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Ion Metabolism in a *Halobacterium*

II. Ion Concentrations in Cells at Different Levels of Metabolism

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Summary. The changes in concentration of K^+, Na^+ and Cl^- are given in growing cultures of a *Halobacterium* species subjected to cold, lack of O_2 or starvation. In growing cells, the ion concentration ratios across the cell membrane were 1,000:1 (inside:outside) for K^+ and 1:2 for Na^+. In bacteria with a low rate of endogenous metabolism induced by 24-hr starvation, the ratios were 500:1 for K^+ and 1:4 for Na^+. O_2 and a substrate were required for K^+ uptake in growing bacteria, but not for the maintenance of K^+ and Na^+ gradients in starving bacteria. The exchange of K^+, Na^+ and Li^+ across the cell membrane of starving bacteria was found to have a time constant for 50% completion of the process of 20 to 30 sec. The exchange of cell Cl^- with NO_3^- was a two-stage process with time constants of approximately 2 min and 2–1/2 hr. The results are explained in terms of the binding of most of the cell K^+ which brings about the distribution of Na^+ and Cl^- according to the Gibbs-Donnan equilibrium; the role of metabolism is to induce changes in the mean ion-activity coefficients.

The classical experiments of Maizels in 1940 proved that ion fluxes were coupled to metabolic reactions in red blood cells. Since then, every kind of animal, plant and bacterial cell studied has demonstrated the important role of metabolism in the regulation of ion gradients across the cell membrane. It therefore seemed a logical starting place, in an investigation of the forces controlling ion movements in a *Halobacterium* species, to study with some thoroughness the role of metabolism in ion regulation. Until now this subject has been little investigated, although measurements of ion concentrations in halophilic bacteria have been made on *H. salinarium* [2]. The metabolism of the halobacteria is said to resemble that of other cell systems [3]. The bacteria are highly aerobic [13]. The metabolic pathways that have been investigated in detail have shown that all enzymes so far isolated from

halophiles have a requirement for 3 to 4 M KCl or NaCl [4, 10] for maximal activity.

In the work to be described, the concentrations and movements of the K^+, Na^+ and Cl^- ions have been studied and contrasted in cultures of bacteria at different levels of metabolic activity. Bacteria growing exponentially were taken to be at a high level of metabolic activity [5]. Growth was stopped by cooling or by deprivation of O_2 or substrate. Starved bacteria metabolized slowly; the rate of metabolism in bacteria deprived of substrate for more than 4 hr was less than 1% of the rate in exponentially growing bacteria, as judged by rates of O_2 uptake and pH change in the ambient medium. Thus the use of exponentially growing and starving cultures served to contrast widely differing levels of metabolic activity. Cooled or anaerobic cultures were in intermediate states.

Bacterial K^+ concentrations of 3 to 4 M were found in all cultures (outside K^+ concentration: 5 mM), no matter what the level of metabolic activity, with the exception of those in an atmosphere of argon in which a slow loss of cell K^+ was detected. The concentration of bacterial Na^+ was always below 3.5 M, the level found in the medium. However, the lowest cell Na^+ was found in bacteria with low rates of metabolic activity, such as those in argon at 0 °C, or in starved bacteria. One possible explanation for these results is that the bacteria have membranes which are highly impermeable to ions; accordingly, a series of experiments to test this point was carried out with starved bacteria. However, it was found that both cations and anions could be transferred across the membrane with rapidity. It was therefore concluded that some other explanation must be sought, and that the most probable reason for the behavior of the cell ions is that the K^+ is almost totally bound within the cell matrix; a part of this cell K^+ may act as a source of positive fixed charges which bring about the distribution of the other cell ions according to the Gibbs-Donnan formula.

Materials and Methods

Methods of culture of the *Halobacterium sp.* used have been described previously [8], together with methods of analysis and calculation.

Metabolic Experiments

Logarithmic phase cultures at an optical density of 0.20 were used (400–465 nm). A 70-ml sample of bacterial suspension was poured from the culture vessel into a jacketed cylindrical vessel with an inner diameter of 8 cm. The vessel was covered with Parafilm. The depth of the suspension was 1.5 cm. It was rapidly stirred by means of a magnetic

stirrer. A combined electrode (Radiometer GK264B), connected to a Radiometer Titrigraph TTTIC, dipped into the suspension and controlled the flow of 0.2 N HCl into the vessel in response to alkalinization of the medium brought about by the bacteria. The response of the electrode used is not significantly affected by high salt concentrations below pH 9. The pH was carefully maintained at 7.0 in all experiments because the bacteria were found to be extremely sensitive to pH changes in the medium. The temperature of the suspension was maintained at 37 ± 0.2 °C by means of water pumped through the jacket of the vessel. A preliminary incubation period of 1 hr was allowed. This was followed by the experimental period which lasted from 1 to 5 hr.

Incubation in Argon

The experimental vessel was closed with a rubber stopper pierced with holes for the electrode and gas inlet and outlet.

The argon was passed through two water towers immersed in water at the same temperature as the cell suspension (either 37 or 0 °C). The absence of O_2 from the experimental set-up was checked by means of an O_2 electrode (Yellow Springs Instrument Co., Inc.) immersed in the cell suspension. In preliminary experiments, a drop of methylene blue was added to the suspension. It was entirely discolored 5 to 10 min after the argon was turned on. Ion measurements were not made on the suspension containing methylene blue.

Experiments Requiring Cell Resuspension

The method of resuspension has been described previously [8]. It took about 20 min for a button of cell material, obtained by centrifugation of a cell suspension, to be resuspended in fresh medium by the action of a rapidly rotating magnetic bar. The first measurement was taken after the cells were completely resuspended and was designated as "time zero".

Measurement of Ion Exchange

To measure ion exchange, we used logarithmic-phase bacteria which had been incubated in the absence of C or N substrate for 24 hr at 37 °C, and then stored at 3 °C until needed. This is the usual procedure for reducing the rate of endogenous metabolism by depriving the bacteria of reserve substrates [12].

The major component of the control medium was 3.5 M NaCl; all solutions also contained 150 mM $MgSO_4$, 5 mM KCl, 1.4 mM $CaCl_2$, and 2.5×10^{-4} mM $MnCl_2$. To study cation exchange, a part or all of the medium Na^+ was replaced by Li^+ or K^+. For complete replacement of medium Na^+ by Li^+ or K^+, given volumes of bacterial suspension in 3.5 M NaCl were spun down and resuspended by aid of a magnetic stirrer in a solution containing Li^+ or K^+, keeping the osmotic concentration of the medium constant at 3.78 osm (the osmoticc oncentration of 3.5 M NaCl). The pH was maintained at 7.1 ± 0.1. Samples were taken for analysis by centrifugation in Beckman Microfuge tubes as soon as the cells appeared to be evenly resuspended – a matter of about 4 min with these cells. The suspensions were shaken in a water-bath at 37 °C and samples taken at regular intervals until equilibrium was attained. Sample times were measured from the moment of mixing to the time at which the centrifuge was turned on; therefore, they slightly underestimate the total time available for mixing of the cells with the ambient medium since they do not take into account the time which elapsed in the centrifuge before the bacteria were separated from the medium.

For partial replacement of medium Na^+ by another cation, a concentrated bacterial suspension in 3.5 M NaCl was diluted with a solution containing the second cation so as to reach the desired concentration. Osmolarity was maintained at 3.78.

For anion exchange, the same methods were used except that medium NaCl was replaced by $LiNO_3$.

Terminology

Cell ion concentrations are expressed in moles per kg cell water, abbreviated as M. Medium ion concentrations are expressed in terms of molarity (M) or molality (MO).

Results

Control Conditions

When cultures of *Halobacterium* at an optical density of 0.2 were incubated in the experimental vessel, there was an initial period of equilibration characterized by fast growth. This period lasted for 1 hr. During this time the parameters measured increased by 16% (mean of 10 cultures). In all of the figures given in this paper, this initial period of equilibration is omitted.

After the period of equilibration, growth proceeded with a generation time of 5 to 7 hr (Fig. 1). This is the rate measured during the logarithmic phase [8]. The changes in parameters measured – pellet volume, bacterial protein, K^+, Na^+ and Cl^- during 3 hr of incubation in the experimental vessel – are shown in Fig. 1.

Protein synthesis did not occur except under conditions as exactly described in Methods; any interference with the aeration of the culture, for instance, such as having a larger volume of suspension, or changing the shape of the vessel, stopped both protein synthesis and K^+ uptake. This supports the statement, made previously by others [13], that *Halobacterium* is an obligate aerobe.

During the period of the experiment, ion concentrations in moles per kg cell water, remained steady at 4.75 ± 0.05 M K^+, 2.34 ± 0.2 M Na^+ and 5.0 ± 0.05 M Cl^-. These values are regarded as typical of metabolizing cells.

Effect of Cold

In nine separate experiments, bacterial cultures in the logarithmic phase were cooled to 0 °C and maintained there for periods of up to 24 hr. They were vigorously aerated for the first 3 hr of the experiment, after which they were stored in a refrigerator overnight. The pH was maintained at 7.0. O_2 uptake was very low (not more than 3% of the rate at 37 °C). It

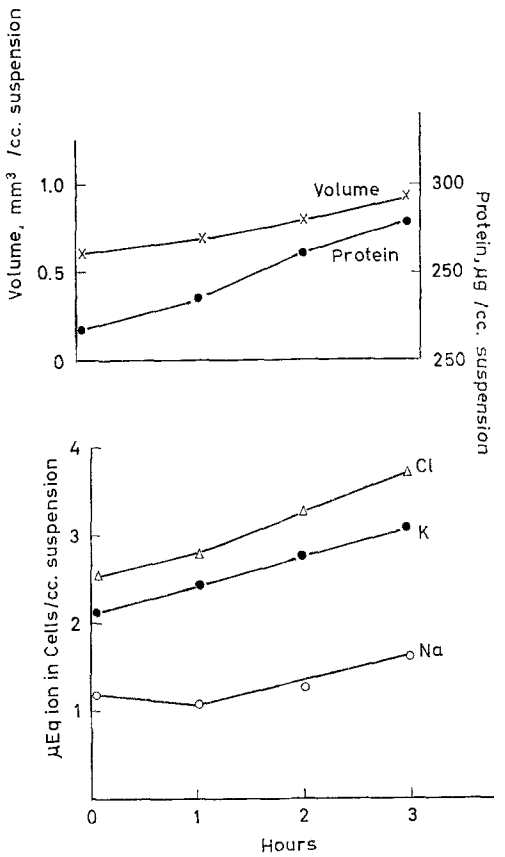

Fig. 1. Growth of *Halobacterium sp.* cultures with C and N substrates at 37 °C with vigorous aeration. Initial optical density: 0.2. Cell ion concentrations throughout experiment: 4.75 ± 0.05 K^+, 2.34 ± 0.2 Na^+, 5.0 Cl, all in moles per kg cell water. Medium NaCl concentration: 3.9 Mo. pH of incubation medium maintained at 7.0. For composition of medium, *see* ref. [8]. ● K; ○ Na; △ Cl

was interesting to observe that when the temperature was dropped from 37 to 0 °C, the bacteria ceased to alkalize the medium and started to acidify it. This acidification lasted for the duration of the experiment, but at a greatly reduced rate after the first hour. There were no increases in protein or K during the experimental period (the amounts after 24 hr were 97 and 104% for protein and K, respectively, of the initial values). It seems probable that metabolism was, therefore, greatly reduced. In contrast, protein synthesis and alkalinization of the medium were resumed immediately on returning the temperature to 37 °C, even after 24 hr at 0 °C. Bacterial protein, after 1 hr at 37 °C following 24 hr at 0 °C, increased by 10% of the original amount. Thus, the bacteria were not killed by the cold exposure.

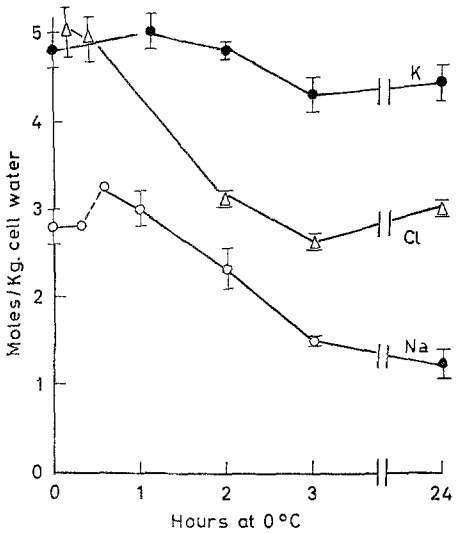

Fig. 2. Effect of incubation at 0 °C on cell ion concentrations of *Halobacterium sp.* The cultures were aerated for the first 3 hr. For composition of medium, *see* ref. [8]. pH of incubation medium maintained at 7.0. Mean of seven experiments. ● K; ○ Na; △ Cl

The effects on ions are summarized in Fig. 2. The cell K^+ concentration fell by 10.5% after 3 hr in the cold (initial, 4.8 ± 0.2 M; after 3 hr at 0 °C, 4.3 ± 0.2 M). The final 24-hr value of 4.45 ± 0.2 M was not, however significantly different from the initial concentration. It is concluded that exposure to cold does not reduce cell K^+ concentration.

Large effects of cold were found on cell Na^+ and Cl^-. Initial concentrations were 2.8 ± 0.2 M Na^+ and 5.5 M Cl^-. During the first hour, a slight increase to 3.2 ± 0.1 M Na^+ was measured. This increase took place suddenly 20 to 40 min after the exposure to cold. As time went on, cell Na^+ was lost; 3 hr after the exposure to 0 °C began, the cell Na^+ concentration reached 1.5 ± 0.03 M Na^+. The 24-hr value of 1.25 ± 0.15 M Na^+ is even lower. The loss in cell Na^+ was accompanied by a parallel loss in cell Cl^-; thus the total losses were 1.5 ± 0.23 M Na^+ and 1.75 ± 0.23 M Cl^- which were lost simultaneously.

It is concluded that cooled cells are characterized by high ratios of K^+ and Na^+ concentrations across the cell membrane despite the lack of metabolism. The K^+ concentration ratio is 1:890 (K_{out}, 5 mM/l; K_{in}, 4.45 M), and the Na concentration ratio is 3.2:1 (Na_{out} 3.9 Mo; Na_{in} 1.25 M).

In a second series of experiments, five bacterial cultures were aerated by vigorous stirring at 2 °C for 24 hr. Results in Table 1 show that no protein synthesis occurred during this time, as in the experiments described in Fig. 2; there were, however, significant increases in the amount of cell

Table 1. *Effect of incubation at 2 °C for 24 hr on Halobacterium sp.*

Cell parameter	% of original value	
	A	*B*
K^+ content	$+144 \pm 2$	104 ± 4
Volume	$+116 \pm 1$	100 ± 1
Protein	100 ± 2	97 ± 2

A. The cultures were stirred vigorously; the medium was not buffered. Mean of five experiments ±S.E.

B. Cultures stirred only for first 3 hr. pH of medium: 7.0.

K^+ and in cell volume. ($144 \pm 2\%$ and $116 \pm 1\%$ of the original values, respectively.) Thus, the cell K^+ concentration actually increased during this time.

For comparison, results are given for cultures aerated only during the initial 3-hr period. These cultures showed no increase in amount of cell K^+. The results demonstrate that aeration is essential for K^+ uptake. It should be remembered that the bacteria and their medium are far from the freezing point, which is −20 °C for 3.5 M NaCl. Thus, the occurrence of some metabolic reactions is not entirely improbable.

Incubation in Argon

Since these bacteria are highly aerobic [13], incubation in argon should entirely abolish metabolism. It was observed, however, that the bacteria continued to alkalinize the medium, and it remains possible that certain metabolic reactions could occur.

There was no protein synthesis in cultures treated with argon. In fact, after the first 3 hr of treatment, the cell protein was $97 \pm 1\%$ of its initial value. Thereafter, a slow decrease was found, likely due to lysis, so that after 5 hr in argon, cell protein was $89 \pm 1\%$ of the original amount.

There was no change in cell volume in cells incubated in argon, and thus any changes in cell ion content which occurred were reflected in changes in ion concentration. Fig. 3 shows the effect of incubation in argon at 37 °C. There is shown to be a fall in cell K^+ concentration. The Na^+ concentration did not change significantly during the period of measurement. There was, however, a slight fall in Cl^- concentration. Thus the loss of 2.0 ± 0.3 M K^+, which occurred during the first 4 hr of the experiments was accompanied by a loss of 0.8 ± 0.2 M Cl^-. Cultures incubated in argon were observed to bring about alkalinization of the medium. It is therefore possible that a portion of the K^+ was lost together with OH^- or was exchanged for H^+.

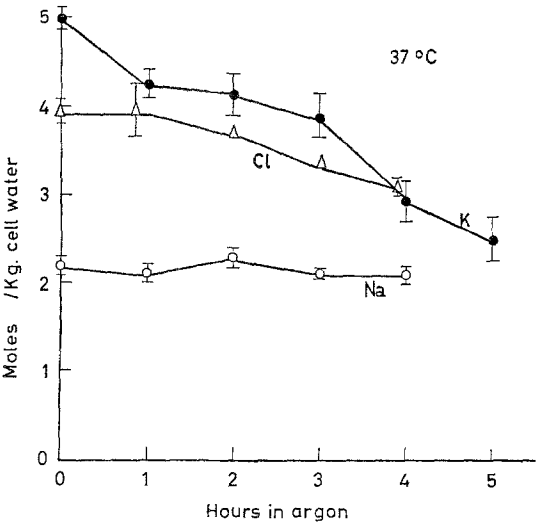

Fig. 3. Effect of incubation at 37 °C under anaerobic conditions on cell ion concentrations of *Halobacterium sp.* Anaerobic conditions obtained by passing a stream of argon through the cultures. For composition of medium, *see* ref. [8]. pH of incubation medium maintained at 7.0. Mean of five experiments. ● K; ○ Na; △ Cl

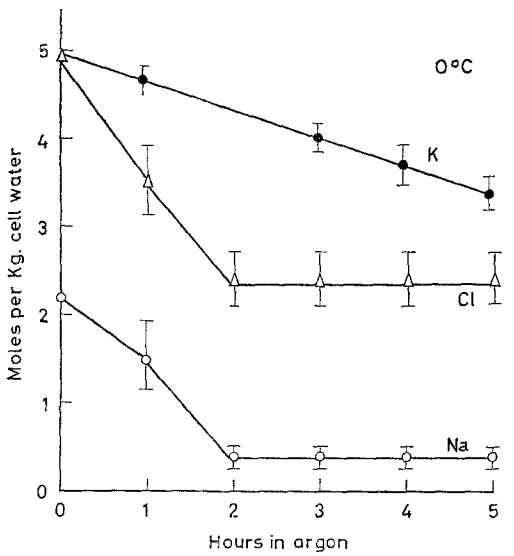

Fig. 4. Effect of incubation at 0 °C under anaerobic conditions on cell ions of *Halobacterium sp.* For other conditions, *see* legend to Fig. 3. ● K; ○ Na; △ Cl

When cultures were incubated at 0 °C in argon, the concentration of Na^+ and Cl^- fell relatively rapidly; the K^+ concentration fell linearly (Fig. 4). The rates of loss of Na^+ and Cl^- were faster and the final values lower in argon than in air (*cf.* Figs. 2 & 4). The final, low values remained

steady for several hours, despite the fact that any metabolic energy output under such circumstances is most unlikely.

The ion concentration ratios in cells after 5 hr at 0 °C in argon are: K^+, 1:660 (K^+_{out} 5 mM; K^+_{in} 3.3 M); Na^+, 10:1; and Cl^-, 1.65:1 ($NaCl_{out}$ 3.9 Mo Na^+_{in} 0.4 M, and Cl_{in} 3.4 M).

Deprivation of Substrate

On resuspending bacteria from cultures at an optical density of 0.25 (i.e., mid-logarithmic stage) in saline solution in the absence of any source of C or N, *Halobacterium sp.* cultures were found to lose K^+ rather slowly. The saline solution contained 3.5 M, NaCl and $MgSO_4$, KCl, $CaCl_2$, and $MnCl_2$ in amounts shown on p. 80. At the end of 24-hr incubation in saline at 37 °C, during which time the cells were continuously aerated and the pH maintained at 7.0, the cell ion concentrations were: K^+ 3.0 ± 0.05 M; Na^+ 1.14 ± 0.1 M; Cl^- 2.48 ± 0.06 M (mean of five experiments for K^+ and Na^+, and three for Cl^-). Cells with this ion composition are referred to as "low-K^+ cells". That the low concentration of K^+ is caused by loss in amount of cell K^+, rather than by cell swelling, is suggested by the low K^+: protein ratio (*see* Table 2, saline incubation).

The ion concentrations of bacteria from cultures at an optical density of 0.06 (i.e., early logarithmic stage), after 24-hr incubation in saline, were: K^+ 3.85 ± 0.05 M; Na^+ 1.3 ± 0.2 M (means ± extreme values of two experiments). These are the ion concentrations of so-called "high-K^+ cells".

Cells suspended in saline continued to absorb O_2 rapidly and to alkalinize their medium for the first 2 hr after their resuspension in saline. Protein synthesis stopped at about this time. Four hours after resuspension, neither

Table 2. *Ratio of K:protein in Halobacterium sp. cultures*

Experimental conditions	Ion concn. (moles/kg cell H_2O) K^+	Na^+	Cl^-	K^+:protein (μequiv/100 μg)	No. of determinations
After 24 hr in saline[a]	3.0 ± 0.05	1.14 ± 0.1	2.48 ± 0.06	1.04 ± 0.04	5
4 addnl. hr in medium	4.9 ± 0.1	2.4 ± 0.25	4.75 ± 0.2	1.46 ± 0.1	4
Maximum ion concentrations (Fig. 11 B)	5.0	2.2 ± 0.3	4.7 ± 0.2	1.63 ± 0.1	4

[a] Initial optical density of culture: 0.20.

was O_2 uptake detected with the O_2 electrode nor did any change in pH of the medium take place. It was concluded that endogenous metabolism had fallen to a low rate.

Time Course of Ion Replacement in Starved Cells

Cations. When starved bacteria were suspended in media in which the Na^+ had been replaced by Li^+, cell Na^+ reached a new steady state within 1–1/2 min of the time of mixing (Fig. 5). The change in cell Na^+ was proportional to the extent of replacement: when one half of the medium Na^+ was replaced by Li^+, cell Na^+ fell to 50% of its former value. Similarly, a 17.5-fold dilution in Na^+ concentration resulted in cell Na^+ levels at approximately 5% of the original value.

No attempt was made to measure the time course of fall in cell Na^+ during the first minute after replacement of medium Na^+. It is clear, however, that since the process was complete 1–1/2 min after Na^+ replacement, even at the highest dilution tried, the time required for the process to reach 50% completion cannot be greater than 20 to 30 sec.

Fig. 6 shows that the cell Na^+ could be replaced equally well by Li^+ or K^+ at all the levels tried. When the medium Na^+ was reduced to 75 mM, the Na^+ content of cell pellets did not differ significantly from the blanks. Similarly, when bacteria were resuspended in 3.9 M KCl, with a residual Na^+ content of 200 mM, the cell Na dropped to 6% of its original value

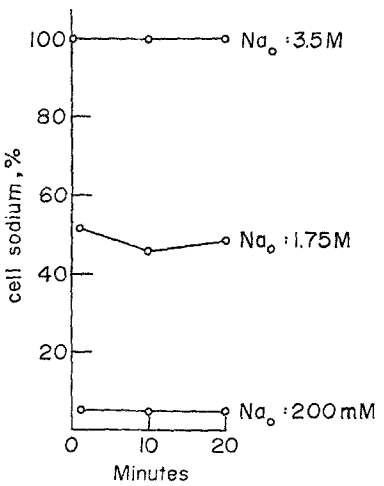

Fig. 5. Effect of concentration of medium Na^+[Na_0] on cell Na^+ of *Halobacterium*, as % of cell Na^+ content in 3.5 M NaCl. At time zero, the medium [Na^+] was brought to the experimental value by addition of a solution containing Li^+ to a concentrated bacterial suspension. Total osmolarity of medium: 3.78 osm

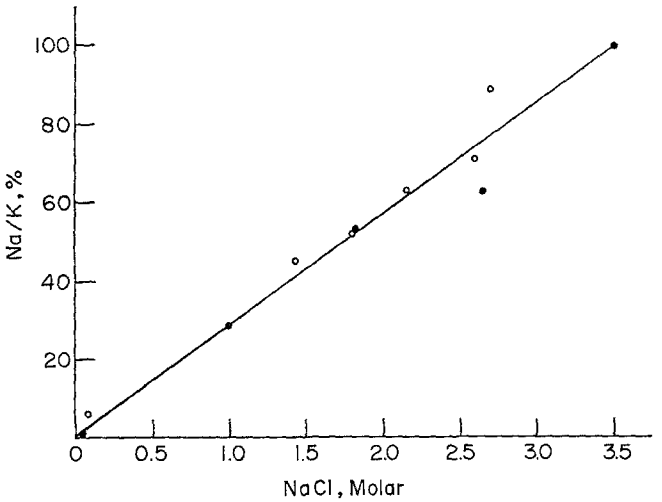

Fig. 6. Effect of replacement of medium Na^+ on Na/K ratio in centrifuged pellets of *Halobacterium sp.* Na^+/K^+ expressed as % of value in 3.5 M NaCl. Medium Na^+ replaced by Li^+ (●) or by K^+ (○). Total osmolarity of medium: 3.78 osm

(mean of three experiments). The expected value was 3% of original, and is within the range of experimental error.

Whatever the mechanism may be, it is clear that the cell Na^+ adjusts itself within a few seconds to the medium Na^+ concentration, after changes in the latter.

Further Experiments on K Transfer. The reversibility of the replacement of cell Na^+ by K^+ was tested by transferring bacteria in 3.9 M KCl (and therefore Na^+-free) back to a 3.5 M NaCl solution. These KCl and NaCl solutions are roughly isoosmotic. After the transferral, it was found that a portion of the total cell K^+ was replaced by Na^+ while the rest had been retained. Furthermore, it was found that the K^+:protein and Na^+:protein ratios were the same as in bacteria not subjected to the series of transfers (Table 3). It was concluded that the cell K^+ originally present in the bacteria behaves differently from the K^+ which had replaced the Na^+; since K^+ is quickly exchangeable in both directions, there is apparently no diffusion barrier to K^+ at the cell boundary. However, the original cell K^+ could not be removed and therefore cannot be free. Preliminary isotope-exchange experiments have indicated that the time for exchange of half of the cell K^+ is well over 24 hr. It will be argued in the Discussion that this cell K^+ is specifically bound to the cell matrix.

Table 3. *Effect on cell K^+ and Na^+ of transferring bacteria from 3.5 M NaCl to 3.9 M KCl and then back again to 3.5 M NaCl. Measurements were made approximately 5 min after suspension in the new medium*

Medium	Cell ions (μequiv/100 μg protein)	
	K^+:protein	Na^+:protein
3.5 M NaCl	1.11 ± 0.01	0.38 ± 0.02
3.9 M KCl	n.m.[a]	0.02
3.5 M NaCl	1.20 ± 0.01	0.41 ± 0.03

[a] not measured.

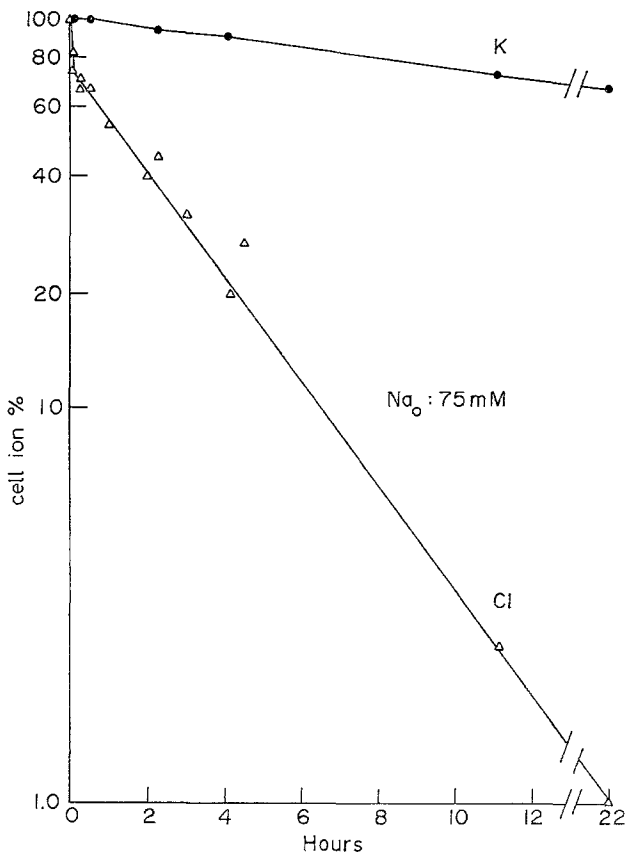

Fig. 7. Time course of loss of cell Cl^- after resuspension of *Halobacterium sp.* in 3.2 M $LiNO_3$. Cell Cl^- expressed as % of value in 3.5 M NaCl

Exchange of Cell Cl. These experiments showed that when NaCl was completely replaced by $LiNO_3$ there were losses in cell Cl^- which proceeded until cell Cl^- was virtually undetectable (Fig. 7). These losses,

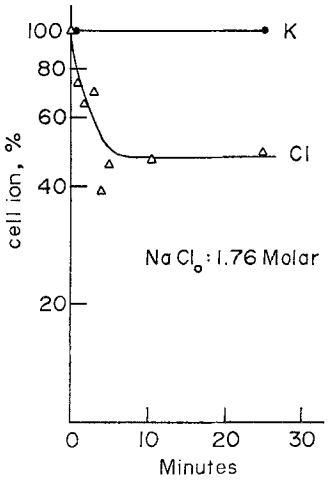

Fig. 8. Time course of loss of cell Cl^- after reduction of medium $[Na^+]$ from 3.5 to 1.76 M, by addition of $[Li^+]$ to suspension. Total osmolarity: 3.78 osm. Cell Cl^- expressed as % of value in 3.5 M NaCl

however, were relatively slow and took about 12 hr to reach completion. Analysis of the loss showed that it occurred in two stages: a rapid stage completed in approximately 15 min and a slower stage with a time constant for 50% completion of 2.2 hr. The extent of the rapid stage was shown to be linked to the loss of cell Na: in five experiments, loss of cell Cl was $98 \pm 2\%$ of the loss of cell Na as measured 15 min after mixing. The rate of this stage is shown more clearly in Fig. 8 which shows the results of an experiment in which bacteria were suspended in 1.75 M NaCl plus $LiNO_3$ (total osmolarity: 3.78 osm). The time required for the process to reach 50% completion is approximately 2–1/2 min. This outward movement of Cl^- was found to be somewhat less rapid than that of the cations K^+, Na^+ or Li^+.

Resuspension of Starved Cells in Complete Medium

The complete medium was similar in composition to the saline solution described on page 80 except for the addition of 10% yeast autolysate.

The bacteria initially responded to resuspension in complete medium by vigorous acidification of the medium. At some stage in the course of the ensuing 4 hr – generally associated with the time at which protein synthesis started – the bacteria started to make the medium alkaline. The pH was maintained at 7.0 by addition of small quantities of acid or base, as required.

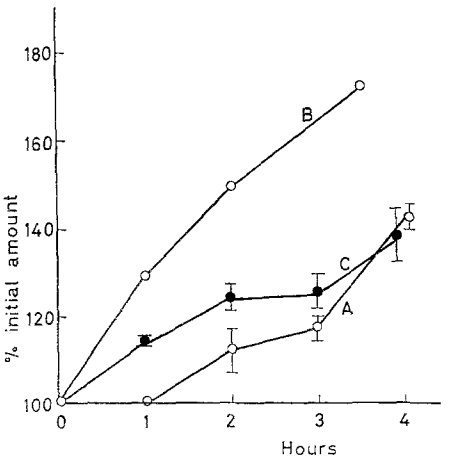

Fig. 9. Increases in volume and protein in *Halobacterium sp.* cultures resuspended in growth medium, after 24-hr period of starvation in saline solution. *A* and *B*, bacterial protein; *C*, pellet volume. Curves *A* and *C* are means of three experiments and pertain to bacteria with low initial ion concentrations (3.0 M K^+, 1.14 M Na^+, 2.48 M Cl^-). Curve *B* is the mean of two experiments and pertains to bacteria with higher initial ion concentrations (3.85 M K^+, 1.75 M Na^+). Temperature of incubation: 37 °C. pH of incubation medium maintained at 7.0

It was observed that the bacteria resumed O_2 uptake immediately on resuspension at a rate comparable to that of bacteria in the logarithmic phase. Therefore a significant proportion must have survived the incubation in saline.

The courses of protein synthesis and volume increase in bacteria with a cell K^+ concentration of 3.0 M are shown in Fig. 9, curves *A* and *C*. There was no increase at all in cell protein for the first hour and only a slight one for the next two hours. For the final hour of the experiment, however, the increase in cell protein was such that, if continued, there would be a doubling of amount in about 3–3/4 hr. This rate of increase would probably not be maintained for long.

The early lag phase is marked by a slight degree of cell swelling (Fig. 9, curve *C*). Curve *B* shows for comparison the rate of protein increase in bacteria with an initial K^+ concentration of 3.85 M. The average generation time over the 3-hr period is 5.5 ± 0.2 hr. There was no initial lag.

Resuspension in complete medium led to a rapid uptake in amount of K^+, no matter what the initial cell K^+ concentration (*see* Fig. 10). Increases in cell K^+ concentration are shown in Figs. 11A and 11B. The final K^+ concentrations were 4.9 ± 0.1 M and 5.0 ± 0.05 M (low and high initial K, respectively).

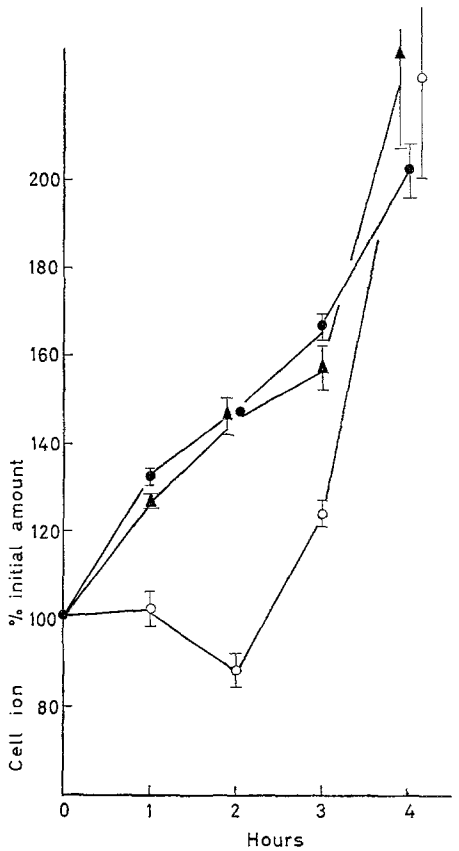

Fig. 10. Increase in ion content in *Halobacterium sp.* cultures resuspended in growth medium after 24-hr period of starvation in saline solution. Temperature of incubation: 37 °C. pH of medium: 7.0. Initial [Na^+] and [Cl^-] were low (1.30 M Na^+; 2.56 M Cl^-). [K^+] 2.75 or 3.85 M. ● K; ○ Na; ▲ Cl

Uptake of cell Na^+ and Cl^- in bacteria with low initial cell ion concentration is shown in Fig. 10. There was no significant Na uptake for the first two hours of the experiment. During the second 2-hr period, the Na^+ concentration increased from 1.5 to 2.4 M (Fig. 11 A). It was in the final hour of the experiment that protein synthesis was accelerated (Fig. 9, curve *A*).

It should be noted that the highest K^+ and Na^+ concentrations measured in these resuspension experiments (e.g., those shown in Fig. 11 B), are close to those measured at the onset of the logarithmic phase of growth [8].

A second point of interest is that a fast rate of protein synthesis is associated with a high cell K^+ concentration; in the cells with *high* initial K^+ concentration, protein synthesis started at once (initial K^+ concentration

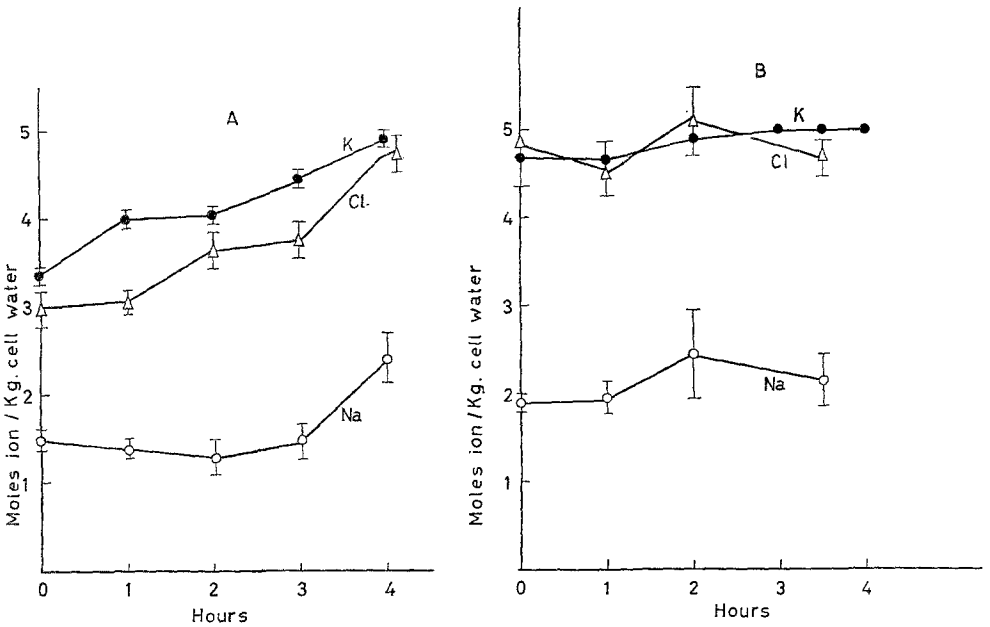

Fig. 11A and B. Change in ion concentrations in *Halobacterium sp.* cultures during resuspension in growth medium after 24-hr period of starvation in saline solution. Temperature of incubation: 37 °C. pH of incubation medium maintained at 7.0. ● K^+; ○ Na^+; △ Cl^-. *A*; low-K^+ cells; *B*; high-K^+ cells

3.85 M). These observations agree with those of a number of workers who have found that many enzymes isolated from halophilic bacteria require a high K^+ concentration for activity [10].

Effects of Metabolic Inhibitors

In contrast to the clear effects obtained by major changes in the environment (e.g., temperature, O_2, substrate), effects of metabolic inhibitors on cell ions have been variable.

NaCN was tested in 14 experiments. This inhibitor arrested O_2 uptake: 50% inhibition was obtained with 1 mM/1. NaCN (Fig. 12). No protein synthesis was observed in cells treated with NaCN at 1 mM or above.

Cell K^+ fell steadily at about the same rate as in argon (*see* Table 4). No effect on cell Na^+ was obtained. A marked but variable effect was observed on cell Cl^-, the amount of which fell by $11 \pm 2\%$ per hour.

No effects of ouabain have been detected in this system. Ouabain was tested at concentrations from 1 to 5 mM.

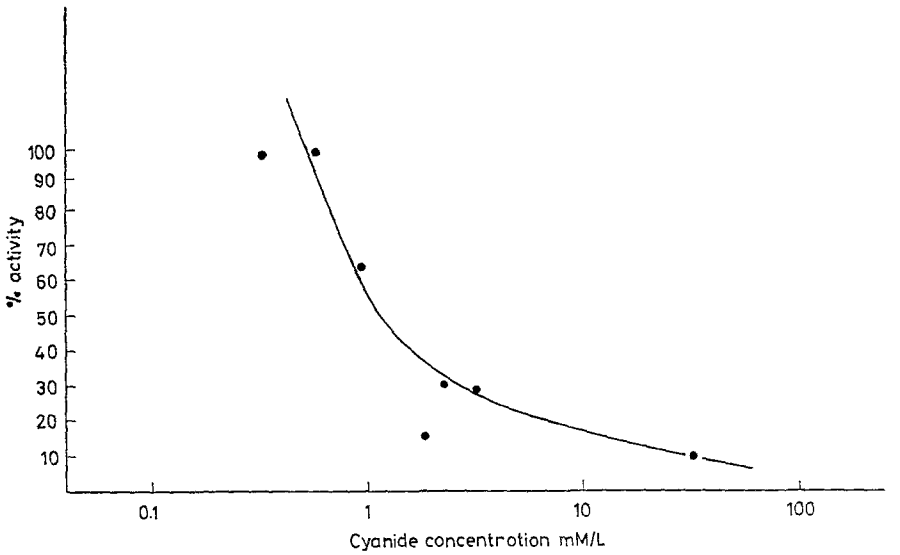

Fig. 12. Effect of NaCN concentration on % O_2 uptake of *Halobacterium sp.* cultures incubated at 37 °C at pH 7.0

Table 4. *Effect of inhibitors on cell* K^+

Inhibitor	% *of initial amount*
	Loss in cell *K* per hour at 37 °C
NaCN (2 mM)	6.8 ± 0.3 (S.E.)
Argon	8.7 ± 0.3

Discussion

In many cells, inhibition of metabolism is accompanied by a progressive reduction and eventual abolishment of the electrochemical potential gradients for ions across the membrane. There is usually a decrease in the ion concentration gradients, a process which may be very slow if the membrane is relatively impermeable to one or more of the ions. The end result of the running-down process may be lysis in cells without a rigid cell wall or else the cell ion concentrations may be the same as in the bathing medium, as in *Escherichia coli* cultures in the stationary state, in which the cells do not lyse because of their wall [15].

In contrast, it has been found in the *Halobacterium* species which has been the subject of this work that large K^+, Na^+ and Cl^- concentration

gradients occurred in cells reduced to a low rate of endogenous metabolism by starvation. According to the usual criteria, active transport processes and thus ion concentration gradients should have been reduced. However, as we have seen, the ion concentrations in bacteria starved for 24 hr were: K^+ 3.0 M; Na^+ 1.14 M; Cl^- 2.48 M (K_{out} 5 mM/L; $NaCl_{out}$ 3.9 M_0).

Protein synthesis was completely arrested in two other cases, namely in cells incubated at 0 °C or in argon (*see* Table 1 and p. 84). O_2 uptake could not occur in bacteria incubated in argon and was much reduced in the cooled bacteria (p. 81). It follows that the rate of metabolism must have been much reduced under these conditions; yet the K^+ concentration remained at nearly 1,000 times its outside value and the cell Na^+ concentration was always lower than that of the medium (e.g., 1.2 M in cells at 0 °C in air; 2.2 M in cells in argon at 37 °C as compared with 3.9 Mo NaCl in the medium). It has been shown previously [8] that in aging cultures in which growth has ceased, there exist the same high concentration differences of Na^+ and K^+ across the membrane. In contrast, in growing cultures, the cell K concentration was only slightly higher (i.e., 4.75 M), and the cell Na concentration of 2.34 M was actually nearer to that found in the medium than was the case in cells from non-growing cultures.

Thus in cultures of *Halobacterium* in which metabolism had been reduced by several means, there were high ion concentration ratios between the cell interior and the medium. Furthermore, these ratios were maintained at steady values for relatively long periods of time and despite the permeability of the membrane to ions, as was demonstrated unequivocally in Figs. 5–8. Now time-invariant states, characterized by the virtual absence of flows of energy and matter through the system, are defined as equilibrium states [11, 14]. Thus it is reasonable to conclude that the ions in starved cells are at equilibrium with ions in the medium.

In order to explain the data described above, it is useful to carry out a thermodynamic analysis of the ion concentrations in starving bacteria. It is assumed that Na^+, K^+ and Cl^- are the only inorganic ions. At equilibrium,

$$\tilde{\mu}_j^{\text{in}} = \tilde{\mu}_j^{\text{out}} \tag{1}$$

where $j = j$-th ion, $\tilde{\mu}_j$ = electrochemical potential, in = inside cell, out = outside cell.

Since $\tilde{\mu}_j = \mu_0 + RT \ln \gamma_j m_j + z_j F \psi + \bar{v}_j P$, where $\mu_0 = \mu$ at standard state, γ = activity coefficient of ion, m = concentration, $\bar{v}$ = partial molar volume, ψ = electrical potential, and R, T, F, P have their usual meanings, in an

equilibrium system in which the cations are Na^+ and K^+, it follows that:

$$\frac{\gamma_K^{in}}{\gamma_{Na}^{in}} = \frac{\gamma_K^{out}}{\gamma_{Na}^{out}} \cdot \frac{m_K^{out} \cdot m_{Na}^{in}}{m_{Na}^{out} \cdot m_K^{in}}. \tag{2}$$

Since $\gamma_K^{out} \simeq \gamma_{Na}^{out}$, and in starving bacteria $m_K^{out} = 5$ mM/L; $m_{Na}^{in} \simeq 1{,}000$ mM/L; $m_{Na}^{out} \simeq 3{,}900$ mM/L; $m_K^{in} \simeq 3{,}000$ mM/L (assuming all the ions are in solution),

$$\frac{\gamma_K^{in}}{\gamma_{Na}^{in}} \simeq \frac{(5)(1{,}000)}{(3{,}900)(3{,}000)} = \frac{1}{2{,}340}.$$

Thus, if the cell K^+ and Na^+ were in solution, the ratio of the activity coefficients of these two cations, $\gamma_K^{in}/\gamma_{Na}^{in}$ would be of the order of 1:2,340. Since this is an unreasonable figure, one is driven to postulate a short-distance (non-coulombic) interaction, such as the specific binding of one of the species, in order to reduce the disparity between γ_K^{in} and γ_{Na}^{in}. The reduction of m_K^{in} to about 1.5 mM, accompanied by the specific binding of the remaining 3 M of cell K, is a necessary condition for equilibrium.

The hypothesis that cell K^+ is bound has been supported by the demonstration (Table 3) that on transferring bacteria from a solution of KCl to one of NaCl, two kinetic pools of K^+ were found, one of which exchanged freely with medium Na^+ and the other of which did not. It is reasonable to suppose that this latter, large, slowly exchangeable pool of K^+ is bound within the bacteria. Experimental evidence concerning the state of K^+ within the bacteria, and supporting the hypothesis that the ion is bound, is awaiting publication.

It can be argued that K^+ binding, which is highly specific, cannot be due to the presence of special compounds, such as, for instance, macrocyclic antibiotics, because there is not enough organic material in the cell to provide for them. The K^+ binding cannot even be accounted for by the cell proteins or other macromolecules. Specific binding might possibly be furnished by the ordering of some of the water around cellular macromolecules, as has been suggested, for example, by Berendsen [1]. That might explain why the Cl^- which is supposed to neutralize a part of the cell K^+ has a relatively long time constant of replacement. A decrease of dielectric constant in the near vicinity of K^+ could account for that.

K^+ binding having been established, our model is based on the postulation that the bound cell K^+ provides a source of positive fixed charges. It can be shown that the cells may possess a *net* positive fixed charge:

$$K_{in}^+ + Na_{in}^+ > Cl_{in}^-.$$

For electroneutrality, $K^+_{in} + Na^+_{in} = Cl^-_{in} + X^-_{in}$, where X^-_{in} represents negative organic ions and small amounts of phosphate, sulfate etc. Since $K^+_{in} > X^-_{in}$, and Na^+_{in} and Cl^-_{in} are taken to be in solution, it follows that K^+_{in} is balanced partly by X^-_{in} and partly by Cl^-_{in}. This latter fraction of the K^+_{in} is referred to as K_{Cl} and must represent the net positive fixed charge.

The separation of cell Cl^- into two fractions is seen in Fig. 7, where the time of replacement of Cl^- by NO_3^- is shown to consist of two compartments with different time constants. The size of the faster compartment equals the amount of cell Na^+. The slower compartment is then equivalent to K_{Cl}.

Whenever one component of a two-phase system is restricted to one of the phases, the final state of the system is a partial equilibrium [9]. When the restricted component is a charged molecule, and the system consists of ions in solution, then the equilibrium is a Gibbs-Donnan system, and the distribution of the other mobile ions between the two phases is unequal. At partial equilibrium, when Na^+ and Cl^- are the only mobile ions,

$$\begin{gathered} \tilde{\mu}^{in}_{Na} = \tilde{\mu}^{out}_{Na}; \\ m^{out}_+ \cdot m^{out}_- = B\, e^{\Delta P(\bar{v}_+ + \bar{v}_-)/RT} \cdot m^{in}_+ m^{in}_-; \\ B = [\gamma \pm^{in} / \gamma \pm^{out}]^2; \end{gathered} \tag{3}$$

ΔP = pressure difference across cell membrane. When $Be^{\Delta P(\bar{v}_+ + \bar{v}_-)} = B'$, Eq. (3) becomes the well-known relationship between inside and outside concentrations:

$$m^{out}_+ \cdot m^{out}_- = B' \cdot m^{in}_+ \cdot m^{in}_-$$

or

$$Na^+_{out} \cdot Cl^-_{out} = B' \cdot Na^+_{in} \cdot Cl^-_{in}. \tag{4}$$

The contribution of the pressure term to B' is unknown. If *Halobacterium* were to be a condensed gel, as is suggested by the low water content, the inside pressure would be very high. Ginzburg and Cohen [6] have shown that there is an internal hydrostatic pressure of 98 atm for resins with 4% cross-linking. When the cross-linking was 20%, the internal pressure rose to ca. 1,000 atm. Should the internal pressure in the *Halobacterium* cell be 100 atm, B' would be increased by 1.2; at 500 atm, B' would be about 2.7 times greater.

When the hydrostatic pressure difference across the membrane is assumed to be zero, then the mean ion activity coefficients within the cell can be calculated from B and from the mean ion activity coefficients in the

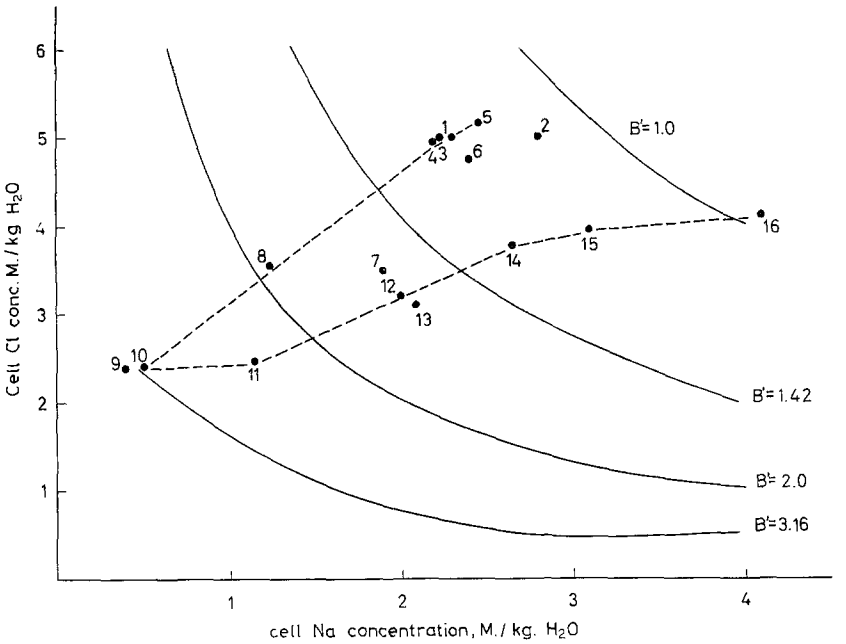

Fig. 13. Variations of cell $[Cl^+]$ and $[Na^+]$ with different B' calculated according to the Donnan equilibrium. B' = ratio of mean ion activity coefficients inside and outside the cells. Outside NaCl concentration: 3.9 M. Solid lines: calculated values. Figures adjacent to experimental points refer to Table 5. Broken line drawn by joining the peripheral points

medium. γ_{in}^{Na} must then be very high indeed. However, it is as yet premature to give an interpretation of B'.

In Fig. 13, Cl_{in}^- is plotted against Na_{in}^+. The solid lines represent values calculated for different B'. It is shown that B' is high in most of the non-growing cells (points 9–13). It should be realized that if B' were to remain at around 1 when Na_{in}^+ is low, Cl_{in}^- would have to approach 30 M.

Let us now consider growing cells. Ion distributions can be interpreted by the coupling of ion flows to chemical reactions, as is customarily done. The pumps required have been described already [8]. An alternative explanation of ion distribution in these growing cells is by the same means as was used for the non-growing state, namely in terms of a Donnan distribution of Na^+ and Cl^- brought about by the large amount of K^+ bound within the cytoplasm. In Fig. 13, the cluster of points representing growing cultures (points 1–6) are at a locus of a mean ion activity coefficient (B') of 1.2 to 1.3. In Fig. 14, K_{Cl} is plotted against Na_{in}^+. As in Fig. 13, points 1–7 represent values obtained from growing cultures; in these cultures, K_{Cl} was between 2 and 3. It is noticed that in stationary-state cultures (point 10)

Table 5. *Ion concentrations in Halobacterium under different experimental conditions*

Conditions	Concn. (moles/kg cell H_2O)				
	K^+	Na^+	Cl^-	Anion deficiency	K_{Cl}: Cl–Na
A. Actively-growing bacteria; pH 7.0					
1. Control (Fig. 1)	4.75	2.34	5.0	2.09	2.66
2. Initial point (Fig. 2)	4.7	2.8	5.0	2.5	2.2
3. Initial point (Fig. 3)	3.95	2.2	4.95	1.2	2.7
4. Initial point (Fig. 4)	4.95	2.2	4.95	2.2	2.75
5. After resuspension (Fig. 11A)	4.9	2.4	4.75	2.65	2.35
6. After resuspension (Fig. 11B)	5.0	2.45	5.15	2.30	2.70
7. Culture at O.D. 0.25[a]	3.75	1.9	3.5	2.15	1.6
B. Stationary bacteria; no lag before resumption of growth					
8. 24 hr, 0 °C.	4.45	1.25	3.55	2.0	1.9
After 24 hr in saline[b]	3.85	1.3	not measured	—	—
C. Stationary bacteria; lag before resumption of growth					
9. 4 hr, argon, 0 °C	3.35	0.4	2.4	1.3	2.0
10. Stationary state[c]	3.9	0.5	2.4	2.0	1.9
11. 24 hr, saline[d]	3.0	1.14	2.48	1.66	1.34
12. 3 hr, 1 mM NaCN	4.0	2.0	3.2	2.8	1.2
13. 4 hr, argon, 37 °C	3.1	2.1	3.1	2.1	1.0
14. At pH 5.0[a]	3.1	2.65	3.78	1.87	1.13
15. At pH 5.5[a]	3.7	3.1	3.95	2.85	0.85
16. At pH 7.8[a]	2.3	4.1	4.1	2.3	0

[a] See Ref. [7].
[b] Bacteria resuspended from cultures at O.D. 0.06.
[c] See Ref. [8].
[d] Bacteria resuspended from cultures at O.D. 0.20.

K_{Cl} is about 2 M, or very close to its value in growing cultures. The fall in Na^+ concentration in the stationary-state cultures is postulated to be accompanied by an increase in B'; otherwise, K_{Cl} would have to approach 30 M.

This interpretation of the *Halobacterium* cell raises some puzzling and interesting questions. What is the nature of the specific binding of cell K^+ within the cell matrix? What changes occur when the rate of metabolism falls? What is the immediate energy source which brings about the efflux of 1 to 2 M NaCl per kg cell water against the concentration gradient?

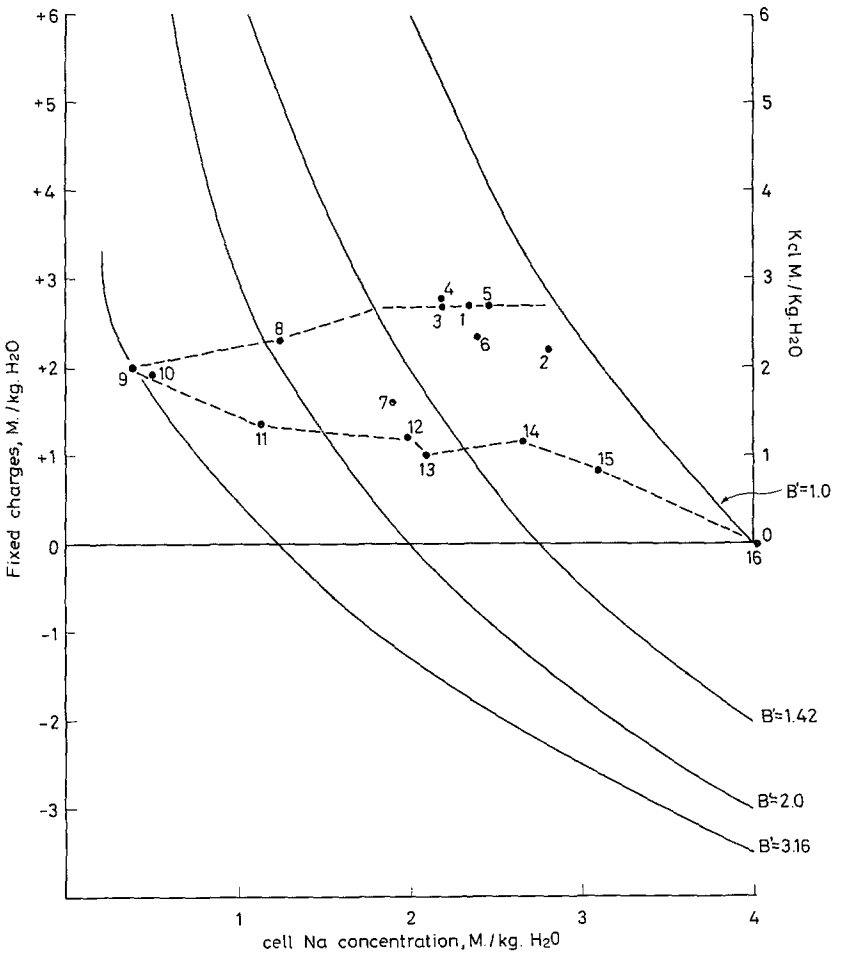

Fig. 14. Effect of positive fixed charge concentration within cells on cell Na^+ concentration. Refer to legend of Fig. 13

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