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State of Water in Extremely Halophilic Bacteria: Freezing Transitions of *Halobacterium halobium* Observed by Differential Scanning Calorimetry

A.D. Brown* and Julian M. Sturtevant

Department of Chemistry, Yale University, New Haven, Connecticut 06520

Summary. The apparent latent heat of fusion (ΔH) of thick pastes of *Halobacterium halobium* was determined by differential scanning calorimetry and compared with values obtained for a control paste of identical composition but containing bacteria already lysed by freezing and thawing. The rationale of the experiment was that, if there were any physico-chemical "abnormality" in the state of intracellular water or K^+, it might be reflected in the freezing behavior of the two types of preparation. Differences were observed, the paste of whole cells consistently giving higher values of ΔH than the lysed bacteria. Furthermore, the differences between the two types of preparation increased with the age of the culture. Scans of whole-cell pastes apparently distinguished between intra- and extracellular water and enabled ΔH to be calculated for those two aqueous compartments. Supplementary measurements were also made on solutions of NaCl, KCl, and mixtures of the two salts. Sodium chloride lowered ΔH whereas KCl raised it. Differences in ΔH between internal and external solutions and between whole and lysed bacteria could be attributed to the different effects of the two salts on freezing behavior. Changing values of ΔH with the age of a culture correlated with changing ratios of K^+/Na^+ in the bacteria. It is concluded that there is no evidence at this level of any physico-chemical abnormality in the halobacterial cytoplasm and no reason for supposing that retention of KCl by these bacteria is achieved by any agency other than the plasma membrane.

The physical nature of intracellular solutions, including some aspects of solute retention within living cells, has been a contentious topic for some years. The conventional opinion is that cytoplasmic solutes are retained entirely by the plasma membrane and that there are no physicochemical "abnormalities" associated with intracellular solutions. There is, however, a minority view championed vigorously by Ling (e.g., Ling, 1962, 1978; Ling & Walton, 1976; Ling et al., 1978) and others (e.g., White, 1976; Edelmann, 1977) that cytoplasmic factors (expressed as "binding", as the "association-induction hypothesis", or as the "polarized multilayer theory" of cell water) are primarily responsible for the retention of small molecules or ions within living cells. An essential part of this type of model is that intracellular solutions differ physico-chemically in some way from non-biological solutions of the same composition.

On balance, however, the evidence favors the conventional wisdom that the physical chemistry of intracellular solutions is essentially "normal" and solutes are accumulated and retained by the cell membrane. Nevertheless, the extremely halophilic bacteria, *Halobacterium* and *Halococcus,* by virtue of their very high intracellular concentrations of potassium, might present a genuinely different set of circumstances. Experimental evidence relevant to this possibility stems from several sources. Cope and Damadian (1970) reported that short transverse nuclear magnetic resonance (NMR) relaxation times for K^+ in *Halobacterium halobium* and suggested that "cell K^+ is complexed by fixed charges and/or is solvated in semicrystalline cell water." Magnuson and Magnuson (1973) reported other apparent peculiarities of potassium in *H. salinarium* (a single narrow continuous wave NMR spectral line for ^{39}K). Edzes et al. (1977) reported nuclear magnetic relaxation measurements of the ions Na^+, and Rb^+ and Cs^+ in a *Halobacterium* isolated from the Dead Sea. They concluded that the results supported a model involving binding of intracellular alkali ions. On the other hand, Shporer and Civan (1977), using pulsed NMR studies of ^{39}K concluded

* *Present and permanent address:* Department of Biology, University of Wollongong, Wollongong, N.S.W. 2500, Australia

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that more than 97% of the intracellular K^+ in *H. halobium* is in free form and that there is a "small ordering factor characterizing all of the intracellular K^+." Ginzburg, Ginzburg and Tosteson (1971) studied the retention of potassium by starving *Halobacterium* sp. and the release of K^+ caused by various anionic detergents. They interpreted their results in terms of selective binding of potassium on sites that are modified by the anion. Lanyi and Silverman (1972), however, measured diffusion of K^+ from frozen-thawed pastes of *H. cutirubrum* and concluded that the ion was not "bound" within the cell.

One of the factors which seems to have restricted discussion of this problem is the consistent contrasting of "bound" and "free" states of an ion or salt within a cell. With salt concentrations in excess of 5 molal, stoichiometric binding to sites within a cell would seem to be impossible since no other substance or reactive group is likely to approach such a concentration.

There are, however, other possibilities which warrant consideration. One is that solvent "structure" is modified by the dominant solute (KCl) and by the ordering of intracellular surfaces in such a way that, for example, the activity coefficient of KCl is effectively lowered and/or the intracellular solution is ordered to such an extent that diffusion of KCl, K^+ or Cl^- is restricted. Conditions of this sort, if they exist, are unlikely to be detected by the measurements of solutes diffusing from cells which had been disorganized by freezing and thawing as in Lanyi and Silverman's (1972) experiments. Furthermore, the low intracellular entropy which characteristically accompanies bacterial growth (Forrest & Walker, 1971) might be expected to affect the organization of the highly concentrated potassium chloride solution in halophils. It is of some interest, therefore, that Edzes and Berendsen (1975), while concluding that, in cells generally, the physical state of ions (and solvent) is no different from that of comparable nonliving systems, admitted some doubt about halobacteria.

Thermodynamic measurements of solution properties offer a potentially useful approach to this problem. The present paper describes measurements of apparent latent heat of fusion during a freezing transition of *H. halobium*.

The underlying assumption was that any changes in solute or solvent activity coefficients imposed by cellular organization would be reflected in the freezing characteristics of the bacteria. Because of the complexity of the system, however, we were unable to predict the direction of any alterations to enthalpy of freezing caused in this way. The approach was essentially an empirical one in which measurements on whole cells were compared with those on a control system comprising the bacteria disrupted by freezing and thawing.

Materials and Methods

Organism

Halobacterium halobium was grown with rotary agitation at 37 °C in a medium containing Bacto Tryptone and yeast extract (each 0.5% wt/vol) plus salts according to Sehgal and Gibbons (1960). This medium contains, among other salts, 250 g sodium chloride and 3 g potassium chloride/liter at 20 °C. Its water content is 75.6% (wt/wt). The concentration of sodium chloride is thus 4.28 M (at 20 °C) or 4.89 molal; the concentration of potassium chloride is 0.040 M or 0.046 molal. The experimental culture was dispensed in volumes of 500 ml into Erlenmeyer flasks of 2-liter capacity and inoculated with a 5% inoculum from a stationary phase culture. At specified times samples were harvested as follows. Two volumes of about 20 ml were transferred to centrifuge tubes, chilled in ice and centrifuged at 0 °C for 20 min at 27,000 × *g*. The supernatant fluid was decanted, and the tubes were allowed to drain onto absorbent paper for exactly 15 min at room temperature (about 20 °C). The tubes were then fitted with lids and returned to the ice bath preparatory to withdrawing weighed samples for analysis.

Samples of 10–12 or 3–4 mg were withdrawn and transferred to a tared Perkin-Elmer sample pan (type 219-0062, for volatile solvents) which was sealed and weighed. Although these pans are supposed to seal hermetically, it was found that occasionally they did not. For that reason, immediately after sealing, they were immersed in clear petroleum jelly, removed, and wiped free of excess grease with a tissue. There was no effect of the residual grease on the calorimetric measurements. The bacterial samples were obtained by inserting the end of a Pasteur pipette into the bacterial paste and subsequently expelling the paste into the sample pan. Three samples were taken for each set of measurements. They were the test sample and two for water determinations. The last were also weighed into the bottom half of a sample pan which was dried overnight at about 80 °C under vacuum or at about 100 °C at atmospheric pressure. Initially "control" samples were also taken and frozen by storage in dry ice. There was some evidence of loss of water by sublimation during storage (notwithstanding the grease seal). Values of apparent latent heat of these samples were consistently lower than the corresponding samples frozen and thawed in the calorimeter and were excluded from the subsequent interpretation.

Calorimetry

The calorimetric measurements were made with a Perkin-Elmer DSC-1B differential scanning calorimeter. The instrument was calibrated with water (melting transition) against a glycerol reference; the same reference sample was used for all scans on the DSC-1B. Some supplementary measurements were made with a Perkin-Elmer DSC-2 (*see* below). Downward scans were normally started at 260 K for bacterial pastes and salt solutions. The scanning rate was 2.5°/min. At the end of a scan the temperature was brought rapidly up to 300 K and held there for 5 min to ensure redissolution of all salts. The ensuing calorimetric measurements revealed that complete dissolution was achieved. The scanning cycle was then repeated once or twice on the same sample. Supplementary scans (melting transitions) at 2.5°/min were sometimes made, but the results so obtained were of little value because of the excessive time required for the dissolution of salts. Moreover, thawing transitions were not considered to be relevant since it was argued that

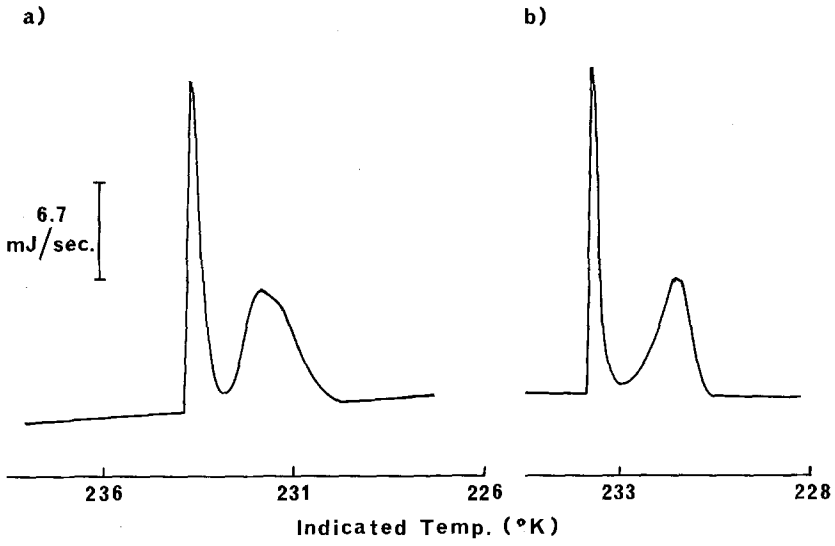

Fig. 1. Freezing transitions of two pastes of whole *Halobacterium halobium*. The two curves indicate approximately the extent of the differences in shape of freezing curves obtained with whole bacteria

any hypothetical anomaly in cell water would have been obliterated by freezing. The purpose of scanning the paste after the first freezing and thawing was to obtain measurements on a "control" paste in which intra- and extracellular solutions had mixed. Preliminary observations had shown that this indeed did happen. Areas of peaks were measured with a planimeter and expressed as apparent latent heat of fusion/mass water. All the values quoted for heats of fusion should be seeen as empirical quantities since they are affected to unknown extents by kinetic factors arising from supercooling and the design of the calorimeter. For this reason, no attempt was made to correct the apparent heat values for the change of the heat of fusion of water with temperature.

The Distribution of Water in the Centrifuged Pellet

The mass of interstitial (extracellular) fluid was determined as follows. Blue dextran was dissolved with warming to a high arbitrary concentration in distilled water, salt was added to give a final concentration of about 28% wt/vol, and this solution was added to a culture of *H. halobium* which was then incubated under the standard growth conditions for 3 hr. The amount of blue dextran solution added to the culture was such as to give an extinction (624 nm) of about 0.5 when the supernatant fluid was diluted 1/20. The culture was then centrifuged in tared centrifuge tubes under the standard conditions and the supernatant solution (S_1) retained. A weighed amount of fresh medium was added, the suspension thoroughly dispersed, and again centrifuged. The second supernatant (S_2) was retained. The water content of the pellet, as well as that of the culture medium as added, was determined by evaporation. The mass of interstitial fluid in the pellet was determined from the equation

$$M_0 = E_2 \cdot M_m (E_1 - E_2)^{-1} \quad (1)$$

where M_o is the mass of interstitial fluid, M_m is the mass of medium added after the first centrifugation, E_1 and E_2 are the extinctions of the supernatants S_1 and S_2, respectively, The masses of intracellular and interstitial water in the pellet were calculated from the equations

$$p = C + W_i + W_o + S_o \quad (2)$$

$$W_i + W_o = W_p \quad (3)$$

$$W_o + S_o = M_o \quad (4)$$

$$W_o = 0.756\ (W_o + S_o) \quad (5)$$

where p is the mass of the wet pellet, C in the mass of the dry cells, W_i is the mass of intracellular water, W_o is the mass of extracellular water in the pellet, W_p is the total mass of water in the pellet, S_o is the mass of extracellular solids and the numeral, 0.756, is the water content of the culture medium expressed as a decimal fraction.

Estimation of Sodium and Potassium

Bacterial pastes were prepared as described above, lysed in distilled water, diluted appropriately, and analyzed for Na^+ and K^+ conventionally by atomic absorption spectrophotometry. Assumptions made in assessing the intra/extracellular distribution of ions are outlined in Results.

Results

During freezing, a paste of whole bacteria gave two exothermic peaks such as shown in Fig. 1. The first peak, interpreted as representing the freezing of the extracellular solution (*see* Discussion), was always sharp. The second peak, attributed to the freezing of the cellular contents, was always broader and lower than the first. Other aspects of the relations of the two peaks, such as the height of the trough above the baseline and apparent freezing temperatures were somewhat variable. Figure 1 *a* and *b* show two examples of the freezing transition and give some indication of the range of shape and freezing temperature which occurred throughout the experiments. Melting transitions, although of little value for the determination of apparent latent heat, did indicate the temperature at which melting began and hence of the extent of supercooling during freezing. Melting temperatures ranged from 243–255 °K. Freezing temperatures of Peak (*i*) ranged from 232–236 °K and of Peak (*ii*) from 231–233 °K. Supercooling was thus in the range

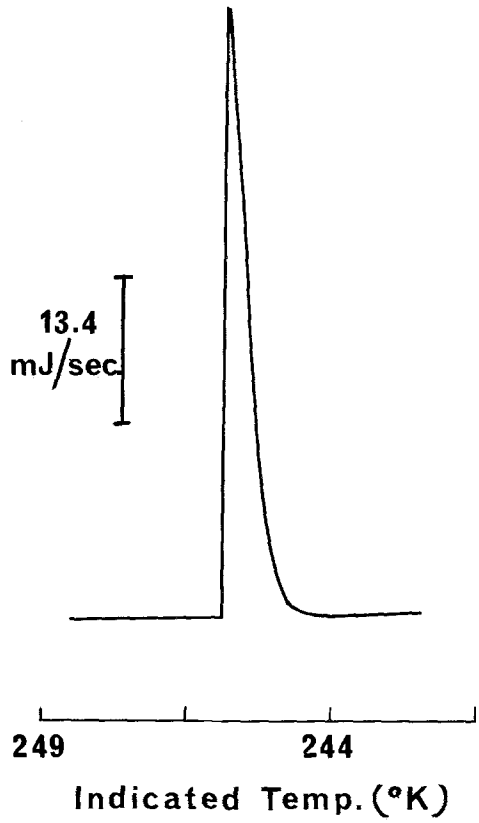

Fig. 2. A freezing transition of a paste of *Halobacterium halobium* previously lysed by freezing and thawing

of 7–24° and was commonly about 15°. Significant supercooling also occurred with the freezing of pure salt solutions.

When a bacterial paste, which had already been frozen and thawed either in the calorimeter or separately in dry ice was again frozen; it gave a single sharp peak (Fig. 2). This froze at a higher indicated temperature than either of the two peaks encountered with whole cells, the range of apparent freezing temperatures being 238–254 °K for pellets of 10–12 mg and 240–246 °K for pellets of 3–4 mg. Moreover, cultures grown for about 116 hr gave pastes which, when lysed as above, froze in the range 235–239 °K (3–4 mg). Thus the frozen and thawed pellets supercooled to a lesser extent than did the intact suspensions.

The Determination of Apparent Latent Heat

There was an effect of sample mass on measured latent heat. For example, samples of 3–4 mg gave apparent latent heats some 8% higher for lysed cells than did samples of 10–12 mg. It is evident, therefore, that the investigation would have benefitted from measurements made with a large enough series of masses to enable extrapolation to zero mass. This was not practicable, and discussion will be confined,

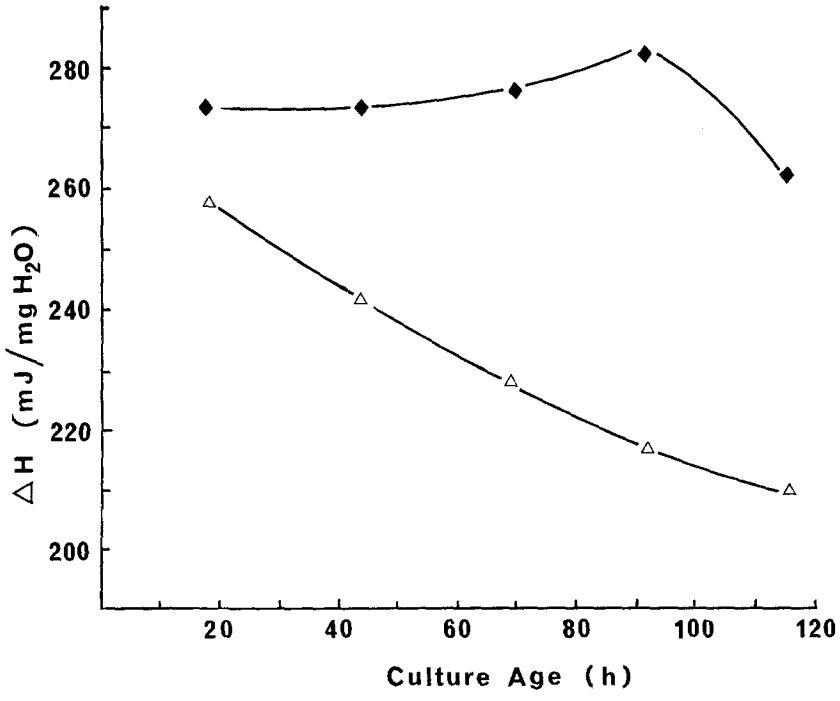

Fig. 3. The effect of culture age on the apparent latent heat of fusion (ΔH) of pastes of whole (◆) and lysed (△) bacteria. A suspension grown for 116 hr as illustrated and then stored at 4 °C for 5 days gave a value of 226 mJ/mg H_2O for a paste of whole bacteria and 116 mJ/mg H_2O after lysis by freezing and thawing

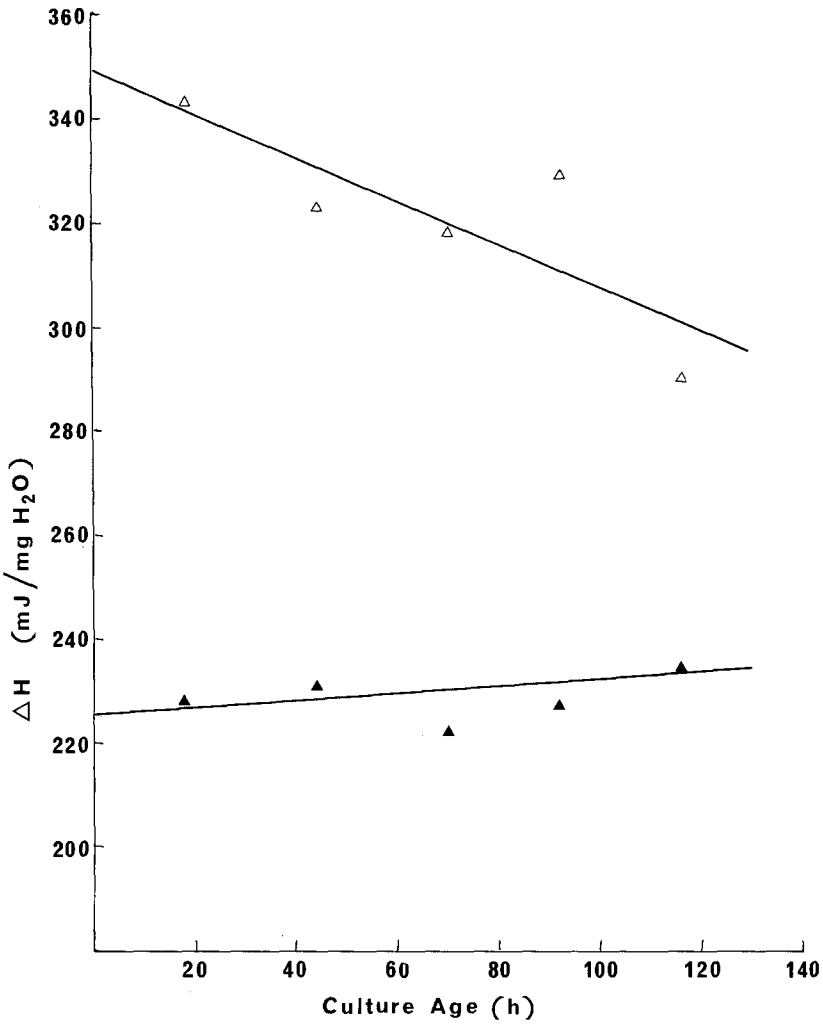

Fig. 4. The effect of culture age on apparent latent heat of fusion (ΔH) of intracellular (△) and extracellular (▲) water

henceforth, to results obtained with the smaller sample size. The results obtained with 10–12 mg, however, were generally similar to those described below.

The total apparent latent heat released during the freezing of whole cell pastes was consistently higher that the corresponding values obtained after freezing and thawing the paste in the calorimeter or obtained with the frozen-thawed control suspension. For example, the apparent latent heat (mJ/mg H_2O) for whole cells was (mean ± SD), 273 ± 8 and, for lysed cell pastes 228 ± 17.

In addition, there was a major effect of culture age on the difference between whole and disrupted bacteria (Fig. 3). It is evident that the effect of age on the difference between whole and disrupted cells was exerted predominantly on the disrupted bacteria. There was, however, a drop in total latent heat of cultures older than about 100 hr; this effect was greatly enhanced in a suspension grown for 116 hr and then stored for 5 days at 0–4 °C.

When the latent heat of the whole bacterial pastes is resolved into values for intra- and extracellular water, a somewhat different impression is gained. Figure 4 shows that the extracellular fluid in these pastes remained about constant, whereas there was a progressive drop in the latent heat of the intracellular water. Cold storage of the culture led to a further drop in both values.

A curious feature of the results is that, although the frozen-thawed paste gave a single peak on freezing, concentrated solutions of sodium chloride gave two peaks which varied in relative size within a range such as illustrated in Fig. 5. Fresh growth medium and culture supernatant each gave double peaks indistinguishable from that of sodium chloride. In contrast, concentrated solutions of potassium chloride normally gave one peak which was sometimes accompanied by one or more small, well separated "blips" (Fig. 6). An approximately equimolar mixture of sodium and potassium chlorides gave peaks

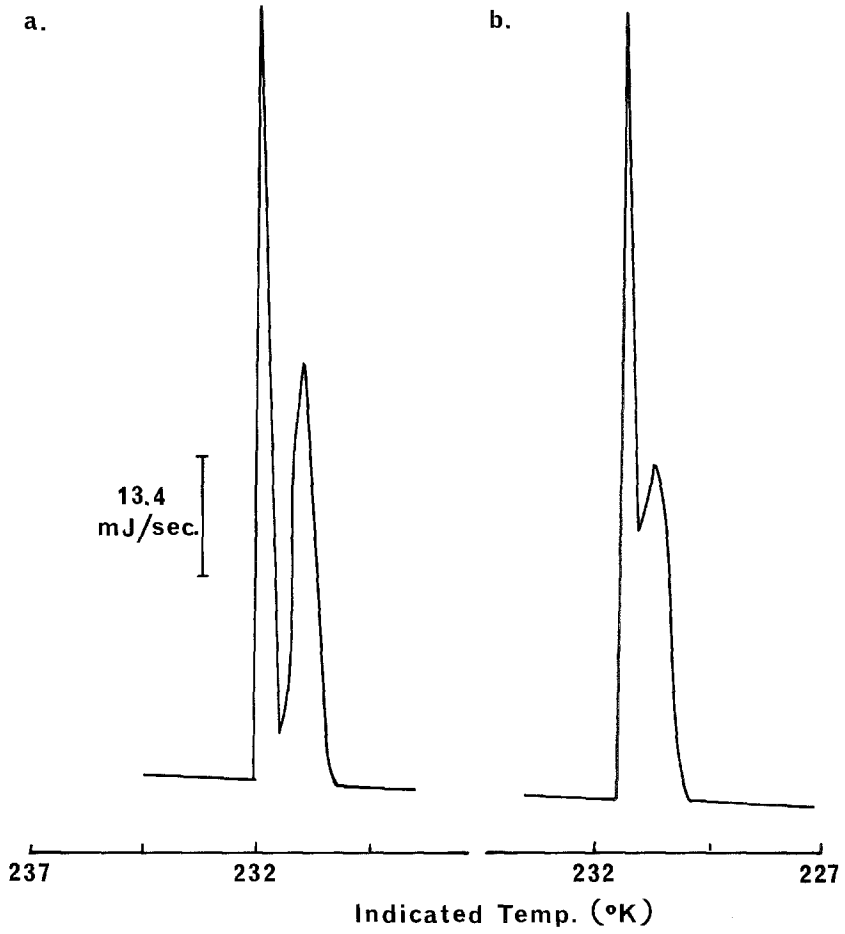

Fig. 5. Two curves depicting freezing transitions of 4.89 molal NaCl. The curves indicate approximately the extent of the difference in shape of freezing curves obtained with this salt

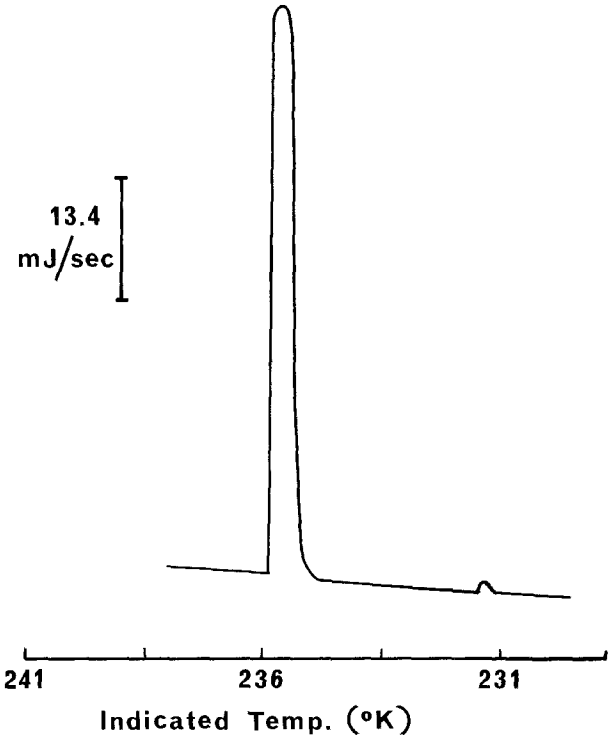

Fig. 6. A freezing transition of 3.69 molal KCl

similar to those of potassium chloride but mixtures of different proportions behaved quite differently in this respect. Some gave two peaks separated by as much as 5.8 indicated degrees. The two salts also had different effects on the apparent latent heat of water. Sodium chloride lowered whereas potassium chloride slightly raised the measured values (Fig. 7).

Changes in sodium and potassium content of the bacterial pellet in the course of a growth cycle are shown in Fig. 8. Although the total cation content of the pellet remained about constant, the proportion of the two ions changed drastically after about 80 hr growth when potassium, initially the dominant ion, dropped sharply and was replaced by sodium. On the assumption that potassium content of the extracellular fluid was negligible and that essentially all the estimated potassium had sedimented within the cells, the highest potassium content shown is equivalent to an intracellular concentration of 6.5 molal and the lowest to 2.7 molal.

Intracellular sodium concentrations were calculated with the assumption that sodium in the growth medium remained constant at 4.89 molal. With that assumption, the sodium concentrations correspond-

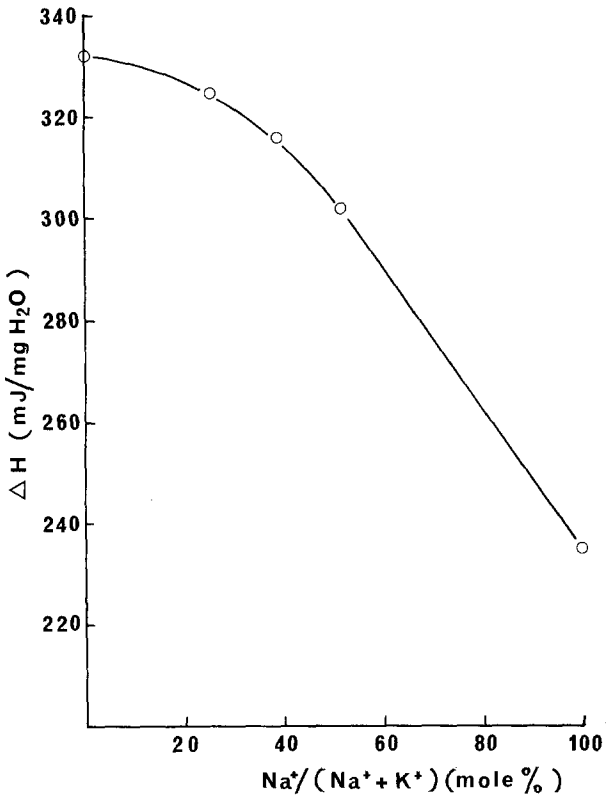

Fig. 7. The effect of salt composition on apparent latent heat of fusion (ΔH, freezing transition) of mixtures of NaCl and KCl at constant molality (4.0 molal). These measurements were obtained with the DSC-2. As with measurements using the DSC-1 B, the instrument was calibrated against a melting transition of water using a value of 333.5 mJ/mg. Freezing transitions of pure water in the DSC-1 b gave values for ΔH of 316 mJ/mg

ing to the foregoing intracellular potassium concentrations were 0.7 and 3.7 molal, respectively. These give total intracellular cation ($Na^+ + K^+$) concentrations of 7.2 and 6.3 molal, respectively, for exponential and 140-hr cultures. The experimental error shown in Fig. 8 suggests that the total salt concentration remained about constant over the growth cycle.

Discussion

Interpretation of these results is based on a set of circumstances in which a paste of whole bacteria consists essentially of two compartments, one an averaged set of intracellular solutions and the other a continuous extracellular occluded solution. When the pastes are frozen and thawed the two compartments mix but the total chemical composition does not change. An important assumption which we have made is that the two peaks obtained by freezing a paste of whole cells are attributable separately to extra- and intracellular water. The assumption is supported by several types of evidence. Intracellular water activity is somewhat lower than that of the growth medium (*see* Brown, 1976) and, without supercooling, the cells should have a lower freezing point than the growth medium. All preparations did supercool, however, and nucleation was presumably a factor in determining the freezing point. Nucleation should occur more readily outside than in the protected and possibly viscous interior of the bacteria. The shape of the two peaks was unlike the double peak given by solutions of sodium chloride and some salt mixtures and was much less variable. The first peak was always sharp, the second always low and relatively broad, indicative of slower freezing. The latent heat of intracellular water, determined on these assumptions, fell into a definite pattern which would not happen if the second peak were merely the second phase of freezing a salt solution. Finally, the two peaks disappeared to be replaced by a single sharp one after the bacteria had frozen and thawed.

An apparent anomaly lay in the absence of the characteristic double peak in the freezing of the extracellular fluid since solutions of that presumed composition invariably gave a double peak when frozen without bacteria. The kinetics of freezing were so complex, however, and so susceptible to factors such as wetting angles that it is not unreasonable to assume that the large area of bacterial surface changed the dynamics of freezing the extracellular solution to the extent of eliminating the bimodal behavior.

A reviewer of this paper made the following comment "... the eutectic composition of the NaCl-water is about 5.1 molal and that of KCl-water about 3.3 molal. Hence in the freezing of 4.9 molal NaCl (Fig. 5) ice crystallization will occur first with the formation of inclusions giving rise to the two observed phases of freezing. Freezing of 3.7 molal KCl implies simultaneous KCl precipitation, presumably preventing the formation of extended ice crystals. The presence of bacteria in a dense system will also prevent extended ice crystallite formation, with accompanying supercooling."

In theory results of the type described should be amenable to thermodynamic analysis. For example, Andronikashvili, Mrevlishvili and Privalov (1969) determined changes in the heat capacity of water, as a function of temperature, in several tissues. They calculated several thermodynamic parameters and the amount of bound water in the tissues. The variability inherent in the present measurements discourages precise analysis. at least initially, but the results do allow an empirical treatment based on comparisons of whole and disrupted bacteria.

Of immediate relevance are the differences in apparent latent heats of fusion of pastes of whole and

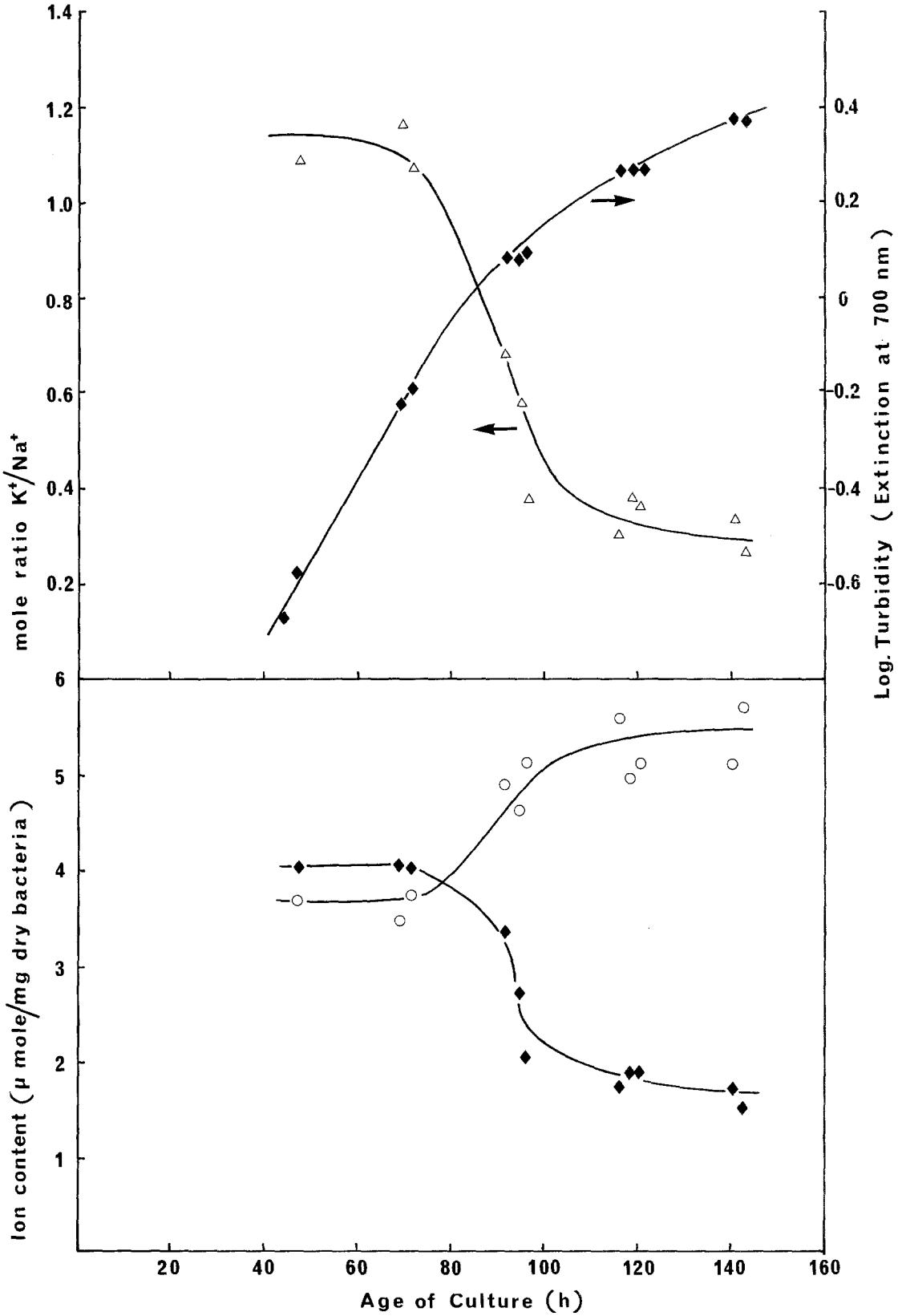

Fig. 8. The effect of culture age on Na^+ and K^+ content of centrifuged pellets of *H.halobium*. *Top panel:* growth curve (turbidity) ◆; ratio K^+/Na^+, △. *Bottom panel:* K^+ content of pellet, ◆; Na^+ content of pellet, ○. *See* text for calculations of intracellular ion contents

lysed bacteria. These differences are considerable and reflect freezing characteristics of two preparations of identical overall composition but with different spatial distribution of their major solutes. Furthermore, the differences are affected to a considerable extent by the age of the culture.

Under the experimental conditions, potassium chloride appears to raise (by about 11% for a 3.69 molal solution) whereas sodium chloride appears to lower (by about 17% for a 4.89 molal solution) the latent heat of fusion of water (*see also* Fig. 7). This is partly explained by the greater heat of aqueous solvation of KCl than of NaCl (about fourfold) since the enthalpy of freezing includes a contribution by the crystallization of salts. Figure 7 is also in general accord with the classification of Na^+ as an ion which lowers the "structural temperature" of water and K^+ as an ion which raises it (Privalov, 1977). Since the dominant intracellular solute in young cultures of halobacteria is potassium chloride and the dominant extracellular one is sodium chloride, it is to be expected that the intracellular fluid should have a higher apparent latent heat of fusion (ΔH) than extracellular fluid, as shown in Fig. 4. Furthermore, the decline with age in ΔH of intracellular fluid of whole cells suggested diminishing potassium content, an expectation which was confirmed by direct analysis (Fig. 8). Thus the difference between the inside and outside of the bacteria in a paste and the effect of age on that difference are consistent simply with differences in salt composition.

The differences between the total values of ΔH obtained for pastes of whole and lysed bacteria are not so simple although composition does provide a large part of the explanation. First, it is clear that cell disruption with the attendant mixing of internal and external solutions lowers ΔH. The effect of age on the difference between whole and lysed cell pastes is exerted mainly on the latter although, as Fig. 4 shows, age does affect the intracellular fluid as well. Nevertheless, it is curious that an effect of age is expressed predominantly on the mélange of disrupted bacteria rather than on the whole cells + extracellular fluid, the combined values of which remained relatively constant.

Again, at least part of the explanation lies in changes in ion content of the ageing bacteria with attendant changes in ion composition of the centrifuged bacterial pellet. The sodium content of the growth medium remains essentially constant throughout a growth cycle, and the potassium content of the growth medium is negligibly low compared with that of the bacteria. Occluded extracellular fluid should therefore contribute little to changes in overall ion composition of the paste. Potassium content drops with age, however (Fig. 8), and this can be explained only by a diminution of intracellular K^+. Figure 8 implies that K^+ lost from the bacteria is replaced by Na^+. Lysis of the bacteria will, therefore, release into the mixture less K^+ and more Na^+ as the culture ages. This is qualitatively consistent with the evidence of Fig. 3.

The highest (log phase) and lowest (stationary phase) intracellular ratios of K^+/Na^+ correspond to mole fractions of Na^+ [$Na^+/(Na+K^+)$] of 9.7% and 57%, respectively. In turn, these fractions indicate, from Fig. 7, values for ΔH of approximately 330 and 293 mJ/mg water for the intracellular solutions in log phase and old bacteria, respectively. The values agree well with those shown in Fig. 4 for the internal water. This agreement is especially noteworthy in view of the different calorimeters used for the two sets of observations.

There is a minor apparent anomaly in that, although changes in salt composition clearly explain the drop in ΔH of lysed cell pastes with increasing culture age, they do not seem able to explain the low absolute values of the lysed pastes of old cultures. For example, the lowest value shown in Fig. 3 is about 210 mJ/mg water whereas 4.9 molal sodium chloride gave a value of about 260 mJ (DSC-1 B) and 4.0 molal about 235 mJ/mg water (DSC-2). The overall precision of measurements on salt solutions was about 5%.

We cannot yet explain this discrepancy, but we assume that the disorganization and release of cellular constituents. especially the disordering of DNA (the lysates were extremely viscous), modified the kinetics of the freezing process as did the assumed lowering of the surface tension which accompanied lysis. The anomaly does not affect the major conclusion, namely, that differences between the apparent latent heat of fusion of pastes of whole and lysed halobacteria are explicable in terms of salt composition, that changes in salt content of the bacteria account for the effect of culture age on observed values, and that there is no evidence at this level of any abnormality in the physical state of either potassium or water in *H. halobium*. One of us (A.D.B.) will report, in due course, results of measurements of the heat of dilution of intracellular KCl which lead to a similar conclusion.

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