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OSMOTIC STRESS DRASTICALLY INHIBITS ACTIVE TRANSPORT OF CARBOHYDRATES BY *Escherichia coli*

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SUMMARY. In intact *Escherichia coli* cells, severe osmotic stress almost totally inhibited active transport of carbohydrate by all of the systems known to transport carbohydrates in *E. coli*: group translocation (glucose), binding-protein mediated transport (maltose), proton symport (lactose), and sodium co-transport (melibiose). Detailed study of glucose transport showed that this inhibition of transport was not secondary to the inhibition of growth by osmotic stress, but rather that the inhibition of transport of a source of carbon and energy was sufficient to cause the complete inhibition of growth observed during severe osmotic upshock. Transport and growth inhibition did not result from cell death; upshocked cells were viable and metabolically active.

"Osmotic upshock" (1), produced by an increase in the osmolarity of the environment, is a stress that inhibits the growth of cells of animals (2), plants (3), and microrganisms (4). Studies of *Escherichia coli* have played a key role in uncovering numerous metabolic responses of the cell to osmotic stress (reviewed in reference 5). Despite this intensive study of *E. coli*, a metabolic defect that can account for the inhibition of growth has not been identified. In this study, we identify a metabolic defect effected by osmotic stress that is sufficient to cause the observed growth inhibition.

METHODS. *E. coli* CA8000 (thi) (6), a K-12 derivative, was kindly provided by Dr. J. Beckwith (Harvard Medical School). The organism was grown aerobically at 32°C in the minimal medium previously described (7). This medium is composed of a solution that contains the salts essential for growth, henceforth referred to as the essential salt solution (165 mOsmol/l), to which was added thiamine (60 μM, final concentration), the source of nitrogen (11.2 mM NH_4Cl, final concentration), and a carbon source. The final concentration of the carbon source in the medium was either 11 mM glucose (reduced to 3.3 or 5.6 mM in experiments to measure glucose utilization), or 5.6 mM maltose, lactose, or melibiose. For growth on melibiose, NaCl was added to the essential salt solution to increase the Na^+ concentration to 10.7 mM in the medium in order to maximally stimulate melibiose transport. Cultures (1 liter) were inoculated with 1.0 ml of cell suspension in Trypticase Soy Broth (Baltimore Biological Laboratories) on the day before each experiment and were grown overnight (17

The abbreviations used are: ppGpp, guanosine 5'-diphosphate 3'-diphosphate; pppGpp, guanosine 5'-triphosphate 3'-diphosphate.

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to 19 hours). Growth of the overnight cultures was followed by measuring the optical density at 450 nm (1-cm light path) as previously described (7).

Protein, glucose, and glycogen were determined and rates of glucose utilization were calculated as described previously (8-10).

Uptake assays were performed in the absence of both a nitrogen source and a metabolizable source of carbon and energy (except for the 0.65 μM [^{14}C]-maltose in the maltose uptake assay). The radioactive subtrates and their specific activities used in the assays were: for the glucose phosphotransferase system, α-methylglucoside (methyl α-D-[U-^{14}C]glucopyranoside, New England Nuclear, 275 mCi/mmol); for the maltose transport system, [U-^{14}C]maltose (Amersham, 604 mCi/mmol); and for both the lactose and melibiose transport systems, methyl-β-thiogalactoside ([methyl-^{14}C]-β-D-thiogalactopyranoside, New England Nuclear, 54.7 mCi/mmol).

For each assay the uptake of substrate was linear over the 3-minute period studied. Samples were collected at 15-second intervals and the rate of uptake and the correlation coefficient (r) were obtained by linear regression analysis of the 12 values of the amount of substrate taken up verus time. Correlation coefficients greater than 0.97 were obtained in all cases except when the rates of uptake were very low (less than about 100 pmol/mg of protein/min), and in these cases values of about 0.90 were usually obtained.

For viable cell counts, cells were suitably diluted with fully supplemented minimal medium and 10 separate portions of the dilution were collected by filtration into separate Bacteriological Monitors (Millipore). After incubation, 50 to 70 colonies per monitor were obtained.

E. coli CA8000 could not metabolize sucrose. We inoculated cells in the minimal medium described above with 20 mM sucrose as the sole source of carbon and energy; even after 72 hours no growth had occurred. Thus, E. coli CA8000 is apparently free of plasmids that allow some E. coli strains to take-up and to metabolize sucrose (11).

RESULTS. We tested the effect of osmotic upshock on four carbohydrate transport systems. These active transport systems represent each of the four types of carbohydrate transport known to occur in E. coli (12): group translocation (glucose), binding-protein mediated transport (maltose), proton symport (lactose), and sodium cotransport (melibiose). Osmotic upshock almost totally inhibited carbohydrate transport by each of these systems (Table 1). The rapidity of this transport inhibition by a hypertonic concentration of an impermeant solute, such as NaCl or sucrose (13,14), is shown for the glucose transport system (Fig. 1). Within 15 seconds after upshock with either 0.8 M NaCl or 0.8 M sucrose, the α-methylglucoside uptake rate decreased drastically. These decreased rates were maintained for at least 3 minutes (Fig. 1).

The severe inhibition of carbohydrate transport by 0.8 M NaCl or 0.8 M sucrose was not the result of cell death. For example, although inhibition of transport occurred within 15 seconds of upshock with 0.8 M NaCl (Fig. 1), the viable cell count 15 minutes after upshock was 12 (± 2) X 10^7 cells/ml (mean

Table 1. Effect of osmotic upshock on active transport of carbohydrates

Transport system	Mechanism of transport	Assay substrate	Protein (μg/ml)	Addition	Rate of uptake (pmol/mg of protein/min)	Inhibition (%)
Glucose phospho-transferase	Group translocation	α-methylglucoside, 1.33 μM	50	None	1030	—
				0.8 M NaCl	60	94
Maltose	Binding-protein mediated	Maltose, 0.65 μM	2	None	9640	—
				0.8 M NaCl	214	98
Lactose	Proton symport	Methyl-β-thio-galactoside, 27.2 μM	240	None	472	—
				0.8 M NaCl	34	93
Melibiose plus lactose[a]	Sodium cotrans-port (melibiose) and proton symport (lactose)	Methyl-β-thio-galactoside, 27.2 μM	90	None	3330	—
				0.8 M NaCl	128	96

E. coli CA8000 was grown overnight on glucose, maltose, lactose, or melibiose, as described in Methods. When an exponentially growing overnight culture reached an optical density of about 0.4, a portion was removed and the cells were collected on a nitrocellulose filter (0.45 μm pore size), washed with 2 volumes of room-temperature (24°C) essential salt solution (described in Methods), resuspended in essential salt solution, and the suspension was chilled on ice. When an experiment was begun, 1 volume of cell suspension was brought to room temperature and was then further diluted with 1.6 volumes of room temperature essential salt solution or essential salt solution containing the amount of upshock agent, NaCl in these experiments, to give a final concentration of 0.8 M. After 15 minutes of room-temperature incubation, the uptake assays, performed at room temperature, were initiated by mixing 0.01 volume (or less) of radioactive substrate with 1 volume of the control or upshocked cell suspension; the final concentrations of cellular protein and of the radioactive substrates are listed below. For each uptake assay, samples were collected, filtered and counted as described in the legend to Fig. 1; rates of uptake were calculated as described in Methods.

[a]Methyl-β-thiogalactoside is transported by both the melibiose and lactose transport systems; growth on melibiose induces both of these transport systems (12). (Growth on lactose induces only the lactose transport system.)

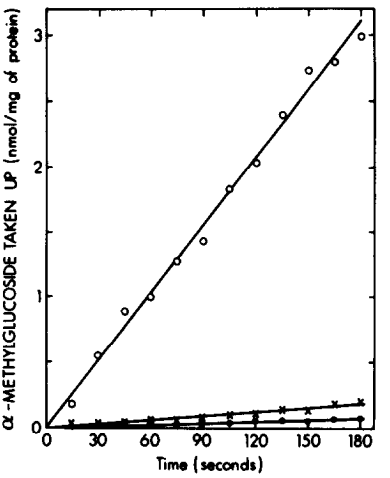


Fig. 1. Effect of osmotic upshock on the initial rate of α-methylglucoside uptake: control (no upshock) (o), 0.8 M NaCl (x), 0.8 M sucrose (•). E. coli CA8000 was grown on glucose and the cells were collected and resuspended as described in the legend of Table 1 except that in this experiment, upshock and initiation of the uptake assays occurred simultaneously. Cell suspensions were brought to room temperature and at zero time, 1 volume of cell suspension was added to 1.6 volumes of essential salt solution that contained α-methyl-[^{14}C]glucoside without or with either NaCl or sucrose. Concentrations of cellular protein and α-methylglucoside in the uptake assays were the same as those shown in Table 1. For each uptake assay, samples were collected on nitrocellulose filters (0.45 μm pore) at 15-sec intervals over a period of 3 min. Each filter was washed immediately at room temperature with 5 ml of the particular isosmotic incubation medium that lacked the labelled substrate. After collection of all 12 of the samples, each filter was washed again, placed in 10 ml of Instagel scintillation fluid (Packard), and allowed to stand for 1.5 h before counting in order to maximally solubilize the labelled material.

± standard deviation) compared to 15 (± 2) X 10^7 before upshock.

The data in Table 1 suggest that in upshocked cells the inhibition of the transport of a source of carbon and energy is sufficient to cause the inhibition of growth by osmotic stress. This concept is supported by our detailed study, presented below, of the inhibition of the transport of glucose, the preferred carbon source (12).

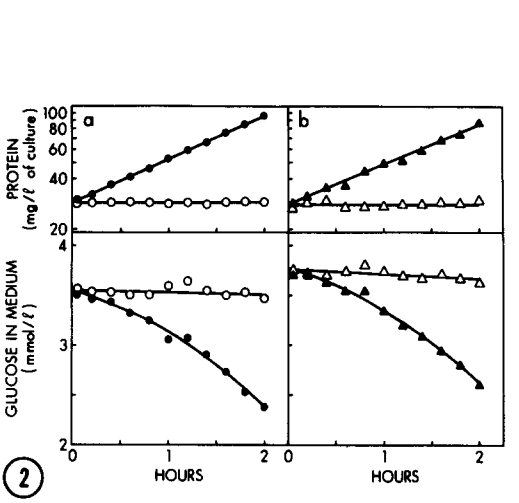
The question of whether the inhibition of glucose transport can account for the inhibition of growth by osmotic stress cannot be answered by studying uptake of the non-metabolizable glucose analogue, α-methylglucoside. Such uptake assays must be performed in the absence of a metabolizable source of carbon and energy and under such conditions the cells cannot grow. However, because of the unique way that glucose is transported in E. coli (by group

translocation) we were able to accurately measure glucose transport in growing cells by an alternative procedure.

In E. coli, extracellular glucose is phosphorylated enzymatically during transport across the cell membrane and is released inside the cell only as the impermeant derivative glucose 6-phosphate (12). Therefore, in E. coli the rate of glucose transport is equal to the rate of glucose utilization, which is the rate of disappearance of the substrate of the transport reaction from the culture medium. When we exposed exponentially growing E. coli cultures to 0.8 M NaCl or 0.8 M sucrose, growth (as assessed by measurement of protein in the culture) and glucose utilization stopped abruptly (Fig. 2). This cessation of glucose utilization by the upshocked cultures shows that they had ceased transporting glucose. The cessation of glucose transport caused by 0.8 M NaCl or 0.8 M sucrose was not the result of cell death. For example, the viable cell count 2 hours after upshock with sucrose was 8 (± 1) X 10^7 cells/ml (mean ± standard deviation) compared to 9 (± 1) X 10^7 before upshock.

Glucose utilization also stops when a nitrogen-starved stationary-phase culture is exposed to 0.8 M NaCl (Fig. 3). The uptake of α-methylglucoside by nitrogen-starved cells is also severely inhibited by osmotic upshock (Table 1, Fig. 1). Thus, glucose transport, whether measured by α-methylglucoside uptake (Fig. 1) or by glucose utilization (Fig. 3), can be inhibited in non-growing cells. These observations unequivocally demonstrate that the inhibition of glucose transport in growing cells is not secondary to the inhibition of growth by osmotic stress.

Although the inhibition of growth could not account for the inhibition of glucose transport, the inhibition of glucose transport was sufficient to account for the inhibition of growth. Inhibition of glucose transport occurred with sufficient rapidity (within 15 seconds after upshock (Fig. 1)) and was of a sufficient magnitude (Table 1, Figs. 1 to 3) to account for the abrupt cessation of growth that followed osmotic upshock (Fig. 2). In addition, osmotically upshocked cells show a pattern of metabolic activity that is



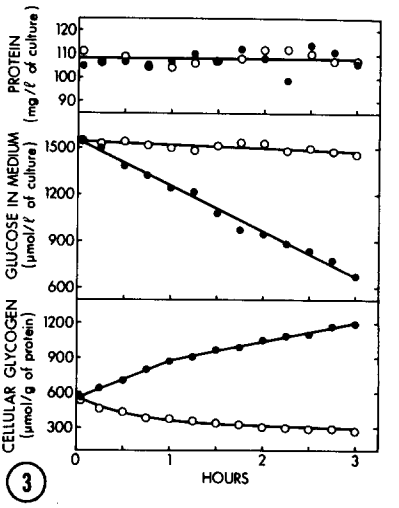


Fig. 2. Effect of osmotic upshock on growth and glucose utilization of exponentially growing E. coli CA8000: a) no upshock agent (●), 0.8 M NaCl (○); b) no upshock agent (▲), 0.8 M sucrose (△). Exponentially growing overnight cultures that had reached an optical density of about 0.4, were diluted 2-fold with pre-warmed (32°C) minimal medium containing thiamine, 5.6 mM glucose, and 11.2 mM NH_4Cl or with an otherwise identical medium that contained the amount of upshock agent necessary to give a final concentration of 0.8 M. The protein and glucose contents of the cultures were determined and rates of glucose utilization were calculated as described in Methods. Rates of glucose utilization in the untreated control cultures were a) 10,500 and b) 11,400 μmol/g of protein/h, essentially equal to the rates previously observed in other E. coli strains during exponential growth (10).

Fig. 3. Effect of osmotic upshock on nongrowing E. coli CA8000: protein content of the culture, glucose utilization, cellular glycogen; no upshock agent (●), 0.8 M NaCl (○). An overnight culture containing a growth-limiting amount of the nitrogen source (2.2 mM NH_4Cl) was allowed to enter the nitrogen-starved stationary phase (occurring at an optical density of about 1.1). One half hour after growth stopped, portions of the culture were diluted 1.2-fold as described in the legend of Fig. 2, except that the diluents contained no NH_4Cl. The protein, glucose, and glycogen contents of the cultures were determined and the rates of glucose utilization were calculated as described in Methods. The rate of glucose utilization in the nitrogen-starved control culture was 2700 μmol/g of protein/h, essentially the same as rates previously observed in nitroten-starved E. coli cultures (15).

characteristic of glucose starvation. An upshocked, nitrogen-starved culture degraded glycogen rather than storing it; storage is the normal response to nitrogen-starvation when an exogenous source of carbon and energy is available (Fig. 3). An even more striking degradation occurred when a culture growing on glucose was upshocked with hypertonic NaCl: cellular glycogen stores fell from 130 to less than 45 μmol/g of protein during the first 20-minute period after upshock. In addition, Harshman and Yamazaki (16) have found that osmotic upshock causes a rapid increase in the cellular level of ppGpp (generated

from ATP (17)), but not pppGpp; this pattern of change is characteristic of glucose starvation (17).

In summary, the extreme inhibition of glucose transport by osmotic upshock is not the consequence of the inhibition of growth, the inhibition of transport is sufficient to account for the inhibition of growth, and the inhibition of transport and of growth is not the consequence of cell death; the upshocked cells are viable and metabolically active.

DISCUSSION. A significant but subsidiary finding of our study is the first demonstration that glucose utilization and α-methylglucoside uptake are equivalent measures of glucose transport.[1] The major finding of the study presented here is the identification of the inhibition of the transport of a source of carbon and energy as a sufficient cause for the inhibition of growth by osmotic upshock. In the succeeding accompanying report we show that reversal of this transport inhibition plays an important role in the adaptive response of the cell to reverse the inhibition of growth owing to osmotic stress; we also provide experimental evidence for an initial working hypothesis to account at the molecular level for the inhibition of transport and its reversal.

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[1] This equivalency is shown by the close agreement of the value of the rate of glucose utilization we observed in a nitrogen-starved E. coli culture (Fig. 3) and the rate of α-methylglucoside uptake we observed with untreated cells in the absence of a nitrogen source (Table 1), when the uptake rate is corrected for the lower temperature and the subsaturating substrate concentration used in the uptake assays. The initial rate of α-methylglucoside uptake was about 1000 pmol/mg of protein/min (Table 1), which is equal to 60 μmol/g of protein/h, and the K_m of the glucose transport system for α-methylglucoside is about 30 μM (18). Thus, the maximum rate of α-methylglucoside uptake is 60[1 + (30/1.33)], or approximately 1400 μmol/g of protein/h. Multiplying by a factor of 2 (reference 19) to correct for the difference in temperature used for α-methylglucoside uptake (24°C) and glucose utilization (32°C) experiments, one obtains a value of 2800 μmol/g of protein/h, essentially identical to the rate of 2700 μmol/g of protein/h observed for glucose utilization in a nitrogen-starved E. coli culture at 32° (Fig. 3). (The rate of glucose utilization was measured under saturating conditions, because the K_m of the glucose transport system for glucose is approximately 50 μM (20) and the concentration of glucose in the culture exceeded that value by at least 10-fold.)

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