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Facilitated Transport of Uracil and 5-Fluorouracil, and Permeation of Orotic Acid Into Cultured Mammalian Cells

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ABSTRACT The mode of permeation of uracil, 5-fluorouracil, and orotic acid into cells has been investigated in four established cell lines (Novikoff rat hepatoma, P388 mouse leukemia, mouse L, and Chinese hamster ovary cells) in attempts to assess the rate-determining step(s) in their incorporation into the nucleotide pool and nucleic acids. Uracil and 5-fluorouracil shared a saturable transport system (K_m = 5 to 15 mM) capable of rapid equilibration of these substrates across the cell membrane ($t_{1/2}$ at 25° in first-order range of concentration = 25 to 58 sec). Thus it seems unlikely that transport is limiting the incorporation of uracil or fluorouracil. Their transport was inhibited by various nucleosides and hypoxanthine. Only the non-ionized form of fluorouracil was a substrate for the transporter; exclusion of charged pyrimidines may explain why orotate was not a substrate at physiological pH. Orotate permeated the cell membrane much more slowly ($t_{1/2}$ = 2890 to 6930 sec); its permeation was apparently non-mediated and rate-determining in the conversion of extracellular orotate to intracellular nucleotides.

5-Fluorouracil is the prototypical antineoplastic drug. It is readily taken up[1] by cultured mammalian cells and incorporated into their nucleic acids—attributes which doubtlessly enhance its toxicity to growing cells (Heidelberger, '75; Jacquez, '62; Kessel and Hall, '69; Wilkinson and Crumley, '77). The natural pyrimidines uracil and orotic acid, in contrast, are utilized only poorly by cultured cells (Jacquez, '62; Gotto et al., '69; Skehel et al., '67; Plagemann, '71; Kessel et al., '69; Long et al., '77; Schneider et al., '74; Ishi and Green, '73; Chan et al., '74; McGarrity et al., '79; Pasternak et al., '61; Schreiber et al., '74; Lea et al., '74; Chen and Jones, '79). In fact, exceptions to the rule of slow uracil uptake have generally been taken as indicative of mycoplasma contamination (Long et al., '77; Schneider et al., '74; McGarrity et al., '79; McIvor and Kenny, '78), although blockage of de novo pyrimidine synthesis (Ishi and Green, '73; Chan et al., '74) or concurrent provision of purine nucleosides (Gotto et al., '69) can also stimulate uracil uptake.

In the present study we have analyzed the mode of permeation of 5-fluorouracil into cultured cells and compared it to that of uracil and orotate as a first step in attempts to elucidate the differences in the utilization of these three pyrimidines by cells. Our data show that fluorouracil, but not orotate, is an efficient substrate for the uracil transporter (Marz et al., '79). Thus differences in rates of uracil and fluorouracil incorporation into the nucleotide pool and into nucleic acids must be due to differences in rates of their intracellular conversion to nucleotides. In contrast, the slow uptake of orotate is attributable to its limited permeation through the cell membrane.

MATERIALS AND METHODS

Cell cultures

Novikoff rat hepatoma cells (subline N1S1-67) were propagated in suspension culture in Swim's medium 67 as described previously (Ward and Plagemann, '69). P388 mouse

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[1]"Uptake" denotes the transfer of radioactivity from exogenous labeled substrate to intracellular space or components regardless of metabolic conversions. "Transport" denotes solely the transfer of unmodified exogenous substrate across the cell membrane as mediated by a saturable, selective carrier. "Incorporation" may be used interchangeably with uptake, but generally is used to denote the transfer of radioactivity from labeled substrate to a specified intracellular material.

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leukemia, mouse L cells, Chinese hamster ovary (CHO) cells, and HeLa cells were propagated in spinner culture in Eagle's minimal essential medium for suspension culture (MEM-S) supplemented (10% by volume) with heat-inactivated fetal calf serum. Cells were enumerated with a Coulter counter.

The cells used in experiments were harvested from late exponential phase cultures. Novikoff cells were resuspended in basal medium 42B (BM42B; Plagemann, '71) and the cells of all other lines in MEM-S.

All cell cultures were screened for mycoplasma contamination by the uracil/uridine incorporation method (Schneider et al., '74) and by testing for mycoplasma growth in two media: PPLO broth (Difco), supplemented with 20% agamma horse serum (Gibco), 1% glucose, 5% fresh yeast dialyzate, and soy-peptone agar (Kenny, '73). In no test was there any evidence of contamination.

Measurements of uracil and 5-fluorouracil zero-trans[2] and equilibrium exchange[2] transport

Our techniques for measuring zero-*trans* influx into cells in suspension have been described in detail elsewhere (Wohlhueter et al., '78a; '79). In brief, samples of 448 μl of cell suspension (2–4 $\times$ 10^7 cells/ml) were mixed with 61 μl of saline containing labeled substrate at 1.5 to 5 second intervals by means of a hand-operated, dual syringe apparatus at ambient temperature (24 to 25°C). The mixtures were dispensed directly into 12 tubes mounted in an Eppendorf micro-centrifuge and containing 200 μl of a silicone oil mixture (density = 1.034 g/cm^3). The centrifuge was started after delivery of the last sample. Under these conditions we have estimated that cells are effectively out of contact with medium in 2 seconds (Wohlhueter et al., '78a).

To estimate total radioactivity in the pellet, the supernatant medium was aspirated, the upper part of the tube flooded with water, and the water, along with most of the oil layer, was aspirated. The cell pellet was then resuspended in 200 μl of 0.5 N trichloroacetic acid, heated at 70°C for 30 minutes, and analyzed by liquid scintillation counting. Total radioactivity was corrected for substrate trapped in the extracellular space of the pellet, as estimated in parallel experiments by [*carboxyl*-^{14}C]inulin, and normalized to intracellular water volume, taken as total ^3H_2O space minus inulin space (Wohlhueter et al., '78a). Intracellular and extracellular volumes obtained in individual experiments are noted in legends; generally intracellular water represented about 85% of total pellet water.

Because the influx of nucleobases is so rapid and initial velocities so shortlived, we fit an integrated rate equation to the entire time course of the attainment of transmembrane equilibrium. The slope of the (best-fitting) curve at zero-time gives initial velocity for a single substrate concentration. Alternatively, when the time courses at a series of substrate concentrations were available, the Michaelis-Menten parameters for transport were computed directly, by treating both time and substrate concentration as independent variables.

The integrated rate equation for transport by a fully symmetrical, carrier-mediated system has been developed on the model of Eilam and Stein ('74), and may be written (Wohlhueter et al., '79);

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

where $S_{2,t}$ = concentration of substrate inside the cell at time t ($S_{2,0} = 0$ for a zero-*trans* experiment), S_1 = exogenous substrate concentration, and is taken as constant, R and K are resistivity and affinity parameters, respectively, which, in the fully symmetrical case, are equivalent to the reciprocal of the maximum velocity and to the Michaelis-Menten constant, respectively. The slope of equation 1 at zero time, for any given S_1 corresponds to the initial velocity of transport at that concentration, $v_{12}^{z,1}$:

$$v_{12}^{z,1} = \frac{S_1/R}{K + S_1} \qquad \text{equation 2}$$

Equation 1 is implicit in the dependent variable $S_{2,t}$, and is most conveniently solved by iterative techniques (to be described in detail elsewhere). Once solved, however, it is amenable to least-squares fitting procedures by standard, nonlinear methods to yield R and K and their associated standard errors of estimate (S.E.). This equation derives from a model which assumes metabolic inertness of the transported substrate. We find, however, that

[2]As defined by Eilam and Stein ('74) "zero-*trans*" designates the transport of a substrate from one side of the membrane to the other side, where its concentration is zero. "Equilibrium exchange" designates the unidirectional flux of radioactively labeled substrate from one side to the other side of the membrane, where substrate is held at equal concentration. Arbitrarily, we designate the outside and inside faces of the membrane as 1 and 2, respectively.

it yields reasonable estimates of initial velocities and/or kinetic parameters of transport of metabolizable substrates, provided that these estimates are based only on early time points, before the accumulation of metabolites becomes significant (Plagemann and Wohlhueter, '80). This proviso is readily met in the case of uracil and fluorouracil, since the rates of metabolism of these substrates are much less than those of transport. Therefore, we have estimated K and R for uracil and fluorouracil transport by fitting equation 1 to a pool of zero-*trans* uptake data comprising usually 12 or 15 early time points at each of six to nine substrate concentrations.

Estimates of initial velocity of uptake at a single substrate concentration were obtained by fitting equation 1 to the data, with K fixed at its experimentally determined value, and then using the K and R values to compute the zero-time slope, according to equation 2.

Equilibrium exchange experiments were conducted and analyzed by methods analogous to those for the zero-*trans* protocol as follows. The cells were preincubated at 37°C for 30 minutes in medium containing nonradioactive substrate at a series of six to eight concentrations. After equilibrating the cell suspensions at ambient temperature the dual syringe was used to mix these preloaded cells with radiolabeled substrate at the same chemical concentration, according to schedule. The time course of exchange of radioactivity into the cell is described by the following integrated rate equation (Wohlhueter et al., '78a).

$$N_{2,t} = N_{2,\infty}\left[1\text{-}\exp\text{-}\left(\frac{V^{ee}t}{K^{ee}+S}\right)\right] \quad \text{equation 3}$$

where $N_{2,t}$ = intracellular concentration of radioactivity at time t, expressed as picomoles of substrate at the original exogenous specific radioactivity per μl cell water; S = chemical concentration of substrate, and K^{ee} and V^{ee} are the Michaelis-Menten constants apparent in an equilibrium exchange experiment. K^{ee} and V^{ee} were estimated directly by fitting equation 3 by the method of least squares to time courses of attainment of isotopic equilibrium, pooled for six to eight substrate concentrations, whereby t and S were treated as independent variables and $N_{2,t}$ as dependent variable.

Long-term uptake of pyrimidines

Suspensions of 0.5 to 4 $\times$ 10^7 cells/ml of BM42B or MEM-S were supplemented with labeled substrate and incubated at 25 or 37°C as indicated in individual experiments. At various times thereafter, the cells from duplicate 0.5 ml samples of suspension were collected by centrifugation through oil and analyzed for intracellular radioactivity as described below.

Fractionation of intracellular radioactivity

Labeled cells were centrifuged through 500 μl of the oil mixture directly into 100 μl of an underlying solution of 0.5 N trichloroacetic acid in 10% (w/v) sucrose (density = 1.04 g/ml). After centrifugation the tubes were immediately placed in ice and the medium was rapidly aspirated. The upper part of the tubes was rinsed, and the aqueous phase beneath the oil extracted twice with ether (to remove residual oil and most of the acid), mixed with 10 μl of 1 M Tris/Cl^- (pH 7.4), and chromatographed on Whatman 3MM paper with a solvent composed of three volumes of 1 M ammonium acetate (pH 5.0) and seven volumes of 95% ethanol (solvent 28).

Materials

Unlabeled nucleobases, nucleosides, and orotic acid were purchased from Sigma Chemical Co. (St. Louis, Missouri). [5-^{3}H]Uracil, 5-fluoro[6-^{3}H]uracil and [5-^{3}H]orotic acid were purchased from Amersham (Arlington Heights, Illinois), and [5-^{3}H]uridine from New England Nuclear (Boston, Massachusetts). Radiochemical purities were judged better than 97% by paper chromatography in solvents 28 and 30B. 6-([4-Nitrobenzyl]thio)-9-β-D-ribofuranosyl purine (nitrobenzylthioinosine) was a gift from Dr. A.R.P. Paterson of the University of Alberta. Other chemicals were reagent grade from standard suppliers.

RESULTS

Transport of uracil and 5-fluorouracil

We have previously characterized kinetically the uracil transport system of Novikoff cells (Plagemann et al., '78; Marz et al., '79) by fitting an integrated rate equation (equation 1, based on the simple, symmetrical carrier model of Eilam and Stein, '74) to the time courses of attainment of transmembrane equilibrium. These experiments encompassed ten concentrations of exogenous uracil ranging from 10 μM to 8 mM, and yielded K = 14.2 mM and V = 164 pmol/μl cell H_2O•sec (Marz et al., '79). We have extended these kinetic studies to uracil transport in two additional cell lines, to fluorouracil transport in Novikoff cells, and to an examination of the symmetry of the transport system in Novikoff cells, which had been assumed previously on the basis of indirect evidence.

The experimental method is illustrated in Figure 1, which shows data for the accumulation of fluorouracil at three concentrations of the seven which comprised this experiment and the theoretical accumulation curves obtained by fitting equation 1 to the combined data. Fitting equation 1 to the data was warranted, since chromatographic analyses of the acid-soluble contents of replicate samples of cells showed that, at all fluorouracil concentrations tested, less than 5% accumulated by the cells was converted to nucleotides during the 5-minute incubation period. Incorporation of fluorouracil into acid-insoluble material during this period was also insignificant. The best-fitting kinetic parameters for fluorouracil influx by Novikoff cells and their standard errors of estimate are tabulated in Table 1 (line 2).

Table 1 also summarizes the results of similar experiments in which uracil was employed as substrate for transport in CHO cells and P388 mouse leukemia cells. Kinetic parameters for transport of uracil are comparable in all three lines, as are those for uracil and fluorouracil in Novikoff cells. The apparent first-order rate constants (k) stated correspond to the attainment of half-equilibrium across the membrane at low (with respect to K) concentrations within 25 to 124 seconds. The permeation of orotic acid is considered in more detail below.

The kinetic parameters for uracil and fluorouracil transport listed in Table 1 were obtained by fitting the rate equation for a symmetrical transport system to zero-*trans* influx data. One method to test for symmetry is to compare the computed apparent Michaelis-Menten constant (K) to that obtained using an equilibrium exchange protocol, in which isotope is exchanged across the membrane at chemical equilibrium (Wohlhueter et al., '79). An equilibrium exchange experiment with uracil as substrate in Novikoff cells is illustrated in Figure 2. The best-fitting Michaelis-Menten parameters were K^{ee} = 17.4 ± 3.4 mM and V^{ee} = 335 ± 49 pmol/μl cell water•sec. K^{ee} and K were thus equal within experimental error—i.e., we did not discern any experimentally significant asymmetry in the uracil transport system.

Specificity of the uracil transporter

The similar kinetics of uracil and fluorouracil transport in Novikoff cells, and the dissimilarity of these to the kinetics of uridine transport (K = 250 μM; Plagemann et al., '78; Plagemann and Wohlhueter, '80), suggested to us that uracil and fluorouracil were probably transported by the same carrier. Proof of this hypothesis is made difficult by the fact that the solubilities of these substrates at physiological pH are not much greater than their K_m's of transport. Nevertheless, we have been able to address the question of specificity in three sorts of experiments.

The first sort is a direct assessment of inhibition of uracil and fluorouracil transport by each

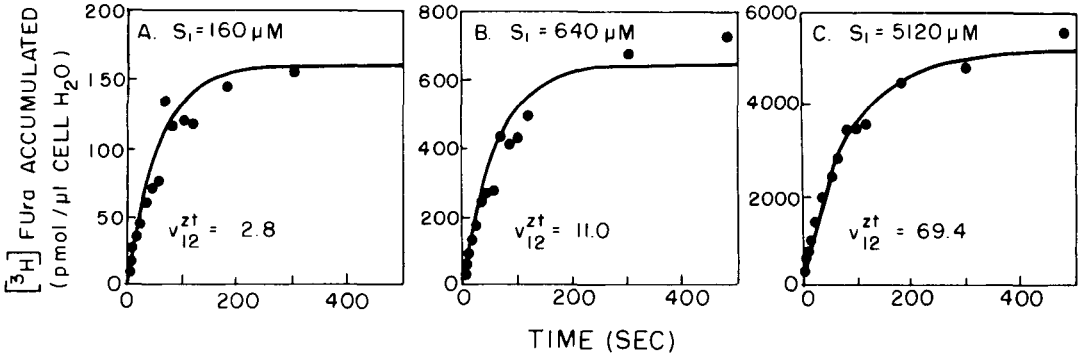

Fig. 1. Zero-*trans* influx of fluorouracil in Novikoff hepatoma cells at 25°C (pH7). The accumulation of 160, 320, 640, 1280, 5120, and 10,240 μM 5-fluoro[^{3}H]uracil (540 cpm/μl, irrespective of concentration) was determined by the rapid kinetic technique as described in Materials and Methods. Radioactivity/cell pellet was corrected for substrate trapped in extracellular space (3.3 μl/cell pellet) and converted to pmol/μl cell H_2O on the basis of an intracellular H_2O space of 21.6 μl/cell pellet. The zero-*trans* integrated rate equation (Eq. 1) was fit to the pooled data. The best fitting parameters were K = 16 ± 2.2 mM and V = 286 ± 30 pmol/μl cell H_2O•sec ($r_{y,\hat{y}}$ = 0.9959). The theoretical curves for fluorouracil accumulation for S_1 = 160, 640, and 5120 μM are illustrated in A–C; the initial zero-*trans* velocities (v_{12}^{zt}, in pmol/μl cell H_2O•sec) were calculated by substituting the experimentally determined values of K and R into equation 2.

TABLE 1. Kinetic parameters for permeation of pyrimidines in various cell lines

Cell Line	Substrate	K	V	$10^3 \times k^1$
		(mM ± S.E.)	(nmol/μl cell H_2O•sec ± S.E.)	(sec $^{-1}$)
N1S1-67	Uracil[2]	14.2 ± 1.1	0.164 ± 0.005	12
	5-Fluorouracil[3]	16.0 ± 2.3	0.286 ± 0.030	18
	Orotic acid	—	—	0.24
CHO	Uracil[4]	10.9 ± 1.6	0.061 ± 0.006	5.6
	Orotic acid	—	—	0.1
P388	Uracil[4]	5.5 ± 0.6	0.151 ± 0.006	28
	Orotic acid	—	—	0.1

[1]Calculated as V/K at 24°, or for orotic acid at 37°C from the data in Figure 5 and similar experiments.
[2]Data from Marz et al., '79.
[3]Data summarized from Figure 1.
[4]From experiments carried out analogously to that shown in Figure 1.

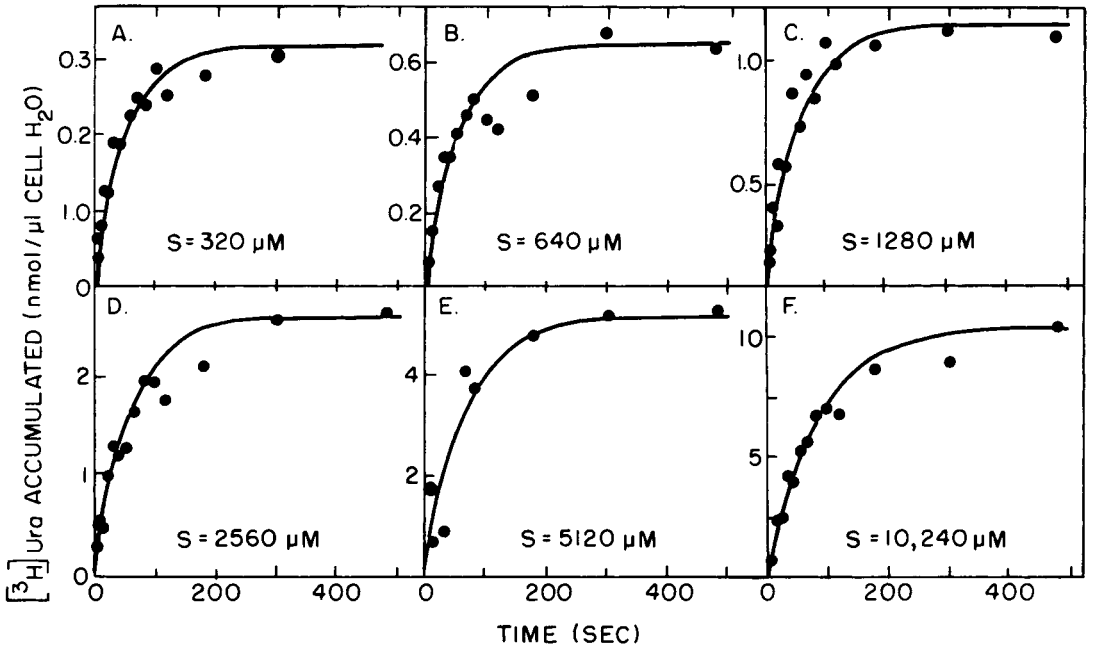

Fig. 2. Equilibrium exchange influx of uracil by Novikoff rat hepatoma cells at 25°C. The accumulation of 160, 320, 640, 1280, 2560, 5120, and 10,240 μM [^{3}H] uracil (412 cpm/μl, irrespective of concentration) by cells preloaded for 30 minutes with the same concentrations of unlabeled uracil was determined by the rapid kinetic technique as described in Materials and Methods. Radioactivity/cell pellet was corrected for substrate trapped in extracellular space (3.2 μl/cell pellet) and converted to radioactivity/μl cell H_2O on the basis of an intracellular space of 10 μl/cell pellet. Equation 3 was fit by nonlinear regression to the pooled data, whereby t and S were treated as independent variables, and $N_{2,t}$ as dependent variable. The best fitting parameters were K^{ec} = 17.4 ± 3.4 mM, and V^{ec} = 335 ± 49 pmol/μl cell H_2O•sec ($r_{y,\hat{y}}$ = 0.9900).

other and by other compounds; the results of these experiments are summarized in Table 2. The K_i's given in the table are only rough approximations, because they were computed on the untested assumption of competitive inhibition, and because the error associated with $v_{12}^{z_1}$ is amplified considerably in the computation of K_i. Still, these data allow the following conclusions: i) Uracil and fluorouracil inhibited the transport of each other to a similar extent. ii) Various nucleosides and hypoxanthine inhibited uracil and fluorouracil transport. The degree of their inhibitions was greater than that of the pyrimidines, but the K_i's of the inhi-

TABLE 2. *Effect of nucleosides and nucleobases on the transport of uracil and fluorouracil*[1]

Inhibitor	Concentration (mM)	Fluorouracil			Uracil		
		$v_{1/2}^{si} \pm$ S.E. (pmol/μl cell H_2O•sec)	Inhibition (%)	$K_{i\ (comp)}$[2] (mM)	$v_{1/2}^{si} \pm$ S.E. (pmol/μl cell H_2O•sec)	Inhibition (%)	$K_{i(comp)}$[2] (mM)
None	5	1.63 ± 0.09	—	—	1.53 ± 0.17	—	—
Fluorouracil	5	—		—	1.39 ± 0.14	9	49
Uracil	5	1.10 ± 0.08	33	10	—	—	—
Uridine	5	0.87 ± 0.11	47	5.5	0.61 ± 0.03	60	3.3
Inosine	5	0.41 ± 0.04	75	1.6	0.60 ± 0.08	61	3.1
Adenosine	5	0.38 ± 0.04	77	1.5	0.60 ± 0.06	61	3.1
Hypoxanthine	2.5	0.85 ± 0.06	48	2.7	0.68 ± 0.06	56	1.9
Adenine	2	1.6 ± 0.09	0	—	ND[3]	—	—

[1]Samples of a suspension of about 2×10^7 N1S1-67 cells/ml of BM42B were analyzed by the rapid kinetic technique for the zero-*trans* influx of 320 μM fluoro[^{3}H]uracil (467 cpm/μl) or 320 μM [^{3}H]uracil (433 cpm/μl). Unlabeled nucleosides or nucleobases were added simultaneously with labeled substrates as indicated. Radioactivity/cell pellet was corrected for substrate trapped in extracellular space (3.5 μl/pellet) and converted to pmol/μl cell H_2O•sec on the basis of an intracellular H_2O space of 16.9 μl/pellet. Equation 1 was fit to each 12 to 15-point uptake time course with K fixed at 16 mM. Initial transport velocities ($v_{1/2}^{si}$) were calculated by substituting K and the computed value of R into equation 2.

[2]K_i was computed on the assumption of competitive inhibition as $K_i = \frac{[I]\,(1-i)}{i(1 + [S]/K)}$ where i = fraction inhibition by inhibitor I and K is the Michaelis-Menten constant for transport of substrate S.

[3]ND = Not determined.

bitions were five to 20 times greater than the K's for their own transport (250, 120, 103, and 350 μM for the transport of uridine, inosine, adenosine and hypoxanthine, respectively; Plagemann and Wohlhueter, '80). iii) Adenine, in contrast, had no significant effect on uracil or fluorouracil transport.

A second sort of experimental approach to this question is a countertransport protocol, in which cells are preloaded with a presumptive (unlabeled) substrate, and the influx of radiolabeled substrate against the unlabeled substrate is measured. Figure 3 shows that 5-fluoro[^{3}H]uracil countertransported against itself, uracil, and hypoxanthine, and to about the same extent also against thymidine, though little, if at all, against adenine. We have demonstrated previously (Plagemann et al., '78) that [^{3}H]uridine countertransports, but only weakly, against uracil.

CHO cells lend themselves to a third approach to the question of specificity. In contrast to the nucleoside transporter of Novikoff cells, that of CHO cells is very sensitive to inhibition by p-nitrobenzylthioinosine (NBTI, Wohlhueter, et al., '78b). The data of Table 3 show that this inhibitor clearly differentiated nucleoside from pyrimidine transport: Forty nM NBTI sufficed to inhibit uridine transport by 80%, while inhibiting uracil and fluorouracil transport by only about 13%. A differential sensitivity to NBTI has also been described for thymidine transport as compared to thymine and hypoxanthine transport in CHO cells (Wohlhueter et al., '78b).

The mode of orotate entry

The velocity and mechanism by which orotate entered Novikoff cells contrasted sharply with those for uracil and fluorouracil, as characterized in the foregoing sections. The initial slope of the curve for accumulation of radiolabeled orotate, Figure 4A, corresponds to a first-order rate constant of 0.00024 second^{-1}, about 2% of that for uracil (cf. last column of Table 1). Even this is about a threefold overestimate, since the experiment of Figure 4 was carried out at 37°C, while uracil transport was measured at 25°C (incubation at 37°C was necessary to obtain significant incorporation of orotate within a reasonable time). Orotate was taken up by a variety of cell types at rates comparable to those in Novikoff cells. At 10 μM orotate and 37°C the rates were 0.0010, 0.0010, 0.0017, and 0.0035 pmol/μl cell water•sec for CHO, L, P388, and HeLa cells, respectively. Rate constants computed from these data are

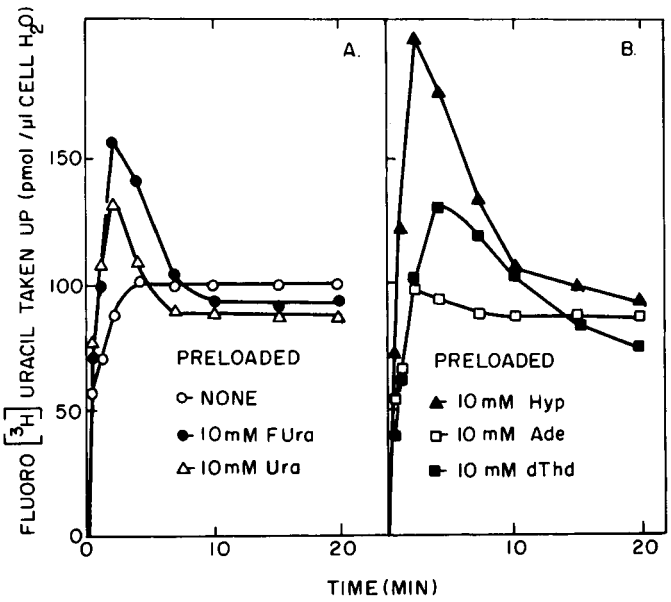

Fig. 3. Counter-transport of 5-fluorouracil in Novikoff hepatoma cells. Samples of a suspension of about 2×10^7 ATP-depleted cells/ml were supplemented where indicated with 10 mM fluorouracil (FUra), uracil (Ura), hypoxanthine (Hyp), adenine (Ade), or thymidine (dThd) and incubated at 37°C for 20 minutes. The cells were then collected by centrifugation and suspended to the same density (0 time) in glucose-free BM42B containing 5 mM KCN, 5mM iodoacetate, and 100 µM fluoro[^{3}H]uracil (5.1 cpm/pmol) at 25°C. At various times thereafter, the cells from duplicate 0.5-ml samples of suspension were collected by centrifugation through an oil layer and analyzed for radioactivity. Average radioactivity/cell pellet was corrected for substrate trapped in extracellular space (4.5 µl/cell pellet) and converted to pmol/µl cell H_2O on the basis of an intracellular H_2O space of 22 µl/cell pellet. Intracellular and extracellular H_2O spaces were estimated independently using the original cell suspensions.

TABLE 3. Lack of effect of nitrobenzylthioinosine on uracil/fluorouracil transport[1]

Labeled substrate	Nitrobenzyl thioinosine (nM)	v_{12}^{z1} ± S.E. (pmol/µl cell H_2O•sec)	Inhibition (%)
Fluorouracil	0	1.45 ± 0.13	0
	40	1.25 ± 0.09	14
Uracil	0	1.19 ± 0.08	0
	40	1.05 ± 0.09	12
Uridine	0	8.0 ± 0.80	0
	40	1.6 ± 0.15	80

[1]One sample of a suspension of 1.4×10^7 CHO cells/ml were supplemented with 40 nM nitrobenzylthioinosine. After 5 minutes of incubation at 25°C this suspension and an untreated suspension of cells were analyzed by the rapid kinetic technique for the zero-*trans* influx of 320 µM fluoro[^{3}H]uracil (519 cpm/µl), 320 µM [^{3}H]uracil (504 cpm/µl), or 320 µM [^{3}H]uridine (606 cpm/µl). Radioactivity per cell pellet was corrected for substrate trapped in the extracellular H_2O space of cell pellets (2.4 µl/pellet) and converted to pmol/µl cell H_2O on the basis of an intracellular H_2O space of 12.1 µl/pellet. Equation 1 was fit to each 12 to 15-time point uptake time course with K fixed at 16 mM for uracil and fluorouracil (see text) and 250 µM for uridine (Plagemann and Wohlhueter, '80). The initial transport velocities (v_{12}^{z1}) were calculated by substituting the computed value for R and K into equation 2.

summarized in Table 1, along with those for uracil and fluorouracil.

Much of the orotate taken up by the cells was phosphoribosylated. This is illustrated in Figure 4B for the 2-hour sample of uptake of 0.4 µM exogenous orotate by Novikoff cells. Eighty percent of the radioactivity in the orotate region of this chromatogram was attributable to extracellular fluid in the cell pellet (see figure legend for extracellular and intracellular volumes), leaving an intracellular contribution corresponding to about 0.04 µM.

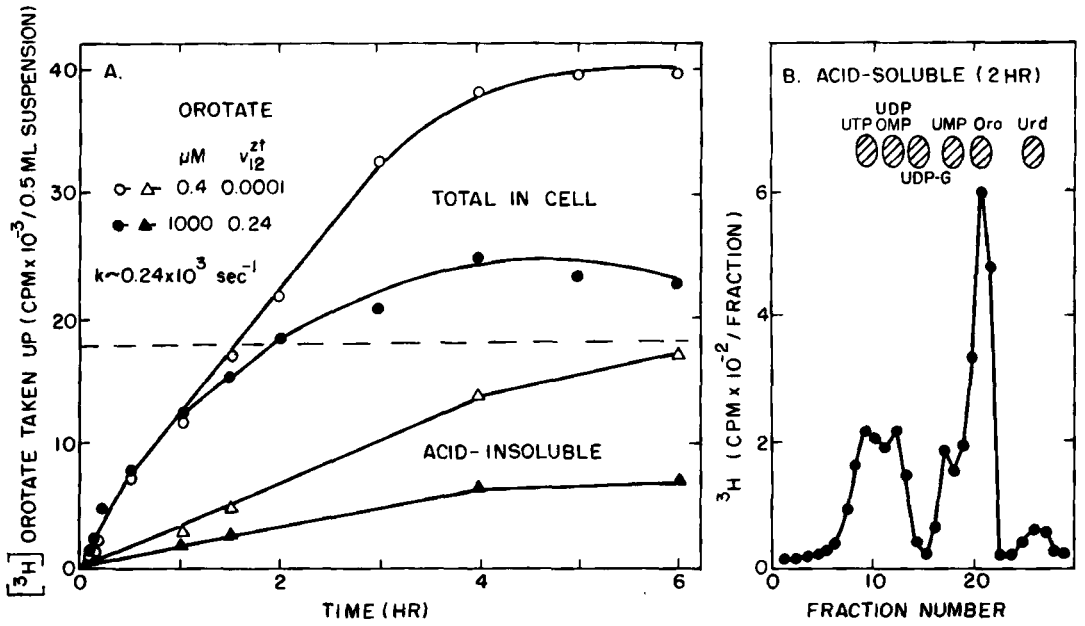

Fig. 4. Orotate uptake by Novikoff cells. Samples of a suspension of about 4×10^7 cells/ml of BM42B were supplemented (0 time) with 0.4 μM or 1 mM [^{3}H]orotate (700 cpm/μl, irrespective of concentration) and incubated at 37°C. At various times, the cells from duplicate 0.5-ml samples of suspension were collected by centrifugation through an oil layer and analyzed for radioactivity. Radioactivity/cell pellet was corrected for substrate trapped in extracellular H_2O space (5.1 μl/sample). All points are averages of the duplicate samples. The broken line indicates the concentration of intracellular radioactivity equal to that in the medium calculated on the basis of an intracellular H_2O content of 25.7 μl/sample. At 1, 2, 4, and 6 hours of incubation the cells from 1-ml samples were collected by centrifugation, extracted with trichloroacetic acid, and the acid extracts chromatographed with solvent 28 as described under Materials and Methods. The chromatogram shown in (B) is of the acid extract from cells incubated with 0.4 μM [^{3}H] orotate for 2 hours.

Figure 4A also shows that the rate constant apparent at 0.4 μM pertained also at 1000 μM. That is, the rate of permeation within this range of concentration was first order with respect to substrate, indicative of nonsaturability of orotate permeation. The decrease in rate at later times and the final cessation of orotate uptake at 1000 μM was presumably a consequence of a limited supply of PRPP.

The finding that orotate uptake (at 10 or 100 μM) was not inhibited by 1 mM uridine or thymidine, nor by 5 mM uracil, hypoxanthine, adenine, or thymine (data not shown) indicated that orotate is not a substrate for the nucleoside or pyrimidine transporter(s) of Novikoff cells. These results suggested that orotate permeation is non-mediated. Stronger evidence for this contention comes from the observation that the relationship between the rate of orotate permeation and its lipophilicity approximates that of several substances believed to enter cells by non-mediated permeation, as shown in Table 4. A theoretical basis for this observation has been supplied by Lieb and Stein ('71), according to whom the permeation coefficient (P) of a substance through a lipid bilayer is a function of the solubility of the substance in the lipid phase relative to that in the aqueous phase (Z)[3], of its diffusion constant within the membrane (D_{mem}), and of the bilayer thickness (ℓ):

$$P = \frac{Z\, D_{mem}}{\ell} \qquad \text{equation 4}$$

D_{mem}, of course, is a function of molecular shape, but to the extent that the shapes of the molecules listed in Table 4 are not vastly different from one another, that the first-order rate constant for entry is proportional to P, and for membranes of equal thickness, k/Z should be constant. This expectation was borne out for

[3]Partition coefficients have generally been designated K (Lieb and Stein, '71). For clarity, we have used Z, since we use K to designate the constant that describes the affinity of a substrate for its transporter.

TABLE 4. Partition coefficients, molecular weights, and first order rate constant for nonmediated permeation of various substances at 37°C

Substrate	Molecular weight	Z (± S.E.)[1]	$10^3 \times k^2$ (sec^{-1})	k/Z
L-glucose	180	0.00158 ± 0.00040	0.067	0.042
Cytosine	111	0.0352 ± 0.00067	2.9	0.082
8-Azaguanine	150	0.173 ± 0.064	6.0	0.035
DMO	129	0.983 ± 0.078	80	0.081
Orotate	156	0.00438 ± 0.00049	0.05–0.24	0.011–0.055

[1]Z = Partition coefficient: Substrate concentration in octanol/aqueous buffer solution as determined by Graff et al. ('77). Data are from Graff et al. ('77) and unpublished. Z for azaguanine and 5',5'-dimethyl-2,4,oxazolidinedione (DMO) is at pH 6.0, all others are independent of pH between 6 and 8.

[2]k = Apparent first-order rate constant for uptake: Values for L-glucose and cytosine are from Graff et al. ('77); those for 8-azaguanine and DMO were determined in the same manner at pH 5.8–6.0 and represent unpublished data. The range of k for orotate was estimated from initial uptake slopes (as shown in Fig. 4) from five separate experiments.

the compounds tested, which embrace a 2,000-fold range of Z.

Molecular charge and transportability of pyrimidines

The striking differences in mode and velocity of uracil and orotate influx apparent in the experiments described above suggested that the negative charge on the orotate carboxylate group disqualified it as a substrate for a presumptive pyrimidine transporter. The disparate ionizability of the three pyrimidines under study provides a simple test of this hypothesis: Orotic acid bears a negative charge throughout the physiological range of pH, because of its carboxylic group with $pK_a \cong 2.8$ (Dawson et al., '69). Uracil has a ring pK_a at 9.5 (Wempen and Fox, '64), and bears essentially no charge at physiological pH. 5-Fluorouracil has a ring pK_a at 8 (Wempen and Fox, '64), and so begins a neutral-to-negative transition well within the physiological range of pH.

The initial velocity of fluorouracil uptake into Novikoff cells is plotted against pH of the medium in Figure 5. Also in this figure are shown the partition coefficients, over a range of pH, between aqueous buffer and octanol (Z_{oct}) of fluorouracil, uracil, and orotic acid. The parti-

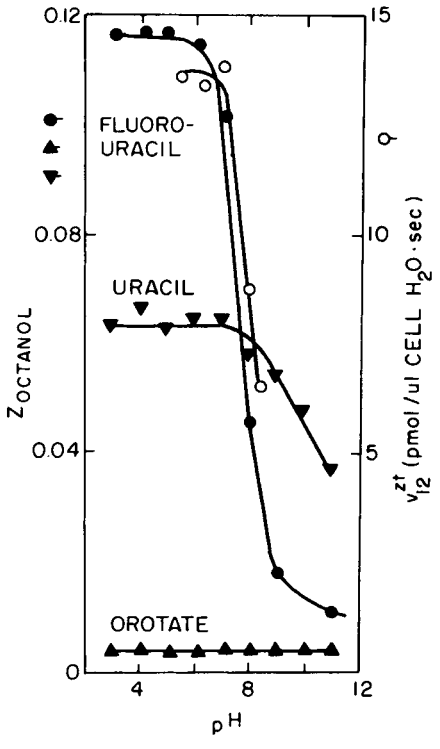

Fig. 5. Partition coefficients of fluorouracil, uracil, and orotate and velocity of fluorouracil transport as a function of pH. The octanol partition coefficient (Z_{oct}) represents the ratio of radioactivity in octanol/radioactivity in balanced salt solution after shaking a mixture of 1 ml of each containing 1 to 3×10^5 cpm ^{3}H-labeled substance/ml at 37°C for 24 hours (see Graff et al., '77). Also samples of a suspension of Novikoff cells were centrifuged sequentially and suspended to about 4×10^7 cells/ml in glucose-free BM42B adjusted to different pH ranging from 5 to 8.8. Immediately each suspension was analyzed for pH and then assayed for the uptake of 640 μM fluoro[^{3}H]uracil (429 cpm/μl) by the rapid kinetic technique as described in Materials and Methods and illustrated in Figure 1 (15 time points were taken over a 6-minute time-period). Radioactivity/cell pellet was corrected for substrate trapped in extracellular H_2O space (6.7 μl/cell pellet) and converted to pmol/μl cell H_2O on the basis of an intracellular H_2O space of 27.9 μl/cell pellet. Equation 1 was fit to each individual time course of fluorouracil accumulation to transmembrane equilibrium with K fixed at 16 mM (see Fig. 1) to obtain an estimate of the value of R. The initial zero-*trans* velocities (v_{12}^z, in pmol/μl cell H_2O•sec) were computed by substituting K and the estimated value of R into Eq. 2 v_{12}^z was plotted as function of the pH determined immediately before the start of each uptake time course.

tion coefficients reflected the charge-state of the three compounds. In particular, the change in Z_{oct} for fluorouracil described a Henderson-Hasselbalch curve with inflexion at pH 7.8. The change in velocity of fluorouracil uptake, necessarily measured over a narrower pH range, was virtually coincidental with the Z_{oct} curve. Uracil influx was also measured in the pH range 5.5 to 8.5, and was constant (data not shown).

(In most other experiments reported in this study pH was not monitored. Our basal medium was routinely adjusted to pH 7.0–7.2. The parameter values reported, therefore, pertain to the largely non-ionized molecule.)

DISCUSSION

Uracil and fluouracil are transported by the same system in Novikoff rat hepatoma cells and, most likely, in other mammalian cells and with similar efficiency. Their transport is saturable and non-concentrative, but the concentrations required for half saturation are so high that entry of either substrate at physiological or pharmacological concentrations is first order. Nevertheless, the transport of either substrate is rapid enough, even at low concentrations, not to be an obstacle to their conversion to intracellular nucleotides. Thus differences in their utilization by cells must be related to differences in their intracellular metabolism.

The specificity of the system transporting uracil and fluorouracil, particularly its relationship to the nucleoside transporter, needs further clarification. In Novikoff hepatoma cells, nucleosides inhibit uracil and fluorouracil transport more strongly than the pyrimidine bases inhibit one another's transport, but not with K_i's expected on a basis of their K_m's with respect to the nucleoside transporter. The finding that nucleosides inhibit pyrimidine transport more strongly than do pyrimidines themselves or hypoxanthine suggests that the effect is not an indirect one resulting from the phosphorolysis of the nucleosides. Furthermore, uridine phosphorolysis in Novikoff cells is relatively slow compared to the rate of pyrimidine transport and thymidine is not phosphorylyzed at all in these cells (Plagemann, '71).

The inhibition of pyrimidine transport by nucleosides might be interpreted as evidence that they share the same transport system, but in CHO cells a clear delineation between base and nucleoside transport systems can be made by means of inhibition of the latter with p-nitrobenzylthioinosine. Hypoxanthine transport is also not inhibited by p-nitrobenzylthioinosine (Wohlhueter et al., '78b). Nucleoside and nucleobase transport have also been distinguished genetically (Cohen et al., '79). A one-step, nucleoside transport-defective mutant of mouse S49 lymphoma cells which is resistant to the toxicity of various natural nucleosides and analogs thereof exhibits normal sensitivity to fluorouracil and 6-thioguanine.

The mechanistic basis for the observed inhibition by nucleosides remains obscure. One might envision that the nucleoside and nucleobase transport systems may be composed of two or more different proteins, not all of which might be shared by the two systems. On the other hand, we cannot exclude the possibility that nucleosides might inhibit pyrimidine and purine transport without themselves being transported by the systems. If this were the case, the concept that countertransport of a transport substrate by another substance represents evidence of a shared carrier (Levine and Levine, '69) needs to be modified. Although the inhibition of pyrimidine transport by nucleosides is of mechanistic interest, it probably is of no physiological importance.

So far no evidence is available to indicate that uracil/fluorouracil and hypoxanthine are not transported by a single system. Adenine, on the other hand, is clearly transported by a carrier which shows no overlapping features with other nucleobase transport systems or the nucleoside transport system (Marz et al., '79).

Our results also show that orotate is not a substrate for the pyrimidine transporter. Judging from the transport behavior of fluorouracil in the vicinity of its pK_a, and from the lack of orotate transport, we conclude that the presence of a negative charge is sufficient to block mediated transport of pyrimidines into cells. Orotate permeation is most likely non-mediated, and almost certainly the rate-determining step in its uptake. Orotate that does permeate the cell membrane is efficiently converted to nucleotides, presumably by the action of orotate phosphoribosyl-transferase. Orotate is reported (Hurlbert and Potter, '52; Hiatt, '62) to be readily incorporated into nucleic acids of rat liver, suggesting that the pyrimidine transporters(s) of this tissue might not discriminate against anionic compounds.

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