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Studies on Halotolerance in a Moderately Halophilic Bacterium

EFFECT OF GROWTH CONDITIONS ON SALT RESISTANCE OF THE RESPIRATORY SYSTEM

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The effect of sodium chloride on the respiratory activity of a moderately halophilic halotolerant bacterium was studied. Irrespective of growth conditions, resting cells oxidized succinate at a low rate unless sodium chloride was included in the assay mixture, maximum respiratory rates being obtained for sodium chloride concentrations between 0·2M and 0·8M. Neither potassium chloride nor sucrose could replace the sodium salt. The response of the respiratory system to sodium chloride concentration above the optimum depended on growth conditions. Respiration of cells harvested from a low-salt medium was almost inhibited completely by 2·0M-sodium chloride, and that of cells grown and washed in the presence of 2·0M-sodium chloride by 30%. After preincubation with a growth medium containing 2·0M-sodium chloride, even with all multiplication suppressed by chloramphenicol, the resistance of the respiratory system of low-salt-grown organisms to high salt concentrations increased considerably and resembled that of their high-salt-grown counterparts. A similar increase in resistance occurred after preincubation with yeast extract or with choline. With labelled choline, energy-dependent accumulation of labelled material occurred, the conditions required for maximum accumulation and retention being the same as those that increased the salt resistance of the respiratory system. The chromatographic behaviour of the labelled material indicated that the substance was not choline but a derivative, possibly betaine.

There is considerable evidence that a close connexion exists between salt requirement for optimum growth and maximum enzyme activity in non-halophilic, marine and extreme halophilic bacteria (Larsen, 1962, 1967; Brown, 1964). Though the above three types of micro-organisms require widely different salt concentrations for growth, the permissible deviation from their respective optima is relatively small. By contrast, the so-called moderately halophilic halotolerant bacteria thrive satisfactorily over an extremely wide range of sodium chloride concentrations.

Several mechanisms by which the halotolerant organism could adjust itself to the milieu are possible, the most important ones being: (*a*) constant favourable intracellular salt content (Christian & Waltho, 1961); (*b*) repression and de-repression of different sets of enzymes in response to growth conditions (Westlake, Horler & McConnell, 1967); (*c*) formation of enzyme proteins with high conformational stability; (*d*) production or accumulation (or both) of protective factors against extreme changes (Maeno, 1965).

The impetus for the present study on halotolerance was given when, during a study of the bacterial population of crude solar salt, a Gram-negative rod was isolated among extreme halophilic species; this rod grew satisfactorily on a 'rich' medium containing only a little sodium chloride, and continued to grow on the same medium after the addition of 4M-sodium chloride. In this bacterium (Ba_1) regulation of the intracellular ion content did not account for the halotolerance, since intracellular concentration and composition were found (D. Rafaeli-Eshkol & R. Emanuel, unpublished work) to vary considerably with the sodium chloride concentration in the growth medium. For elucidation of the factors involved in the resistance of bacterium Ba_1 to high salt concentrations, a study of the response of its respiratory system to salts was relevant. Being an obligate aerobic organism, bacterium Ba_1 was

expected to possess a respiratory system that was functional over the wide range of salt concentrations that permit growth. A study of the respiratory system offered the additional advantage of an enzymic system lending itself to convenient measurement both in the intact cells and in cell-free extracts.

In the present paper and in its sequel (Rafaeli-Eshkol & Avi-Dor, 1968), evidence is presented that the salt resistance of the respiratory system in bacterium Ba_1 can be increased by certain low-molecular-weight substances such as choline and betaine, and that identical or similar factors may also be involved in the adaptation of growing cells to changes in the ion environment.

MATERIALS AND METHODS

[*Me*-^{3}H]Choline chloride was obtained from The Radiochemical Centre, Amersham, Bucks. Other chemicals were of analytical grade.

Organism. An unidentified, moderately halophilic halotolerant bacterium, referred to as bacterium Ba_1 and isolated by the author from a crude salt sample collected at the Dead Sea evaporation ponds, was used in this study. It is an obligate aerobic Gram-negative rod (Rafaeli-Eskhol, 1968).

Medium. The standard liquid medium used contained (per l. of water): 2·0g. of yeast extract (Difco); 2·5g. of Casitone (Difco); 30·0g. of $MgCl_2,6H_2O$. Its pH was adjusted to 6·7 before sterilization; NaCl (sterilized separately) was added to the sterilized standard medium when necessary.

Growth conditions and preparation of bacterial suspensions. Cultures of bacterium Ba_1 were maintained, by transfers, on slants of the standard liquid medium to which 2% (w/v) agar (Difco) and 24% (w/v) NaCl were added before sterilization. Cells from an agar-slant culture were transferred to 50ml. of standard medium containing 4 molal NaCl and incubated for 24–48hr. at 37° on a rotary shaker. Samples (1ml.) of the growing ('starter') culture were transferred either into 1l. of the standard liquid medium or into 1l. of standard medium to which 2 moles of NaCl had been added. The two cultures thus obtained [low-salt-grown (Ba_{1LS}) and high-salt-grown (Ba_{1HS}) cells] were incubated in Erlenmeyer flasks at 37° on a rotary shaker until the end of the exponential phase, and the cells harvested by centrifugation in the cold. Ba_{1LS} cells were washed with cold 0·15M-$MgCl_2$ and Ba_{1HS} cells with cold 0·15M-$MgCl_2$–2·0M-NaCl.

Assay conditions. Manometric experiments were carried out by the conventional Warburg technique. Each Warburg vessel contained, in the main compartment (unless otherwise stated), 'buffered succinate' containing 33mM-tris–HCl buffer, pH 7·4, 10mM-succinate, 10mM-$MgCl_2$ and 3·3mM-KCl; the total volume was 3·0ml. Unless otherwise stated, Ba_{1LS} cells equivalent to 0·5–1·0mg. of protein/cup or Ba_{1HS} cells equivalent to 1·0–2·0mg. of protein/cup were introduced into the reaction mixture before the 10min. thermal equilibration. Manometric readings were taken every 10–20min., depending on the rate of respiration. Specific respiratory rates (μl. of O_2/hr./mg. of protein) were calculated from respiration in the steady state.

For the determination of radioactive material accumulated in the cells after preincubation with labelled compounds, measured volumes of the assay mixture were withdrawn and centrifuged in the cold. Unless otherwise stated, the sedimented bacteria were washed twice with a volume of cold 0·15M-$MgCl_2$–2·0M-NaCl equal to four times the original volume of the sample. Subsequently, the cells were resuspended in a small volume of the washing solution from which known volumes were transferred to 10ml. of Bray's (1960) scintillation liquid and counted.

Ascending one-dimensional chromatography on Whatman no. 1 paper was carried out at room temperature, with ethanol–water (8:1, v/v) as the solvent mixture. The paper was air-dried and the spots were developed with iodine vapour. To determine the distribution of labelled material on the chromatogram, the paper was subsequently cut into 2cm. strips, which were wetted with 0·5ml. of water. Bray's (1960) scintillation liquid (15ml.) was added to each strip before counting.

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) with the bacterial cells solubilized by boiling with N-NaOH for 20min. Radioactivity was measured in a Packard Tri-Carb model 3003 scintillation spectrometer.

RESULTS

Effect of salt concentration in the assay medium on succinate oxidation by low-salt-grown and high-salt-grown Ba_1 cells. Ba_1 cells were grown in the standard medium either in the absence of added salt (Ba_{1LS} cells) or in the presence of 2·0M-sodium chloride (Ba_{1HS} cells). The respiratory activity of Ba_{1LS} and Ba_{1HS} cells was assayed with various substrates (succinate, glutamate, glucose, ethanol) as a function of the salt concentration in the assay medium. Since the nature of the substrate had no influence on the respiration pattern, only the results obtained with succinate are described in detail.

When the effects of sodium chloride, potassium chloride and sucrose on respiration were compared, only sodium chloride supported the oxygen uptake (Fig. 1). In the range of concentration that stimulated respiration, the effect of sodium chloride on Ba_{1LS} and Ba_{1HS} cells was similar, maximum rates of oxygen uptake being reached at approx. 0·2M-sodium chloride in the assay mixture. However, the specific rate of oxygen uptake (μl./hr./mg. of protein) was, in each experiment, considerably higher for Ba_{1LS} than for Ba_{1HS} cells. When the sodium chloride concentration exceeded that giving maximum stimulation, a difference was seen in the response of the two types of resting cells. The respiration of Ba_{1HS} cells showed a plateau up to a sodium chloride concentration of 1·2M, and even with 2·0M-sodium chloride the rate was still 70% of the maximum; the respiration of Ba_{1LS} cells was inhibited by salt concentrations above 0·8M, and with 2·0M-sodium chloride these cells practically ceased to oxidize succinate.

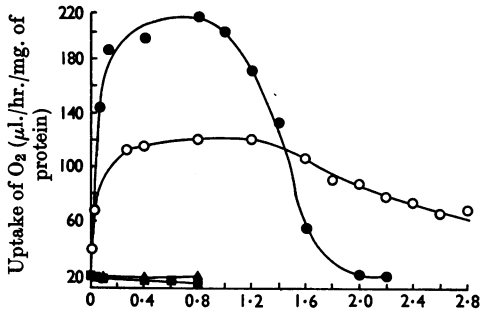

Fig. 1. Effect of NaCl, KCl and sucrose in the assay medium on succinate oxidation by Ba_{1LS} and Ba_{1HS} cells. In addition to the components described in the Materials and Methods section, the Warburg vessels contained NaCl, KCl or sucrose in the concentration indicated: ○ and ●, NaCl; ▲, sucrose; ■, KCl. ○, O_2 uptake of Ba_{1HS} cells; ●, ▲ and ■, O_2 uptake of Ba_{1LS} cells.

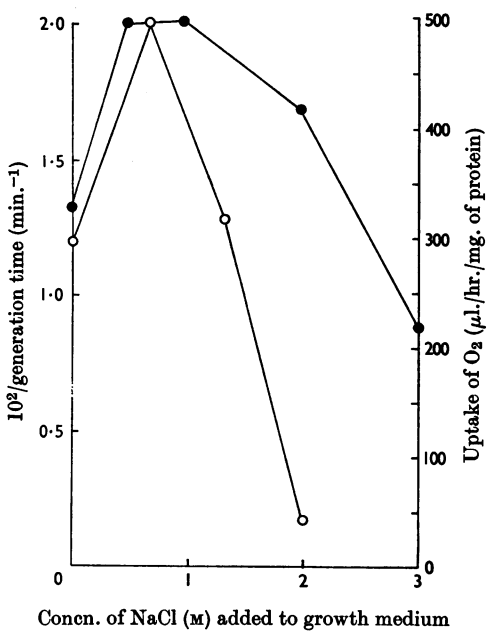

Fig. 2. Comparison of the effect of NaCl concentration on growth of bacterium Ba_1 and on respiration of Ba_{1LS} cells. ○, O_2 uptake of Ba_{1LS} cells suspended in growth medium supplemented with 165μg. of chloramphenicol/ml. and different NaCl concentrations; ●, growth rate expressed as the reciprocal of generation time in the presence of the NaCl concentration indicated.

The effect of different sodium chloride concentrations on the respiration of Ba_{1LS} cells was also tested with the growth medium supplemented with 165μg. of chloramphenicol/ml., which was found to suppress cell multiplication completely, replacing the buffered succinate as source of substrates. Fig. 2 shows the respiratory pattern obtained, compared with a curve representing the generation time of Ba_1 cells as a function of the salt concentration. The generation time found was the same irrespective of whether the inoculum was of Ba_{1LS} or Ba_{1HS} cells. Since the aerobic bacterium Ba_1 was not expected to multiply without the energy derived from respiration, the lack of correlation between the slopes of the descending branches of the two curves in Fig. 2 requires some explanation. Presumably under the growth conditions in question there was an adjustment enabling the cells to respire in the presence of high sodium chloride concentrations. This assumption was put to test in experiments described below.

Effect of cell concentration on respiratory rate. With Ba_{1LS} cells the effect of cell concentration on the respiratory rate of cells was examined either in buffered succinate (Fig. 3*a*) or in the growth medium (Fig. 3*b*). Cells respiring on succinate (Fig. 3*a*) behaved in the expected manner; that is, the oxygen uptake increased linearly with cell concentration, the specific rate thus remaining unchanged. Fig. 3(*b*) shows that a 'normal' curve was obtained for cells in the growth medium containing 0·7M-sodium chloride. A completely different pattern, however, was observed when the concentration of sodium chloride in the growth medium was 2·0M; the curve passed through a maximum. When the specific rates were plotted as a function of cell concentration they decreased with an increase in the amount of bacteria in the system. This anomalous behaviour was not due to a limitation in oxidizable substrates in the medium. These experiments suggested that the growth medium contained a component capable of stimulating oxygen uptake in 2·0M-sodium chloride, and that the observed fall in the specific rate occurs when the high bacterial concentration makes the amount of the component available per cell the limiting factor in respiration.

Conditions for the attainment of increased salt resistance of the respiratory system in Ba_{1LS} cells. In addition to the bacterial concentration (see the preceding paragraph), other factors influencing the attainment of increased salt resistance of the respiratory system were investigated. The possibility that cells, having acquired the ability to respire at an appreciable rate in the presence of 2·0M-sodium chloride, would retain it even when removed from the growth medium, washed and

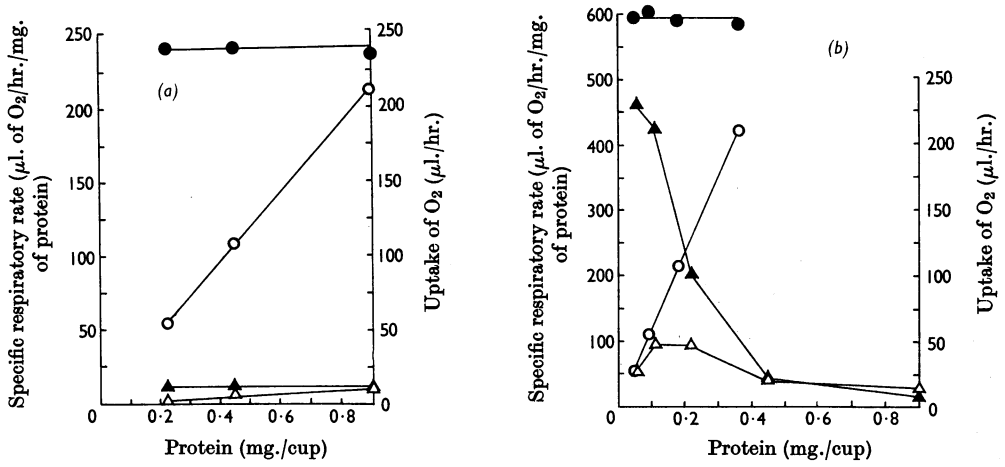

Fig. 3. Effect of cell concentration on respiratory rate. Ba_{1LS} cells were suspended either in buffered succinate (*a*) or in the growth medium (*b*). Each Warburg vessel was supplemented with 500 μg. of chloramphenicol. ○ and ●, 0·7 M-NaCl in assay medium; △ and ▲, 2·0 M-NaCl in assay medium. ○ and △, O_2 uptake; ● and ▲, specific respiratory rates.

Table 1. *Effect of conditions of preincubation and subsequent washing on respiration of Ba_{1LS} cells in buffered succinate in the presence of 2·0 M-sodium chloride*

The preincubation media contained 165 μg. of chloramphenicol/ml. and 2·0 M-NaCl unless otherwise stated. The supernatant obtained by centrifuging the contents of flask no. 4 after preincubation was designated as 'depleted' growth medium. Preincubation time was 90 min. For the composition of the buffered succinate medium used for preincubation, and for the respiratory assay, see the Materials and Methods section.

	Conditions of preincubation			Respiration of preincubated Ba_{1LS} cells on buffered succinate + 2·0 M-NaCl (μl. of O_2/hr./mg. of protein)	
Flask no.	Medium of preincubation	Concn. of bacteria (μg. of protein/ml.)	Temp.	Cells washed with 0·15 M-$MgCl_2$–2·0 M-NaCl	Cells washed with 0·15 M-$MgCl_2$
1	Growth medium	50	37°	110	0
2	Growth medium, no NaCl added	50	37	0	5
3	Growth medium	50	0	0	0
4	Growth medium	500	37	0	—
5	Depleted growth medium	50	37	3	—
6	Buffered succinate	50	37	4	0
7	No preincubation			—	7

transferred into buffered succinate was also examined.

Increased salt resistance was indeed induced as a result of preincubation with the medium and retained after transfer into buffered succinate, provided that certain conditions were observed: low cell concentration, temperature maintained at 37°, and 2·0 M-sodium chloride included both in the medium used for preincubation and in the washing medium of the preincubated cells (Table 1). In contrast, cells preincubated with buffered succinate, or with the growth medium 'depleted' by prior incubation with bacterial cells equivalent to 500 μg. of protein/ml., did not acquire increased salt resistance (Table 1).

Other experiments showed that yeast extract alone also induced increased salt resistance of the respiratory system. As yeast extract is known to be a source of vitamins, the effect of water-soluble vitamins on the respiration of Ba_{1LS} cells was also

Table 2. *Effect of choline on succinate oxidation by Ba_{1LS} cells*

Each Warburg vessel contained 33mM-tris–HCl buffer, pH 7·4, 10mM-$MgCl_2$, 3·3mM-KCl, 165μg. of chloramphenicol/ml., and other components as indicated. Other details are given in the Materials and Methods section.

Additions to assay medium		Uptake of O_2 (μl./hr./mg. of protein)	
NaCl (M)	Succinate (mM)	No choline	0·5mM-Choline
0·8	10	148	230
0·8	0	8	10
2·0	10	10	150
2·0	0	6	20

Table 3. *Retention of choline-induced respiratory stimulation*

The preincubation mixture contained buffered succinate supplemented with 2·0M-NaCl, 0·5mM-choline, 165μg. of chloramphenicol/ml. and Ba_{1LS} cells equivalent to 0·2mg. of protein/ml. Preincubated cells were washed as indicated and transferred to Warburg vessels containing buffered succinate supplemented with 165μg. of chloramphenicol/ml. and NaCl as indicated. Other details are given in the Materials and Methods section.

	Uptake of O_2 (μl./hr./mg. of protein)	
Concn. of NaCl (M)	Cells washed with 0·15M-$MgCl_2$–2·0M-NaCl	Cells washed with 0·15M-$MgCl_2$
0·8	204	126
2·0	140	14

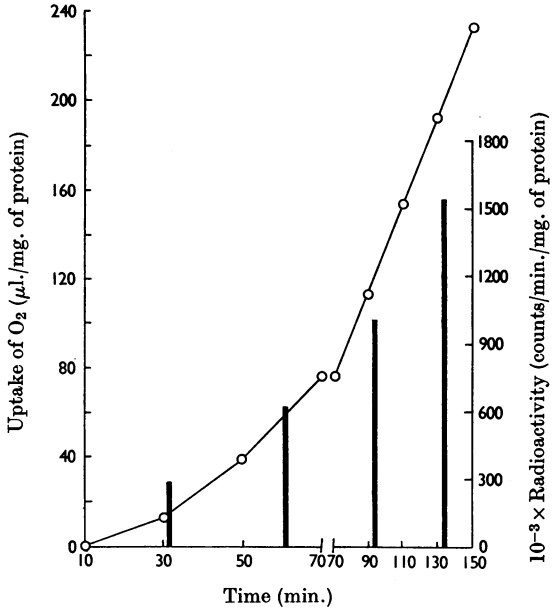

Fig. 4. Interrelation between rate of O_2 uptake and accumulation of labelled substances in Ba_{1LS} cells respiring in the presence of [*Me*-^{3}H]choline. Each Warburg vessel contained buffered succinate supplemented with 2·0M-NaCl and unlabelled 0·5mM-choline. Labelled choline was added in an amount yielding $1{\cdot}6 \times 10^6$ counts/min./μmole of choline. Experimental points indicate O_2 uptake; the block bars indicate the amount of radioactive material (assayed as described in the Materials and Methods section) found to be associated with the cells at the time indicated.

tested. A mixture of eight water-soluble vitamins including choline caused a significant increase in respiration on succinate in the presence of 2·0M-sodium chloride, and choline apparently accounted for most of the respiratory stimulation.

Examination of certain parameters associated with choline-induced salt resistance. As the factor affecting the salt resistance of Ba_{1LS} cells present in the growth medium was found to be replaceable by choline, the action of choline was investigated in more detail. Choline was found to accelerate succinate oxidation both at 0·8M- and at 2·0M-sodium chloride, but whereas with the former the rate of oxidation was only doubled, with the latter it was increased 15-fold (Table 2). If choline is oxidized by the bacteria, the contribution to oxygen uptake is negligible (Table 2).

The choline-induced effect, like that mediated by the growth medium, was retained by Ba_{1LS} cells after removal of choline from the supernatant (Table 3). As with the factor in the growth medium, inclusion of 2·0M-sodium chloride in the washing medium was essential for retention of the effect.

One explanation of the results in Table 3 is that choline accumulated during preincubation but was released from the cells unless 2·0M-sodium chloride was included in the washing medium. This explanation was verified by using [*Me*-^{3}H]choline. Ba_{1LS} cells were incubated with labelled choline in buffered succinate containing 2·0M-sodium chloride. At intervals, cells were sedimented by centrifugation and washed with 0·15M-magnesium chloride–2·0M-sodium chloride, and the residual radioactivity was counted. This is shown in Fig. 4, which also shows the change in the rate of succinate oxidation with time of incubation. Accumulation of the labelled choline and increase in respiratory rate were relatively slow processes, though the radioactivity accumulated in the cells continued to increase even after the oxygen uptake had reached a steady rate.

Some other factors influencing accumulation of

Table 4. *Accumulation of labelled material by Ba_{1LS} cells incubated with [Me-^{3}H]choline*

Ba_{1LS} cells were incubated with shaking in a medium containing tris buffer, pH 7·4, $MgCl_2$, KCl and chloramphenicol as in Table 2, and unlabelled 0·5 mM-choline; succinate and NaCl were added as indicated. In Expt. 1 the cell concentration corresponded to 0·8 mg. of protein/ml., the time of incubation was 150 min. and labelled choline was added in amount yielding $1·4 \times 10^6$ counts/min./μmole of choline; in Expt. 2 the corresponding values were 0·25 mg. of protein/ml., 120 min. and $0·7 \times 10^6$ counts/min./μmole of choline.

	Additions to incubation medium			$10^{-3} \times$ Radioactivity associated with cells (counts/min./mg. of protein)	
Expt. no.	NaCl (M)	Succinate (mM)	Temp. of incubation	Cells washed with 0·15 M-$MgCl_2$–2·0 M-NaCl	Cells washed with 0·15 M-$MgCl_2$
1	2·0	10	37°	980	97
	2·0	10	0	9	—
	2·0	0	37	470	82
2	0	10	37	25	—
	0·4	10	37	275	—
	0·8	10	37	520	—
	2·0	10	37	1160	—

the labelled material were studied (Table 4). A sodium chloride concentration of 2·0 M, in both the incubation medium and the washing medium, was the most favourable for accumulation. A source of energy also seemed to be required, since the amount of accumulated labelled substance was decreased by 50% when succinate was omitted from the incubation medium, and accumulation was almost completely inhibited by lowering the temperature to 0°.

Paper chromatography of the labelled material washed out of cells preincubated with radioactive choline. To find out whether the labelled material released from Ba_{1LS} cells on washing them with 0·15 M-magnesium chloride was choline or a derivative, 50 μl. of the wash fluid, equivalent to 50–100 ng. of cell protein, was chromatographed together with choline. The strip in which most of the radioactivity resided did not coincide with the one containing the choline spot. On repeating the chromatography with both unlabelled choline and its oxidation product betaine as markers, the radioactivity was found to reside in the same strip as the betaine spot.

DISCUSSION

The finding that accumulation and release of labelled material derived from choline in Ba_{1LS} cells corresponds respectively to acquisition and loss of salt resistance shows that of the various mechanisms enumerated in the introduction likely to play a role in halotolerance, at least one, namely the presence of protective substances, is involved in the resistance of the respiratory system of Ba_1 cells to high salt concentrations. The substances (or choline, which substitutes for them) are able to equalize the salt resistance of the respiratory system of low-salt-grown and high-salt-grown cells. Thus a difference determined by growth conditions that possibly may seem to be due to differences on the enzymic level is likely to be associated with the presence or absence of protective substances.

In addition to the differences in salt resistance between the low-salt-grown and high-salt-grown organisms, the former were found to have higher specific rates of respiratory activity when respiration was measured at optimum sodium chloride concentration. Since, as pointed out by various authors (White & Smith, 1964; White, 1962; Smith, 1954; Gray, Wimpenny & Mossman, 1966; Scholes & Smith, 1968), micro-organisms can vary the absolute and relative amount of respiratory enzymes in response to changes in the growth medium, it is likely that the low-salt-grown organism produces larger amounts of all (or some) of the respiratory enzymes to compensate for their low activity under growth conditions dictated by the low Na^+ concentration in the medium.

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

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