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FACILITATED TRANSPORT OF INOSINE AND URIDINE IN CULTURED MAMMALIAN CELLS IS INDEPENDENT OF NUCLEOSIDE PHOSPHORYLASES

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Summary

The zero-*trans* uptake of uniformly and base-labeled inosine and uridine was measured at 25°C in suspensions of Novikoff rat hepatoma cells, Chinese hamster ovary cells, mouse L cells, mouse S49 lymphoma cells and a purine-nucleoside phosphorylase-deficient subline thereof (NSU-1), and in monolayer culture of mouse 3T3 and L cells. The initial velocities of uptake of both nucleosides were about the same in all cell lines investigated, regardless of the position of the label or of the substrate concentration between 3 and 300 μM or whether or not the cells possessed uridine or purine-nucleoside phosphorylase activity. The kinetic parameters for the facilitated transport of uridine and inosine were also similar in phosphorylase positive and negative cell lines (K = 120—260 μM and V = 6—40 pmol/μl cell water per s) and the transport activities of the cells exceeded their total phosphorylase activities by at least 10-fold for uridine and 1—2-fold for inosine. Chromatographic fractionation of the intracellular contents and of the culture fluid showed that the free nucleosides appeared intracellularly prior to and more rapidly than their phosphorolysis products. During the initial 20—60 s of uptake of U-^{14}C-labeled nucleosides the rates of intracellular appearance of ribose-1-*P* and base were about the same. After several minutes of incubation, on the other hand, the main intracellular component was ribose-1-*P* whereas the base attained a low intracellular steady-state concentration and accumulated in the medium due to exit transport. Other nucleosides, dipyridamole and nitrobenzylthioinosine, specifically inhibited the transport of uridine and inosine, and depressed the intracellular accumulation of ribose-1-*P* and the formation of base commensurate with that inhibition. The data indicate that the metabolism of inosine and uridine by the various cell lines can be entirely accounted for by the facilitated transport

of unmodified nucleoside into the cell followed by intracellular phosphorolysis.

Introduction

In previous studies [1—5] we have measured the transport of uridine, thymidine, adenosine and deoxycytidine into cells of various lines in which their conversion to nucleotides was blocked, either by chemical treatment or because of a deficiency in metabolizing enzymes. These studies documented that the nucleosides are transported by a single, simple, symmetrical carrier, although probably not with the same efficiency (for reviews see Refs. 6 and 7). Michaelis-Menten constants in Novikoff rat hepatoma cells range from about 150 μM for adenosine [5] to 200—300 μM for thymidine [2,4,6] and to about 600 μM for deoxycytidine [6]. Similar values have been observed for other cell lines [6] (Wohlhueter, R.M. and Plagemann, P.G.W., unpublished data). Transport is very rapid; the half-time ($t_{1/2}$) for transmembrane equilibration in the first-order range of substrate concentration is 6—15 s, which is at least 20-times more rapid than their non-mediated permeation [6]. The long-term (over minutes) accumulation of radioactivity from these nucleosides in cells active in phosphorylation ('uptake' *), on the other hand, was shown to reflect the accumulation of nucleotides and to involve the tandem operation of facilitated transport and intracellular phosphorylation [7].

In contrast to our results, Hochstadt and her collaborators [8—13] have reported that membrane vesicles from L_{929} cells and from transformed and untransformed 3T3 cells lack a facilitated transport system for inosine. Instead, these investigators concluded that inosine is degraded by membrane-bound purine-nucleoside phosphorylase (EC 2.4.2.1), whereby the ribosyl moiety of inosine is transferred to P_i and appears in the intravesicular space as ribose-1-*P* (group translocation), while the hypoxanthine moiety is released to the extravesicular space. This conclusion was based mainly on the finding that during 10—20 min of incubation of the membrane vesicles with [U-^{14}C]-inosine, only labeled ribose-1-*P* seemed to accumulate in the vesicles, whereas no inosine was detected intravesicularly, and hypoxanthine accumulated only in the extravesicular fluid.

Preferential intravesicular accumulation of labeled ribose-1-*P* was also observed with [U-^{14}C]uridine in vesicles from L_{929} cells, and led to the conclusion that a ribosyl group translocation is also catalyzed by membrane-associated uridine phosphorylase (EC 2.4.2.3) with the extravesicular release of uracil [10]. It has also been concluded from studies with rat liver slices [14] and intact rats [15] that the rapid phosphorolysis of uridine observed in the circulation occurs mainly in the plasma membrane of liver cells without entry of either uridine or uracil into cells. In the present study we have assessed the extent to which such group translocations of ribose-1-*P* from uridine

* 'Uptake' denotes the accumulation of radioactivity, derived from exogenous, labeled nucleosides, within the cell regardless of metabolic conversions. 'Transport' strictly denotes the transfer of a substance across the cell membrane as mediated by a saturable, selective carrier.

and inosine might be operating in cells of a number of mammalian cell lines including those of which the vesicles have been reported to exhibit such activity. Our results indicate that a phosphorolysis of uridine and inosine occurs primarily intracellularly subsequent to their transport into the cell via the broadly specific, facilitated transporter of mammalian cells.

Materials and Methods

Cell culture. Novikoff rat hepatoma cells (N1S1-67) and hypoxanthine/guanine phosphoribosyltransferase-deficient (1-9-1; Ref. 16) and uridine kinase-deficient (1-14-6; Ref. 2) strains thereof were propagated in suspension culture in Swim's medium 67 as previously described [17]. P388 mouse leukemia, mouse L (except L_{929}) and Chinese hamster ovary (CHO) cells were propagated in spinner culture in Eagle's minimal essential medium for suspension culture supplemented with 2% (v/v) fetal calf serum, 4% (v/v) horse serum and 4% (v/v) calf serum (all heat-inactivated at 56°C for 30 min). S49 mouse T lymphoma cells and a purine-nucleoside phosphorylase-deficient strain thereof (NSU-1), kindly supplied by Dr. D.W. Martin, Jr., were propagated in stationary suspension culture in 650-ml plastic tissue culture flasks (Costar) in unmodified Eagle's minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. Cells to be used in experiments were harvested from exponential phase cultures by centrifugation at $400 \times g$ for 1 min and suspended as indicated in complete or glucose-free basal media without serum [18].

We have used four lines of mouse L cells in these studies: L-67, propagated in our laboratory for the last 15 years [19]: Ltk^-, a thymidine kinase-deficient strain of L cells originally isolated by Kit et al. [20]; L_{929}, obtained from the American Type Culture Collection; and L_{241}, a strain of L_2 (a subline of L_{929}) which has been selected for sensitivity to mouse hepatitis virus [21], obtained from Dr. Martin V. Haspel. L_{929} and Balb/c 3T3 cells were propagated in monolayer culture in 60-mm or 150-mm plastic petri dishes (Lux Scientific Corp.) in Eagle's minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. Cells were enumerated with a Coulter counter. Cultures of all cell lines were ascertained to be free of mycoplasma as determined by the uridine-uracil incorporation method [22].

Nucleoside phosphorylase activity measurements in cell extracts. Cell extracts were prepared in 145 mM NaCl and 10 mM phosphate (pH 7.4) as described previously [5]. In the assay of phosphorolytic activity, the final reaction mixtures contained 200 μM [U-^{14}C]inosine, [2-^{3}H]inosine or [U-^{14}C]-uridine (5—10 cpm/pmol) and 25 mM potassium phosphate (pH 7.8). To assay uridine phosphorylase activity based on the conversion of uracil to uridine, the final reaction mixture contained 1 mM [5-^{3}H]uracil (3 cpm/pmol), 2 mM ribose-1-*P* and 50 mM triethanolamine hydrochloride (pH 7.8). Reaction mixtures were incubated at 37 or 25°C and five replicate samples of 100 μl were removed at various time intervals, heated in a boiling water bath for 1 min, clarified by centrifugation, and substrate and products were separated chromatographically on 3MM Whatman paper with a solvent composed of 6 vol. of butanol and 1 vol. of H_2O (solvent 30) as described previously [18].

At equivalent substrate concentrations and a pH between 7.4 and 7.8, the rate of conversion of uracil to uridine was about 1.3-fold greater than the rate of phosphorolysis of uridine.

Measurement of zero-trans uptake of nucleosides by whole cells. The zero-*trans* * uptake of radioactively labeled uridine and inosine into cells in suspension ($1-4 \cdot 10^7$/ml of basal medium) was measured at 25°C by a rapid mixing-sampling technique described in detail previously [4,6,24] which allows sampling of cell suspensions in intervals as short as 1.5 s. Where indicated, the cells were depleted of ATP (and *P*-Rib-*PP*) by preincubation in glucose-free basal medium containing 5 mM KCN and 5 mM iodoacetate at 37°C for 10—15 min [25].

Nucleoside uptake in confluent monolayer cultures of 3T3 and L_{929} cells in 6-cm plastic petri plates was determined as described previously [2]. After various times of incubation (2 s to 10 min) with radioactively labeled nucleosides at 25°C, the medium was aspirated and the cell layer rapidly washed four times with 4 ml of ice-cold balanced salt solution containing 50 μM dipyridamole [2]. The cells were analyzed for radioactivity and the values converted to pmol/μl cell H_2O on the basis of intracellular H_2O values estimated by equilibrium labeling with 3-*O*-methyl-D-[^{3}H]glucose [2].

The time course of intracellular substrate accumulation to transmembrane equilibrium as mediated by the simple, completely symmetrical nucleoside carrier of cultured mammalian cells [4—6] in the zero-*trans* mode is described by the following integrated rate equation [6]:

$$S_{2,t} = S_1\left[1 - \exp\left(-\frac{tV + (1 + S_1/K)\,S_{2,t}}{K + 2S_1 + S_1^2/K}\right)\right] \tag{1}$$

Where $S_{2,t}$ = concentration of substrate inside the cell at time t ($S_{2,0} = 0$); S_1 = exogenous substrate concentration (and is taken as constant); V is the maximum velocity and K is equivalent to the Michaelis-Menten constant.

Eqn. 1 is strictly applicable only to substrate uptake data when no substrate metabolism occurs, but we find it yields reasonable estimates of initial velocities and kinetic parameters when fitted to the early portion of the time courses of substrate uptake in metabolizing cells, provided the rate of substrate metabolism is much lower than that of transport [6,7]. K and V for inosine and thymidine transport were estimated by fitting Eqn. 1 by the method of least squares to zero-*trans* uptake data pooled for six to nine substrate concentrations [4—6]. To analyze time courses of nucleoside uptake at a single concentration, Eqn. 1 was fitted to the data, whereby K was fixed at its experimentally determined value to yield an estimate of V. In both cases, initial zero-*trans* velocities (v_{12}^{zt}) were calculated for a given concentration (S_1) as the slope of the best-fitting curve at $t = 0$, given by:

$$v_{12}^{zt} = \frac{S_1 V}{K + S_1} \tag{2}$$

* As defined by Eilam and Stein [23], zero-*trans* denotes the transport of a substrate from one side of the membrane to the other side where its concentration at zero time is zero. Arbitrarily, we designate the outside and inside faces of the membrane as 1 and 2, respectively.

All computations were conducted as described by Wohlhueter et al. [4] with some refinements (Wohlhueter, R.M. and Plagemann, P.G.W., unpublished data). Computed values are stated ±S.E. of estimate.

To determine time courses of nucleoside uptake by cell suspensions stretching over 5—15 min ('long-term' uptake), suspensions of $1—4 \cdot 10^7$ cells/ml were supplemented with substrate and after various times of incubation at 25°C the cells from 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for intracellular radioactivity [2,4,5].

When analysis of the culture fluid was required, samples thereof were removed immediately after centrifugation of cell suspensions or directly from monolayer cultures, heated in boiling water bath for 1 min, and subjected to chromatography with solvent 30.

When fractionation of intracellular radioactivity was required, suspended labeled cells were centrifuged through oil directly into an acid solution [24]. Rinsed, labeled monolayer cultures were extracted with 0.2 ml of 0.5 M trichloroacetic acid. The acid extracts were processed as described previously [2—5] and subjected to chromatography with solvent 30.

To monitor the oxidation of inosine to CO_2, samples of 5 ml of cell suspension supplemented with [U-^{14}C]inosine (see above) were incubated in CO_2 collection flasks [26] at 25°C. At various times, 1 ml of 1 M trichloroacetic acid was added per flask. The flasks were kept at 0°C for 30 min and the radioactivity in CO_2 was determined [26].

Materials. Unlabeled nucleosides were purchased from Sigma Chemical Co. [U-^{14}C]-, [8-^{14}C]- and [2-^{3}H]inosine were purchased from Amersham Corp. (Arlington Heights, IL) and Moravek Biochemicals (City of Industry, CA), [U-^{14}C]uridine and [5-^{3}H]uracil from Amersham, [2-^{14}C]uridine from ICN (Irvine, CA) and [5-^{3}H]uridine from New England Nuclear (Boston, MA). The isotopes were diluted to the desired specific radioactivity with unlabeled substance. Dipyridamole (Persantin) and 6-([4-nitrobenzyl]thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine) were gifts from Geigy Pharmaceuticals and Dr. A.R.P. Paterson (University of Alberta), respectively.

Results

Uridine uptake by uridine kinase-deficient Novikoff cells and by other cell lines

The results in Fig. 1A and B show that the initial time courses of uptake of radioactivity from [U-^{14}C]- and [2-^{14}C]uridine by uridine kinase-deficient Novikoff cells were about the same at 10 and 250 μM substrate. The use of [U-^{14}C]uridine enabled us to monitor, by chromatographic fractionation, the intracellular and extracellular appearance of metabolites of both the sugar and base moieties of uridine. During the first 30 s of incubation with either 10 or 250 μM [U-^{14}C]uridine, over 90% of the intracellular radioactivity was associated with free uridine. Time periods of minutes were required to detect significant formation of uracil and ribose-1-*P*. Results from such an experiment with 250 μM [U-^{14}C]uridine in Novikoff hepatoma cells are illustrated in Fig. 2. Consistent with the measured transport velocity (Fig. 1B), an intracellular steady-state concentration of free uridine, which was

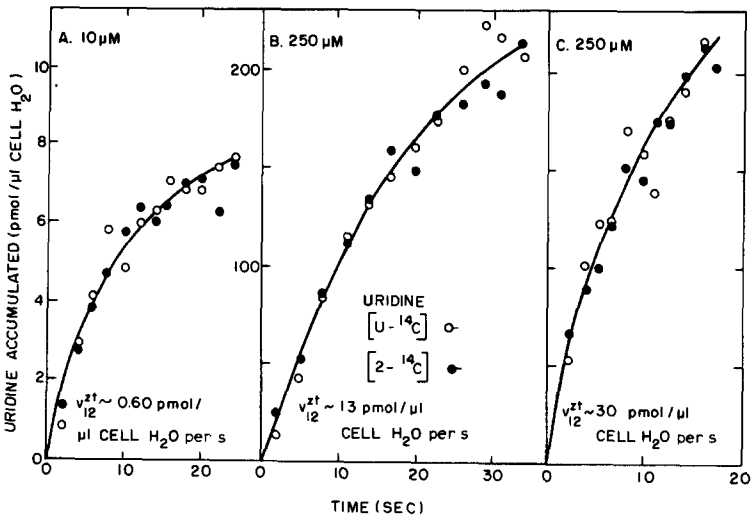

Fig. 1. Comparison of [U-^{14}C]- and [2-^{14}C]uridine uptake by uridine kinase-deficient Novikoff (A and B) and Ltk$^-$ (C) cells. Time courses of radioactivity uptake from 10 or 250 μM [U-^{14}C]- (590 cpm/μl) and [2-^{14}C]uridine (each between 500 and 600 cpm/μl) were determined by the rapid kinetic technique described in Materials and Methods. The zero-*trans* integrated rate equation (Eqn. 1) was fitted to each time course with K fixed at 250 μM to yield an estimate of V [4,6]. The curves shown are those generated by the combined data with both substrates. The initial velocities (v_{12}^{zt}) in pmol/μl H_2O per s were calculated by substitution of V and K into Eqn. 2.

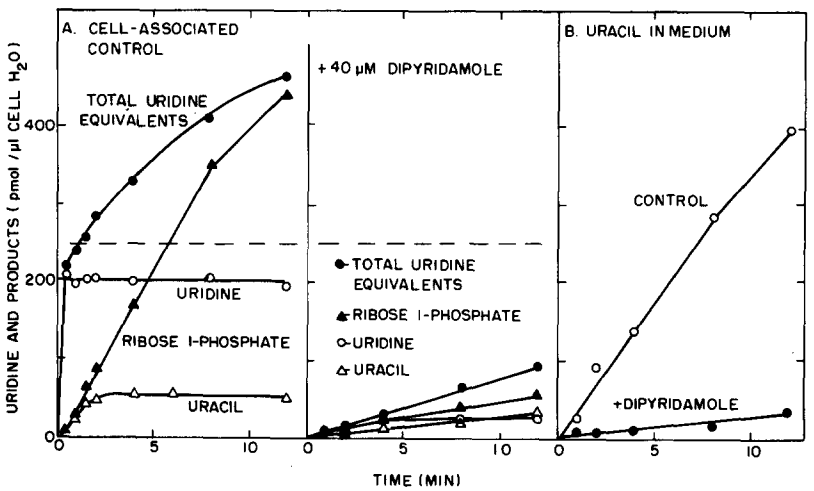

Fig. 2. Uptake and metabolism of [U-^{14}C]uridine by uridine kinase-deficient Novikoff cells and effect of dipyridamole. (A) Samples of a suspension of $3.9 \cdot 10^7$ cells/ml were supplemented (zero time) with 250 μM [U-^{14}C]uridine (4.4 cpm/pmol) and where indicated with 40 μM dipyridamole. At various times of incubation at 25°C, the cells from 0.5-ml samples of suspension were separated from the medium by centrifugation through an oil layer and analyzed for radioactivity. The values were corrected for radioactivity trapped in extracellular H_2O space in cell pellets ([^{14}C]inulin space = 5.5 μl/cell pellet) and converted to pmol uridine equivalents taken up/μl cell H_2O (●——●) on the basis of an intracellular H_2O space of 32.7 μl/cell pellet. Cells from duplicate 0.5-ml samples of suspension were centrifuged through oil into an acid/sucrose mixture and the acid extracts were subjected to chromatography with solvent 30. The intracellular concentrations of ribose-1-*P* (▲——▲), uracil (△——△) and uridine (○——○) were calculated on the basis of these chromatographic separations, the total uridine equivalents recovered in intracellular H_2O space and the fact that the specific radioactivity of the products, ribose-1-*P* and uracil, were 55 and 45%, respectively, of that of uridine. The dashed lines indicate the initial extracellular concentration of uridine expressed in pmol/μl medium. (B) Results of chromatographic analyses of culture fluid with solvent 30. For comparative purposes the data were expressed as the amount of uracil released from cells/μl of cell H_2O.

equivalent to about 80% of the extracellular uridine concentration was attained by 30 s of incubation. The intracellular concentrations of labeled uracil and ribose-1-*P* increased at only about 5% of the initial rate of entry of uridine (0.7 pmol/μl cell H_2O per s). Furthermore, the concentrations of both increased initially at about the same rate. The rate of intracellular accumulation of uracil, however, decreased with time until a steady-state concentration was attained after about 4 min of incubation. This concentration probably reflected an equilibrium between the rate of uridine phosphorolysis and the rate of exit of uracil, since uracil continued to accumulate in the culture fluid after 2 min (Fig. 2B). Re-entry of released uracil could not have been significant under the conditions of the experiment, since the extracellular H_2O space exceeded that inside the cells by about 16-fold and thus acted as a trap. The other product of phosphorolysis, ribose-1-*P*, on the other hand, was retained inside the cells (Fig. 2A).

The presence of 40 μM dipyridamole, a known inhibitor of nucleoside transport [6], in the medium reduced uridine transport into the cell to 0.11 pmol/μl cell H_2O per s (about 99% inhibition; see also Ref. 2), and diminished to about the same extent the intracellular accumulation of ribose-1-*P* (Fig. 2A, right-hand frame) and of uracil in the medium (Fig. 2B). In contrast, 100 μM dipyridamole had no effect on the phosphorolysis of 250 μM [U-^{14}C]-uridine in cell-free extracts (data not shown).

The maximum velocity of uridine phosphorylase in extracts of Novikoff cells was only about 20% of that of uridine transport (Table I). The Michaelis-Menten constants of the phosphorylase for uracil or uridine were about 200 μM (Ref. 27; and Wohlhueter, R.M. and Plagemann, P.G.W., unpublished data), which is slightly higher than that reported for other types of cell [28,29].

Overall, the patterns of uptake of uridine in four lines of L cells were similar to that observed with uridine kinase-deficient Novikoff cells, except that in

TABLE I

COMPARISON OF KINETIC PARAMETERS FOR URIDINE TRANSPORT IN VARIOUS CELL LINES WITH URIDINE PHOSPHORYLASE ACTIVITY OF CELL EXTRACTS THEREOF

Uridine transport: values are for ATP-depleted cells at 25°C and have been computed from previously reported data (Ref. 2, Table I) by fitting Eqn. 1 to the data. All kinetic constants are reported ±S.E. of estimate. They are from single experiments for each cell line, but similar values have been obtained in repeated experiments [6]. $r_{y,\hat{y}}$ = correlation coefficient. Uridine phosphorylase activity: values represent ranges of activities with 1 mM uracil at 25°C observed in the number of experiments indicated in parentheses and have been reported in preliminary form [27].

Cell line	Uridine transport		$r_{y,\hat{y}}$	Uridine phosphorylase activity (pmol/μl cell H_2O per s)
	K (mM)	*V* (pmol/μl cell H_2O per s)		
N1S1-67	260 ± 17	14.7 ± 0.4	0.9889	1.6—3.2 (5)
L-67	252 ± 10	8.2 ± 0.1	0.9844	0.7—2.9 (5) *
P388	230 ± 17	19.2 ± 1.7	0.9565	<0.03 (3)
CHO	169 ± 12	5.6 ± 0.2	0.9304	<0.03 (5)
HeLa	187 ± 5	13.4 ± 0.1	0.9913	0.8—2.8 (8)

* Although analyzed in less detail, uridine phosphorylase activity of L_{241}, L_{929} and Ltk$^-$ cells fell in the same range as that observed for L-67 cells.

L cells uridine became also phosphorylated (data not shown). Particularly, the initial time courses and rates of intracellular accumulation of radioactivity from [U-^{14}C]uridine and [2-^{14}C]uridine were about the same, just as observed for Novikoff cells. The data for Ltk$^-$ cells are illustrated in Fig. 1C. The L cell lines possessed uridine transport and phosphorylase activities similar to those observed in Novikoff cells (Table I). However, equal uptake of radioactivity from [U-^{14}C]- and [2-^{14}C]uridine was also observed with Chinese hamster ovary cells (data not shown) which lack uridine phosphorylase (Table I). The kinetic parameters for uridine transport (K and V) were similar for all cell lines investigated whether or not they possessed uridine phosphorylase (Table I).

Inosine uptake and transport by various cell lines

Qualitatively, the results for inosine uptake by the various cell lines resembled those for uridine uptake. Fig. 3A demonstrates congruency of the initial time courses of accumulation of intracellular radioactivity from [8-^{14}C]- and [U-^{14}C]inosine (both at 80 μM) by ATP-depleted, wild-type Novikoff cells. The time courses of radioactivity accumulation for the two types of labeled inosine diverged only upon longer incubation (greater than 1 min; Fig. 3B). In ATP-depleted cells, intracellular radioactivity from [8-^{14}C]inosine rapidly reached a maximum level close to that in the medium, whereas radioactivity from [U-^{14}C]inosine accumulated against a concentration gradient, reflecting the formation of labeled ribose-1-*P*. Fractionation of the intracellular radioactivity showed that about 60% was associated with ribose-1-*P* after 25 min of incubation with [U-^{14}C]inosine, whereas only labeled inosine and hypoxan-

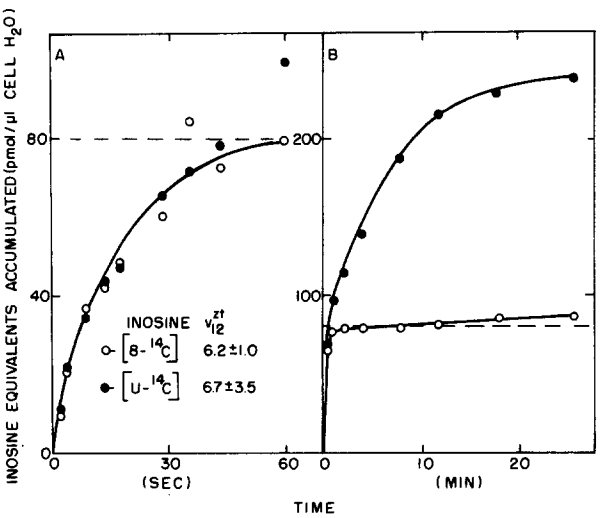

Fig. 3. Comparison of [U-^{14}C]- and [8-^{14}C]inosine uptake by ATP-depleted, wild-type Novikoff cells. The short-term (A) and long-term (B) time courses of radioactivity uptake from 80 μM [U-^{14}C]- and [8-^{14}C]inosine (250 cpm/μl) were determined as described under Materials and Methods. Eqn. 1 was fitted to each short-term time course with K fixed at 150 μM (see Table III) to yield an estimate of V. Initial velocities of uptake (v_{12}^{zt}) in pmol/μl cell H_2O per s were calculated by substitution of V and K into Eqn. 2. The short-term curves shown resulted from fitting Eqn. 1 to the combined data with both labeled inosines. The dashed lines indicate the initial extracellular concentration of inosine in pmol/μl medium.

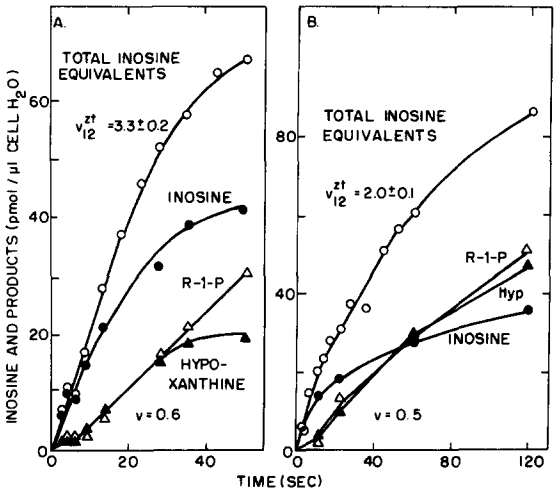

Fig. 4. Uptake and metabolism of [U-^{14}C]inosine by hypoxanthine phosphoribosyltransferase-deficient Novikoff cells (A) and ATP-depleted CHO cells (B). Time courses of radioactivity uptake from 80 µM [U-^{14}C]inosine (300 and 216 cpm/µl in A and B, respectively) were determined by using the rapid kinetic technique (○———○) and the initial velocities of uptake (v_{12}^{zt}) were computed as described under Materials and Methods. Additional samples of cells were collected by centrifugation through oil into an acid/sucrose mixture. The supernatant medium was rapidly removed, heated at 100°C for 1 min, and an acid extract was prepared from the bottom layer and both were subjected to chromatography. The concentrations of inosine (corrected for non-specific trapping), ribose-1-*P* (R-1-P) and hypoxanthine were calculated on the basis of the chromatographic separations, the total inosine equivalents accumulated and the fact that the specific radioactivities of hypoxanthine and ribose-1-*P* were 50% of that of inosine. The steady-state velocities (v; in pmol/µl cell H_2O per s) of intracellular phosphorolysis of inosine were approximated from the linear portions of the hypoxanthine/ribose-1-*P* curves.

thine were present in [8-^{14}C]inosine-labeled cells. Similar results were obtained with hypoxanthine phosphoribosyltransferase-deficient Novikoff cells not treated with ATP (data not shown). Inosine was not converted to nucleotides in either type of cell, since Novikoff cells lack inosine kinase activity and the phosphoribosylation of hypoxanthine was blocked either because the cells were depleted of ATP (as well as *P*Rib-*PP*) or lacked transferase activity.

More detailed time courses of uptake and metabolism of [U-^{14}C]inosine (at 80 µM) by untreated, transferase-deficient Novikoff cells are illustrated in Fig. 4A. During the first 20 s of incubation inosine was the main intracellular labeled component. The rates of intracellular accumulation of ribose-1-*P* and hypoxanthine were about the same (0.6 pmol/µl cell H_2O per s) and significantly lower than the initial rate of inosine accumulation (3.3 pmol/µl cell H_2O per s).

Hypoxanthine accumulation leveled off rapidly due to efflux as indicated by its accumulation in the medium (data not shown). Similar results were obtained with CHO cells in which hypoxanthine phosphoribosylation was blocked due to depletion of the cells of ATP and *P*Rib-*PP* (Fig. 4B). The only difference was that hypoxanthine accumulated to higher levels in CHO than Novikoff cells, presumably because the former transport hypoxanthine significantly slower than do Novikoff cells [30], whereas the nucleoside transport capacity of these cells is similar [6]. With both cells the rate of intracellular accumulation of ribose-1-*P* and hypoxanthine increased during the first

20 s of incubation concomitant with an increase in intracellular concentration of inosine. It should be noted that the rate of accumulation of these compounds probably somewhat underestimates the rate of their formation, since hypoxanthine is subject to efflux and ribose-1-*P* to metabolism. Intracellular metabolism of ribose-1-*P* was indicated by the formation of labeled ribose and the evolution of $^{14}CO_2$ (data not shown). $^{14}CO_2$ evolution was approximately linear for 60 min, and can be attributed to the oxidation of the ribose moiety, since none was evolved when [8-^{14}C]inosine served as substrate. The rate of $^{14}CO_2$ formation in Novikoff cells from [U-^{14}C]inosine varied from 0.03 to 0.10 pmol/μl cell H_2O per s, which accounted for as much as 25% of the rate of ribose-1-*P* formation from 80 μM extracellular inosine, assuming complete oxidation of the ribose moiety.

The initial time courses of uptake of 200 μM [U-^{14}C]- and [8-^{14}C]inosine were also the same in S49 mouse T lymphoma cells and cells of a mutant clone thereof, NSU-1 (Fig. 5), which possess less than 1% of the purine-nucleoside phosphorylase of wild-type S49 cells [31]. We have confirmed the purine-nucleoside phosphorylase deficiency of the NSU-1 cells (Table II). The initial uptake rates for both labeled forms of inosine were somewhat higher in S49 than in NSU-1 cells, but this difference was variable and smaller in other experiments (see Table II) and correlated with differences in initial rates of uptake of thymidine by these two lines. Virtually the same initial time courses of radioactivity accumulation from 200 μM [U-^{14}C]- and [8-^{14}C]inosine were also observed in L-67 and 3T3 cells (data not shown).

The initial velocities of inosine uptake (at 200 μM inosine) observed in the

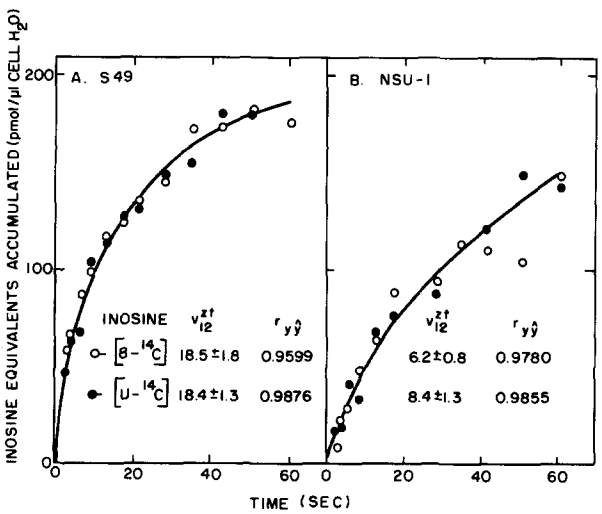

Fig. 5. Comparison of [U-^{14}C]- and [8-^{14}C]inosine uptake by wild-type (S49) and purine-nucleoside phosphorylase-deficient (NSU-1) mouse T cell lines. The time course of radioactivity uptake from 200 μM [U-^{14}C]- and [8-^{14}C]inosine (278 and 110 cpm/μl, respectively) were determined at 25°C by using the rapid kinetic technique and the initial velocities (v_{12}^{zt}) of uptake in pmol/μl cell H_2O per s were computed as described in Materials and Methods. The curves shown are those generated by fitting Eqn. 1 to the combined data with both subtrates. Cells from replicate time points were collected by centrifugation into an acid/sucrose layer. Samples of processed acid extracts as well as of the culture fluid were analyzed chromatographically and the results summarized in the text.

TABLE II

COMPARISON OF INITIAL VELOCITIES OF INOSINE UPTAKE (v_{12}^{zt}) BY WHOLE CELLS AND PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY OF CELL LYSATES

Inosine uptake: initial velocities of uptake of 200 μM inosine (v_{12}^{zt}) at 25°C were measured as described in Figs. 3—5. Values represent ranges of velocities observed in at least three independent experiments. Purine-nucleoside phosphorylase activity was measured at 25°C with 200 μM inosine as substrate as described in Materials and Methods. Values are from single experiments for each cell line.

Cell line	Inosine uptake (v_{12}^{zt}) (pmol/μl cell H_2O per s)	Purine-nucleoside phosphorylase activity (pmol/μl cell H_2O per s)
N1S1-67	4—12	1.8
S49	8—24	3.8
NSU-1	6—10	<0.12
L-67	4— 8	5.3
3T3	4—10	2.8
CHO	4—10	6.6

various cell lines were in general higher than the total purine-nucleoside phosphorylase activity of the cells (Table II). A kinetic analysis for the nucleoside phosphorylase in a crude extract from Novikoff cells (subline 1-9-1) yielded an apparent K_m value for inosine of 109 ± 9.8 μM and a V value of 4.6 ± 0.2 pmol/μl cell H_2O per s at 25°C. These values are similar to those recently reported for Novikoff cells and rat liver [32] and not unlike those reported for the enzyme from human erythrocytes [33,34], bovine liver [35] and cultured hamster cells [36].

The availability of the purine-nucleoside phosphorylase-deficient NSU-1 cells also made it possible to assess unequivocally the kinetics of inosine transport, since in populations of these cells inosine metabolism was minimal. The

TABLE III

KINETIC PARAMETERS FOR THE TRANSPORT OF INOSINE AND THYMIDINE IN VARIOUS CELL LINES

The accumulation of 20, 40, 80, 160, 320 and 640 μM [8-^{14}C]inosine (244 cpm/μl, irrespective of concentration) by NSU-1 cells to transmembrane equilibrium was determined by using the rapid kinetic technique as described under Materials and Methods. Radioactivity per cell pellet was corrected for substrate trapped in extracellular space and converted to pmol/μl cell H_2O on the basis of an experimentally determined intracellular ^3H_2O space. Eqn. 1 was fitted to the pooled data whereby time (t) and the extracellular concentration (S_1) were treated as independent variables and the intracellular concentration ($S_{2,t}$) as a dependent variable [4]. The kinetic parameters for the transport of inosine in S49 and hypoxanthine/guanine phosphoribosyltransferase-deficient Novikoff cells (1-9-1) and for thymidine transport in S49 cells were estimated in the same manner, except that Eqn. 1 was fitted to only the early time points (first 10—20 s).

Cell line	Substrate	K (μM)	V (pmol/μl cell H_2O per s)	Correlation coefficient ($r_{y,\hat{y}}$)
NSU-1	Inosine	135 ± 9	13 ± 0.3	0.9767
		182 ± 27	30 ± 1.5	0.9308
S49	Inosine	122 ± 8	42 ± 0.9	0.9915
	Thymidine	161 ± 13	37 ± 1.0	0.9899
N1S1-67 (1-9-1)	Inosine	150 ± 9	13 ± 0.3	0.9715

best-fitting kinetic parameters for inosine transport obtained by integrated rate analysis of complete time courses of zero-*trans* accumulation of inosine to transmembrane equilibrium at six concentrations (Eqn. 1, Materials and Methods) in two experiments are stated in Table III. Similar values were obtained for inosine and thymidine transport in S49 cells and for inosine transport in N1S1-67 cells (Table III). For the latter cell-substrate combinations, the estimation of the kinetic parameters was necessarily based on the early portions of the uptake curves (first 10—20 s; see Figs. 3A and 5A), since Eqn. 1 is not strictly applicable because of the metabolism of the substrates in these cells (see Materials and Methods).

We have also probed the mode of radioactivity accumulation from [U-^{14}C]-inosine by determining the effect of known inhibitors or alternate substrates of the nucleoside transporter on inosine uptake by whole cells and on their purine nucleoside activity. Fig. 6A shows that 5 mM uridine and 40 μM dipyridamole reduced the initial velocity of uptake of [U-^{14}C]inosine by transferase-deficient Novikoff cells by about 80 and 93%, respectively. The intracellular accumulation of ribose-1-*P* was inhibited to a similar extent (Fig. 6B), whereas 8 mM uridine or 100 μM dipyridamole had no effect on the purine-nucleoside phosphorylase activity in lysates of these cells (data not shown). The accumulation of hypoxanthine in the medium (Fig. 6C) was inhibited by uridine and dipyridamole to an even greater extent than the uptake of radioactivity and

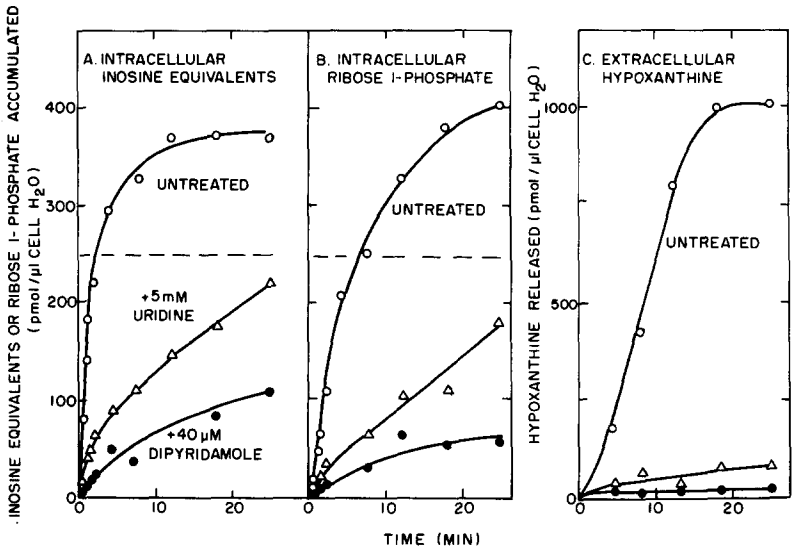

Fig. 6. Effect of uridine and dipyridamole on the uptake and metabolism of [U-^{14}C]inosine by hypoxanthine phosphoribosyltransferase-deficient Novikoff cells. Samples of a cell suspension were supplemented with 250 μM [U-^{14}C]inosine (550 cpm/μl) and where indicated at the same time (0 min) with 5 mM uridine or 40 μM dipyridamole. At the indicated times of incubation at 25°C, the cells from 0.5 ml of suspension were separated from the medium by centrifugation through oil and analyzed for radioactivity (A). An acid extract was prepared from a replicate sample of cells and analyzed chromatographically for ribose-1-*P* (B). All values are expressed in pmol/μl cell H_2O on the basis of an intracellular H_2O space of 45.3 μl/cell pellet and those in (A) were corrected for inosine trapped in extracellular H_2O space (4 μl/cell pellet). The amounts of hypoxanthine released into the medium per μl cell H_2O (C) were estimated from chromatographic analysis of samples of culture fluid.

the intracellular accumulation of ribose-1-*P*, but this finding is not unexpected, since uridine and dipyridamole also inhibit the transport of hypoxanthine [30], and thus its release from the cells. Nitrobenzylthioinosine, on the other hand, is quite specific in its inhibition of nucleoside transport in mammalian cells, and does not affect hypoxanthine transport [37]. Nitrobenzylthioinosine at 10 nM inhibited the uptake of 200 μM [U-^{14}C]inosine by 3T3 cells by about 60%, and to the same extent the intracellular accumulation of labeled ribose-1-*P* and the accumulation of labeled hypoxanthine in the medium, whereas the phosphorolysis of 200 μM [^{3}H]inosine by a cell-free extract from 3T3 cells was not inhibited by 40 nM nitrobenzylthioinosine (data not shown).

Discussion

Our results indicate that inosine and uridine enter mammalian cells mainly unmodified via the nucleoside-facilitated diffusion system and that their phosphorolysis occurs subsequent to their transport into the cells. In fact, no evidence for the occurrence of ribosyl group translocation with extracellular release of the base moiety was apparent in any of the six cell lines examined. These included the two cell lines (3T3 and L), the vesicles of which have been claimed to be active in such membrane-associated, phosphorylase-catalyzed group translocations [9—14]. Similar results were obtained with four strains of L cells. Our evidence can be summarized as follows.

1. The initial velocities of uptake of uniformly and base-labeled nucleosides are about the same in all cell lines tested. If ribosyl group translocations with a concomitant extracellular release of base had occurred at a significant rate, radioactivity from U-^{14}C-labeled nucleosides should have accumulated intracellularly more rapidly than the radioactivity from base-labeled nucleosides. In fact, little if any base formed by phosphorolysis at the cell surface would be expected to be taken up by the cells under our experimental conditions because of its rapid dilution in extracellular H_2O which exceeded that inside the cells by 14—20-fold.

2. In the zero-*trans* procedure, the free nucleoside appears intracellularly prior to an more rapidly than its phosphorolysis products.

3. During the initial time period of incubation with U-^{14}C-labeled nucleosides, the time courses of intracellular appearance of ribose-1-*P* and base are about the same. In fact, the initial accumulation of nucleoside and its products exhibits a typical precursor-product relationship.

4. A specific inhibition of nucleoside transport depresses the intracellular accumulation of ribose-1-*P* and the formation of base to about the same extent as the transport of the unmodified nucleosides is inhibited.

5. The capacity of the cells to transport uridine and inosine is unrelated to whether or not they possess the respective phosphorylases. Even for cells that possess such activity, their capacity to transport nucleosides in unmodified form is greater, particularly in the case of uridine, than the total phosphorylase activities of the cells. That most of the phosphorolysis of uridine and inosine occurred intracellularly rather than on the surface of the various cell lines is indicated by the good correlation between phosphorolysis activities of whole cells and cell lysates.

In corroboration of these conclusions, Cohen et al. [38] have shown that a nucleoside transport-deficient mutant of the S49 line (AE_1) has simultaneously lost the capacity to take up inosine and all other nucleosides tested. Inosine uptake by this line is at least 98% lower than in wild-type cells, while its purine-nucleoside phosphorylase activity is equal to that of the parental line.

The significance of nucleoside phosphorylase-dependent group translocations in procaryotes [39,40] has also been recently reexamined [41]. By measuring nucleoside uptake in *Escherichia coli* mutants that lack specific nucleoside-metabolizing enzymes, it has been shown that the transport of nucleosides into whole cells or vesicles thereof can take place independently of catabolizing enzymes and that the presence of these enzymes is not sufficient for transport to take place. Thus, a tandem operation of nucleoside transport and intracellular metabolism also seems to be the primary, if not the only, mode of uptake of nucleosides by procaryotes. The main difference between procaryotes and eucaryotes in this process relates to the nature of the transporter, active in procaryotes and facilitated in eucaryotes.

The results reported from several laboratories [8—13,42,43], in apparent support of the occurrence of nucleoside phosphorylase-catalyzed ribosyl group translocations in mammalian cells, need to be re-evaluated in view of the rapidity of nucleoside and nucleobase transport in these cells; intracellular steady-state levels of free nucleoside or base are attained in 10—40 s of incubation depending on substrate concentration and temperature [6,7]. Taking the rapid entry of nucleosides and rapid exit of nucleobases into account, most of the reported data are readily interpretable in terms of the tandem operation of facilitated transport of nucleosides and their intracellular phosphorolysis.

The kinetic parameters for the zero-*trans* influx of inosine in Novikoff and mouse lymphoma cells determined in the present study are similar to those for other nucleosides in various lines of cultured mammalian cells [6]. All evidence available at present indicates that ribo- and deoxyribonucleosides are transported by a single carrier with broad specificity [6]. Most convincing in the matter of substrate specificity is the isolation of a single-step mutant of the S49 human T cell line which is deficient in the uptake of all nucleosides examined [38]. It is also of interest that the Michaelis-Menten constants of nucleoside kinases are at least one order lower than those for nucleoside transport, whereas those for the nucleoside degradative enzymes are similar to those for transport. These correlations are probably of physiological significance, insuring that at low extracellular nucleoside concentrations, entering nucleosides are preferentially phosphorylated rather than phosphorolyzed.

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