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Effect of osmotic pressure on membrane energy-linked functions in *Escherichia coli*

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Osmotic upshock of *E. coli* cells in NaCl or sucrose medium resulted in a large decrease in the cytoplasmic volume and the inhibition of growth, of the electron transfer chain and of four different types of sugar transport system: the lactose proton symport, the glucose phosphotransferase system, the binding-protein dependent maltose transport system and the glycerol facilitator. In contrast to NaCl and sucrose, the permeant solute glycerol had no marked effect. These inhibitions could be partially relieved by glycine betaine. Despite these inhibitions, the internal pH, the protonmotive force and the ATP pool were maintained. It is concluded that inhibition of electron transfer and of sugar transport is the consequence of conformational changes caused by the deformation of the membrane. It is also concluded that the arrest of growth observed upon osmotic upshock is not due to energy limitations and that it cannot be explained by the inhibition of carbohydrate transport.

Introduction

Osmotic shock, produced by an increase in the osmolarity of the medium, inhibits bacterial growth [1,2]. The reason for this inhibition is still unknown. In recent years, attention has focused on the different mechanisms involved in the adaptation of microorganisms, particularly of *E. coli*, to media of high osmolarity. The first response to an osmotic upshock is the accumulation of K^+ [3]. However, when external osmolarity exceeds 600 mosM, internal K^+ ions are no longer able to counterbalance the increase in osmotic pressure. The bacteria are able to accumulate several osmoprotectants, principally glycine betaine [4]. The synthesis of the high-affinity transport system for glycine betaine, ProU, is triggered by the increase in intracellular potassium induced by the osmotic shock [5,6]. When glycine betaine is present in the medium, it is accumulated by the bacteria and not catabolized [7]. In consequence the reversal of the osmotic stress by glycine betaine has been ascribed to a partial restoration of the turgor pressure. Nevertheless, a direct protective effect of glycine betaine on intracellular enzymes has also been suggested on the basis of in vitro experiments showing that inhibition of malate dehydrogenase by high ionic strength can be reversed by the addition of glycine betaine [8]. Other responses to an osmotic upshock include synthesis of glycine betaine from choline [9], when this solute is present in the medium, and synthesis of large amounts of glutamate and trehalose [10,11].

In contrast to the aforementioned mechanisms of adaptation, the effects of an increase in external osmolarity on membrane functions have received less attention. It was shown by Roth et al. [12] that an increase in osmolarity (0.8 M NaCl), drastically inhibits four different sugar transport systems in *E. coli*, namely the glucose phosphotransferase system, the binding protein mediated maltose transport, the lactose-proton symport and the melibiose-sodium symport. In a subsequent paper, the same authors showed that addition of glycine betaine to cells induced for glycine betaine transport can restore the transport of glucose under conditions of high osmolarity [13]. The inhibitory effects induced by hypertonic stress were explained in terms of conformational changes of the different membrane proteins caused by the deformation of the membrane. In addition, they suggested that inhibition of the active trans-

Abbreviations: α-MG, methyl α-D-glucopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMS, phenazine methosulfate; PTS, phosphotransferase system; TPP^+, tetraphenyl phosphonium ion.

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port of carbohydrates was sufficient to account for the observed growth inhibition following osmotic stress.

Inhibition of sugar active transport could, indeed, result from conformational changes, or it could be a secondary consequence of a possible decrease in the different energy forms involved in active transport. This led us to determine the effect of osmolarity on the bioenergetics of *E. coli* (rate of respiration, protonmotive force, ATP content) before reinvestigating the inhibition of different sugar active transport systems (lactose proton symport, PTS, maltose transport). Transport by facilitated diffusion via the glycerol facilitator (a protein channel) was also studied as a function of the external osmolarity. The protective effect of glycine betaine on these membrane functions was investigated.

Increasing the external osmolarity with a permeant or an impermeant solute results in all cases in a decrease in the activity of water, but, in addition, impermeant but not permeant solutes affect the osmotic pressure across the cytoplasmic membrane [14]. In order to distinguish between these two effects, we systematically compared permeant (glycerol) and impermeant (NaCl or sucrose) solutes. The use of sucrose in parallel with NaCl allowed us to rule out a possible effect of an increase in the ionic strength of the medium.

A preliminary report of this work has been presented elsewhere (Ghazi, A., Houssin, C., Eynard, N. and Shechter, E., Abstr. 5th European Bioenergetics Conference, 1988, p. 200).

Materials and Methods

Growth conditions and cell treatment

The strains of *E. coli* used in this study are ML 308-225 (i^-, z^-, y^+, a^+), pop 1000 ($MalQ^-$, His^- [15]) and K10 [7]. Cells were grown aerobically at 37°C in minimal medium M9, supplemented with 0.4% glycerol as the sole carbon source and 40 μg/ml histidine for the pop 1000 strain. Bacteria were harvested by centrifugation at an absorbance of 0.5 at 650 nm.

To induce cells for glycine betaine transport, growing bacteria ($A_{650} = 0.3$) were subjected to an osmotic upshock by addition of 0.65 M NaCl (final concentration) to the medium and harvested 2 h later.

For membrane potential measurements, and in order to render the membrane permeable to TPP^+, cells were treated with EDTA according to Ref. 16.

In all cases, cells were resuspended at $A_{650} = 100$ (50 mg dry weight/ml) in 10 mM Tris-HCl/150 mM NaCl/1 mM KCl (pH 7.6), kept on ice and used within 5 h.

All assays were performed at room temperature in 10 mM Tris-HCl buffer (pH 7.6) containing 1 mM KCl and 100 μg/ml chloramphenicol. The osmolarity of the assay medium was varied by addition of NaCl or sucrose or glycerol. We used the following corresponding between the osmolarity of the medium and the concentrations of these solutes [34].

Osmolarity (osmol/l)	NaCl (M)	Sucrose (M)	Glycerol (M)
0.28	0.15	0.25	0.25
0.56	0.30	0.48	0.55
1.13	0.60	0.83	1.00
1.50	0.80	1.00	1.30
1.88	1.00	1.20	1.50

Except when otherwise stated, cells were incubated in the assay medium, 20 min prior to any measurements, in the absence or in the presence of 1 mM glycine betaine.

Determination of cytoplasmic volume

The cytoplasmic volume was determined with ^3H_2O (0.12 MBq/ml) and [^{14}C]sucrose (6.9 μM, 20 GBq/mmol) according to Ref. 17, using catalase (5000 U/ml) and H_2O_2 (0.53 mM) to prevent anaerobiosis. [^{14}C] Sucrose was biologically purified before use, to eliminate glucose contamination, in the following way: ML 308-225 cells grown in glucose were incubated at a final concentration of 5 mg dry weight/ml, 10 min, in the presence of 15 μl [^{14}C]sucrose (0.46 mM, 20 GBq/mmol). The cell suspension was then centrifuged for 2 min on a Microfuge TH12 and the supernatant containing the purified sucrose was kept for further use. To determine the cytoplasmic volume, cells (5 mg dry weight/ml) were incubated in the assay medium (1.5 ml) in the presence of tritiated water, catalase and H_2O_2 in the presence or absence of glycine betaine. Purified [^{14}C]sucrose was added just before centrifugation. After centrifugation (2 min in a Microfuge) an aliquot (100 μl) of the supernatant was removed and transferred to a counting vial containing a similar pellet of non-radioactive bacteria (100 μl). The remaining supernatant was discarded and the microcentrifuge tube wall was carefully dried. The pellet was completely resuspended in 200 μl of the assay medium and transferred to a counting vial. Vials were counted for radioactivity on the preset of a $^3H/^{14}C$ program of a liquid scintillation counter. All measurements were made in triplicate.

Determination of $\Delta\Psi$ and ΔpH

$\Delta\Psi$ was determined from the accumulation of [^{3}H]TPP^+ [17]. EDTA-treated cells were resuspended in the assay medium at a final concentration of 1 mg dry weight/ml and incubated with agitation for 20 min in the presence of 10 μM [^{3}H]TPP^+ (3.7 GBq/mmol). Aliquots (100 μl) were then removed, diluted with 4 ml of the assay medium and filtered on Whatman glass microfiber filter (GF/F). The filters were washed with 4 ml of the same medium and counted for radioactivity. TPP^+ uptake was corrected for nonspecific binding by

subtracting a blank obtained under the same conditions except that the cells were pretreated with 25 μM CCCP.

ΔpH was estimated from the accumulation of [^{14}C]benzoate (10 μM, 24 GBq/mmol) in the presence of tritiated water (0.12 MBq/ml) as a marker of the total water content [17]. Cells were preincubated in the assay medium at a concentration of 1 mg dry weight/ml in the presence of radioactive probes, catalase (5000 U/ml) and H_2O_2 (0.53 mM). The method of separation was centrifugation and the experimental procedure was the same as that of the cytoplasmic volume determination. All $\Delta\Psi$ and ΔpH measurements were made in triplicate.

Transport of carbohydrates

Initial rate of influx. [^{14}C]Lactose (0.5 mM, 36.9 kBq/mmol), [^{14}C]α-MG (1.33 μM, 5.55 GBq/mmol) or [^{14}C]maltose (2.1 μM, 925 MBq/mmol), was added to 100 μl of cell suspension (1 mg dry weight/ml) and the sample was filtered 10 s later as described for $\Delta\Psi$ measurements.

Time course of lactose accumulation. [^{14}C]Lactose (2.11GBq/mmol) was added to the cell suspension (1 mg dry weight/ml), at a final concentration of 3.5 μM. Aliquots (100 μl) were withdrawn as a function of time, immediately diluted with 4 ml of the assay medium and filtered as above.

Initial rate of facilitated diffusion. In order to obtain a high concentration of bacteria in the assay medium, cells were first centrifuged and resuspended in this medium at a final concentration of 100 mg dry weigth/ml. In the case of lactose measurements, cells were also pretreated with 40 μM CCCP. Initial rates of uptake were determined by filtration 10 s after the addition of [^{14}C]lactose (8.33 mM, 12.3 kBq/mmol) or [^{14}C]xylitol (100 mM, 3.1 MBq/mmol) to 10 μl of the concentrated bacterial suspension.

In all cases (lactose, α-MG, maltose and xylitol), filtrations were made in triplicate and uptakes were corrected by subtracting blanks obtained under the same conditions except that the sample was filtered immediately after addition of the radioactive sugar and that, in the case of lactose uptake, cells were pretreated with 25 μM CCCP and 300 μM *p*-chloromercuriphenylsulfonate (a thiol reagent which blocks the permease).

Rate of respiration

Oxygen consumption was determined polarographically with a Gilson oxygraph using a Clarke oxygen electrode. Cell concentration was 1 mg dry weight/ml.

ATP measurement

10 μl of cell suspension were first diluted with 90 μl dimethylsulfoxide; 4.9 ml of sterile H_2O was then added and 100 μl of this suspension were withdrawn for ATP measurement using the luciferin/luciferase assay system, with a luminometer (Lumac). All measurements were made in triplicate.

Protein content

Protein was determined by the method of Lowry et al. [18].

Material

Strain K10 was a generous gift from Dr Le Rudulier. Strain pop 1000 was obtained from the Pasteur Institute (Laboratory of Dr M. Schwartz). [^{3}H]TPP^{+}, [^{14}C]sucrose [^{14}C]benzoate and tritiated water were from CEA, France. [^{14}C]Lactose, [^{14}C]α-MG, [^{14}C]maltose and [^{14}C]xylitol were from Amersham, U.K. All other materials were of reagent grade and obtained from commercial sources.

Results

Growth of strain ML308-225 and induction of glycine betaine transport

We verified that this strain is inducible for glycine betaine transport. Addition of 0.65 M NaCl to ML308-225 growing cells resulted in a nearly complete inhibition of growth. When 1 mM glycine betaine was present in the medium, growth resumed after a lag period of 2 h with a doubling time similar to that of the control (data not shown). The same result was obtained by Le Rudulier et al. with the strain K10, for which it was shown that restoration of growth is due to the induction of glycine betaine transport and to the subsequent accumulation of this solute [7].

When the same experiment was performed using sucrose (0.85 M) instead of NaCl, a different behavior was observed: after a slow decrease in absorbance, which lasted some 80 min, growth resumed at a rate similar to that of the control (data not shown). This pattern is best explained by a slow permeation of sucrose: the decrease in absorbance would correspond to a reswelling of the bacteria upon entry of sucrose. The resulting decrease in the osmotic pressure leads to a recovery of growth. This slow permeation of sucrose is not specific to our strain, since we observed the same phenomenon with *E. coli* K10. Despite this, we used sucrose in the rest of this study, in parallel with NaCl, to raise the external osmolarity. The duration of the experiments was short enough for sucrose to be considered as an osmoticant. The similarity of the inhibitory effects obtained with sucrose and NaCl, and the fact that this inhibitory effect could be prevented by glycine betaine in both cases (see below), justify a posteriori this assumption.

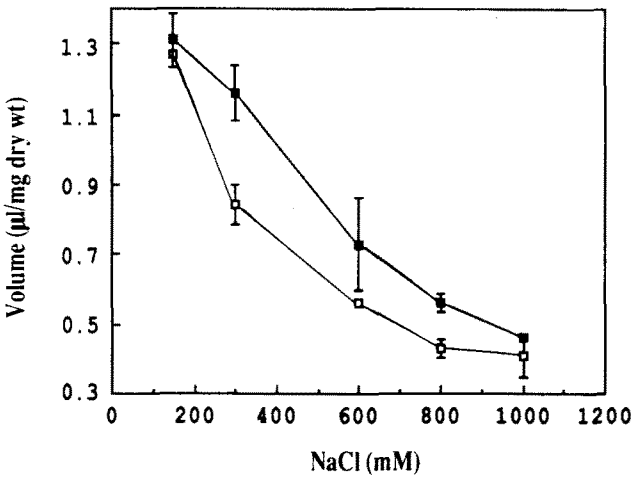

Fig. 1. Cytoplasmic volume of *E. coli* ML 308-225 cells as a function of external NaCl concentration. The determination was carried out with cells induced for glycine betaine transport, in the absence (□) or in the presence (■) of 1 mM glycine betaine. All volume measurements (i.e., centrifugation of bacterial suspension, see methods) were made 20 min after the osmotic upshock.

Cytoplasmic volume

Cytoplasmic volume was determined using the exclusion of radioactive sucrose. The sucrose concentration that we used (6.9 µM) and the time of incubation (less than 1 min) minimize the possibility of a systematic error due to the slow permeation of sucrose mentioned above. In all cases the values which we report must be considered as minimal values of the internal volume.

The cytoplasmic volume was measured with bacteria induced for glycine betaine transport. The bacteria were resuspended either in the absence or in the presence of 1 mM glycine betaine. In the presence of 150 mM NaCl an internal volume of 1.3 µl/mg dry weight was found in the presence as well as in the absence of glycine betaine. Fig. 1 shows that raising the external NaCl concentration up to 1 M resulted in a decrease in the cytoplasmic volume by a factor of 3. For intermediate concentrations of NaCl, the presence of glycine betaine allowed a partial restoration of the cytoplasmic volume (typically a 40% increase in the volume at 300 mM NaCl). Glycine betaine was no longer effective at 1 M NaCl external concentration. The same measurements could not be done when sucrose was used to vary the external osmolarity, since a sizable fraction of the bacteria could not sediment at high sucrose concentration.

In the rest of this study, the values that we used for the cytoplasmic volume in the determination of the protonmotive force and of different metabolite concentrations, were those reported in Fig. 1.

Respiration

High NaCl concentrations inhibited endogenous respiration as well as respiration stimulated by exogenous substrates: succinate, D-lactate, glycerol and the artificial electron donnor ascorbate PMS (data not shown). The pattern of inhibition was roughly similar in all cases. A comparable inhibition of respiration was observed upon addition of increasing concentration of sucrose. When osmolarity was varied by increasing the concentration (up to 1.5 M) of glycerol, a permeant solute, no inhibition was observed. In EDTA-treated cells, which were used for determination of $\Delta\Psi$, inhibition was complete at 1 M NaCl, while non-EDTA treated cells could still retain 15% respiratory activity at this NaCl concentration. When the rate of respiration was measured in bacteria induced for glycine betaine transport, addition of 1 mM glycine betaine in the assay medium partially prevented the inhibition of respiration obtained by increasing osmolarity with NaCl (Fig. 2A) as well as with sucrose (Fig. 2B).

Protonmotive force

Surprisingly, the membrane potential was not affected by an increase in external osmolarity: in glycerol-energized cells $\Delta\Psi$, measured 20 min after the osmotic upshock, was about 160 mV in a NaCl medium (from 150 mM to 1 mM external concentration) and 200 mV in a sucrose medium (from 250 mM to 1.2 M external concentration). This high membrane potential

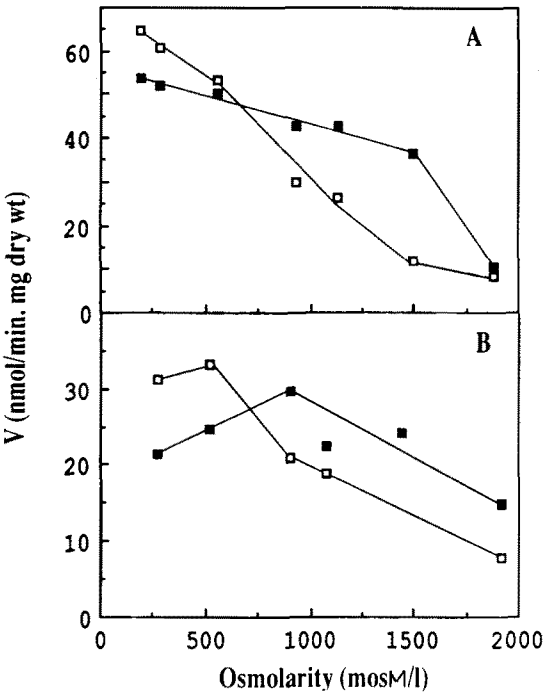

Fig. 2. Rate of respiration in *E. coli* ML 308-225 as a function of osmolarity. Osmolarity was varied by increasing external NaCl (A) or sucrose (B) concentrations. Rate of respiration (nmol O_2/min per mg dry weight) was measured in the presence of 0.4% glycerol, in bacteria induced for glycine betaine transport, 20 min after the osmotic upshock, in the absence (□) or in the presence (■) of 1 mM glycine betaine.

could be maintained at least for 40 min after the osmotic upshock. The experiments reported in this study were performed at pH 7.6. For this external pH there is no ΔpH, and $\Delta\Psi$ is the sole component of the protonmotive force. 20 min after the osmotic upshock, no ΔpH could be detected, using benzoic acid as a probe, at 150 mM and at 1 M NaCl external concentration. When the experiment was performed at pH 6, ΔpH was found to be 1.6 at 150 mM and at 1 M NaCl. Thus, the external osmolarity had no marked effect on internal pH and on ΔpH.

Taken as a whole, external osmolarity, up to 2 osmol/l does not affect the protonmotive force.

ATP

When glycerol-energized cells were subjected to an osmotic upshock (800 mM NaCl) ATP concentration first increased (due to the decrease of the cytoplasmic volume) and then decreased within minutes to a level similar to that of control cells (Fig. 3). When cells were energized with succinate, after the initial increase, the ATP concentration decreased slowly below the level of the control (ATP was decreased by a factor of 3 after 30 min, data not shown).

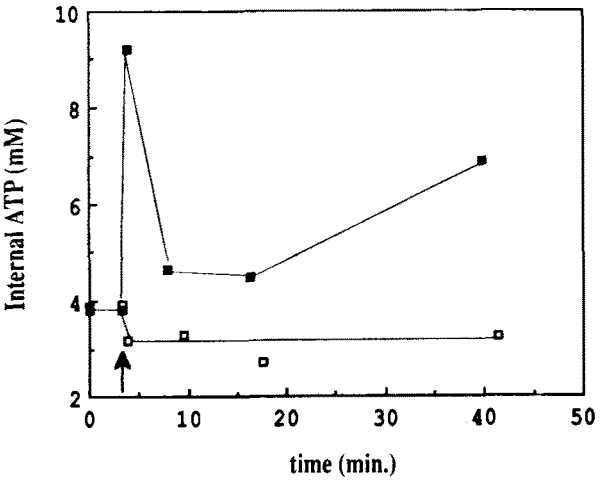

Fig. 3. Effect of osmotic upshock on internal ATP concentration in *E. coli* ML 308-225 cells. (□): cells were incubated in the assay medium (containing 150 mM NaCl) in the presence of 0.4% glycerol (control cells). (■): cells were incubated as for control cells and, at the time indicated by an arrow, subjected to an osmotic upshock by increasing the external NaCl concentration from 150 to 800 mM. The error on the ATP determination is less or equal to the size of the symbols.

Carbohydrate transport systems

Active transport. A sudden increase in the NaCl concentration of the medium (0.6 M) resulted in a nearly complete inhibition of the initial rate of transport of α-MG, an analog of glucose for the glucose PTS. The inhibition was obtained within 15 s of the raise in osmolarity and remained unchanged for 150 min.

The effect of increasing concentrations of NaCl (from 0.15 M to 1 M) or sucrose (from 0.25 M to 1.2 M) or glycerol (from 0.25 M to 1.5 M) was assayed on the activity of three different transport systems. The cells were induced for glycine betaine transport and the experiments were performed in the presence or in the absence of glycine betaine, after 20 min incubation in the presence of the osmoticant. The results obtained for selected values of the NaCl concentration are given in Table I. Although the pattern differed from one transport system to the other, in the three cases, increased NaCl concentrations resulted in a drastic decrease of transport. Sucrose had a similar effect. In contrast, increased glycerol concentrations had only a minor effect: in 1.5 M glycerol buffer, rates of sugar transport were decreased by 30 to 40% as compared to nearly complete inhibition in 1 M NaCl buffer, a medium of similar osmolarity. Inhibition of these transport systems by NaCl and sucrose could be partially relieved by the presence of 1 mM exogenous glycine betaine (Table I). Nevertheless, at high NaCl or sucrose concentrations, glycine betaine was no longer able to prevent the inhibitory effect.

TABLE I

Effects of different NaCl concentrations on the initial rate of sugar transport in the absence or in the presence of 1 mM glycine betaine

Values are given ± S.D. ($n = 3$). External sugar concentrations were: lactose, 0.5 mM; α-MG, 1.3 μM; maltose, 2.1 μM; xylitol, 100 mM. GB: glycine betaine.

NaCl (M)	GB (1 mM)	Lactose transport [a] (nmol/min per mg d.wt.)	α-MG transport (pmol/min per mg d.wt.)	Maltose transport [a] (nmol/min per mg d.wt.)	Xylitol transport (nmol/min per mg d.wt.)
0.15	−	79.4 ± 2.5	111.9 ± 2.2	1.29 ± 0.07	95.1 ± 15.9
0.15	+	71.6 ± 5.3	99.4 ± 7.9	1.43 ± 0.13	n.d.
0.6	−	49.8 ± 5	1.32 ± 0.38	0.095 ± 0.02	6.5 ± 5.6
0.6	+	65.3 ± 9.6	71.8 ± 6.9	0.52 ± 0.02	n.d.
0.8	−	31.4 ± 7.9	1.2 ± 0.9	0.058 ± 0.019	14 ± 3.4
0.8	+	57.5 ± 1.3	0.4 ± 0.3	0.22 ± 0.03	n.d.
1	−	8.6 ± 0.7	< 0.2	0.046 ± 0.01	n.d.
1	+	25.4 ± 1.4	< 0.2	0.091 ± 0.009	n.d.

[a] Cells were energized with 0.4% glycerol. n.d., not determined.

The activity of the different transport systems was systematically lower in cells induced for glycine betaine transport than in non-induced cells, probably due to the growth conditions necessary for induction, but the patterns of inhibition by NaCl or sucrose were very similar in the two cases (not shown). Glycine betaine had no protective effect in non-induced cells.

We studied the time-course of lactose accumulation at 150 mM and 600 mM NaCl. The time necessary to reach state was longer at high osmolarity (some 60 min vs. 15 min) but importantly, similar accumulation ratios were obtained in both cases.

Facilitated diffusion. The initial rate of facilitated diffusion of lactose through the *lac* permease (i.e., transport in the presence of an uncoupler which abolishes the protonmotive force) was also inhibited at increasing osmolarity. Nevertheless, the pattern of inhibition was different from that of active transport. Up to 600 mM NaCl, no inhibition was found. At 1 M NaCl inhibition was only 50% of the maximum rate.

In *E. coli*, glycerol is transported by passive diffusion through the lipids. It can also be transported by facilitated diffusion via the glycerol facilitator. The glycerol facilitator has been postulated to be a channel in view of its very high rate of transport [19]. Diffusion of glycerol via the facilitator is too rapid to be measured by conventional filtration techniques and glycerol is metabolized. We therefore followed the uptake of radioactive xylitol, another substrate of the glycerol facilitator, which is not metabolized and which has a much slower rate of permeation. The possibility cannot be excluded that xylitol is also transported by other transport systems. As in the procedure described in Ref. 19, we used a high concentration of xylitol (100 mM). A putative contribution of other transport system is then negligible, since transport via the glycerol facilitator is nonsaturable. Under these conditions, transport of xylitol was shown to be independent of temperature, further support for a channel-like nature for this transport system [19]. We checked that such was the case in ML 308-225: entry and equilibration of xylitol were identical at 25° C and 2° C (data not shown). Increasing concentrations of NaCl strongly inhibited the initial rate of xylitol transport (Table I).

Inhibition of growth by osmotic upshock is not due to energy limitations

Roth et al. [12] proposed that the arrest of growth observed upon osmotic upshock is due to the inhibition of the sugar transport systems. Although our study confirms that osmotic upshock inhibits sugar transport, the finding that neither the protonmotive force nor the ATP level were markedly modified, in vitro, by an increase in osmolarity of the medium, contradicts this proposal. This led us to investigate, directly during growth, the evolution of protein synthesis in parallel with the ATP content in cells subjected to an osmotic upshock. It was observed that protein synthesis stopped with minutes of NaCl addition, while the ATP content still continued to increase for some 20 min (Fig. 4).

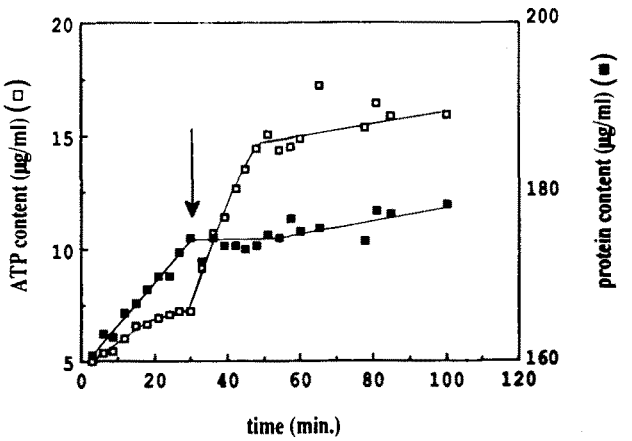

Fig. 4. Effect of osmotic upshock on protein synthesis and ATP content of *E. coli* ML 308-225 growing cells. Cells were grown at 37° C on minimal medium M9, with 0.4% glycerol as an energy source, and subjected to an osmotic upshock by addition of 0.65 M NaCl in the medium (arrow). At intervals, aliquots (400 μl) were removed and assayed for protein (■) and ATP content (□).

Discussion and Conclusion

The inhibitory effects on membrane functions which are reported in this study can be ascribed to an increase in the osmotic pressure across the cytoplasmic membrane: the permeant solute glycerol had little or no effect as compared to the impermeable solutes NaCl or sucrose, thus ruling out that a decrease in the activity of water could be the cause of the observed inhibitions. In addition, the fact that NaCl and sucrose have similar inhibitory effects, which can be relieved, at least partially, in both cases by the osmoprotectant glycine betaine, indicates that the effect of NaCl is not due to the increase in the ionic strength of the medium.

Plasmolysis of *E. coli* in moderately hyperosmotic medium has been reported, using the sucrose method [20,11], or by following the optical absorbance of the bacterial suspension [21]. Our measurements show that the cytoplasmic volume starts decreasing as soon as the osmolarity increases. That glycine betaine, added to cells which can transport it, induces a partial restoration of the cell volume, confirms one of the role attributed to this solute: its accumulation counterbalances the external osmotic pressure. Above 1 M NaCl glycine betaine has no protective effect. This could be explained by the limited capacity of the cells to accumulate glycine betaine. The efficiency of glycine betaine to reverse plasmolysis (and to restore respiration and transport) may depend on the composition of the external medium. In particular, it is probable that the potassium present

in the medium, even at this low concentration (1 mM), exerts also a protective effect at intermediate values of the osmolarity [3,6]. The presence of potassium could not be avoided, since respiration is severely impaired in the total absence of exogenous potassium [22].

We observed that respiration is strongly inhibited at high osmolarity. The fact that different substrates have a similar pattern of inhibition indicates that the electron transfer chain per se is inhibited. This conclusion is supported by the inhibition of the oxidation of ascorbate PMS, which has no specific carrier and is not metabolized but injects its electrons directly to the respiratory chain. An inhibition of respiration by high osmolarity has been reported in other bacterial species: *Azobacter vinelandii* [23] and the halotolerant bacterium Bal [24].

Despite this inhibition of respiration, we found that neither the membrane potential nor the internal pH is modified up to an external osmolarity of 1 M NaCl. Similarly, it was recently reported by Dinnbier et al. [11] that, under growth conditions, a moderate upshock (0.5 M NaCl) resulted only in a transient alkalinisation of the internal medium of *E. coli* cells and a transient decrease of $\Delta\Psi$ by about 25 mV. In principle, maintenance of the protonmotive force could be achieved by ATP hydrolysis via the ATPase. It is also possible that under high osmotic pressure, the permeability of the membrane to protons is lowered. It is also conceivable that a slow discharge of preformed Na^+ and K^+ gradients could contribute to maintain the protonmotive force as hypothetized by Skulachev [25].

Roth et al. [12] reported that addition of 0.8 M NaCl to a suspension of *E. coli* cells drastically inhibits three different types of transport system: the lactose permease and the melibiose permease, the maltose transport system and the glucose transport system. Our study confirms these results and shows that the glycerol transport system (facilitated diffusion) is also inhibited.

It has been proposed that these inhibitions can be attributed to conformational changes of the different carriers [12]. Another possibility, not taken into account by Roth et al. [12], is that inhibition of sugar transport is the consequence of a possible abolition of the energy sources involved in active transport. Our results allow us to exclude this second possibility. (i) Maltose transport and glucose transport are powered by chemical energy sources. Maltose transport is believed to be driven by ATP [26], the level of which is not markedly changed after osmotic shock. Phospho*enol*pyruvate, the energy source for glucose transport, was not measured in our experiments. Nevertheless, we checked that inhibition of α-MG transport is complete within 15 s of

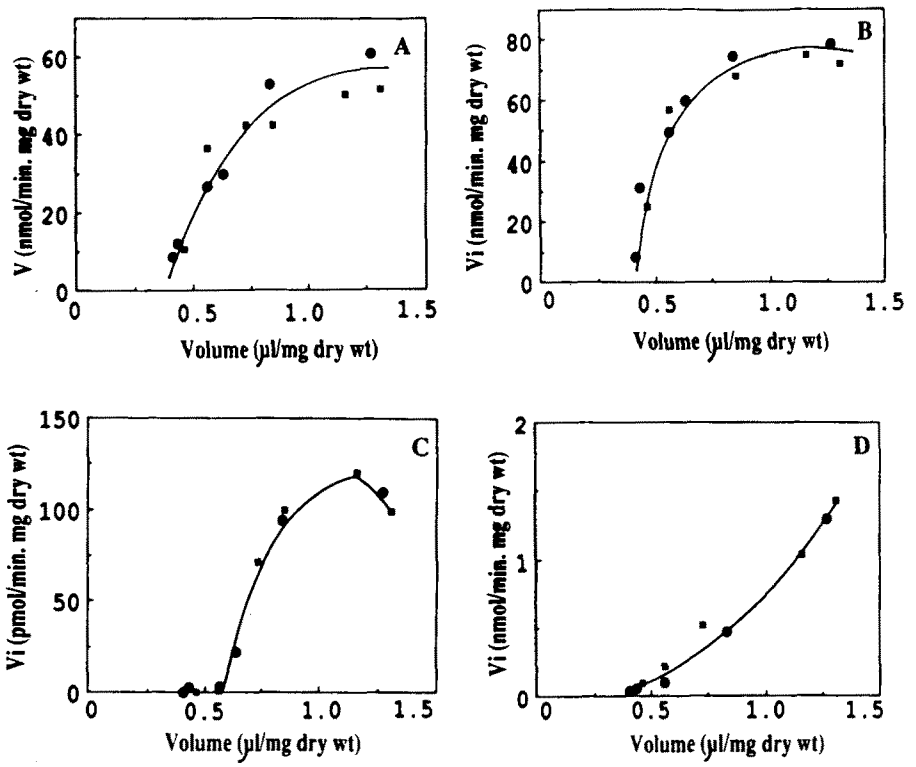

Fig. 5. Respiratory and transport activities as a function of cytoplasmic volume. All measurements were made on cells induced for glycine betaine transport, in the presence (■) or in the absence (●) of 1 mM glycine betaine. Data for cytoplasmic volume and respiration were taken from Figs. 1 and 2, respectively. Transport activities (i.e., initial rates of influx) were determined as described in Materials and Methods section, using NaCl as the external osmoticant. Each value of sugar transport represents the average of three different measurements. (Some of these data are given in Table I.) (A) respiration; (B) lactose transport; (C) α-MG transport; (D) maltose transport.

the addition of 1 M NaCl (data not shown); in such a short time depletion of phospho*enol*pyruvate cannot occur. (ii) The protonmotive force, which is the driving force for lactose transport, is not affected. (iii) The transport of xylitol does not involve energy. In consequence the inhibitory effects that we observed are best explained in terms of conformational changes of the different membrane proteins induced by the deformation of the membrane.

This hypothesis is strengthened by the following observation: for each membrane function which is inhibited, when the activity is plotted against the cytoplasmic volume, for cells incubated in the presence of glycine betaine (which partially reverse both the inhibition and the decrease in cytoplasmic volume) and for cells incubated in its absence, one obtains a unique relationship (Fig. 5). In all cases, the activity increases with cytoplasmic volume. In the case of respiration, lactose transport and glucose transport, the activity levels off for a volume of some 1 μl/mg dry weight and then becomes more or less independent of the volume, while no such maximum of activity is observed in the case of maltose transport. Thus, in a certain range of cytoplasmic volume, the activity is solely determined by the cytoplasmic volume and hence by the stress on the membrane which dictates this volume. In addition, this observation indicates that the protective effect of glycine betaine results from its ability to counterbalance the external osmotic pressure, rather than from a direct interaction with the proteins [8]. This conclusion calls for several remarks.

Maltose and glucose transport systems include non-membrane components. It is unlikely that the periplasmic maltose binding proteins could be inhibited by both sucrose and NaCl, but the possibility cannot be excluded that cytoplasmic components of the glucose PTS are sensitive to an increase in the osmolarity of the medium.

The difference in sensitivity between active transport and facilitated diffusion of lactose is difficult to explain in view of the consensus which states that both modes of transport are catalyzed by the same site and occur in symport with proton. We previously proposed [27], on the basis of kinetic observations, that active transport and facilitated diffusion of lactose are catalyzed by different sites and that facilitated diffusion could occur without proton. This hypothesis, which could account for the above results, nevertheless has to be proved.

While all the membrane activities which we have studied are inhibited by osmotic pressure, the sensitivity and the pattern of inhibition are clearly different in each case as is apparent from Fig. 6. Chavez et al. [28] carried out a similar study in mitochondria and concluded that only the ATP/ADP translocase was inhibited by increasing sucrose concentration. Since the higher sucrose concentration in this study is 0.5 M, it could be that other systems are inhibited at higher osmotic pressure.

Though osmotic pressure across the membrane is seen here to decrease the activities of some membrane systems, it can activate others, such as the transport systems for potassium [3], glycine betaine [29], or ionic channels [30–32], also undoubtedly via conformational changes.

Transport systems of the carrier type are inhibited by both low temperature and increased osmotic pressure. In contrast, channel-like systems as the glycerol facilitator are only inhibited by increased osmotic pressure: this emphasizes the differences in the state of membrane induced at low temperature or high pressure.

Roth et al. [13] observed a partial recovery of growth and glucose transport over 2 h in growing cells subjected to a moderate osmotic upshock (0.6 M NaCl). Under our conditions (in vitro measurements in the presence of chloramphenicol) and for the same NaCl concentration, inhibition of α-MG transport was nearly complete within 15 s of osmoticant addition and remained unchanged for 2.5 h. A possible explanation for this discrepancy is that recovery of transport (and growth) requires protein synthesis.

When growing cells were subjected to an osmotic upshock, protein synthesis stopped immediately whereas the ATP content was observed to increase for another 20 minutes. A similar rise in the ATP content upon osmotic shock has been reported previously by Ohwada and Sagisaka [33]. The reason for the continued rise in the ATP level of the cells is unclear. A possible explanation could be that, after growth inhibition, the energy requirements are reduced, while ATP is still synthesized. We could not test, in this study, whether the ATPase/ATPsynthase is inhibited by increased osmotic pressure, but in all cases ATP can still be synthesized by substrate level phosphorylation. Whatever the explanation, this observation demonstrates that inhibition of growth by osmotic upshock is not due to energy limitations. In Ref. 12 it was proposed that inhibition of nutrient uptake induced by osmotic upshock could be the cause of the inhibition of growth, via a shortage of energy supply of the cells. These results demonstrate that, in spite of the drastic effect of increased osmotic pressure on membrane functions, the arrest of growth cannot be the result of carbohydrate transport inhibition. In consequence, an explanation of growth inhibition upon osmotic upshock has to be sought elsewhere.

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