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Nucleoside Transport in Cells and Membrane Vesicles from *Escherichia coli* K12*

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Osmotic shock treatment of cells of *Escherichia coli* K12 caused a reduction in the transport of nucleosides into the cells. The strains used carried mutations in the nucleoside catabolizing enzymes. This indicated that the decrease in transport capacity was not due to loss of these enzymes during the shock treatment.

Membrane vesicles, prepared from the same strains, showed a limited transport of cytidine, deoxycytidine, and uridine. Transport of purine nucleosides and of thymidine was very low in vesicles lacking the appropriate nucleoside phosphorylases and no significant stimulation was observed if the nucleoside phosphorylases were present in the membrane vesicles.

These results all indicate that components outside the cytoplasmic membrane are important for nucleoside transport.

Selection for resistance to fluorodeoxycytidine yielded mutants which were unable to transport any nucleoside, even when the nucleoside phosphorylases were present in high amounts. This finding is consistent with a requirement for a specific transport process prior to the initial enzymatic attack on the incoming nucleoside.

Nucleosides are readily taken up and catabolized by cells of *Escherichia coli*. It was shown previously that an energy-requiring nucleoside transport may take place independent of subsequent catabolism (1). This was demonstrated in mutant cells, unable to metabolize the nucleosides, and concentrating effects of 300- to 500-fold were estimated for uridine and cytidine (1).

Several lines of evidence have indicated the existence of at least two different nucleoside transport systems in *E. coli* (2-5). For further reference see Ref. 5. The two systems are termed the *nupC* and the *nupG* systems[1, 2] and they may be distinguished by their different resistance to the uridine analogue showdomycin, since the *nupC* system is strongly inhibited by this antibiotic while the *nupG* system is not.

Gene(s) coding for the *nupC* system have been located at 50 min on the revised *E. coli* chromosome, 65% co-transducible with *ptsI*.[3] This system transports nucleosides except guanosine (and deoxyguanosine) and it is regulated (5) by the *cytR* gene product, which is also a repressor in the control of the nucleoside-catabolizing enzymes, listed in Table I (6).

Gene(s) coding for the *nupG* system map at 64 min on the revised *E. coli* chromosome, 25% co-transducible with *metC*.[3] This system is subject to a double control, since it is regulated by the *cytR* and the *deoR* gene products (5). The *deoR* repressor also participates in the control of the nucleoside-catabolizing enzymes (6).

Komatsu and Tanaka (4, 7) demonstrated the transport of adenosine and deoxycytidine in membrane vesicles of *E. coli* K12, wild type, or a showdomycin-resistant mutant. Similarly, Hochstadt and co-workers (8, 9) prepared vesicles from *Salmonella typhimurium*, which were able to transport uridine, inosine, and adenosine. In this work, it was suggested that the transport consisted in a group translocation of the nucleoside, catalyzed by the appropriate nucleoside phosphorylase, *i.e.* uridine phosphorylase or purine nucleoside phosphorylase.

On the other hand, there is evidence that extensive transport of nucleosides takes place in strains lacking the nucleoside phosphorylases (1, 5). Moreover, a mutant was described which was unable to transport nucleosides although it contained normal amounts of the nucleoside-catabolizing enzymes (5). This indicated separate systems for transport and catabolism, and further evidence for such separate systems is presented below. There is, however, an intimate coupling between transport and catabolism of nucleosides, and for reasons mentioned below this coupling has been a serious complication in the detailed measurements of nucleoside transport.

The main feature in the catabolism of nucleosides is a phosphorolytic cleavage of the *N*-glycosidic bond between the base and pentose moieties of the nucleosides. In these reactions, which are catalyzed by specific phosphorylases, a pentose phosphate is formed, which is then channelled into the pentose metabolism, while the bases may be used for synthesis of nucleoside monophosphates. This synthesis requires 5'-phosphoribosyl-1-pyrophosphate and is catalyzed by specific phosphoribosyltransferases. If the strains lack the appropriate phosphoribosyltransferase, the base is quantitatively excreted, but even in the presence of the enzyme a substantial amount is lost to the medium (1).

As an example, the catabolism of cytidine and uridine is shown in Fig. 1. In the case of cytidine (and deoxycytidine), a

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[1] These genes were previously termed *cru* and *gru*, respectively (4).

[2] The abbreviations and symbols used are:

nupC	gene(s) coding for the system, which transports nucleosides except guanosine
nupG	gene(s) coding for the system which transports all nucleosides
cytR	regulatory gene for the following enzymes: cytidine deaminase, uridine phosphorylase, deoxyriboaldolase, thymidine phosphorylase, deoxyribomutase, and purine nucleoside phosphorylase
deoR	regulatory gene for the *deo* enzymes: deoxyriboaldolase, thymidine phosphorylase, deoxyribomutase, and purine nucleoside phosphorylase
DNP	2,4-dinitrophenol
FdCyd	5-fluoro 2'deoxycytidine
FdUrd	5-fluoro 2'deoxyuridine
PMS	phenazine methosulfate
PRPP	phosphoribosyl-1-pyrophosphate

[3] B. Mygind, unpublished.

TABLE I

Enzymes, catalyzing the initial catabolic attack on exogenously added nucleosides

Nucleoside added	Initial enzyme reaction	Enzyme
Cytidine	Deamination (to uridine)	Cytidine deaminase
Deoxycytidine	Deamination (to deoxyuridine)	Cytidine deaminase
Uridine	Phosphorolysis	Uridine phosphorylase
Adenosine	Deamination (to inosine) or phosphorolysis	Adenosine deaminase Purine nucleoside phosphorylase
Guanosine	Phosphorolysis	Purine nucleoside phosphorylase
Inosine	Phosphorolysis	Purine nucleoside phosphorylase
Thymidine	Phosphorolysis	Thymidine phosphorylase
Deoxyuridine	Phosphorolysis	Thymidine phosphorylase

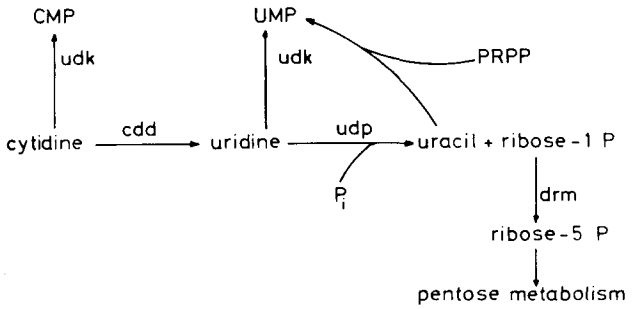

FIG. 1. Metabolism of cytidine (and uridine) in *E. coli*.

deamination reaction must occur, before the phosphorolytic cleavage takes place. A deamination of adenosine may also occur initially, but is not required for the phosphorolysis to take place.

Table I schematizes the primary enzymic attacks exerted by the cells on exogenously added nucleosides. The enzymes are all inducible and, with the exception of adenosine deaminase, they are regulated together in two complex control systems, governed by the *cytR* and *deoR* genes (6).

The phosphorolytic attack on exogenously added nucleosides takes place with a high velocity, probably due to a localization of the enzymes at or near the cell surface (10–13). The above mentioned partial or complete excretion of the bases occurs with a similar high velocity (1), and this tends to disturb the standard measurements of transport rates, since in the commercially available ^{14}C-labeled nucleosides the bases always carry a radioactive label. A variable amount of counts is therefore lost from the cells into the medium when the nucleosides are cleaved phosphorolytically. For this reason, strains with point mutations or deletions in the nucleoside phosphorylases have been used in our previous experiments whenever possible. Likewise, the strains contained mutations in the cytidine-uridine kinase (1, 5).

In the present study, such mutants have been used to show that osmotic shock treatment of the cells reduces the ability of the cells to transport nucleosides. Membrane vesicles, prepared from the same strains, are shown to be capable of a limited transport of nucleosides. This transport is only slightly stimulated, if the vesicles are prepared from strains containing the appropriate nucleoside phosphorylases.

Results with a transport negative mutant containing the enzymes support the contention that for significant utilization of the nucleosides by the cells a transport process must occur prior to catabolism.

EXPERIMENTAL PROCEDURES

Materials—Nucleosides, showdomycin, and other fine chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Radioactive isotopes were from the Radiochemical Centre Ltd. (Amersham, England).

Isolation of Mutants and Growth of Cells—The strains employed are listed in Table II. Mutants containing the *nupC* system or the *nupG* system were obtained by mating and recombination in a transport negative strain Sϕ 587 as reported previously (5).

In the isolation of the mutant strains, used for uptake measurements, advantage was taken of the map position of the two regulatory genes, *cytR* and *deoR*, close to *metB* and *clmA*, respectively (6).

Strain Sϕ 1130 was isolated from strain Sϕ 430 *cdd, metB, udk* (14) in the following way: a showdomycin-resistant derivative Sϕ 659 was selected and used as recipient in a mating with Sϕ 818, *HfrCavalli, cytR*, Δ*deo*, selecting for Met$^+$. A *cytR*, showdomycin-resistant recombinant was mated with Sϕ 694 *Hfr H, clmA, deoR* (15), selecting for resistance to chloramphenicol (7.5 μg/ml). Sϕ 1130 was a recombinant from this mating with the genotype *cdd, udk, cytR*, Δ*deo, nupC*.

Sϕ 1131 was derived from Sϕ 659. A mutant, resistant to FdUrd was first isolated. This strain, which was negative for transport of all nucleosides, was mated with Sϕ 694, selecting for growth on thymidine as a carbon source. A chloramphenicol-resistant, *deoR* recombinant was used as a recipient for the introduction of *cytR* and Δ*deo* mutations by mating with Sϕ 818, selecting for Met$^+$.

Sϕ 1138, Δ*deo, tsx*, and Sϕ 1139, Δ*deo* were constructed by mating

TABLE II

List of strains

Strain no.	Relevant genotype	Source or reference
Sϕ 587	F$^-$, *argA, lysA, metB, upp, udk, nupC, nupG*	(5)
Sϕ 910	F$^-$, *proA, thr, tsx*	Identical to AB 1157 (16)
Sϕ 285	F$^-$, *upp, udk, nupC, cytR, deoR*, Δ*deo*[a]	Identical to AM 234 (5)
Sϕ 861	F$^-$, *upp, udk, nupC, nupG,*[b] *cytR, deoR*, Δ*deo*	Sϕ 285, resistant to FdCyd (10 μg/ml)
Sϕ 1135	F$^-$, *upp, udk, nupC, nupG,*[b] *cytR, deoR*	Sϕ 694 × Sϕ 861
Sϕ 304	F$^-$, *upp, udk, nupG, cytR, deoR*, Δ*deo*	Identical to AM 240 (5)
Sϕ 1145	F$^-$, *upp, udk, nupC,*[b] *nupG, cytR, deoR*, Δ*deo*	Sϕ 304, resistant to FdCyd
Sϕ 1132	F$^-$, *upp, udk, nupC, cytR, deoR*, Δ*deo*	Isolated like Sϕ 285
Sϕ 1133	F$^-$, *upp, udk, nupC, cytR, deoR*	Sϕ 694 × Sϕ 1132
Sϕ 1130	F$^-$, *cdd, udk, nupC, cytR, deoR*, Δ*deo*	See text
Sϕ 1131	F$^-$, *cdd, udk, nupG,*[b] *cytR, deoR*, Δ*deo*	See text
Sϕ 1142	*HfrH, upp, udk, udp, tpp, cytR, deoR*	See text
Sϕ 1138	F$^-$, *tsx*, Δ*deo*	See text
Sϕ 1139	F$^-$, Δ*deo*	See text
Sϕ 694	*HfrH, clmA, deoR*	(15)
Sϕ 818	*HfrCavalli, cytR*, Δ*deo*	
Sϕ 1137	*HfrCavalli*, Δ*deo*	

[a] This deletion covers the genes for thymidine phosphorylase, deoxyribomutase, and purine nucleoside phosphorylase. The original strain, harboring this deletion, was a gift from Dr. V. Sukhodoletz (Moscow).

[b] The FdCyd resistance mutations behave phenotypically as *nupG* or *nupC* mutations but they have not been characterized genetically.

of Sϕ 910 (16) with Sϕ 1137 as donor, selecting for Pro^+ and Thr^+.

The *udp* mutation in strain Sϕ 1142 was obtained in a *tpp, upp, cytR* background selecting for resistance to fluorouracil (2.5 μg/ml) + adenosine (200 μg/ml) (17). The *deoR* mutation was introduced by mating with Sϕ 694, *Hfr, deoR*.

The strains were grown routinely in phosphate-buffered minimal medium: $(NH_4)_2SO_4$ (15 mM), Na_2HPO_4 (42 mM), NaCl (50 mM), KH_2PO_4 (22 mM), $MgCl_2$ (2 mM), $CaCl_2$ (0.1 mM), and $FeCl_3$ (0.003 mM) and supplemented with the necessary nutritional requirements. Glycerol (0.2%) was used as a carbon source unless otherwise stated, and 1% Luria broth was routinely added to all growth media.

Mating and recombination procedures were as described by Miller (18).

Osmotic Shock Treatment—Cells were grown in minimal medium, containing 0.2% glycerol, and harvested in the exponential phase of growth. The procedure for osmotic shock treatment was that described by Heppel (19) for cells in the exponential phase. The EDTA concentration in the sucrose solution was 10^{-4} M. Osmotic shock treatment was carried out with distilled H_2O.

Preparation of Membrane Vesicles—Vesicles were prepared from strains containing either of the two nucleoside transport systems. Preliminary experiments had indicated that nucleoside transport was most efficient in vesicles prepared from strains with mutations in the two regulatory genes, *cytR* and *deoR*, which have been shown to control the nucleoside transport systems as well as the synthesis of the nucleoside-catabolizing enzymes. These two regulatory mutations were therefore introduced in all strains used.

Membrane vesicles were also prepared from two transport negative strains, Sϕ 861, containing a deletion mutation covering the purine nucleoside phosphorylase and thymidine phosphorylase genes, and Sϕ 1135, a derivative of Sϕ 861, containing the intact enzyme genes.

The cells were grown in minimal medium with glycerol (0.2%) as a carbon source to a density of approximately 10^9 cells/ml. They were then harvested by centrifugation and vesicles were prepared according to Kaback (20) (omitting the final sucrose gradient centrifugation). The vesicle suspensions were stored in plastic vials in liquid nitrogen. Before each experiment, the vesicle suspension was thawed at room temperature and allowed to equilibrate 5 to 10 min.

Transport Experiments—Unless otherwise stated, the nucleosides used for transport experiments were uniformly ^{14}C-labeled. Specific activities were the following: [*U*-^{14}C]deoxycytidine (95 Ci/mol), [*U*-^{14}C]cytidine (88 Ci/mol), [*U*-^{14}C]uridine (95 Ci/mol), [*U*-^{14}C]adenosine (87 Ci/mol), [*U*-^{14}C]thymidine (54 Ci/mol), [*U*-^{14}C]proline (290 Ci/mol), and [2-^{14}C]uridine (60 Ci/mol).

Transport in whole cells was measured as described previously (1). The substrate concentrations were 1.7 μM.

Transport in vesicles was measured at room temperature at substrate concentrations of 3 to 4 μM. The artificial electron donor system phenazine methosulfate + ascorbate (final concentration of 0.15 mM and 30 mM, respectively) was added immediately before the experiment to a mixture of labeled substrate, potassium phosphate buffer (0.1 M, pH 6.5) and $MgSO_4$ (0.1 M). At time zero, the vesicle suspension (final concentration 0.4 to 0.8 mg of protein/ml) was added to give a total volume of 200 μl, the mixture was vigorously shaken, and, at the times indicated, samples of 50 μl were pipetted into 3 ml of cold LiCl (0.1 M) and immediately filtered through Millipore filters (pore size 0.45 μ).

When inhibition by *N*-ethylmaleimide or DNP was tested, the vesicle suspension was preincubated 5 min with the inhibitor before the transport assay. Other inhibitors were present in the substrate mixture, when the vesicle suspension was added.

The filters were washed with 3 × 1 ml of LiCl, dried 1 to 2 h at 65°C, and counted in a Packard Tri-Carb liquid scintillation spectrometer model 3375, using Permablend III in toluene as scintillation liquid.

Identification of Intracellular Nucleosides—For this purpose, cell suspensions were exposed to nucleosides of high specific activities (400 to 600 Ci/mol) under the usual assay conditions. At 40 s, 2 aliquots were filtered and washed. One filter was dried and counted directly as a control while the other was immediately extracted with 200 μl of chilled 0.33 M formic acid. The extract was centrifuged and 50-μl aliquots were chromatographed on polyethyleneimine cellulose plates, using H_2O (for guanosine and thymidine) or *n*-butyl alcohol:H_2O (86:14) (for cytidine, deoxycytidine, and uridine) as separating solvents. The radioactive spots were located by autoradiography, cut out, and counted. The yield of the formic acid extraction of the membrane filters was 87 to 93% of the total radioactivity, measured in the control filter.

Enzyme Assays—The procedures used for enzyme assays on cell extracts have been described previously (21), and these procedures were also applied when the enzymes were determined at the different stages during preparation of the vesicles. The suspensions of spheroplasts and of membrane vesicles were subject to sonication before the assays in order to release the enzymes, while the centrifuged cell lysates and the supernatants from spheroplast formation were used directly in the assay mixture.

One enzyme unit is defined as the amount of enzyme which converts 1 nmol of substrate per min at 37°C.

RESULTS

Transport and Accumulation of Free Nucleosides

The strains used in the present work contain various mutations in the enzymes which catalyze nucleoside metabolism (cf. Table II). The chemical state of the intracellular nucleosides after transport was identified for each nucleoside in strains carrying the appropriate mutation. Uridine was measured in Sϕ 1142, and deoxycytidine, cytidine, guanosine, and thymidine in strain Sϕ 1130 *cdd, udk, Δdeo*. Sϕ 1142 cannot metabolize uridine, while Sϕ 1130 is inert towards deoxycytidine and cytidine, besides being unable to phosphorolyze guanosine and thymidine. No mutations in the guanosine kinase or the thymidine kinase genes have been available, but, as shown in Table I, very little of these nucleosides is converted to phosphorylated compounds during the time interval of the transport assay. Cytidine, deoxycytidine, and uridine appear nearly intact.

Parallel experiments were performed with cells pretreated with dinitrophenol (1 mM). These cells accumulated less than 10% of the activity found in the control cells and chromatography of the cell extracts did not yield discrete radioactive spots. Thus, the nucleosides may be transported and accumulated in a chemically intact state, provided the cells carry mutations in the appropriate catabolizing enzymes.

Function of Components Outside the Cytoplasmic Membrane in Nucleoside Transport

There have been reports that components outside the cytoplasmic membrane participate in the transport of nucleosides. Thus, the T_6 receptor protein (the *tsx* gene product) stimulates the utilization of certain nucleosides (16, 22). The specificity of this system is seen in Fig. 2. All nucleosides tested except cytidine and deoxycytidine are affected by the absence of the *tsx* product. The effect is most pronounced when nucleosides are present at low (0.1 μM) concentration. Strains lacking the *tsx* gene product grow well on agar plates with nucleosides as sole carbon sources (1 mM concentration), provided the necessary enzymes for nucleoside catabolism are present in the cells (22).

Osmotic shock treatment, carried out according to the procedure of Heppel (19), causes a reduction in the transport of all nucleosides. Two strains, Sϕ 1130 and Sϕ 1131, containing the *nupG* and the *nupC* system, respectively, were investigated and both systems were impaired (Table IV). Transport

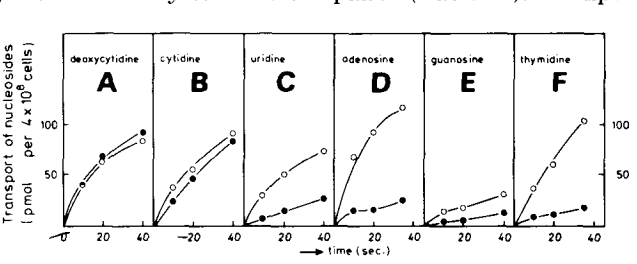

FIG. 2. Transport of different nucleosides into cells with or without a functional T_6 receptor protein. Transport assay was carried out at standard conditions, except that the substrates were present in 10^{-7} M concentrations. ○—○, wild type strain; ●—●, *tsx* mutant.

of glycine before and after osmotic shock treatment was used as a control, since transport of this amino acid occurs independent of shock-releasable proteins (23). The rate of glycine transport is reduced approximately 50% after the shock treatment (Table IV). This probably reflects partial damage of cells, while the more extensive (80 to 90%) reduction of nucleoside transport suggests that the shock treatment releases factors which are essential for the transport of nucleosides.

Osmotic shock treatment causes partial release of the nucleoside-catabolizing enzymes (5), but since the strains used in the experiments in Table III carry mutations in these enzymes, the reduced transport cannot be ascribed to a loss of the enzymes during the shock procedure.

Enzymatic Properties of Membrane Vesicles

Routinely, the strains used for transport measurements harbored a deletion in the genes which code for thymidine phosphorylase and purine nucleoside phosphorylase. Some of the strains also contained mutations in cytidine deaminase or uridine phosphorylase (Table II). It has been suggested, however, that nucleoside phosphorylases perform transport functions in membrane vesicles through a group translocation process (8, 9). To test the influence of the enzymes on transport, membrane vesicles also were prepared from a strain containing the *nupG* system and carrying the intact genes for the nucleoside phosphorylases.

Due to the *cytR* and *deoR* gene mutations, the enzymes, when present, were synthesized in maximally derepressed amounts. These enzymes are soluble proteins and it seemed important to know to what extent they were retained in the final vesicle preparations. Table V shows the distribution of enzyme activities as measured at each step of the procedure in the preparation of vesicles from two transporting strains and from two negative strains. The activities of the three nucleoside phosphorylases as well as of cytidine deaminase were determined, since these four enzymes catalyze the initial, specific cleavage of the transported nucleosides (Table I).

Three important features emerge from the table.

TABLE III

Distribution of radioactivity from transported, U-^{14}C-labeled nucleosides in thin layer chromatograms of cell extracts

Cells were filtered and washed 40 s after addition of the labeled nucleoside. Extracts were prepared and chromatographed as described under "Experimental Procedures." Phosphorylated compounds do not move in the chromatographic solvents used. The radioactivity at the origin therefore represents any phosphorylated compound formed from the nucleosides.

Strain	Nucleoside	Radioactivity in nucleoside spot	Radioactivity in origin (phosphorylated compounds)	Total radioactivity applied	% recovery of nucleoside
Sϕ 1130	Deoxycytidine	2390	15	2500	96
	Cytidine	2821	170	3020	93
	Guanosine	1595	90	1760	91
	Thymidine	445	106	610	73
Sϕ 1142	Uridine	2267	363	2647	86

TABLE IV

Effect of osmotic shock treatment on nucleoside transport

The shock treatment was carried out according to Heppel (19). Shocked cells and control cells were suspended in medium containing 50 μg of chloramphenicol/ml. Transport assays were performed under standard conditions.

Strain	Substrate	Rate of transport		Relative rate
		Control cells	Shocked cells	
		pmol/s/4 × 10⁸ cells		%
Sϕ 1130 *nupC, cytR, deoR cdd, Δdeo*	Deoxycytidine	35.0	7.4	21
	Cytidine	35.3	5.6	16
	Guanosine	21.6	5.4	25
	Adenosine	15.1	3.5	23
	Thymidine	19.2	4.1	21
	Glycine	43.0	23	54
Sϕ 1131 *nupG, cytR, deoR cdd, Δdeo*	Deoxycytidine	20.2	2.5	12
	Cytidine	15.0	2.8	19
	Adenosine	7.4	<2	
	Thymidine	11.5	2.1	18
	Glycine	40	18	45

TABLE V

Release of nucleoside-catabolizing enzymes during preparation of membrane vesicles

The results are normalized and given in percentage of enzyme activities present in the original cell culture. Figures in parentheses indicate specific activities (enzyme units/mg of protein).

Strain no.	Genotype	Enzyme[a]	Original cell culture	Spheroplast formation		Spheroplast lysate	Membrane vesicles
				Spheroplasts	Supernatant		
Sϕ 285	*nupC, cytR deoR, Δdeo*	Cytidine deaminase	100 (2810)	39	2.4	37	<1 (<10)
		Uridine phosphorylase	100 (1570)	72	2.2	70	<1 (27)
Sϕ 861	*nupC, nupG cytR, deoR Δdeo*	Cytidine deaminase	100 (2980)	42	5.4	36	<1 (38)
		Uridine phosphorylase	100 (1050)	50	3.8	46	<1 (64)
Sϕ 1133	*nupC cytR, deoR*	Cytidine deaminase	100 (3620)	37	5.3	32	<1 (36)
		Uridine phosphorylase	100 (1590)	73	5.0	66	<1 (53)
		Purine nucleoside phosphorylase	100 (2570)	90	5.2	78	<1 (51)
		Thymidine phosphorylase	100 (6500)	59	3.2	46	<1 (50)
Sϕ 1135	*nupC, nupG cytR, deoR*	Cytidine deaminase	100 (3900)	41	7.8	37	<1 (27)
		Uridine phosphorylase	100 (1020)	51	5.7	39	<1 (46)
		Purine nucleoside phosphorylase	100 (1865)	42	7.6	34	<1 (21)
		Thymidine phosphorylase	100 (5800)	52	11.9	41	<1 (43)

[a] The enzymes used were: purine nucleoside phosphorylase or purine nucleoside:orthophosphate (deoxy)ribosyltransferase (EC 2.4.2.1); thymidine phosphorylase or thymidine:orthophosphate deoxyribosyltransferase (EC 2.4.2.4); uridine phosphorylase or uridine:orthophosphate ribosyltransferase (EC 2.4.2.3); cytidine deaminase or (deoxy)cytidine aminohydrolase (EC 3.5.4.5).

1. Very little enzyme activity is released in the medium, when spheroplasts are formed by lysozyme-EDTA treatment. The bulk of activity does not appear in the medium until after lysis. This supports the notion that the enzymes are located inside the cytoplasmic membrane rather than in the periplasmic space (10).

2. Small, but significant enzyme activities are retained by the vesicles, but no enrichment of the enzyme is observed. Specific activities drop 50- to 100-fold, compared to crude extracts. Thus, there is no evidence that the nucleoside phosphorylases are integrated in the cytoplasmic membrane.

3. The residual activities in the membrane vesicles of the four enzymes are the same in vesicles derived from the transport negative strains, and from the corresponding transporting strains.

Comparison between Nucleoside Transport in Whole Cells and Membrane Vesicles

The nupG System—Cells of Sϕ 285, which contains the *nupG* system, but lacks thymidine phosphorylase and purine nucleoside phosphorylase, transport all nucleosides efficiently (Fig. 3*A*). The ratio between intracellular and extracellular concentrations may be calculated, assuming an intracellular water volume of 0.05% of the cell suspension volume (24) at a cell density of 2×10^8 cells/ml. At the substrate concentration employed (1.8×10^{-6} M), a ratio of 590 was found for deoxycytidine, in agreement with values found previously for cytidine (1). For the amino acid proline, a ratio of 122 was calculated.

Membrane vesicles prepared from this strain retained some ability to transport cytidine, deoxycytidine, and uridine. Based on the assumption that the inner volume of membrane vesicles from *E. coli* K12 is 5 μl/mg of protein (25), the concentrating effects for membrane vesicles were calculated to be 10-fold for deoxycytidine and 28-fold for proline. Thus, the ability of the vesicles to transport and concentrate proline was retained to a higher extent than the ability to transport the nucleosides.

Transport of guanosine, adenosine, and thymidine was negligible in membrane vesicles from strain Sϕ 285, which lacks the appropriate nucleoside phosphorylases, *i.e.* purine nucleoside phosphorylase and thymidine phosphorylase. When membrane vesicles were prepared from strain Sϕ 1135, containing the enzymes in high amounts, some enzyme activity was retained (Table V) but this activity was not able to stimulate the transport of purine nucleosides or thymidine significantly (Fig. 3*B*).

A transport negative mutant, Sϕ 861, was derived from Sϕ 285 by selection for resistance to FdCyd (10 μg/ml). The mutant strain was unable to transport any nucleoside in whole cells as well as in membrane vesicles (Fig. 3, *A* and *B*). A derivative of this mutant, Sϕ 1135, containing all nucleoside-catabolizing enzymes in high amounts (Table V) did not show significant transport, neither in whole cells nor in membrane vesicles. Transport of the amino acid proline was unaltered in cells and vesicles of this mutant strain, compared to the parent strain (Fig. 3, *A* and *B*).

The nupC System—Cells of strain Sϕ 304, containing the same deletion mutation in purine nucleoside phosphorylase and thymidine phosphorylase as strain Sϕ 285, transport all nucleosides except guanosine (5). The transport pattern in membrane vesicles from this strain was similar to that found for the *nupG* system. Some transport of cytidine, deoxycytidine, and uridine was observed, but the rate was reduced compared to whole cells (Fig. 4). Only insignificant transport of purine nucleosides or thymidine could be detected in the membrane vesicles (not shown).

Also here, a transport negative derivative, Sϕ 1145, resistant

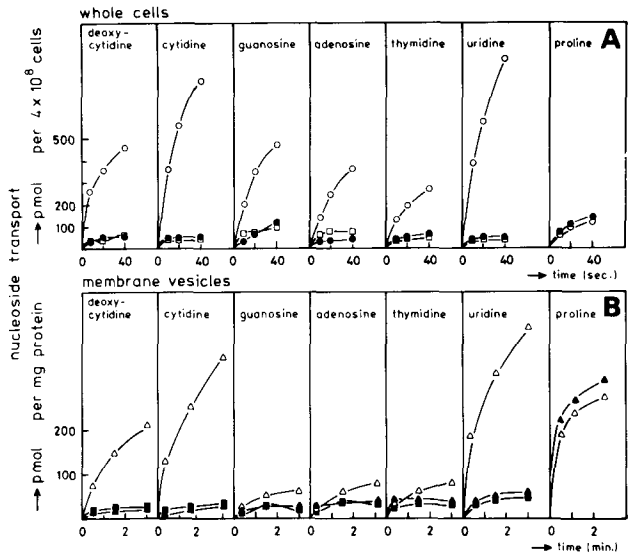

FIG. 3. Nucleoside transport in strain Sϕ 285, containing the *nupG* system, and in its transport negative derivatives, Sϕ 861 and Sϕ 1135. Transport was measured under standard conditions in (*A*) whole cells and (*B*) membrane vesicles. ○——○, Sϕ 285, cells; △——△, Sϕ 285, vesicles; ●——●, Sϕ 861, cells; ▲——▲, Sϕ 861, vesicles; □——□, Sϕ 1135, cells; ■——■, Sϕ 1135, vesicles.

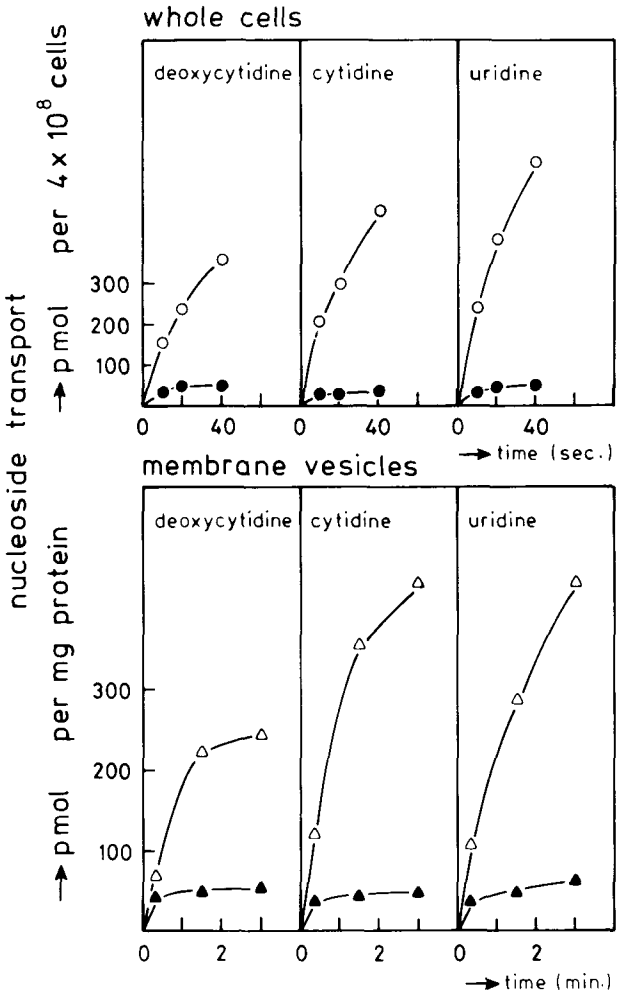

FIG. 4. Nucleoside transport by strain Sϕ 304, containing the *nupC* system and by its transport negative derivative, Sϕ 1145. Transport was measured under standard conditions in whole cells and in membrane vesicles. ○——○, Sϕ 304, cells; △——△, Sϕ 304, vesicles; ●——●, Sϕ 1145, cells; ▲——▲, Sϕ 1145, vesicles.

to FdCyd, was isolated and tested. None of the nucleosides were transported by this mutant strain, neither in whole cells nor in vesicles (shown for pyrimidine nucleosides only (Fig. 4)). The levels of cytidine deaminase and uridine phosphorylase were unaltered in the mutant strain.

Characteristics of the Two Transport Systems

Transport of deoxycytidine was examined in more detail in membrane vesicles containing either of the two transport systems. To ensure that catabolism did not interfere with the results, these vesicles were prepared from two strains containing a mutation in cytidine deaminase in addition to the previously mentioned enzyme mutations (Table II). Only the results for deoxycytidine are shown, but transport of cytidine and uridine responds to the various inhibitors in a manner similar to that of deoxycytidine transport.

As shown in Fig. 5, the two transport systems may be distinguished from each other in whole cells by their behavior towards guanosine or showdomycin as inhibitors of deoxycytidine (or cytidine) transport. Thus, in cells containing the *nupC* system, the transport is not inhibited by guanosine (20 μM), but strongly inhibited by showdomycin (0.1 mM). Conversely, deoxycytidine transport in the *nupG* system is strongly inhibited by guanosine, but much less affected by showdomycin (Fig. 5).

The difference in behavior towards showdomycin and guanosine is very pronounced in whole cells (Fig. 5, *A* and *C*), but it is not very evident in vesicles (Fig. 5, *B* and *D*). Here, both systems are inhibited by showdomycin as well as by guanosine and the difference observed is only quantitative.

The inhibition by guanosine and showdomycin in the vesicles is specific for nucleosides. Neither of the two compounds affects proline transport significantly (not shown).

In other respects, the two transport systems showed the same properties in the membrane vesicles as found in whole cells. In both systems, the transport was energy-requiring as shown by the stimulation by the artificial electron donor, PMS + ascorbate. It was inhibited by dinitrophenol (0.1 mM) and—to a lesser extent—by *N*-ethylmaleimide (4 mM) (Fig. 5). Adenosine, uridine, and thymidine, added in 10-fold excess, were strong inhibitors (not shown).

If unlabeled substrate is added in excess 3 min after addition of the labeled substrate, the major part of the radioactivity is lost from the vesicles (Fig. 5). This indicates that the nucleoside is transported and accumulated without appreciable degradation.

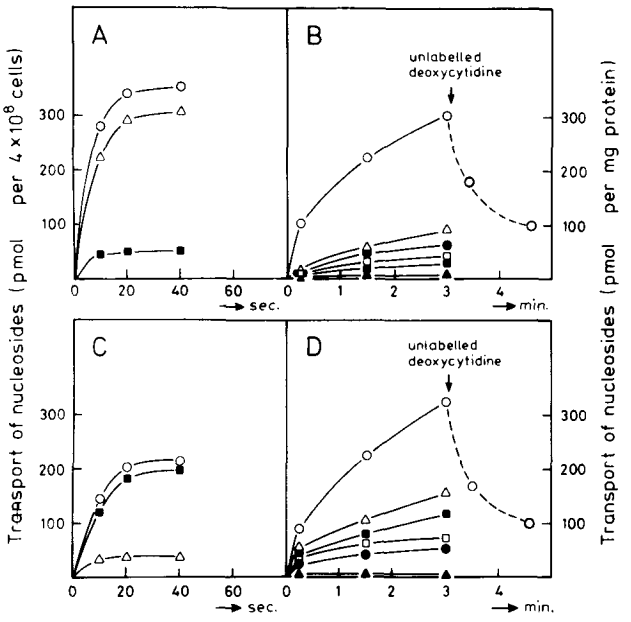

FIG. 5. Effect of various inhibitors on deoxycytidine transport in whole cells and vesicles, containing either of the two nucleoside transport systems. Transport assays and inhibitor addition as described under "Experimental Procedures." *A*, Sϕ 1130 *nupC* (cells); *B*, Sϕ 1130 *nupC* (membrane vesicles); *C*, Sϕ 1131 *nupG* (cells); *D*, Sϕ 1131 *nupG* (membrane vesicles). ○——○, control; △——△, +showdomycin (0.1 mM); ■——■, +guanosine (20 μM); ●——●, +*N*-ethylmaleimide (4 mM); ▲——▲, +DNP (0.1 mM); □——□, PMS + ascorbate omitted.

K_m

In a previous paper, a K_m of 0.3 μM for cytidine transport in whole cells was determined (7). K_m for transport of cytidine and deoxycytidine in membrane vesicles was estimated to 0.5 to 0.8 μM, *i.e.* the same order of magnitude as that found in whole cells.

Uridine Transport

In both transport systems, uridine transport exhibits the same properties as deoxycytidine transport, but the measurements require special consideration, since the membrane vesicles contain some uridine phosphorylase activity (Table V), which is sufficient for a rapid phosphorolytic splitting of uridine. This is concluded from Fig. 6, where transport is measured of uridine, labeled either uniformly or in the base moiety alone. In the transport assay, only [*U*-^{14}C]uridine gives rise to accumulation of radioactivity in the membrane vesicles. In both cases, the uridine must be transported and phosphorylized and the ribose moiety retained by the vesicles, probably as ribose 1-phosphate (9). Since the strains contain *upp* mutations (Table II), no uracil phosphoribosyltransferase is available to form uridine monophosphate, and the free uracil is excreted into the medium. When uniformly labeled uridine is used as a substrate, the radioactivity from the labeled ribose is retained by the vesicles (Fig. 6*A*). If [2-^{14}C]uridine is employed, the pentose is unlabeled and no radioactivity is accumulated (Fig. 6*B*). Only in membrane vesicles prepared from a strain lacking uridine phosphorylase is it possible to demonstrate a transport of [2-^{14}C]uridine which equals that of [*U*-^{14}C]uridine. This uridine transport is energy-requiring and shows saturation kinetics (Fig. 6, *A* and *B*).

If the same measurements are performed in vesicles prepared from the transport negative strain Sϕ 861, neither of the labeled uridines causes any accumulation of radioactivity (Fig. 6), although uridine phosphorylase is present in these

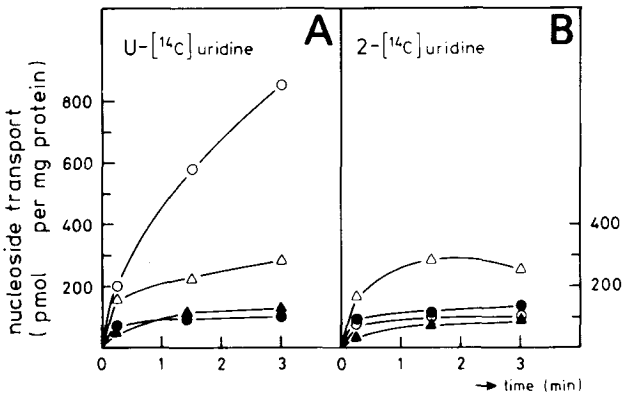

FIG. 6. Uridine transport in membrane vesicles from strain Sϕ 285 containing the *nupG* system; from strain Sϕ 861, its transport negative derivative, and from strain Sϕ 1142, containing both transport systems, but lacking uridine phosphorylase. Transport assays as described under "Experimental Procedures." ○——○, Sϕ 285; ●——●, Sϕ 861; △——△, Sϕ 1142; ▲——▲, Sϕ 1142, PMS + ascorbate omitted.

vesicles in the same amounts as in the vesicles prepared from transporting cells (Table V).

DISCUSSION

Transport Systems in Whole Cells and Membrane Vesicles—The two nucleoside transport systems in *E. coli*, the *nupC* and the *nupG* systems, are strongly affected by osmotic shock treatment, indicating that in both cases extramembraneous components are important for the transport. One outer membrane protein, the T_6 receptor, has been shown to stimulate nucleoside transport, but the loss of this protein after osmotic shock cannot explain the general reduction in nucleoside transport, since the receptor was shown to have no influence on the transport of cytidine and deoxycytidine (Fig. 2).

The present results further demonstrate that membrane vesicles from cells containing either of the two transport systems are able to carry out some nucleoside transport. The transport rates are low compared to whole cells, but in this low efficiency range the nucleosides seem to fall in two categories. Cytidine, deoxycytidine, and uridine are transported in significant amounts, while purine nucleosides and thymidine are poorly transported under the standard assay conditions employed in this investigation. The presence of residual amounts of nucleoside phosphorylases does not stimulate this transport significantly.

Transport of Cytidine, Deoxycytidine, and Uridine—These nucleosides are transported by both transport systems, in cells as well as in vesicles, but in vesicles the two systems exhibit properties that are in some respects different from those found in whole cell experiments. The transport data for deoxycytidine (Fig. 5) show that the vesicles containing the *nupC* system have lost partially the resistance to guanosine, which is characteristic for this system, when deoxycytidine transport is measured in whole cells. Similarly, the resistance to showdomycin is partially lost in vesicles, containing the *nupG* system.

The change in response to inhibitors in membrane vesicles, as well as the strongly reduced transport in osmotically shocked cells, indicates that components of the two transport systems are lost during the preparation of vesicles or during the osmotic shock treatment. These components are important, but not essential for nucleoside transport, since membrane vesicles retain the ability to carry out some transport. The stimulation of this transport by PMS + ascorbate (Fig. 5) implies that the nucleoside transport systems are able to derive their energy directly from the energized membrane.

The data available at present do not allow a decision between the following two possibilities: 1) membrane vesicles, prepared from strains containing either the *nupG* or the *nupC* system, each carry a separate transport system or 2) one common membraneous translocation system serves two different components or systems, located outside the cytoplasmic membrane.

Transport of Purine Nucleosides and Thymidine—The inability of the membrane vesicles to support significant transport of these nucleosides confirms the participation in nucleoside transport of protein factors localized outside the cytoplasmic membrane. It also indicates that transport of purine nucleosides and thymidine requires one or more components different from those functioning in the transport of cytidine nucleosides and uridine. Such components might be lost during the vesicle preparation procedure due either to instability or to a localization outside the cytoplasmic membrane. So far, all attempts to isolate binding protein(s) for nucleosides have failed.

Separate Systems for Transport and Catabolism—There is abundant evidence that transport of nucleosides into whole cells may take place independent of the nucleoside-catabolizing enzymes. Thus, in strains devoid of thymidine phosphorylase and purine nucleoside phosphorylase, an efficient transport and accumulation of thymidine, guanosine, and adenosine are observed (5) (Fig. 3*A* and Table III). Furthermore, strains deficient in cytidine deaminase or uridine phosphorylase, the first enzymes to attack cytidine and uridine, respectively, retain the ability to transport these nucleosides although no catabolism takes place (1, 2) (Table III). Also, the regulation of the transport process by the *cytR* and *deoR* repressors occurs independent of the presence of enzymes (5).

In the present paper, it is shown that the presence of the nucleoside-catabolizing enzymes is not sufficient for transport to take place. Selection for resistance to FdCyd in a transporting strain, containing the *nupG* system, yielded a mutant, Sϕ 861, which is unable to transport any nucleoside (Fig. 2*A*). A derivative of this mutant, containing all nucleoside-catabolizing enzymes in high amounts did not show significant nucleoside transport either (Fig. 3*A*).

Experiments with membrane vesicles corroborate the results obtained with whole cells. Cytidine and deoxycytidine are transported in significant amounts by membrane vesicles containing either of the two transport systems, but lacking cytidine deaminase, while no transport can be measured in membrane vesicles prepared from the transport negative strain (Fig. 3*B*). Similarly, uridine is transported by vesicles, lacking uridine phosphorylase, while vesicles from the transport negative strain, which contain uridine phosphorylase, are unable to support transport (Fig. 6). In the case of purine nucleosides and thymidine, the presence of the corresponding nucleoside phosphorylases does not stimulate transport in vesicles from the transport negative strain (Fig. 3*B*).

The experiments on uridine transport in membrane vesicles demonstrate the intimate coupling between transport and phosphorolytic cleavage of the nucleoside (Fig. 6). Here the transported uridine is quantitatively phosphorylized by the uridine phosphorylase even at the first sampling at 10 s. A similar rapid phosphorolytic cleavage of uridine by membrane vesicles from *S. typhimurium* has been interpreted as a group translocation of the nucleoside through the cytoplasmic membrane, catalyzed by uridine phosphorylase (8, 9). In the present study, the lack of transport in whole cells and vesicles from Sϕ 861 and Sϕ 1135 (Fig. 3, *A* and *B*) speaks against such a mechanism as the major way of nucleoside transport, since these strains contain uridine phosphorylase in the same amounts as the parent strain. Moreover, if the uridine phosphorylase was spanning the cytoplasmic membrane as suggested (9), an enrichment of this enzyme would be expected in the membrane vesicles as compared to the whole cells. Instead, a substantial loss in specific activity occurs (Table V).

The nature of the mutation to transport negativity is being investigated. The simultaneous loss of transport function for all nucleosides but not for proline indicates that the mutation is in a component essential and specific for nucleoside transport. Since the transport negativity is expressed in both whole cells and membrane vesicles, the mutated component probably resides in the cytoplasmic membrane.

In summary, the present results provide strong evidence that cells of *E. coli* contain energy-requiring transport systems which will transport nucleosides into the cells independent of the catabolic process. This does not exclude the possibility that in wild type cells the nucleoside phosphorylases may mediate some transport. The failure of these enzymes to support transport in strain Sϕ 1135, *nupG*, *nupC*, is consistent, although, with the hypothesis that a transport is required prior to the initial enzymatic attack on the incoming nucleo-

side. This also places the nucleoside catabolizing enzymes inside the permeability barrier of the cell, a location which agrees with the failure of EDTA-lysozyme treatment (Ref. 10 and this paper) to cause their release.

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