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Glycine Betaine Transport in *Escherichia coli*: Osmotic Modulation

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Exogenous glycine betaine highly stimulates the growth rate of various members of the *Enterobacteriaceae*, including *Escherichia coli*, in media with high salt concentrations (D. Le Rudulier and L. Bouillard, Appl. Environ. Microbiol. 46:152–159, 1983). In a nitrogen- and carbon-free medium, glycine betaine did not support the growth of *E. coli* either on low-salt or high-salt media. This molecule was taken up by the cells but was not catabolized. High levels of glycine betaine transport occurred when the cells were grown in media of elevated osmotic strength, whereas relatively low activity was found when the cells were grown in minimal medium. A variety of electrolytes, such as NaCl, KCl, NaH_2PO_4, K_2HPO_4, K_2SO_4, and nonelectrolytes like sucrose, raffinose, and inositol triggered the uptake of glycine betaine. Furthermore, in cells subjected to a sudden osmotic upshock, glycine betaine uptake showed a sixfold stimulation 30 min after the addition of NaCl. Part of this stimulation might be a consequence of protein synthesis. The transport of glycine betaine was energy dependent and occurred against a concentration gradient. 2,4-Dinitrophenol almost totally abolished the glycine betaine uptake. Azide and arsenate exerted only a small inhibition. In addition, *N,N'*-dicyclohexylcarbodiimide had a very low inhibitory effect at 1 mM. These results indicated that glycine betaine transport is driven by the electrochemical proton gradient. The kinetics of glycine betaine entry followed the Michaelis-Menten relationship, yielding a K_m of 35 μM and a V_{max} of 42 nmol min^{-1} mg of $protein^{-1}$. Glycine betaine transport showed considerable structural specificity. The only potent competitor was proline betaine when added to the assay mixtures at 20-fold the glycine betaine concentration. From these results, it is proposed that *E. coli* possesses an active and specific glycine betaine transport system which is regulated by the osmotic strength of the growth medium.

Many organisms have evolved sophisticated mechanisms to live in a world deficient in available water. Several members of the *Enterobacteriaceae* exposed to increased osmolarity in their growth media are able to avoid dehydration by taking up molecules which act as osmotic balancing agents. For example, in *Salmonella oranienburg*, exogenous L-proline stimulates growth rates in media of inhibitory osmotic strength (4), and similar enhancement has been demonstrated in *Salmonella typhimurium* (7), *Escherichia coli* (9), and *Klebsiella pneumoniae* (25). In addition, we have recently reported that glycine betaine, the trimethylated derivative of glycine, is a much better osmoprotectant compound than proline (22–24). However, although the transport systems for L-proline are well understood in *S. typhimurium* (2, 8, 31) and *E. coli* (17, 28), there is no information available concerning the transport of glycine betaine in bacteria.

The current study was undertaken to characterize the glycine betaine transport system of *E. coli*. To demonstrate the existence of this system, we showed cytoplasmic accumulation of the unaltered substrate, specific inactivation of transport by metabolic inhibitors, saturation kinetics, and substrate specificity. Modulation of glycine betaine transport by osmotic strength was also reported. The present paper reports evidence that glycine betaine is a molecule which plays an important role in osmoregulation in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture media. *E. coli* K-10 was generously supplied by R. C. Valentine, University of California, Davis. Strain C600 (Δ*trp leu lacY hsdM hsdR recA rif*) was obtained from J. Collins, G.B.S., Braunschweig, Stöcheim. The rich medium used was LB medium (10). The inoculum culture was grown aerobically overnight in LB medium at 37°C and used at a concentration of 1%. The minimal medium used was medium 63 (5) consisting of 100 mM KH_2PO_4, 75 mM KOH, 15 mM, $(NH_4)_2SO_4$, 0.16 mM $MgSO_4$, and 3.9 μM $FeSO_4$. The carbon source was 10 mM D-glucose. Media of elevated osmotic strengths were attained by adding NaCl, other electrolytes, or nonelectrolytes at concentrations indicated. When necessary, the pH was readjusted to that of the minimal medium (pH 7.2). The osmotic pressure of the different media was measured by freezing point depression by using a microosmometer (model H. Roebling; Bioblock Scientific, Illkirch, France). Readings in milliosmoles per kilogram of water were converted into bars. The minimal medium had an osmotic pressure of 7.4 bars. When 0.15, 0.3, 0.5, 0.65, 0.8, or 1 M NaCl was added to this medium, the osmotic pressure was 14.5, 20.4, 29.4, 36.2, 43.0, and 52.9 bars, respectively. Solutions of glycine betaine were sterilized by filtration. When glycine betaine was used as the sole nitrogen source, the $(NH_4)_2SO_4$ in the medium 63 was replaced with 15 mM K_2SO_4, and glycine betaine was added at 10 mM.

The cells were grown at 37°C with shaking at 200 rpm. Growth was monitored turbidimetrically with a spectrophotometer (model 24; Beckman Instruments, Inc., Fullerton, Calif.) at 420 nm after suitable dilution with the growth medium. An absorbancy at 420 nm (A_{420}) of 1.0 was equivalent to a cell density of 0.125 mg of protein ml^{-1}. A correction factor was applied when the cells were grown in the presence of high concentrations of solutes.

Measurement of intracellular volume. Cell volumes were determined by the technique of Stock et al. (35) using [*carboxyl*-^{14}C]dextran (0.2 mg ml of cell suspension^{-1}; 0.5

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μCi mg^{-1}) and ^3H_2O (1 μCi ml^{-1}) as radioactive markers. Radioactivities from the supernatant and from the cell pellet were determined in a liquid scintillation spectrometer (model 33-80; Packard Instrument Co., Rockville, Md.). The dry weight of bacteria was determined after drying to constant weight at 65°C in a vacuum oven, ashing for 12 h at 550°C, and correcting for the weight of salt in the ashed material.

Preparation of [^{14}C]glycine betaine. [*methyl*-^{14}C]glycine betaine and [1,2-^{14}C]glycine betaine were prepared from [*methyl*-^{14}C]choline and [1,2-^{14}C]choline, respectively, as described by Ikuta et al. (15). [^{14}C]choline was enzymatically oxidized by choline oxidase from *Alcaligenes* sp. To isolate [^{14}C]glycine betaine, the reaction mixture was subjected to high-voltage electrophoresis for 90 min at 40 V cm^{-1}, with Whatman 3MM paper previously moistened in 0.75 N formic acid (pH 2.0). The [^{14}C]glycine betaine was completely eluted with 0.01 N HCl which was then removed by rotary evaporation. Radioactive glycine betaine thus obtained was pure as judged by coelectrophoresis and cochromatography with pure glycine betaine. Samples were run in two different systems of paper electrophoresis (0.75 N formic acid [pH 2.0] and pyridine-acetic acid-water [10:4:986] [pH 5.3]) and paper chromatography (*n*-butanol-acetic acid-water [12:3:5] and ethanol-water-ammonia [20:4:1]). Fractions containing radioactive glycine betaine were sterilized by filtration and frozen until needed.

Transport assays. When the culture density reached an A_{420} of 0.4 to 0.6, the cells were harvested by centrifugation (8,000 × *g*, 10 min, 10°C), washed twice with the growth medium, and suspended to a concentration of ca. 0.1 mg of cell protein ml^{-1} in the same solution. Individual uptake experiments were performed aerobically (200 rpm) at 37°C in prewarmed glass tubes (1.5 by 11.0 cm) containing 25 μl of [^{14}C]glycine betaine (63,800 dpm per assay) and 25 μl of nonradioactive glycine betaine to produce the appropriate specific radioactivity. Organisms and components of the incubation mixture were thermoequilibrated at 37°C. Transport was initiated by adding 1 ml of cell suspension. The suspension was mixed, and the reaction was terminated by rapid filtration through membrane filters (type HA, 0.45-μm pore size, 25-mm diameter; Millipore Corp., Bedford, Mass.). The cells were then quickly washed twice at room temperature (20°C) with 1 ml of the corresponding culture medium containing 50 mM $MgCl_2$ and 0.5 M sucrose (27). Under these conditions, no loss of internal labeled substrate was observed. The filters were solubilized in scintillation vials containing 6 ml of ACS liquid (Amersham Corp., Amersham, England), and radioactivities were determined ca. 24 h later in a Packard liquid scintillation spectrometer. In some cases, the reactions was terminated by centrifugation of cells. Cell pellets were counted by liquid scintillation. Values for glycine betaine uptake agreed within 10% for the two methods.

In all experiments, the total concentration of glycine betaine in the transport assay solution was adjusted so that no more than 10% of the substrate was taken up during the course of the reaction. In inhibitory assays, the cells were preincubated for 30 min at 37°C with the inhibitor before the addition of [^{14}C]glycine betaine. When chloramphenicol was used, the preincubation time wa 1 h. In competition exper-

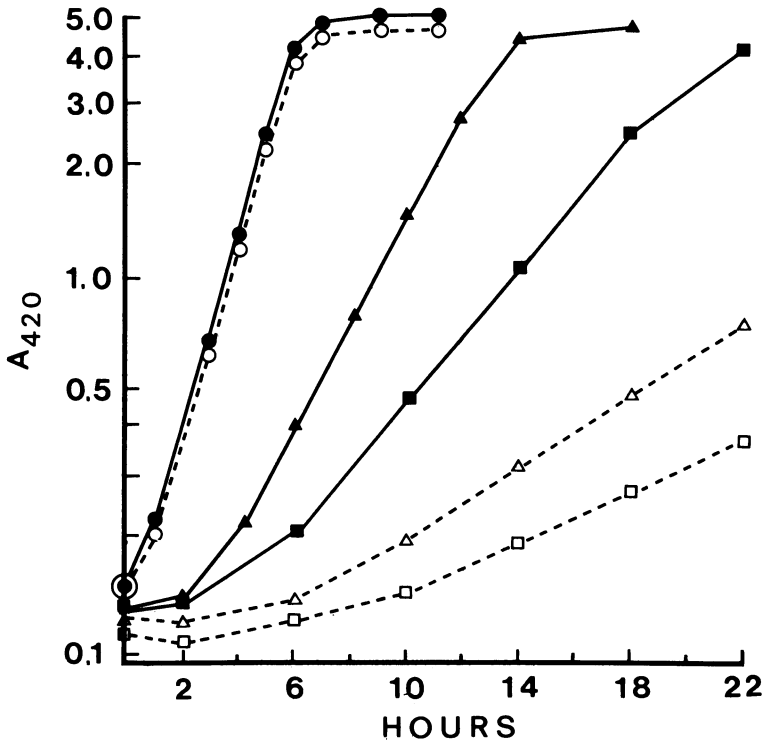

FIG. 1. Effect of exogenous glycine betaine on the growth of *E. coli* K-10 in media of various concentrations of NaCl. The cells were grown aerobically at 37°C in the medium 63 described in the text. Cell growth was estimated turbidimetrically. A_{420} values are plotted as a function of incubation time. The cells were grown in the presence of 1 mM glycine betaine (solid symbols) or in the absence of glycine betaine (open symbols). Glycine betaine was added to the media at the concentration which caused maximal stimulation. Symbols: ● and ○, minimal medium; ▲ and △, minimal medium plus 0.65 M NaCl; ■ and □, minimal medium plus 0.8 M NaCl.

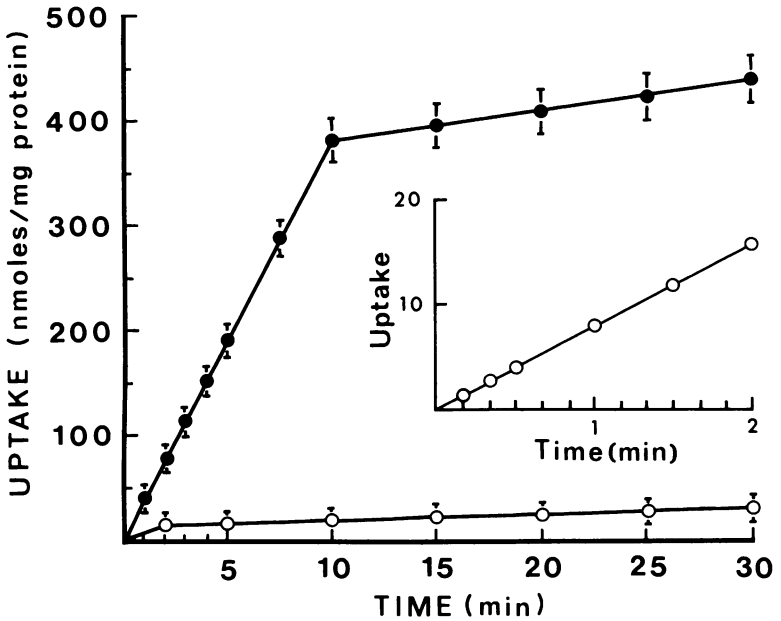

FIG. 2. Time course of glycine betaine uptake by *E. coli* K-10. Cells (0.1 mg of cell protein ml^{-1}) were incubated at 37°C with [*methyl*-^{14}C]glycine betaine (250 μM) as described in the text. Reactions were terminated by filtration at the times shown. Values for glycine betaine uptake are expressed as nanomoles per milligram of cell protein. The points shown are the mean values of duplicate assays from three different experiments. Vertical bars indicate the ±SD. The inset shows glycine betaine uptake during the first 2 min. Symbols: ○, cells grown in minimal medium; ●, cells grown in minimal medium plus 0.5 M NaCl.

iments, cells were added to a mixture of labeled substrate and unlabeled analogs. The competitors were prepared in the transport medium and, when necessary, adjusted to the desired pH by addition of NaOH or HCl.

The initial rates of uptake were calculated from the amount of radioactive glycine betaine accumulated by the cells in 1 min. During this period, uptake was linear with time. Unless otherwise indicated, all data are mean values derived from duplicate replications from at least three experiments, and results agreed within less than 10%. All results were expressed as nanomoles per milligram of cell protein as determined by the method of Lowry et al. (26).

Chemicals. [*carboxyl*-^{14}C]dextran (0.5 mCi g^{-1}) was obtained from New England Nuclear Corp., Boston, Mass. [*methyl*-^{14}C]choline (58 mCi $mmol^{-1}$) was obtained from the Radiochemical Centre, Amersham, England, [1,2-^{14}C]choline (45 mCi $mmol^{-1}$) and ^3H_2O (10 mCi ml^{-1}) were obtained from the C.E.A., Gif-sur-Yvette, France. Choline oxidase (EC 1.1.3.17), catalase (EC 1.11.1.6), 2,4-dinitrophenol (DNP), sodium azide, chloramphenicol, glycine betaine, glycine betaine aldehyde, sarcosine, choline, trigonelline, and all amino acids were purchased from Sigma Chemical Co., St. Louis, Mo. Potassium cyanide, sodium arsenate, sodium arsenite, all sugars, and *N*,*N*'-dicyclohexylcarbodiimide (DCCD) were obtained from E. Merck AG, Darmstadt, Germany. The latter compound was prepared as methanolic solutions, and appropriate controls were run to account for any effects of methanol alone. Dimethylglycine and *N*-ethylmaleimide were obtained from Fluka AG, Buchs, Switzerland. Homarine was kindly provided by R. G. Wyn Jones, University College North Wales, Bangor Gwynedd, Wales. β-Alanine betaine (homobetaine) was a gift of F. Larher, University of Nancy, France. Proline betaine (stachydrine) and *N*-methylproline were synthesized from the sodium salt of proline, and pipecolate betaine (homostachydrine) was prepared from pipecolic acid by the method of Corti (6) modified by Ikutani (16). γ-Butyrobetaine (γ-trimethylaminobutyrate) was synthesized as described by Anderson et al. (1). All other reagents used were of analytical grade.

RESULTS

Growth of *E. coli* in the presence of glycine betaine. Increasing the salt concentration of the minimal medium reduced the growth rate and the growth yield. NaCl at a concentration of 0.65 M decreased the growth rate of *E. coli* K-10 from 0.96 generation h^{-1} in the minimal medium to 0.14 generation h^{-1}. The addition of glycine betaine alleviated the inhibitory effects (Fig. 1), and maximal stimulation was obtained with a glycine betaine concentration as low as 1 mM (22). Similar stimulatory effects were also observed in the presence of other solutes, such as KCl, K_2SO_4, K_2HPO_4, NaH_2PO_4, and sucrose (data not shown), added at a concentration osmotically equivalent to 0.65 M NaCl. In a nitrogen- and carbon-free minimal medium, addition of glycine betaine did not support growth. Incorporation of glucose into this medium also failed to promote growth.

Evidence for active transport of glycine betaine. The time course of glycine betaine uptake in low-salt-grown and high-salt-grown *E. coli* K-10 cells is shown in Fig. 2. Cells grown in minimal medium showed a small glycine betaine transport activity. The uptake was linear during only 2 min with an initial rate of 7 nmol min^{-1} mg of $protein^{-1}$. After 30 min, a level of 35 nmol mg of $protein^{-1}$ was achieved. The internal water volume of *E. coli*, determined from the distribution of ^3H_2O and [^{14}C]dextran, was 3.65 ± 0.26 μl mg of $protein^{-1}$ (mean ± standard error; six separate measurements). Similar values have been obtained previously for *E. coli* and *S. typhimurium* grown in medium 63 (35). Assuming that glycine betaine is present in solution in the intracellular fluid, we estimated the intracellular concentration of this solute to be 9.5 mM, a concentration gradient of 1:38 at an external concentration of 0.25 mM. Since the uptake of glycine betaine by cells grown in minimal medium was

almost totally abolished (95% inhibition) by DNP (5 mM), uptake was not due to passive diffusion. When the cells were grown in high-salt medium (0.5 M NaCl), a strong uptake was noted. The initial rate of uptake by whole cells was directly proportional to cell concentration (data not shown). This rate was constant during ca. 10 min with a maximal velocity of 42 nmol min^{-1} mg of $protein^{-1}$. Glycine betaine was taken up by the cells in a biphasic manner; the initial rapid rate was followed by a markedly slower one. Consequently, after 30 min, the transported quantity of glycine betaine was 440 nmol mg of $protein^{-1}$ which was 13 times greater than that of cells grown in minimal medium. The cellular water volume in 0.5 M NaCl-grown cells was 2.50 ± 0.22 μl mg of $protein^{-1}$ and the internal concentration of glycine betaine was 176 mM, corresponding to a concentration gradient of 1:705.

To investigate the energy source, transport was measured in 0.5 M NaCl-grown cells treated with various inhibitors (Fig. 3). Uncouplers of oxidative phosphorylation, such as DNP, which dissipate the electrochemical gradient of protons across the cytoplasmic membrane (14, 34), effectively reduced the initial rates of glycine betaine transport: 70% inhibition by 1 mM. Equally severe inhibition was apparent by the respiratory inhibitor potassium cyanide. Sodium arsenite and the sulfhydryl group reagent *N*-ethylmaleimide had lesser effects on glycine betaine transport, 55 and 38% inhibition by 1 mM, respectively. Sodium azide, an inhibitor of cytochrome oxidase, caused only 35% inhibition at a concentration as high as 5 mM. Moreover, sodium arsenate, which inhibits reactions involving high-energy phosphate bonds and causes a drastic depletion of intracellular ATP levels (20), was relatively ineffective: inhibition by 2 mM sodium arsenate was less than 20%. In addition, *N*,*N*'-dicyclohexylcarbodiimide, a membrane-bound ATPase inhibitor (32), exerted a slight stimulation when present at a concentration of 100 μM or less. At a higher concentration of 1 mM, this molecule produced a very small decline in glycine betaine transport (5% inhibition). The effect of low temperature was also investigated: uptake was greatly diminished at 4°C (90% inhibition).

These results provide further evidence on the dependency of glycine betaine transport on the availability of energy. They suggest that the main driving force in uptake is the electrochemical proton gradient generated by respiration.

Fate of intracellular glycine betaine. *E. coli* K-10 cells grown in minimal medium or in minimal medium with additional 0.05 M NaCl were incubated with [^{14}C]glycine betaine (5 μM) as described for transport assays. After 60 min and 5 h, samples were filtered through a membrane filter and washed. The filters were immediately placed into 10 ml of 70% (vol/vol) ethanol, and the cells were extracted (22). Incorporation of [^{14}C]glycine betaine itself or incorporation of a derivative into the trichloroacetic acid-insoluble product was never detected. All of the cell-bound radioactive material could be extracted by 70% ethanol. Chromatographic and electrophoretic studies (22) revealed only a single ^{14}C-labeled compound which moved to the same positions as pure glycine betaine. Similar results were obtained with [*methyl*-^{14}C]glycine betaine and [1,2-^{14}C]glycine betaine. These experiments suggested that intracellular glycine betaine is free in the cytoplasm and never catabolized. This conclusion was consistent with the observations

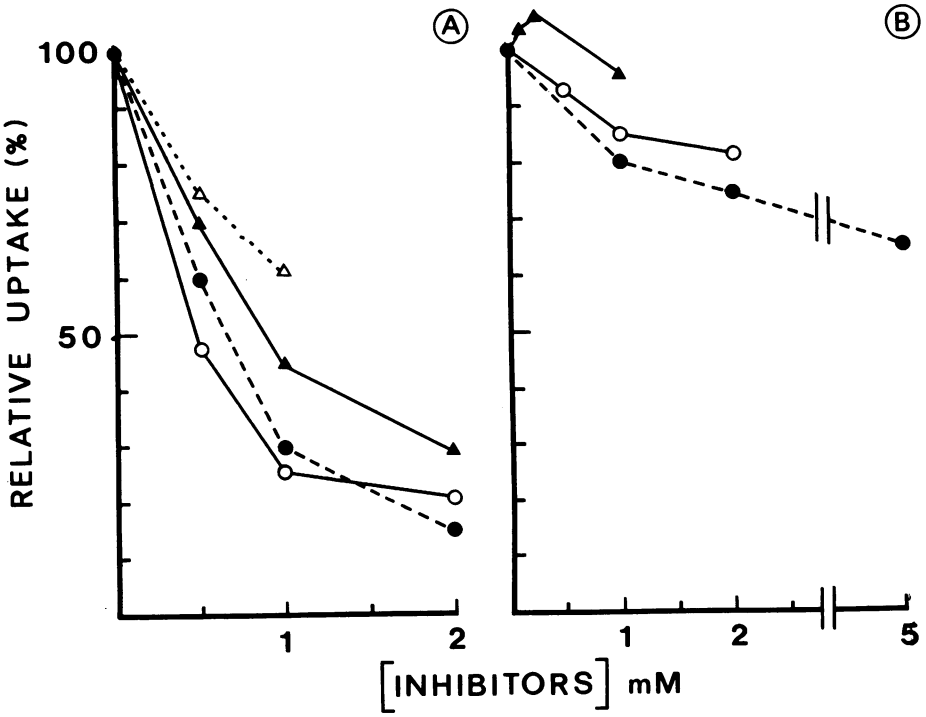

FIG. 3. Effect of metabolic inhibitors on glycine betaine uptake by *E. coli* K-10. Cells were shaken in minimal medium plus 0.5 M NaCl at 37°C until the culture density reached an absorbance of 0.6 at 420 nm. The cells were then washed twice with the growth medium and incubated with the inhibitors at the indicated concentrations for 30 min before the uptake was initiated. Uptake of [*methyl*-^{14}C]glycine betaine (250 μM) was tested as described in the text. The specific activity of the uninhibited control cells was 40 nmol min^{-1} mg of $protein^{-1}$. Symbols: (A) ●, DNP; ○, KCN; ▲ , sodium arsenite; △ , *N*-ethylmaleimide; (B) ●, sodium azide; ○, sodium arsenate; ▲ , DCCD.

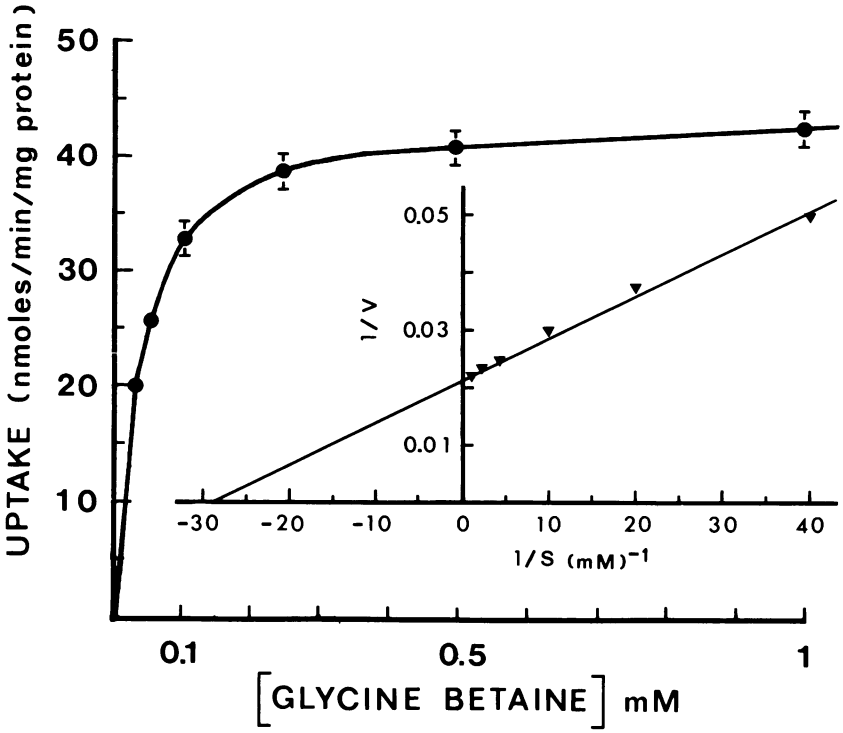

FIG. 4. Kinetics of glycine betaine transport by *E. coli* K-10. Cells were grown in minimal medium with 0.5 M NaCl at 37°C. Glycine betaine uptake was measured as described in the text. Insert is the Lineweaver-Burk plot of the initial rate of glycine betaine transport. The points shown are the mean values of three to five determinations, and the line drawn is that derived from regression analysis of these data.

that glycine betaine cannot serve as a nitrogen source for *E. coli*.

Kinetics of glycine betaine transport. To determine the concentration needed to saturate the uptake system, the kinetic parameters of glycine betaine transport by *E. coli* were determined with 0.5 M NaCl-grown cells. Initial rates were calculated at 2, 3, and 5 min at substrate concentrations, from 25 μM to 1 mM. Transport was a saturable function of substrate concentration (Fig. 4). Double-reciprocal plots gave a straight line, indicating that the uptake follows typical Michaelis-Menten kinetics. The line of best fit was performed by using a least-squares linear regression. The apparent K_m was 35 ± 2 μM, and the maximal velocity (V_{max}) was 42 ± 2.5 nmol min^{-1} mg of protein^{-1}. With minimal medium-grown cells, the apparent K_m showed a quite similar value. For subsequent uptake studies, a concentration of seven times the K_m was usually used.

Effect of increased osmotic strength on glycine betaine transport. *E. coli* K-10 cells were grown in presence of inhibitory concentrations of several electrolytes, such as NaCl, KCl, NaH_2PO_4, K_2HPO_4, and K_2SO_4, and nonelectrolytes like sucrose, inositol, and raffinose. All solutes were added to the minimal medium at concentrations osmotically equivalent to 0.5 M NaCl, except in the case of raffinose which was osmotically equivalent to 0.3 M NaCl. It was verified that none of the sugars examined supported the growth of this strain. The data for glycine betaine uptake are shown in Table 1. Initial rates of transport ranged from 23 to 44 nmol min^{-1} mg of protein^{-1} in the presence of K_2HPO_4 or K_2SO_4, respectively. Cultures in which the salt was replaced by sucrose gave the highest transport rate, ca. twofold greater than that of NaCl-grown cells. Significant stimulation of the transport in *E. coli* C600 (*lacY*) was also observed when the osmolarity of the growth medium was increased by addition of lactose: 8 ± 1 nmol min^{-1} mg of protein^{-1} in cells grown in minimal medium and 45 ± 3 nmol min^{-1} mg of protein^{-1} in cells grown in the presence of 0.7 M lactose. These experiments showed that the cells displayed no requirement for a specific ion to stimulate glycine betaine transport. The data suggested that glycine betaine uptake was stimulated as a consequence of elevated external osmotic pressure, rather than as a consequence of any given solute.

TABLE 1. Effect of various solutes added to the growth medium on glycine betaine uptake by *E. coli* K-10[a]

Addition to minimal medium[b]	Concn (M)	Glycine betaine transport[c]
None		7 ± 0.5
NaCl	0.50	38 ± 2
KCl	0.43	33 ± 2
NaH_2PO_4	0.56	41 ± 1
K_2HPO_4	0.52	23 ± 1
K_2SO_4	0.44	44 ± 1
Sucrose	0.72	91 ± 5
Inositol	0.92	42 ± 2
Raffinose	0.43	36 ± 2

[a] Cells were grown in minimal medium plus the indicated electrolyte or nonelectrolyte until the culture density reached an absorbance of 0.6 at 420 nm. The cells were then washed twice with the growth medium.

[b] The concentrations of electrolytes and nonelectrolytes added to the minimal medium were osmotically equivalent to 0.5 M NaCl, except in the case of raffinose which was osmotically equivalent to 0.3 M NaCl.

[c] Uptake of [*methyl*-^{14}C]glycine betaine (250 μM) was performed as described in the text. Transport is expressed as nanomoles of glycine betaine uptake per minute per milligram of protein. Initial rates were determined by 1-min assays. Mean results of duplicate from three separate experiments are shown ± the standard deviation of the mean.

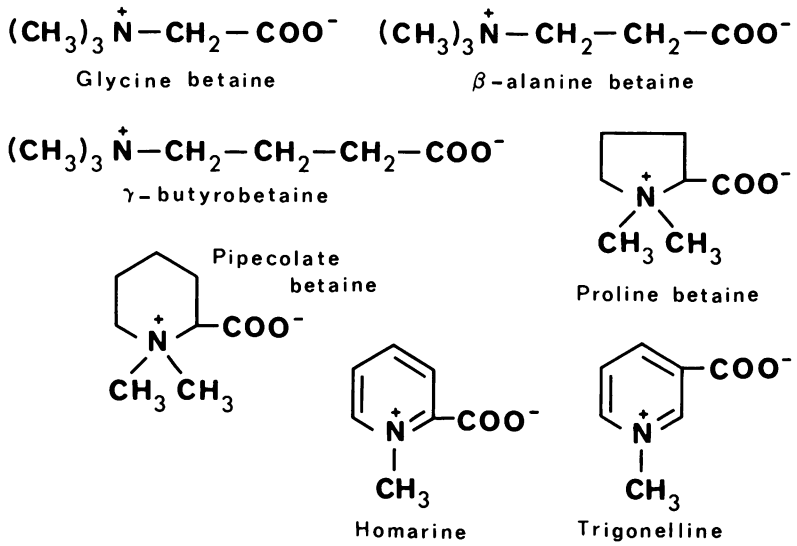

FIG. 5. Structure of various betaines used as competitors in glycine betaine uptake assays.

Second, we tested whether the glycine betaine uptake by *E. coli* K-10 depends on the value of the osmotic strength of the medium. Cells were grown in media with increased osmotic pressure obtained by the addition of NaCl (Table 2). Over an external osmotic pressure of 14.5 to 36.2 bars, minimal medium with 0.15 or 0.65 M NaCl, respectively, the initial rate of glycine betaine uptake did not substantially vary, whereas the intracellular glycine betaine levels exhibited a ca. sixfold variation. The greater the increase in osmolarity, the longer was the linear period of uptake: 2 min in 0.15 M NaCl-grown cells to 15 min in 0.65 M NaCl-grown cells.

Third, cells grown in minimal medium were subjected to a sudden osmotic upshock realized by the addition of NaCl (final concentration, 0.5 M). Samples were withdrawn at intervals of 10 min over a period of 1 h, and glycine betaine uptake was determined by 1-min assays. Within 10 min after the addition of NaCl, there was a strong increase in uptake from 7 to 25 nmol min^{-1} mg of $protein^{-1}$. The maximal rate (40 ± 4 nmol min^{-1} mg of $protein^{-1}$) was ca. sixfold that measured before the osmotic shock and was attained ca. 30 min after the addition of NaCl. Then, this rate remained almost constant, and it should be noted that the stable value obtained was very similar to that observed when the cells were grown in the presence of 0.5 M NaCl. To find out whether this increased uptake rate was due to formation of new protein(s), the effect of chloramphenicol was investigated. When cells grown in minimal medium or minimal medium plus 0.5 M NaCl were incubated for 1 h in the presence of a chloramphenicol concentration (100 μg ml^{-1}) that quite effectively blocked growth and inhibited protein synthesis, the glycine betaine uptake was not reduced compared with that of untreated cells (data not shown). On the other hand, when cells grown in minimal medium and incubated during 1 h with chloramphenicol were subjected to the osmotic upshock as described above, the glycine betaine uptake rate measured 10 min after the addition of NaCl showed only 20% stimulation. However, this slight increase was followed by a decrease, and 10 min later, uptake reached a constant value of 6 ± 2 nmol min^{-1} mg of protein $^{-1}$. Furthermore, when cells grown in low-NaCl medium were shifted to high-NaCl medium (0.5 M) in the presence of chloramphenicol, a fourfold stimulation of the initial rate of glycine betaine uptake was observed. In spite of that, the stimulation ceased rapidly, and within 30 to 45 min, the initial rate of uptake dropped back to the value routinely measured in low-salt-grown cells. The synthesis of new protein(s) in medium of high osmotic strength, prevented by the addition of chloramphenicol, was apparently needed to obtain the uptake of glycine betaine at a maximum rate. Nevertheless, this phenomenon should be studied further before definitive conclusions are reached.

TABLE 2. Effect of the osmotic pressure of the growth medium on glycine betaine uptake by *E. coli* K-10[a]

Concn (M) of NaCl added to minimal medium	Osmotic pressure (bars)[b]	Glycine betaine uptake[c]		Intracellular glycine betaine level (mM)[d]
		Initial rates	Glycine betaine accumulated	
None	7.4 ± 0.5	7 ± 0.5	30 ± 5	9.5 ± 3
0.15	14.5 ± 0.5	35 ± 2	100 ± 10	29 ± 6
0.3	20.4 ± 0.5	39 ± 2	230 ± 14	82 ± 12
0.5	29.4 ± 0.5	38 ± 2	440 ± 20	176 ± 24
0.65	36.2 ± 0.5	34 ± 2	580 ± 22	246 ± 35

[a] Cells were grown at 37°C in minimal medium with the indicated NaCl concentration.

[b] Osmotic pressure of the different media was measured by freezing point depression with a microosmometer.

[c] Cells were incubated at 37°C with [*methyl*-^{14}C]glycine betaine (250 μM) as described in the text. Initial rates were determined by 1-min assays and expressed as nanomoles of glycine betaine per minute per milligram of protein. Glycine betaine accumulated was measured after 30 min of uptake and expressed as nanomoles per milligram of protein. Data are ± the standard error of the mean (from three separate experiments).

[d] The cellular water volume was determined as described in the text and shown to range from 3.65 ± 0.26 μl mg of $protein^{-1}$ in cells grown in minimal medium to 2.35 ± 0.24 μl mg of $protein^{-1}$ in cells grown in the presence of added 0.65 M NaCl.

Specificity of glycine betaine uptake. The specificity of the transport was investigated by studying the inhibitory effect of various unlabeled compounds competing with the uptake of [*methyl*-^{14}C]glycine betaine by 0.5 M NaCl-grown *E. coli* K-10 cells. The uptake of radioactive glycine betaine was measured by experiments in which structural analogs were introduced into the assay mixtures at 20- and 40-fold the glycine betaine concentration. Figure 5 shows the structure

of some compounds used in this study. Glycine betaine uptake was not inhibited by 40-fold-higher concentrations of amino acids such as L-glycine, γ-aminobutyric acid or L-alanine (Table 3). However, L-proline exerted a 35% inhibition when present in 10 mM. An interesting finding was that addition of methyl groups on these amino acids rendered the molecule more inhibitory in terms of glycine betaine transport. Thus, dimethylglycine, γ-butyrobetaine (γ-trimethylaminobutyrate), and β-alanine betaine (β-trimethylaminopropionate) at a concentration of 10 mM reduced the uptake by 38, 24, and 66%, respectively. In addition, *N*-monomethylproline was a fairly potent inhibitor (52% inhibition by 5 mM), and proline betaine (*N*-dimethylproline) was the most effective competitor (91% inhibition by 5 mM). The size of the molecules used as competitors was also considered. When the length of the carbon chain was increased, the initial uptake rate of glycine betaine was decreased: 52 and 8% inhibition were achieved when β-alanine betaine or γ-butyrobetaine were present in 20-fold excess (5 mM), respectively. Similar results were observed with cyclic molecules: 91% inhibition by 5 mM proline betaine, and only 51% inhibition by 5 mM pipecolate betaine (*N*-dimethylpipecolate). A carboxyl group seems to be very important, since there was no inhibition by glycine betaine aldehyde and a notable stimulation with choline (23% by 5 mM). It would also appear that the relative position of the methyl and the carboxyl groups is of importance, since homarine (α-*methyl*) was more potent inhibitor (24% inhibition by 5 mM) than trigonelline (β-*methyl*, only 3% inhibition by 5 mM).

These data indicate that the glycine betaine transport system of *E. coli* possesses considerable structural specificity, but suggest that this system may also function in the transport of a few analogs, such as proline betaine.

DISCUSSION

The results presented here demonstrate the existence in *E. coli* K-10 of an active transport system for glycine betaine, a compound which has been shown to remove the inhibition of growth of *Enterobacteriaceae* members in high-salt media (22). This conclusion is based on the following evidence. (i) Glycine betaine is concentrated in the cells to over several hundred times the extracellular concentration (Table 2). Intracellular glycine betaine is not metabolized. In aerobic conditions, the cells are not able to form trimethylamine from glycine betaine; they can neither degrade this compound to *N*,*N*′-dimethylglycine, acetate, and butyrate as has been shown in *Eubacterium limosum* (29). (ii) Glycine betaine transport is greatly reduced at low temperatures and highly inhibited by 2,4-dinitrophenol, a proton-conducting uncoupler, and also by potassium cyanide, a respiratory inhibitor. In contrast, as demonstrated in Fig. 3, the inhibitor of membrane-bound ATPase, DCCD, sodium azide, and sodium arsenate had only a slight effect or none at all. All of these results are very similar to those obtained for proline transport in *E. coli* (3) and also in a moderately halophilic halotolerant bacterium (30). They suggest that the electrochemical proton gradient, generated by respiration, is the main driving force for glycine betaine transport. ATP or a metabolic derived from ATP might not be used directly as an energy source. Further studies using cytoplasmic membrane vesicles will be required, however, to determine the mode of energy coupling. (iii) The kinetic data indicate that glycine betaine transport is substrate saturable and exhibits a Michaelis-Menten relationship, observations which support the involvement of a transport protein. The possibility of several distinct transport systems for glycine betaine operating individually, as previously shown for proline transport in *E. coli* and *S. typhimurium* (2, 8, 31), could not be suggested without further investigations. The small difference in the apparent K_m values of cells grown on low-salt or high-salt medium are insignificant and could not be related to different transport systems.

Structure-function studies with structural analogs of glycine betaine (Table 3) reveal that the uptake system is specific for glycine betaine. Analogs lacking a carboxyl group such as choline or glycine betaine aldehyde are not transported, indicating a role for the carboxyl group in binding to the permease. Esterification or amidation of the carboxyl group of glycine betaine might give further information, since it is very important to know whether the carboxyl group per se is essential for recognition by the permease binding site. In the case of proline transport system in *E. coli*, it was suggested that binding may be achieved by hydrogen bonding between the carboxyl moiety of the carboxyl group and an appropriate group in the binding site (33). Partial or total demethylation, as in sarcosine or glycine, rendered compounds inactive as inhibitors of glycine betaine uptake, suggesting the *N*-trimethyl group of glycine betaine is also involved in the interaction of substrate and permease carrier.

It is also of interest that the glycine betaine uptake is stimulated in cells grown in media of high salt concentration. Since this molecule is not used as a source of both carbon and nitrogen, it is advantageous to the cell to be able to enhance its uptake in response to high osmolarity. In this case, glycine betaine is preserved to function as an osmoprotectant and is able to partially compensate the increment of external osmolarity. The intracellular glycine betaine in *E. coli* cells grown in the presence of 0.65 M NaCl was found to be 246 ± 35 mM, assuming that this solute was in solution in the intracellular fluid. However, the cells can accumulate more than that. When they are transferred from a minimal medium to the same medium with 0.65 M NaCl and allowed to grow for six generations in the presence of 1 mM glycine betaine, the intracellular concentration reached 500 mM. A

TABLE 3. Effect of unlabeled competitors on [*methyl*-^{14}C]glycine betaine uptake by *E. coli* K-10[a]

Inhibitor	% Inhibition at concn of[b]:	
	5 mM	10 mM
None	0	0
Glycine	ND	−14
Sarcosine	−17	3
Dimethylglycine	29	38
γ-Aminobutyric acid	2	−12
γ-Butyrobetaine	8	24
Alanine	1	5
β-Alanine betaine	52	66
Proline	32	35
Monomethylproline	52	66
Proline betaine	91	95
Pipecolate betaine	51	57
Glycine betaine aldehyde	7	0
Choline	−23	−14
Homarine	24	28
Trigonelline	3	14

[a] Bacteria were grown in minimal medium plus 0.5 M NaCl, and uptake assays were carried out as described in the text with [*methyl*-^{14}C]glycine betaine (250 μM) and 5 or 10 mM inhibitor.

[b] The data are given as the percent reduction of the uninhibited uptake rate, which was 40 nmol min^{-1} mg of protein^{-1}. ND, Not determined.

similar value was previously obtained in *K. pneumoniae* (22). This stimulation of the uptake is due primarily to elevated osmotic strength rather than to the presence of any given solute. Similar effects have been found for potassium transport in *E. coli* (11) and also for proline transport in cell-free membrane vesicles of *E. coli* (17). It should be noted that both potassium and proline are also known to play a crucial role in osmoregulation. The control of glycine betaine transport by osmotic strength is most convincingly shown by experiments in which the osmotic pressure of the medium is suddenly increased by the addition of NaCl. A rapid upshock was always followed by a strong stimulation of the uptake. In cells treated by chloramphenicol at a concentration which blocks growth and inhibits protein synthesis, no stimulation of the transport by a sudden upshock was observed. Thus, the synthesis of new protein(s) might be needed for glycine betaine uptake at maximum rates. These results suggest that osmotic stress alone somehow activates or causes the expression of this uptake system. It is premature to use the word induction since the cellular level at which activation takes place is not yet known. The signal probably comes from a differential osmometer or manometer sensing the difference in hydrostatic pressure inside and outside the cell. The osmosensory system which is perhaps best understood is with the outer membrane porin proteins OmpF and OmpC. The osmolarity of the medium strikingly affects the proportionate amounts of these two porins, such as the *ompC* gene is preferentially expressed in cells grown in a medium of high osmolarity, whereas the *ompF* gene is expressed in cells grown in a medium of low osmolarity (18, 36). It has been suggested that an osmosensory protein embedded in the envelope functions to trigger a regulatory cascade involving a second cytoplasmic regulatory protein which determines the expression of the desired outer membrane protein (12, 13). Epstein and his collaborators have also suggested that a membrane-bound osmosensing protein regulates potassium transport genes as a function of osmotic strength (21). It is proposed that turgor pressure regulates K^+ transport at two different levels; it controls both the expression of the *kdp* operon and the function of K^+ transport systems. In addition, Kennedy (19) has proposed that an osmotic sensor protein presumably localized in the inner membrane of *E. coli* plays a crucial role in modulating the biosynthesis of membrane derived oligosaccharides whose production is geared to the osmotic strength of the medium. It will be of considerable interest to determine whether the osmotically modulated glycine betaine uptake system characterized in this study share a common osmosensor with any of the systems discussed above. In view of the potential importance of glycine betaine uptake in *E. coli*, we are currently isolating mutants defective in the uptake system. Such mutants should allow a more complete analysis of glycine betaine transport and a more definitive assessment of the role of glycine betaine in the cellular adaptation to osmotic stress.

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