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ADENOSINE UPTAKE BY ISOLATED MEMBRANE VESICLES FROM *ESCHERICHIA COLI* K-12

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SUMMARY

1. Three different adenosine-uptake systems were found in isolated membrane vesicles of *Escherichia coli* K-12 cells: the first system was strongly inhibited by adenine nucleosides, pyrimidine nucleosides and the antibiotic showdomycin but weakly inhibited by guanine nucleodises and inosine; the second system was strongly inhibited by adenine nucleosides, guanine nucleosides and inosine but weakly inhibited by pyrimidine nucleosides and showdomycin; and the third system was strongly inhibited by adenine.

In isolated membrane vesicles of a showdomycin-resistant mutant (Shm^r-001), the first system was no longer detectable but the second and the third systems were found to remain active.

2. The adenosine uptake by these systems was stimulated by the artificial electron-donor system, ascorbate–phenazine methosulfate. The reduced phenazine methosulfate-stimulated uptake of adenosine by these systems was inhibited by sodium azide.

3. Caffeine markedly inhibited the uptake of adenosine by the second system, but stimulated that of adenosine by the first system.

4. Showdomycin strongly inhibited the uptake of adenosine by the first system but only slightly inhibited that of adenosine by the second system. Further, the antibiotic produced a rapid efflux of adenosine from previously loaded membrane vesicles of *Escherichia coli* K-12 in the presence of adenine and caffeine. The reduced phenazine methosulfate-stimulated uptake of [^{14}C]-showdomycin by membrane vesicles from *Escherichia coli* K-12 cells was strongly inhibited by adenosine and deoxycytidine but only slightly inhibited by guanosine.

These results suggest that showdomycin is taken up by the first system of adenosine uptake in the membrane vesicles from *Escherichia coli* K-12 cells.

INTRODUCTION

The uptake of the antibiotic showdomycin [2-(β-D-ribofuranosyl)maleimide] by cells of *Escherichia coli* K-12 is competitively inhibited by adenosine[1].

Showdomycin-resistant mutants have an impaired ability to take up not only showdomycin but also adenosine[2].

These facts suggested that the transport of showdomycin in *Escherichia coli* K-12 is closely related to that of adenosine.

Earlier studies with intact cells[3] suggested that adenosine is transported into the cells of *Escherichia coli* K-12 by at least two different systems in the presence of a high concentration of caffeine: one is strongly inhibited by adenine nucleosides and pyrimidine nucleosides but only slightly inhibited by guanine nucleosides, the other system is only slightly inhibited by all these nucleosides. The former system was not detected in a showdomycin-resistant mutant Shm^r-001 but the latter remained active.

Subsequent studies[4] demonstrated that two different deoxycytidine-uptake systems which are strongly inhibited by adenosine are present in isolated membrane vesicles from *Escherichia coli* K-12 cells: one was strongly inhibited by showdomycin, the other was not significantly inhibited. In isolated membrane vesicles from the showdomycin-resistant mutant (Shm^r-001), the former system was no longer detectable but the latter was found to remain active. These facts suggested that not only deoxycytidine but also adenosine can be taken up by these deoxycytidine-uptake systems in the membrane vesicles from *Escherichia coli* K-12 cells.

In the present paper, data are presented which demonstrate that three different adenosine-uptake systems are present in isolated membrane vesicles from *Escherichia coli* K-12 cells, and that one of these systems is closely related to the uptake of showdomycin.

MATERIALS AND METHODS

Preparation of cells

Escherichia coli K-12 and a showdomycin-resistant mutant, Shm^r-001 (derived from K-12)[2,3], were grown with aeration in a jar fermentor, using a glucose–mineral medium[5] supplemented with 0.025% (w/v) Difco casamino acid. Cells were harvested at approximately the end of the logarithmic phase of growth, and stored at −85 °C.

Preparation of membrane vesicles

Membrane vesicles were prepared from lysozyme and EDTA-induced spheroplasts of K-12 and Shm^r-001 essentially as described by Kaback[6]. They were stored at −85 °C in 0.1 M potassium phosphate buffer, pH 6.6, at concentrations of 0.93–1.49 mg protein/ml, and rapidly thawed just before use. Each membrane preparation was checked by microscopy. Contamination with intact cells was not observed.

Measurement of the uptake of adenosine and other labeled compounds by membrane vesicles

Portions of the membrane suspensions (either 40 μl or 80 μl) preequilibrated at 25 °C for 6–10 min were added to prewarmed tubes containing $MgSO_4$ (10 mM final concentration) and the respective labeled compounds (17.5–104 μM final concentration) as stated in the legends to the tables and figures. The mixtures (final volume of either 100 μl or 200 μl) were then incubated at 25 °C. After a given time, each reaction mixture was diluted with 2 ml of ice-cold 0.1 M LiCl solution, filtered through Millipore filters of 0.45-μm pore size, and washed with 4 ml of the same solution. The filters were dried and counted with a liquid scintillation spectrometer using toluene–phosphor solution (7 ml). In control experiments the radioactive compounds were added to the membrane suspension in 2 ml of ice-cold 0.1 M LiCl

solution just prior to filtration. Correction was made to each experimental value by subtracting the corresponding value obtained in the control experiment.

Measurements of adenosine metabolism by membrane vesicles

Membrane vesicles of *Escherichia coli* K-12 cells and the showdomycin-resistant mutant (Shm^r-001) cells were incubated with [8-^{3}H]adenosine and various additions in a final volume of 100 μl at 25 °C. After 1 min, the incubation mixtures were quickly pipetted into 400 μl ice-cold methanol. The supernatants were mixed with the appropriate non-labeled reference compounds, and spotted on Whatman 3MM paper strips (2.5 cm × 40 cm). The paper strips were developed for 4 h at 22 °C by ascending chromatography with solvent III[3] (water adjusted to pH 10 with 1 M NH_4OH). To identify the radioactive substrate and products on the paper chromatograms more conveniently, the spots of non-labeled reference compounds were located under an ultraviolet lamp, after which the chromatograms were cut into pieces (0.5 cm) and counted in a toluene–phosphor solution by a liquid scintillation spectrometer.

Chemicals and reagents

Purified iodoacetic acid was kindly supplied by Dr N. Yoshida; cytosine arabinoside was purchased from the Upjohn Company; *N*-ethylmaleimide was from Nakarai Chemicals Ltd; phenazine methosulfate was from Calbiochem; the labeled compounds were from the Schwarz BioResearch Corp.; the unlabeled nucleosides and bases were from the Sigma Chemical Corp. and the Schwarz BioResearch Corp.

RESULTS

Ability of isolated membrane vesicles to take up adenosine

As shown in Figs 1A and 1B, [^{3}H]adenosine uptake by membrane vesicles from a showdomycin-resistant mutant (Shm^r-001) of *Escherichia coli* K-12 and from the parent strain was markedly stimulated by the artificial electron donor system, ascorbate–phenazine methosulfate. Physiological electron donors such as D(−)-lactate, α-glycerol phosphate, NADH and succinate caused similar though weaker stimulation of adenosine uptake (Table I, second and fourth columns).

Effects of unlabeled adenosine, adenine and caffeine on the uptake of [8-^{3}H]adenosine by membrane vesicles from parent and mutant cells

It can be seen in Figs 1A and 1B that the addition of excess unlabeled adenosine did not cause significant loss of the accumulated radioactivity (derived from [8-^{3}H]-adenosine) from preloaded membrane vesicles from both parent and mutant cells.

As indicated in Table II, unlabeled adenosine, at a concentration 48-fold higher than that of [8-^{3}H]adenosine, produced only a minimal effect on the uptake of the labeled adenosine by membrane vesicles from mutant cells (third column, line 2), and produced a moderate effect on that of the labeled adenosine by membrane vesicles from parent cells (fifth column, line 2).

Moreover, unlabeled adenine, at a concentration 97-fold higher than that of [8-^{3}H]adenosine, produced only a minimal effect on the uptake of the labeled adenosine by membrane vesicles from mutant cells (third column, line 3).

In combination with adenine (2 mM), however, unlabeled adenosine strongly

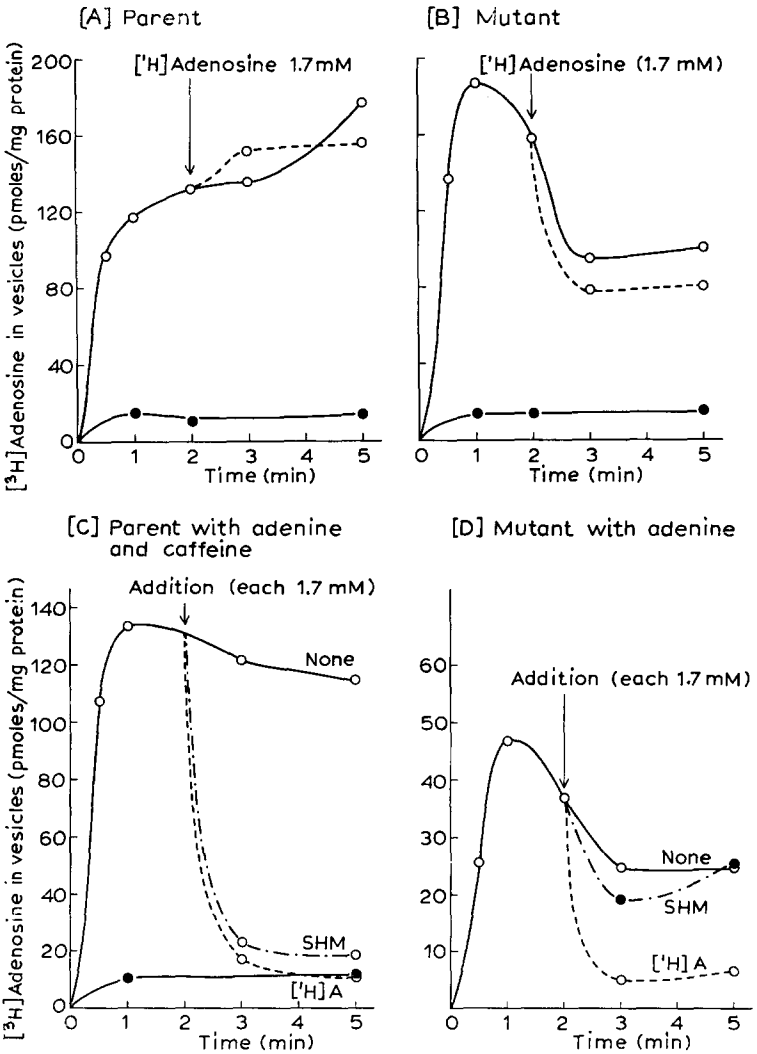

Fig. 1. Time courses of the uptake at 25 °C of [^{3}H]adenosine by membrane vesicles from parent and mutant cells under various conditions. (A) Membrane suspensions of the parent (1.49 mg membrane protein per ml) were allowed to equilibrate at 25 °C for 10 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration) and [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM). The mixtures were with (○—○) or without (●—●) sodium ascorbate (20 mM) and phenazine methosulfate (0.1 mM). Total volume of each incubation mixture was 100 μl. The incubations were continued at 25 °C for the indicated times, then terminated and assayed as described in Materials and Methods. The arrow indicates the time of addition of 1.7 mM unlabeled adenosine (○---○). (B) [^{3}H]Adenosine uptake by membrane vesicles of mutant cells (1.06 mg per ml) was assayed as described in A. (C) [^{3}H]Adenosine uptake by membrane vesicles of parent cells (0.93 mg per ml) was assayed as described in A except that the incubation mixtures contained phenazine methosulfate at a concentration of 0.2 mM, adenine (2 mM) and caffeine (20 mM). The arrow indicates the time of addition of 1.7 mM unlabeled adenosine ○---○) or 1.7 mM showdomycin (SHM) (○-·-○). (D) [^{3}H]Adenosine uptake by membrane vesicles of mutant cells (1.41 mg per ml) was assayed as described in A except that the incubation mixtures contained phenazine methosulfate at a concentration of 0.2 mM and adenine (2 mM). The arrow indicates the time of addition of 1.7 mM unlabeled adenosine (○---○) or 1.7 mM showdomycin (●-·-●).

TABLE I

EFFECT OF VARIOUS ELECTRON DONORS ON THE UPTAKE OF [^{3}H]ADENOSINE BY MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS

Membrane suspensions from the parent [1.49 (in Expt I) or 0.93 (in Expt II) mg membrane protein/ml] or mutant cells (1.41 mg/ml) were allowed to equilibrate at 25 °C for 6 (in Expt I) or 8 (in Expts II and III) min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM) and one of the electron donors being tested [each at a final concentration of 20 mM except phenazine methosulfate (PMS), which was at 0.1 (in Expt I) or 0.2 (in Expts II and III) mM] with adenine (2 mM) (in Expt III), with adenine (2 mM) and caffeine (20 mM) (in Expt II), or neither adenine and caffeine (in Expt I). Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined.

Electron donor added (mM)	*[^{3}H]Adenosine uptake (ratio)*			
	(Expt I) Parent	*(Expt II) Parent with adenine and caffeine*	*(Expt I) Mutant*	*(Expt III) Mutant with adenine*
None	1.00*	1.00**	1.00***	1.00†
PMS (0.1)+ascorbate (20)	5.70	—	3.08	—
PMS (0.2)+ascorbate (20)	—	14.64	—	4.28
D(−)-Lactate (20)	2.87	6.70	1.86	2.80
α-Glycerol phosphate (20)	2.56	2.93	1.29	2.72
NADH (20)	1.91	2.09	1.34	1.97
Succinate (20)	1.31	2.17	1.42	1.66

* 1.00= 15.11 pmoles/mg membrane protein per min.
** 1.00= 10.84 pmoles/mg membrane protein per min.
*** 1.00= 16.67 pmoles/mg membrane protein per min.
† 1.00= 9.89 pmoles/mg membrane protein per min.

inhibited the uptake of [8-^{3}H]adenosine by membrane vesicles from mutant cells (third column, line 4).

These results suggest that adenosine-uptake activity of membrane vesicles from the mutant cells is divisible into two different uptake systems: one (designated, for convenience's sake, as the second adenosine-uptake system) directly transports adenosine across the membrane and accumulates it intravesicularly, the other (designated, for convenience's sake, as the third adenosine-uptake system) rapidly degrades adenosine to adenine without transporting adenosine across the membrane, and then transports adenine across the membrane.

Even at a high concentration of caffeine (20 mM) significant amounts (21.9%) of [8-^{3}H]adenosine were taken up by the membrane vesicles from mutant cells (fourth column, line 5). This caffeine-resistant portion of adenosine uptake was markedly inhibited by the addition of adenine (2 mM) (fourth column, line 7) but only slightly inhibited by the addition of unlabeled adenosine (1 mM) (fourth column, line 6).

These results indicate that the second adenosine-uptake system is very sensitive to caffeine so that in the presence of caffeine (20 mM) the uptake of adenosine by the system is no longer observable.

TABLE II

EFFECT OF UNLABELED ADENOSINE, ADENINE AND CAFFEINE ON THE UPTAKE OF [8-^{3}H]ADENOSINE BY MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS

Membrane suspensions from mutant (1.41 mg membrane protein per ml) or parent cells (0.93 mg per ml) were allowed to equilibrate at 25 °C for 8 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), [8-^{3}H]adenosine [604.7 Ci/mole, 20.7 μM (in Expt I and III) or 904.9 Ci/mole, 22.2 μM (in Expt II)], sodium ascorbate (20 mM), phenazine methosulfate (0.2 mM) and neither, either, or both unlabeled adenosine (1 mM) and adenine (2 mM), with or without caffeine (20 mM). Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined.

Line	*Addition (mM)*	*[8-^{3}H]Adenosine uptake (%)*		
		(Expt I) Mutant	*(Expt II) Mutant*	*(Expt III) Parent*
1	None	100*	100**	100***
2	Adenosine (1)	78.3	—	47.6
3	Adenine (2)	63.5	—	—
4	Adenosine (1) + Adenine (2)	7.2	—	—
5	Caffeine (20)	—	21.9	49.7
6	Caffeine (20) + adenosine (1)	—	15.7	—
7	Caffeine (20) + adenine (2)	—	2.4	61.2
8	Caffeine (20) + adenosine (1) + adenine (2)	—	—	2.1

* 100= 70.72 pmoles/mg membrane protein per min.
** 100= 108.88 pmoles/mg membrane protein per min.
*** 100= 186.81 pmoles/mg membrane protein per min.

In the presence of caffeine (20 mM) and adenine (2 mM) the uptake of [8-^{3}H]-adenosine by membrane vesicles from mutant cells was no longer observable (fourth column, line 7), whereas under the same conditions large amounts (61.2%) of [8-^{3}H]-adenosine uptake by the membrane vesicles from parent cells was observed (fifth column, line 7). This adenine and caffeine-resistant portion of adenosine uptake was strongly inhibited by the addition of excess unlabeled adenosine (fifth column, line 8).

These results suggest that one more system for adenosine uptake (designated, for convenience's sake, as the first adenosine-uptake system) is present in membrane vesicles from parent cells, and that [8-^{3}H]adenosine uptake by this system is markedly reduced by unlabeled adenosine but not reduced by unlabeled adenine.

Properties of the first and the second adenosine-uptake systems

[8-^{3}H]Adenosine uptake by the membrane vesicles from parent cells in the presence of caffeine (20 mM) and adenine (2 mM) was compared with uptake by the membrane vesicles from mutant cells in the presence of adenine (2 mM) to obtain information on the first and second adenosine-uptake systems in the absence of each other.

Fig. 1C shows the time course of [8-^{3}H]adenosine uptake by membrane vesicles from parent cells in the presence of caffeine and adenine with or without ascorbate–phenazine methosulfate. [8-^{3}H]Adenosine was taken up with a higher initial rate

and to a higher internal concentration than in the absence of the electron donor system. The addition of excess unlabeled adenosine resulted in rapid loss of the majority of the accumulated radioactivity from preloaded membrane vesicles from parent cells.

Fig. 1D shows the time course of [8-^{3}H]adenosine uptake by the membrane vesicles from mutant cells in the presence of adenine with ascorbate–phenazine methosulfate. [8-^{3}H]Adenosine was taken up with a high initial rate and to a high internal concentration. The addition of excess unlabeled adenosine resulted in rapid loss of the majority of the accumulated radioactivity from preloaded membrane vesicles from the mutant cells.

TABLE III

EFFECT OF VARIOUS NUCLEOSIDES ON THE UPTAKE OF [8-^{3}H]ADENOSINE BY MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS UNDER VARIOUS CONDITIONS

Membrane suspensions of parent [1.49 (in Expt I) or 0.93 (in Expt II) mg membrane protein per ml] or mutant cells [1.06 (in Expt I) or 1.41 (in Expt III) mg per ml] were allowed to equilibrate at 25 °C for 10 (in Expt I) or 8 (in Expts II and III) min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), sodium ascorbate (20 mM), phenazine methosulfate [0.1 (in Expt I) or 0.2 (in Expts II and III) mM], [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM), and one of the nucleosides being tested (each at a final concentration of 1 mM) with adenine (2 mM) (in Expt II), with adenine (2 mM) and caffeine (20 mM) (in Expt III), or neither adenine and caffeine (in Expt I). Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined.

Nucleoside added (each 1 mM)	*[8-^{3}H]Adenosine uptake (%)*			
	(Expt I) Parent	*(Expt II) Parent with adenine and caffeine*	*(Expt I) Mutant*	*(Expt III) Mutant with adenine*
None	100*	100**	100***	100†
Adenosine	47.6	3.4	55.5	11.3
Deoxyadenosine	29.5	4.2	63.0	14.3
Guanosine	27.7	57.0	75.2	8.2
Deoxyguanosine	—	54.5	—	4.0
Inosine	58.7	43.7	68.9	17.9
Xanthosine	53.4	100.5	67.8	65.7
Uridine	100.6	5.7	103.7	44.8
Deoxyuridine	84.3	15.9	113.2	44.2
Cytidine	62.0	2.9	140.1	50.1
Deoxycytidine	60.3	14.7	78.4	44.0
Deoxythymidine	41.5	2.9	—	38.8
Showdomycin	—	16.8	—	82.1
Cytosine arabinoside	—	102.2	—	—
5-Iodouridine	—	2.8	—	—

* 100= 149.49 pmoles/mg membrane protein per min.
** 100= 114.32 pmoles/mg membrane protein per min.
*** 100= 64.75 pmoles/mg membrane protein per min.
† 100= 44.93 pmoles/mg membrane protein per min.

As summarized in Table I, physiological electron donors such as D(−)-lactate, α-glycerol phosphate, NADH and succinate caused similar though weaker stimulation of the uptake of adenosine by membrane vesicles from parent cells both in the presence of caffeine and adenine (third column), and by membrane vesicles from mutant cells in the presence of adenine (fifth column).

As shown in Table III, [8-^{3}H]adenosine uptake by the membrane vesicles from parent cells in the presence of adenine (2 mM) and caffeine (20 mM) was markedly inhibited by unlabeled adenine nucleosides, pyrimidine nucleosides, the artificial pyrimidine nucleoside iodouridine, and showdomycin, but was inhibited to a lesser degree by guanine nucleosides and inosine. Xanthosine and cytosine arabinoside did not inhibit the uptake at all (third column).

[8-^{3}H]Adenosine uptake by the membrane vesicles from mutant cells in the presence of adenine (2 mM) was markedly inhibited by unlabeled adenine nucleosides, guanine nucleosides and inosine but was inhibited to a lesser degree by pyrimidine nucleosides, xanthosine and showdomycin (Tabe III, fifth column).

Fig. 2B shows in detail the effect of showdomycin on the uptake of [8-^{3}H]-adenosine by membrane vesicles. Reduced phenazine methosulfate-stimulated uptake of [8-^{3}H]adenosine by the membrane vesicles from parent cells in the presence of adenine (2 mM) and caffeine (20 mM) was strongly inhibited by showdomycin,

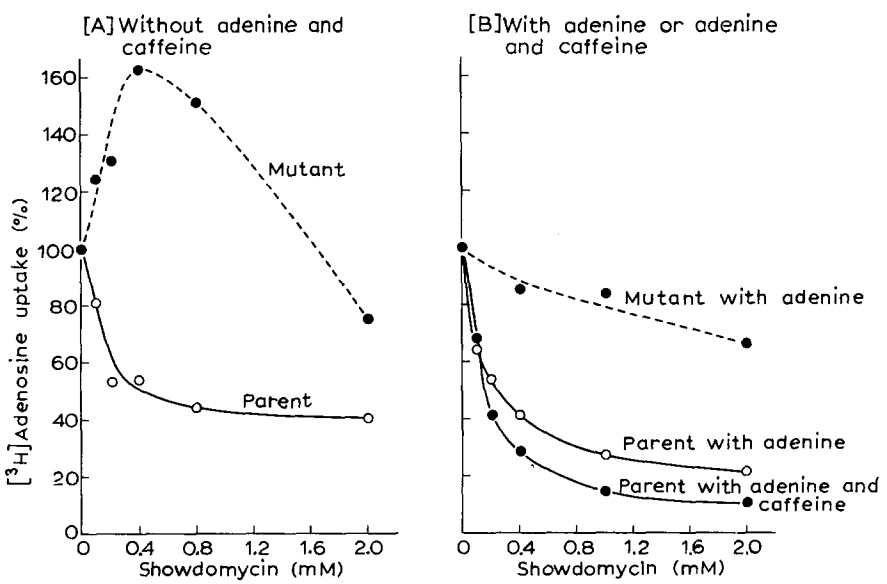

Fig. 2. Effect of showdomycin on the uptake of [^{3}H]adenosine under various conditions by membrane vesicles from parent and mutant cells. (A) Membrane suspensions of the parent (1.49 mg membrane protein per ml) or mutant cells (1.06 mg per ml) were allowed to equilibrate at 25 °C for 10 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), sodium ascorbate (20 mM), phenazine methosulfate (0.1 mM), [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM) and the cited amounts of showdomycin. Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined. (B) [^{3}H]Adenosine uptake by membrane vesicles of parent (0.93 mg per ml) or mutant cells (1.41 mg per ml) was assayed as described in A except that the incubation mixtures contained adenine (2 mM) or adenine (2 mM) and caffeine (20 mM).

whereas such uptake by the membrane vesicles from mutant cells in the presence of adenine (2 mM) was not greatly affected by the antibiotic. [8-^{3}H]Adenosine uptake by the membrane vesicles from parent cells in the presence of adenine (2 mM) and caffeine (20 mM) was not at all inhibited by *N*-ethylmaleimide, an analogue of the maleimide moiety of showdomycin (Table V, second column, line 2).

It can also be seen that the addition of showdomycin resulted in rapid loss of the majority of the accumulated radioactivity (derived from [8-^{3}H]adenosine) from the preloaded membrane vesicles from parent cells in the presence of adenine (2 mM) and caffeine (20 mM) (Fig. 1C). The addition of showdomycin to preloaded membrane vesicles from the mutant cells in the presence of adenine (2 mM) did not cause significant loss of the accumulated radioactivity (Fig. 1D).

As shown in Table IV, reduced phenazine methosulfate-stimulated [^{14}C]showdomycin-uptake activity was found exclusively in the membrane vesicles from parent cells, and this uptake activity was strongly inhibited by adenosine and deoxycytidine but only slightly inhibited by guanosine.

TABLE IV

[^{14}C]SHOWDOMYCIN UPTAKE BY THE MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS

Membrane suspensions of the parent (1.49 mg membrane protein per ml) or mutant cells (1.06 mg per ml) were allowed to equilibrate at 25 °C for 10 min, then 80-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration) and [^{14}C]showdomycin (1.90 Ci/mole, 104 μM) with or without additions indicated. Total volume of each incubation mixture was 200 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined. For further explanation, see text. PMS, phenazine methosulfate. Results are expressed as pmoles per mg membrane protein per min.

Addition (mM)	[^{14}C]*Showdomycin uptake*				
	Parent			*Mutant*	
	pmoles	*Ratio*	*%*	*pmoles*	*Ratio*
None	209.4	1.00	—	95.7	1.00
PMS (0.1) + ascorbate (20)	741.4	3.54	100	113.7	1.19
PMS (0.1) + ascorbate (20) + adenosine (1)	256.4	—	34.6	—	—
PMS (0.1) + ascorbate (20) + deoxycytidine (1)	267.0	—	36.0	—	—
PMS (0.1) + ascorbate (20) + guanosine (*2*)	655.8	—	88.5	—	—

[8-^{3}H]Adenosine uptake by the membrane vesicles from parent cells in the presence of adenine (2 mM) was stimulated by caffeine at higher concentrations, whereas at similar caffeine concentrations uptake by the membrane vesicles from mutant cells in the presence of adenine (2 mM) was strongly inhibited (Fig. 3).

As summarized in Table V, second column, [8-^{3}H]adenosine uptake by the membrane vesicles from parent cells in the presence of adenine (2 mM) and caffeine (20 mM) was resistant to sulfhydryl reagents such as *N*-ethylmaleimide, iodoacetic acid and *p*-chloromercuribenzoic acid at the concentrations examined. [8-^{3}H]Adenosine uptake by the membrane vesicles from mutant cells in the presence of adenine

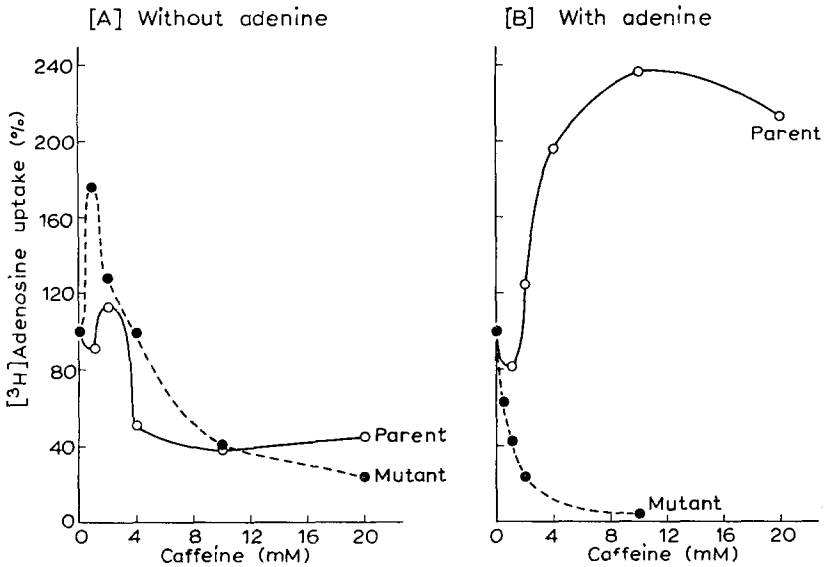

Fig. 3. Effect of caffeine on the uptake of [^{3}H]adenosine with or without adenine by membrane vesicles from parent and mutant cells. (A) Membrane suspensions of the parent (1.49 mg membrane protein per ml) or mutant cells (1.06 mg per ml) were allowed to equilibrate at 25 °C for 10 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), sodium ascorbate (20 mM), phenazine methosulfate (0.1 mM), [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM) and the cited amounts of caffeine. Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined. (B) [^{3}H]Adenosine uptake by membrane vesicles of parent (0.93 mg per ml) or mutant cells (1.41 mg per ml) was assayed as described in A except that the incubation mixtures contained phenazine methosulfate at a concentration of 0.1 mM and adenine (2 mM).

(2 mM) was moderately sensitive to iodoacetic acid and *p*-chloromercuribenzoic acid while it was considerably resistant to *N*-ethylmaleimide (Table V, third column).

[8-^{3}H]Adenosine uptake by the membrane vesicles from parent cells in the presence of adenine (2 mM) and caffeine (20 mM) and uptake by the membrane vesicles from mutant cells in the presence of adenine (2 mM) were almost completely inhibited by sodium azide at the concentration examined (Table V, line 5).

Existence of the second adenosine-uptake system in the membrane vesicles from parent cells

If the second adenosine-uptake system functions in the membrane vesicles from parent cells in addition to the first (and the third) adenosine-uptake system(s), then the uptake of adenosine by membrane vesicles from parent cells in the presence of excess adenine (2 mM) should consist of two components: one which is inhibited by guanosine and caffeine and another which is only slightly inhibited by these compounds. As shown in Fig. 4, uptake by the membrane vesicles from parent cells in the presence of adenine (without caffeine) decreased with increasing guanosine concentration to about 32% of the maximal value, no further decrease being observed. The guanosine-sensitive component was no longer observed when caffeine (20 mM) was added to the membrane vesicles.

TABLE V

EFFECT OF VARIOUS METABOLIC INHIBITORS ON THE UPTAKE OF [8-^{3}H]-ADENOSINE BY MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS UNDER VARIOUS CONDITIONS

Membrane suspensions from the parent (0.93 mg membrane protein per ml) or mutant cells (1.41 mg per ml) were allowed to equilibrate at 25 °C for 8 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), sodium ascorbate (20 mM), phenazine methosulfate (0.2 mM), the cited amounts of the metabolic inhibitor being tested and [8-^{3}H]-adenosine (604.7 Ci/mole, 20.7 μM) or L-[U-^{14}C]proline (260.0 Ci/mole, 17.5 μM), with adenine (2 mM) (in Expt II), with adenine (2 mM) and caffeine (20 mM) (in Expt I), or neither adenine and caffeine (in Expt III). Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined. Results are expressed as pmoles per mg membrane protein per min. NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid.

Inhibitor added (mM)	[^{3}H]*Adenosine uptake*			[^{14}C]*Proline uptake*	
	(Expt I) Parent with adenine and caffeine	*(Expt II) Mutant with adenine*	*(Expt III) Mutant*	*(Expt III) Parent*	*(Expt III) Mutant*
	pmoles (%)	*pmoles* (%)	*pmoles* (%)	*pmoles* (%)	*pmoles* (%)
None	106.9 (100)	42.2 (100)	75.0 (100)	262.7 (100)	187.3 (100)
NEM (2)	110.4 (103)	37.3 (88)	98.0 (131)	86.7 (33)	60.2 (32)
Iodoacetic acid (2)	94.7 (89)	23.2 (55)	123.1 (164)	— —	— —
PCMB (0.1)	111.6 (104)	25.0 (59)	76.3 (102)	— —	— —
Sodium azide (50)	3.6 (3)	2.5 (6)	23.5 (31)	— —	— —

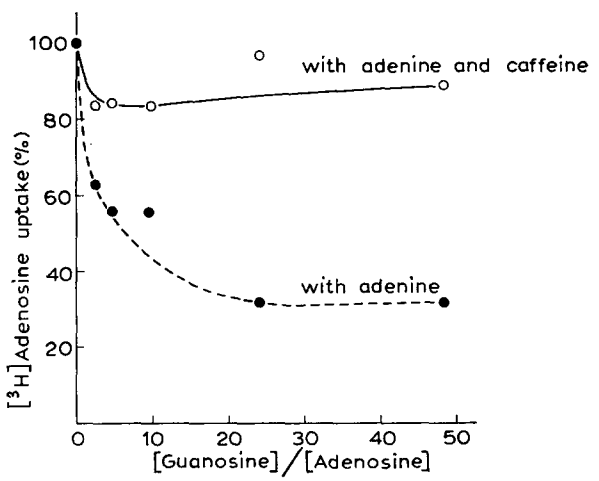

Fig. 4. Effect of guanosine on the uptake of [^{3}H]adenosine by membrane vesicles from parent cells in the presence of adenine or adenine and caffeine. Membrane suspensions of the parent cells (0.93 mg membrane protein per ml) were allowed to equilibrate at 25 °C for 8 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), sodium ascorbate (20 mM), phenazine methosulfate (0.2 mM), adenine (2 mM), [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM) and the cited amounts of guanosine. The incubation mixtures were with (○—○) or without (●---●) caffeine (20 mM). Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined.

Properties of the third adenosine-uptake system

To determine whether purine nucleoside phosphorylase (EC 2.4.2.1) is present on our membrane vesicles, the fate of ^3H label was examined after the membrane vesicles had been exposed to [8-^{3}H]adenosine under the conditions where uptake of [8-^{3}H]adenosine by membrane vesicles took place (Table VI). It can be seen that rapid conversion of adenosine to adenine occurred in membrane vesicles from both parent and mutant cells, regardless of the presence of reduced phenazine methosulfate.

TABLE VI

CLEAVAGE OF ADENOSINE TO ADENINE BY MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS

Membrane suspensions from the parent (1.49 mg membrane protein per ml) or mutant cells (1.06 mg per ml) were allowed to equilibrate at 25 °C for 10 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), [8-^{3}H]adenosine (604.7 Ci/mole, 20.7 μM) and additions indicated. Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the incubation mixtures were quickly pipetted into 400 μl ice-cold methanol. The supernatants (40 μl each) were applied on strips of Whatman 3MM paper and chromatographed with solvent III at 22 °C for 4 h by the ascending method. For further explanation, see text.

Source of membrane vesicles	*Addition (mM)*	*[^{3}H]Adenine formed (%)**	*[^{3}H]Adenosine remaining (%)**
Parent	None	4.1	92.3
Parent	PMS (0.1) + ascorbate (20)	5.3	83.7
Mutant	None	3.9	92.1
Mutant	PMS (0.1) + ascorbate (20)	6.5	80.9

* % of total count.

To determine whether group translocation of adenine mediated by membrane adenine phosphoribosyltransferase[7] (EC 2.4.2.7) is present in our membrane vesicles or whether a respiration-linked adenine transport system is present, the effects of 5-phosphoribosyl pyrophosphate, a substrate for adenine phosphoribosyltransferase, and ascorbate–phenazine methosulfate, the artificial electron donor system for various respiration-linked transport systems, on the uptake of [8-^{14}C]adenine by membrane vesicles from both parent and mutant cells were examined (Table VII). As can be observed from the data, 5-phosphoribosyl pyrophosphate alone has essentially no effect on the uptake of adenine by membrane vesicles from both parent and mutant cells (second and third column, line 2). However, ascorbate–phenazine methosulfate markedly stimulated the uptake of adenine by these membrane vesicles as well as that of adenosine by the first and second adenosine-uptake system (line 3).

As indicated in Table III, [8-^{3}H]adenosine uptake by membrane vesicles from mutant cells in the presence of adenine was strongly inhibited by unlabeled adenine nucleosides, guanine nucleosides and inosine (fifth column), whereas uptake by the same membrane vesicles in the absence of adenine was only slightly inhibited by these nucleosides, and was not inhibited at all by pyrimidine nucleosides (fourth column).

TABLE VII

EFFECT OF 5-PHOSPHORIBOSYL PYROPHOSPHATE AND REDUCED PHENAZINE METHOSULFATE ON THE UPTAKE OF [8-^{14}C]ADENINE AND [8-^{3}H]ADENOSINE BY MEMBRANE VESICLES FROM PARENT AND MUTANT CELLS

In Expt I membrane suspensions from the parent (0.93 mg membrane protein per ml) or mutant cells (1.41 mg per ml) were allowed to equilibrate at 25 °C for 8 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM final concentration), [8-^{14}C]adenine (58.0 Ci/mole, 20.0 μM) and neither, either, or both 5-phosphoribosyl pyrophosphate (10 mM) and ascorbate (20 mM)-phenazine methosulfate (0.2 mM). In Expts II, III and IV membrane suspensions from the parent (0.93 mg per ml) or mutant cells (1.41 mg per ml) were allowed to equilibrate at 25 °C for 8 min, then 40-μl samples were added to tubes containing $MgSO_4$ (10 mM), [8-^{3}H]adenosine [604.7 Ci/mole, 20.7 μM (in Expts II and III) or 505.3 Ci/mole, 19.8 μM (in Expt IV)] and neither, either, or both 5-phosphoribosyl pyrophosphate (10 mM) and ascorbate (20 mM)-phenazine methosulfate (0.2 mM) with adenine (2 mM) (in Expt III), with adenine (2 mM) and caffeine (20 mM) (in Expt II), or neither adenine and caffeine (in Expt IV). Total volume of each incubation mixture was 100 μl. After a 1-min incubation at 25 °C, the membrane vesicles were filtered, washed, and their total radioactivity was determined. PRPP, 5-phosphoribosyl pyrophosphate; PMS, phenazine methosulfate.

Addition (mM)	[^{14}C]*Adenine uptake*		[^{3}H]*Adenosine uptake*		
	(Expt I) Parent	*(Expt I) Mutant*	*(Expt II) Parent with adenine and caffeine*	*(Expt III) Mutant with adenine*	*(Expt IV Mutant*
None	1.00†	1.00††	1.00*	1.00**	1.00***
PRPP (10)	1.27	1.00	1.86	1.16	1.87
PMS (0.2) + ascorbate (20)	7.95	5.57	14.64	4.28	6.84
PMS (0.2) + ascorbate (20) + PRPP (10)	8.16	14.47	11.99	3.37	11.21

† 1.00= 34.76 pmoles/mg membrane protein per min.
†† 1.00= 31.98 pmoles/mg membrane protein per min.
* 1.00= 10.84 pmoles/mg membrane protein per min.
** 1.00= 9.89 pmoles/mg membrane protein per min.
*** 1.00= 7.00 pmoles/mg membrane protein per min.

[8-H^3]Adenosine uptake by membrane vesicles from mutant cells in the presence of adenine was inhibited by iodoacetic acid (Table V, third column, line 3) and caffeine (Fig. 3B), whereas at similar concentrations uptake by the same membrane vesicles in the absence of adenine was stimulated by iodoacetic acid (Table V, fourth column, line 3) and caffeine (Fig. 3A).

DISCUSSION

Three different systems for adenosine uptake have been found in isolated membrane vesicles from *Escherichia coli* K-12 cells.

Adenosine uptake by the first adenosine-uptake system exhibits similar properties with those demonstrated previously for respiration-linked transport systems of amino acids and sugars[8–15].

Kaback[16] reported that the transport systems of lactose and amino acids by

membrane vesicles from *Escherichia coli* cells are sensitive to sulfhydryl reagents such as *p*-chloromercuribenzoic acid and *N*-ethylmaleimide even in the presence of ascorbate–phenazine methosulfate. The uptake of adenosine by the first adenosine-uptake system was very resistant to these sulfhydryl reagents in the presence of ascorbate–phenazine methosulfate, adenine and caffeine (Table V). This indicates either that, unlike carriers for sugar and amino acids, the adenosine carrier in the first adenosine-uptake system does not contain a functional sulfhydryl group, or that access of these sulfhydryl reagents to a functional sulfhydryl group present is hindered.

The uptake of [8-^{3}H]adenosine by the first adenosine-uptake system was strongly inhibited by both the ribosides and 2′-deoxyribosides of adenine and a wide variety of pyrimidine bases. This suggests that replacement of the 2′-hydroxyl group of the ribose moiety in the nucleosides by hydrogen is fairly well tolerated by the transport system of the first adenosine-uptake system. However, inversion of that group is not tolerated by the system, since cytosine arabinoside (the epimer of cytidine) did not inhibit the uptake of [8-^{3}H]adenosine at all. Guanine nucleosides and inosine were all less inhibitory to [8-^{3}H]adenosine uptake, and xanthosine was not inhibitory at all. This suggests that, although the nature of the aglycone moiety in nucleosides is generally not particularly critical for affinity for the transport system, one exception is the low tolerance to replacement of the 6-amino group in the adenine moiety by a hydroxyl group. The transport system is tolerant to a bulky group at the position 5 in the uracil moiety, since 5-iodouridine inhibited the [8-^{3}H]adenosine uptake quite strongly. These properties are very like those observed with the uptake system of showdomycin in intact cells[1].

The adenosine transport system of the first adenosine-uptake system has a high affinity for showdomycin and can transport the antibiotic across the membrane.

The first adenosine-uptake system was no longer observed in the membrane vesicles from the showdomycin-resistant mutant (Shm^r-001) cells (Table II and Fig. 2B).

Properties observed for the first adenosine-uptake system are very like those observed for the first deoxycytidine-uptake system[4]. Thus, it is very likely that the transport system of the first adenosine-uptake system and that of the first deoxycytidine-uptake system are identical or share a common component(s). It would seem that in the membrane vesicles from the showdomycin-resistant mutant (Shm^r-001) cells this common component(s) has been lost or inactivated.

Adenosine uptake by the second adenosine-uptake system also exhibits properties similar to those demonstrated previously for respiration-linked transport systems[8–15] (Tables I and V).

Unlike the adenosine transport system of the first adenosine-uptake system, that of the second adenosine-uptake system would seem to have a high affinity for adenine nucleosides, guanine nucleosides and inosine but a low affinity for pyrimidine nucleosides and showdomycin.

Properties observed for the second adenosine-uptake system are like those observed for the second deoxycytidine-uptake system[4]. Thus, it is likely that the transport system of the second adenosine-uptake system and that of the second deoxycytidine-uptake system are identical or share a common component(s). This system remained active in the membrane vesicles from the showdomycin-resistant mutant (Shm^r-001) cells.

In conclusion then, it can be postulated that at least two transport systems which are common to a wide variety of nucleosides and are linked to the respiration chain are present in cytoplasmic membrane of *Escherichia coli* K-12: one (designated as adenine-pyrimidine nucleoside transport system) has a high affinity for adenine nucleosides, pyrimidine nucleosides and showdomycin, but a low affinity for guanine nucleosides, inosine and xanthosine; the other (designated as purine nucleoside transport system) has high affinity for adenine nucleosides, guanine nucleosides and inosine, but a low affinity for pyrimidine nucleosides, xanthosine and showdomycin. It should be noted that adenine nucleosides can be transported very effectively by both systems. The adenine–pyrimidine nucleoside transport system was lost and the purine nucleoside transport system remained active in the membrane vesicles from the showdomycin-resistant mutant (Shm^r-001) cells.

Caffeine stimulated adenosine uptake by the first adenosine-uptake system, whereas it markedly inhibited uptake by the second adenosine-uptake system (Fig. 3B). It has been postulated by Peterson and Koch[17] that caffeine is a competitive inhibitor of the inosine transport mechanism in *Escherichia coli*. The main transport system for inosine would seem to be the purine nucleoside transport system. Therefore, it is postulated that caffeine causes a competitive inhibition of adenosine entry by the purine nucleoside transport system into the membrane vesicles, resulting in the marked inhibition of adenosine uptake by the second adenosine-uptake system, and that caffeine also causes a competitive inhibition of the efflux of adenosine (which entered into the membrane vesicles by the adenine–pyrimidine nucleoside transport system) from the membrane vesicles by the purine nucleoside transport system, resulting in the marked stimulation of adenosine accumulation. Other mechanisms of inhibition, however, cannot completely be excluded as possible causes of the inhibition by caffeine, since a high concentration (20 mM) of caffeine caused significant inhibition of v-[^{14}C]proline uptake by the membrane vesicles from parent cells (data not shown).

Properties observed for the third adenosine-uptake system resemble Hochstadt-Ozer's findings[7] with respect to the first reaction (extramembranal cleavage of adenosine to adenine by membrane purine nucleoside phosphorylase). However, the second reaction (group translocation of adenine by membrane adenine phosphoribosyltransferase) was not observed with our membrane vesicles (Table VII).

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