Betaine-Induced Stimulation of Respiration at High Osmolarities in a Halotolerant Bacterium

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It was shown that non-penetrating solutes at high concentrations inhibit the respiration of the halotolerant bacterium *Ba₁*. Betaine relieved the inhibition caused by osmotic stress and exhibited in this respect a considerable structural specificity. The rate of oxidation of various substrates was stimulated to different extents. It stimulated the rates of both respiration and growth to a similar extent, leaving the energy yield essentially unchanged. In cells pre-loaded with labelled glutamate, betaine also stimulated the rate of oxidation of this intracellular substrate. Betaine was accumulated by respiring cells, and the maximum amount taken up was correlated with the osmolarity of the medium. As judged by chromatography, accumulated intracellular betaine underwent no chemical modification, and this accumulated betaine could not be exchanged with the betaine in the medium or released by passive efflux when respiration was inhibited. Intracellular betaine caused no stimulation of respiration, whereas betaine added to the medium increased the respiratory rate to the same extent in cells pre-loaded with betaine as that in the non-loaded cells. The above observations suggest that iso-osmotic adjustment is not involved in the anti-osmotic effect of betaine, and that betaine exerts its action on the cellular membrane from the outside.

Betaine, the trimethylated derivative of glycine, is a product of the oxidation of choline in certain bacteria (Shieh, 1965) and in animal mitochondria (Jellinek *et al.*, 1959). Although it is widely distributed in plants and animals (Guggenheim, 1951), relatively little is known about its biological functions. It is involved in transmethylation as a methyl donor (Ericson *et al.*, 1955). Dulaney *et al.* (1968) have identified it as the factor in yeast extract which relieves the inhibition of the growth of *Vibrio parcolans* and *Erwinia carotovora* by high sucrose concentrations. Betaine has also been reported to relieve the inhibition of respiration by high NaCl concentration in the moderately halophilic bacterium, *Ba₁* (Rafaeli-Eshkol & Avi-Dor, 1968). Both sucrose and NaCl are non-penetrating solutes for many organisms; they cause shrinkage of the cytoplasmic membrane in bacteria, and, as a corollary, an inhibition of respiration (Henneman & Umbreit, 1964; Knowles & Smith, 1971).

The experiments described in the present paper were designed to test whether the inhibition of respiration of cells of bacterium *Ba₁* by high concentrations of NaCl is a specific effect of this solute, or is due to the stress exerted on the cells by high osmolarity. We wished to elucidate both the nature of the inhibition and the mechanism by which betaine is able to relieve it.

Experimental

Materials

Special materials were obtained from the following sources: ATP, carbonyl cyanide *m*-chlorophenylhydrazine and firefly lanterns from Sigma Chemical Co., St. Louis, Mo., U.S.A.; catalase (A grade, 40000 units/mg of protein) from Calbiochem, Los Angeles, Calif., U.S.A.; [*Me*-¹⁴C]betaine hydrochloride (1.5–2 mCi/mmol) from New England Nuclear Corp., Boston, Mass., U.S.A.; L-[U-¹⁴C]glutamic acid (225 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; L-carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate), dimethylglycine (2-*N*-dimethylaminoacetate) and sarcosine (2-*N*-monomethylaminoacetate) from Fluka A.G., Buchs, Switzerland; propiobetaine (3-*N*-trimethylaminopropionate) and dimethylthetin (2-*S*-trimethylthioacetate) were a generous gift from Dr. Y. Shalitin, The Technion, Haifa, Israel. Nutrient broth was obtained from Difco Laboratories, Detroit, Mich., U.S.A.

Other materials were of analytical grade.

Organism

Ba₁ is a halotolerant, obligately aerobic, Gram-negative rod-shaped bacterium, isolated by Rafaeli-

Eshkol (1968b) from a crude salt sample collected at the Dead Sea evaporation ponds.

Media

Unless otherwise indicated, bacterium Ba₁ was cultivated in a liquid medium containing (per litre of water): 8.0g of nutrient broth, 11.7g of NaCl, 20.3g of MgCl₂·6H₂O and 3.7g of KCl. The pH was adjusted to 6.8 with NaOH.

In the experiment shown in Fig. 2, a medium of defined composition was used which contained (per litre of water): 5.0g of NH₄Cl, 1.0g of NH₄NO₃, 2.0g of Na₂SO₄, 18.5g of K₂HPO₄, 2.6g of KH₂PO₄, 37.2g of KCl, 87.6g of NaCl and 7.1g of succinic acid. Its pH was adjusted to 6.8.

Growth conditions and preparation of bacterial suspensions

The conditions were those described by Rafaeli-Eshkol (1968a) for low-salt-grown cultures, except that the washing fluid contained 100mM-MgCl₂ and 250mM-KCl. The concentration of the cells suspended in the washing medium was approx. 70–90mg of protein/ml.

Respiration

Manometric experiments were carried out by the conventional Warburg technique. The temperature of incubation was 37°C. The centre well contained 0.1ml of 20% (w/v) KOH. Readings were taken every 10min. Polarographic measurements of O₂ uptake were carried out with a Clark oxygen electrode and recorded with a Goerz model 520 recorder. In those experiments in which the solute used for the adjustment of the osmotic pressure was other than NaCl, the latter was also included in the assay medium, at a concentration of 40mM. This was found to be the concentration of Na⁺ which saturates the specific requirement of the respiratory system (Shkedy, 1974). A similar specific requirement for Na⁺ has been demonstrated for various marine bacteria by MacLeod (1965).

Differentiation between 'penetrating' and 'non-penetrating' solutes by turbidity measurements

A portion of bacterial suspension (10μl) was injected into a cuvette which contained 10mM-Tris-HCl buffer (pH8.5) and the appropriate solute (NaCl, KCl, sucrose, glycerol or dimethyl sulfoxide) at a concentration up to 2.0M, in a final volume of 3.0ml. At 30s after the addition of the bacteria, the absorbance at 700nm was measured in a Cary model 118 spectrophotometer against a reference cuvette which contained an assay mixture of identical com-

position except that the bacterial suspension was omitted. The *E*₇₀₀ of the suspension in water was about 0.2, and a maximum increase of 30% in turbidity was reached in approx. 0.8M-NaCl, -KCl or -sucrose. No change in turbidity occurred with glycerol or dimethyl sulfoxide.

Determination of the accumulation of [Me-¹⁴C]-betaine

Cells of bacterium Ba₁, in an assay medium containing the components described in Fig. 6, were preincubated in a water bath at 37°C for 30min, with shaking. The labelled betaine and the carrier were then added. At various time-intervals, portions (0.4ml) were withdrawn and centrifuged at 15000 rev./min (7000g) for 5min in a Beckman Microfuge (type 152) at room temperature (approx. 22°C). A 0.2ml portion of the supernatant was then added to 10ml of Triton-X scintillant [see Turner (1969)] for the determination of the rate of disappearance of the labelled material from the medium. A slice of the pellet formed at the bottom of the plastic centrifuge tube was transferred into 0.5ml of water and dispersed by trituration. A 0.3ml portion of the suspension formed was used for the determination of the amount of labelled material associated with the cells, and 0.5ml was used for protein determination. The amount of betaine which was found to be taken up from the supernatant was in all experiments recovered in the fraction associated with the cells.

Preparation of cells loaded with [¹⁴C]glutamate

Cells of bacterium Ba₁, equivalent to 10mg of protein/ml, were incubated for 120min at 37°C, with shaking, in a medium containing 66mM-Tris-HCl buffer (pH8.5), 100mM-MgCl₂, 50mM-KCl, 2.0M-NaCl, 50mM-ethanol, 30mM-glutamate and [U-¹⁴C]-glutamate (to yield 1700c.p.m./μmol of glutamate). The cells were then sedimented (10min, 15000g, 0°C), washed by centrifugation with the incubation from which ethanol and glutamate had been omitted, and resuspended in a small volume of the washing medium to yield a suspension containing approx. 90–100mg of protein/ml and approx. 0.7μmol of intracellular glutamate/mg of protein. The amount of radioactive material associated with the cells was determined in an essentially similar way to that described in the preceding paragraph for the determination of intracellular betaine.

Measurement of exchange, passive efflux and preparation of betaine-loaded cells

Cells of bacterium Ba₁ were allowed to accumulate [Me-¹⁴C]betaine in the presence of 2.0M-KCl, under

the conditions described in Fig. 4, until the amount of radioactive material associated with the cells reached a plateau. This cell suspension was then used in the following experiments.

(a) *Exchange between intracellular and extracellular betaine.* To 3.0ml of the cell suspension, 0.3ml of unlabelled 2.0M-betaine was added, and the sample was incubated with shaking at 37°C. At various time-intervals, samples (0.4ml) were withdrawn, and the amount of radioactive material which remained associated with the cells was measured as described above for the accumulation of betaine.

(b) *Passive efflux of intracellular betaine.* A portion of the cell suspension (10.0ml) was centrifuged at 0°C for 10min at 15000g. The sedimented pellet was washed with the same medium as that used for the loading of the cells, except that ethanol and betaine were omitted. The washed pellet was resuspended with a volume of the washing medium equal to that of the original sample. After addition of 10mM-KCN (to prevent re-accumulation of the betaine released into the medium), the sample was incubated at 37°C, with shaking. At various time-intervals, samples (0.4ml) were withdrawn and the amount of radioactive material which remained associated with the cells was measured as described above.

(c) *Preparation of betaine-loaded cells.* The cells were prepared in the same way as for measuring passive efflux (see above), except that the volume of the washing medium used for the final resuspension was one-thirtieth of that of the original sample taken. Control cells were prepared in a similar way, except that betaine was omitted.

Chromatography of the labelled material accumulated in the cells

The technique used was that described by Rafaeli-Eshkol (1968a).

Radioactivity measurements

The samples were diluted with Turner's (1969) scintillation liquid, based on toluene-Triton X-100 (Triton-X scintillant), and counted for radioactivity in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer.

Protein

This was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Betaine-dependent relief of respiratory inhibition induced by high osmolality of the medium

Since betaine is known to relieve the respiratory inhibition caused by NaCl, it was of interest to determine whether the betaine effect was NaCl-specific, or more general, in that it relieves inhibition caused by hyperosmoticity. To establish which solutes can be considered, for cells of bacterium *Ba*₁, as non-penetrating and penetrating respectively, we used the turbidity method, which is based on the observation that, in suspensions of Gram-negative micro-organisms, a rise in the osmotic pressure of the

Fig. 1. Effect of penetrating and non-penetrating solutes on the rate of oxidation of ethanol by cells of bacterium *Ba*₁

The Warburg vessels contained cells of bacterium *Ba*₁ in an amount equivalent to 2.0mg of protein/ml. $\circ$, KCl; Δ , sucrose; $\blacksquare$, NaCl; $\bullet$, glycerol; $\blacktriangle$, dimethyl sulphoxide, at the concentrations indicated, in 66mM-Tris-HCl buffer (pH 8.5), 100mM-MgCl₂ and 53mM-ethanol. Respiration was measured in the presence and in the absence of 60mM-betaine. In (a), only the results in the absence of betaine are shown. For the sake of clarity, the results obtained with betaine (b) are presented as the ratio between the rate of respiration with (R^*) and without (R) betaine. For other details, see the Experimental section.

medium causes an increase in the light-scattering (Mager *et al.*, 1956; Packer & Perry, 1961). The turbidity measurements were carried out as described in the Experimental section, and by this criterion, NaCl, KCl and sucrose were classified as 'non-penetrating' and glycerol and dimethyl sulphoxide as 'penetrating' solutes. Fig. 1(a) shows that by plotting the respiratory rate as a function of the solute concentration, a bell-shaped curve was obtained with non-penetrating solutes, and a line, parallel to the abscissa, with penetrating solutes. Betaine was found to stimulate the rate of O₂ uptake whenever the concentration of one of the non-penetrating solutes exceeded the optimum for respiration, and the stronger the inhibition, the greater was the relative relief (Fig. 1b). Betaine had no stimulatory effect in the presence of the penetrating solutes. Thus in the cells of bacterium Ba₁, as in several other Gram-negative bacteria (Henneman & Umbreit, 1964; Knowles & Smith, 1971), the respiratory system was sensitive to changes in the osmolarity of the medium. However, the concentration of solutes which inhibited the respiration of the halotolerant bacterium Ba₁ was higher than in non-halotolerant species. The effect of betaine is to relieve osmotic stress, since it only stimulated respiration under hyperosmotic conditions. The extent of the betaine-dependent relief of respiratory inhibition was found to vary with the nature of the respiratory substrate. For example, in the presence of 2.0M-KCl, and under the conditions described in the legend to Fig. 1, the betaine-induced stimulation (expressed as the ratio of O₂ uptake in the presence of betaine to that in its absence) was as follows: ethanol, 2.40; glutamate, 1.52; pyruvate, 2.71; glucose, 0.90.

Table 1. Comparison between betaine and its analogues in stimulating respiration

Each Warburg vessel contained Tris buffer, MgCl₂, ethanol, NaCl and cells of bacterium Ba₁, as in Fig. 1, and also 1.8M-KCl. Betaine or its analogues were added at a concentration of 60mM. The temperature of incubation was 37°C. For other details see the legends to Fig. 1 and the Experimental section. R* is the rate of O₂ uptake in the presence of betaine or an analogue; R is the rate of O₂ uptake in their absence.

Additions	R*/R
Betaine	2.0
Dimethylglycine	1.7
Sarcosine	1.5
Glycine	1.1
Propiobetaine	1.5
Dimethylthetin	2.5
L-Carnitine	1.0
Tetramethylammonium chloride	1.0

To test the specificity of betaine as an agent which is able to relieve the inhibition of respiration caused by hyperosmolarity, we compared its effect with that of other structurally related compounds. The results summarized in Table 1 show that its sulphur analogue (dimethylthetin), and its homologue (propiobetaine), were effective, and that in the *N*-methylated glycine series, the effectiveness decreased with the number of methyl substituents. L-Carnitine and tetramethylammonium chloride proved to be ineffective. Although the number of compounds tested do not allow the exact structural requirements for the stimulation to be established, it is apparent that these are fairly stringent.

Comparison between the effect of betaine on respiration and on growth

Since betaine affects resting cells, it was decided to test whether betaine would also stimulate the respiration of actively dividing cells of bacterium Ba₁ under

Fig. 2. Effect of betaine on the growth and respiration of Ba₁ cells cultured in a defined medium of high osmolarity

Cells were cultivated in a medium of defined composition as described in the Experimental section. When the turbidity reached approx. 40 Klett units (660nm filter), samples (2.7ml) were transferred into Warburg vessels, half of which contained 0.2ml of 1.0M-betaine (closed symbols) and the other half, 0.2ml of water (open symbols). Incubation (with shaking) was continued in the Warburg apparatus, and manometric readings were taken (○, ●). At the time-intervals indicated, a pair of vessels, one with and one without betaine, was removed from the bath, and 0.2ml of 60% (w/v) trichloroacetic acid was added. The contents of these vessels were used for protein determination (Δ, ▲).

hyperosmotic conditions, and whether its anti-osmotic effect would also be reflected by a stimulation of growth. Therefore cells of bacterium *Ba*₁ were cultivated in a medium of defined composition containing 1.5M-KCl. Samples of the culture were transferred in the middle of the exponential phase of growth to Warburg vessels, and betaine was added to half of the samples. The respiration was measured manometrically, and the growth by determining the increase in the amount of bacterial protein (Fig. 2). Fig. 2 shows that betaine stimulated the rates of both respiration and growth. The increment in the amount of protein (expressed in μg) which occurred during a time-interval (between 4 and 5h) was divided by the O_2 consumption during the same period of time (expressed in μmol) and yielded the ratios of 1.9 and 2.1 in the presence and in the absence of betaine respectively. The constancy of these ratios implies that the stimulatory effect of betaine on growth is correlated with, and results from its effect on,

respiration. Considering that the growth yield in several species of bacteria was shown to be proportional to the calculated amount of ATP produced from an energy source (Senez, 1962), the constancy of the ratios also indicates that the stimulatory effect of betaine on the rate of respiration is not accompanied to any appreciable extent by a change in the effectiveness of coupling between respiration and ATP synthesis.

Effect of betaine on the rate of oxidation of intracellular [^{14}C]glutamate

According to Henneman & Umbreit (1964), inhibition of respiration under hyperosmotic conditions is due to an impeded penetration of the respiratory substrate through the cytoplasmic membrane of the bacterial cell. Thus the betaine-induced

Fig. 3. Effect of betaine on the rate of oxidation of intracellular [^{14}C]glutamate

Cells of bacterium *Ba*₁, loaded with [^{14}C]glutamate, were prepared as described in the Experimental section and added to give a final concentration of 5mg of protein/ml. This amount of cells contains radioactive glutamate equivalent to 3.5 μmol . In addition to the cells, each Warburg vessel contained Tris buffer, MgCl_2 and NaCl as described in Fig. 1, and 2.0M-KCl. The set of vessels were arranged in pairs, such that one contained 60mM-betaine (closed symbols) and the other did not (open symbols). No exogenous respiratory substrate was added. Incubation was at 37°C. After each manometer reading at the times indicated (○, ●), a pair of vessels was removed from the bath and their contents were used for the determination of the amount of radioactive material associated with the cells (△, ▲), as described in the Experimental section.

Fig. 4. Time-course of [$\text{Me-}^{14}\text{C}$]betaine accumulation by cells of bacterium *Ba*₁ as a function of KCl concentration in the medium

Cells of bacterium *Ba*₁ equivalent to 10mg of protein/ml were incubated at 37°C in a medium which contained Tris buffer, MgCl_2 , ethanol and NaCl as described in Fig. 1, 15mM-[^{14}C]betaine (750c.p.m./ μmol), and KCl at the following concentrations: ○, 0.2M; △, 0.4M; □, 0.8M; ●, 1.2M; ▲, 2.0M. The time-course of [$\text{Me-}^{14}\text{C}$]betaine accumulation was followed as described in the Experimental section.

Fig. 5. Simultaneous measurement of respiration and betaine uptake

The polarographic measurement (continuous line) and the measurement of uptake of labelled betaine (○) were carried out in parallel simultaneous experiments. In both experiments, the temperature of incubation was 37°C, the total volume was 4.0 ml, the assay medium contained Tris buffer, MgCl_2 , ethanol and NaCl as described in Fig. 1, 2.0 M-KCl, and cells of bacterium Ba_1 equivalent to 0.3 mg of protein/ml. In the polarographic experiment, the incubation was carried out under conditions in which the access of air was excluded. The assay medium also contained 40 units of catalase to liberate O_2 from H_2O_2 on the addition of the latter (as indicated). In the uptake experiment, $[\text{Me-}^{14}\text{C}]\text{betaine}$ (1.6×10^4 c.p.m./ μmol) was added and the incubation mixture shaken in a water bath. At the times indicated, a 0.4 ml portion was withdrawn to determine the rate of disappearance of the labelled material from the medium as described in the Experimental section.

relief of osmotic inhibition might be a result of facilitated substrate penetration. One way to test this assumption is to determine whether endogenous substrates would respond to betaine. For this purpose, cells loaded with $[\text{U-}^{14}\text{C}]\text{glutamate}$ were prepared and assayed in a medium not containing exogenous substrates. In the experiment shown in Fig. 3, the rate of O_2 uptake, and the rate of decline in the amount of labelled material associated with the cells, were measured simultaneously. Both of these parameters are influenced similarly when betaine is present. During the same time of incubation, O_2 uptake in the control cells not loaded with glutamate was negligibly small. Moreover, practically no radioactivity was lost from respiration-inhibited cells incubated under similar conditions.

These results indicate that betaine also stimulated the rate of respiration when the penetration of a

respiratory substrate could not be the rate-limiting step.

Betaine accumulation and respiratory stimulation

An active adjustment of the intracellular osmotic pressure to changes in the osmolarity of their surroundings occurs in many organisms. Such adjustment prevents major changes in the state of hydration of the cells. Amino acids and their derivatives, among them betaine, play an important role in this co-called 'iso-osmotic intracellular regulation' (see Florkin & Schoffeniels, 1969). The cells of bacterium Ba_1 were shown to accumulate betaine in the presence of 2 M-NaCl (Rafaeli-Eshkol & Avi-Dor, 1968). We therefore investigated the possibility that osmotic re-adjustment, connected with accumulation of betaine against the concentration gradient,

Fig. 6. Effect of betaine flux on the respiratory rate

Cells of bacterium *Ba*₁, in an amount equivalent to 8.2 mg of protein/ml, were incubated at 37°C in a medium which contained Tris buffer, MgCl₂, ethanol and NaCl as described in Fig. 1, 2.0M-KCl and 15mM-[Me-¹⁴C]-betaine (1150 c.p.m./μmol). In one experiment, the accumulation of betaine (○) was measured as described in Fig. 4. Simultaneously, the respiratory rate was measured manometrically in another experiment, under identical conditions, in the presence (■) and in the absence (□) of betaine. When the accumulation of betaine reached a plateau, the betaine-loaded cells were used for the determination of passive efflux of intracellular betaine (●), and for an assay of the rate of exchange between intra- and extra-cellular betaine (▲). The two latter assays were started at the time shown by an arrow and conducted as described in the Experimental section.

Table 2. Effect of extracellular betaine on the respiration of cells pre-loaded with betaine

Each Warburg vessel contained Tris buffer, MgCl₂, ethanol, NaCl (as in Fig. 1) and also 2.0M-KCl. Betaine-loaded and non-loaded (see the Experimental section) cells were added in an amount equivalent to 2.0 mg of protein/ml. The temperature of incubation was 37°C. For other details see the Experimental section.

Additions	Rate of O ₂ uptake (μl/mg of protein)	
	Non-loaded cells	Loaded cells
None	36.8	36.5
60mM-betaine	71.5	92.0

is the mechanism by which the latter counteracts the deleterious effect of high osmolality on the respiratory system and on growth. The time-course of the accumulation of [Me-¹⁴C]betaine by cells of bacterium

*Ba*₁, respiring on ethanol in a medium containing various concentrations of KCl, was studied (Fig. 4). Fig. 4 shows that the amount of labelled betaine associated with the cells increased with the osmolality, and in 2.0M-KCl it reached an amount equivalent to approx. 2 μmol/mg of protein. The accumulated labelled material was released from the cells by osmotic shock, examined by chromatography as described by Rafaeli-Eshkol (1968a), and identified as betaine. Rafaeli-Eshkol (1968b) found that 1 mg of bacterial protein corresponds to a cell volume of 2.7 μl (determined with [¹⁴C]inulin) in 2.0M-NaCl. A rough calculation shows that the cells loaded to their maximum capacity contained over 800 mM-betaine. When further net accumulation ceased, the concentration of betaine in the medium fell to approx. 2–3 mM (not shown in Fig. 4). Therefore the accumulation of betaine, which has been shown to require energy (Rafaeli-Eshkol & Avi-Dor, 1968), took place against a steep concentration gradient.

If the betaine-mediated relief of the inhibition of respiration is indeed a result of iso-osmotic adjustment, one might expect that the rate of respiration should increase gradually during the process of betaine accumulation, and should reach its maximum when the intracellular concentration of the latter does. Moreover, the respiration of betaine-loaded cells should be more resistant to hyperosmotic conditions than that of non-loaded ones, and should not be affected by extracellular betaine. All these assertions were experimentally tested and the results are shown in Figs. 5 and 6 and in Table 2.

The polarographic experiment (Fig. 5) shows that on the addition of a small amount of betaine there was an instantaneous rise in the rate of O₂ uptake, and this increased rate persisted until practically all the betaine was taken up from the medium. The respiration reverted then to the initial rate. Further addition of betaine renewed the stimulation of respiration.

As an extension of the experiment shown in Fig. 5, we measured the rate of respiration manometrically as a function of accumulation of betaine (Fig. 6), under conditions in which the amount of betaine added to the medium exceeded that which the cells were able to take up. Fig. 6 shows that the extent of the betaine-induced stimulation of respiration was constant throughout the experiment, including the time-interval in which no more net flux of betaine could be observed. Fig. 6 also shows that at the time when the net uptake of betaine ceased, neither an exchange between the intra- and extra-cellular betaine, nor an efflux of the intracellular betaine from respiration-inhibited cells into a betaine-free medium, occurred; thus, stimulation of respiration by betaine is not connected with fluxes of the latter.

These results indicate that betaine in the extra-cellular space, rather than its accumulation by the

cells or its flux, was responsible for the relief of inhibition of respiration under hyperosmotic conditions. Support for this contention also comes from the finding that when the rates of respiration of betaine loaded and non-loaded cells were compared in presence of 2.0M-KCl (Table 2), no differences were revealed between the two. Extracellular betaine stimulated the O₂ uptake in the loaded cells even slightly more than in the non-loaded ones.

Discussion

Non-penetrating solutes at high concentrations are known to inhibit respiration in certain Gram-negative micro-organisms (Henneman & Umbreit, 1964; Knowles & Smith, 1971). The peculiarity of the Gram-negative halotolerant bacterium Ba₁ is that the inhibition becomes only manifest at a much higher range of solute concentrations than in other micro-organisms, and that betaine is able partly to relieve the respiratory inhibition. The underlying cause for this inhibition is, according to the above-mentioned authors, the shrinkage of the cytoplasmic membrane. Hence one mechanism by which betaine might relieve this inhibition is by an adjustment of the osmolarity of the cytosol to that of the surroundings.

However, if any re-adjustment of the water balance occurred in connexion with the accumulation of betaine by cells of bacterium Ba₁, it is unlikely to have played a major role in the abolition of the effect of hyperosmolarity on the respiration. This is indicated by the finding that, whereas the accumulation of betaine was a relatively slow process, the stimulation of respiration promptly occurred on adding betaine to the respiring cells. The observation that cells which were fully loaded with betaine still responded to extracellular betaine also argues against an involvement of iso-osmotic regulation. Thus the relief of inhibition by betaine can be classified as a desensitization of the respiratory system to the effect of hyperosmolarity. This mechanism of adaptation recalls the findings of Knowles & Smith (1971), who observed that phosphate increased the resistance of the respiratory system to hyperosmolarity in *Azobacter vinelandii* without reversing the shrinkage of the cells induced by osmotic stress.

The respiratory chain in bacteria is located in the cytoplasmic membrane (Gelman *et al.*, 1967). However, the connexion between the shrinkage of the latter at high osmolarities, and the inhibition of

respiration, is still obscure. Henneman & Umbreit (1964) suggested that the structural changes in the osmotic barrier might limit the accessibility of the respiratory substrate to the site of dehydrogenation. In the present work it was shown that cells of bacterium Ba₁ which were loaded with labelled glutamate and which did not release the labelled material into the medium under hyperosmotic conditions, oxidized the intracellular substance at a higher rate in the presence of betaine than in its absence. Thus it seems unlikely that facilitation of substrate penetration plays a major role in the anti-osmotic effect of betaine.

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