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MEMBRANE TRANSPORT OF NUCLEOSIDES IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES

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SUMMARY

The membrane transport of nucleosides in rabbit polymorphonuclear leukocytes has been examined by use of a rapid sampling technique. Both purine and pyrimidine nucleosides are transported by a single saturable system as indicated by the identity of their K_m's and K_i's against a spectrum of nucleosides. The specificity of the carrier was examined in detail. Adenosine (K_m = 0.010 mM, v_{max} approx. 10 pmoles/10^6 cells per 45 sec) has the highest affinity for the system. Its fate after uptake is deamination and subsequent conversion to nucleotide. The most critical structural requirements for binding include the pyrimidine base moiety and a 3'-OH configuration on pentose, but other groups make significant contributions to binding. From an analysis of the substrate specificity it is argued that changes in the conformation of the carrier active site are induced by the substrate.

INTRODUCTION

Nucleosides such as adenosine and its analogs can induce profound changes in animal cells. Transport of these compounds into cells occurs by a carrier-mediated process[1-4]. However, a detailed characterization of the specificity of nucleoside transport has not been previously undertaken. The development in this laboratory of a rapid-sampling technique employing cell monolayers[5] has made possible such an analysis in the rabbit polymorphonuclear leukocyte. The results appear to have significant implications for the mechanism of carrier–substrate interactions and for the use of nucleoside analogs to affect intracellular processes.

MATERIALS AND METHODS

Chemicals

[^{3}H]Adenosine, [^{3}H]thymidine and [^{14}C]adenosine were bought from Schwarz Bioresearch. β-Arabinosyladenosine, β-xylosyladenosine, allosyladenosine and L-adenosine were obtained courtesy of Dr. Leon Goodman through the services of Dr. Harry B. Wood, Jr., National Service Center, National Cancer Institute. Tubercidin and psicofuranine were gifts of the Upjohn Company. Formycin was obtained through the generosity of Dr. Makoto Hori, Institute of Microbial Chemistry, Kamiosaki, Shinagawa-Ku, Tokyo, Japan. Puromycin and puromycin nucleoside were bought

from Nutritional Biochemicals Corporation, and other nucleosides from Sigma Chemical Company and Cyclo Chemical Corporation. [^{3}H]Adenosine was purified by ascending paper chromatography in water adjusted to pH 10.5 with NH_4OH. The strips of paper containing adenosine were cut out and the purine nucleoside eluted with modified Hanks solution (see below)at 4°. [^{3}H]Thymidine was purified in the same manner except that the solvent used for ascending paper chromatography was ethyl acetate–formic acid–water (70:20:10, v/v/v).

Animals

New Zealand white rabbits of either sex weighing between 1 and 2 kg were used.

Preparation of leukocytes

Polymorphonuclear neutrophilic leukocytes were obtained from sterile peritoneal exudates in rabbits by the method of KAISER AND WOOD[6]. The neutrophils were centrifuged at 200 × *g* for 5 min at 4°, and the ascitic fluid discarded. The cells were resuspended in modified Hanks solution which consists of the following: 139 mM NaCl, and 10 mM potassium monohydrogen phosphate adjusted to pH 7.4 ± 0.05 with HCl. The neutrophils were kept in cold modified Hanks solution until used; and under these conditions more than 95 % remained viable, as judged by their impermeability to eosin Y, for longer than 3 to 4 h which was the duration of the experiment.

Column chromatography

The mixtures to be analyzed for products of adenosine metabolism were adjusted to approximately pH 2 with perchloric acid. After centrifuging in the cold for 5 min at 2000 × *g* to remove denatured protein, the supernatants were passed over 5 cm × 0.5 cm columns of the cation exchange resin Dowex 50 (H form). The nucleotides were eluted with 4.0 ml water and the nucleosides and purine bases with 6.0 ml of 1.2 M NH_4OH.

Paper chromatography

The products of adenosine metabolism were analyzed by ascending paper chromatography in *n*-butanol–acetic acid–water (50:25:25, v/v/v). Standard spots of purines, nucleosides, and nucleotides were cochromatographed. The chromatogram was cut into strips which were eluted with 0.05 M KOH, and the eluates counted by liquid scintillation in Bray's solution. The products of thymidine metabolism were analyzed by ascending paper chromatography in ethyl acetate–formic acid–water (70:20:10, v/v/v). The chromatogram was cut into 1 cm × 2 cm strips which were placed in vials and counted by liquid scintillation in a toluene-based solution.

Determination of nucleoside uptake by a rapid sampling technique

In this study we used the rapid sampling technique developed by HAWKINS AND BERLIN[5]. Neutrophil monolayers were made on circular coverslips by incubating 0.5-ml aliquots of a suspension containing $4 \cdot 10^6$ leukocytes per ml for 30 min at 37°. After the monolayers were formed, the coverslips were drained, and covered with approximately 0.4 ml of modified Hanks solution, prewarmed to 37°, containing radioactive nucleoside and other compounds to be tested. When the incubations were completed, the coverslips were drained, and rinsed consecutively in four beakers

containing cold modified Hanks solution, which removed extracellular radioactivity. The coverslips were then prepared for counting by liquid scintillation. Samples were done in quintuplicate and the results averaged.

An examination of the rate of uptake using 0.007 mM adenosine indicated transport of this nucleoside to be linear over a 2-min period. The rate of uptake of 0.04 mM thymidine was linear through 45 sec. Unless otherwise stated, the incubations in this study were of 45 sec duration. This uptake is considered the initial rate.

The loss of labeled compound during the rinses in cold modified Hanks solution was determined to be negligible by washing coverslips for varying periods of time. After labeling cells by incubation with 0.007 mM adenosine for 45 sec, there was a loss of only 9 % of radioactivity when coverslips were rinsed for intervals between 25 and 120 sec. When 0.04 mM thymidine was used, there was a 6 % loss of label between 25 and 120 sec. A 25-sec rinse was employed routinely.

Correction of raw data

Theoretically, the radioactivity recovered from the coverslips after rinsing may arise from three processes: (1) carrier-mediated, or (2) diffusion mediated, transport into the leukocyte, or (3) extracellular contamination.

When blank coverslips (no cells) were incubated with 10 mM [^{3}H]adenosine and rinsed there was no detectable radioactivity adherent to the glass. Therefore extracellular contamination is negligible.

It was observed using a supersaturating concentration of 10 mM adenosine (1000 times the K_m) that the amount of radioactivity recovered increased linearly with time through 135 sec. Thus a diffusion mediated process is operable and the radioactivity recovered from coverslips after rinsing reflects both carrier-mediated and diffusion mediated transport. To determine that component representing the carrier-mediated process, in each experiment the uptake of radioactivity from a control 10 mM adenosine solution was determined, and the value subtracted from the total radioactivity taken up by experimental monolayers. This correction amounted to only 1–5 % of the carrier-mediated transport.

RESULTS

Nucleoside metabolism by leukocytes

The intracellular fates of adenosine and thymidine were examined after their incubation with monolayers for 45 sec. After incubation with adenosine, 86 % of the intracellular radioactivity was nucleotide as determined by chromatography on Dowex 50 (see METHODS). Acid hydrolysis of the intracellular nucleotides and paper chromatography showed that approx. 20 % contained the base adenine. A preliminary analysis of the cell content of monolayers incubated with 0.04 mM thymidine indicated a variety of metabolic products, only 17 % of the total radioactivity occurring in thymidine.

It is of considerable importance for the subsequent discussion that the first step in adenosine metabolism be identified. There are three possibilities: direct phosphorylation to AMP, deamination to inosine and subsequent hydrolysis to hypoxanthine, or immediate hydrolysis to adenine. The lattermost pathway is extremely unlikely: no adenine was detected in chromatograms of cell extracts and no adenosine

phosphorylase was found in cell extracts although abundant inosine phosphorylase was demonstrable. This is in keeping with the known poor affinity of adenosine for phosphorylases[7]. Direct phosphorylation to AMP seems unlikely in these cells since the bulk of labeled nucleotides do not contain adenine.

The most direct evidence that the initial step in the metabolism of adenosine is deamination to inosine, with subsequent hydrolysis to hypoxanthine, was provided by experiments in which a double isotope technique was used to follow the separate metabolic fates of the purine and ribose moieties of adenosine. [^{3}H]Adenosine labeled only in the base, and [^{14}C]adenosine labeled 50 % in the base and 50 % in ribose were mixed so that the ratio of disint./min ^{3}H to disint./min ^{14}C was equal to 10. Neutrophil monolayers were incubated for 45 sec with this mixture and sufficient cold adenosine to make the concentration up to 0.007 mM. If the adenosine were directly phosphorylated to AMP then the ratio of disint./min ^{3}H/^{14}C in adenine nucleotides should remain equal to 10. However, if the adenosine were deaminated to inosine and hydrolyzed to hypoxanthine the ratio of disint./min ^{3}H/^{14}C should increase. The cell content was analyzed by paper chromatography, as described under methods. Under the experimental conditions employed for counting the eluates from chromatograms, the only adenine compound having sufficient ^{14}C disint./min to permit an accurate calculation was ATP. The ratio of ^{3}H/^{14}C in this compound was 25 (average of two experiments), approximately what would be expected if all the adenosine were first

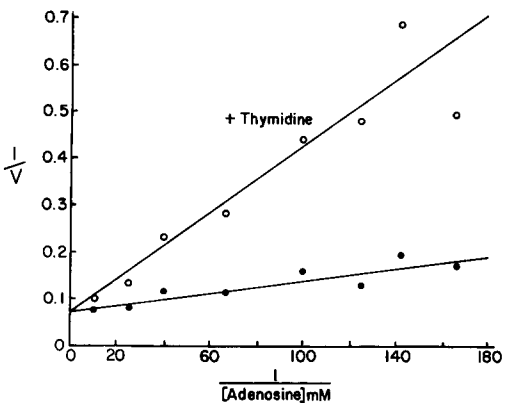


Fig. 1. Double reciprocal plot of initial rate of adenosine transport against the concentration of adenosine in extracellular fluid. $1/v$ expressed as pmoles^{-1}·45 sec^{-1}·10^6 cells^{-1}.

TABLE I

KINETICS OF NUCLEOSIDE TRANSPORT

The values for the K_m and v_{max} are derived from double reciprocal plots of the data. The K_m value is an average of the number of independent determinations indicated in parentheses.

Substrate	K_m (*mM*)	v_{max} (*pmoles/10^6 cells*)
Adenosine	0.010 (4)	9.3, 6.6, 4.6, 14.7
Inosine	0.021 (1)	4.0
Thymidine	0.04 (2)	9.1, 14.5

cleaved into purine base and sugar moieties, and the purine base then reincorporated into nucleotide.

Kinetics of nucleoside transport

Initial rates of adenosine influx were measured at concentrations of substrate between 0.006 and 0.1 mM. The number of dpm per coverslip was corrected for extracellular contamination as described, converted to pmoles transported per 10^6 cells, and the data were expressed in a double reciprocal plot. A typical experiment is plotted in Fig. 1, bottom line, and the results are summarized in Table I. Four separate determinations were made and an average K_m value of 0.010 mM (range, 0.008–0.01 mM) derived. The v_{max} of adenosine transport is more variable. Using the same technique, the K_m for thymidine was found to be 0.04 mM (average of two determinations), and the K_m for inosine was found to be 0.021 mM (one determination). These values were confirmed by the K_i values obtained for thymidine and inosine when these compounds were used as competitive inhibitors of adenosine transport (see below).

Specificity of the nucleoside transport system

The availability of a large number of nucleosides which have been synthesized as potential chemotherapeutic agents made possible a detailed study of transport specificity. It was first shown that representative analogs altered in either sugar, base, or both sugar and base were competitive inhibitors of adenosine transport. The analogs selected for detailed study encompassed the range of structural modifications: inosine (6-position of the purine base modified), arabinosyl adenine (sugar moiety modified, 2-epimer of ribose), and thymidine (pyrimidine base and sugar moiety modified, 2-deoxyribose). Monolayers were incubated at various adenosine concentrations between 0.006 and 0.1 mM with and without inhibitor. The concentrations of inhibitor used were: inosine, 0.5 mM; arabinosyladenine, 0.8 mM; and thymidine, 0.1 mM. The data for both inhibited and uninhibited transport of adenosine were corrected as discussed above (see kinetics of adenosine transport), and expressed in double reciprocal plots of pmoles transported versus concentration of substrate. In all three cases the line for inhibited adenosine transport intersected the line for uninhibited adenosine transport on the ordinate, thus indicating competitive kinetics. As an example, Fig. 1 shows a plot for adenosine transport inhibited by 0.1 mM thymidine.

There is further evidence that thymidine and adenosine share a common membrane carrier. If the transport carrier is the same, and K_m and K_i represent simple equilibrium constants, then the K_m value for adenosine should be the same as its K_i value when adenosine is tested as an inhibitor of thymidine transport. Also, a given competitive inhibitor (in this case tubercidin and uridine were used) should have the same K_i value when tested against either adenosine or thymidine[8]. The results are summarized in Table II and indicate the existence of a single transport system for all nucleosides. The K_i's for tubercidin (or uridine) against adenosine and thymidine are equal within experimental error. The K_m for adenosine (or thymidine) and its K_i against thymidine (or adenosine) are also equal.

Characterization of carrier specificity by the effects of analogs on adenosine transport

The foregoing results establish the competitive nature of inhibition by a variety

TABLE II

EVIDENCE FOR A COMMON NUCLEOSIDE CARRIER BASED ON THE KINETICS OF TRANSPORT

A comparison of K_m and K_i values for adenosine and thymidine, and of K_i values for tubercidin and uridine against adenosine and thymidine.

Substrate	K_m (*mM*)	K_i (*mM, inhibitor*)			
		Adenosine	*Thymidine*	*Tubercidin*	*Uridine*
Adenosine	0.010	—	0.055	0.017	0.030
Thymidine	0.040	0.014	—	0.016	0.020

of adenosine analogs, modified structurally in either sugar, base, or both sugar and base. Consequently, it is assumed that numerous related compounds also exert competitive inhibition on adenosine transport. Thus their K_i's, which characterize their affinity for the carrier may be determined from the inhibition at a single concentration of adenosine. In practice 0.007 mM adenosine (approximately its K_m) and a concentration of inhibitor yielding roughly 30–60 % inhibition were used in triplicate experiments. The K_i for the test compound could be obtained from a simple formula*.

Firstly, it was shown that the affinity of the nucleoside transport system for the free bases, including adenine, is extremely poor. The K_i's for adenine and xanthine, the only bases with detectable affinity, were approx. 10 mM or four orders of magnitude greater than adenosine. This clearly separates the nucleoside transport system from either the adenine or xanthine transport systems previously described[5].

The remaining results are grouped according to categories of molecular structure. In Table III are listed purine ribonucleosides in descending order of affinity. Substitutions of similar bulk to the amino group of adenine are well-tolerated (II–IV). However, the relatively poor affinities of purine riboside (VIII), with the least bulk at the 6-position, and of xanthosine (IX) with substitutions similar in bulk to guanosine (III), indicate that conjugated sites at other regions of the molecule play a significant role in the binding of these molecules to the carrier.

The imidazole moiety is nonessential, as shown in Tables IV and V which summarize results with pyrimidine nucleosides and various analogs of adenosine. Uridine is comparable in affinity to the strongest purine inhibitors.

* $K_i = \dfrac{I}{[v/v_i(1 + K_m/S) - 1]S/K_m - 1}$

where I and S are the concentrations of inhibitor and substrate, respectively, and v and v_i are the velocities with and without inhibitor. This is derived by solving Eqn. 1 for v_{max} substituting in Eqn. 2 and solving for K_i:

$$v = \frac{v_{max}}{1 + K_m/S} \quad (1)$$

$$v_i = \frac{v_{max}}{1 + K_m/S(1 + i/K_i)} \quad (2)$$

Under the conditions of these experiments we may substitute 0.010 mM for the K_m and 0.007 mM for S.

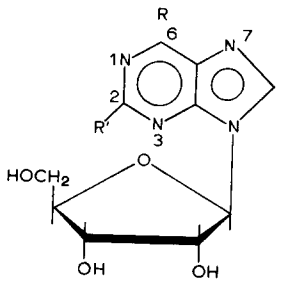
$$K_i = \frac{I}{(2.43v/v_i - 1)0.7 - 1} = \frac{I}{1.7(v/v_i - 1)}$$

In general, the pyrimidine moiety of the bicyclic purine structures would seem the essential base component. Thus, in Table V it is seen that tubercidin (XIV) which is modified in the 5-membered ring is the best inhibitor of all analogs tested, whereas AICA (XVI) in which the imidazole portion is intact (pyrimidine, eliminated) binds very poorly.

With respect to the linkage between base and sugar, the relatively poor affin-

TABLE III

BINDING TO THE ADENOSINE CARRIER BY PURINE RIBOSIDES

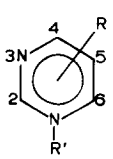


	Compound	*R*	*R'*	K_i (*mM*)
(I)	Adenosine	NH_2	H	0.010*
(II)	Isoguanosine	NH_2	OH	0.013
(IIA)	Inosine	OH	H	0.022
(III)	Guanosine	OH	NH_2	0.033
(IV)	6-Mercaptopurine riboside	SH	H	0.038
(V)	6-Methyladenosine	$NHCH_3$	H	0.107
(VI)	6-Chloropurine riboside	Cl	H	0.12
(VII)	6-Dimethyladenosine	$N(CH_3)_2$	H	0.18
(VIII)	Purine riboside	H	H	0.22
(IX)	Xanthosine	OH	OH	0.91

* K_m.

TABLE IV

BINDING TO THE ADENOSINE CARRIER BY PYRIMIDINE NUCLEOSIDES

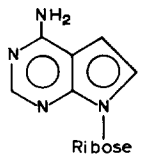
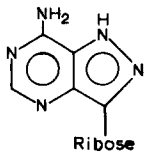
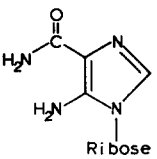


	Compound	*R*	*R'*	K_i (*mM*)
(X)	Uridine	2,4-Dihydroxy	1-Ribose	0.03
(XI)	Cytidine	2-OH, 4-NH_2	1-Ribose	0.05
(XII)	Thymidine	2,4-Dihydroxy-5-methyl	1-(2'-Deoxy)ribose	0.06
(XIII)	Pseudouridine	2,4-Dihydroxy-5-ribose	H	0.28

ities of pseudouridine (XIII) and formycin (XV) in which the linkage is carbon to carbon rather than nitrogen to carbon, suggests nitrogen to carbon bond specificity. However, in each case, in addition to the alteration in base–sugar bonding, there are distortions or modifications at ring sites which may be equally critical.

TABLE V

BINDING TO THE ADENOSINE CARRIER BY AZALOGS OF ADENOSINE

Compound	*Structure*	K_i (*mM*)
(XIV) Tubercidin (7-deazaadenosine)		0.017
(XV) Formycin (8-aza-9-deazaadenosine)		0.203
(XVI) AICA (4-amino-5-imidazole) carboxamide riboside		0.62

There is a high degree of specificity for the substituent at the 9-position as illustrated by a series of pentosyl derivatives. The results are grouped in Table VI. The 3′-position is clearly more important in determining binding than is the 2′. Thus, the 2′-deoxy compound (XVII) has an affinity nearly 50 times greater than the 3′-deoxy analog, cordycepin (XIX). Similarly, the 2′-epimer, arabinosyladenine (XVIII), is a much better inhibitor than the 3′-epimer, xylosyladenine (XXII). Comparison of dimethylaminopurine nucleoside (VII) with puromycin nucleoside (XXIV) shows that a 10-fold loss in affinity accompanies substitution of an amino for an hydroxyl group at the 3′-position. Further substitution of the 3′-amino group as in puromycin itself (XXV) leads to an additional though small, further decrease in affinity. The 3′-OH in the α-configuration is clearly an important determinant in the affinity of nucleosides for the transport protein.

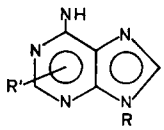
It may be noted that certain 9-alkyl derivatives show appreciable inhibition of adenosine transport. 9-Cyclopentyl and 9-cyclohexyladenine have K_i's of 0.7 mM 9-*n*-propyladenine, 3.2 mM, whereas 9-methyladenine gives no detectable inhibition.

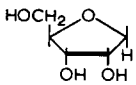
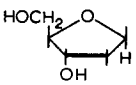
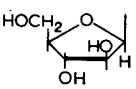
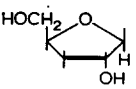
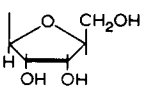
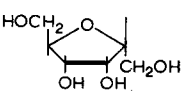
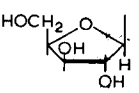
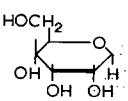
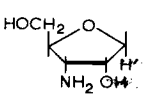
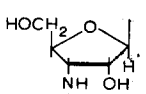
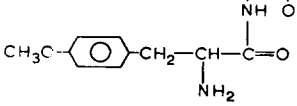
Effect of borate ion on nucleoside transport

The formation of reversible complexes of borate ion with sugars containing adjacent *cis*-hydroxyl groups[9] (in the case of ribose derivatives, at positions 2′ and 3′) can produce an apparent change in the selectivity of the carrier. The borate

TABLE VI

BINDING TO THE ADENOSINE CARRIER BY 9-PENTOSYLADENINE DERIVATIVES



	Compound	*R*	*R'*	K_i (*mM*)
(I)	Adenosine			0.010
(XVII)	2'-Deoxyadenosine			0.037
(XVIII)	9-(β-D-Arabinofuranosyl)-adenine			0.298
(XIX)	9-(β-D-3'-Deoxyribofuranosyl)-adenine, cordycepin			1.94
(XX)	L-Adenosine			1.99
(XXI)	9-(β-D-Psicofuranosyl)-adenine, psicofuranine			3.04
(XXII)	9-(β-D-Xylofuranosyl)-adenine			4.36
(XXIII)	9-(β-D-Allofuranosyl)-adenine			∞
(XXIV)	Puromycin nucleoside		6-$N(CH_3)_2$	1.88
(XXV)	Puromycin	 	6-$N(CH_3)_2$	2.47

complex introduces a bulky negatively charged group near to the critical ribose 3'-position, which interferes with binding to the carrier. Table VII shows the results of an experiment in which the effect of borate on the transport of a ribose compound (adenosine) was compared with its effect on a 2'-deoxyribose compound (thymidine). As anticipated, since the borate ion complexed only with the ribose derivative (line 2) and not with the deoxyribose derivative (line 4), the affinity of adenosine is altered but not that of thymidine.

This observation was extended by testing the effect of borate ion on the ability of uridine to inhibit thymidine transport (line 6). The borate–uridine complex should be a poorer inhibitor than uridine itself, and this is indicated by a comparison of line 6 with line 5 of Table VII. Assuming that a nucleoside–borate complex has no affinity for the carrier, line 2 allows calculation of an equilibrium constant for the nucleoside–borate complex. If this equilibrium constant is identical for both purine and pyrimidine ribonucleosides the effect of borate on uridine inhibition of thymidine transport may be predicted. This theoretical value agrees well with the experimental value*.

DISCUSSION

The foregoing results indicate that adenosine and other nucleosides enter the polymorphonuclear leukocyte by a specific saturable process. The immediate fate of

* The equilibrium constant for the reaction adenosine (RAd) + borate $\rightleftarrows$ adenosine–borate can be determined from the data of Table VII, lines 1 and 2, assuming that the borate complex is completely inert and that borate itself is non-inhibiting. From the velocity indicated in line 1, the K_m of 0.01 mM and the substrate concentration, the v_{max} of the cells in these experiments is calculated after rearrangement of the Michaelis–Menten equation

$$1/v_{max} = \frac{1}{(1 + K/S)v} = \frac{1}{(1 + 0.01/0.007)4.4}$$

$$v_{max} = 10.25 \text{ pmoles}/10^6 \text{ cells}$$

The effective concentration of RAd in the presence of borate is (again after rearrangement of the same equation)

$$S = \frac{K_m}{(v_{max}/v - 1)} = \frac{0.01}{(10.25/1.65 - 1)} = 0.0019$$

Thus, the equilibrium constant

$$K_{BorRAd} = \frac{(RAd\text{–}Bor)}{(RAd)\,(Bor)} = \frac{(0.007 - 0.0019)}{(0.0019)\,(20)} = \frac{0.0051}{0.038} = 0.134$$

Assuming this constant is alike for all ribose compounds, then the concentration of free uridine, ψ, available in the experiment of line 6 is

$$\frac{(0.05) - \psi}{(\psi)\,(20)} = 0.134$$

$$\psi = 0.0136 \text{ mM}$$

Using the K_i uridine determined in the experiment of line 5, (see footnote on p. 11) and assuming strict competitive kinetics against thymidine (K_s, affinity constant for thymidine),

$$v_i = \frac{1}{1 + K_s/S(1 + \psi/K_i)}$$

the theoretical inhibition is $v_i/v \times 100 = 22\%$, as compared with the 29% observed inhibition in the experiment of line 6.

adenosine in these cells is its deamination. Considerable kinetic evidence indicates that the entry and metabolism of adenosine are separate events: (a) the K_m for adenosine uptake, 0.01 mM and deamination, 0.073 mM, are clearly different (Table VIII) (b) the specificities of the transport system and of adenosine deaminase are not the same (Table VIII). While some variation in specificity with enzyme source may be expected, striking differences are found wherever data are available. We have found (unpublished results) that pyrimidine nucleosides are poor inhibitors of the adenosine leukocyte deaminase whereas they have high affinity for the nucleoside transport system (Table IV). These differences between transport and the first enzyme of metab-

TABLE VII

THE EFFECT OF BORATE ION–RIBOSE COMPLEXES ON THE AFFINITY OF NUCLEOSIDES FOR THE CARRIER

Uptake of [^{3}H]adenosine or [^{3}H]thymidine was determined as in METHODS. The pH of the incubation media containing 20 mM sodium borate was adjusted to 7.4 with HCl. Values are the average of three experiments.

Composition of medium	*pmoles/10^6 cells*	% *Inhibition*
(1) 0.007 mM [^{3}H]Adenosine	4.40	—
(2) 0.007 mM [^{3}H]Adenosine + 20 mM borate*	1.65	64
(3) 0.04 mM [^{3}H]Thymidine	4.33	—
(4) 0.04 mM [^{3}H]Thymidine + 20 mM borate	4.08	7.3
(5) 0.04 mM [^{3}H]Thymidine + 0.05 mM uridine	1.97	52
(6) 0.04 mM [^{3}H]Thymidine + 0.05 mM uridine + 20 mM borate	3.11	29

* 5 mM $Na_2B_4O_7$ assuming complete dissociation and hydrolysis of the borate anion in solution.

TABLE VIII

COMPARISON OF AFFINITIES OF SELECTED COMPOUNDS FOR THE NUCLEOSIDE TRANSPORT SYSTEM AND FOR ADENOSINE DEAMINASE

	Compound	K_m *or* K_i *(mM)*		*Reference*
		Transport	*Deaminase*	
(I)	Adenosine	0.010	0.042	10
			0.083	11
			0.073*	12
(II)	Inosine	0.022	>0.3	13
(IV)	6-Mercaptopurine riboside	0.038	0.37	14
(V)	6-Methyl adenosine	0.107	0.0053	14
(VIII)	Purine riboside	0.22	0.0088	14
(XIV)	Tubercidin	0.017	>0.4	14
(XVII)	2'-Deoxyadenosine	0.037	0.015	10
(XXII)	Xylosyladenosine	4.36	0.394	15
(XXVI)	Adenine	14.5	0.28	13

* K_m for leukocyte adenosine deaminase was determined to be 0.02 mM. A crude extract from sonified cells was used. The activity was determined by employing adenosine substrate in the absence of phosphate, and determining the ratio of inosine to adenosine after ascending paper chromatography in a solvent system containing *tert.*-butanol–methyl ethyl ketone–water–conc. NH_4OH (40:30:20:10, v/v/v/v).

olism are characteristic of animal cells[16]. This fact should be considered in assessing the mechanism of action of certain nucleosides, which by competition for adenosine deaminase inhibit the degradation of a second nucleoside. Thus nucleoside A may inhibit the deamination of nucleoside, B, thereby enhancing its activity. However, A may also inhibit the transport of B and thereby decrease its effective intracellular concentration.

Evidence for a flexible transport carrier protein

It is sometimes assumed that the specificity of the transport protein is determined by an active site which is rigid in conformation. The results of the present study, however, are most consistent with the concept that the carrier molecule is quite flexible and that conformational changes are induced by interaction with the substrate at the active site, analogous to the induced–fit hypothesis of enzyme–substrate interactions advocated by KOSHLAND AND NEET[17]. This conclusion is based on a comparison of the binding affinities of purine *versus* pyrimidine nucleosides. Essentially, it can be shown that in order for the active site to accommodate both purine and pyrimidine nucleosides with comparable affinity, there must be considerable flexibility. (We assume that the mutually competitive kinetics imply the same active site for all nucleosides.) There is a marked difference in the spatial relationship of ribose to pyrimidine as compared with ribose to purine (see Fig. 2). However, the transport protein appears to bind quite specifically, at both ribose and base, to groups which are separated by much shorter distances than the displacement of the pyrimidine ribose with respect to the purine ribose.

A specific example will make the point clear. The bases of the nucleosides are rigid planar molecules[18]. It seems to follow that isoguanosine (II) and cytidine (XI) should have similar affinities (which they do, K_i equal to 0.013 and 0.05 mM, resectively) since the nuclei of atoms in the pyrimidine compound are nearly superimposable on those of the pyrimidine moiety of the purine derivative (Fig. 2). How-

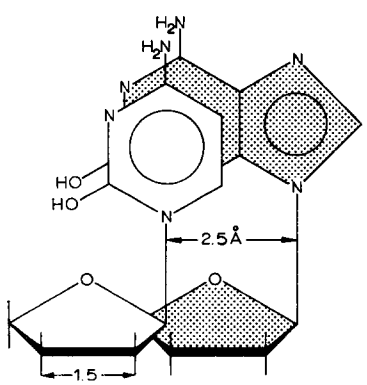


Fig. 2. Schematic representation of cytidine and isoguanosine structures. Cytidine is shown with the atomic nuclei of its pyrimidine moiety superimposed on the corresponding pyrimidine moiety of guanosine. After superposition, the carbon to nitrogen glycosidic bonds of the two nucleosides are separated by 2.5 Å. Individual hydroxyl groups of ribose which make distinguishable contributions to the binding of the nucleosides to the carrier protein are separated by only 1.5 Å (ref. 19). The representation does not indicate the difference in C–N bond angles with the bases, nor the preferred rotations of the ribose with respect to the plane of the bases[20].

ever, the base–ribose bonds are displaced approx. 2.5 Å and rotated 11 degrees with respect to one another[19]. (The most probable configuration appears to be one in which the ribose is rotated perpendicular to the plane of the paper[20].) Small rotations or slightly less probable configurations are possible; however, these cannot alter the large displacement of the glycosidic bonds. The resulting shift in the position of the ribose is equivalent to major translocations of specific binding groups. Adjacent hydroxyl groups on ribose which, as indicated in Table VI, make highly specific contributions to the binding affinity are separated by only 1.5 Å. A 2.5-Å displacement would be expected to lead to drastic reductions in affinity if the binding site were conformationally rigid. One could postulate, of course, that the sugar were bound at a rigid site, but if the ribose moieties of purine and pyrimidine nucleosides are superimposed, a corresponding displacement of the bases is also evident.

These findings lead us to hypothesize that there is an induced fit of a flexible carrier about the substrate. Specificities for ribose and pyrimidine moieties are preserved perhaps by relatively fixed constellations of contact amino acid residues. Operationally, these constellations may be moved with respect to one another by 2.5 Å. This may be achieved with some strain in the carrier protein, but the similar affinities of purine and pyrimidine nucleosides such as cytidine and isoguanosine suggest that the substrate-induced conformations are of similar energy level.

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