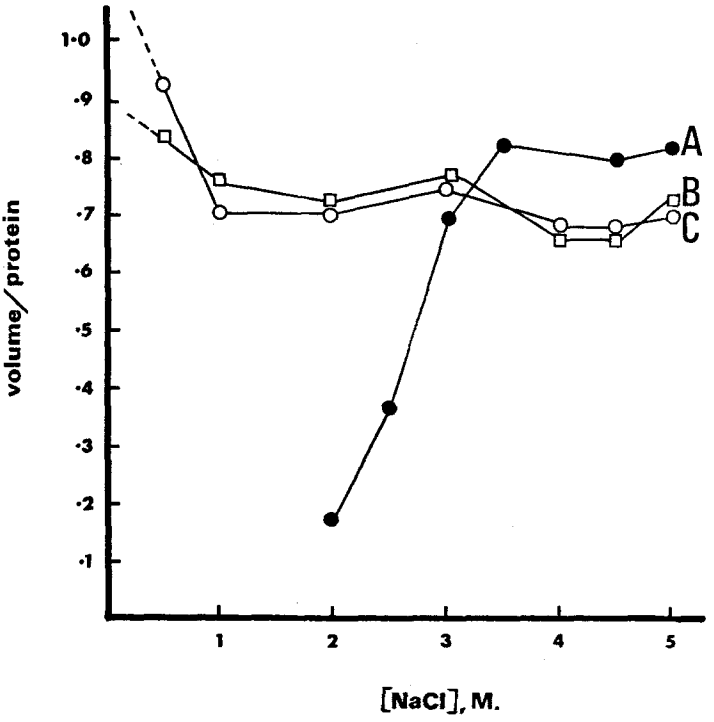

Fig. 1. Effect of NaCl concentration in outside medium on cell volume in *Halobacterium sp.* (*A*) Bacteria from stationary-state cultures grown at 37 °C at the concentration of NaCl indicated. (*B*, *C*) Starved bacteria after 2-hr equilibration at 20 °C in NaCl and LiCl, respectively. Volume/protein: mm^3 packed cells per 100 μg cell protein

is an approximately fivefold increase in the size of cells in 3.5 M NaCl over those in 2 M NaCl (0.18 mm^3/100 μg protein and 0.83 mm^3/100 μg protein in bacteria at 2 M and 3.5 M, respectively).

The increase in cell size must be due to increases both in the relative amounts of cell water and of salts: this emerges from the measurements of dry-weight content, as per cent total fresh weight, given in Table 1. There are no significant differences in these per cent dry weight values of bacteria grown at the different NaCl concentrations.

The above-mentioned parameters can be used to calculate the concentrations of ions, given measurements of cell ion content. Measurements of cell K, Na and Cl concentrations in stationary phase bacteria grown at 3.5 M or 2 M NaCl are given in Table 2. In both types of culture the cell Na and Cl concentrations were lower than in the external medium; $Na_{out}/Na_{in} \cong 7$ for both cultures (7.58 at 2 M and 6.3 at 3.5 M).

Table 2. Summary of ion concentrations inside and outside bacteria

Bacteria	1	2	3	4	5	6	7	8	9	10	Relative volume
	pH	$[NaCl]_o$ (M)	$[K]_i$ (M)	$[Na]_i$ (M)	$[Cl]_i$ (M)	$\sum C_i$ (M)	$[Na]_i + [Cl]_i$	$\sum C_o$	$\sum C_i - \sum C_o$	$[Na]_i + [Cl]_i - \sum C_o$	
I. Starved	7.0	3.5	3.0	1.5	4.1	8.6	5.6	7.8	0.8	−2.2	85
		3	2.7	2	4.2	8.9	6.2	6.6	2.3	−0.4	91
		2	2.1	1.2	2.9	6.3	4.1	4.2	2.1	−0.1	85
		1	1.2	0.45	0.9	2.55	1.35	2.1	0.45	−0.65	85
		0.5	0.5	0.90	1.1	2.5	2.0	1.0	1.5	1	112
II. Stationary state	7.0	3.5	2.73	0.6	2.4	5.73	3	7.8	−2.07	−4.8	100
		3	2.51	0.5	2.0	5.01	2.5	6.6	−1.6	−4.1	85
		2.5	2.9	0.5	2.4	5.8	2.9	5.2	−0.4	−2.3	45
		2.2	2.3	0.3	1.2	3.8	1.5	4.5	−0.7	−3.0	–
		2.0	1.15	0.3	–	–	–	4.2			22
III. Logarithmic phase	7.0	3.5	3.9	1.8	4.5	10.2	6.3	7.8	2.4	−1.5	100
	6.0	3.5	4.0	2.3	5.4	11.7	7.7	7.8	3.9	−0.1	66
	5.5	3.5	4.3	3	5.5	12.8	8.5	7.8	5	0.7	50

In these stationary state bacteria the rate of O_2 uptake is appreciable; in 72-hr cultures the O_2 uptake per unit of cell protein was found to be half that of the rate measured in logarithmic-phase cultures. In starving bacteria, on the other hand, the rate of O_2 uptake fell to an undetectable level (Ginzburg *et al.*, 1971). Bacteria grown and starved at 3.5M NaCl, and then transferred to NaCl solutions at some other concentration, were found not to change in size except below 1M NaCl; at 0.5M NaCl the bacteria swelled (Fig. 1, curve *B*) and at lower concentrations they lysed. A similar volume curve was obtained with bacteria suspended in solutions of LiCl varying in concentration from 0.5 to 5M (Fig. 1, curve *C*). The dry-weight content of starved bacteria fell markedly as the NaCl concentration of the suspending solution was lowered (Table 1); thus, 45.1 % of the total fresh weight of bacteria at 4M NaCl consisted of solid matter while the figure for bacteria at 1M NaCl was only 36.1 %. This difference can be entirely accounted for in terms of losses of potassium chloride and sodium chloride (Ginzburg & Ginzburg, 1975).

Effect of pH on Cell Volume and Ion Concentrations

When bacteria in the logarithmic phase of growth and suspended in growth medium at pH 7.0 were incubated at a pH between 5.5 and 7.0,

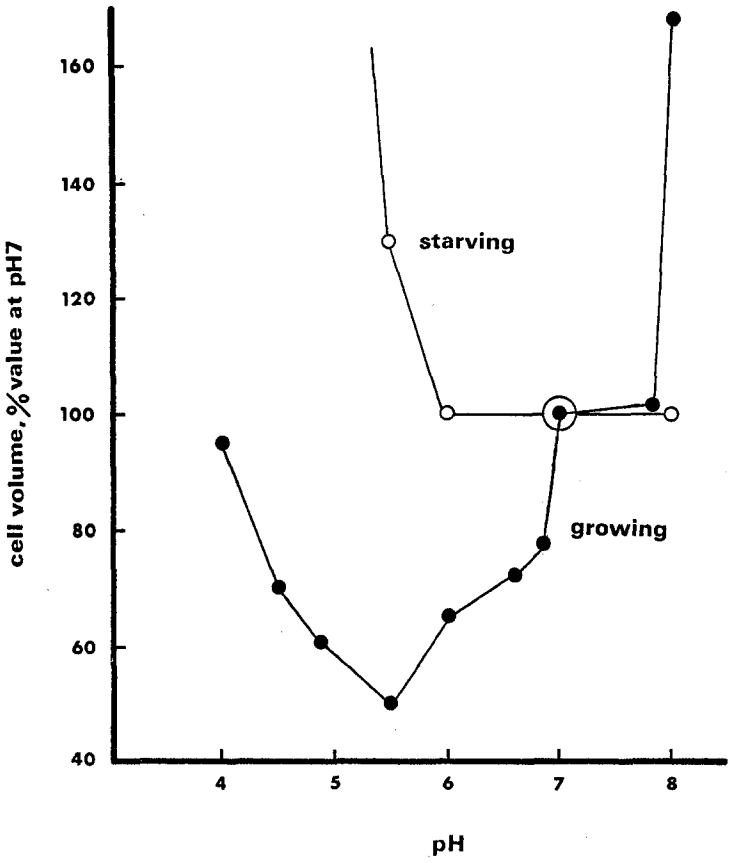

Fig. 2. Cell volume of *Halobacterium sp.* as function of the pH of suspending medium, expressed as % cell volume at pH 7. Suspending medium consisted of complete salt solution (*see* Materials and Methods) + 10% yeast autolysate for the growing bacteria. pH of medium controlled by addition of small amounts of acid or base. Measurements made 2 hr after adjustment of pH. Temperature 37 °C. All points are averages of not less than four separate experiments

the volume of packed pellets was observed to decrease, and it was concluded that the bacteria had shrunk in size (Fig. 2). This phenomenon was observed in detail at pH 6.0 (Fig. 3) and at pH 5.5 (Fig. 4 *A*). Fig. 3 illustrates the sequence of events that occurred when bacteria, originally at pH 7.0, were exposed to medium at pH 6.0 for 90 min, after which the pH was returned to 7.0. During the initial hour of incubation at pH 7 the bacteria grew steadily, as is revealed by the increases shown in all the parameters measured (protein, volume, K, Na, Cl). During the period that the pH was at 6.0, no increase in cell protein occurred, indicating that growth had stopped. Nearly all the other parameters were affected by the low pH:

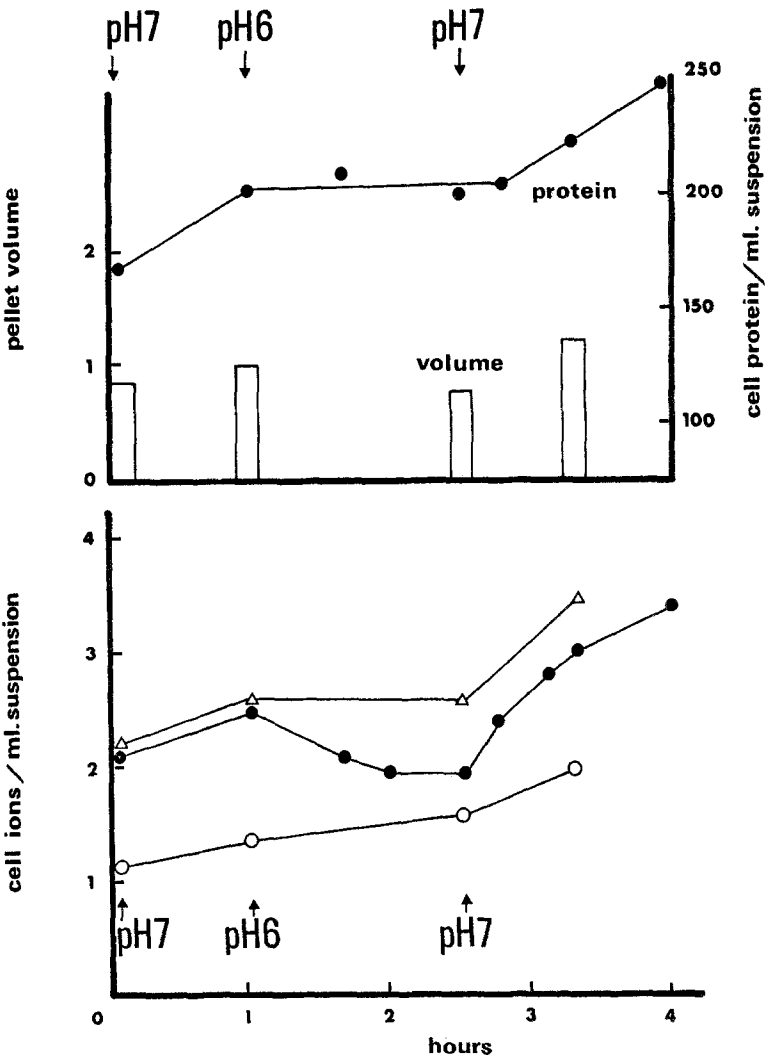

Fig. 3. Effect of change of pH on protein, volume and ion content of *Halobacterium sp.* in logarithmic phase. From hour 0–1 cultures were at pH 7; from hour 1–2½ they were maintained at pH 6; at hour 2½ the pH was returned to 7.0. Temperature: 37 °C. Pellet volume and protein: mm^3 and μg, respectively, both per ml suspension. Ions: K ●; Na ○; Cl △ in μEquiv ion per ml suspension. Mean of two experiments

the pellet volume and cell K fell and the cell Na rose very slightly, though insufficiently to balance the cell K lost. The amount of cell Cl did not change and it appears that most of the cell K which was lost must have been exchanged for H^+. The amount of base effectively lost by the bacteria during the period of adaptation to the low pH was 2–3 times larger than

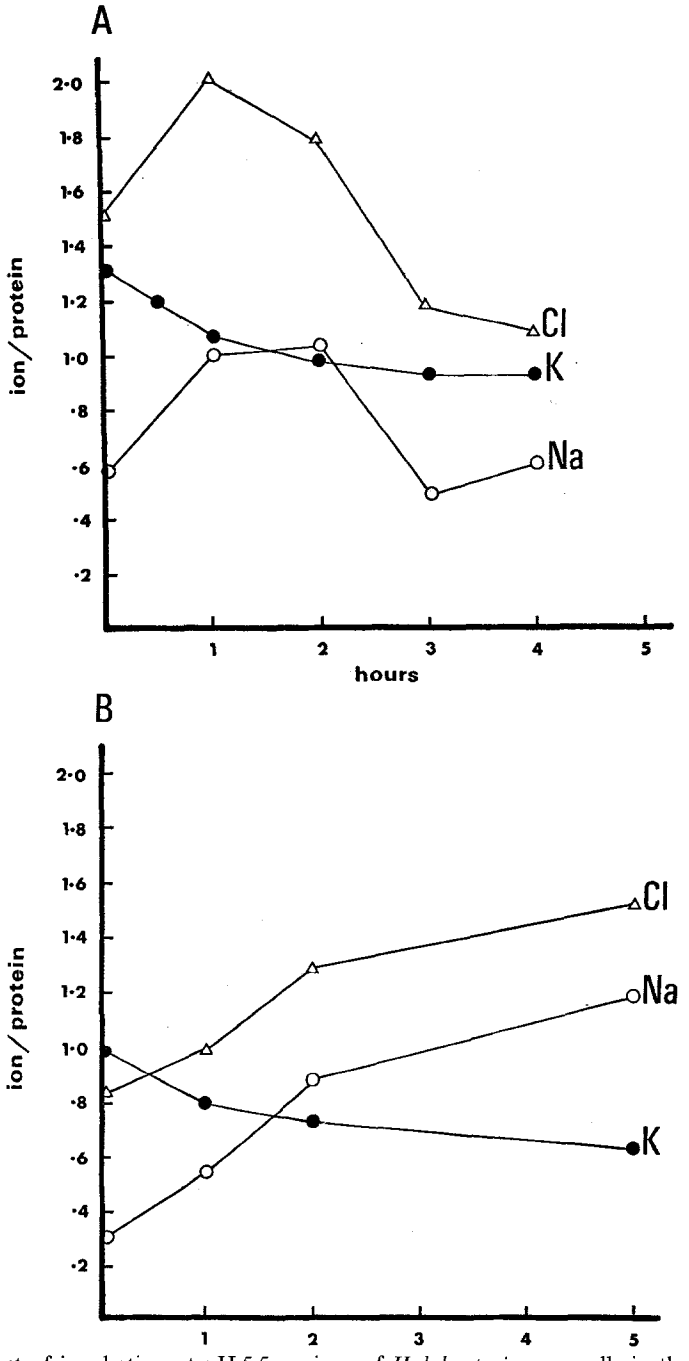

Fig. 4. Effect of incubation at pH 5.5 on ions of *Halobacterium sp.* cells in the logarithmic phase (*A*) or in a state of starvation (*B*). Temperature: 37 °C. Cultures were incubated at pH 7.0 for one hour before the pH was lowered to 5.5. Initial period at pH 7.0 is not shown; i.e. hour 0 represents moment at which pH was lowered. Ion/protein: μEquiv ion per 100 μg protein. (*A*) mean of three experiments; (*B*) one experiment representative of three

the K lost. After the pH was returned to 7.0, all of the K lost was regained and the cells swelled, as indicated by the increase in pellet volume.

Similar, though more pronounced changes were observed in bacteria exposed to pH 5.5 (Fig. 4*A*). In this Figure the initial period of equilibration is not shown, and the ions are expressed directly in terms of the protein content. The pH remained at 5.5 throughout the experiment: no period of

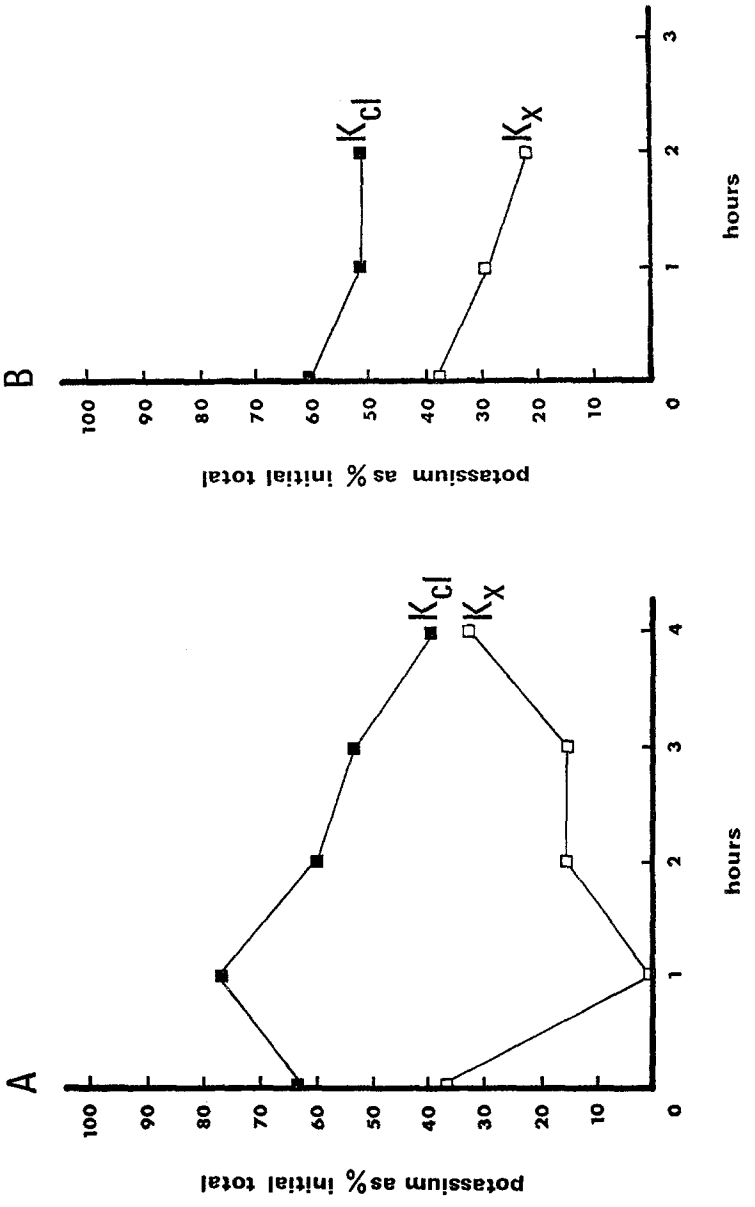

Fig. 5. *Effect of incubation at pH 5.5 on potassium of Halobacterium sp.* cells in the logarithmic state (*A*) or in a state of starvation (*B*). Same conditions as for Fig. 4. K_{Cl}: that part of the total cell K balanced by Cl; K_x: remaining cell K presumed to be balanced by fixed organic anions. *A* and *B* are means of three experiments each

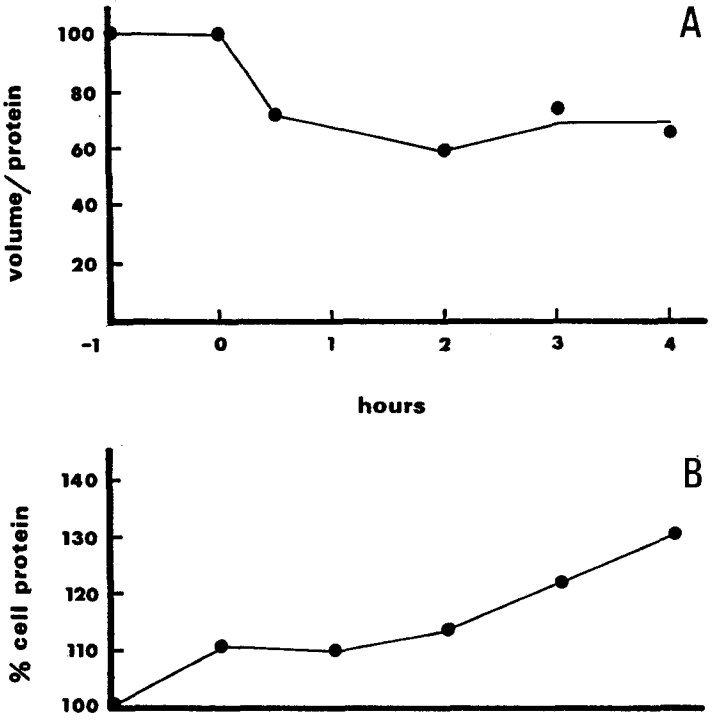

Fig. 6. Effect of incubation at pH 5.5 on volume and protein content of *Halobacterium sp.* cells in the logarithmic phase of growth. Hour −1 to 0 represents period of incubation at pH 7.0; at hour 0 the pH was lowered to 5.5 and was maintained there until the end of the experiment. Temperature: 37 °C. Volume/protein expressed as % of amount before pH was lowered. Cell protein expressed as % of the amount at hour −1

recovery at pH 7.0 was allowed. The Figure shows that loss of cell K proceeded until 69 % of the original amount was left (i.e. 0.92/1.33 μEquiv K/100μ g protein). The loss of cell K was accompanied by a rapid gain of Na^+ and Cl^- for the first hour at pH 5.5. Later, first Cl^- and then Na^+ were lost until the amount of Na^+ left was no different from the start. During the first hour of the experiment, i.e., during the period of parallel increases in Na^+ and Cl^-, the K^+ lost must have been exchanged for H^+; later, however, there was a recovery of K_x at the expense of K_{Cl} (Fig. 5*A*); this coincides with the arrest of the entry of Na^+ into the cells and its subsequent efflux.

The changes in pellet volume shown in Fig. 6*A* reflect the change in size of the individual bacteria. These shrank for the first two hours of their exposure to pH 5.5, and later swelled somewhat, or perhaps shrank for one hour and then didn't change. The shrinking phase is seen to coincide

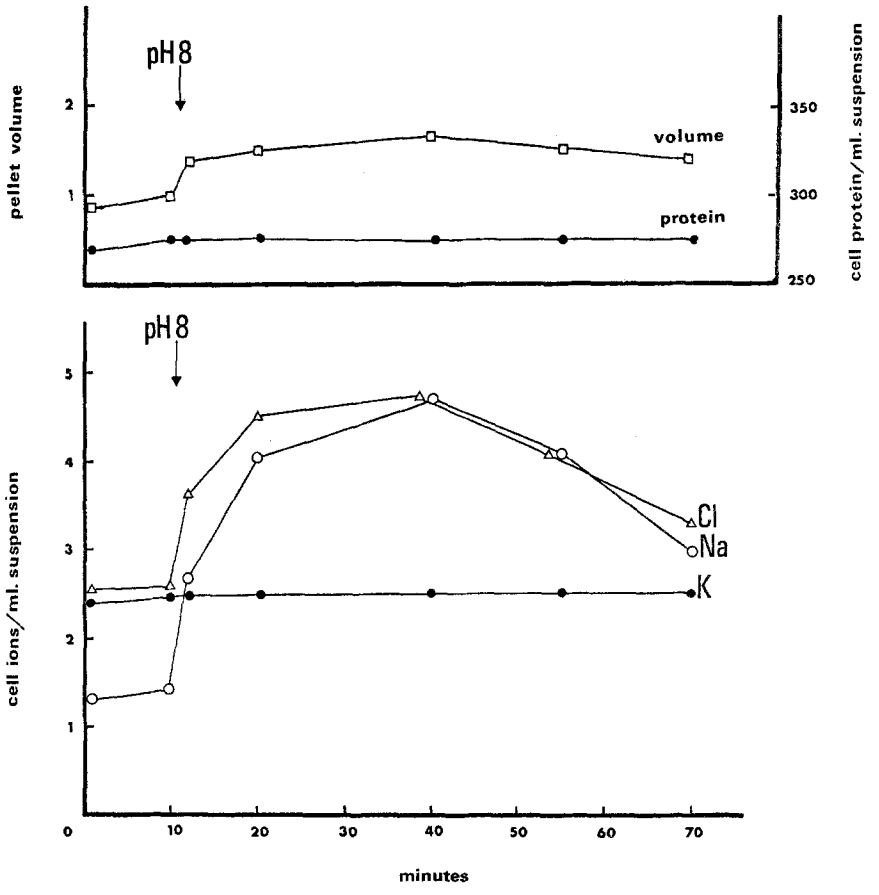

Fig. 7. Effect of incubation at pH 8 on protein, volume and ion content of *Halobacterium sp.* in the logarithmic phase of growth. For the first 10 min the suspension was at pH 7.0. At 10 min the pH was changed to 8.0 and was maintained there until the end of the experiment. Pellet volume, mm^3, and cell protein, μg, both per ml suspension. Cell ions: μEquiv K, Na, or Cl per ml suspension. Temperature of experiment: 37 °C. Mean of four experiments

with the period of loss of K, and the swelling with the renewal of protein synthesis (Fig. 6*B*) and loss of Na^+ (Fig. 4*A*).

When the pH of suspensions of bacteria in the logarithmic phase of growth was altered to a value below 5.5 or above 7.8, the bacteria were observed to swell (Fig. 2). The sequence of events that occurred in bacteria at pH 8 is described in Fig. 7. The swelling – marked by a virtual doubling in pellet volume – was accompanied by an inrush of Na and Cl. The reaction took about 30 min to reach completion, after which there was some reversal of all the observed changes (i.e., Na^+ and Cl^- were lost; the pellets shrank).

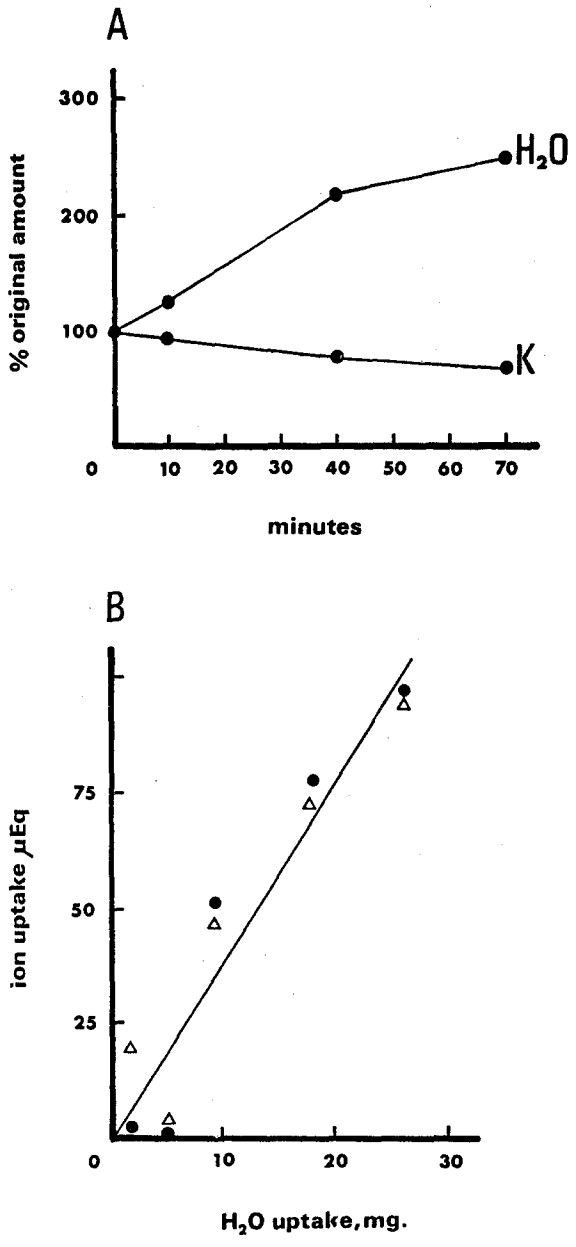

Fig. 8. Effect of incubation at pH 5.0 on starving *Halobacterium sp.* (*A*) Time curves of loss of cell K and gain in water, both as % initial amounts at pH 7.0, after pH had been lowered to 5.0 at time 0. (*B*) Plot to show relation between uptake of water and that of Na (●) and Cl (△). The line drawn shows what the relative uptakes of water and NaCl would be if undiluted NaCl solution were to enter the bacteria from the outside medium

Starving bacteria behaved quite differently from growing bacteria when subjected to changes in pH of the suspending salt solution. In starving bacteria shrinkage of the pellet volume was not observed at any pH, neither did the bacteria swell at pH 8.0 (Fig. 2). They did, however, swell rapidly and irreversibly at low pH (*see* Fig. 8). At pH 5.0 there was the usual progressive loss of K which amounted to 15 % of the initial content after 70 min in the experiments in question. This was accompanied by a rapid uptake of water, which amounted to 270 % of the initial value after 70 min. Water uptake is plotted against gains of Na^+ and Cl^- in Fig. 8*B*. The line represents what the relative uptakes of ions and water would be if undiluted salt solution at 3.9 molal were to enter the cells. It is evident that the measured values fit the calculated line quite closely.

Similar but slower changes were observed in starving bacteria incubated at pH 5.5. A representative experiment is shown (Fig. 4*B*). There was loss of 36 % of the total initial potassium accompanied by a progressive uptake of Na^+ and Cl^-. The proportion of potassium lost was the same as in the bacteria from the growing cultures, but the constant, unimpeded increases in Na^+ and Cl^- observed in the starving bacteria contrast markedly with the changes which occurred in the growing bacteria (*cf.* Figs. 4*A* and 4*B*). There are other differences: K lost from starving was lost largely in exchange for H^+ (in the three experiments in which this parameter was measured the proportions of K_x to total K lost were 86 %, 42 %, and 97 %), whereas in growing bacteria there is an initial loss of K_x followed by its recovery at the expense of K_{Cl} (Figs. 5*A* and 5*B*). It is in the reactions controlling these differences that the mechanism responsible for controlling the cell Na^+, Cl^- and water content is thought to lie.

Discussion

A. *Osmotic Behavior of Halobacterium sp.*

For a biphasic system such as a cell suspension at equilibrium

$$\mu_o = \mu_i \tag{1}$$

μ = chemical potential of water and subscripts o and i refer to water outside and inside the cell, respectively.

$$\mu_o - \mu_i = \Delta\mu.$$

μ can be split into several components, depending upon the interactions occurring in the system:

$$\mu = \mu^* + \mu^p + \mu^c + \mu^{\text{poly}}. \tag{2}$$

μ^* = under standard conditions; μ^p = hydrostatic pressure term; μ^c = dependent on cell solutes; μ^{poly} = dependent on water-polyelectrolyte interactions.

When $\mu^{\text{poly}} = 0$ one can calculate the Van't Hoff osmotic pressures π_i and π_o:

$$\pi_o = RT \sum \phi_o C_o$$

$$\pi_i = RT \sum \phi_i C_i$$

where ϕ = osmotic coefficient and C = solute concentration.

Then

$$RT \sum \phi_o C_o = RT \sum \phi_i C_i - \Delta P. \tag{3}$$

If $\Delta P = 0$, then

$$\sum \phi_o C_o = \sum \phi_i C_i. \tag{4}$$

Since $C_i = N_i/(v-b)$ it is possible to rewrite Eq. (4) as

$$\sum \phi_o C_o (v-b) = \sum \phi_i N_i \tag{5}$$

where v = cell volume, b = nonosmotic component of v, and N_i = moles solute in cell.

Eq. (4) describes the behavior of an ideal osmometer. If the experimental system under consideration does not behave according to Eq. (4), one should consider whether the parameters ΔP or μ^{poly} have values other than zero.

In the first stage of an analysis of the osmotic behavior of the halophilic bacteria described in this paper, it should be assumed that the major ions to be taken into account are K^+, Na^+, and Cl^-; furthermore there are assumed to be no significant interactions involving polyelectrolytes (i.e., $\mu^{\text{poly}} = 0$). By examining Table 2, which summarizes the data needed for an assessment of a halophilic cell as an ideal osmometer, we can see that $\sum C_o \neq \sum C_i$ (column 9): in groups I and III $\sum C_i > \sum C_o$, whereas in group II $\sum C_o > \sum C_i$. Thus, for Eq. (4) to hold, ϕ_o cannot equal ϕ_i. In the concentration range 0.5–4 M, ϕ_{NaCl} and ϕ_{KCl} are 0.68–0.79 and 0.61–0.58, respectively (Robinson & Stokes, 1959); thus the differences between

ϕ_{NaCl} and ϕ_{KCl} are not great. Since the major salt in the outside medium is NaCl, $\phi_o \cong \phi_{NaCl}$. On assuming ϕ_i to have the same value for NaCl and for KCl, then for bacteria in groups I and III $\phi_i = 0.6\text{–}0.9\ \phi_o$ while for bacteria in group II $\phi_i = 1.2\text{–}1.5\ \phi_o$. In groups I and II ϕ_i falls as the outside NaCl concentration is lowered. Since it seems unreasonable to postulate an *increase* in ϕ_i for one group of bacteria and a *decrease* in ϕ_i for the other two groups, one must reject the possibility that a common value of ϕ_i applies to the cell KCl and NaCl. Instead, let us assume that NaCl is the only osmotically active species within the cell, K being bound, and that $(\phi_o)(\mathrm{NaCl})_o = (\phi_i)(\mathrm{NaCl})_i$ according to Eq. (4). On this assumption, we find that $\phi_i = \phi_o$ for bacteria in groups I and III and that $\phi_i = 2\text{–}3\,\phi_o$ for group II, again an unacceptable result.

Let us now consider whether ΔP might have a finite value; i.e., whether the cell ions might be in agreement with Eq. (3). One finds that if this equation were to hold and all the cell ions were osmotically active, there would be a ΔP of over 100 atm for groups I and III and a negative ΔP for group II. Alternatively, should only NaCl be osmotically active within the cell (i.e. not KCl) one must postulate that $\Delta P = 0$ for group I and $\Delta P < 1$ for group II while for group III ΔP is variable. These all seem untenable, and one must conclude that Eq. (3) does not apply to the bacteria.

The next model to consider is represented by Eq. (5) in which cell volume is present as an explicit parameter. It is possible to consider that a change in external osmotic pressure might bring about a change in cell volume (v) or in cell solute content (N_i). For bacteria of group I there is no need to suggest a model more complex than that represented by Eq. (4) to describe the phenomena observed. In groups II and III, on the other hand, there were considerable changes in cell volume and K content. However, whether the cell ions are assumed to be osmotically active or partly bound, there is no way to describe the observed volume changes by an osmotic model of the type described by Eq. (5).

It must be concluded that none of the models examined up to now is sufficient to describe the behavior of the various groups of bacteria in Table 2. So far the term μ^{poly} has been neglected. In a former paper (Ginzburg & Ginzburg, 1975) a model of the cell was presented showing two phases of cell water; a *w* phase consisted of water with bulk properties and an *sw* phase consisted of water interacting strongly with the surfaces of the acidic proteins. In such a model μ^{poly} must be introduced into Eq. (2) at equilibrium. In such a case,

$$\mu_o^c = \mu_i^c + \mu_i^{poly}.$$

μ_i^{poly} may act either directly through the interaction of water and polyelectrolytes, or indirectly by the binding of ions by the cell polyelectrolytes (Noy-Meir & Ginzburg, 1967).[1]

B. *Factors Controlling Cell Na Content*

Studies of the changes in cell ion content which occurred as a result of displacements of the pH or NaCl concentration of the medium have thrown light on the mechanism whereby the cell Na content is controlled.

When the pH of suspensions of starved bacteria was lowered to pH 5, there was an inrush of NaCl and water into the bacteria even though a minor part only of the total cell K was lost (Fig. 8). At this pH it was hard to tell whether the cell K was lost together with Cl^- or in exchange for H^+ (i.e., whether K_{Cl} or K_x was lost), but at pH 5.5 it was apparent that most of the cell K lost must have been exchanged for H^+, i.e., consisted of the K_x fraction (Fig. 5*B*). This is presumably true at pH 5.0 also; it suggests that in starved bacteria K_x plays a special part in the regulation of the cell NaCl and that the loss of this cell K fraction is accompanied by movement of Na, Cl and finally water into the bacteria. All three substances were gained at pH 5.0 (Fig. 8); only Na and Cl increased at pH 5.5, indicating that the gain of these two precedes that of water.

In starving bacteria the only change in volume recorded was the swelling which preceded lysis. This type of change was seen also in metabolizing bacteria at pH values just above 4 (Fig. 2) or transferred to NaCl solutions below 2 M. However, the reactions of metabolizing bacteria to pH values around 5.5 were more complex than those of starving bacteria (*cf.* Figs. 4*A* and 4*B*): both types of bacteria reacted in the same way for the first hour by losing K^+ and gaining Na^+ and Cl^-; later, however, the same ion movements continued in starving bacteria whereas the levels of Na^+ and Cl^- levelled off in the metabolizing bacteria and subsequently dropped so that by the fourth hour they had returned to values close to the original ones.

Figs. 4*A* and 5*A* demonstrate that movements of Na and Cl into and out of the cell are correlated with changes in the relative proportions of K_x and K_{Cl}; when K_x fell, the levels of Na and Cl rose abruptly. Later, the

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1 *Also*, Edzes, H.T., Ginzburg, M., Ginzburg, B.Z., Berendsen, H.J.C. The state of water in a *Halobacterium*. An NMR study. *(To be published).*

reconversion of K_x at the expense of K_{Cl} was accompanied by the efflux of Na^+ and Cl^-.

Thus, in both starving and metabolizing bacteria the loss of K_x led to the entry of Na and Cl into the cell. The difference between these two types of bacteria is that in the metabolizing (growing) bacteria there is a conversion of $K_{Cl} \rightarrow K_x$, a reaction which did not take place in starving bacteria and which is presumably under metabolic control. Thus, metabolizing bacteria must possess a mechanism for the active extrusion of Na^+ and Cl^- from the cell interior, by the replenishment of K_x from K_{Cl}.

We now wish to see how far these results go toward providing an explanation of the cell Na and its regulation. We have suggested that K_{Cl} plays no direct role in the regulation of cell Na. A loss of K_x, on the other hand, has always been found to be correlated with the entry of Na^+, Cl^-, and eventually water into the cell. In starving bacteria, loss of K_x could not be made up, whereas metabolizing bacteria possessed a means of converting K_{Cl} to K_x and hence of replenishing any K_x lost due to the low pH of the medium.

These findings should now be related to the conclusions of section A, namely that interactions between cell water and cell polyelectrolytes must be invoked to explain the osmotic relationships of the cell. Since the K_x fraction is postulated to be balanced electrostatically by COO^- groups belonging to cell proteins, there would appear to be a phase of cell water affected by interactions both with polyelectrolytes and potassium (*sw* phase). This model has been described in detail elsewhere (Ginzburg & Ginzburg, 1975); it suggests that sodium is excluded from the *sw* phase because the energy of hydration of the Na ion which is high relative to that of K^+, does not enable it to fit within an ordered layer of H_2O molecules. Thus, cell sodium would be restricted to the non-*sw*, or *w* phase where its concentration would be much higher than that calculated on using the total cell-water as a basis for calculation and could approach the Na concentration found outside. The amount of cell Na would then reflect the size of the *w* phase which would be determined by the strength of the interactions in the *sw* phase; i.e., a high cell Na content would imply that the *w* phase is large, relative to *sw* which would be small because of the relatively weak polyelectrolyte-potassium-water interactions in it. In support of this hypothesis the experimental findings described in this paper have shown that there is an inverse correlation between K_x (i.e., a component of the *sw* phase) and cell Na (the major postulated component of the *w* phase).

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