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Apparently Unidirectional Polyamine Transport by Proton Motive Force in Polyamine-Deficient *Escherichia coli*

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A transport system for polyamines was studied with both intact cells and membrane vesicles of an *Escherichia coli* polyamine-deficient mutant. Polyamine uptake by intact cells and membrane vesicles was inhibited by various protonophores, and polyamines accumulated in membrane vesicles when D-lactate was added as an energy source or when a membrane potential was imposed artificially by the addition of valinomycin to K^+-loaded vesicles. These results show that the uptake was dependent on proton motive force. Transported [^{14}C]putrescine and [^{14}C]spermidine were not excreted by intact cells upon the addition either of carbonyl cyanide *m*-chlorophenylhydrazone, A23187, and Ca^{2+} or of an excess amount of nonlabeled polyamine. However, they were excreted by membrane vesicles, although the degree of spermidine efflux was much lower than that of putrescine efflux. These results suggest that the apparent unidirectionality in intact cells has arisen from polyamine binding to nucleic acids, thus giving rise to a negligible free intracellular concentration of polyamines. Polyamine uptake, especially putrescine uptake, was inhibited strongly by monovalent cations. The Mg^{2+} ion inhibited spermidine and spermine uptake but not putrescine uptake.

Several studies have led to the conclusion that polyamines participate in a wide variety of growth processes (3, 21, 23). Thus, it is important to understand the mechanism by which the cellular polyamine concentration is regulated. Although the biosynthesis of polyamines has been studied extensively (2, 22), the transport of polyamines has not. In eucaryotes, it has been reported that different polyamines appear to share the same transport system (17) and that their uptake is sodium dependent (18). In procaryotes, an energy-dependent polyamine uptake system has been shown in *Escherichia coli* B (20), and two transport systems for putrescine have been demonstrated in *E. coli* K-12 grown in a low-osmolarity medium (16).

Since the *E. coli* cells used in the studies described above can synthesize polyamines, there is a possibility that such polyamines, especially putrescine, may be excreted from the cells during the uptake of polyamines added to the medium (16, 20). If this occurs, an exact analysis of polyamine transport would be difficult. To clarify the characteristics of polyamine transport further, we used cells unable to synthesize polyamines and membrane vesicles of a polyamine-deficient mutant for our experiments. Polyamine transport was apparently unidirectional, and the uptake was dependent on proton motive force.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The polyamine-requiring mutant of *E. coli* used in this experiment, MA261 (*speB speC thr leu ser thi*), was kindly supplied by W. K. Maas. The cells were grown in medium B (6) without putrescine. When growth was sufficient to yield an A_{540} of 0.30, the cells were harvested by centrifugation at 12,000 × *g* for 10 min. The cells were washed once with buffer A, which contained 0.4% glucose, 62 mM potassium phosphate (pH 7.0), 1.7 mM sodium citrate, 7.6 mM $(NH_4)_2SO_4$, and 0.41 mM $MgSO_4$, centrifuged as described above, and suspended in buffer A to yield a protein concentration of 0.1 mg/ml. Protein was measured by the method of Lowry et al. (14) after trichloroacetic acid precipitation of cells.

Polyamine uptake by intact cells. The cell suspension (0.48 ml), prepared as described above, was preincubated at 30°C for 5 min, and the reaction was started by the addition of 20 μl of 250 μM [^{14}C]polyamine (10 mCi/mmol). After incubation at 30°C, the cells were collected on membrane filters (type EH, 0.5 μm; Millipore Corp.), and the filters were washed three times at room temperature with a total of 6 ml of buffer A. In the case of spermine, the filters were washed with buffer A containing 50 μM spermine to remove the adsorbed [^{14}C]spermine. The amount of amine adsorbed to the cell surface and filter was less than 0.1% of the added amine under these experimental conditions. The radioactivity on the filter was assayed with a liquid scintillation spectrometer.

Preparation of right-side-out membrane vesicles. The polyamine-requiring mutant of *E. coli* used in this experiment, DR112 (*speA speB thi*), was kindly supplied by D. R. Morris. The cells were cultured without putrescine by the method of Linderoth and Morris (13). The cultures were harvested in the mid-exponential phase by centrifugation. Membrane vesicles were prepared by the procedure of Kaback (10), except that the concentration of lysozyme was decreased from 500 μg/ml to 50 μg/ml. Potassium-loaded vesicles were prepared by the method of Hirata et al. (5).

Polyamine uptake by membrane vesicles. The reaction mixture (0.1 ml), containing 20 mM potassium phosphate buffer (pH 6.6), 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.6), 5 mM $MgSO_4$, 20 mM D-lactate (lithium salt), and approximately 100 μg of membrane vesicle protein, was preincubated at 37°C for 3 min. The reaction was started by the addition of 2 μl of 400 μM [^{14}C]putrescine (104 mCi/mmol) or [^{14}C]spermidine (85 mCi/mmol). After incubation at 37°C, membrane vesicles were collected on membrane filters (type EH, 0.5 μm; Millipore Corp.), washed, and counted as described above.

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TABLE 1. Components of intact cells and membrane vesicles

Material tested	Amt of:					
	DNA[a]	RNA[a]	Phospholipids[a]	ATP[b]	Putrescine[b]	Spermidine[b]
Intact cells	29.4	286	97.1	4.72	0.35	0.91
Membrane vesicles	4.9	15.3	401	0.28	0.01	0.01

[a] In micrograms per milligram of protein.
[b] In nanomoles per milligram of protein.

For polyamine uptake by potassium-loaded membrane vesicles, the reaction mixture (1 ml), containing 100 mM Tris-maleate (pH 7.0), 13 mM $MgSO_4$, 280 mM sucrose, 8 μM [^{14}C]putrescine (104 mCi/mmol) or [^{14}C]spermidine (85 mCi/mmol), and approximately 1 mg of membrane vesicle protein, was preincubated at 30°C for 3 min. The reaction was started by the addition of 2 μl of valinomycin in ethanol (1 mg/ml), and incubation was carried out at 30°C. At intervals, 0.1-ml aliquots were filtered and washed.

Measurement of DNA, RNA, phospholipid, ATP, and polyamines. The amounts of DNA and RNA in cells and membrane vesicles were measured by the method of Schneider (19). Phospholipids were extracted with chloroform-methanol (2:1, vol/vol), and their concentration was calculated by measuring phosphorus by the method of Morrison (15). ATP was assayed with a luciferase enzyme system (11) with some modifications (8). Polyamines were measured as described previously (4).

Chemicals. [^{14}C]Putrescine dihydrochloride, [^{14}C]spermidine trihydrochloride, and [^{14}C]spermine tetrahydrochloride were purchased from New England Nuclear Corp. *N*-Ethylmaleimide, *p*-chloromercuribenzoic acid, putrescine, spermidine, and spermine were obtained from Nakarai Chemicals. D-Lactate (lithium salt), valinomycin, nigericin, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma Chemical Co. Ionophore A23187, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF6847) were purchased from Eli Lilly & Co., Fluka AG, and Wako Pure Chemical Industries, respectively. Tetrachlorosalicylanilide was a kind gift from F. M. Harold.

RESULTS

Analysis of intact cells and membrane vesicles. The amounts of putrescine and spermidine in the polyamine-deficient cells were 0.35 and 0.91 nmol/mg of protein, respectively, which corresponded to approximately 1 and 8% of the levels found in normal cells (*E. coli* B) (Table 1). When the cells were incubated with 10 mM Na_2HAsO_4 at 30°C for 20 min, the ATP concentration decreased from 4.72 to 1.24 mmol/mg of protein. This degree of ATP decrease was less than that reported by Berger and Heppel (1). The DNA and RNA contents were approximately threefold greater than the phospholipid content. Membrane vesicles consisted mainly of phospholipids and proteins. Insignificant amounts of putrescine and spermidine and only small amounts of DNA, RNA, and ATP were found in membrane vesicles.

Energy donor in polyamine uptake. Na_2HAsO_4, an inhibitor of ATP synthesis, did not significantly inhibit polyamine uptake by intact cells (Table 2). The uptake was inhibited greatly by cyanide, an inhibitor of respiration, and completely by CCCP and FCCP, inhibitors of proton circulation (Table 2). Therefore, polyamine uptake by intact cells appeared to be energized by proton motive force and not by ATP.

This result was confirmed by experiments with membrane vesicles. Polyamines accumulated in membrane vesicles when D-lactate was added as an energy source (Fig. 1). The uptake was inhibited by various protonophores (CCCP, FCCP, SF6847, and tetrachlorosalicylanilide) (Table 3). Polyamines accumulated in membrane vesicles when a membrane potential was imposed artificially by the addition of valinomycin to K^+-loaded vesicles (Fig. 2). Thus, it is evident that the energy source in polyamine transport is proton motive force.

The results in Fig. 2 show that polyamines were transported by the electrical potential only, if this was great enough as the driving force. In the system to which D-lactate was added as an energy source, valinomycin, acting as an inhibitor of the electrical potential, and nigericin, acting as an inhibitor of a pH gradient, both inhibited polyamine uptake (Table 3). These results suggest that both the electrical potential and a pH gradient are involved in polyamine transport. The same results were obtained with serine and proline transport systems (data not shown).

Apparently unidirectional transport of polyamines by intact cells. It has been reported that accumulated [^{14}C]spermidine or [^{14}C]spermine does not exchange with nonlabeled external spermidine or spermine, although small amounts of accumulated [^{14}C]putrescine exchange with nonlabeled external putrescine at an equilibrium state (20). Since the absence of such an exchange is characteristic of polyamine transport, unidirectional transport was reexamined (Fig. 3). When either CCCP, A23187 (an ionophore for Ca^{2+}), and 5 mM Ca^{2+} or an excess amount of nonlabeled putrescine was added at 6 or 10 min, respectively, after the onset of the reaction, no efflux of [^{14}C]putrescine from polyamine-deficient cells was observed. Similar results were obtained with spermidine and spermine transport systems. These results suggest that not only spermidine and spermine transport but also putrescine transport is unidirectional.

When membrane vesicles were used instead of intact cells, transport was bidirectional. Although putrescine accumulated in membrane vesicles was not excreted in the presence of CCCP alone, A23187 plus CCCP, or 5 mM Ca^{2+} plus CCCP, it was excreted when either CCCP, A23187, and Ca^{2+} or an excess amount of putrescine was added to the reaction mixture (Fig. 4A). Accumulated putrescine was also released after the treatment of vesicles with toluene. Therefore, we suggest that (i) putrescine is truly transported into

TABLE 2. Effect of energy production inhibitors on the uptake of polyamines by intact cells[a]

Inhibitor	Preincubation time (min)	Uptake (nmol/min per mg of protein) of:		
		Putrescine	Spermidine	Spermine
None	5	1.39	0.505	0.111
Na_2HAsO_4 (10 mM)	10	1.34	0.437	0.093
Na_2HAsO_4 (10 mM)	20	1.25	0.412	0.090
KCN (10 mM)	5	0.44	0.260	0.062
CCCP (40 μM)	5	0.06	0.065	0.020
FCCP (20 μM)	5	0.07	0.060	0.022

[a] The inhibitors were added during preincubation at 30°C, and incubation was carried out at 30°C for 5 min.

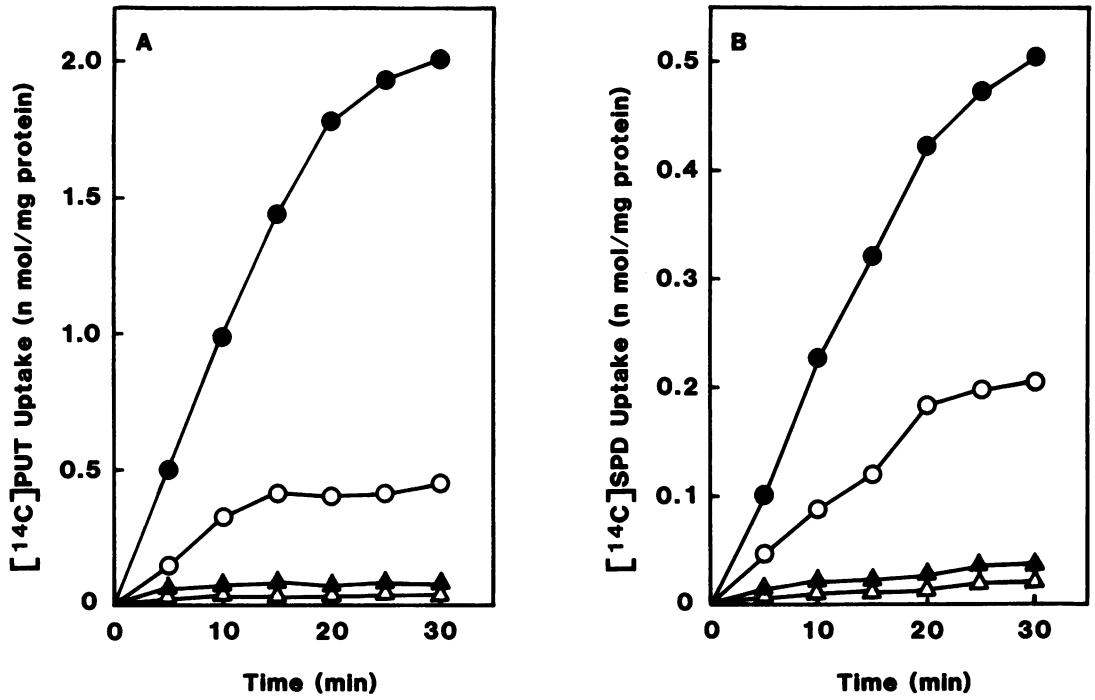

FIG. 1. D-Lactate-dependent polyamine transport by membrane vesicles. (A) Putrescine (PUT) transport; (B) spermidine (SPD) transport. Symbols: ○, no addition; ●, 20 mM D-lactate; △, 40 μM CCCP; ▲, 20 mM D-lactate and 40 μM CCCP.

the intravesicular space, (ii) transported putrescine is bound to phospholipids, and (iii) bound putrescine is released in the presence of Ca^{2+}, which enters the intravesicular space via A23187 in the presence of CCCP, or in the presence of nonlabeled putrescine. Since the binding of spermidine to phospholipids is stronger than that of putrescine, putrescine was released completely by the addition of either CCCP, A23187, and Ca^{2+} or an excess amount of nonlabeled putrescine, whereas spermidine was released only partially under the same conditions (Fig. 4B). When another uncoupler, tetrachlorosalicylanilide, was used instead of CCCP, similar results were obtained (data not shown).

TABLE 3. Effect of inhibitors on the uptake of polyamines by membrane vesicles

Inhibitor	Uptake (pmol/min per mg of protein)[a] of:	
	Putrescine	Spermidine
None	654	113
CCCP (40 μM)	0 (0)	4 (3.5)
FCCP (20 μM)	6 (0.9)	5 (4.4)
SF6847 (10 μM)	12 (1.8)	7 (6.2)
Tetrachlorosalicylanilide (10 μM)	15 (2.2)	5 (4.4)
Valinomycin (8 μM)	38 (5.8)	9 (8.0)
Nigericin (10 μM)	68 (10)	13 (12)
KCN (10 mM)	341 (52)	57 (50)
Na_2HAsO_4 (10 mM)	659 (101)	93 (82)
N-Ethylmaleimide (5 mM)	7 (1.0)	0 (0)
p-Chloromercuribenzoic acid (0.1 mM)	18 (2.8)	0 (0)

[a] Numbers in parentheses represent percent uptake.

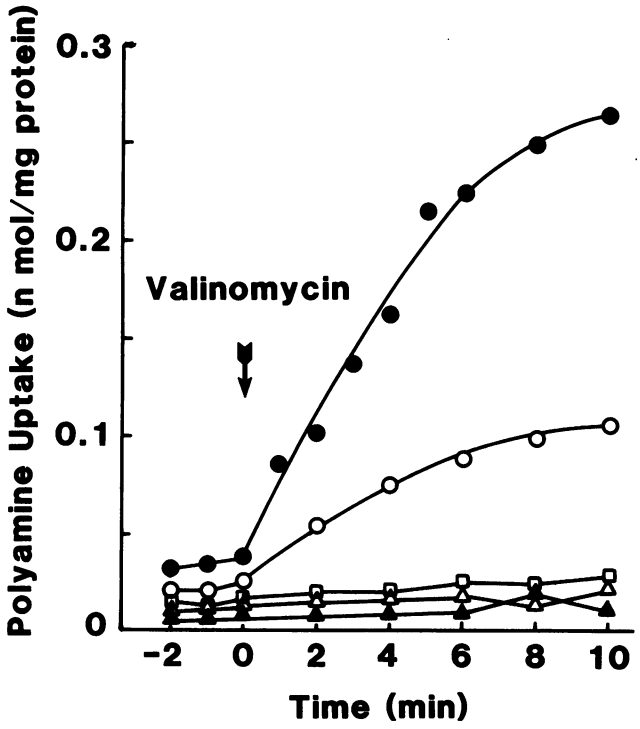

FIG. 2. Uptake of polyamines by K^+-loaded membrane vesicles. Symbols: ●, [14C]putrescine; ○, [14C]spermidine; ▲, [14C]putrescine and 40 μM CCCP; △, [14C]spermidine and 40 μM CCCP; □, [14C]putrescine and 5 mM *N*-ethylmaleimide.

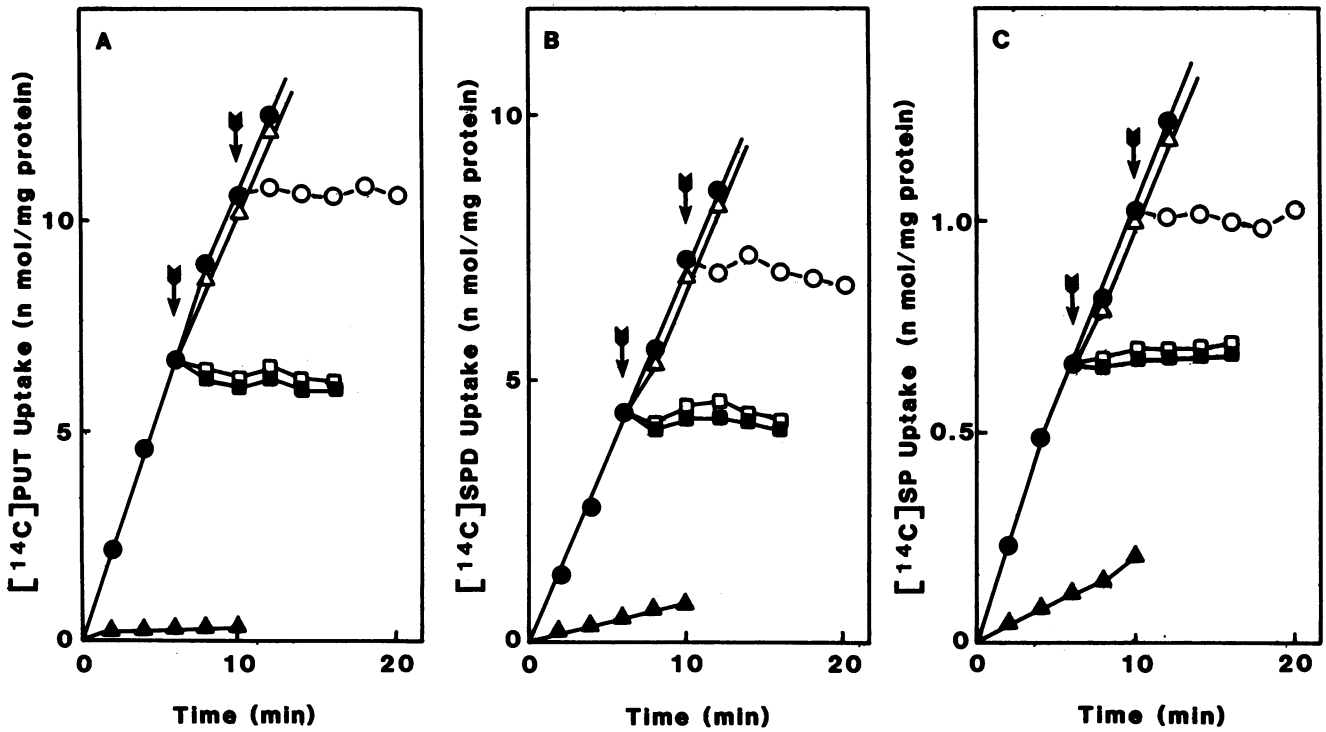

FIG. 3. Effect of CCCP and nonlabeled polyamine on ^{14}C-polyamine excretion by polyamine-deficient cells. (A) [^{14}C]putrescine (PUT) transport; (B) [^{14}C]spermidine (SPD) transport; (C) [^{14}C]spermine (SP) transport. Symbols: ●, no addition; ▲, CCCP added at the onset of the reaction; □, CCCP added 6 min after the onset of the reaction; ■, CCCP, 5 mM Ca^{2+}, and 2 μg of A23187 per ml added 6 min after the onset of the reaction; △, 5 mM Ca^{2+} and 2 μg of A23187 per ml added 6 min after the onset of the reaction; ○, 500 μM nonlabeled polyamine added 10 min after the onset of the reaction.

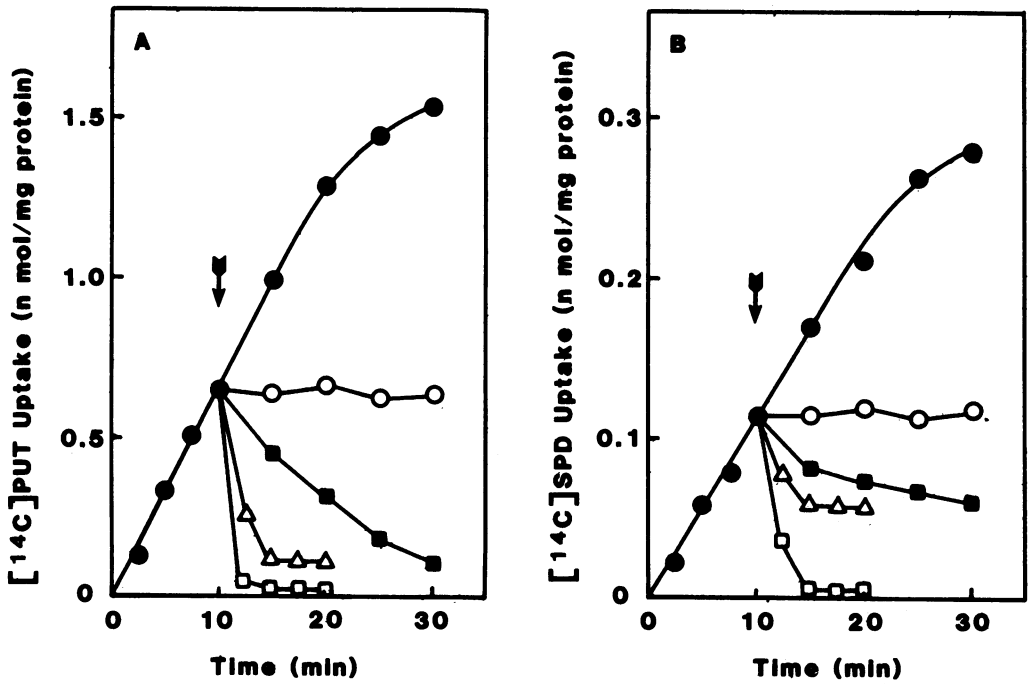

FIG. 4. Effect of CCCP and nonlabeled polyamine on ^{14}C-polyamine excretion by membrane vesicles. (A) [^{14}C]putrescine (PUT) transport; (B) [^{14}C]spermidine (SPD) transport. At 10 min after the onset of the reaction, the following additions were made to the reaction mixtures: ●, no addition; ○, 40 μM CCCP; ■, 40 μM CCCP, 5 mM Ca^{2+}, and 2 μg of A23187 per ml; △, 500 μM nonlabeled putrescine (A) or spermidine (B); □, 1% toluene. The value without D-lactate was subtracted for each point.

These results suggest that the apparent unidirectionality in intact cells has arisen from polyamine binding to nucleic acids rather than phospholipids.

No efflux of [^{14}C]putrescine was observed when either CCCP, A23187, and 5 mM Ca^{2+} or an excess amount of nonlabeled putrescine was added to a reaction mixture containing MA261 cells grown in the presence of 100 μg of putrescine per ml (data not shown). The rate of putrescine uptake was about 50% of the rate observed in cells grown in the absence of putrescine.

Other characteristics of polyamine uptake by membrane vesicles. Polyamines are known to replace the function of monovalent cations (12) and Mg^{2+} (7). Putrescine uptake was inhibited more strongly by KCl than was spermidine uptake (Table 4). The Mg^{2+} ion inhibited spermidine uptake but not putrescine uptake.

The addition of *N*-ethylmaleimide and *p*-chloromercuribenzoic acid abolished polyamine uptake (Fig. 2 and Table 3); this result suggests that a sulfhydryl group is involved in the polyamine transport system.

The substrate specificity of transport was then studied. Neither spermidine nor spermine inhibited putrescine uptake, and putrescine did not inhibit spermidine uptake. Spermidine uptake was inhibited by spermine. These results suggest that the putrescine transport system differs from the spermidine and spermine transport systems and that spermine is transported by the same system as spermidine. These results are in agreement with those previously reported for intact cells (20).

Although the rate of polyamine uptake in membrane vesicles was much slower than the rate in intact cells (Fig. 1 and 3), the K_m values of putrescine and spermidine uptake were 1.43×10^{-6} and 1×10^{-7} M, respectively. These values are very similar to those previously reported for intact cells (20).

DISCUSSION

Although it has been reported that there is energy-dependent polyamine uptake in *E. coli* (20), an energy donor in polyamine uptake has not yet been determined. Polyamine uptake in intact cells and membrane vesicles was inhibited by various protonophores, and polyamines accumulated in membrane vesicles when D-lactate was added as an energy source or when a membrane potential was imposed artifically by the addition of valinomycin to K^+-loaded vesicles.

TABLE 4. Effect of cations on polyamine uptake by membrane vesicles

Addition	Uptake (pmol/min per mg of protein)[a] of:	
	Putrescine	Spermidine
None	684	104
Putrescine (40 μM)		94 (90)
Putrescine (80 μM)		88 (85)
Spermidine (40 μM)	690 (101)	
Spermidine (80 μM)	685 (100)	
Spermine (80 μM)	704 (103)	58 (56)
Spermine (160 μM)	671 (98)	29 (28)
KCl (100 mM)[b]	312 (46)	72 (69)
KCl (200 mM)	205 (30)	48 (46)
$MgCl_2$ (10 mM)[b]	631 (92)	70 (67)
$MgCl_2$ (20 mM)	610 (89)	55 (53)

[a] Numbers in parentheses represent percent uptake.
[b] The concentration shown was that added to the standard reaction mixture.

These data show that polyamine transport is driven by proton motive force. It is possible that polyamines are transported into the cells by high-affinity transport carriers without invoking any driving force, since the free intracellular concentration of polyamines seems to be negligible. However, such a possibility is unlikely on the basis of our data.

Polyamine transport in intact cells was apparently unidirectional. It is known that the binding of polyamines to nucleic acids is stronger than that to phospholipids (9), that polyamine binding to phospholipids is inhibited by Ca^{2+} (unpublished data), and that membrane vesicles do not contain enough nucleic acids to bind polyamines (Table 1). Since transported [^{14}C]putrescine and [^{14}C]spermidine were excreted from membrane vesicles upon the addition either of CCCP, A23187, and Ca^{2+} or of an excess amount of nonlabeled polyamine, we suggest that the apparent unidirectionality in intact cells has arisen from polyamine binding to nucleic acids rather than phospholipids. Thus, it is clear that the apparent unidirectionality is due to the negligible amount of free intracellular polyamines and not to a unidirectional operation of the transport system itself. The low rate of uptake in membrane vesicles also may be related to the fact that membrane vesicles contain only a small amount of nucleic acids, which bind polyamines strongly. The apparent unidirectionality was true even in putrescine transport when the cells were grown in the presence of 100 μg of putrescine per ml; this contradicts previous results (20). Cells grown in the presence of putrescine contained approximately 70 nmol of putrescine and 800 nmol of RNA phosphorus per mg of protein. Our results seem to be reasonable, since there are sufficient sites for putrescine binding on RNA. In addition, the amount of putrescine adsorbed to the cell surface and filter was less than 0.1% of the added amine under our experimental conditions; this amount was low in comparison with that reported previously (20). This result may be related to the discrepancy.

Our data suggest that polyamines accumulated in cells bind immediately to nucleic acids. Therefore, one might argue that the K_m values that we obtained are the binding constants of polyamines for nucleic acids but not the affinity constants of the transport system. However, this is unlikely because the binding constants of polyamines for rRNA in cells calculated as described previously (9) are 1×10^{-3} to 5×10^{-4} M^{-1}. In addition, the binding constants of polyamines for nucleic acids are in the order spermine, spermidine, and putrescine, and the K_m values of polyamine transport are in the order spermine, putrescine, and spermidine.

Cells grown in a low-osmolarity medium have been shown to take up putrescine at rates severalfold higher than cells grown in a high-osmolarity medium (16). This is thought to be one of the mechanisms of osmotic adaptation of *E. coli*. We also have shown that polyamine uptake is affected by monovalent cations and the Mg^{2+} ion. It is of interest that the Mg^{2+} ion inhibited spermidine and spermine uptake but not putrescine uptake, whereas monovalent cations inhibited the uptake of all three polyamines. This result appears to be reasonable because the Mg^{2+} ion in cells was replaced by polyamines and because an effective concentration of spermidine or spermine was much lower than that of putrescine for polyamine replacement of the Mg^{2+} ion.

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