BBA 75648

NUCLEOSIDE TRANSPORT BY NOVIKOFF RAT HEPATOMA CELLS GROWING IN SUSPENSION CULTURE

SPECIFICITY AND MECHANISM OF TRANSPORT REACTIONS AND RELATIONSHIP TO NUCLEOSIDE INCORPORATION INTO NUCLEIC ACIDS

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(Received December 18th, 1970)

SUMMARY

The transport of adenosine, guanosine, inosine, uridine and cytidine by Novikoff rat hepatoma cells (subline N1S1-67) is competitively inhibited by each of the other nucleosides, thymidine, persantin and phenethyl alcohol. Comparisons of the transport kinetics of the various nucleosides (K_m and v_{max}), of the K_m/K_i ratios for the inhibitions and of the effect of heat shock (47.5°, 5 min) on nucleoside transport suggest that guanosine and inosine are transported by a single system, whereas different specific systems transport adenosine and the pyrimidine nucleosides. Uridine and cytidine also seem to be transported by a single system. All systems are inactivated to about the same extent by treatment of the cells with *p*-chloromercuribenzoate. Incubation of the cells in media containing 2% trypsin or chymotrypsin, 1% neuraminidase or 0.025% phospholipase C for 15 min has no significant effect on uridine transport, and energy poisons have an effect only at relatively high concentrations.

The incorporation of each of the nucleosides into nucleic acids by the cells also follows simple Michaelis–Menten kinetics and the apparent K_m for each nucleoside is similar to that of its transport into the cell. Competitive inhibition of nucleoside transport by heterologous nucleosides, persantin or phenethyl alcohol results in an apparent competitive inhibition of nucleoside incorporation into nucleic acids and the apparent K_i values for the inhibitions are similar for both processes. The results suggest that the rate-limiting step in the incorporation of each of the nucleosides into nucleic acids is its transport into the cell.

INTRODUCTION

Results from previous studies[1–3], have shown that at concentrations below 50 μM, uridine is taken up by cultured Novikoff hepatoma cells mainly by a transport

Abbreviations: PCMB, *p*-chloromercuribenzoate.

system, while at concentrations above 100 μM, simple diffusion becomes the predominant mode of entry. Further, the transport of uridine into the cells is the rate-limiting step in the incorporation of uridine into the nucleotide pool and into RNA. The cells contain an excess of uridine kinase, so that immediately upon entry into the cell, uridine becomes trapped inside the cell by being phosphorylated. Thus, the kinetics of uridine incorporation into the nucleotide pool by intact cells are those of the transport reaction and not of the uridine kinase[1].

It has been repeatedly observed that ribonucleoside incorporation into RNA[4–9] or into the nucleotide pool[10] of cells is inhibited by the presence of high concentrations of other nucleosides in the medium. Our results discussed above and those of STECK *et al.*[11], however, indicate that these effects are mainly due to the inhibition of nucleoside transport into the cell and do not reflect an inhibition of RNA synthesis or of nucleoside phosphorylation. On the basis of the observation that the incorporation of each nucleoside was inhibited by all other nucleosides tested, STECK *et al.*[11] concluded that a common transport site or class of sites is shared by the nucleosides. The more detailed kinetic analyses of the present investigation, however, suggest that various pyrimidine and purine nucleosides are transported by different systems.

MATERIALS AND METHODS

Cell culture

Novikoff rat hepatoma cells (subline NISI-67) were propagated in Swim's medium 67 in suspension culture and enumerated as described previously[12,13]. For studies on nucleoside incorporation, cells were collected by centrifugation from cultures in the exponential phase of growth at about $2 \cdot 10^6$ cells per ml and suspended to the same cell concentration in basal medium 42 (ref. 12).

Reagents

Reagents were purchased as follows: Phenethyl alcohol from Matheson, Coleman and Bell; *p*-chloromercuribenzoate (PCMB) from Calbiochem, [5-^{3}H]uridine from Dohm Products Ltd; [5-^{3}H]cytidine and [8-^{3}H]guanosine from Schwarz Bio-Research; [G-^{3}H]inosine and [8-^{3}H]adenosine from Amersham/Searle; trypsin (1:250) from Difco; neuraminidase and β-chymotrypsin from Worthington Biochemical Corp; phospholipase C from Sigma Chemical Co.; persantin (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido (5,4-*d*) pyrimidine) was a gift from Geigy Pharmaceuticals.

Solutions of ^{3}H-labeled nucleosides with decreased specific radioactivity were prepared by addition of the appropriate unlabeled nucleoside. All labeled nucleoside preparations as received from the suppliers were tested for purity by paper chromatography (see refs. 3, 14). The specific radioactivities of the nucleoside preparations were corrected for the presence of small amounts of labeled impurities as follows: 5 % for [5-^{3}H]uridine, 7 % for [5-^{3}H]cytidine and 3 % for [8-^{3}H]adenosine. No significant amounts of labeled impurities were detected in the [8-^{3}H]guanosine and [^{3}H]-inosine preparations.

Incorporation of labeled nucleosides

Suspensions of cells were supplemented with labeled nucleosides as indicated in the appropriate experiments and incubated on a gyrotory shaker at 37°. Replicate

0.5 or 1-ml samples of suspension were analyzed for radioactivity in total cell material (acid-soluble *plus* acid-insoluble) and for radioactivity in acid-insoluble material as described previously[1].

Preparation of acid-extracts from cells and chromatographic analysis

The procedures have been described in detail elsewhere[3,14]. Briefly, labeled cells were collected by centrifugation, washed twice in balanced salt solution and then extracted with perchloric acid. The acid extracts were neutralized and analyzed by ascending paper chromatography. The papers were developed at 30° for 18 h with a mixture of 3 vol. 1 M ammonium acetate (pH 5.0) and 7 vol. 95 % ethanol. The developed chromatograms were cut at right angles to the direction of migration into 1-cm segments. The segments were eluted with water and the eluates were analyzed for radioactivity.

Uridine kinase assay

Cell-free extracts were prepared and their uridine kinase activity assayed as described previously[14].

RESULTS

Competitive inhibition of nucleoside transport by heterologous nucleosides, persantin and phenethyl alcohol

The Lineweaver–Burk plots in Fig. 1 demonstrate that the transport of adenosine, guanosine, inosine, uridine and cytidine was inhibited in a simple competitive manner by heterologous purine or pyrimidine ribonucleosides, thymidine, persantin and phenethyl alcohol. The apparent K_m and K_i values and K_m/K_i ratios calculated from the data in Fig. 1 are summarized in Table I. Very similar values were obtained in a duplicate experiment. The apparent K_m's were very similar for the transport of the various nucleosides ranging from about 8 μM for adenosine transport to 23 μM for cytidine transport. The theoretical v_{max} values were also similar for all the nucleosides tested (1–2 nmoles/10^6 cells per 10 min).

The K_m/K_i ratios (Table I) indicate the following:

(1) the K_m/K_i ratios for the inhibition of guanosine transport by inosine and of inosine transport by guanosine were both close to one, indicating that in both cases the inhibitor had approximately the same affinity for the transport system as the substrate. The results suggest that inosine and guanosine are transported by the same system. This conclusion is supported by the finding that guanosine and inosine transport are inhibited to about the same extent by other nucleosides, persantin and phenethyl alcohol (see K_m/K_i ratios, Table I). Further, the rates of inosine and guanosine transport were similarly affected by changes in substrate concentration in the medium and the theoretical v_{max} values were also the same for both nucleosides.

In a separate experiment inosine and guanosine transport were compared in a single experiment, since the apparent K_m and v_{max} values for the same nucleoside varied somewhat between individual experiments with different cell populations. In this experiment, the apparent K_m for the transport of both guanosine and inosine was 13 μM and the v_{max} was 1.4 nmoles/10^6 cells per 10 min. On the other hand, the effect of inosine and guanosine on the transport of adenosine and the pyrimidine

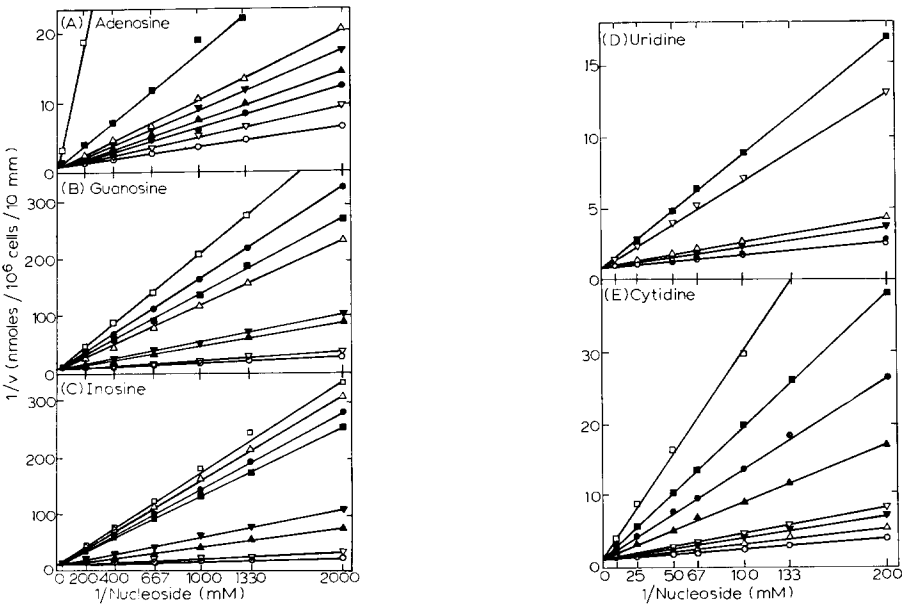


Fig. 1. Lineweaver–Burk plots of the incorporation of adenosine (A), guanosine (B), inosine (C), uridine (D), and cytidine (E) into total cell material in the absence and presence of heterologous nucleosides, persantin and phenethyl alcohol. Each frame (A–E) represents a separate experiment. In each, portions of a suspension of $2 \cdot 10^6$ cells per ml of basal medium 42 were mixed with the various inhibitors as indicated below* and immediately thereafter, 10 ml samples of each portion were supplemented with various concentrations of ^{3}H-labeled nucleoside also shown below**. The suspensions were incubated on a gyrotory shaker at 37° and after 5 min, duplicate 1-ml samples of suspension were analyzed for radioactivity in total cell material. All points represent averages of the duplicate samples. Incorporation of each of the nucleosides into total cell material is approximately linear for 5–10 min. After 10 min of incubation, other samples were analyzed for radioactivity in acid-insoluble material (see Table IV) and acid-extracts were prepared from the remaining cells (see text).

Frame	* *Inhibitor (mM)*											
	None	*Adenosine*	*Guanosine*	*Inosine*	*Uridine*	*Cytidine*	*Thymidine*	*Persantin*	*Phenethyl alcohol*
A	○–	—	1.0 ●–	0.25 △–	0.5 ▲–	1.0 ▽–	0.5 ▼–	0.1 □–	20 ■–
B	○–	0.05 ●–	—	0.1 △–	0.5 ▲–	1.0 ▽–	0.5 ▼–	0.05 □–	20 ■–
C	○–	0.05 ●–	0.1 △–	—	0.5 ▲–	1.0 ▽–	1.0 ▼–	0.05 □–	20 ■–
D	○–	—	0.2 ●–	0.2 △–	—	0.5 ▽–	0.5 ▼–	0.05 □–	16 ■–
E	○–	0.5 ●–	1.0 △–	0.5 ▲–	0.01 ▽–	—	0.5 ▼–	0.1 □–	20 ■–

	** *Labeled nucleoside*	*μM*	*Counts/min per pmole*	*μM*	*Counts/min per pmole*
A	[^{3}H]adenosine	0.5, 0.75, 1.0, 1.5, 2.5, 5.0	127	25	25
B	[^{3}H]guanosine	0.5, 0.75, 1.0, 1.5, 2.5, 5.0	140	25	28
C	[^{3}H]inosine	0.5, 0.75, 1.0, 1.5, 2.5, 5.0	130	25	26
D	[^{3}H]uridine	5, 10, 15, 20, 40, 100	20.0		
E	[^{3}H]cytidine	5, 7.5, 10, 15, 20, 40	14.2	100	7.1

TABLE I

APPARENT KINETIC CONSTANTS FOR THE INHIBITION OF NUCLEOSIDE TRANSPORT BY HETEROLOGOUS NUCLEOSIDES, PERSANTIN AND PHENETHYL ALCOHOL

The K_m and K_i values were estimated from the Lineweaver–Burk plots in Fig. 1.

Labeled nucleoside	K_m (μM)	K_i *of inhibitor* (μM)							
		Adenosine	*Guanosine*	*Inosine*	*Uridine*	*Cytidine*	*Thymidine*	*Persantin*	*Phenethyl alcohol*
Adenosine	8.8	—	1060	107	390	1800	290	3.8	3600
Guanosine	12	5.8	—	16	300	3000	250	4.3	2730
Inosine	11	6.4	10	—	340	2800	430	5.3	2900
Uridine	14	150	6000	220	—	6.2	700	4.0*	3500*
Cytidine	23	68	2850	120	10	—	510	5.8	1600
		K_m/K_i							
		Adenosine	*Guanosine*	*Inosine*	*Uridine*	*Cytidine*	*Thymidine*	*Persantin*	*Phenethyl alcohol*
Adenosine		—	0.0083	0.082	0.023	0.0049	0.030	2.7	0.0024
Guanosine		2.1	—	0.75	0.040	0.0040	0.048	2.8	0.0044
Inosine		1.7	1.1	—	0.033	0.0039	0.026	2.1	0.0038
Uridine		0.091	0.0023	0.062	—	2.2	0.019	3.5	0.0040
Cytidine		0.30	0.0081	0.19	2.2	—	0.045	4.0	0.014

* from ref. 1.

nucleosides, differed markedly, inosine having a much greater effect than guanosine (see K_m/K_i ratios, Table I).

(2) Adenosine had at least as great an affinity for the guanosine–inosine transport system as these substrates themselves (Table I). However, adenosine was probably not transported by the latter system to any significant extent, but rather by a separate system, since guanosine and inosine had relatively little effect on adenosine transport. As indicated by the relatively high K_m/K_i ratios, adenosine also inhibited markedly the transport of the pyrimidine nucleosides.

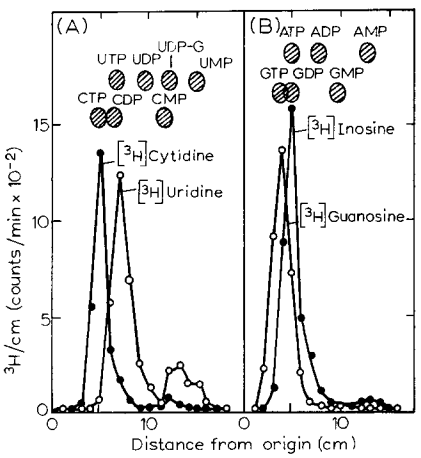
(3) The K_m/K_i ratios for the inhibition of uridine transport by cytidine and of cytidine transport by uridine were also close to one. These data suggest that one system transports both uridine and cytidine. However, we observed consistently that the K_m for cytidine transport (22–23 mM) was significantly higher than that for uridine transport (13–17 mM), even when compared with the same cell suspension, whereas the v_{max} was slightly larger for uridine transport than for cytidine transport. Cytidine transport was also inhibited about 3 times more effectively by adenosine, guanosine, inosine, thymidine and phenethyl alcohol than uridine transport (see K_m/K_i ratios, Table I).

Chromatographic analysis of acid extracts from 10-min labeled cells showed that most of the intracellular acid-soluble label derived from the various nucleosides in the medium was located in the corresponding nucleoside triphosphate regardless of the nucleoside concentration in the medium; 80–85 % of the adenosine and inosine labels was in ATP, about 95 % of the guanosine label in GTP, about 85 % of the cytidine label in CTP and 70–75 % of the uridine label in UTP and about 16 % in UDP-sugars[3]. Representative chromatograms are shown in Fig. 2. Significant amounts of labeled nucleosides were not present in the acid extracts. The relative distribution of label among the various nucleotides in the intracellular pool was the same whether or not transport was inhibited by the presence of heterologous nucleosides, persantin or phenethyl alcohol.

Effects of heat shock and treatment with PCMB on nucleoside transport

The following experiments were undertaken to obtain further information on the specificity and nature of the nucleoside transport systems. The data in Fig. 3A and C demonstrate that treatment with PCMB or heating the cells at 47.5° for 5 min markedly reduced the capacity of the cells to incorporate uridine into total cell material. The following observations indicate that the effect of heat shock or PCMB treatment on uridine incorporation into the nucleotide pool was due to an inactivation of the uridine transport system and not to an overall change of cell permeability or an inactivation of the uridine kinase. (1) No significant morphological differences could be detected between untreated and treated cells by light microscopy. In all suspension, less than 2 % of the cells were permeable to trypan blue at the end of the experiment. (2) As shown later, uptake of uridine by simple diffusion was not affected by the treatments. (3) The uridine kinase activity of the cells as measured in cell-free extracts was only reduced 10–15 % by heating cells at 47.5° for 5 min or PCMB treatment. (4) The distribution of label among the uridine nucleotides in the pool was about the same in treated and untreated cells. Between 65 and 75 % of the label was present in UTP plus UDP (mostly UTP, see Fig. 2), 15–20 % in UDP-sugars and the remainder in UMP. Further, the incorporation of uridine into acid-insoluble material was reduced

by treatment with the various concentrations of PCMB to about the same extent as the incorporation into the nucleotide pool (compare Fig. 3A and B; see Table II). This finding suggests that the reduction of RNA labeling by PCMB treatment was simply a consequence of the inactivation of the uridine transport system. In contrast,



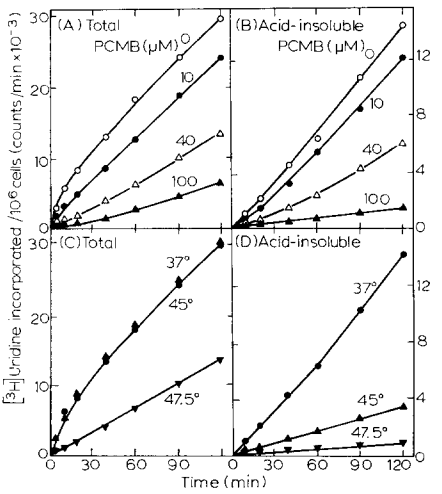


Fig. 2. Chromatographic analysis of acid-extracts prepared from cells after labeling with [^{3}H]-uridine or [^{3}H]cytidine (A), or with [^{3}H]guanosine or [^{3}H]inosine (B). The details of the experiment are described in the legend to Fig. 1. Acid extracts were prepared from $1 \cdot 10^7$ cells after 10 min of labeling with 5 μM [^{3}H]uridine or [^{3}H]cytidine or with 25 μM [^{3}H]guanosine or [^{3}H]inosine and 50-μl samples of acid extract were analyzed by ascending paper chromatography as described in MATERIALS AND METHODS. Each frame is a composite of independent chromatograms.

Fig. 3. Effect of treatment with PCMB (A and B) or heat-shock (C and D) on [^{3}H]uridine incorporation into total cell material or acid-insoluble material. Samples of $4 \cdot 10^7$ cells were collected by centrifugation from an exponential phase culture and treated as follows: (A and B) The cells were suspended to $4 \cdot 10^6$ cells per ml in basal medium 42 (○—○) or basal medium 42 containing 10 μM (●—●), 40 μM (△—△) or 100 μM (▲—▲) PCMB. The suspensions were incubated on a gyrotory shaker at 37° for 10 min, and the cells were again collected by centrifugation. (C and D) The cell pellets were incubated for 5 min at 37° (●—●), 45° (▲—▲), or 47.5° (▼—▼). Following these treatments, the cells were suspended to $2 \cdot 10^6$ cells per ml in basal medium 42 containing 2.5 μM [^{3}H]uridine (400 μC/μmole). The suspensions were incubated at 37° and duplicate 0.5-ml samples were analyzed for radioactivity in total cell material (A and C) or acid-insoluble material (B and D). All points represent averages of the duplicate determinations.

heat shock markedly affected RNA synthesis. Heating cells at 45° for 5 min reduced uridine incorporation into nucleic acid by about 80% (Fig. 3D), in spite of the fact that the treatment had little effect on uridine incorporation into the nucleotide pool (Fig. 3C), and heating at 47.5° almost completely abolished any subsequent incorporation of uridine into nucleic acids (Fig. 3D; see Table II).

The conclusion that heating at 47.5° and PCMB treatment caused a reduction in uridine incorporation into the nucleotide pool by inactivating the transport system is further supported by the kinetic data in Fig. 4. The data confirmed results from preliminary experiments which indicated that the apparent degree of inhibition of uridine incorporation by heat-shock or PCMB treatment differed markedly depending on the uridine concentration used in the assay. In the experiment illustrated in Fig. 4A, a suspension of cells supplemented with 40 μM PCMB was incubated at 37° and

TABLE II

INHIBITION OF INITIAL RATES OF NUCLEOSIDE INCORPORATION INTO TOTAL CELL MATERIAL AND ACID-INSOLUBLE MATERIAL BY TREATMENT OF THE CELLS AT 47.5° FOR 5 min OR 40 μM PCMB FOR 10 min

Cells were collected from an exponential phase culture and samples were treated with PCMB or heated as described in the legend to Fig. 3. Immediately thereafter the cells were suspended in basal medium 42 to $2 \cdot 10^6$ cells per ml and samples of each suspension were supplemented with 6.25 μM [^{3}H]uridine (9.5 counts/min per pmole) or [^{3}H]cytidine (15 counts/min per pmole), 2.5 μM [^{3}H]adenosine (80 counts/min per pmole), or 2.5 μM [^{3}H]guanosine (100 counts/min per pmole) or [^{3}H]inosine (100 counts/min per pmole). The suspensions were incubated at 37° and at various time intervals, duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in total cell material or in acid-insoluble material. The initial rates of incorporation were estimated from the linear portions of the incorporation curves.

Nucleoside	*Inhibition (%)**			
	47.5°, 5 min		*40 μM PCMB, 10 min*	
	Total	*Acid-insoluble*	*Total*	*Acid-insoluble*
Uridine	90	98	72	70
Cytidine	90	98	76	77
Adenosine	50	97	77	76
Guanosine	75	97	71	80
Inosine	75	97	71	73

* As compared to untreated controls.

at 2, 8 and 17 min, samples of the suspension were supplemented with various concentrations of [^{3}H]uridine and the initial rates of incorporation into the nucleotide pool were determined. The data show that a maximum degree of inhibition was attained after about 8 min of incubation with PCMB and that the rate of the remaining PCMB-resistant incorporation was directly proportional to the uridine concentration of the medium. This behavior is characteristic for uptake by simple diffusion and was one experimental finding[3] that suggested that at concentrations above 100 μM in the medium uridine is taken up by NiSi-67 cells mainly by simple diffusion (see Fig. 4B). This conclusion was further supported by the finding that at these high concentrations, uridine incorporation is little affected by changes in temperature, whereas incorporation at concentrations below 50 μM exhibits a Q_{10} of about 1.8 (ref. 2). The data in Fig. 4B demonstrate that heating at 47° or pretreatment with PCMB almost completely abolished uridine transport without significantly affecting simple diffusion. Thus, while overall uridine incorporation was reduced by the treatments about 80–90 % at 5 μM uridine in the medium, it was reduced only 20–30 % at 500 μM.

In other experiments, the effects of heat shock and PCMB treatment on the capacity of the cells to transport various nucleosides was compared. To make the test as sensitive as possible, nucleoside incorporation was measured at concentrations in the medium at which heat and PCMB-resistant simple diffusion does not play a major role in nucleoside uptake. The results showed that PCMB treatment reduced the initial rates of transport of all nucleosides to about the same extent, whereas heat shock reduced the transport of both uridine and cytidine about 90 %, of adenosine about 50 % and of both guanosine and inosine about 75 % (Table II).

In other experiments the effect of *N*-ethyl maleimide on uridine transport was investigated. Incubation of cells in basal medium 42 containing 10 μM *N*-ethyl maleimide for 12 min had no significant effect on the subsequent incorporation of uridine into total cell material for at least 210 min. Treatment with 40 μM *N*-ethyl maleimide markedly and progressively reduced uridine incorporation, but also rendered about 50 % of the cells permeable to trypan blue within 60 min of incubation following the treatment.

Effect of inhibitors of energy metabolism on uridine transport

The data in Fig. 5 demonstrate that the presence of NaCN, iodoacetate and 2,4-dinitrophenol in the medium markedly inhibited the incorporation of uridine

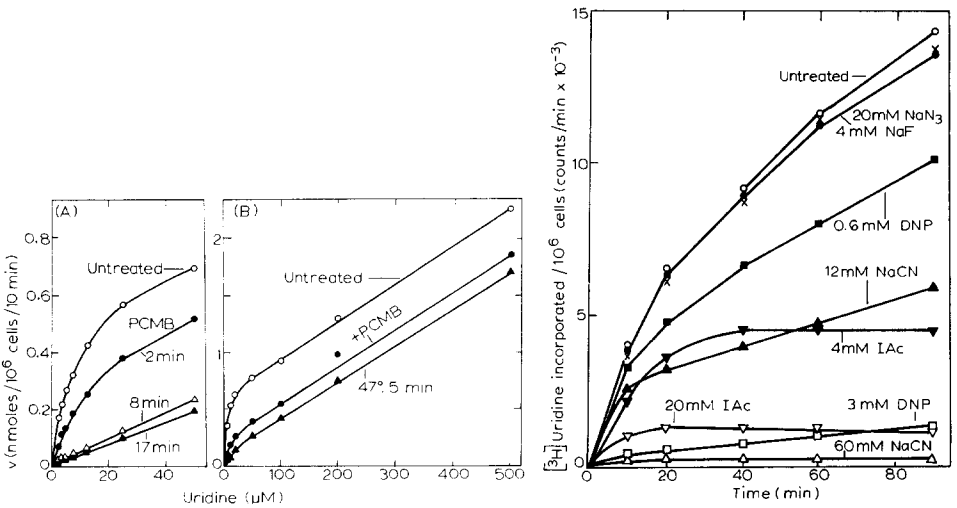


Fig. 4. Rate of uridine incorporation into the nucleotide pool by untreated, PCMB-treated or heat-shocked cells as a function of uridine concentration. (A) Portions of a suspension of $2 \cdot 10^6$ cells per ml of basal medium 42 were supplemented with 40 μM PCMB and incubated for 2 (●—●), 8 (△—△) or 17 min (▲—▲) on a gyrotory shaker at 37°. Then 10-ml samples of these suspensions and an untreated portion (○—○) were supplemented with the indicated concentrations of [^{3}H]uridine (7 counts/min per pmole). After 5 min of incubation at 37°, duplicate 1-ml samples of each suspension were analyzed for radioactivity in total cell material. (B) Samples of $2 \cdot 10^8$ cells were collected from an exponential phase culture. One cell pellet was incubated for 5 min at 37° (○—○) and another at 47° (●—●). The cells of a third sample were suspended to $4 \cdot 10^6$ cells per ml in basal medium 42 containing 40 μM PCMB, incubated at 37° for 10 min and then again collected by centrifugation. The cells of each portion were suspended in 100 ml of basal medium 42 and 10-ml samples of each suspension were supplemented with 5, 7.5, 12.5, 20 or 50 μM [^{3}H]uridine (11 counts/min per pmole) or with 50 μM [^{3}H]uridine *plus* unlabeled uridine to 100, 200 or 500 μM. After 5 min of incubation at 37°, duplicate 1-ml samples of each suspension were analyzed for radioactivity in total cell material. All points represent averages of the duplicate determinations.

Fig. 5. Effects of various energy poisons on uridine incorporation into the nucleotide pool. One sample of a suspension of $2 \cdot 10^6$ cells per ml of basal medium 42 remained untreated (○—○); other samples were supplemented with 4 mM NaF (●—●) or 12 mM (▲—▲) or 60 mM (△—△) NaCN; or 4 mM (▼—▼) or 20 mM (▽—▽) iodoacetate (IAc); or 20 mM NaN_3 (×—×); or 0.6 mM (■—■) or 3 mM (□—□) DNP. Within 2 min thereafter, each suspension was supplemented with 0.5 μM [^{3}H]uridine (2000 μC/μmole). The suspensions were incubated at 37° and at various time intervals, duplicate 0.5-ml samples were analyzed for radioactivity in total cell material. All points represent averages of the duplicate samples. Other samples were analyzed for radioactivity in acid-insoluble material (not shown).

into the nucleotide pool of the cells. However, relatively high concentrations of these inhibitors (60 mM NaCN, 20 mM iodoacetate, 3 mM 2, 4-dinitrophenol) were required to effect a rapid or significant inhibition. The inhibitions were probably not primarily due to an inhibition of the phosphorylation reactions, since the distribution of the incorporated label among the nucleotides in the pool was relatively little affected by the presence of the inhibitors (Table III). In contrast 4 mM NaF and 20 mM NaN_3 had no significant effect on uridine incorporation for at least 90 min (Fig. 5). Higher concentrations of NaF could not be employed, since they caused rapid lysis of the cells.

TABLE III

DISTRIBUTION OF ^3H AMONG URIDINE NUCLEOTIDES AFTER LABELING WITH [^3H]URIDINE IN THE ABSENCE AND PRESENCE OF NaF, NaCN, IODOACETATE, SODIUM AZIDE OR DINITROPHENOL

The details of the experiment are described in the legend to Fig. 5. After 90 min of labeling with [^3H]uridine in the presence of the indicated inhibitors, $1 \cdot 10^7$ cells were collected, washed twice with balanced salt solution, extracted with perchloric acid and the acid-extracts were analyzed chromatographically as described in MATERIALS AND METHODS.

Inhibitor	*Total ^3H of chromatogram (counts/min)*	*^3H in nucleotides (% of total)*		
		UTP + UDP	*UDP-sugars*	*UMP*
None	4150	69	17	14
NaF, 4 mM	5310	91	3	6
NaCN, 12 mM	1320	74	16	10
NaCN, 60 mM	330	45	19	36
Iodoacetate, 4 mM	1340	65	13	22
Iodoacetate, 20 mM	360	42	16	42
Sodium azide, 20 mM	4200	73	17	10
Dinitrophenol, 0.6 mM	3050	75	15	10

Uridine incorporation after enzyme treatments

Attempts were made to gain further information on the cell surface components involved in nucleoside transport by determining the effect of pretreatment of the cells with various enzymes on uridine incorporation. Cells were collected by centrifugation and suspended to $4 \cdot 10^7$ cells per ml in basal medium 42 or basal medium 42 containing 3 % (w/v) trypsin, 2 % (w/v) chymotrypsin, 1 % (w/v) neuraminidase or 0.025 % (w/v) phospholipase C. The suspensions were incubated at 37° for 15 min and then diluted 20-fold with basal medium 42. The suspensions were supplemented with [^3H]uridine and incorporation into total cell material was determined. None of the treatments had any significant effect on uridine incorporation. Treatment of the cells with neuraminidase and subsequently with trypsin or chymotrypsin was equally ineffective. Treatment with higher concentrations of phospholipase C markedly reduced the incorporation of uridine by a population of cells. This effect, however, was probably mainly due to the death of a proportion of the cells, since it correlated with an increase in the number of trypan blue-permeable cells in the suspension. Treatment of cells with 0.025 % phospholipase C for 15 min and subsequently with 0.5 % chymotrypsin resulted in the complete dissolution of a large proportion of the cells with the release of their nucleic acids.

TABLE IV

APPARENT KINETIC CONSTANTS FOR THE INHIBITION OF NUCLEOSIDE INCORPORATION INTO ACID-INSOLUBLE MATERIAL BY HETEROLOGOUS NUCLEOSIDES, PERSANTIN AND PHENETHYL ALCOHOL

The details of the experiment are described in the legend to Fig. 1. Samples of cell suspensions supplemented with the indicated heterologous nucleosides, thymidine, persantin or phenethyl alcohol were incubated for 10 min at 37° with various concentrations of the indicated ^{3}H-labeled nucleosides and analyzed in duplicate for radioactivity in acid-insoluble material. The initial velocities of incorporation were estimated from these 10-min values, since the rates of incorporation are approximately constant for at least 40 min (ref. 3, and P. W. G. PLAGEMANN, unpublished data). The K_m and K_i values were estimated from conventional Lineweaver–Burk plots of the data.

Labeled nucleoside	K_m (μM)	K_i *of inhibitor* (μM)							
		Adenosine	*Guanosine*	*Inosine*	*Uridine*	*Cytidine*	*Thymidine*	*Persantin*	*Phenethyl alcohol*
Adenosine	13	—	1130	100	500	2600	370	4.5	3700
Guanosine	11	2.5	—	12	450	4400	440	3.8	2000
Inosine	6	2.7	4.2	—	240	3900	260	5.8	1800
Uridine	10	100	5000	360	—	6.8	560	4.0	2000
Cytidine	14	51	2500	236	15	—	530	2.4	1400

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Effect of inhibition of transport on nucleoside incorporation into nucleic acids

It has been demonstrated previously[1,3,15–17] that uridine and adenosine incorporation into cellular or mengovirus RNA follows normal Michaelis–Menten kinetics, that the K_m's for these incorporations are similar to those for the transport of the respective nucleoside into the cell and that the inhibition of uridine transport into the cell by adenosine, azauridine, persantin or phenethyl alcohol results in a proportional reduction in uridine incorporation into RNA without interfering with RNA synthesis *per se*. Kinetic analyses showed that the initial rates of incorporation of cytidine, guanosine and inosine into acid-insoluble material also followed normal Michaelis–Menten kinetics (not shown). The apparent K_m values were very similar to those obtained in the same experiment for the incorporation of each of the nucleosides into the nucleotide pool (compare Table I and IV). Further, the incorporation of all these nucleosides into acid-insoluble material was "competitively" inhibited by the heterologous nucleosides, persantin or phenethyl alcohol in the medium and the apparent K_i values were also very similar to the corresponding values for the inhibition of nucleoside transport (compare Table I and IV). The results indicate that the inhibition of nucleoside incorporation into nucleic acids was simply a consequence of the inhibition of their transport into the cell. Inactivation of the transport systems by treatment with PCMB (Table II) or inhibition of uridine incorporation into the nucleotide pool by various energy poisons (Fig. 5) also resulted in a proportional suppression of nucleoside incorporation into nucleic acids.

DISCUSSION

The overall results suggest that guanosine and inosine are taken up by NISI-67 cells by the same transport system, whereas adenosine and the pyrimidine nucleosides are transported by different systems. This conclusion is based on differences or similarities among the nucleosides with respect to their transport kinetics, the inhibition of their transport by heterologous ribonucleosides, thymidine, persantin and phenethyl alcohol and the differential effect of heat shock on the various transport systems. Similar results have been reported for the nucleoside transport systems of *Escherichia coli*[18,19]. It cannot be concluded unequivocally at present that uridine and cytidine are transported by a single system. The findings that the K_i values for the inhibition of their transport by each other are approximately the same as the K_m's for their transport and that their transport is affected to the same extent by heat-shock would be consistent with such hypothesis. Uridine and cytidine transport, however, exhibit slightly different K_m's and are inhibited to a different degree by heterologous nucleosides and persantin. It is of interest that mutants have been isolated from *Saccharomyces cerevisiae* that have lost the capacity to transport cytidine while the uridine transport system was unaltered[20].

Purines, pyrimidines or ribose have no effect on nucleoside transport by either bacteria[19] or mammalian cells[11] (P. G. W. PLAGEMANN, unpublished data), indicating that the transport systems are specific for nucleosides, but the specificities of the nucleoside transport systems are difficult to explain on a purely structural basis. However, they are similar to the specificities of various enzymes involved in nucleoside metabolism. For instance, purine nucleoside phosphorylase (EC 2.4.2.1) is active on guanosine and inosine, but not adenosine (see ref. 21). Adenosine kinase (EC 2.7.1.20) and guano-

sine kinase fail to phosphorylate guanosine and adenosine, respectively[21,22], whereas the same kinase (EC 2.7.1.48) seems to phosphorylate both uridine and cytidine[22,23]. The specificities of the transport systems probably reflect those of nucleoside receptor sites or permeases, but the chemical nature of the transport systems is difficult to assess at present. The finding that PCMB causes a rapid inactivation of the transport systems suggests that –SH groups are involved. The transport systems are relatively sensitive to inactivation by heat, but are highly resistant to the action of various hydrolytic enzymes. It seems likely that a nucleoside inhibits the transport of a heterologous nucleoside by competitively binding to the receptor site or permease for the latter, without being transported itself. This is suggested by the fact that the K_i values for the inhibition of nucleoside transport by heterologous nucleosides are generally much higher than the K_m for the transported nucleoside or the K_m of the inhibiting nucleoside. One exception is adenosine, which has as great an affinity for the guanosine–inosine transport system as for its own, whereas guanosine and inosine have relatively little effect on adenosine transport.

The energy requirements for nucleoside transport are uncertain. PETERSON and coworkers[18,19] concluded that nucleoside transport by *E. coli* is probably coupled to energy metabolism, since it is inhibited about 75 % by 60 mM NaN_3, 20 mM NaCN or 3 mM 2,4-dinitrophenol. Similarly, CUNNINGHAM AND PARDEE[24] concluded that uridine uptake by 3T3 cells is energy-dependent, since it is inhibited over 90 % within 15 sec of addition of 2 mM NaCN or 10 mM iodoacetate. Uridine transport by NISI-67 cells appears to be significantly more resistant to energy poisons than the above systems, even when measured at very low uridine concentrations in the medium (0.5 μM). Further, although 3 mM 2, 4-dinitrophenol or 60 mM NaCN almost completely inhibited uridine uptake, another inhibitor of cytochrome oxidase, NaN_3 at 20 mM, had no effect. Similarly, while 4 mM iodoacetate had some delayed effect on uridine incorporation, 4 mM NaF was ineffective. Interpretation of the data is further complicated by the lack of information on the relative amounts of ATP produced by oxidative phosphorylation and substrate phosphorylation.

The overall results also indicate that transport into the cell is the rate-limiting step in the incorporation of all the nucleosides tested into the nucleotide pool and into nucleic acids. Thus, as pointed out previously[1,3], caution is required in equating the rate of nucleoside incorporation into acid-insoluble material with the rate of nucleic acid synthesis.

ACKNOWLEDGEMENTS

This investigation was supported by U. S. Public Health Service Research Grant AI07250. Some of the experiments on the effect of hydrolytic enzymes on uridine transport were conducted by James R. Buckstein.

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