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# The Effect of Anions on $K^+$-Binding in a *Halobacterium* Species

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*Summary.* Trinitrocresolate (TNC) at a concentration of $2 \times 10^{-3}$ M brings about rapid loss of K from starving *Halobacterium* cells. Higher concentrations of other anions such as salicylate, thiocyanate, and perchlorate produce a similar effect. The TNC-induced K loss is not significantly reversed when TNC is removed from the ambient medium. The rate of K loss in the presence of $2 \times 10^{-3}$ M TNC is only slightly increased by the temperature in the ranges of 30 to 40 °C and 0 to 20 °C; between 20 and 30 °C, however, the rate increases 10-fold. The K loss was partly replaced by $Na^+$. These data are interpreted in terms of the hypothesis that K is retained in starving *Halobacterium sp.* not by active transport, but rather by selective binding on loci which are modified by TNC.

It has been known for some time that at least two species of *Halobacterium* contain high concentrations of potassium [1, 2] while living in a medium rich in Na and poor in K. Since this is true even in bacteria subjected to prolonged periods of starvation [3], it does not appear that the cell potassium is retained by active transport, especially as the ion exchanges rapidly across the cell membrane.

It is, therefore, necessary to consider some other way whereby high concentrations of the potassium ion can be maintained within the bacteria against low external concentrations. A possibility which has been considered on thermodynamic as well as on experimental grounds [3] is that there might be some mechanism of specific binding which would permit the retention of 3 to 4 M potassium within the bacteria against an external concentration of 5 mM/liter KCl and 3.5 M NaCl.

It is not possible to postulate any special binding compound, such as macrocyclic antibiotic, since the dry weight content of the bacteria is not high enough to accomodate sufficient quantities of such molecules. There is not even enough cell protein to allow one to postulate a binding mechanism

depending exclusively on some configuration involving amino acid radicles and potassium.

In the course of the present work, designed to examine further the status of K within the *Halobacterium* species in question, it has not been found possible to isolate bound K from disrupted cells. In all attempts in which bacteria were broken and the contents dispersed into the ambient medium, there was liberation of free potassium. It has, however, been found possible to bring about loss of potassium from intact bacteria by exposure to various anions. The order of effectiveness, as judged by the anion concentration required to liberate at least one-third of the total K, is: $ClO_4 < SCN <$ salicylate < trinitrocresolate (TNC). TNC has also been found to be a particularly soluble anion in hydrocarbon solvents [7] and to affect profoundly the permeability of erythrocytes to anions and cations [4].

Presently, no adequate explanation exists for the relative effects which different anions (e.g. Hofmeister or lyotropic series) may have on certain systems. However, we discuss some of the kinds of phenomena involved and suggest how these might affect binding of potassium by *Halobacterium sp.*

## Materials and Methods

*Organism and Method of Culture.* The organism used in the course of this investigation was a species of *Halobacterium* isolated from the Dead Sea. The methods of isolation and subsequent culture have been described in an earlier paper [2].

*Preparation of Starving Bacteria.* Cultures were grown for about 24 hr, at which time they were in the logarithmic phase of growth. The bacterial suspension was centrifuged, and the resulting bacterial pellets were resuspended in saline solution consisting of 3.5 M NaCl, 0.15 M $MgSO_4$, $1.4 \times 10^{-3}$ M $CaCl_2$, $2.5 \times 10^{-7}$ M $MnCl_2$, and $5.10^{-3}$ M KCl. This solution is referred to as Complete Salt Solution (CSS). Resuspension was brought about by rapid stirring with a magnetic bar. The pH of the suspension was monitored with a joint glass electrode (Radiometer GK 264 B which is largely insensitive to high salt concentrations at pH values below 10) and was maintained at 7.0 against initially rapid acidification brought about by the bacteria by additions of small amounts of 0.01 N NaOH in 3.5 M NaCl. After 3 to 4 hr, no further pH changes were observed and the bacterial suspension was left overnight in a warm room at 37 °C where it was rapidly stirred. The suspension was then stored at 3 °C and was used for experiments for periods of up to three weeks.

*Preparation of Reagent Solutions.* Solutions were made up in CSS; the pH was adjusted to 7.0.

*Method of Experiments.* Three ml portions of bacteria suspended in CSS (pH 7.0) were equilibrated by shaking in a water bath at 37 °C for one hr prior to addition of the experimental substance. At given time intervals after addition of the test substance, 75 µliter samples were removed with a micro-pipette and centrifuged in a Beckman Microfuge. The treatment of samples has been described earlier [2].

*Measurement of Na and Cl in Bacteria Treated with Trinitrocresolate (TNC).* After treatment with TNC, bacteria were resuspended in a 3.2 M $LiNO_3$ solution isoosmotic with 3.5 M NaCl. The residual NaCl content of the $LiNO_3$ was measured after 2, 10, and 17 min to ensure that equilibrium had been reached, since it had already been established that the Na of these halophilic bacteria comes to equilibrium with the Na of the ambient medium in 1 to 2 min [3].

The Na and Cl content of control bacteria not treated with TNC was determined in the same way.

The method of measuring total pellet Na and Cl has been described earlier [2].

*Analysis of Ions.* K and Na were analyzed by means of an atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, Conn., Model 303) after lysis of the bacterial pellets in a solution containing $7.4 \cdot 10^{-3}$ M CsCl, 5 ml concentrated $NH_4OH$, and 0.0313 ml Non-Ion-Ox per liter. Non-Ion-Ox was obtained from ALOE Scientific Co., St. Louis, Mo. Cl was analyzed by means of a Cotlove Chloridometer.

*Protein Analysis.* Protein was determined by the method of Lowry, Rosebrough, Farr and Randall [6].

*Sonication.* Bacteria were sonicated with a Branson Ultrasonic Corporation Sonifier, Model LS-75.

*K Electrode.* K activity was measured with a Beckman liquid K-sensitive electrode.

*Abbreviations.* The following abbreviations are used in the course of this work:

| | |
|---|---|
| NEM | N-ethylmaleiamide |
| DNP | dinitrophenol |
| PCMBS | p-chloromercuribenzene sulfonate |
| CCCP | m-chlorocarbonyl cyanide phenyl hydrazone |
| TNC | trinitrocresol |
| TNBS | trinitrobenzene sulfonate. |

## Results

### *Maintenance of K in Starved Bacteria*

The K content of starved bacteria remains constant for at least three weeks at 3 °C and for about two days at 37 °C. There is little or no loss of K from starved bacteria incubated at 37 °C for 5 hr – the maximum length of any experiment described in this paper.

### *Attempts to Isolate Bound K from Starved Bacteria*

Bacteria suspended in several kinds of media were disrupted by various mechanical means, and the total K content, as determined by flame photometry, was compared with the K content measured with a K-sensitive electrode. The media in which bacteria were suspended included distilled water, 3.12 M urea, 3.5 M NaCl buffered at pH values from 1 to 8, 4 M KCl and ethyl alcohol. Bacteria were broken by sonication, freezing, or by homogenization in a hand-homogenizer.

No indication of any complexed K was obtained by these methods. On disruption of the cells, the bacterial protein released into the solution, cal-

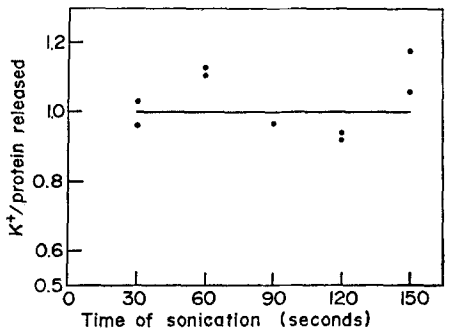


Fig. 1. Proportion of free K to soluble protein liberated as a result of sonicating suspensions of *Halobacterium sp.* for times varying from 30 to 150 sec. The proportion of K or protein liberated by sonication, as % of total K or protein present in the suspension, rose from 30% after 30 sec to 80% after 150 sec. K/protein expressed in μequiv K/100 μg protein

culated as a proportion of the total bacteria protein, was accompanied by an equal proportion of the total K (Fig. 1). All of the K released into the solution was free (ionic) potassium as judged by the response of the K electrode.

### *Effect of Specific Chemical Reagents on K in Starved Bacteria*

Table 1 describes the results of experiments designed to explore the effect of more or less specific agents on K retention by starved *Halobacterium*. The thiol agents PCMBS, NEM and glutathione did not bring about release of bacterial K. Valinomycin and the uncoupling agents DNP and CCCP were equally ineffective.

Table 1. *Measurement of K/protein ration in Halobacterium cells after treatment with various substances*[a]

| Compound | Concentration | K/protein as % control |
|---|---|---|
| NEM | $5 \cdot 10^{-4}$ | 102 (4) |
| PCMBS | $2.5 \cdot 10^{-3}$ | 100 (4) |
| Glutathione (oxid.) | $10^{-3}$ | 98 (3) |
| Glutathione (red.) | $10^{-3}$ | 99 (1) |
| DNP | $5 \cdot 10^{-3}$ | 100 (2) |
| CCCP | $10^{-6}$ | 100 (1) |
| Valinomycin | $10^{-6}$ | 111 (3) |

[a] Duration of treatment: two hr. Temperature: 37 °C. Mean K/protein ratio of untreated controls was $1.09 \pm 0.21$ μequiv/100 μg (mean ± SE of 15 cultures). Numeral in parantheses refers to number of determinations.

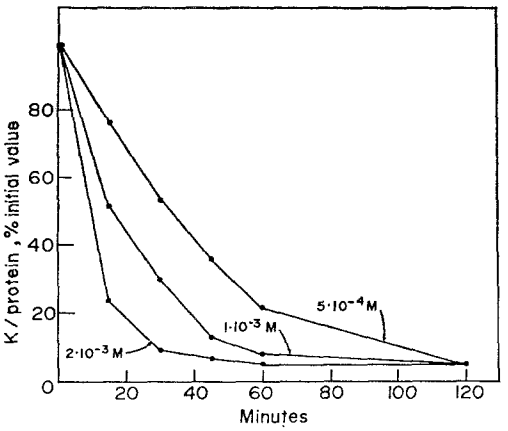


Fig. 2. Effect of incubation at various TNC concentrations on K/protein ration of *Halobacterium sp.* Temperature: 37 °C

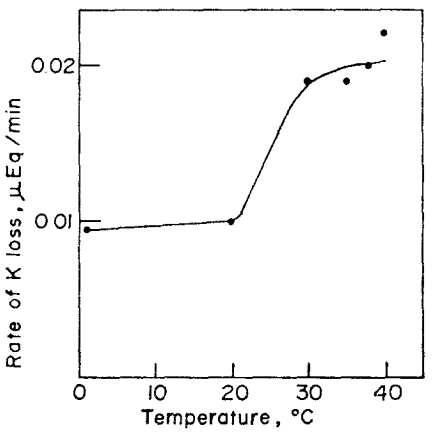


Fig. 3. Effect of temperature on mean rate of K loss in *Halobacterium sp.* during the first 30 min after addition of $1.8 \cdot 10^{-3}$ M TNC to cultures

## *Effect of TNC*

TNC at $2 \cdot 10^{-3}$ M produced rapid loss of K without loss of protein (Fig. 2). Lower TNC concentrations also caused complete loss of K from starved bacteria, although the rate of loss was lower at lower concentrations of the aromatic anion.

The rate of loss was temperature-dependent (Fig. 3), being highest above 30 °C and falling off markedly at lower temperatures. The inset to Fig. 4 shows that the time required for loss of 50% bacterial K is directly proportional to the reciprocal of the TNC concentration employed. Thus K

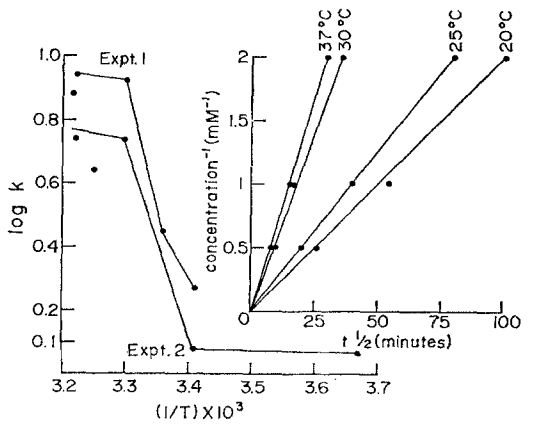


Fig. 4. Arrhenius diagram of $\log_{10}$ of the rate constant ($k$) times $10_5$ of loss of potassium *vs.* the reciprocal absolute temperature (1/T). *Inset:* Plot of reciprocal of concentration *vs.* time required for loss of half of bacterial potassium ($t_{1/2}$). Each $t_{1/2}$ was obtained from a semi-logarithmic plot of K/protein *vs.* time

loss follows first-order kinetics. The disjunct nature of the Arrhenius plot is similar to that in many systems in which a phase transition is observed [5].

To study the reversibility of the TNC effect, bacteria in which the amount of K had been reduced by treatment with TNC were resuspended in CSS containing $5 \cdot 10^{-3}$ M KCl but without TNC. In the course of the subsequent incubation, which lasted for two hr, the K/protein ration rose by 0.05 to 0.09 μequiv K/100 μg protein (control level: 0.95 μequiv K/100 μg protein). Thus the loss of K, which had been induced by TNC, was only slightly reversed during a subsequent relatively prolonged incubation in the absence of TNC.

Loss of K was not accompanied by gain of protons. This was shown by noting pH changes in the suspension while the bacteria were losing K. The suspension was observed to become slightly alkaline during the experimental period, but the amount of HCl required to bring the pH back to its original value was less than one-tenth of the total K lost.

On the other hand, the TNC-induced loss of K was accompanied by gain of Na which could not be washed out in NaCl-free medium (Table 2). $X^-$ was calculated as the difference between the sum of $(K^+ + Na^+)$ and $Cl^-$. It includes the cell phosphate and sulfate, etc., but is thought to be due in large part to fixed negative charges present on protein and nucleic acid molecules within the cell [3]. Although the interpretation of $X^-$ is open to question, the high value of (K + Na − Cl) has been measured by others [1] as well as by us [2, 3].

Table 2. *Measurement of Na and Cl in Halobacterium treated with* $1.8 \cdot 10^{-3}$ M *TNC for one hr*[a]

| Medium | Units (μequiv ion/100 μg protein) | | | |
|---|---|---|---|---|
| | K | Na | Cl | $X^-$ |
| Control | 0.90 ±0.1 | 0.06±0.05 | 0.49±0.02 | 0.47 |
| TNC-treated | 0.065±0.05 | 0.48±0.05 | 0.33±0.01 | 0.25 |

[a] Na and Cl contents were measured after transfer of the pellets to 3.2 M $LiNO_3$ (for details *see* Materials and Methods). $X^-$ is the calculated difference between (K + Na) and Cl. Mean of 2 experiments.

Table 3. *Effect of analogs of TNC and certain inorganic salts on retention of K in Halobacterium sp.*[a]

| Substance | Concn. (M) | $t_{1/2}$ (min) |
|---|---|---|
| TNC | $2 \cdot 10^{-3}$ | 15 |
| Trinitrobenzene | $2 \cdot 10^{-3}$ | ∞ |
| Trinitrobenzoate | $2 \cdot 10^{-3}$ | ∞ |
| Trinitroresorcinol | $2 \cdot 10^{-3}$ | ∞ |
| Picrate | $3 \cdot 10^{-3}$ | ~20 |
| Trinitrobenzene sulfonate | $2 \cdot 10^{-3}$ | ~20 |
| Salicylate | $1 \cdot 10^{-1}$ | 7 |
| Thiocyanate | 2.8 | 30 |
| Perchlorate | 2.8 | 180 |
| Sulfate | 1.4 | 240 |
| Lithium nitrate | 3.2 | ∞ |

[a] Temperature of experiments: 37 °C. $t_{1/2}$: time required for K/protein ratio to fall to 50% of control value. ∞ indicates no K loss in 2-hourly treatment period. The Na salts were used unless otherwise indicated. Each result is the mean of at least 2 experiments.

It is known that when normal bacteria are suspended in 3.2 M $LiNO_3$, cell Na comes to equilibrium within one min after resuspension, and little or no Na is found in these bacteria [3]. The gain of Na was not as great as the loss of K, and was accompanied by loss of $Cl^-$ and $X^-$. It thus appears that TNC acts in part by causing an exchange of Na for K and in part by reducing the total amount of cation in the cells.

Picric acid and TNBS, both at $3 \cdot 10^{-3}$ M, were found to cause loss of 82 to 85% of bacterial K in two hr and were thus almost as effective as TNC (Table 3). Other analogs of TNC, notably trinitrobenzoic acid, trinitrobenzene, and trinitroresorcinol were ineffective. Total loss of K was also caused by $1 \cdot 10^{-1}$ M socium salicylate, while partial loss was also observed with 2.8 M sodium perchlorate. Several inorganic sodium salts,

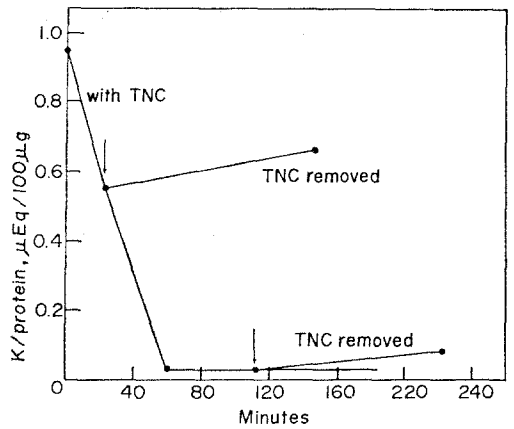


Fig. 5. Reversibility of TNC effect on K/protein ratio of *Halobacterium sp.* Initial TNC concentration: $1.8 \cdot 10^{-5}$ M. At points marked by arrows a portion of the bacterial suspension was washed free of TNC and the bacteria were incubated in CSS containing $5 \cdot 10^{-3}$ M KCl until the end of the experiment

such as sodium sulfate and sodium thiocyanate, caused lysis; therefore, it was not certain that the mechanism of K loss was the same as in bacteria treated with TNC, which caused no lysis. It thus appears that anions effective in bringing about loss of bacterial K can be arranged in a series in the following way: $Cl = NO_3 <$ perchlorate $<$ salicylate $<$ picric acid $<$ TNC.

## Discussion

The present work has shown that K is released when starving (non-metabolizing) *Halobacterium sp.* are treated with certain aromatic or inorganic anions. The most potent of these was TNC, a substance which also reduces anion permeability and increases cation permeability in sheep erythrocytes [4]. The same TNC concentration range was found effective both on cation permeability in sheep erythrocytes and on K depletion in *Halobacterium.*

Wieth [8] has shown that cation permeability in human erythrocytes is increased by several anions, of which salicylate was the most potent. Gunn and Tosteson [4] consider TNC to be an extreme example of the anionic series described by Wieth. They found TNC to be 20 times more effective than salicylate in promoting $Na^+$ influx in LK sheep erythrocytes. It is interesting to observe that in *Halobacterium* the loss of K induced by $1 \cdot 10^{-1}$ M salicylate was approximately twice as fast as that induced by $2 \cdot 10^{-3}$ M TNC, and thus the effect of TNC at 37 °C is approximately

25 times that of salicylate. The relative effectiveness of TNC and salicylate is thus similar in the erythrocyte and in the *Halobacterium* system. As in sheep and human erythrocytes, thiocyanate was much less effective than was TNC or salicylate.

Thus, cation permeability in erythrocytes and loss of K in *Halobacterium* are promoted by the same anions in the same order of effectiveness. Even the quantitative relationships between TNC and salicylate appear the same. There must, however, be important differences between the two, since TNC modifies the permeability of the erythrocyte membrane to ions whereas such a membrane effect is unlikely in *Halobacterium*, in which the membrane is highly permeable to cations, and K is apparently retained within the cell by some kind of specific binding [3]. It is possible that a similar general mechanism is involved in both cases; thus, the effect in erythrocytes has been explained as being due to the absorption of the test anions (TNC, salicylate, etc.) onto ion binding sites in the membrane. Conceivably, the *Halobacterium* results can be explained by postulating an interaction between these test anions and ion binding sites located in the bulk of the cell [3].

A major observable difference between the anion-induced release of K in *Halobacterium sp.* and the increased cation permeability in erythrocytes is in the effect of temperature on these phenomena. In erythrocytes, maximal effects occurred at 0 and 37 °C with a minimum at 23 °C [4, 8]; this is thought to represent a summation of two processes with different temperature coefficients. In the *Halobacterium sp.*, on the other hand, the rate of K release increased monotonically with temperature, with a sharp discontinuity at around 20 °C in the amount of total K released at equilibrium. In other systems this discontinuity indicates a phase transition [5]. Possibly some sort of phase transition occurs in *Halobacterium* between 20 and 30 °C. If so, it will be necessary to consider the interrelations of cations with cellular macro-molecules and possibly ordered water in these bacteria.

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## References

1. Christian, J. H. B., Waltho, J. A. 1962. Solute concentrations within cells of halophilic and non-halophilic bacteria. *Biochim. Biophys. Acta* **65**:506.
2. Ginzburg, M., Sachs, L., Ginzburg, B. Z. 1970. Ion metabolism in a *Halobacterium*. I. Influence of age of culture on intracellular concentrations. *J. Gen. Physiol.* **55**:187.
3. — — — 1971. Ion metabolism in a *Halobacterium*. II. Ion concentrations in cells at different levels of metabolism. *J. Membrane Biol.* **5**:78.

4. Gunn, R. B., Tosteson, D. C. 1971. The effect of 2,4,6 trinitro-m-cresol on cation and anion transport in sheep red blood cells. *J. Gen. Physiol.* **57**:593.
5. Hippel, P. H. von, Schleich, T. 1969. The effects of neutral salts on the structure and conformation stability of macro-molecules. *In:* Structure and Stability of Biological Macro-molecules. S. N. Timasheff and G. D. Fasman, editors. p. 417. Marcel Dekker, Inc. New York.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265.
7. Tosteson, D. C. 1971. Macrocyclic compounds as ion carriers in thick and thin lipid membranes. *Proc. Internat. Microbiol. Cong.* In press.
8. Wieth, J. O. 1970. Paradoxical temperature dependence of sodium and potassium fluxes in human red cells. *J. Physiol.* **207**:563.