Ion Metabolism in a *Halobacterium*

I. *Influence of age of culture on intracellular concentrations*

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ABSTRACT Work is described on the changes in cell ions during growth of cultures of a species of *Halobacterium* isolated from the Dead Sea. Cell K concentration fell from 5.5 to 3.8 moles per kg cell water during the logarithmic phase of growth and maintained the latter value during the stationary phase (initial medium concentration, 7 mM). Cell Na and Cl followed a complex series of roughly parallel changes. The logarithmic phase ion concentrations were: Na, 1.0-2.3 moles/kg cell water; Cl, 2.3-3.7 moles/kg cell water. The final stationary phase values were: Na, 0.5 moles/kg cell water; Cl, 2.3-2.9 moles/kg cell water (medium NaCl concentration, 3.9 Molal). It is suggested that most of the K^+ is bound within the cytoplasm.

INTRODUCTION

It has been known for some time that halophilic bacteria, living in an environment containing 2 M NaCl or more, themselves contain large amounts of salt, including 3 M potassium/liter cell water (1). It is very puzzling to know how any organism can tolerate such high internal salt concentration and how the mechanisms for ion transport can operate under such conditions of high external salinity. Thus an investigation of ion transport in halophilic bacteria is certain to be rewarding. It could not be undertaken, however, until methods had been developed for the study of ion concentrations in single-celled cultures and in small unicellular organisms. Such methods have been developed recently and have been applied in particular to *Escherichia coli* and yeast. Work on *E. coli* has been published in a series of papers starting in 1961 (2). Rothstein (3) has summarized work on yeast up to 1964. Other unicellular systems studied include *Streptococcus fecalis* (4), *Chlorella* (5), and the HeLa cell (6).

Up to the present, methods for studying ion transport in cell suspensions have depended on the isolation of uniform pellets centrifuged from concentrated cell suspensions or on the filtration, followed by washing, of undisturbed cell suspensions. An important feature of the work to be described here is that

ion concentrations have been measured in dilute suspensions of bacteria, by separation of the bacteria from the medium without any washing procedure. Such a technique was necessary since halophilic bacteria are extremely sensitive to the usual experimental procedures (e.g. pipetting, washing with sucrose solutions) which cause cell breakage and loss of a portion of the cell population (7, 8).

It is the purpose of this paper to show how intracellular K, Na, and Cl concentrations in a strain of halophilic bacteria change during the course of growth.

ISOLATION AND IDENTIFICATION OF ORGANISM

A sample of water from the Dead Sea was centrifuged and the residue streaked onto an agar plate containing the substances listed in Table I, with the addition of 5% Bacto-tryptone. After several days round pinkish colonies were observed. One of these was restreaked onto agar and then inoculated into a liquid medium of the same composition. The resulting bacterial culture was found to grow with a generation time of 5–6 hr.

TABLE I

COMPOSITION OF GROWTH MEDIUM

	Millimolal
Na^+	3900
Mg^{++}	150
Ca^{++}	1.4
Mn^{++}	$2.5 \cdot 10^{-4}$
K^+	7 ± 0.5
Cl^-	3900
PO_4^{---}	1.25
SO_4^{--}	150
Yeast autolysate	10%
Initial pH, 7.0	

Preliminary trials established that the optimum concentration of NaCl for growth was 3.5-4.0 M. 3.5 M NaCl was chosen as a working concentration. No growth was observed at concentrations lower than 2.0 M NaCl.

The bacteria were highly pleomorphic but showed a tendency towards the rod shape. The color of suspensions was pink, the color deepening as the cultures aged. These two characteristics together with the obligately halophilic nature of the bacteria, show them to belong to the genus *Halobacterium* (Bergey's Manual of Determinative Bacteriology. The Williams & Wilkins Co., Baltimore. 7th edition. p. 207).

The species has not yet been determined.

CHEMICALS

All salts used were of analytical reagent grade. The deionized water used throughout the course of this investigation had a conductivity of less than 1 μmho/cm.

METHODS OF CULTURE

Medium

The composition of the growth medium is shown in Table I. It was prepared by dissolving all the salts except Mg in water. Yeast autolysate was added and the pH adjusted to 0.3 pH unit above the value desired for the final medium. The addition of $MgSO_4$ at this point lowered the pH to the desired final value. $MgSO_4$ was added last because any alkalinization of the medium in its presence caused precipitation of Mg compounds.

The medium was sterilized by filtration through autoclaved Millipore filters HAWP (pore diameter 0.45 μ).

Yeast autolysate was prepared by heating equal weights of baker's yeast and water at 80°C for 10 min. The cooled mixture was centrifuged and the supernatant filtered through Millipore filters until the solution was clear. Only 2 wk supply was prepared at any one time, since the autolysate was found to deteriorate with time.

The yeast autolysate was the sole source of carbon and nitrogen.

Methods of Culture

150 ml of medium were poured in a sterile manner into 500 ml Erlenmeyer flasks with an inserted sidearm, consisting of a Klett tube, to permit measurement of the optical density of the growing culture. The flasks were incubated in a water bath at 37°C on a horizontal shaker. Growth was measured by means of a Klett photometer (blue filter, 400–465 mμ).

Maintenance of Bacterial Stock

Stocks were carried on agar slants consisting of growth medium solidified with 1.5% agar. The stocks were transferred every 2 months onto fresh slants. A new stock was taken for experiments every 2 wk. Periodically, the purity of the culture was checked by plating out very dilute suspensions.

RESUSPENSION OF BACTERIA

When it was necessary to resuspend the bacteria, they were centrifuged at 5900 *g* in a Sorvall refrigerated centrifuge (model RC2-B) at 20°C, for 10 min. The button of red bacterial mass, which had collected at the base of the tube, was transferred with a spatula to the required volume of fresh medium. Rapid stirring of the medium by means of a magnetic bar brought about resuspension of the bacteria within 20 min. On resuspension, such cells resumed growth, as measured by protein synthesis, at their former rate.

Lysed cells did not sediment during centrifugation.

Resuspension and concentration of bacterial suspensions were required for measurement of the dry weight content and trapped volume. The bacteria were not resuspended for any other measurement described in this paper.

Resuspension brought about swelling of the bacterial cells. The degree of swelling

could be measured by comparing cell protein or K content with cell volume before and after resuspension.

MEASUREMENTS OF CELL MASS

The following criteria were used as measurements of bacterial mass: (*a*) protein, (*b*) volume of pellet after centrifugation under standard conditions, (*c*) per cent dry weight. Protein and pellet volumes were measured in the course of each experiment. The per cent dry weight was determined at various stages of the growth cycle of the culture. Measurements (*b*) and (*c*) required correction for supernatant trapped within the pellet during centrifugation, in order for the volume and per cent dry weight of the cell material to be calculated.

(a) *Protein*

250 μl samples of suspension were centrifuged in polyethylene tubes in a Beckman Microfuge for 5 min. The tip of the tube containing the bacteria was cut off with a clean razor blade and dropped into a glass test tube to which was added 0.5 ml 1 N NaOH. The protein was determined according to Lowry's method (9), using bovine serum albumin as standard. Protein determinations on whole cells extracted with NaOH and on trichloroacetic acid precipitates of whole cells agreed to within 1%. Thus, routinely, whole cells extracted with NaOH were used. As a check on the Lowry method, the same trichloroacetic acid precipitate was used both for a set of Lowry determinations and for measurement of nitrogen, assuming that nitrogen was 14.7% by weight of total protein. In two separate determinations the protein, as measured by the Lowry method, was 81.5 ± 1.5% of the amount of protein calculated from the measurement from total N. The protein results quoted throughout this paper are those obtained by use of the Lowry method. In any one determination the protein samples were measured in triplicate. Agreement between replicates was to within 3%.

(b) *Volume*

The volume of bacterial pellets was determined by centrifuging bacterial suspensions in cytocrit tubes consisting of a glass bulb of 3–4 ml capacity connected to a precision-bore capillary tube of 1 mm diameter (Chance Bros., Smethwick, England). The tube was sealed at the base; to overcome the deformity due to the sealing at the extreme base of the tube, a drop of mercury was introduced into the base.

3 ml of bacterial suspension were introduced into the tube by means of a syringe. The tubes were centrifuged at 13,000 *g* for 30 min. The length of the resultant cell column was measured by means of an ocular scale in the eyepiece of a binocular microscope. The volume of the pellet was calculated from its length, the diameter of the tube being known.

The horizontal centrifuge was designed and built by Mr. E. Sochatchewer.

(c) *Fresh Weight and Dry Weight of Bacterial Pellets*

1. BY EXTRUSION OF PELLET ONTO DISC

This method was first described by Schultz and Solomon (2). A bacterial suspension (not less than 2% of total volume occupied by cells) is centrifuged in cytocrit tubes

similar to those described in the previous section, but with the bases closed by Teflon plugs. After centrifugation, portions of the resultant cell columns are extruded onto tared aluminum discs and weighed on a Misco quartz helix balance (Microchemical Specialties Co., Berkeley, Calif.). The weighed pellets are dried at 90°C to constant weight and reweighed. The ratio of the dry weight to total fresh weight can then be calculated.

Several determinations from bacteria in the early logarithmic phase were made by this method (see Table II).

2. BY MEASUREMENT OF PELLET WITHIN CYTOCRIT TUBE

This method has been described by Bentzel and Solomon (10). The bacterial suspension (about 2% cells per total volume) is centrifuged in a tared tube of the same shape as described above. After centrifugation, the supernatant is drawn out by means of a syringe, and the portion of the tube above the cells is rinsed with water and dried with acetone. The tube is then weighed, to obtain the fresh weight of the pellet, and dried for 48 hr at 90°C. It is reweighed to obtain the dry weight of the pellet.

Measurement of Supernatant Trapped in Centrifuged Cytocrit Pellets

The methods used for measuring volume of supernatant trapped within bacterial pellets after centrifugation of suspensions in cytocrit tubes, have been described elsewhere (11). The volume of medium trapped after 30 min centrifugation at 13,000 *g* was 21.1% ± 0.5% (SE) of the total pellet volume (mean of 14 determinations). The trapped volume was measured with dextran-^{14}C.

ION MEASUREMENTS

At the beginning of the investigation ion measurements were made on pellets extruded from cytocrit tubes. Owing to the lengthy period of centrifugation needed and the difficulties involved in extruding the poorly packed pellets, the method was abandoned and use was made of the Beckman Microfuge. This enabled ion determinations to be made on small (250 μl) samples of dilute cell suspensions which could be tested rapidly, since the period of centrifugation is 5 min or less.

Total Potassium

250 μl of bacterial suspension were introduced into 400 μl polyethylene tubes and centrifuged in a Beckman Microfuge for 5 min. The tip containing the cells was cut off with a clean razor blade and transferred to a Vycor Corning glass tube. 5 ml deionized water were added and the tube shaken on a Vortex mixer (Scientific Products, Evanston, Ill.) until the cells (which are red in color) were seen to leave the cut tip. A clear liquid was obtained. First, the total Na was measured with a flame photometer (Eppendorf Model 700) using a propane-butane flame. K standards containing the concentrations of Na measured in the samples were prepared. This precaution is necessary since high Na concentrations interfere with K determinations made on this flame photometer. The K concentration was measured, and the amount of K present in each sample calculated.

Samples for total K determination were taken in triplicate, and agreed to ±0.7% of the total K.

Sodium and Chloride

For the measurement of these ions, samples of bacterial suspension were introduced into Microfuge tubes containing a 50 μl drop of dimethylphthalate (sp gr 1.191). On centrifugation, the cells, accompanied by 25.6% of their volume of supernatant, sedimented below the phthalate which is of lower specific gravity. The measurement of the amount of supernatant trapped within the cell pellet is described elsewhere (11). The supernatant (apart from the amount accompanying the cells) stayed above the ester. This technique was first used by Ballentine and Burford (12) for red blood cells.

The tube tip containing the bacteria was rinsed in distilled water and cut with a new razor blade, precautions being taken to avoid contamination. The tip was dropped into a Vycor silica glass tube and the cells dispersed in 5 ml distilled water. The K and Na concentrations of the solution thus obtained were measured with the flame photometer. The ratio of the Na:K concentrations was then calculated. 3 ml of the same solution were removed to a separate vial for measurement of Cl. This ion was measured by means of a Buchler-Cotlove chloridometer. An additional 1 ml portion was heated by dryness, ashed with 200 μl concentrated HNO_3, and the K concentration of the dissolved ash measured. The ratio of the Cl:K concentrations in the original solution was calculated.

Samples were taken in quadruplicate; the standard deviations were ±6% of the mean for Na:K and ±5% of the mean for Cl:K.

The ashing step was needed to eliminate the dimethyl phthalate which tended to reduce flame photometer readings without affecting chloridometer readings. In fact, the dimethylphthalate affected readings only in samples with low K concentrations (10 μEq/liter or less), or when the solutions had been shaken particularly vigorously for extraction of the cell pellet from the tube tip.

Even after ashing, K as measured after centrifugation of cells with dimethyl phthalate was lower than that measured without the phthalate. This is because the cells of lowest density did not centrifuge through the phthalate, and hence were not included in the cell pellet. K, as measured after ashing, was 80–90% of the K measured in the absence of dimethyl phthalate. The bacteria represented by this large fraction of the total cell K were assumed to be representative of the total population.

Checks on Methods of Extraction

(A) IN THE ABSENCE OF DIMETHYL PHTHALATE

A series of cell pellets was prepared in the usual way and dispersed in distilled water. 1 ml portions of the resultant solutions were heated to dryness and ashed with 200 μl HNO_3 at 140°C. The ash was dissolved in 1 ml distilled water. The K concentrations were read in the solutions obtained before and after ashing and were found to agree to within 1.6% (mean of five samples). It was concluded that water extraction of the pellets liberated all the cell K.

(B) IN THE PRESENCE OF DIMETHYL PHTHALATE

The above procedure was repeated on pellets obtained by centrifugation of samples of bacterial suspension with dimethyl phthalate. The Na/K ratios were as follows:—

	Water extraction	After ashing
Experiment 1	0.73 ± 0.02 (mean of four replicates)	0.77 ± 0.06 (mean of four replicates)
Experiment 2	0.93 ± 0.08 (mean of five replicates)	0.94 ± 0.08 (mean of five replicates)

It was again concluded that it sufficed to extract the pellets with water in order to extract all the K and Na present.

(c) FOR CHLORIDE

Chloride was checked according to the method of Cotlove (13) who mentions that protein and peroxide are the major substances interfering with determination of chloride. Protein was destroyed by boiling for 30 min with 0.6 N NaOH and then centrifuging off the precipitate obtained after addition of acid zinc sulfate. In samples in which the protein had been hydrolyzed in this way, the chloride content was 104.6% of the water-extracted samples. Peroxides were destroyed by treatment with sodium perborate. In such samples, the chloride content was 98.0% of that measured in the water-extracted samples. It was concluded that extraction of samples with water was sufficient.

CALCULATIONS OF FRESH AND DRY WEIGHTS OF CELLS

These are calculated by correcting the measured dry/fresh weights of bacterial pellets for the trapped volume of supernatant:

$$\frac{A}{B} = \frac{(D_p)\ (V_p)\ (P_p)/100 - (Vs)\ (Ds)}{(V_p)\ (P_p) - (Vs)\ (Ps)}$$

A/B = dry/fresh weights of cells

D_p = *measured* dry:fresh weights of pellet, per 100mg fresh weight of pellet

V_p = volume of fresh pellet, i.e. 100

P_p = density of fresh pellet, i.e. 1.18, obtained from *measured* ratios of weights and volumes of pellets before drying

Vs = trapped volume of supernatant, i.e. 32.1% of total volume

Ds = weight of dried residue of supernatant, i.e. 0.24 mg/ml, as measured on ml samples of supernatant heated to dryness

Ps = density of supernatant, i.e. 1.153, as measured on 1 ml samples of supernatant

$(Vs)\ (Ps)$ = weight of trapped supernatant.

$(V_p\ (P_p)$ = fresh weight of pellet

The dry:fresh weight ratio thus obtained is that of the cells of the concentrated cell suspension on which measurements were made. In order to know the dry:fresh weight ratio of cells of the original suspension before it was concentrated by centrifugation, the ratio must be corrected for the cell swelling which occurs during centrifugation. This swelling amounted to 13 ± 1% of the original volume (mean of five experiments done at different phases of the growth cycle). Values corrected for swelling are given in the fourth column of Table II.

CALCULATIONS INVOLVING IONS

The errors involved in the calculation of amounts and concentrations of ions have been calculated according to Wilson (14):

$$\sigma^2 = \frac{(\delta F)^2}{\delta x_i}\,\sigma_i^2$$

σ^2 = variance of final result

F = known functional form

x_i = component i

σ_i^2 = variance of ith component

Total K, Na, and Cl

The amount of total K can be calculated directly from the K concentration measured with the flame photometer. A small correction must be made for the amount of K present in the supernatant above the cells but cut off with the tip of the tube, and which is estimated from the Na concentration measured at the same time (see below).

Total Na and Cl are calculated by multiplying the Na:K and Cl:K ratios by the total K.

(A) TOTAL K

Volume of bacterial suspension centrifuged in Microfuge tube: 250 μ. Cell pellet dispersed in 5 ml distilled water.

Na concentration, 1.5 mM/liter $\quad\therefore$ Amount of Na = 7.5 μEq

K concentration, 202 $\pm$ 4 μEq/liter $\quad\therefore$ Amount of K = 1.01 $\pm$ 0.02 μEq

Supernatant Na concentration = 3.5 moles per liter
= 3.5 μEq per μl

On the assumption that all the measured Na is due to supernatant included above the cells, then the *volume* of this included supernatant:

$$\frac{7.5\text{ Eq}}{3.5\text{ Eq/liter}} = 2.2\ \mu\text{l}$$

Supernatant K concentration = 5 mM per liter
= 0.005 μEq per μl

$\therefore$ Maximum possible amount of K in supernatant above the cells
= (2.2)(0.005) μEq
= 0.01 μEq

Since cell K = pellet K − trapped K

$$\therefore \text{ cell K} = 1.01 - 0.01\ \mu\text{Eq}$$
$$= 1.00 \pm 00.02\ \mu\text{Eq in cells in 250 } \mu\text{l suspension}$$
$$\therefore \text{ cell K} = 4.00 \pm 0.08\ \mu\text{Eq in cells/ml suspension}$$

(B) TOTAL NA

Volume of bacterial suspension centrifuged in Microfuge tube: 250 μl. Cell pellet dispersed in 5 ml distilled water.

Na concentration, $167 \pm 4\ \mu$Eq (mean of four samples)
K concentration, $149 \pm 1\ \mu$Eq (mean of four samples)
$$(\text{Na}):(\text{K}) = 1.12 \pm 0.3$$
$$\therefore \text{ Total Na} = (\text{cell K})([\text{Na}]:[\text{K}])$$
$$= 4.48 \pm 0.12\ \mu\text{Eq in pellet from 1 ml suspension}$$

(C) TOTAL CL

Same sample as for total Na.

Amount of Cl in 3 ml portion $= 0.827 \pm 0.007\ \mu$Eq (mean of three samples)
$$\therefore \text{ concentration} = 276 \pm 2\ \mu\text{Eq}$$
K concentration after ashing $= 182 \pm 3\ \mu$Eq
$$(\text{Cl}):(\text{K}) = 1.52 \pm 0.02$$
$$\therefore \text{ Total Cl} = (\text{cell K})([\text{Cl}]:[\text{K}])$$
$$= 6.08 \pm 0.08\ \mu\text{Eq in pellet from 1 ml suspension}$$

Calculation of Cell Na and Cl

It is first necessary to measure the amount of supernatant trapped within Microfuge pellets. The relative contributions of supernatant and cells to total Na and Cl in these pellets are then calculated.

The measurement of trapped supernatant in Microfuge pellets has been described elsewhere (11). It was established that the volume of supernatant trapped within Microfuge pellets after a centrifugation period of 5 min, was $25.5 \pm 0.8\%$ (SE) of the total pellet volume obtained when the same suspensions were centrifuged in cytocrit tubes (mean of 45 determinations).

An example of the calculation of cell Na is given below.

Total Na $= 4.48 \pm 0.12\ \mu$Eq in pellet from 1 ml suspension
Pellet volume $= 1.8\ \text{mm}^3$/ml suspension
$$\therefore \text{ volume of trapped supernatant} = (1.8)(0.256 \pm 0.008)\ \text{mm}^3$$
Amount of Na in this volume $= (1.8)(0.256 \pm 0.008)(3.5)\ \mu$Eq
(since Na concentration is $3.5\ \mu\text{Eq}/\mu\text{l}$)
$$= 1.64 \pm 0.05\ \mu\text{Eq}$$
$$\text{Cell Na} = \text{total Na} - \text{trapped Na}$$
$$= (4.48 \pm 0.12) - (1.64 \pm 0.05)\ \mu\text{Eq}$$
$$= 2.84 \pm 0.13\ \mu\text{Eq in cells in 1 ml suspension}$$

The amount of cell Cl is calculated in the same way and is 4.44 ± 0.09 μEq in cells in 1 ml suspension.

Calculation of Cell Water

Cell water was calculated from the measured pellet volume according to the following formula

$$X = v(1 - Vs)(Pc)(1 - A/B)$$

X = cell water in milligrams
v = measured volume of a given pellet. See *Volume* in section entitled Measurements of cell mass.
Pc = cell density

The cell density is measured by preparing different mixtures of dimethyl phthalate and octoil (Consolidated Vacuum Corporation, Rochester, N. Y.). The specific gravity of the dimethyl phthalate is 1.191 and that of the octoil 1.064. Both substances are miscible with each other and hence mixtures of known specific gravity can be prepared. The cell density equals the density of the mixture which permits the centrifugation through it of 50% of the cells.

At 37°C, rather more than half (80–90%) of the cells from logarithmic phase suspensions sedimented through pure dimethyl phthalate. The average cell density was therefore slightly greater than 1.191. A value of 1.2 was therefore arbitrarily taken, since the error introduced by the uncertainty at the second decimal place is unlikely to affect the result by more than 5% of the total value.

Specimen calculation

Pellet volume = 1.8 mm^3/ml suspension

Since 32.1 ± 0.5% of the total pellet volume is occupied by supernatant, the cell volume is 1.22 ± 0.02 mm^3

Cell fresh weight = (1.22)(1.2) mg
= 1.46 ± 0.024 mg

Weight of cell H_2O = (1.46 ± 0.024)(0.596 ± 0.0014) mg, since cell H_2O is 59.6% of cell fresh weight at this stage of growth (Table II).

= 0.87 ± 0.04 mg

Calculation of Ion Concentrations

Specimen calculations

$$\text{Cell K concentration} = \frac{4.00 \pm 0.08}{0.87 \pm 0.04} \mu\text{Eq per mg cell } H_2O$$
$$= 4.60 \pm 0.22 \text{ moles per kg cell water}$$

$$\text{Cell Na concentration} = \frac{2.84 \pm 0.13}{0.87 \pm 0.04}\ \mu\text{Eq per mg cell } H_2O$$
$$= 3.26 \pm 0.2 \text{ moles per kg cell water}$$
$$\text{Cell Cl concentration} = \frac{4.42 \pm 0.09}{0.87 \pm 0.04}\ \mu\text{Eq per mg cell } H_2O$$
$$= 5.08 \pm 0.26 \text{ moles per kg cell water}$$

Limits of Error in Calculating Cell Na and Cell Cl Concentrations

The error involved in the calculation of total Na and Cl is shown to be relatively small. A larger uncertainty is involved in the calculation of the trapped Na and Cl; the final mean cell Na and Cl have standard deviations of 7% and 5%, respectively of the total amounts.

In the calculation of cell Na and Cl concentrations, the parameter of

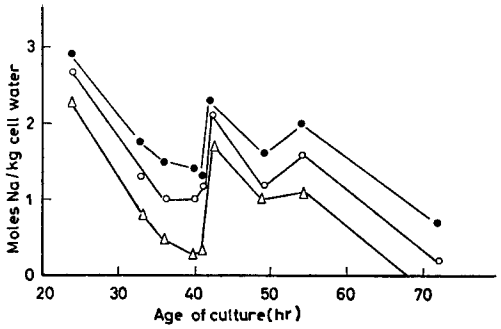


FIGURE 1. Effect of value taken for calculation of trapped volume on cell Na concentration. Solid circles, 20% of total volume assumed to be supernatant; open circles, 30%; open triangles, 40%.

trapped volume is used twice, first for calculation of the amount of cell ion and second for the amount of cell water. Thus, any overestimation of the trapped space value would lead to a *low* estimate of cell Na and a *low* estimate of cell H_2O, and hence a cell Na concentration closer than expected to the true value. The influence of trapped space on concentrations of Na is shown in Fig. 1 for cells throughout the growth phase. In Fig. 1 Na concentrations are calculated on the assumption that 20, 30, or 40% of the total pellet volume is occupied by trapped supernatant. The 40% value is a clear overestimate since in the stationary phase, it leads to a calculated trapped Na larger than the total measured Na. The 20 and 30% estimates never differ by more than ± 0.2 mole per kg cell water.

RESULTS

Dry Weight Content of the Bacteria

The dry weight contents of the bacteria at various stages of growth are described in Table II. In cultures at an optical density of 0.2, the dry weight

content was measured by both methods used (see Methods); these yield results of 46.1 ± 1.0% (method 2) and 49.8 ± 0.3% (method 1). The agreement between the two methods is probably within the limit of error of the methods used. In the midlogarithmic phase the dry weight content is at its lowest (40.4%) and increases at later stages of growth, reaching a value of 45.4 ± 1.2% in the stationary phase. Christian and Waltho (1) found a dry weight of 50% for a related species at the same phase of growth.

TABLE II

DRY WEIGHT, AS A PROPORTION OF TOTAL FRESH WEIGHT OF *Halobacterium* sp. AT VARIOUS STAGES OF GROWTH

Age of culture		Dry weight		No. of determinations
Time	Method	Pellet	Cells	
hr		% ± *SE*		
24	2	33.5 ± 0.3	46.1 ± 0.3	4
24	1	35.8 ± 1.0	49.8 ± 1.0	15
37	2	30.9 ± 0.14	40.4 ± 1.2	14
72	2	38.3 ± 0.8	45.4 ± 1.2	20

TABLE III

COMPOSITION OF CELL MATERIAL OF *Halobacterium* sp. AT DIFFERENT STAGES OF GROWTH

Stage of growth	Early logarithmic	Late logarithmic	Stationary
Dry weight, %	46.1	40.4	45.4
Dry matter, *g/kg cell* H_2O	852	666	850
K + Na + Cl, *g/kg cell* H_2O*	254–425	290–352	239–266
Protein, *g/kg cell* H_2O‡	280	265	265
Unaccounted for, *g/kg cell* H_2O	320–147	161–47	346–319
Total	852	666	850
Salts as % dry matter	29–51	44–52	28–31

* Calculated from Table IV.

‡ Calculated with the aid of Fig. 2.

The salt content of the bacteria accounts for 28-52% of the total dry weight (Table III).

Growth of Culture of Halobacterium

The growth curves of the halophilic bacteria, typical of 17 cultures measured throughout the growth period are shown in Fig. 2. An initial inoculum of about 10^7 cells/ml was used. Although the cultures were inoculated from mother cultures in the logarithmic phase of growth, there was an initial lag. The first measurements of optical density, protein content, and volume of the

cells were made 20–24 hr after inoculation. After this, growth proceeded logarithmically until 40 hr after the start of the culture. During this phase the generation time was 6.5 hr, as calculated from all the parameters measured. This appears to be the shortest generation time recorded for a *Halobacterium*. Larsen (7) has written: "The extreme halophiles multiply slowly even under

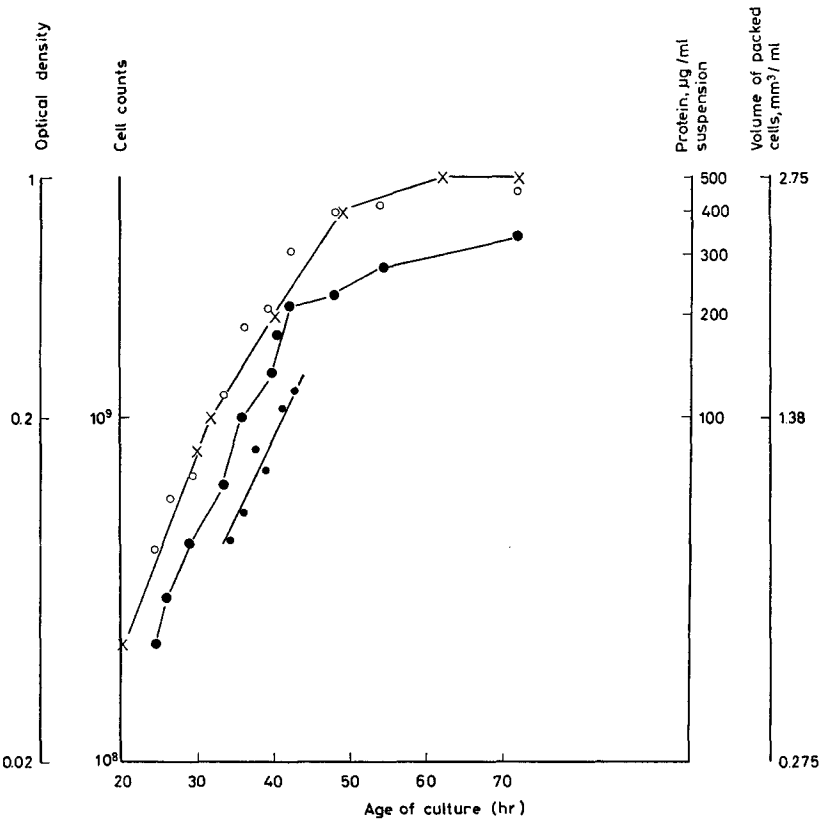


FIGURE 2. Growth of *Halobacterium* sp. as measured by (a) volume of cell material, × ----- ×; (b) total protein, o——o. The line is drawn by joining the volume measurements; (c) optical density, large solid circles; (d) cell counts, small solid circles. Initial pH of medium, 7.0. For composition of growth medium, see Table I.

the most favorable conditions designed. The shortest time obtained for the halobacteria is not much less than 7 hours."

A comparison was made of cell counts with a Petroff-Hausser bacteria counter and viable counts by dilution and plating on agar plates. It was concluded that in 60 hour cultures (stationary phase) at least 90% of the total cells yielded colonies.

The original pH of the medium was 7.0. For the first 28 hr of growth the pH fell, due presumably to the activity of the bacteria. The lowest value

reached was pH 6.4. Thereafter, the pH rose to a final value of 7.5 which was reached at the end of the experiment.

The rates of oxygen consumption per unit of protein are shown in Fig. 3. The oxygen consumption remained relatively constant throughout the logarithmic phase, despite changes in pH. When the growth rate decreased, the oxygen consumption fell to about a third of its initial rate.

Ions in Growing Cultures

The total amounts of ion in the cells in 1 ml of suspension are plotted in Figs. 4 a, 4 b, and 4 c. From these figures can be calculated the net influx of ion during the growth phase. The net K influx is 50 $\times$ 10^{-12} moles/cm^2 $\times$ sec

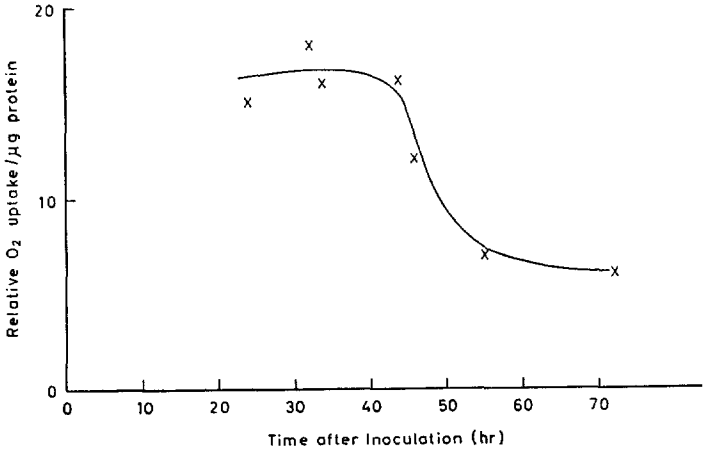


FIGURE 3. O_2 consumption of *Halobacterium* sp. during period of growth. For conditions of culture, see legend to Table I.

assuming a cell radius of 0.5 μ. This is four to five times higher than the net flux calculated from Schultz's results (2) for *E. coli*.

No net decrease in the amount of cell K occurred in the stationary phase (Fig. 4 a). However, a net decrease in Na was observed at this time (Fig. 4 b).

Ion concentrations are shown in Figs. 5 a and 5 b which are the results of two separate experiments performed in July, 1968, and January, 1969. An essential similarity between the two experiments is clear, although there are differences in timing. Except for the 20 and 24 hour points the K concentration stayed steady at 3.7 $\pm$ 0.2 moles per kg cell water throughout the growth phase, even in the stationary phase at a time when the medium K concentration was under 1 mM/liter.

The Na concentration is also seen to fall in the period from 20–40 hr of growth, and reaches minimum values of 1.2 moles/kg cell H_2O in experiment

1 and 1.0 mole in experiment 2. There is then a rapid increase to 3 M/kg cell water (experiment 1) and 2.4 moles (experiment 2), followed by a slow decline throughout the stationary phase to a final concentration of 0.5 M/kg cell water, well below the medium Na concentration of 3.9 M.

The Cl concentrations follow the pattern of Na concentrations.

Fig. 5 demonstrates that the sum of the Na + K concentrations is always greater than the Cl with an average anion deficiency of 2 moles per kg cell H_2O. The PO_4 concentration was measured on two sets of samples in the logarithmic phase and was found to be 125 ± 25 mM/kg cell water. These results imply that excess cation charges must be balanced by organic anions.

Thus, ion concentrations in cultures of these halophilic bacteria are not characterized by any one set of values; even in cultures growing and respiring at steady rates there may be wide fluctuations in concentrations of Na and Cl. On the other hand, stationary-phase cultures in which no net growth occurs are characterized by a K concentration nearly 5000 times higher than in the outside medium (K_{in} 3.75 moles/kg cell water; K_{out} 0.75 mM) and a Na concentration lower than in the outside medium (Na_{in} 0.50 mole/kg cell water; NaCl outside 3.9 M).

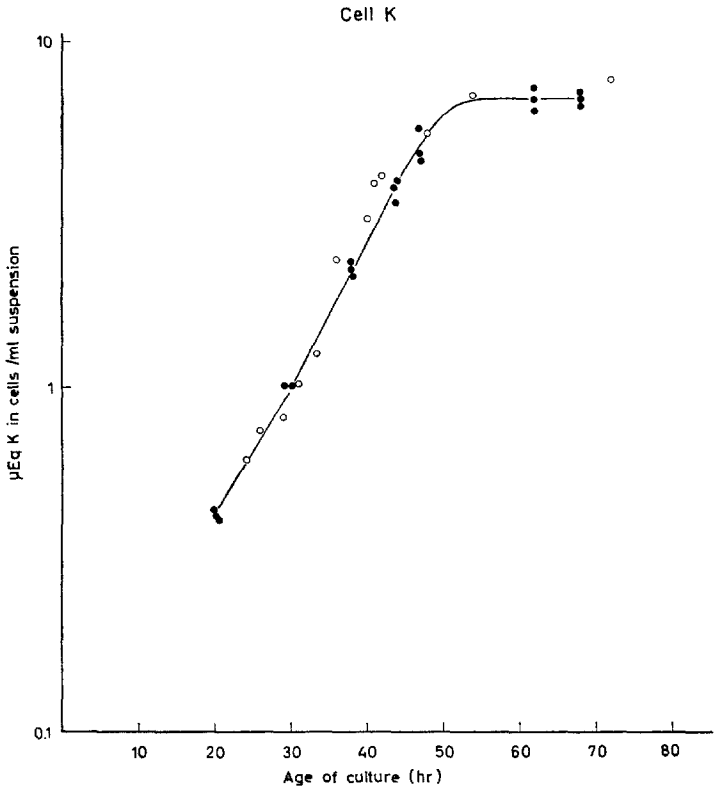
DISCUSSION

The ion metabolism of *Halobacterium* sp. has been shown to bear some features of resemblance to that of other organisms so far studied. For example, Na, K, and Cl are ions of major importance in the cell interior, as is the case in most other organisms. Furthermore, the cell concentrations of these ions differ from those in the medium, implying that metabolic, electric, and other activities are involved in the regulation of the cell ion concentration. The distribution of these ions is summarized in Table IV. The nature—passive or active—of the ion fluxes will now be considered in the light of the potential difference which may exist across the cell membrane.

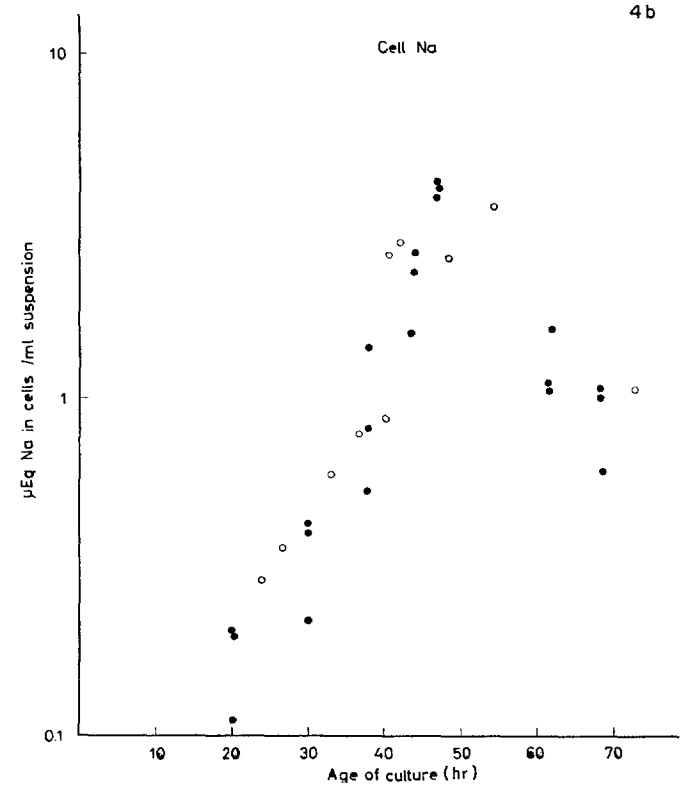
Should the PD be close to zero, the Cl ion would be distributed passively when the Cl chemical concentrations are equal on both sides of the membrane. K influx and Na efflux would have to be coupled to some metabolic reactions. Such a situation is found in many animal cells and in *E. coli* (2). It does not necessarily imply the existence of a Na-K exchange pump, nor do all the cell ions have to be in solution.

An alternate model assumes K to be passively distributed through the existence of a high potential (up to 200 mv, inside negative with respect to the exterior). Such high PD's have been found in *Neurospora* by Slayman (15). When such is the case, Na efflux must be active as before, but more energy is required to bring about the active efflux because the electrochemical potential gradient is higher than in the first model. Cl influx in this second

4a


Cell K
10
1
0.1
µEq K in cells /ml suspension
10
20
30
40
50
60
70
80
Age of culture (hr)

4b


Cell Na
10
1
0.1
µEq Na in cells /ml suspension
10
20
30
40
50
60
70
Age of culture (hr)

model would be active. Active Na effluxes and Cl influxes have been detected in *Nitella* (16) and *Chlorella* (5), for example. This picture could apply to the metabolizing *Halobacterium* cell.

If ion movements are indeed coupled to metabolic reactions which maintain gradients in ion concentrations, then the inhibition of metabolism should lead to the reduction and eventual abolition of ion concentration gradients.

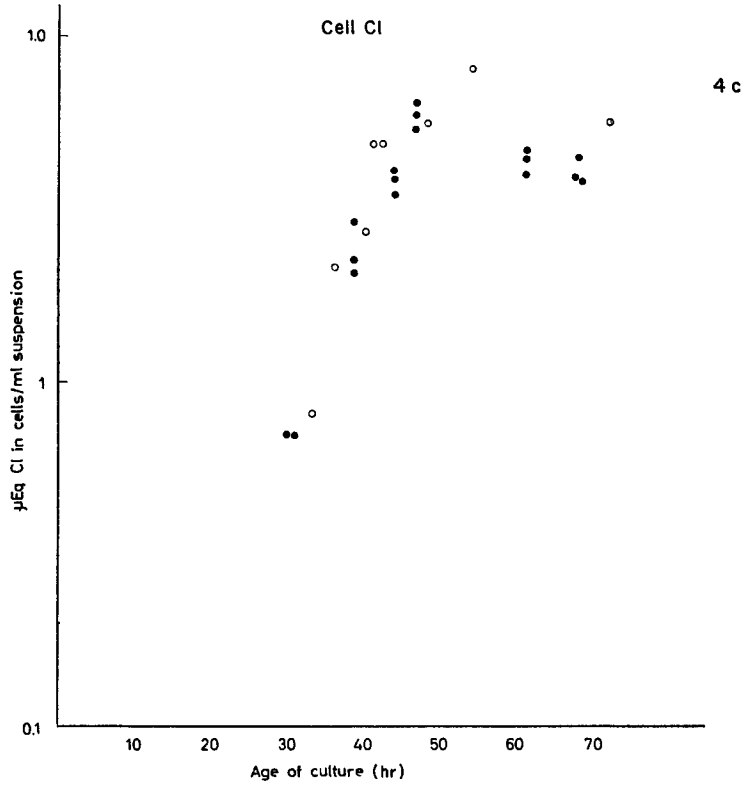


FIGURE 4. Ion content of bacteria contained in 1 ml suspension throughout period of growth. For conditions of culture, see legend to Fig. 2. Open and solid circles refer to two separate experiments.

This prediction has been generally fulfilled in most tissues and organisms studied up to now. Quite on the other hand, higher gradients of Na and K were observed in stationary-state cells than in logarithmic-phase cells even though the rate of respiration per unit of protein was lower. High gradients have been observed in experiments in which O_2 uptake was completely abolished (unpublished observations). Thus it may be argued that, in *Halobacterium*, the mechanism of ion movements is not due to "active transport" reactions in the usual sense of the word.

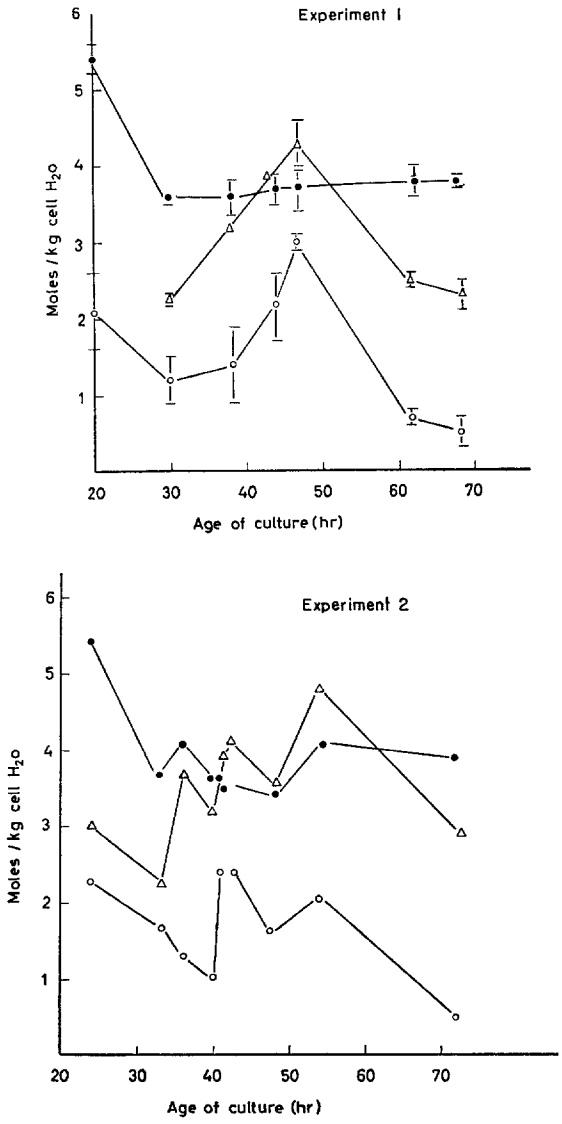


FIGURE 5. Ion concentrations of *Halobacterium* sp. throughout growth phase. For conditions of culture see legend to Fig. 1. Experiment 1, each point is the mean ± SD of three cultures measured at the same time. Experiment 2, each point represents a single culture selected randomly out of 11 cultures incubated together. Solid circles, K; open circles, Na; open triangles, Cl.

There is another major difference between ion metabolism in *Halobacterium* sp. and that of most organisms so far studied. It appears to be the usual case that cell ions, or the greater part of them, diffuse freely within the cell water (17). There are several types of evidence against this being the

case in *Halobacterium* sp. First, Table III shows that in growing cells large differences in total ionic content exist between the cell interior and the external medium. It follows that, if the cell ions were in solution, they would exert an osmotic pressure up to several hundred atmospheres during the logarithmic phase of growth. Thus a high hydrostatic pressure would be exerted against the cell envelope. For cells to tolerate so high a hydrostatic pressure, a rigid, mechanically strong cell wall would be needed. But, according to Larsen, who has summarized the evidence (7), the halophilic bacteria

TABLE IV

SUMMARY OF BIOPHYSICAL PARAMETERS OF *Halobacterium* sp. AT DIFFERENT STAGES OF GROWTH

	Early logarithmic	Late logarithmic	Stationary
A. Ion concentrations, *mM*			
K cell	3770-5500	3700-4000	3700-4000
medium	4-7	1-4	0.75
Na cell	1200-3000	1600-2100	500-700
medium	3900	3900	3900
Cl cell	2300-4200	3200-4100	2300-2900
medium	3900	3900	3900
B. Selectivity*	1000-3000	2500-10,000	33,000
C. Potential difference (calculated)			
K	−180	−200	−220
Na	(+31)-(+8)	(+23)-(+17)	(+54)-(+44)
Cl	(−17.0)-(+2.5)	(−4.0)-(+2.0)	(−17.0)-(−7.0)
D. Osmotic pressure			
ΔC	(−400)−(+6100)	(+700)−(+2400)	(−1300)−(−200)
$\Delta\pi$, atmospheres (approximate)	−10-250	22-75	−27.5-0

* Selectivity $= \dfrac{(K_{in})/(Na_{in})}{(K_{out})/(Na_{out})}$.

have no polysaccharide cell wall. It is generally agreed that the halobacteria are bounded by a cell envelope which differs from the cell walls of most other bacteria in that it does not contain muramic acid, nor any other mucopeptide which might confer rigidity on the structure (18). We have observed large changes in cell volume brought about by changes in medium pH (data to be published). Since it is difficult to envisage a mechanically strong cell wall which is also highly elastic, the results presented here make it likely that halophilic bacteria do not possess a rigid cell wall and that there is little or no hydrostatic pressure across the envelope. In this case, a portion of the cell ions must have a reduced osmotic coefficient. A second argument in

favor of reduced ion activity coefficients comes from the high cell ion concentrations themselves. KCl solutions of 4–5 M are almost saturated; it is physically impossible to make an aqueous solution of 4-5 M KCl + 1-3 M NaCl. Yet these are the amounts of ion present in the bacteria. It can only follow therefore that the activity of a part of these ions is reduced.

The large amounts of cell K, together with the relative inertness of this ion in stationary phase cells and in the cold, suggest that it is this cation whose activity is reduced. Restricted K mobility is also suggested by the extreme permeability of the cell membrane to moderately large molecules such as inulin and serum albumin (11). It is hard to see how large amounts of K could be retained within so leaky a cell unless the K were specifically bound onto an intracellular molecule.

In conclusion, the *Halobacterium* cell has been found to consist of K-rich cytoplasm bounded by a permeable cell membrane. The K is presumed to be retained within the cytoplasm by specific binding.

We wish to thank Professor Aharon Katchalski, who first suggested the problem to us, for his enthusiastic interest and help.

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