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Permeation and Salvage of Dideoxyadenosine in Mammalian Cells

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SUMMARY

Transmembrane equilibration of 2′,3′-dideoxyadenosine (ddAdo) was measured by rapid kinetic techniques in deoxycoformycin-treated P388 and L1210 mouse leukemia cells and human erythrocytes, at 25°. It was only about 10% as rapid as that of other purine nucleosides that are known substrates for the nucleoside transporters of these cells. ddAdo entry was nonsaturable up to a concentration of 1 mM and was not inhibited by other nucleosides or two nucleoside transport inhibitors, dipyridamole and nitrobenzylthioinosine. Thus, ddAdo permeation was mainly nonmediated. It was relatively rapid because of the high lipid solubility of ddAdo. ddAdo entered the cells at least 100 times more rapidly than dideoxycytidine but less rapidly than trideoxythymidine, with an even greater lipophilicity than ddAdo. ddAdo was not phosphorylated in human erythrocytes, but there was some phosphorylation in deoxycoformycin-treated P388 and L1210 cells. *In situ* conversion of 10 μM ddAdo to ddATP, however, was slow and ceased after 5–10 min at 25° or 37°. Cessation of net uptake was not due to turnover of dideoxy-ATP or deamination of dideoxy-AMP. The results suggest that ddAdo salvage in the absence of deamination is limited by feedback inhibition of its phosphorylation, perhaps by deoxycytidine kinase. Permeation into the cells was not rate limiting to ddAdo salvage. In P388 and L1210 cells that had not been treated with deoxycoformycin, ddAdo was salvaged at least 100 times more efficiently than in deoxycoformycin-treated cells and converted to nucleoside triphosphates, but the end-products and pathways of salvage have not been resolved entirely. Salvage of ddAdo required deamination but was not primarily via dideoxyinosine→hypoxanthine→IMP, as is the case for 2′-deoxyadenosine salvage, because [^{3}H]ddAdo salvage was only little inhibited by unlabeled hypoxanthine, whereas it was strongly inhibited by 2′-deoxyadenosine, adenosine, and adenine.

2′,3′-Dideoxynucleosides are being investigated as potential antiviral agents, especially as inhibitors of retrovirus replication (1–5). In order to exert their antiviral effect, which is thought to be mediated at the level of viral DNA synthesis (1–5), dideoxynucleosides need to be salvaged by the cells. Nucleoside salvage in mammalian cells, in its simplest form, can be viewed as a two-component system composed of carrier-mediated, nonconcentrative transport or nonmediated permeation across the plasma membrane followed by intracellular phosphorylation (6–8). The facilitated nucleoside transporter of mammalian cells exhibits a broad substrate specificity, but the Michaelis-Menten constant differs greatly for different nucleosides, ranging from 20 μM for 2-chloroadenosine to several millimolar for cytidine (6–8). Nucleoside kinases, on the other hand, exhibit much more restricted substrate specificities and, in general, higher affinities for their natural substrates than the nucleoside transporter (6).

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Only limited information is available concerning the mode of entry and intracellular metabolism of dideoxynucleosides in mammalian cells. ddCyd and ddAdo have been shown to be phosphorylated by cultured human lymphoidal cells (3, 9–14), but the efficiency of phosphorylation seems to be low and ddAdo is much more rapidly deaminated than phosphorylated (11, 14). Genetic evidence indicates that ddCyd is phosphorylated primarily or solely by dCyd kinase (3, 12, 13). ddAdo seems to be mainly phosphorylated by this kinase, as well as by Ado kinase (10, 11, 14, 15), but additional pathways for its conversion to nucleotides may exist (10). Previous indirect evidence suggested that the facilitated nucleoside transporter plays a primary role in the entry of ddCyd into mammalian cells (12, 13), but our recent direct transport studies have shown that ddCyd is transported by the carrier <1% as efficiently as natural nucleosides, such as Urd and dCyd (16). Because of its low lipid solubility, nonmediated permeation through the membrane seems to play only a minor role in ddCyd entry (16). In contrast, our present study shows that, because of their higher lipid solubilities, nonmediated permeation of ddAdo and

ABBREVIATIONS: ddCyd, 2′,3′-dideoxycytidine; ddAdo, 2′,3′-dideoxyadenosine; ddIno, dideoxyinosine; dddThd, 3′,5′-trideoxythymidine; dAdo, 2′-deoxyadenosine; Ado, adenosine; dCyd, 2′-deoxycytidine; dIno, 2′-deoxyinosine; dThd, thymidine; Urd, uridine; chloroAdo, 2-chloroadenosine; NBTI, nitrobenzylthioinosine; ddATP, dideoxy-ATP.

dddThd is very rapid. In fact, it is the main route of entry of these nucleosides into mammalian cells. The intracellular phosphorylation of ddAdo in deoxycoformycin-treated L1210 and P388 cells, although more rapid than that of ddCyd, is also very inefficient, as also observed in human lymphoidal cells (10, 11, 14). In contrast, the salvage of ddAdo by P388 cells not treated with deoxycoformycin is very rapid and efficient but the metabolic pathways involved are more complex than previously suggested for human T lymphocytes (11, 14).

Materials and Methods

Cells. P388 and L1210 mouse leukemia cells were propagated in suspension culture as described previously (17, 18), harvested from late exponential phase cultures, and suspended in basal medium 42B (BM42B; Ref. 19) to 8×10^6 to 3×10^7 cells/ml. Erythrocytes from freshly drawn human blood were kindly supplied by Dr. J. Kersey (Department of Pathology, University of Minnesota), washed thrice in cold saline that contained 5 mM Tris·HCl, pH 7.4 (Tris-saline), and suspended in the same to 5×10^8 to 8×10^8 cells/ml. Cells were enumerated with a Coulter counter.

Measurement of ^{3}H-nucleoside uptake. Initial time courses of uptake of ^{3}H-labeled nucleosides were measured at 25° by rapid kinetic techniques (12 time points) using a dual syringe apparatus as described previously (7, 20). For longer time points, samples of cell suspension were supplemented with labeled nucleoside and then sampled manually at appropriate times. In both approaches, the cells were separated from the medium by centrifugation through an oil layer and were analyzed for radioactivity as described previously (18, 20, 21). Values for radioactivity in cell pellets were corrected for the amount of radioactivity trapped in the extracellular space, which was estimated by the use of [^{14}C]inulin (20) and, where appropriate, were converted to pmol/μl of cell water on the basis of the specific radioactivity of the substrate and the intracellular water space, determined by the use of ^3H_2O (20). Where appropriate, an integrated rate equation for equilibrium exchange by a symmetrical carrier was fitted to time courses of transmembrane equilibration of [^{3}H]Urd with the Michaelis-Menten constant (K^{cr}) fixed at 500 μM (8, 22), and the slope at time $t = 0$ was taken as the initial transport velocity (v^{cr}). In the case of ddAdo and dddThd uptake, the first-order rate equation $S_{2,t} = S_1 (1\text{-}e^{-kt})$ (Eq. 1) was fitted to the data, where $S_{2,t}$ is the intracellular concentration of substrate at time t, S_1 is the extracellular concentration of substrate, and k is the first-order rate constant. The initial velocity was calculated as $v_o = k/S_1$. Incorporation of the substrates into DNA and RNA was determined as described previously (19).

For an analysis of the intracellular radiolabeled components, samples of cells were collected by centrifugation through oil and the cell pellet was extracted with 60% (v/v) ethanol at −20°. Acid extraction was avoided because dAdo and ddAdo and their nucleotides are highly unstable in 0.2–0.5 N trichloroacetic acid (data not shown). The cell extracts as well as the culture fluid remaining above the oil layer were analyzed by ascending paper chromatography, as described previously (18, 19), with solvent 28 (30 ml of 1 M ammonium acetate, pH 5, and 70 ml of 95% ethanol), which separates nucleoside tri-, di-, and monophosphates and nucleosides (plus nucleobases), or solvent 40 (39 ml of butanol, 22 ml of ethyl acetate, 22 ml of ammonium hydroxide, and 17 ml of methanol), which separates nucleotides, hypoxanthine, ddIno, and ddAdo.

Materials. [5-^{3}H]Urd, [2,8-^{3}H]Ado, [2,8-^{3}H]dAdo, [8-^{3}H]chloroAdo, [*methyl*-^{3}H]dThd, [5-^{3}H]dCyd, [G-^{3}H]formycin B, [2,8-^{3}H]ddAdo, and [5-^{3}H]ddCyd, at specific radioactivities ranging from 2 to 60 Ci/mmol, were purchased from Moravek Biochemicals (Brea, CA). They were >95% pure, as assessed by chromatography with solvents 28 and 40. Unlabeled ddCyd and ddAdo were purchased from Pharmacia-P.L. Biochemicals (Piscataway, NJ) and from Sigma Chemical Co. (St. Louis, MO). ddATP, unlabeled nucleosides, and nucleobases were obtained from Sigma and NBTI from Calbiochem (San Diego, CA). Dipyridamole was a gift from Geigy Pharmaceuticals (Yonkers, NY). L-Alanosine (NSC 153353, Na salt) was supplied by the Division of Cancer Treatment, National Cancer Institute, dddThd was synthesized as described by Hogenkamp (23). First, the chloronucleoside derivative was synthesized from the parent dThd (24), followed by dehalogenation as described by Wang *et al.* (25). The reaction products were purified by recrystallization and high pressure liquid chromatographic analysis on an Altex C_{18} column (ODS-18, 5 μm, 4.6 × 250 mm), using a 10 mM $(NH_4)HPO_4$, pH 4.7/methanol gradient system at 42°. The unlabeled dddThd, which was synthesized, was analyzed and its identity was confirmed by NMR spectrophotometry, which was kindly performed by Professor Hogenkamp. [^{3}H]dddThd was synthesized in the same way using [^{3}H]dThd as starting material, which was diluted with unlabeled dThd to a specific radioactivity of 1 mCi/mmol. The final product was >95% pure, as assessed by paper chromatography with solvents 28 and 40.

Results and Discussion

Fig. 1 compares the initial time courses of uptake of ddAdo, ddCyd, dddThd, and a number of other nucleosides that are transported by the nucleoside carrier with high efficiency (Urd, Ado, dAdo, dThd, dCyd, formycin B, and chloroAdo) by deoxycoformycin-treated human erythrocytes. Chromatographic analysis of the extracellular fluid with solvent 40, which separates ddAdo, ddIno, and hypoxanthine, showed that the deoxycoformycin treatment completely inhibited the deamination of Ado (26), dAdo, and ddAdo (data not shown). The uptake of ddAdo by the deoxycoformycin-treated red cells was fairly rapid but still much slower than that of the nucleosides that are known to be substrates for the nucleoside transporter. Furthermore, no saturation of the initial rate of ddAdo uptake was observed, up to a concentration of 1 mM (Fig. 2A; the concentration of radioactivity was kept constant, while the specific radioactivity was decreased by addition of unlabeled ddAdo). Higher concentrations of ddAdo were difficult to test because of the relative insolubility of this compound in aqueous solutions. Furthermore, the uptake of 10 μM [^{3}H]ddAdo was inhibited little, if at all, by other nucleosides or by dipyridamole and NBTI (Fig. 2B), two potent inhibitors of the facilitated transport of nucleosides in these cells (7, 8).

These results suggest that ddAdo is at best a very poor substrate for the nucleoside transporter of human erythrocytes and that its entry is mainly not carrier mediated. The relative rapidity of ddAdo permeation across the membrane is explained by its lipid solubility, which we estimated as the ratio of its solubility in octanol over that in water ($Z_{oct} = 0.609 \pm 0.021$; Table 1). Our estimate was in agreement with the value previously reported for [2′,3′-^{3}H]ddAdo (27). From the data in Fig. 2A, we estimated a first-order rate constant (k) for the permeation of ddAdo of 1.87 ± 0.053 min^{-1}, which yielded a ratio of $k/Z_{oct} = 3.1$. This value was 1–2 log units lower than those for other purine and pyrimidine nucleosides that are efficient substrates for the nucleoside carrier (Table 1), but it falls in the upper range of values observed for the nonmediated permeation of nucleosides and nucleobases and for the permeation of other substances with similar molecular weights that are believed to enter mammalian cells by nonmediated permeation (8). However, the k/Z_{oct} ratio for ddCyd entry into cells is even lower than that of ddAdo permeation (Table 1), in spite of the fact that ddCyd permeation is mainly, although very inefficiently, facilitated by the nucleoside transporter (13, 16). This fact could only be established because the nonmediated per-

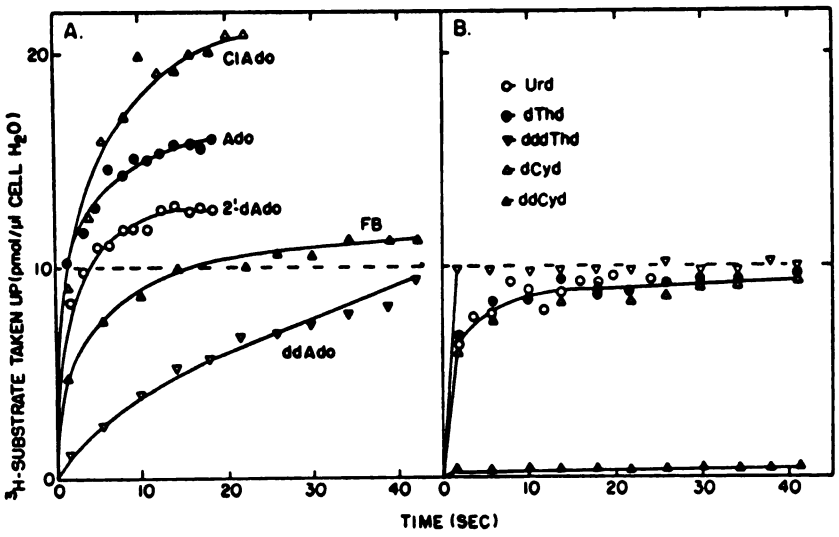

Fig. 1. Comparison of the initial time course of uptake of [^{3}H]ddAdo and [^{3}H]ddCyd with those of other ^{3}H-labeled nucleosides by deoxycoformycin-treated human erythrocytes at 25°. A suspension of 6 × 10^8 erythrocytes/ml of Tris-saline was supplemented with 25 μM deoxycoformycin and then the zero-*trans* entry of 10 μM [^{3}H]ddAdo, [^{3}H]chloroAdo, [^{3}H]Ado, [^{3}H]dAdo, [^{3}H]formycin B, [^{3}H]Urd, [^{3}H]dThd, [^{3}H]dddThd, [^{3}H]dCyd, and [^{3}H]ddCyd (specific radioactivity varied between 10 and 20 cpm/pmol) was measured by rapid kinetic techniques in samples of this suspension as described in Materials and Methods. Radioactivity/cell pellet was corrected for that trapped in extracellular space and converted to pmol/μl of cell water on the basis of the experimentally determined cell water space. The *broken lines* indicated the intracellular concentration of substrate equivalent to that in the medium (i.e., 10 μM).

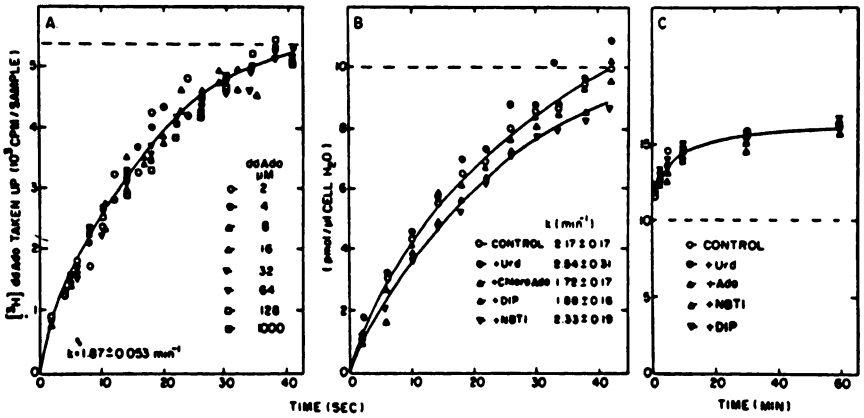

Fig. 2. Zero-*trans* entry of various concentrations of ddAdo into deoxycoformycin-treated human erythrocytes (A) and effect of other nucleosides and nucleoside transport inhibitors on the short term (B) and long term (C) uptake of ddAdo. Suspensions of 5.5 × 10^8 (A) and 8 × 10^8 (B) red cells/ml of Tris-saline were supplemented with 25 μM deoxycoformycin and then the uptake of the indicated concentrations of [^{3}H]ddAdo (282 cpm/μl, irrespective of concentration) or of 10 μM [^{3}H]ddAdo (30 cpm/pmol) (B) was measured by rapid kinetic techniques at 25°. Where indicated in B, 1 mM Urd or Ado were added simultaneously with the [^{3}H]ddAdo or the samples of cell suspension were preincubated for at least 2 min with 1 μM NBTI or 10 μM dipyridamole (*DIP*). Eq. 1 was fitted to each uptake time course. In B, the first-order rate constants (k) ± standard error of the estimate are listed. The k value in A represents the mean of all estimated values ± standard error (eight experiments). In C, samples of a suspension of 7 × 10^8 deoxycoformycin-treated erythrocytes/ml were supplemented with 10 μM [^{3}H]ddAdo (20 cpm/pmol) and, where indicated, with 1 mM Urd, 1 mM Ado, 1 μM NBTI, or 10 μM dipyridamole. At the indicated times of incubation, the cells from duplicate 0.5-ml aliquots of suspension were collected by centrifugation through oil and were analyzed for radioactivity. In B and C, radioactivity/cell pellet was converted to pmol/μl of cell water. The *broken lines* indicate the intracellular concentrations of ddAdo equivalent to those in the medium.

meation of ddCyd is also very slow (16). Its equilibration across the membrane by combined transport and permeation at 25° requires more than 60 min (16) (for initial time course of uptake, see Fig. 1B). The reason for these unusually slow rates of both transport and nonmediated permeation of ddCyd is unclear but, in the case of nonmediated permeation, it suggests that factors in addition to lipid solubility and molecular weight (28, 29) may play a rate-determining role.

Nevertheless, the importance of lipid solubility in determining the rate of nonmediated permeation is further indicated by the uptake of dddThd. Uptake was so rapid in both human erythrocytes (Fig. 1B) and P388 cells (data not shown) that it equilibrated across the plasma membrane at 25° in less than 2 sec. This rapid permeation of dddThd was consistent with its high Z_{oct} of 2.6 (Table 1). In order to measure the time course of transmembrane equilibration of dddThd, we had to reduced the temperature to 5°. Under these conditions, the first-order rate constant of permeation ± SE (five experiments) in P388 cells was 1.85 ± 0.55 min^{-1} and uptake of 50 μM dddThd was inhibited <10% by other nucleosides at 1 mM, 1 μM NBTI, or 10 μM dipyridamole (data not shown). Thus, permeation of dddThd was also primarily nonmediated.

It is obvious, however, that any ddAdo or dddThd transport by the nucleoside carrier, if it were as inefficient as that of ddCyd, would be difficult to detect under our experimental conditions, because of the rapid nonmediated permeation of these deoxynucleosides. The data in Table 2 are in agreement with this conclusion. They show that 1 mM ddAdo and ddCyd inhibited the equilibrium exchange of 500 μM Urd in human erythrocytes to a similar extent, but only slightly and far less than 1 mM dThd, cytidine, ddCyd, dAdo, Ado, and chloroAdo, which are efficiently transported by the nucleoside transporter

TABLE 1

First-order rate constants for the transport or permeation of various nucleosides in human erythrocytes at 25° and octanol partition coefficients for these nucleosides

The first-order rate constants (k) for the transport of chloroAdo, Ado, dAdo, formycin B, dThd, Urd, dCyd, and cytidine (Cyd) were calculated from previously presented data (8, 22, 31, 32, and submitted for publication) as the ratio of maximum velocity/Michaelis-Menten constant for equilibrium exchange (V^{eq}/K^{eq}). The k values for ddAdo and dddThd permeation were estimated from the data in Fig. 2, A and B, and Fig. 1B, respectively, and the k value for ddCyd permeation from previously presented data (16). All the values are averages of at least two independent experiments. The octanol partition coefficients (Z_{oct}) represent the ratios of the concentration of substrate in octanol to that in balanced salt solution at equilibrium and have been determined previously (see Refs. 8 and 27), except for that for ddAdo, which was determined in the same manner in the present study.

Substrate	k	Z_{oct}	k/Z_{oct}
	min^{-1}		
ChloroAdo	27.6	0.510	54
Ado	32.4	0.123	263
2′-dAdo	13.5	0.219	62
Formycin B	7.2	0.0307	235
dThd	32.4	0.0753	1055
Urd	22.8	0.0149	1530
dCyd	12.0	0.0220	545
Cyd	6.9	0.0120	575
dddThd	≥60	2.6	≥23
ddAdo	1.9	0.609	3.1
ddCyd	0.016	0.048	0.33

TABLE 2

Effects of various nucleosides on the equilibrium exchange of Urd in human erythrocytes

Time courses of equilibrium exchange of 500 μM [^{3}H]Urd were measured by rapid kinetic techniques in suspensions of 6×10^8 erythrocytes/ml of Tris-saline, as described previously (8, 22). Where indicated, other nucleosides in unlabeled form were added to final concentrations of 400 or 800 μM, simultaneously with the [^{3}H]Urd. The results for 400 and 800 μM competitor are from two independent experiments. The initial velocities of Urd exchange were estimated by integrated rate analysis of the uptake time courses, as described in Materials and Methods.

Competitor	V^{eq}	
	400 μM	800 μM
	pmol/μl of cell H_2O·sec	
None	95 ± 12	85 ± 15
ddCyd	85 ± 9	72 ± 9
dCyd	62 ± 4	49 ± 5
Cyd[a]	48 ± 5	22 ± 3
dddThd	64 ± 17	31 ± 8
dThd	27 ± 2	13 ± 1
ddAdo	86 ± 5	ND[b]
dAdo	11 ± 0.5	9 ± 0.7
Ado	10 ± 0.8	6 ± 0.5
ChloroAdo	7 ± 0.2	3 ± 0.2

[a] Cyd, cytidine.
[b] ND, not determined.

(8).[1] We had previously found that dddThd inhibits the transport of dThd in Novikoff rat hepatoma cells, but rather inefficiently (30). This has been confirmed for Urd exchange in human erythrocytes. As shown in Table 2, the inhibition of Urd equilibrium exchange in human red cells by dddThd was only slightly higher than that by ddCyd and ddAdo. Because 3′-dAdo (cordycepin), 2′-dAdo, and Ado are transported by the nucleoside transporter with similar efficiency (7, 31) our data suggest that at least one OH group needs to be present at the 2′ or 3′ position for an efficient recognition of a nucleoside by the nucleoside transporter.

It also was apparent that ddAdo accumulated in deoxycoformycin-treated human erythrocytes to about 160% the extracellular concentration (Fig. 2C). This concentrative accumulation was not due to formation of phosphorylated products; <5% of the intracellular ^{3}H present after 10–60 min of incubation with various concentrations of [^{3}H]ddAdo was associated with nucleotides. This slight concentrative accumulation of ddAdo was not unexpected, because we have made similar observations for all natural purine nucleosides and several purine nucleoside analogs we have analyzed, as well as for adenine and hypoxanthine (32–34)[1] (see Fig. 1A). We have ruled out active transport and partitioning into cell lipids for this observation (32). It seems to reflect binding of the purine derivatives to some intracellular components(s). The binding seems to be purine specific, because all natural pyrimidine nucleosides and bases strictly equilibrate with the intracellular water space (32) (Fig. 1B).

The inefficient phosphorylation of ddAdo in human erythrocytes suggests that ddAdo is a poor substrate for the Ado kinase of human erythrocytes, because these cells express high *in situ* activity (V_{max} ~0.1 pmol/μl of cell water·sec at 25°; Refs. 33 and 35), while lacking kinases for the phosphorylation of pyrimidine nucleosides. ddAdo phosphorylation by partially purified Ado kinase from cultured human lymphoblasts was also found to be very inefficient (14).

Fig. 3 illustrates initial time courses of ddAdo uptake by P388 cells. As in human erythrocytes, entry was nonsaturable

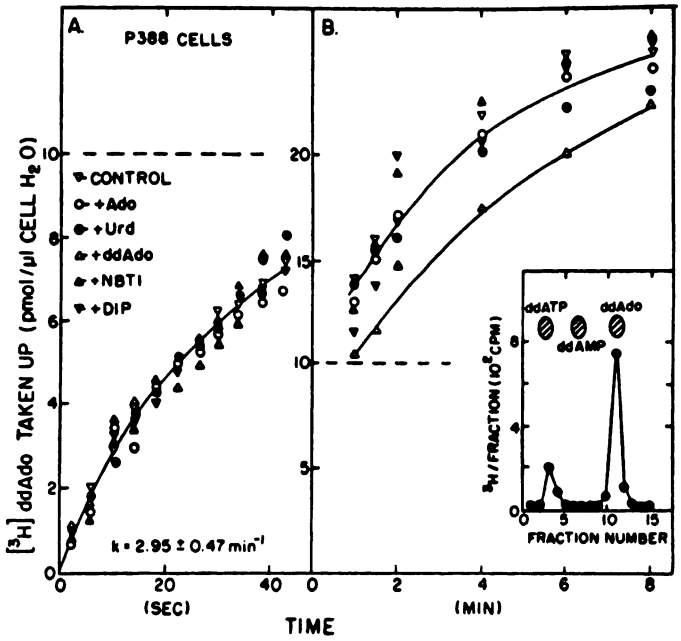

Fig. 3. Zero-*trans* entry of ddAdo into deoxycoformycin-treated P388 mouse leukemia cells. A suspension of 1×10^7 cells/ml BM42B was supplemented with 25 μM deoxycoformycin and then the uptake of [^{3}H]ddAdo (60 cpm/pmol) was measured at 25° by rapid kinetic techniques (A) or by normal sampling of the cell suspension (B), as described in Materials and Methods. Where indicated, 1 mM unlabeled Urd, Ado, or ddAdo were added simultaneously with the [^{3}H]ddAdo or samples of the cell suspension were preincubated for at least 2 min with 1 μM NBTI or 10 μM dipyridamole (*DIP*). Eq. 1 was fitted to each time course in A. The k value listed represents the mean of all estimated values ± standard error. The *broken lines* indicate the intracellular concentration of ddAdo equivalent to that in the medium. After 8 min of incubation, the ethanol-soluble pools were extracted from replicate samples of cells and chromatographed with solvent 28. A representative chromatographic profile (that of the control cells) is shown in B, *inset*.

[1] Unpublished data.

up to a concentration of 1 mM and was not inhibited by other nucleosides, dipyridamole, or NBTI. Thus, as in human red cells, permeation through the plasma membrane was mainly nonmediated. In fact, the first-order rate constant for entry (k = 1.99 ± 0.26 min^{-1}; mean of five independent experiments ± SE) was comparable to that observed for the red cells. Similar results were obtained with L1210 cells (data not shown).

Chromatographic analysis of cell extracts showed that a small proportion of the entering ddAdo was converted to a phosphorylated product(s) (Fig. 3B, *inset*). The primary product comigrated with authentic ddATP but has not been identified unequivocally. However, ddATP is the likely product because the cells were pretreated with 25 μM deoxycoformycin, which completely inhibited any deamination of ddAdo, as ascertained by chromatographic analysis of the culture fluid. Thus, conversion of ddAdo to AMP or GMP via ddIno→hypoxanthine→IMP (14) (Fig. 4) did not play a role. Because little radioactivity was recovered in the nucleoside monophosphate region of the chromatograms (Fig. 3B, *insert*) and no significant amounts of labeled ddIno and hypoxanthine were detected in the culture fluid, deamination of ddAMP (10) was also not a major metabolic reaction in P388 and L1210 cells.

In another experiment, we have measured the uptake of 10 μM [^{3}H]ddAdo by both deoxycoformycin-treated and untreated P388 and L1210 cells, over a 60-min period at 37° and compared it with the uptake of 10 μM [^{3}H]dAdo under identical experimental conditions (Fig. 5). dAdo uptake was very rapid in P388 and L1210 cells that had not been treated with deoxycoformycin (Fig. 5, A and C). In fact, salvage was so rapid that the medium became depleted of [^{3}H]dAdo by 10 min of incubation and net uptake ceased. However, under these conditions dAdo became almost exclusively converted to ribonucleotides via dAdo→dIno→hypoxanthine→IMP and was incorporated into

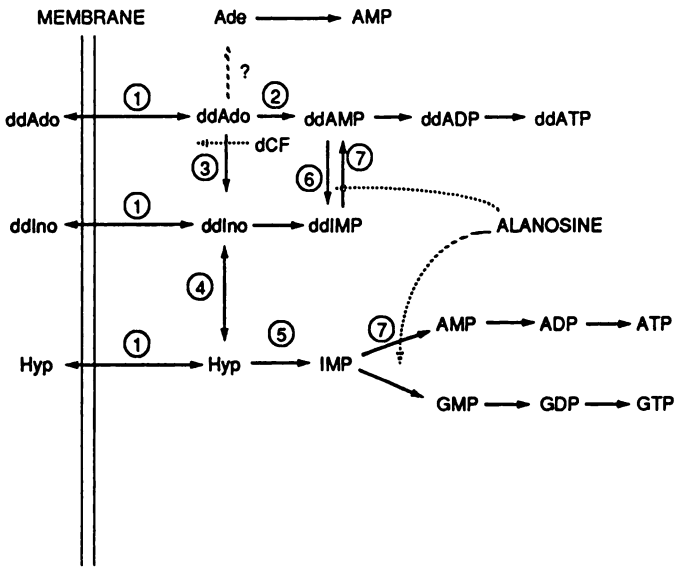

Fig. 4. Known pathways for the metabolism of ddAdo (modified from Ref. 11 and 27). The numbers refer to: 1) transport or permeation across the membrane; 2) direct phosphorylation of ddAdo by dCyd and Ado kinases; 3) Ado deaminase (inhibited by deoxycoformycin, *dCF*), 4) purine nucleoside phosphorylase; 5) hypoxanthine (*Hyp*) phosphoribosyl transferase; 6) AMP deaminase; and 7) adenylsuccinate synthease/lyase, which is inhibited by alanosine. The enzyme responsible for the conversion of ddIno to ddIMP has not been clearly identified. There is no evidence that ddAdo is phosphorylized to adenine.

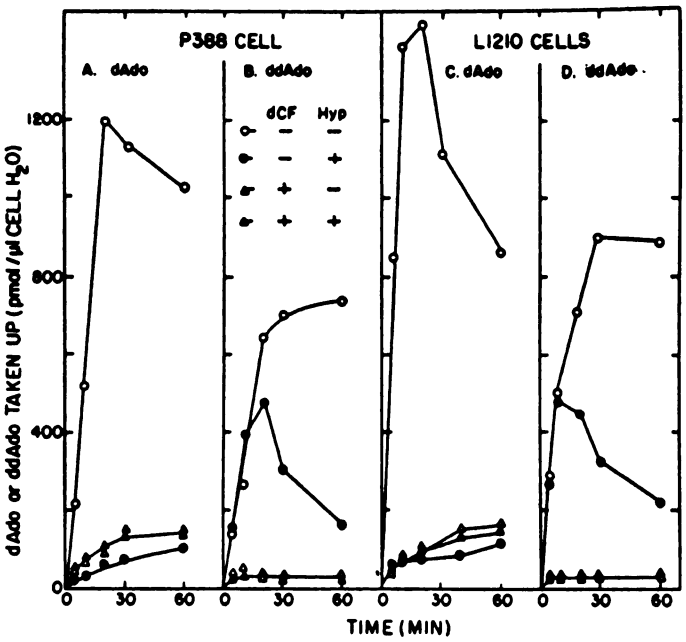

Fig. 5. Long term salvage of [^{3}H]dAdo (A and C) and [^{3}H]ddAdo (B and D) by P388 and L1210 cells in the absence and presence of deoxycoformycin (*dCF*) and hypoxanthine (*Hyp*). Samples of suspensions of about 1.7 × 10^7 cells/ml BM42B were treated with 20 μM deoxycoformycin and after at least 5 min these and untreated control suspensions were supplemented with 10 μM [^{3}H]dAdo (27 cpm/pmol) or 10 μM [^{3}H]ddAdo (24 cpm/pmol). Where indicated, 500 μM unlabeled hypoxanthine was added simultaneously with the labeled substrates. At various times of incubation at 37°, the cells from duplicate 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity. Radioactivity/cell pellet was converted to pmol/μl of cell water on the basis of an experimentally determined cell water space. All values are averages of the duplicate samples. After 60 min of incubation, the ethanol-soluble pools were extracted from replicate samples of cells and chromatographed with solvent 28 (see text). Samples of the culture fluid were chromatographed with solvent 40.

RNA (data not shown; see Ref. 36 and Fig. 4). In agreement with this conclusion is the almost complete inhibition of [^{3}H]dAdo salvage by these cells in the presence of 500 μM unlabeled hypoxanthine (Fig. 5, A and C). Because the K_m for hypoxanthine phosphoribosyltransferase falls in the low micromolar range (36), the presence of 500 μM hypoxanthine saturates the enzyme and greatly reduces the phosphoribosylation of the [^{3}H]hypoxanthine formed from [^{3}H]dAdo via dIno. Pretreatment of the P388 and L1210 cells with deoxycoformycin greatly reduced their salvage of dAdo (Fig. 5, A and C). Under these conditions dAdo was mainly converted to dATP and incorporated into DNA (data not shown; see Ref. 37), which explains that [^{3}H]dAdo salvage by deoxycoformycin-treated cells was not significantly affected by 500 μM unlabeled hypoxanthine (Fig. 5, A and C).

ddAdo was also very rapidly salvaged by P388 and L1210 cells that had not been treated with deoxycoformycin (Fig. 5, B and D) and was converted to a nucleoside triphosphate, which has been tentatively identified as ATP on the basis of its chromatographic behavior (see Fig. 6B), acid stability (see later) as compared with that of ddATP, and results from similar studies with human lymphocyte lines (11, 14). However, the salvage of ddAdo by P388 and L1210 cells was not only via ddAdo→ddIno→hypoxanthine→IMP (see Fig. 4), as is the case for dAdo and as has been postulated for ddAdo metabolism by human lymphocytes (11, 14), because the presence of 500 μM

hypoxanthine affected the salvage of ddAdo by these cells only after about 10 min (Fig. 5, B and D). At this time the cells had already accumulated about 400 μM ATP from 10 μM ddAdo. In addition, it has been shown that ddIno is converted to hypoxanthine by purine nucleoside phosphorylase of P388 cell extracts only 10% as rapidly as inosine and only 0.1% as rapidly as ddAdo is deaminated (10). This low rate of phosphorolysis of ddIno seems incompatible with the rapid conversion of ddAdo to ATP by P388 cells. The data in Fig. 6 support this conclusion. In agreement with the finding that ddAdo is as efficient a substrate for Ado deaminase as Ado (11, 38), chromatographic analysis of the medium from P388 cells that were incubated with 10 μM [^{3}H]ddAdo showed that ddAdo was rapidly deaminated by the cells (Fig. 6C). The 10 μM ddAdo in the medium became almost completely deaminated in 60 min of incubation at 37°, resulting in the accumulation of ddIno, but little hypoxanthine, in the medium (Fig. 6C).

The experiment depicted in Fig. 6 was conducted to inquire into potential alternate pathways for the rapid conversion of ddAdo to nucleotides in P388 cells that were not treated with deoxycoformycin. This conversion clearly involved Ado deaminase because treatment of the cells with deoxycoformycin reduced the salvage of ddAdo >90% (Fig. 5, B and D, and Fig. 6 A). Chromatographic analyses of the ethanol extracts of the cells showed that, after 5 min of incubation, about 75% of the intracellular radioactivity was associated with a nucleoside triphosphate, which comigrated with ATP (Fig. 6B, *left*) and which was completely stable in 0.5 N trichloroacetic acid at 0° for several hours. The remaining intracellular radioactivity was associated with a compound comigrating with ddAdo (Fig. 6B, *left*) but chromatography with solvent 40 showed that it was practically all ddIno (data not shown). Significant amounts of labeled hypoxanthine were not detected. After 60 min of incubation, the control cells had salvaged about 50% of the ddAdo present in the medium originally (Fig. 6A) and >95% of the intracellular ^{3}H was recovered in the nucleoside triphosphate fraction (Fig. 6B, *right*).

In agreement with the data in Fig. 5B, the presence of 500 μM unlabeled hypoxanthine had no effect on the conversion of ddAdo to nucleotides during the first 5 min of incubation (Fig. 6B). During this time, the cells had accumulated ~100 μM ATP. Longer incubation resulted in inhibition of ddAdo salvage (Fig. 6A) and of its conversion to nucleotides (Fig. 6B). The presence of unlabeled hypoxanthine also resulted in the accumulation of [^{3}H]hypoxanthine in the medium (data not shown). It had no effect on the rate of deamination of ddAdo but, after 60 min of incubation, about 35% of the ^{3}H in the medium was recovered in hypoxanthine. This effect was probably due to the inhibition of the phosphoribosylation of the [^{3}H]hypoxanthine formed slowly from [^{3}H]ddIno, due to substrate dilution.

The presence of 500 μM Urd or dThd had no significant effect on ddAdo salvage (Fig. 6A) and its conversion to nucleoside triphosphates or deamination (data not shown). On the other hand, 500 μM dCyd caused a delayed inhibition of ddAdo salvage (Fig. 6A) and conversion to nucleotides (data not shown). Even more effective were 500 μM concentrations of dAdo, Ado, adenine, and formycin B, a C-nucleoside analog of inosine (Fig. 6A), which were tested as potential intermediates in alternate pathways of ddAdo metabolism or as regulators of some metabolic steps. They inhibited the conversion of ddAdo to nucleosides >90%, as exemplified for dAdo in Fig. 6B. None of these compounds had any effect on the rate of deamination of ddAdo (data not shown). The mechanism of these inhibitions of ddAdo salvage has not been resolved but it cannot be explained on the basis of the established pathways of ddAdo metabolism (Fig. 4). Understanding the mechanism might be important in determining the pathway(s) of ddAdo conversion to ribonucleotides in these cells. The delayed effect (Fig. 6A) suggests that some metabolic products of the inhibitors are responsible. On the other hand, formycin B was quite effective as a delayed inhibitor, even though it is only very slowly and inefficiently phosphorylated in P388 cells (32).

It has been reported that ddIno is converted in human T cell lines to ddATP (14, 27, 39), probably via a conversion of ddIMP to ddAMP by adenylosuccinate synthase/lyase (Fig. 4) because the conversion of ddIno as well as of ddAdo to ddATP was inhibited by alanosine (27), an inhibitor of this reaction (40). However, this conversion seemed to be very inefficient, because

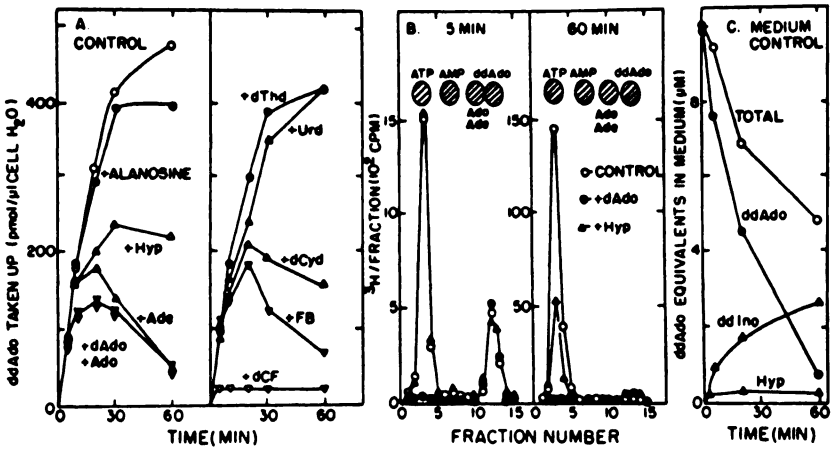

Fig. 6. Effects of L-alanosine and various nucleosides and nucleobases on the uptake of ddAdo by P388 cells. Samples of a suspension of 1.5 × 10^7 P388 cells/ml of BM42B were supplemented with 10 μM [^{3}H]ddAdo (25 cpm/pmol) and, where indicated, simultaneously with 500 μM unlabeled dAdo, Ado, dThd, Urd, dCyd, formycin B (*FB*), adenine (*Ade*), or hypoxanthine (*Hyp*). Other samples of suspension received 50 μM L-alanosine or 25 μM deoxycoformycin (*dCF*) about 5 min before addition of [^{3}H]ddAdo. At various times of incubation at 37°, the cells from duplicate 0.5-ml aliquots of suspension were collected by centrifugation through oil and analyzed for radioactivity. Radioactivity/cell pellet was converted to pmol/μl cell water on the basis of an experimentally determined cell water space. All values are averages of the duplicate samples. After 5 and 60 min of incubation, replicate samples of cells were extracted with ethanol and the cell extracts were chromatographed with solvents 28 and 40. Representative chromatographic profiles for cells incubated with [^{3}H] ddAdo in the absence (○–○) and the presence of unlabeled dAdo (●–●) and hypoxanthine (▲–▲) obtained with solvent 28 are shown in (B). Samples of cell-free culture fluid were chromatographed with solvent 40 and representative results for the control cells are shown in (C).

well over 90% of the ddIno and ddAdo became converted to hypoxanthine, ddIno, AMP, ADP, and ATP during a 4-hr incubation period (27). Furthermore, it has not been explained how ddIno is converted to ddIMP. A conversion of ddAdo to ddATP via dideoxy-IMP→dideoxy-AMP does not account for its rapid salvage by P388 cells that had not been treated with deoxycoformycin. As shown in Fig. 6A, the uptake of ddAdo by these cells was affected little, if at all, by the presence of 50 μM alanosine, whereas this treatment inhibited the salvage of hypoxanthine (data not shown), just as previously demonstrated for Novikoff rat hepatoma cells (40). Furthermore, ddAdo salvage was not via adenine→AMP (Fig. 4). This conclusion is indicated by the findings that deoxycoformycin strongly inhibited ddAdo salvage and that ddAdo salvage was more strongly inhibited by dAdo and Ado than adenine.

In comparison with the rapid salvage of ddAdo by cells that had not been pretreated with deoxycoformycin, its salvage in deoxycoformycin-treated P388 and L1210 cells was extremely inefficient (Fig. 5, B and D, and Fig. 6A). Salvage ceased after about 5 min of incubation at 37°, ddAdo accumulated in the cells to only twice the concentration in the medium, and even after 60 min of incubation only 15–20% of the intracellular radioactivity was associated with ddAdo, which was equivalent to about 3–4 μM (data not shown, see Fig. 3B, *inset*). The remainder of the intracellular ^3H was retained in unmodified ddAdo. In contrast, the cells not treated with deoxycoformycin accumulated up to 900 μM nucleotides from ddAdo (Fig. 5, B and D). The results indicate that ddAdo is only very poorly phosphorylated by cellular kinases. dAdo is phosphorylated by both dCyd and Ado kinases from various types of cells (41–43), including P388 and L1210. We have observed that in cell-free extracts of P388 and L1210 cells, both kinases play about equal parts in phosphorylating 20 μM dAdo.[1] The lack of phosphorylation of ddAdo in human erythrocytes, in spite of high Ado kinase activity, might suggest that it is dCyd kinase that primarily catalyzes the phosphorylation of ddAdo in P388 and L1210 cells; studies with kinase-deficient variants of human T cell lines have led to a similar conclusion (10, 14). However, dCyd kinase is subject to feedback control by dCTP and perhaps other nucleotides (43, 44), and such feedback control may account for the relatively slow salvage of dAdo by deoxycoformycin-treated cultured mammalian cells (see Fig. 5, A and C), when compared with the salvage of other nucleosides. Thus, the poor salvage of ddAdo by the P388 and L1210 cells may be a result of a combination of poor substrate activity of ddAdo for cellular nucleoside kinases in general and feedback inhibition of dCyd kinase. Relief of such regulatory control might enhance the conversion of ddAdo to ddATP and, thus, its efficacy in inhibiting human immunodeficiency virus replication. Other experiments showed that ddAdo exhibited very low or no cytotoxicity for P388 and L1210 cells. The growth of deoxycoformycin-treated cells over a 2-day period was inhibited about 20% by 300 μM ddAdo, but this concentration of ddAdo had no effect on the growth of cells not treated with deoxycoformycin.

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