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TRANSPORT OF TWO NON-METABOLIZED NUCLEOSIDES, DEOXYCYTIDINE AND CYTOSINE ARABINOSIDE, IN A SUB-LINE OF THE L1210 MURINE LEUKEMIA

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SUMMARY

Transport of cytosine arabinoside and deoxycytidine was examined in a sub-line of the L1210 murine leukemia unable to metabolize either nucleoside. At 37°, uptake of these nucleosides was unaffected by metabolic inhibitors, but apparently saturable. A cell/medium distribution ratio of 1 was achieved, within 1 min of incubation, except at the highest nucleoside level tested (25 mM). Kinetic studies were mainly carried out at 0°, at which temperature nucleoside uptake was slowed and both saturability and structural specificity of nucleoside transport could readily be demonstrated.

Nucleoside exit was non-linear at both 0° and 37°, *i.e.* a rapid phase of loss was followed by a much slower phase. The fast phase was inhibited by UO_2^{2+} at either temperature. At 0°, the rapid phase exit of cytosine arabinoside or deoxycytidine could be inhibited by high intracellular levels of certain other nucleosides indicating that a process with structural specificity and saturability was involved.

INTRODUCTION

Although detailed studies on nucleoside transport by *Escherichia coli* have been reported[1,2], similar studies on mammalian cells are complicated by rapid cellular nucleoside metabolism, leading to difficulties in interpretation of flux data. JACQUEZ[3] found evidence to suggest "passive" (but apparently mediated) transport of several pyrimidine nucleosides by Ehrlich ascites carcinoma cells; the nucleosides were extensively hydrolyzed to bases which then diffused from the cells. Most mammalian cells also show a high capacity for conversion of nucleosides to nucleotides[4–6], even at low temperatures[7].

In studies of conversion of the nucleoside cytosine arabinoside to nucleotides by animal leukemia cells[8], L1210/CA, a sub-line of the L1210 leukemia resistant to cytosine arabinoside[9], was found unable to phosphorylate either deoxycytidine or cytosine arabinoside. Deamination[10,11] of cytosine arabinoside or deoxycytidine did not occur in L1210/CA cells nor did phosphorolysis of these compounds. The L1210/CA cell line was therefore useful for characterization of transport of these two non-

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metabolized nucleosides, just as, for example, a peptidase-deficient bacterial mutant was helpful in studies of peptide transport[12].

MATERIALS AND METHODS

Nucleosides

[^{3}H]Cytosine arabinoside, labeled mainly in the pyrimidine ring (3 C/mmole) was purchased from Schwarz Bio-Research Corp., and was provided by the Cancer Chemotherapy National Service Center of the National Cancer Institute. [2-^{14}C]-Deoxycytidine (200 mC/mmole) was purchased from New England Nuclear Corp. Other nucleosides were purchased from Schwarz Bio-Research and Calbiochem. Non-radioactive cytosine arabinoside and uracil arabinoside were provided by Dr. C. SMITH of the Upjohn Co. These compounds, and labeled cytosine arabinoside, were purified by descending paper chromatography (Whatman No. 1) for 20 h using *n*-butanol–formic acid–water (77:10:13, by vol.).

Tumor cells

The cytosine arabinoside-resistant line L1210/CA was provided by Mr. I. WODINSKY of the A. D. LITTLE Corp., Cambridge, Mass. Cells were removed from tumor-bearing DBA/2 mice 7 days after inoculation with 10^6 cells.

Incubations

Tumor cells were collected as described before[8] and resuspended in a medium buffered with 75 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–HCl (Calbiochem) at pH 7.2 (ref. 13) and containing, at levels found in Ehrlich ascites fluid[14], 71 mM NaCl, 20 mM KCl, 1.5 mM $MgCl_2$, 1.3 mM $CaCl_2$ and 1 mM Na_2HPO_4. Another medium[8] was also used for some studies. Incubations were carried out in 10 mm × 30 mm siliconized glass tubes, which contained 200-μl aliquots of 5–8% cell suspensions and additions (< 10 μl) of labeled nucleosides and other compounds. Incubations were terminated by centrifugation of tubes for 30 sec in a Misco model 5500 micro-centrifuge (Microchemical Specialties Co., Berkeley, Calif.) for 30 sec at 300 × *g*; the supernatant fluid was discarded.

To measure uptake of labeled compounds, the cell pellets were quickly blotted with pointed strips of filter paper, taken up in 250 μl of 0.9% NaCl, and a 200-μl portion of this suspension used for determination of cellular radioactivity by liquid scintillation techniques[8]. The minimum time from the addition of labeled nucleosides until the decantation of the supernatant fluid was 1 min.

To measure exodus of labeled nucleosides after a loading incubation, the cells were collected by centrifugation, quickly blotted, then resuspended in 50 vol. of fresh medium at either 0° or 37°. After a measured interval, the cells were again collected by centrifugation, the supernatant fluid was removed, and a portion retained for measurement of radioactivity. The cell pellets were taken up in 0.9% NaCl and cellular radioactivity measured as described above.

Determination of intracellular space

The amount of fluid trapped in cell pellets was determined by incubation of cells in medium containing labeled sulfate (1 mM) under experimental conditions.

The cells were collected by centrifugation, blotted dry, and resuspended in 0.9 % NaCl for determination of radioactivity. The total intracellular *plus* extracellular water in the pellets was determined by measuring the dilution of tritiated water. Under experimental conditions employed here, 200-μl aliquots of a 5 % cell suspension yielded pellets of 10 mg of cells (wet weight) which contained 3.5 μl of intracellular water and 4 μl of extracellular water. After incubation at 37° for 15 min, the extracellular space apparently increased by 10 % at the expense of intracellular space. Appropriate corrections were made for these data in computation of the distribution of nucleosides between cell water and extracellular water.

Identification of cellular radioactive compounds

After incubation in medium containing labeled cytosine arabinoside or deoxycytidine (0.05 mM), cell pellets were collected, then uniformly suspended in 10 vol. of water and frozen rapidly to —80°, then thawed to 4°. After two more freeze–thaw cycles, the cell debris, which contained negligible radioactivity, was removed by high-speed centrifugation. The supernatant fluid was slowly percolated through acid washed charcoal[15]; a 1-ml charcoal column removed 95 % of the radioactivity from 10 ml of cell extract. The column was eluted with 50 % ethanol–5 % NH_3–45 % water until 95 % of the radioactivity was removed. The eluate was taken to dryness *in vacuo*, then dissolved in water. The resulting solution was analyzed by paper chromatography using solvents capable of resolving likely constituents. 5 % conversion could be detected.

Presentation of data

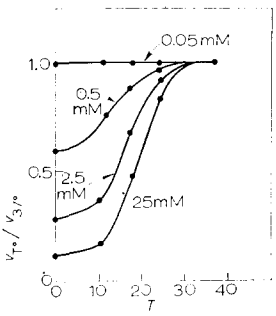
Results from representative experiments are shown. The data were reproducible with other cell preparations to $\pm$ 10 % of these values. Observations described here were generally carried out with labeled cytosine arabinoside, but could be duplicated ($\pm$ 5 %) using labeled deoxycytidine, in the same cell preparations.

RESULTS

Uptake of cytosine arabinoside and deoxycytidine

The observed uptake of either nucleoside, over 1 min (the shortest incubation time feasible under conditions employed here), as a function of temperature and external nucleoside level, is shown in Fig. 1 and Table I. Saturability of uptake, although demonstrable at higher temperatures, could clearly be shown at 0°. The steady-state cell/medium nucleoside distribution achieved at 37°, over a broad range of external nucleoside levels, was approx. 1 (Fig. 2). In contrast, the distribution ratio achieved after incubations at 0° for 45 min varied with the external nucleoside level (Fig. 3). Approximate kinetic constants derived from rate measurements at 0° indicate a K_m of 7.5 mM and a v_{max} of 2 mmoles/min, for uptake of either nucleoside at 0°.

The uptake of cytosine arabinoside or deoxycytidine, at 0°, could be inhibited by any of a variety of other purine or pyrimidine nucleosides, but not by free uracil, cytosine or arabinose (Table II). There was no evidence of inhibition of cytosine arabinoside or deoxycytidine uptake by dinitrophenol (1 mM), KCN (10 mM), *N*-ethylmaleimide (1 mM), or by iodoacetate (1 mM). Addition of uranyl nitrate at 1–5 mM



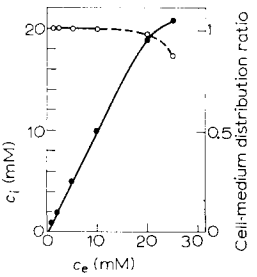


Fig. 1. Relative rate of uptake (as % of rate at 37°) of cytosine arabinoside from different extracellular nucleoside concentrations. The actual rates observed are shown in Table I.

Fig. 2. Effect of the extracellular concentration on steady-state distribution ratio (○—○) and intracellular level (●—●). Cells were incubated for 15 min at 37°.

TABLE I

NUCLEOSIDE UPTAKE DURING 1-min INCUBATIONS

Data are expressed in terms of mmoles/kg cell water.

External level (mM)	*Incubation temperature*				
	0°	*12°*	*18°*	*24°*	*37°*
0.05	0.05	0.05	0.05	0.05	0.05
0.5	0.3	0.375	0.45	0.48	0.5
2.5	0.68	0.9	1.7	2.3	2.5
25	0.7	1.0	3	5.2	6.2

TABLE II

INHIBITION OF UPTAKE OF LABELED CYTOSINE ARABINOSIDE BY STRUCTURAL ANALOGS

Cells were incubated with 0.05 mM labeled nucleosides + 12.5 or 25 mM non-labeled test compounds for 20 min at 0°.

Additions	*Inhibition of uptake of labeled nucleoside* (%)	
	12.5 mM inhibitor	*25 mM inhibitor*
Cytosine arabinoside	50	75
Deoxycytidine	50	75
Cytidine	48	73
Uridine	52	76
Uracil arabinoside	53	75
Thymidine	48	75
5-Fluorouridine	47	72
Adenosine	48	74
Guanosine	49	
Deoxyguanosine	52	
Pseudouridine	40	
5-Fluorodeoxyuridine	43	
Cytosine	5	8
Uracil	3	5
Arabinose (D or L)	2	5
Cytidylic acid	6	9

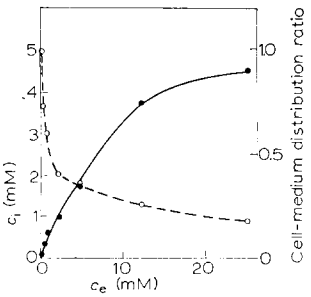
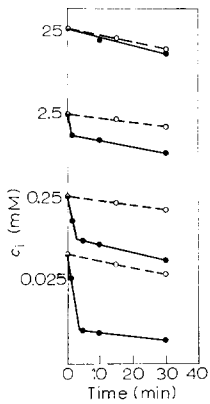


Fig. 3. Effect of extracellular concentration on the cell/medium distribution ratio (○—○) and in intracellular level (●—●) 45 min of incubation at 0°.



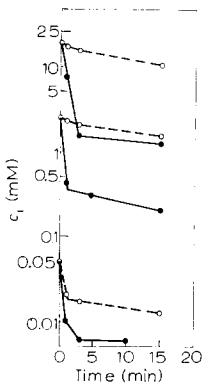


Fig. 4. Effect of initial intracellular nucleoside level on the subsequent rate of loss at 0°. Cells were first incubated with labeled cytosine arabinoside at 37° for 15 min. UO_2^{2+} (5 mM) was present during the measurement of efflux in experiments indicated by the dashed lines.

Fig. 5. Effect of initial intracellular nucleoside level on subsequent loss at 37°. See legend to Fig. 4 for further details.

failed to alter the initial rate of nucleoside uptake at any temperature, but did affect nucleoside loss, and thereby changed the distribution ratio, as shown later.

Nucleoside exit

L1210/CA cells were incubated with labeled cytosine arabinoside or deoxycytidine at 37° for 15 min to load the cells with labeled nucleosides. Subsequent rates of nucleoside loss were measured at 0° or at 37°. Aliquots of the cell suspensions were used to find the initial nucleoside levels before measurement of efflux. The data of Figs. 4 and 5 were obtained with cells which initially contained 0.05–20 mM of labeled cytosine arabinoside. A rapid phase of loss was not detectable at 0°, when the initial nucleoside level was 20 mM. Addition of 1–5 mM of UO_2^{2+} to the medium abolished the rapid phase of nucleoside loss. At the 5 mM level, uranium salts precipitated from the medium. Therefore, studies on efflux into 0.9 % NaCl containing 5 mM UO_2^{2+} were used for data of Figs. 4 and 5. In the absence of UO_2^{2+}, the kinetics of nucleoside loss into the usual medium and into 0.9 % NaCl were identical. In other experiments, not shown here, it was found that *N*-ethylmaleimide, at 5 mM, also inhibited the rapid

phase of nucleoside loss, but to only 20 % of the extent found with UO_2^{2+} at 5 mM.

The rapid phase of loss of labeled nucleosides also showed some specificity with respect to nucleoside inhibitors. For these studies, cells were first incubated for 15 min at 37° with 0.05 mM of labeled deoxycytidine or cytosine arabinoside *plus* non-labeled compounds at 12.5 or 25 mM. The intracellular level of labeled nucleosides was not significantly altered by the addition. Loss of labeled nucleosides at 0° was markedly slowed if pyrimidine nucleosides had been present during the first incubation (Table III). Since this same effect could be demonstrated if non-labeled deoxycytidine or cytosine arabinoside were used, it was concluded that phosphorylation of the test nucleosides during the first incubation was not related to the slowing of loss of radioactivity from cells.

TABLE III

EFFECT OF OTHER NUCLEOSIDES ON LOSS OF INTRACELLULAR CYTOSINE ARABINOSIDE OR DEOXYCYTIDINE

Cells were first incubated with 0.05 mM labeled nucleoside (cytosine arabinoside or deoxycytidine) and test compounds at 25 mM for 15 min at 37°; then resuspended at 0° for 5 min, and the cellular level of labeled nucleoside measured.

Additions	*Retained labeled nucleoside (mM)*	*Inhibition of loss (%)*
None	0.006	0
Adenosine	0.009	7
Deoxyadenosine	0.008	5
Guanosine	0.008	5
Deoxyguanosine	0.007	7
Uridine	0.032	60
Deoxyuridine	0.028	50
Cytidine	0.035	66
Deoxycytidine	0.030	55
Uracil arabinoside	0.030	55
Cytosine arabinoside	0.030	55
Thymidine	0.035	66
Pseudouridine	0.020	32
5-Fluorouridine	0.030	55
Cytosine	0.006	0
Arabinose (D or L)	0.006	0

Extracellular nucleoside levels reached during efflux studies

Measurements of the level of labeled nucleosides in the medium during studies of nucleoside exit showed this to be insufficient to account for the intracellular nucleoside levels present after the rapid phase of loss was over. Suspension of cell pellets in fresh medium resulted in a 70-fold dilution of the total water space of the pellets.

Apparently concentrative uptake of nucleosides

If uranyl ion were present in the incubation medium, uptake of cytosine arabinoside or of deoxycytidine was apparently concentrative, even at 0° (Table IV). In the absence of uranyl ion, concentrative nucleoside uptake could not be demonstrated under the experimental conditions employed here. Active transport was not considered demonstrated by this finding, as will be discussed.

TABLE IV

EFFECT OF UO_2^{2+} ON NUCLEOSIDE ACCUMULATION

Cells were incubated with labeled cytosine arabinoside in medium containing 1 mM UO_2^{2+} under specified conditions.

External nucleoside level (mM)	*Incubation conditions*	*Cell/medium nucleoside distribution*
0.05	1 min, 37°	2.7
2.5	1 min, 37°	2.5
25	1 min, 37°	2.4
0.05	45 min, 0°	2.6

Identification of radioactive intracellular materials

After incubation of cells with labeled cytosine arabinoside or deoxycytidine, the intracellular radioactivity was shown to be the unchanged starting material, as described under METHODS. After such incubations, either at 0° or at 37°, the labeled material which was lost from the cells, either at 0° or at 37°, was similarly found to be the starting material. No chromatographic evidence for deamination of either nucleoside, or of phosphorolytic cleavage to cytosine, was found.

DISCUSSION

These studies were designed to characterize transport of two non-metabolized nucleosides in L1210/CA, a sub-line of the L1210 murine leukemia lacking in deoxycytidine kinase[8]. No significant metabolic alteration of either nucleoside was observed under conditions employed here. Earlier studies of nucleoside transport by mammalian cell types were complicated by reactions which resulted in rapid cleavage or phosphorylation[3–7]. With the parent L1210 cell line, and other animal leukemias tested, very rapid phosphorylation resulted in formation of cellular pools of nucleoside phosphates, but almost no free nucleosides, even at temperatures near 0° (D. KESSEL, unpublished experiments).

The present data permit some conclusions concerning the nature of nucleoside uptake and loss in L1210/CA. Saturation kinetics and structural specificity were clearly demonstrable at temperatures near 0°. At higher temperatures, nucleoside uptake was too rapid to enable careful measurements of flux to be made by methods used here. The present methods were selected to facilitate study of nucleoside exodus and identification of intracellular radioactive compounds. More rapid sampling procedures could yield data which would clarify the question of temperature-sensitivity of nucleoside transport. The use of incubation temperatures substantially below 37° resulted in the delineation of the nucleoside transport process which, at 37°, might be mistaken for a simple (non-facilitated, non-saturable) diffusion. In this regard, it should be noted that entry of thiourea, which is said to occur by simple diffusion[17], is also slowed at temperatures near 0° (ref. 18).

At 0°, both purine and pyrimidine nucleosides, but not free bases or arabinose, inhibited uptake of deoxycytidine or cytosine arabinoside. These data provide further evidence that a mediated process is involved in nucleoside entry.

When exodus of labeled nucleosides was studied, a two-phase process was found.

(1) Rapid, inhibited by UO_2^{2+}, possibly a saturable process, or representing loss from an intracellular nucleoside pool of limited capacity which is larger at 37° than at 0°. (2) Slow ($t_{0.5}$ = 30 min), not affected by UO_2^{2+}.

It is noteworthy that UO_2^{2+} inhibited only nucleoside exit, not entry. This ion, which presumably binds to the cell surface[19], also interferes with sugar[20] and nucleoside[1] transport.

The capacity of L1210/CA cells to apparently concentrate nucleosides after treatment with UO_2^{2+} might represent active transport demonstrable in a system with impaired nucleoside exit. This finding could, however, also represent nucleoside–uranyl interaction and cannot, therefore, be taken to demonstrate active transport, without further studies.

Nucleoside transport in L1210/CA is therefore found to represent a facilitated diffusion, *i.e.*, a non-concentrative process with demonstrable saturation kinetics and structural specificity. Under physiological conditions, at 37°, the cells rapidly take up nucleosides, although this is slowed at lower temperatures. There may be a diffusion component of the entry process, but this was difficult to delineate, since the facilitated process was not abolished at 0°, nor affected by metabolic inhibitors. A rapid phase of nucleoside exodus could be shown with demonstrable saturability, structural specificity, and temperature-dependence, although these could not be ascribed with certainty to a trans-membrane transport process, and could also be explained by the presence of different intracellular nucleoside compartments. Uptake of deoxycytidine and cytosine arabinoside could be inhibited, under appropriate conditions, by either purine or pyrimidine nucleosides, but only the latter inhibited the rapid phase of nucleoside loss.

Interpretation of these data must take into account possible consequences of the derivation of the L1210/CA cell line, which was selected for resistance to cytosine arabinoside. This might have resulted in other mutations in addition to the deletion of deoxycytidine kinase.

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