The Specific Requirement for Sodium Chloride for the Active Uptake of L-Glutamate by *Halobacterium salinarium*

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1. Uptake of L-glutamate by *Halobacterium salinarium* is dependent on high concentrations of sodium chloride in the environment. When the sodium chloride is replaced by isomolar concentrations of potassium chloride, sodium acetate or potassium acetate, only negligible uptake occurs. 2. Most of the glutamate taken up can be shown to be in the cells in the free state and at a concentration of at least 50 times that in the medium. Sodium chloride is therefore required for an active transport of the glutamate into the cells. 3. The question whether sodium chloride is essential for the actual migration of glutamate across the cell envelope or for the mechanism supplying energy for this migration is discussed on the basis of experiments on endogenous respiration and with inhibitors.

Halobacterium salinarium (Harrison *et* Kennedy) Volcani belongs to the group of extremely halophilic bacteria and has a requirement of 4–5M-sodium chloride for growth. The requirement of the extremely halophilic bacteria for sodium chloride is specific; attempts to grow the organism on media in which other salts substituted for the sodium chloride failed. This work has been reviewed by Larsen (1963).

Studies of the function or functions of sodium chloride in these organisms have revealed that the salt is required to activate a number of enzymes and to protect the cells from lysis. Sodium chloride is not, however, the only salt which can act in these capacities. A number of others, notably potassium salts, activate the enzymes (Gibbons, 1958; Baxter, 1959; Larsen, 1963). Many of the salts which interact weakly with proteins, e.g. potassium chloride and sodium acetate, protect the cells from lysis when they replace sodium chloride in isomolar concentrations (Abram & Gibbons, 1961; Mohr & Larsen, 1963; Boring, Kushner & Gibbons, 1963). The biochemical aspect of the specific requirement for sodium chloride for the growth process has thus not been understood. In the present work evidence is presented that sodium chloride is required for the uptake of glutamate by *H. salinarium*, and that for this process it cannot be replaced by other salts as described above.

MATERIALS AND METHODS

Growth conditions. *Halobacterium salinarium*, strain 1, was grown in liquid cultures at 30° on a reciprocating shaker

as described by Mohr & Larsen (1963). In experiments involving glutamate uptake by the bacterium, the growth medium was supplemented with 1% (w/v) of sodium glutamate. The experiments were all conducted on cultures incubated for about 70 hr., i.e. close to the end of the exponential growth phase.

Incubation with [U-¹⁴C]glutamate. Cultures were diluted with 4.3M-NaCl to give a cell concentration of approx. 1.0 mg. of salt-free dry wt./ml. (i.e. a reading of 0.3 in an EEL Portable Colorimeter, with 5 ml. standard cuvettes and filter no. 608). A portion (12 ml.) of this suspension was centrifuged in the cold for 20 min. at 5000g, and the sedimented cells were carefully resuspended in 6.0 ml. of a solution of 4.3M-NaCl, 2.5 mM-tris buffer (adjusted to pH 7.0 with 0.1N-HCl), and L-[U-¹⁴C]glutamic acid (The Radiochemical Centre, Amersham, Bucks.; 0.2 µC/µmole or 6.35 µC/µmole depending on the experiment) in concentrations as given in the Results section. In some experiments different concentrations of NaCl and salts other than NaCl were used, as described in the Results section; in each case the initial dilution of the cultures was made in the appropriate salt solution. The mixtures were incubated in 50 ml. flasks on a reciprocating shaker (220 oscillations/min., 4.0 cm. excursion) at 30° for appropriate times.

Determination of incorporated radioactivity. Samples of 0.5 or 1.0 ml. were withdrawn from the incubation mixtures, filtered through membrane filters (type MF group 2, Membranfilter A.-G., Göttingen), and the retained cells washed rapidly with 3 × 1.0 ml. of 4.3M-NaCl or a solution containing the concentration of NaCl or other salt used in the incubation mixture. The filter area was 0.95 cm.² and, after filtering through 0.5 ml. of cell suspension (5 × 10⁸ cells/ml.), the layer on the filter would be approximately 20 cells thick. The filters were dried flat on aluminium planchets and assessed directly for radioactivity in a gas-flow counter (Frieske and Hoepfner, type FH49).

Identification of incorporated radioactive material. The incubation mixtures described above were filtered in 0.5 or 1.0 ml. portions and washed on membrane filters as

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described in the preceding section. The filters were then plunged into a small volume of hot ($>90^\circ$) de-ionized distilled water and allowed to soak for 15 min. After this time the filters were removed and washed carefully with de-ionized distilled water, the washings being added to the main cell extract. The filters were dried and their radioactivity was assessed on planchets.

On being immersed in water, the cells on the filters lysed. The resulting solution was acidified with acetic acid and run through miniature Dowex 50 W (X 8; 200–400 mesh) cation-exchange columns according to the method of Harris, Tigane & Hanes (1961). The amino acid fraction was eluted with aq. 2N-NH₃ soln. and this NH₃ was removed afterwards under vacuum. Solutions to be assessed for radioactivity were evenly spotted directly on to aluminium planchets. These were then dried and their radioactivities determined. (The film of material on the planchets was so thin that effects due to self absorption of radioactivity could be neglected.) The amino acid mixture was resolved in two ways. (1) In a one-dimensional, descending paper-chromatographic system with butanol-acetic acid-water (4:1:2) as developing solvent; the amino acid spots were developed with ninhydrin spray; radio-active areas were identified by cutting out the paper and counting the areas directly, or by radioautography with Kodirex X-ray film. (2) On an Amberlite ion-exchange column according to Moore, Spackman & Stein (1958); the amino acids were determined colorimetrically by reaction with ninhydrin by the method described by Yemm & Cocking (1955).

Measurement of endogenous respiration. A portion (40 ml.) of culture was centrifuged, carefully washed and re-suspended in a solution containing 4.3M-NaCl (or other salts as given in the Results section), 20 mM-MgSO₄, 13 mM-KCl, 0.9 mM-CaCl₂, 2.5 mM-tris buffer, adjusted to pH 7.0 with 0.1N-HCl. The oxygen uptake of these suspensions was measured aerobically at 30° in a conventional Warburg apparatus. By using sufficiently dilute suspension of cells (15 mg. dry wt. of cells/ml.) it was assured that the rate of diffusion of oxygen into the strongly saline solutions did not limit the oxygen uptake.

RESULTS

Influence of salts on uptake of L-[U-¹⁴C]glutamate. In 4.3M-sodium chloride the organism took up L-[U-¹⁴C]glutamate as illustrated in Fig. 1. When 4.3M-potassium chloride, sodium acetate or potassium acetate was used instead of sodium chloride as the suspending medium, only a negligible accumulation of radioactivity occurred in the cells upon incubation with L-[U-¹⁴C]glutamate (Table 1). It was noted that replacing sodium chloride by sodium acetate or potassium acetate did not cause any change in the microscopic appearance of the cells. Replacing sodium chloride by potassium chloride, however, caused some distortion of the rod-shaped cells; after an hour of incubation with the latter most cells appeared club-shaped.

When the cells were suspended in concentrations of sodium chloride lower than 4.3M their ability to incorporate glutamate was impaired (Fig. 2).

Fig. 1. Uptake of [U-¹⁴C]glutamate by *H. salinarium* when suspended in 4.3M-NaCl. The suspending medium contained 0.01 mM-L-[U-¹⁴C]glutamate (6.35 μ C/ μ mole) and 2.5 mM-tris buffer, pH 7.0.

Table 1. Uptake of L-[U-¹⁴C]glutamate by *H. salinarium* in different salt media after incubation for 1 hr. at 30°

Incubation media consisted of 0.05 mM-L-glutamate (0.2 μ C/ μ mole), 2.5 mM-tris buffer, pH 7.0, and 4.3M-salt solution.

Suspending salt (at 4.3M)	Radioactivity (counts/min./ml. of cell suspension)
NaCl	1573 $\pm$ 13
KCl	12 $\pm$ 2
Sodium acetate	14 $\pm$ 2
Potassium acetate	14 $\pm$ 2

In a series of experiments sodium chloride was partially replaced by potassium chloride, sodium acetate and potassium acetate, whilst the total molarity of the salt mixture was maintained at 4.3. The results are given in Fig. 2 and show that the higher the degree of replacement of sodium chloride by the other salts the smaller is the uptake of glutamate by the cells. However, it should be noted that in the partial replacement of sodium chloride by potassium chloride or sodium acetate, greater uptake occurs than if the 4.3M-sodium chloride solution is simply diluted with water to the same extent. Thus potassium chloride and sodium acetate appear able to replace sodium chloride to a certain degree. Potassium acetate is clearly inhibitory to the uptake mechanism; when it partially replaces sodium chloride a stronger inhibition of the glutamate uptake occurs than when simply diluting the 4.3M-sodium chloride with water.

Identification of incorporated radioactive material. After incubating the cells in 4.3M-sodium chloride and 0.01 mM-[U-¹⁴C]glutamate (6.35 μ C/ μ mole) for 1 hr., a substantial amount of the total radioactivity taken up was identified as free glutamate in the cells. The proportion of the total incorporated radioactivity found as free glutamate varied with the procedure used in extraction. If the cells,

having been filtered from the incubation medium and washed thoroughly to remove traces of medium, were not plunged directly into boiling water, a much smaller proportion of radioactivity could be identified with the glutamate. Enzymic processes must be stopped and the isolation of the cell constituents done in minimum time.

With care, some 80% of the glutamate taken up can be recovered again in the free state. On incubation a total of 25000 counts/min. of radioactivity were incorporated by the cells contained in 6 ml. of medium. The radioactivity was measured from the dried cells on the filters. After extraction and purification, the glutamic acid fraction had an activity of 21000 counts/min.

The extracted glutamic acid was shown to co-chromatograph with an authentic sample of L-glutamic acid on the one-dimensional paper-chromatographic system used. Radioautograms of the paper strips showed that the blackened area of the film corresponded exactly, in position and shape, to the ninhydrin-stained glutamic acid spot. As additional proof of the identity of the radioactive compound with glutamic acid, samples were chromatographed, together with authentic L-glutamic acid as carrier, on a Stein and Moore Amberlite column. The fractions collected were assessed for radioactivity and amino acid content. The radioactivity was seen to accompany the glutamic acid peak (Fig. 3). The recovery of glutamic acid and of radioactivity from the column was 100%.

Fig. 2. Uptake of L-[U-¹⁴C]glutamate by *H. salinarium* when suspended in solutions of NaCl and of various salt mixtures: ■, KCl; ▲, sodium acetate; ○, no replacing salt; △, potassium acetate. Uptake was assessed as radioactivity and is given as % of maximum. Glutamate concentration was as in Table 1.

Fig. 3. Column chromatography of an amino acid fraction extracted from *H. salinarium* after incubation with [U-¹⁴C]glutamate. ●, Amino acid concentration; ○, radioactivity. The peak at fraction no. 89 is that due to glutamic acid.

Inhibition studies of glutamate uptake. In similar incubation experiments to those described above with 4.3M-sodium chloride and 0.01mM-[U-¹⁴C]-glutamate (6.35 μ C/ μ mole), but without shaking the suspension during the incubation period, the rate of uptake of radioactivity by the cells was 80–90% of that observed with shaking. In an atmosphere of nitrogen the uptake was 20% of that with an ample supply of air. Chloramphenicol caused a 50% reduction in uptake at 1mM and complete inhibition at 2.25mM. 2,4-Dinitrophenol caused 50% and 100% inhibition of uptake at 0.1mM and 1mM concentration respectively.

Endogenous respiration. In both sodium chloride and potassium chloride at a concentration of 4.3M the cells displayed an endogenous oxygen uptake of 10 μ l. of oxygen/hr./mg. dry wt. When suspended in sodium acetate and potassium acetate at the same concentration, no oxygen uptake by cells could be observed.

DISCUSSION

The results presented in the preceding section demonstrate the specific requirement for sodium chloride for the uptake of L-glutamate by *Halo-bacterium salinarium*. Neither sodium acetate nor potassium chloride can replace sodium chloride.

Up to 80% of the glutamate taken up in the presence of sodium chloride could be shown to be present in the cells in the free state. This suggests that sodium chloride is essential for the actual penetration of the glutamate through the cell envelope into the cells. The penetration is an 'active transport' in the sense that the glutamate is taken up by the cells against a concentration gradient. With 0.01mM-glutamate in the suspending medium, a cell concentration of 5×10^8 cells/ml., and assuming a mean cell volume of $1.7 \mu^3$ (Mohr & Larsen, 1963), it can be calculated from the uptake experiments that a concentration ratio of at least 50:1 in favour of the cells will ensue.

'Active transport' involves the actual migration of a substance into or across the cell envelope, and a supply of energy to mediate this migration. It must be assumed that this energy is supplied by the metabolic activity of the cells, and possibly the sodium chloride could be specifically required for the energy production and not necessarily the migration mechanism itself. The observation that glutamate uptake is severely retarded when these obligate aerobic cells are exposed to anaerobic conditions indicates that uptake is linked to the oxidative metabolism of the cells. This contention is strengthened by the experiment in which 50% inhibition of glutamate uptake occurred when 0.1mM-dinitrophenol was included in the incubation medium. Endogenous respiration proceeds

freely in the presence of both sodium chloride and potassium chloride. Therefore sodium chloride is not specifically required to activate the respiratory apparatus and oxygen uptake alone is insufficient for driving the active transport of glutamate. The sodium chloride could, however, have a function in the mechanism of oxidative phosphorylation.

Payne (1960) and Rhodes & Payne (1962) have concluded that a function of the Na⁺ ions in some marine bacteria studied by them lies in the induction of a mechanism for the transport of substrate across the cell envelope. Also, Drapeau & MacLeod (1963), studying another marine bacterium, found that Na⁺ ions are involved in the transport mechanism. The latter workers concluded, however, that sodium is specifically required for the function rather than the formation of the transport mechanism since chloramphenicol did not prevent uptake of substrate by cells grown in the absence of that substrate. In the present investigation the cells were grown in a medium containing glutamate, and the transport mechanism could thus be expected to be already formed. The observations that glutamate is taken up without an apparent lag and that chloramphenicol had little effect on the glutamate uptake are in agreement with this view. The present investigation thus demonstrates the specific requirement of sodium chloride in the function of a preformed mechanism of active transport, and suggests in this respect a similarity between *H. salinarium* and the marine bacterium studied by Drapeau & MacLeod (1963).

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