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Studies on Halotolerance in a Moderately Halophilic Bacterium

EFFECT OF BETAINE ON SALT RESISTANCE OF THE RESPIRATORY SYSTEM

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The role of betaine as a factor influencing the salt resistance of the respiratory system in resting cells of the moderately halophilic halotolerant bacterium Ba_1 was studied. Betaine accelerated succinate oxidation in cells obtained from low-salt medium, and stimulation of the respiratory rate was stronger the higher the sodium chloride concentration in the assay medium. The stimulatory effect also depended on the ratio of betaine concentration to the amount of bacteria present. Accumulation of labelled betaine by the bacterial cells was demonstrated; like the respiratory stimulation, it was favourably influenced by an increase in the sodium chloride concentration of the medium. In cells harvested from a high-salt medium and washed with 2·0M-sodium chloride, betaine caused no increase in the respiratory rate, nor was the already high salt resistance of the respiratory system further improved by the addition of betaine. When, however, these cells lost their salt resistance as a result of washing in the absence of sodium chloride, betaine was able to restore it to its original level. In contrast with respiration in low-salt-grown bacteria, that in high-salt-grown cells was not affected by betaine, even after they were washed in the absence of sodium chloride, when the sodium chloride concentration was optimum.

The preceding paper (Rafaeli-Eshkol, 1968*b*) described the response of the respiratory system of resting cells of the moderately halophilic halotolerant bacterium Ba_1 to changes in the sodium chloride concentration of the assay medium. It was shown that interference by high salt concentration with oxidative processes was much less in high-salt-grown than in low-salt-grown cells. The salt sensitivity of the latter could, however, be decreased by preincubation with choline under appropriate conditions. During the preincubation a derivative of choline, whose behaviour on a paper chromatogram resembled that of betaine, accumulated in the cells and was apparently responsible for the changed salt sensitivity.

The present paper describes the results of a study designed to clarify the role of betaine as a factor regulating the salt resistance of the respiratory system of bacterium Ba_1.

MATERIALS AND METHODS

Chemicals. [1,4-$^{14}C_2$]Succinic acid was obtained from The Radiochemical Centre, Amersham, Bucks., and [*Me*-^{14}C]betaine hydrochloride was from the New England Nuclear Corp., Boston, Mass., U.S.A. Other chemicals used were of analytical grade.

Organism, medium, growth conditions and preparation of bacterial suspensions. Unless otherwise stated, these were as described by Rafaeli-Eshkol (1968*b*). Ba_{1LS} and Ba_{1HS} denote respectively cells grown in low-salt or high-salt media.

Assay conditions. With ^{14}C-labelled succinate as substrate, the respiratory $^{14}CO_2$ was trapped on paper moistened with aq. KOH in the Warburg vessel centre well, and counted in toluene–ethanol scintillation liquid as described by Buhler (1962). All other assay conditions were as described by Rafaeli-Eshkol (1968*b*).

RESULTS

Effect of betaine on succinate oxidation by resting Ba_1 cells. The effect of betaine on succinate oxidation in Ba_{1LS} cells in the presence of different sodium chloride concentrations was studied (Table 1 and Fig. 1). In the experiment presented in Table 1, ^{14}C-labelled succinate was used as substrate to establish unequivocally that any increase in oxygen uptake observed on adding betaine was due to stimulation of succinate oxidation. Betaine at 0·3mM approximately doubled the rate of $^{14}CO_2$ release from succinate with 0·8M-sodium chloride

Table 1. *Effect of betaine on the rate of* $^{14}CO_2$ *liberation from* [1,4-$^{14}C_2$]*succinate*

The assay medium contained 33mM-tris–HCl buffer, pH7·4, 10mM-$MgCl_2$, 3·3mM-KCl, 165μg. of chloramphenicol/ml., and 3·3mM-succinate labelled by adding [1,4-$^{14}C_2$]succinate yielding 4×10^4counts/min./μmole; NaCl and betaine were added as indicated. Liberation of $^{14}CO_2$ during 120min. incubation at 37° was determined as described in the Materials and Methods section. Ba_{11S} cells equivalent to 1·1mg. of protein were added to each Warburg cup.

Concn. of NaCl (M) in assay medium	$^{14}CO_2$ liberated (counts/min.)	
	No betaine	0·3mM-Betaine
0	8291	5575
0·8	35828	66362
2·0	547	13845

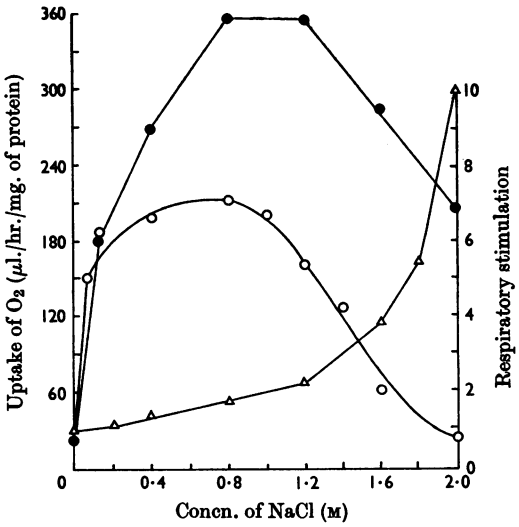

Fig. 1. Effect of betaine on succinate oxidation at different NaCl concentrations. The assay medium contained buffered succinate supplemented with 165μg. of chloramphenicol/ml. and NaCl as indicated. Uptake of O_2: ○, no betaine added; ●, 70mM-betaine. △, Extent of respiratory stimulation by betaine (ratio of uptake with 70mM-betaine/uptake without betaine).

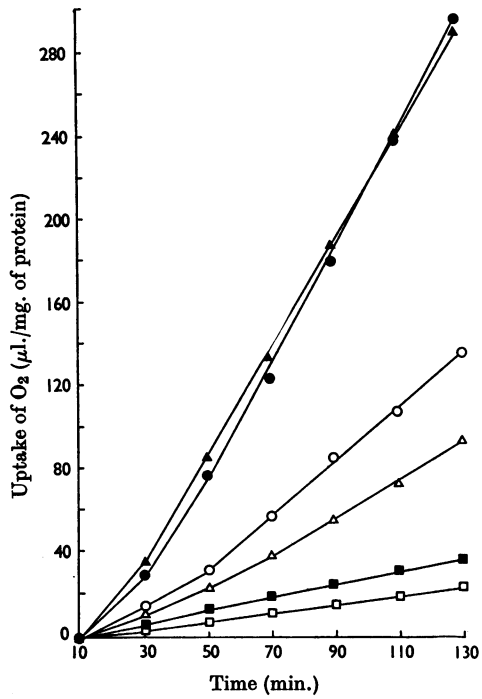

Fig. 2. Effect of relation between betaine and protein concentration on specific respiratory rate. The assay medium contained buffered succinate, 165μg. of chloramphenicol/ml. and 2·0M-NaCl. Bacterial protein per vessel: ○ and ●, 0·7mg.; △ and ▲, 1·4mg.; □ and ■, 14mg. Betaine concentration: ○, △ and □, 0·1mM; ●, ▲ and ■, 1mM.

but increased it 20-fold with 2·0M-sodium chloride. Betaine inhibited, rather than accelerated, respiration in the absence of added sodium chloride.

The effect of sodium chloride concentration on betaine-induced stimulation of the respiratory rate was examined in more detail (Fig. 1). Betaine stimulated the rate of succinate oxidation only if the sodium chloride concentration in the medium exceeded 0·1M. With higher sodium chloride concentrations the ratio of the respiratory rate observed to that without betaine increased progressively with the sodium chloride concentration, becoming especially high once the curves themselves had passed the maximum. In other words, the effect of betaine seemed to be salt-dependent.

Effect of variations in the concentration of bacterial cells on betaine-induced respiratory stimulation. The kinetics of oxygen uptake with a constant sodium chloride concentration (2·0M) with succinate as substrate were followed, with betaine and bacterial concentrations as variables. At a betaine concentration of 0·1mM the steady rate of oxygen uptake was reached only gradually, the lag being the more pronounced the lower the protein concentration (Fig. 2). The specific rates varied inversely with the protein concentration, being highest with 0·25 and lowest with 5·0mg. of protein/ml. At a betaine concentration of 1mM, the lag was much less pronounced; the maximum rate exceeded that observed with 0·1mM-betaine, and became less

Table 2. *Accumulation of labelled material by Ba_{1LS} cells incubated with [Me-^{14}C]betaine*

The reaction mixture contained tris–HCl buffer, pH 7·4, $MgCl_2$, KCl and chloramphenicol as described in Table 1; NaCl, succinate and betaine were added as indicated. The betaine was labelled by adding [*Me*-^{14}C]betaine, yielding 2×10^5 counts/min./μmole in Expt. 1 and $5{\cdot}6 \times 10^5$ counts/min./μmole in Expt. 2. Incubation in Expt. 2 was with shaking. Labelled material associated with the cells was determined as described in the Materials and Methods section.

		Additions to the assay medium				Radioactivity associated with cells (counts/min./mg. of protein)	
Expt. no.	Temp.	NaCl (M)	Betaine (mM)	Succinate (mM)	Uptake of O_2 (μl./hr./mg. of protein)	Cells washed with 0·15M-$MgCl_2$–2·0M-NaCl	Cells washed with 0·15M-$MgCl_2$
1 (0·2mg. of protein/ml.; 130min.)	37°	2·0	0	10	40	—	—
	37	2·0	0·7	10	127	18220	—
	37	2·0	0·7	10	117	—	926
	37	2·0	0·7	0	10	8110	—
	37	2·0	0·7	0	6	—	636
2 (0·33mg. of protein/ml.; 150min.)	37	0	0·5	10	—	180	—
	37	2·0	0·5	10	—	27000	—
	37	2·0	0·5	0	—	10500	—
	0	2·0	0·5	10	—	1810	—

dependent on bacterial concentration. A fall in rate was observed only at the highest protein concentration (5mg./ml.).

Conditions for accumulation and retention of betaine by Ba_{1LS} cells. The effect of sodium chloride concentration and of the availability of energy on betaine accumulation and retention by low-salt-grown Ba_1 cells was examined by using ^{14}C-labelled betaine. Table 2 shows that at 37° maximum accumulation of labelled material took place with 2·0M-sodium chloride and succinate in the medium. Omission of succinate inhibited accumulation only partially, whereas lowering the temperature to 0° or omission of sodium chloride from the medium inhibited it almost completely. Retention of the labelled betaine also required a concentration of 2·0M-sodium chloride in the washing medium. It was also found that cells incubated under conditions favouring maximum accumulation of labelled betaine retained increased salt resistance after being washed with magnesium chloride–sodium chloride and transferred to betaine-free respiratory assay medium.

Specificity of the betaine effect. An attempt was made to replace betaine as a factor stimulating succinate oxidation at high sodium chloride concentrations by substances structurally related to it, namely glycine, sarcosine and tetramethylammonium chloride. Of these substances only sarcosine had a significant influence on the rate of succinate oxidation with 2·0M-sodium chloride, and even then its effect was observed only at the highest concentration tested (70mM); at 1% of that concentration only betaine was effective.

Table 3. *Effect of betaine on the rate of $^{14}CO_2$ liberation by Ba_{1HS} cells from [1,4-$^{14}C_2$]succinate*

The reaction mixture contained tris–HCl buffer, pH 7·4, $MgCl_2$, KCl and chloramphenicol as described in Table 1, and 2·0M-NaCl. Other additions were: 3·3mM-succinate labelled by adding [1,4-$^{14}C_2$]succinate yielding $3{\cdot}4 \times 10^4$ counts/min./μmole; betaine as indicated. Liberation of $^{14}CO_2$ during 120min. incubation at 37° was determined as described in the Materials and Methods section. To each Warburg cup Ba_{1HS} cells equivalent to 1·4mg. of protein were added.

Medium for washing harvested cells	Concn. of betaine (mM)	$^{14}CO_2$ liberated (counts/min.)
0·15M-$MgCl_2$	0	1280
	0·35	9620
	0·70	11890
	70·0	17300
0·15M-$MgCl_2$–2·0M-NaCl	0	25570
	0·35	30580
	0·70	32000
	70·0	29000

Effect of betaine on succinate oxidation in Ba_{1HS} cells. The above experiments show the effect of betaine on the respiratory system of Ba_{1LS} cells, whose salt resistance was relatively low. The question arose whether bacteria grown at 2·0M-sodium chloride (Ba_{1HS} cells), known to have relatively high resistance to high salt concentrations, would also respond to betaine.

After being harvested, Ba_{1HS} cells were washed either with 0·15M-magnesium chloride–2·0M-sodium

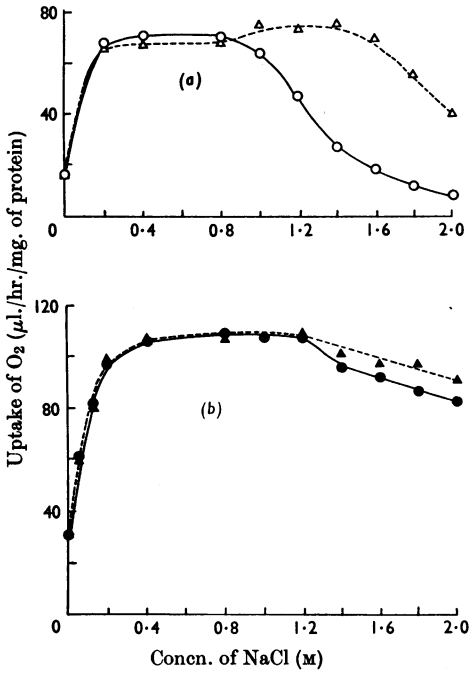

Fig. 3. Effect of betaine on succinate oxidation in Ba_{1HS} cells. The assay medium contained buffered succinate, 165µg. of chloramphenicol/ml. and NaCl as indicated. ○ and ●, No betaine added; △ and ▲, 70mM-betaine added. (*a*) (○ and △), cells washed, after being harvested, with 0·15M-$MgCl_2$; (*b*) (● and ▲), cells washed with 0·15M-$MgCl_2$–2·0M-NaCl.

chloride or with 0·15M-magnesium chloride. The washed cells were added to the assay medium containing different concentrations of betaine, and the rate of oxidation of succinate in the presence of 2·0M-sodium chloride was assessed from the rate of liberation of $^{14}CO_2$ from labelled succinate. Betaine stimulated $^{14}CO_2$ liberation in cells washed with magnesium chloride alone, and the stimulation increased with betaine concentration; by contrast, cells washed with magnesium chloride–sodium chloride were not influenced by betaine and respired at a high rate even in its absence (Table 3).

The effect of betaine on the respiration of cells washed with magnesium chloride alone (Fig. 3*a*) and with magnesium chloride–sodium chloride (Fig. 3*b*) suspended in buffered succinate containing different concentrations of sodium chloride was also examined. Comparison of Figs. 3(*a*) and 3(*b*) reveals that absence of sodium chloride from the washing medium caused a decrease in salt resistance. Addition of betaine had no effect on the 0·15M-magnesium chloride–2·0M-sodium chloride-washed cells, but restored the fall in salt resistance induced by omission of sodium chloride from the washing medium. Washing without sodium chloride also caused an overall depression of the respiratory rate, which was not influenced by betaine. Thus a significant difference existed between the effect of betaine on Ba_{1LS} cells and that on Ba_{1HS} cells: with the former the effect was apparent over almost the whole range of sodium chloride concentrations tested, whereas with the latter it is restricted to concentrations above the optimum.

DISCUSSION

The present study shows that betaine increased the salt resistance of the respiratory system in Ba_1 cells under certain specified conditions. To demonstrate the betaine effect, it was necessary either to cultivate the cells in a low-salt medium (Ba_{1LS} cells) or to wash high-salt-grown cells (Ba_{1HS} cells) in a sodium chloride-free medium. Apparently the prerequisite for the betaine effect is that the cells be free of, or contain little of, a genuine factor associated with salt resistance, and betaine acts by replacing it. Therefore the high-salt-grown cells, not influenced by betaine, presumably contained the genuine factor, which they may have acquired either through accumulation from their environment or by a process of synthesis and retention. Ba_{1HS} cells influenced by betaine after exposure to low-salt conditions had probably lost the protecting factor through the treatment. The low-salt-grown cells may lack the factor because their growth conditions had been unfavourable for its synthesis, or because the low-salt environment had prevented its accumulation or retention or both.

The metabolic relationship between choline and betaine (Goldstein, 1959; Shieh, 1964, 1965), the chromatographic data on the material accumulated in the bacterial cells during incubation with choline (Rafaeli-Eshkol, 1968*b*) and the present finding that betaine itself is effective suggest that choline owes its salt-resistance activity to its oxidation by the bacteria to betaine. The betaine effect was greatly enhanced by an increase in sodium chloride concentration of the medium. Since it was shown that betaine accumulation was also salt-dependent, it seems that salt resistance was directly related to intracellular betaine concentration. The difference in the response of Ba_{1LS} cells and magnesium chloride-washed Ba_{1HS} cells to betaine is also significant. Whereas betaine stimulated the respiration of Ba_{1LS} cells over a wide range of sodium chloride concentrations (including that in which maximum oxygen uptake occurred), with magnesium chloride-washed Ba_{1HS} cells the effect was restricted to the inhibitory concentration range. Since in the absence of added betaine the

maximum specific respiratory rate of Ba_{1HS} cells was also much lower than that of Ba_{1LS} cells, the lack of effect of betaine on the optimum rate in the former case may be due to a deficiency in certain component(s) of the respiratory system, that become(s) rate-limiting at optimum sodium chloride concentration.

Any suggestion on the mechanism of the betaine effect would be highly speculative, as it is not known whether the active substance is betaine itself or a derivative (Shieh, 1966). Considering the well-known role of betaine in transmethylation (Muntz, 1950; Durell, Anderson & Cantoni, 1957), it may be that betaine serves only as methyl donor for the active substance. In that case, however, the active substance cannot be a membrane-bound component, as it is removable from the cells by washing.

If betaine itself is involved in the effect on the respiratory system of bacterium Ba_1, an explanation of its mode of action should also account for its specificity. The two main features of the betaine molecule are its dipole character at physiological pH values, and the crowding of three methyl groups at the positively charged end of the molecule. Its being a zwitterion may facilitate its passage through the permeability barrier without the need for either an accompanying counterion or simultaneous ejection of intracellular ions, while still not violating the requirement of electroneutrality. Once inside the cell, the betaine molecule may contribute to the conformational stability of enzymes and structural moieties by binding on to charged groups and even forming cross-links between positively charged and negatively charged sites. Its binding on to negatively charged sites, in spite of the abundance of Na^+ ions, may be attributed to the presence of the methyl groups, which may promote binding of the positive pole by formation of hydrophobic bonds as well. Thus other structurally related dipolar molecules (such as glycine and sarcosine) would respectively either lack this advantage over Na^+ ions or possess it to a smaller extent.

An alternative explanation of the effect induced by betaine is that accumulation of betaine, unaccompanied by efflux of other molecules, would result in increased intracellular osmotic pressure, which may in itself be of advantage for cells exposed to high sodium chloride concentrations (Miller & Avi-Dor, 1958; Avi-Dor, Kuczynski, Shatzberg & Mager, 1956).

Other experiments (Rafaeli-Eshkol, 1968*a*) have shown that Ba_1 cells can grow in the presence of high sodium chloride concentrations even in a minimal medium. Assuming that a factor similar to that described in the present work accounts for salt resistance under those conditions as well, Ba_1 cells should be able to synthesize it from simple precursors.

The subject of this paper forms part of a doctoral thesis (Rafaeli-Eshkol, 1968*a*) submitted by D.R.-E. to the Department of Chemistry, Technion–Israel Institute of Technology.

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