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TRANSPORT OF DEOXYCOFORMYCIN IN HUMAN ERYTHROCYTES

MEASUREMENT BY ADENOSINE DEAMINASE TITRATION AND RADIOISOTOPE ASSAYS

SHIH-FONG CHEN,* JOHANNA D. STOECKLER and ROBERT E. PARKS, JR.
Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

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Abstract—The assay of residual adenosine deaminase (ADA) activity was used as a sensitive measure of the transport of deoxycoformycin (dCF) into human erythrocytes. Contrary to prior reports from this laboratory, the inactivation of intraerythrocytic ADA by dCF was linear rather than log-linear, with time. Linear inactivation rates were also seen when erythrocytes were preloaded with a 5-fold excess of calf intestinal ADA. The uptake of tritium-labeled dCF molecules and the rate of inactivation of ADA molecules showed an approximate 1:1 stoichiometry. The nucleoside transport inhibitors, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) and dipyridamole, and the permeant, uridine, inhibited dCF transport with K_i values of 35 nM, 45 nM, and 340 μM respectively. The affinity of dCF for the nucleoside transporter was low with a K_i of approximately 10 mM for the inhibition of adenosine influx.

The antibiotics coformycin (CoF†) and deoxycoformycin (dCF, Covidarabine, Pentostatin) are naturally occurring tight-binding inhibitors of the enzyme adenosine deaminase (ADA) [1–3]. Recently, ADA and the use of ADA inhibitors were reviewed by Agarwal [4]. Since deficiency in ADA is associated with a heritable severe combined immunodeficiency disease [5], inhibitors of this enzyme are potential immunosuppressive agents. Also, a number of analogs of adenosine that display chemotherapeutic activities are converted to their inactive inosine analogs by ADA [6]. Enhanced analog nucleotide formation and striking synergistic cytotoxicity are seen when adenosine analogs are used in combination with an ADA inhibitor [3, 7–11]. These considerations and others have led to the introduction of dCF into clinical trials as a single agent. Therapeutic responses have been observed in patients with both T-lymphocyte-related disorders, i.e. T-cell leukemias and mycosis fungoides, and with B-cell malignancies [12–15], although unexpected toxicities encountered have limited extensive clinical study [15–17].

To understand better the behavior of CoF and dCF, this laboratory has examined in detail their interactions with partially purified enzymes [1–3] and ADA in intact cells [18, 19]. Both compounds are tight-binding inhibitors of mammalian ADA (K_i values: CoF = 1×10^{-11} M; dCF = 2.5×10^{-12} M). The $T_{1/2}$ values for dissociation of the inhibitor-ADA complexes are 8 hr and 25–30 hr for CoF and dCF respectively [3]. However, studies of the effects of dCF on ADA in intact Sarcoma 180 cells and human erythrocytes revealed a rate of inactivation of ADA that was 300- to 500-fold slower than that seen with isolated enzyme preparations [18, 19]. Also, no reactivation of intracellular dCF-inhibited ADA occurred for prolonged periods, whereas, upon hemolysis and incubation with charcoal, activity was restored with a $T_{1/2}$ of about 25 hr. These observations demonstrated the key role played by the cellular membrane in the actions of these inhibitors in intact cells and provided the fundamental rationale for measurement of dCF transport by ADA titration. Furthermore, evidence was obtained that dCF enters erythrocytes via the nucleoside transport system. The inactivation of ADA by dCF was blocked by the permeant uridine and by the nucleoside transport inhibitors 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) and dipyridamole [18, 19]. In these prior studies it appeared that the rate of intracellular ADA inactivation by dCF was exponential with time as observed with the isolated enzyme. Based on this assumption, a number of kinetic interpretations were made that now require a major reassessment. Double-reciprocal plots of k_λ (the pseudo first-order rate constant for the inactivation of ADA) versus the dCF concentrations gave a K_m value for dCF of 6×10^{-7} M and predicted a maximal $T_{1/2}$ of about 115 sec. This surprisingly low K_m value (the K_m for the natural substrate, adenosine, is about 2×10^{-6} M) [20, 21] indicated that dCF should be a reasonably potent competitive inhibitor of the nucleoside transporter, a prediction that was not verified experimentally by others [22–24]. Also, we

* Author to whom all correspondence should be addressed.

† Abbreviations: ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); CoF, coformycin, 3-β-D-ribofuranosyl-6,7,8-trihydroimidazo[4,5-*d*][1,3]diazepin-8(*R*)-ol; dCF, 2′-deoxycoformycin, Covidarabine, Pentostatin, or 3-(2′-deoxy-β-D-erythropentofuranosyl)-6,7,8-trihydroimidazo[4,5-*d*][1,3]diazepin-8(*R*)-ol; NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine, 6-(*p*-nitrobenzyl)mercaptopurine ribonucleoside; and PCA, perchloric acid.

found that when erythrocytes are incubated with a high concentration of dCF (100 μM), the enzyme is wholly inactivated within seconds, i.e. the postulated maximal $T_{1/2}$ is invalid. These latter observations indicated that the exponential rates observed with intact erythrocytes in the earlier studies were the results of misinterpretations of data derived from imprecise measurements. This necessitated a reevaluation of experimental procedures, especially those for stopping the inactivation reaction in intact cells.

The studies presented below confirm the earlier postulate that dCF enters erythrocytes principally via the nucleoside transporter but show that the uptake is linear with time and the affinity of dCF for the nucleoside transporter is much lower than previously believed [19]. A preliminary report of these findings has been presented [25].

MATERIALS AND METHODS

dCF was provided by the Drug Development Branch of the National Cancer Institute, Bethesda, MD. NBMPR was a gift from A.R.P. Paterson of the University of Alberta, Edmonton, Alberta. Dipyridamole (Persantin), uridine, adenosine, 1-bromododecane, Triton X-100, and calf intestinal mucosa adenosine deaminase (Type III) were purchased from the Sigma Chemical Co., St. Louis, MO. Trypsin was obtained from GIBCO Laboratories, Grand Island, NY. Dilazep (Carmelian) was a gift from Hoffmann-La Roche Inc., Nutley, NJ. [G-^{3}H] dCF (sp. act. 7 Ci/mmole) and [18-^{14}C]adenosine (52 mCi/mmole) were purchased from Moravek Biochemicals Inc., Brea, CA. Aqueous Counting Scintillant (ACS) was purchased from the Amersham Corp., Arlington Heights, IL. The concentrations of solutions were determined spectrophotometrically: dCF (ε_{282} in $H_2O = 8 \times 10^3$) [26], NBMPR (ε_{290} in $H_2O = 2.5 \times 10^4$) [27], adenosine (ε_{260} in $H_2O = 14.9 \times 10^3$), and uridine (ε_{263} in $H_2O = 10.1 \times 10^3$). Human erythrocytes were obtained from healthy adults, age 20–32.

Preparation of cells. Human erythrocytes were separated from plasma and buffy coat by washing three to four times in Standard Medium (potassium phosphate buffer, 50 mM, pH 7.4; NaCl, 75 mM; $MgCl_2$, 2 mM; glucose, 10 mM) and were then suspended in medium to give 20% (v/v) final cell suspensions. Hematocrits were determined on an Adams Autocrit centrifuge, Clay-Adams, Inc., New York, NY.

Entrapment of calf intestinal ADA in resealed human erythrocytes. A slight modification of the procedure of Dale *et al.* [28] was used to entrap calf intestinal ADA in human erythrocytes. Ten milliliters of a suspension of washed erythrocytes [76% (v/v) in normal saline] and 80 units of calf intestinal ADA were placed in a section of seamless cellulose dialysis tubing (16 mm in diameter and 6 cm in length). The tubing was tied, leaving an air space of about 3 ml inside the bag. The bag was then secured to a glass rod and placed in a bottle containing 500 ml of cold hypotonic buffer (5 mM potassium phosphate buffer, pH 7.4). The bottle was rotated end-on-end at 4–5 rpm at 4° for 1 hr. The dialysis bag was then placed in a bottle containing resealing solution, 0.15 M NaCl and 5 mM potassium phosphate buffer, pH 7.4, and rotated at the same speed for 1.5 hr at room temperature. The resealed erythrocytes were diluted with 20 ml of cold isotonic saline and washed repeatedly in a clinical centrifuge at 1300 *g* at 4° until ADA activity was not detectable in the washings. The human erythrocytes containing entrapped calf intestinal ADA were then resuspended in Standard Medium to a concentration of 20% for dCF transport assays. In a control experiment, exposure of intact cells to 80 units of ADA, followed by the identical washing procedure, showed that no ADA activity was absorbed to the cell surfaces.

Purification of [^{3}H]dCF. The purity of [^{3}H]dCF was determined by high performance liquid chromatography (HPLC) (Waters Associates, LCS-III; column, μBondapack C_{18}; mobile phase, isocratic 23% methanol in H_2O; flow rate, 1 ml/min). Less than 2% degradation of tritiated dCF was detected 1 year after purchase. However, 40–50% of the radioactivity was detected in degradation products 2 years later (retention times, 2.2 to 4.4 min) and was removed from the undegraded dCF (retention time, 9.3 min). The purified dCF fraction was lyophilized and reconstituted with appropriate medium. The concentration of radioactive dCF was determined from its peak area on the chromatogram, compared to that of a standard dCF solution, using a Hewlett–Packard 3390A Integrator.

Determination of ADA activity in hemolysates. The activity of ADA in hemolysates was determined by measuring the ammonia liberated from adenosine as described previously [2]. One unit of ADA liberates 1 μmole NH_3/min in Standard Medium containing 1 mM adenosine at 30°.

Measurement of dCF transport into human erythrocytes by the ADA titration assay: NBMPR-stop method. Suspensions (20%) of washed human erythrocytes in Standard Medium were incubated at 30° on a shaking water bath (120 oscillations/min) with various concentrations of dCF. At appropriate time intervals, 0.5-ml aliquots of the reaction mixtures were withdrawn and mixed thoroughly with 2 ml of ice-cold Standard Medium containing 5 μM NBMPR. The cells were centrifuged at 1300 *g* at room temperature for 1 min. After removal of the supernatant fluid by aspiration, the packed cells were washed three times with 2 ml of cold Standard Medium containing 5 μM NBMPR (when higher concentrations of dCF were used, more washings were needed), and once with 2 ml of Standard Medium without NBMPR. The packed cells were then resuspended in 2.15 ml of Standard Medium and hemolyzed by two freeze–thaw cycles. After addition of 0.25 ml of 10 mM adenosine to give a final concentration of 1 mM, the ADA activities were determined by the ammonia liberation procedure.

Measurement of dCF transport into human erythrocytes by the ADA titration assay: adenosine-stop method. As an alternative stopping method, 0.5-ml aliquots of the reaction mixtures were withdrawn at appropriate time intervals and mixed thoroughly with 2 ml of Standard Medium containing 10 mM adenosine. The cells were centrifuged immediately at 1300 *g* at room temperature for 1 min. After removal of the supernatant fluid by aspiration, the

packed cells were washed five times with 2 ml of Standard Medium containing 10 mM adenosine. The packed cells were then resuspended twice in the same medium and let stand each time for 1 hr at room temperature. The cells were next washed three times with cold Standard Medium containing 10 mM adenosine and twice with cold Standard Medium. The packed cells were finally resuspended in 2.15 ml of Standard Medium. The remaining ADA activities were determined as described above.

Construction of rapid mixing pipetter. A modified version of a one-hand dual-syringe apparatus [29] was designed for rapid transport studies. As shown in Fig. 1, a Plexiglas chamber which enables rapid

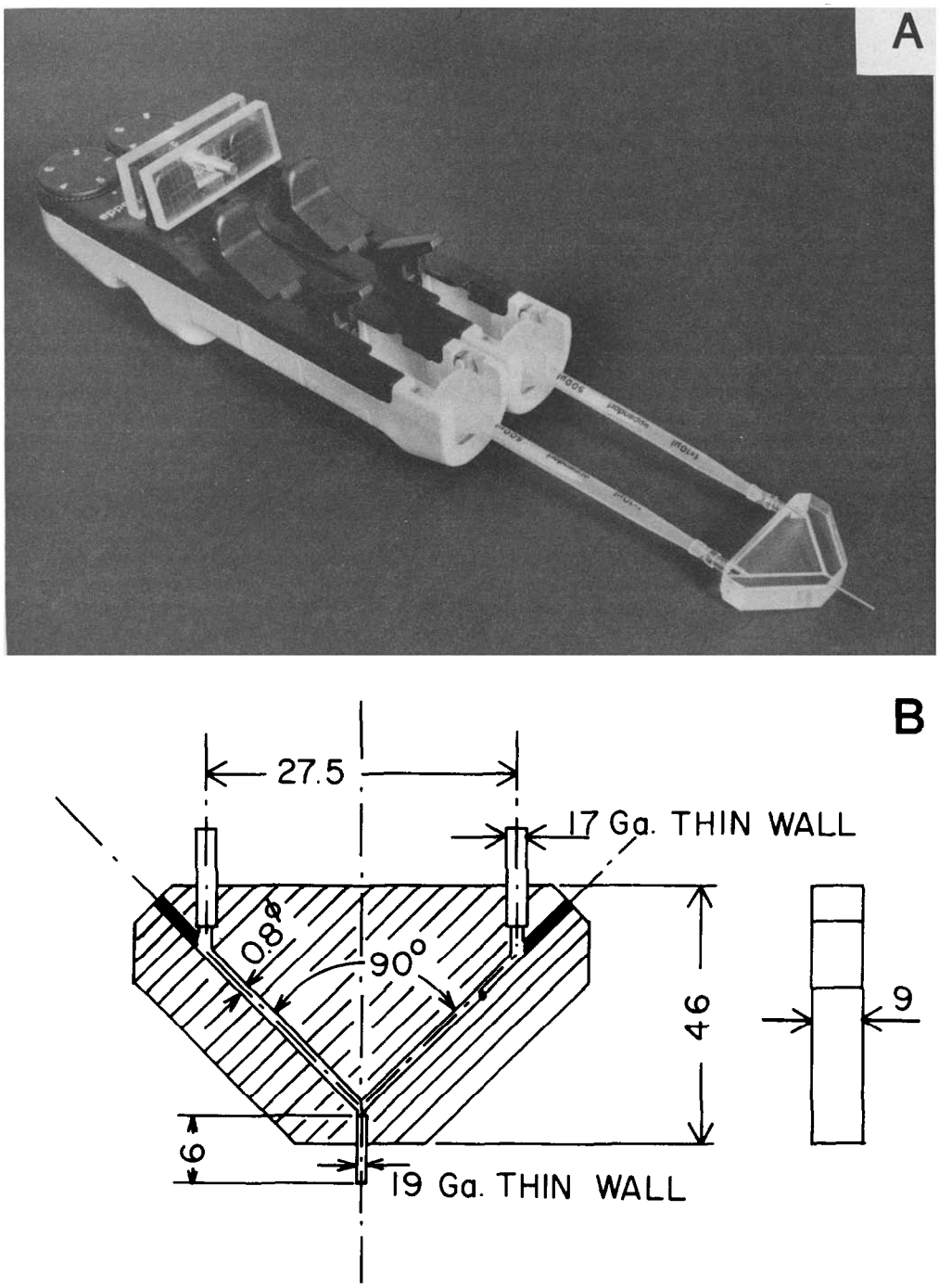

Fig. 1. Rapid mixing pipetter. (A) Photograph of the assembled rapid mixing pipetter. (B) Diagram showing the dimensions of the rapid mixing chamber. The 17- or 19-gauge thin wall stainless steel tubings are press fitted in the Plexiglas. All dimensions are in mm. The drill holes are filled with Epoxy where indicated (■). The mixing chamber is attached to the Combitips through two short pieces of Tygon tubing.

mixing of the erythrocyte suspensions and radioactive dCF solutions is constructed to fit two Eppendorf Repeater pipettes 4780 (Brinkmann Instruments, Inc., Westbury, NY), which are taped side by side. The pipetting levers are clamped between two Plexiglas bars held by a central screw. After withdrawal of the cell suspensions and test solutions into separate Combitips, the mixing chamber is attached to the cones of the Combitips. With each depression of the joined pipetting levers, the two solutions are injected simultaneously through the mixing chamber. The 19-gauge thin wall steel tube has a volume of 3 μl. This results in a 3% carry-over from one sample to the other when a total volume of 100 μl is delivered. The percent carry-over can be minimized by injecting a larger volume of test solutions. However, larger volumes may result in longer delivery times.

Direct measurement of the transport of [^{3}H]dCF into human erythrocytes. The rapid transport assay employing the mixing chamber was used to determine the transport of [^{3}H]dCF into the intact cells for time intervals of less than 30 sec. Microcentrifuge tubes containing 0.25 ml of 1-bromododecane were placed in the rotor of an Eppendorf Microcentrifuge model 5414 (Brinkmann Instruments, Inc.). The caps of the tubes had 0.5-cm holes which permitted rapid aliquot delivery into the tubes but prevented spillage during centrifugation. The washed erythrocytes in Standard Medium and Standard Medium containing various concentrations of [^{3}H]dCF were ejected at timed intervals through the mixing chamber into the microcentrifuge tubes. After the last injection, the transport process was terminated within 2 sec by centrifuging the cells through 1-bromododecane. The medium remaining above the 1-bromododecane was removed by aspiration. The absence of hemoglobin in the medium and oil layers indicated that no hemolysis occurred during the experiment. The inner walls of the microcentrifuge tubes above the 1-bromododecane were washed gently with about 1 ml of 0.9% NaCl, avoiding contact between the sedimented cells and the washing solution. After removing the saline and most of the 1-bromododecane, the erythrocytic pellets were incubated overnight with 0.75 ml of tissue solubilizer (2 g ammonium bicarbonate, 1 g Triton X-100, and 1 g trypsin in 100 ml H_2O) [30]. The centrifuge tubes were then placed in scintillation vials, 1 ml of toluene was added to each tube to prevent foaming, and 0.5 ml of 30% H_2O_2 was added for decolorization of the samples. The samples were held at room temperature for 2 hr to ensure complete decomposition of the peroxide. After mixing with 10 ml of ACS, the radioactivity was determined in a Packard TriCarb 460 Liquid Scintillation Spectrometer.

To determine the [^{3}H]dCF transport over longer time intervals, i.e. greater than 30 sec, reaction mixtures containing 20% (v/v) erythrocytes and [^{3}H]dCF in Standard Medium were incubated at 30° on a shaking water bath. At timed intervals, 0.5-ml aliquots of the cell suspensions were mixed thoroughly with 2 ml of cold Standard Medium containing 5 μM NBMPR. After removal of the extracellular dCF as described under the NBMPR-stop procedure, the cell pellets were lysed with 0.5 ml of cold 4.8% PCA. The tubes were kept on ice for 15 min and occasionally vortexed. Precipitated protein was removed by centrifugation, and the supernatant fluids (400 μl) were neutralized with 5 N KOH (45 μl) at 4°. After removal of the precipitated potassium perchlorate, the supernatant fluids (400 μl) were mixed with 10 ml ACS, and the radioactivity was determined as described above. The PCA extraction was used in these later experiments because it was less time-consuming than use of tissue solubilizer and gave identical results.

Determination of inhibition constants of uridine, NBMPR and dipyridamole for dCF transport in human erythrocytes. Washed erythrocytes were preincubated on a shaking water bath with the desired concentrations of inhibitor or permeant for 15 min before addition of various concentrations of dCF. The rate of ADA inactivation was determined as described above. Double-reciprocal plots were constructed of the rate of ADA inactivation versus the dCF concentration at the various fixed inhibitor concentrations. The inhibitor constants (K_i) were estimated from replots of the slopes versus inhibitor concentrations. The weighted non-linear regression analysis program of Cleland [31] was modified by Dr. S. Cha to calculate the K_i values on a Wang computer.

Inhibition of [^{14}C]adenosine transport into human erythrocytes by dCF. The rapid transport assay was used to approximate the K_i value of dCF for adenosine transport in erythrocytes. Equal volumes (50 μl) of erythrocyte suspensions and [^{14}C]adenosine plus appropriate dCF concentrations were injected through the mixing chamber at 2-sec time intervals as described above. The final reaction mixtures contained: 1% (v/v) erythrocytes, 2 μM adenosine and 1–10 mM dCF in Standard Medium. The transport process was stopped by centrifugation through 1-bromododecane, and the radioactivity remaining in the cell sediments was determined as described above.

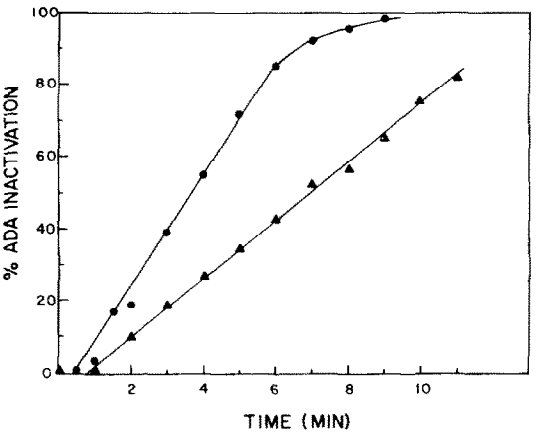

Fig. 2. Determination of the rate of ADA inactivation by dCF in intact human erythrocytes. Washed human erythrocytes (20%) were incubated at 30° with 0.4 μM (▲) and 0.8 μM (●) dCF. Residual ADA activity was measured as described in Methods.

Table 1. Rate of ADA inactivation in different individuals by 0.6 μM dCF

Donor	Number of determinations	ADA activity* Average	ADA activity* Range	ADA inactivation rate† Average	ADA inactivation rate† Range
1	2	0.169	0.159–0.179	0.045	0.041–0.048
2	1	0.174		0.037	
3	1	0.188		0.032	
4	2	0.200	0.198–0.201	0.115	0.100–0.131
5	1	0.217		0.045	
6	2	0.240		0.053	0.049–0.057
7	1	0.248		0.043	
8	4	0.250	0.234–0.262	0.035	0.033–0.039
9	3	0.252	0.243–0.269	0.127	0.086–0.170
10	1	0.259		0.046	
11	1	0.275		0.052	
12	1	0.316		0.052	

* ADA activity is expressed in units/ml of packed cells.
† The rate of ADA inactivation is expressed as units ADA inactivated/min per ml of packed cells.

RESULTS

Inactivation of ADA as a measure of dCF transport. As shown in Fig. 2, with use of the revised procedures for stopping uptake, the percentages of intraerythrocytic ADA inactivated were directly proportional to the incubation times. In most experiments, the rate of ADA inactivation was linear until 80–90% of the enzyme was inhibited. Over a dCF concentration range of 0.2 to 1.0 μM, the rate of ADA inactivation increased proportionally with dCF concentration. When a high concentration of dCF (100 μM) was used in the incubation mixture, the intraerythrocytic ADA activity was abolished within 1–2 sec. These findings indicate that the plot of log % ADA inactivation versus time is not linear. Thus, the assumptions that led to an estimated maximal $T_{1/2}$ of 115 sec [19] are invalid.

In the course of these studies, blood samples were obtained from a number of volunteers, a few on more than one occasion. As seen in Table 1, the dCF transport activity as measured by the rate of ADA inactivation differed over a 5-fold range. When donors 4 and 9 were excluded, the mean transport rate of 0.043 ± 0.008 (S.D.) units of ADA inactivated/min per ml of packed cells indicated a relatively narrow range of activities among the remaining donors. The results obtained from this limited number of samples show no correlation between the ADA activity and the rate of ADA inactivation.

As reported earlier, the inactivation of ADA in intact erythrocytes is blocked by nucleoside transport inhibitors [18]. The rate of ADA inactivation by 10 μM dCF was reduced by 98% in the presence of high concentrations (5 and 10 μM) of the nucleoside

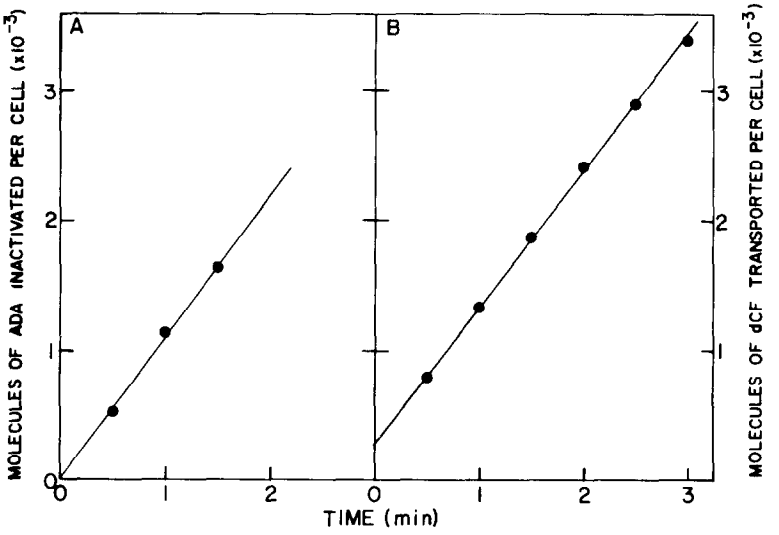

Fig. 3. Comparison of the rates of dCF uptake and ADA inactivation in intact human erythrocytes. The erythrocytes were prepared and incubated under identical conditions with 2 μM [^{3}H]dCF in both assays. Panel A: aliquots were withdrawn and assayed for residual ADA activity. Panel B: aliquots were withdrawn and the intraerythrocytic radioactivity was determined.

transport inhibitors NBMPR, dilazep or dipyridamole. These observations illustrate the key role played by nucleoside transport in the inhibition of ADA by dCF in human erythrocytes and indicate that, under these conditions, simple diffusion can account for only about 2% of the uptake.

Comparison of rates of dCF uptake and rates of ADA inactivation. The influx of [^{3}H]dCF was determined by both the NBMPR-stop method and by centrifugation through 1-bromododecane. Identical rates were obtained by these methods. Furthermore, the rate of uptake of [^{3}H]dCF into erythrocytes was compared directly with the rate of ADA inactivation. Under identical incubation conditions, 1.80×10^{-11} moles of [^{3}H]dCF were taken up and 0.181 units of ADA were inactivated/min per ml of packed cells. If one assumes the molar equivalency of ADA to be 1×10^{-10} moles per unit [1], 1 ml of packed erythrocytes (1×10^{10} cells) with an ADA activity of 0.314 units contains 3.14×10^{-11} moles of ADA. Therefore, the rate of inactivation was about 1.81×10^{-11} moles of ADA/min per ml of cells. It may be estimated that a single erythrocyte contains about 1.9×10^5 ADA molecules. Based on these calculations, under the conditions used in Fig. 3, about 1082 molecules of dCF are taken up and about 1092 molecules of ADA are inactivated per min per erythrocyte. Thus, by use of these assumptions there is an approximate 1:1 stoichiometry.

The experiments of Figs. 2 and 3 employed the NBMPR-stop procedure which caused an immediate block of dCF influx but entailed a prolonged time-lapse (usually 1 hr or more) before ADA measurements were made. This procedure stops transport but does not inhibit further binding of dCF to ADA within the cell. Although Fig. 3 indicates a 1:1 stoichiometry between dCF influx and ADA inactivation, it does not provide information on the rate at which dCF associates with ADA after entering the cell. Therefore, the NBMPR-stop method was compared with the adenosine-stop procedure. The concentration of adenosine used (10 mM) was about 400-fold greater than the K_m of adenosine with ADA [6] and about 5000-fold greater than its high-affinity K_m [20, 21] with the nucleoside transporter. Thus, the adenosine-stop procedure should block both the influx of dCF and its further binding to intracellular ADA. Figure 4 shows that the rate of uptake of radiolabeled dCF determined with the adenosine-

Table 2. Comparison of dCF transport rates determined by the NBMPR-stop and adenosine-stop procedures*

	ADA activity†	% ADA inactivation‡
Control	0.246 ± 0.004	0
NBMPR-stop	0.053 ± 0.002	78.54 ± 1.96
Adenosine-stop	0.076 ± 0.005	69.25 ± 2.55

* Suspensions (20%) of washed human erythrocytes were incubated at 30° with 2 μM dCF for 90 sec. The transport processes were terminated by the NBMPR-stop or adenosine-stop method. After thorough washing, the cells were hemolyzed, and the ADA activities were determined by the ammonia liberation procedure. Values are means ± S.D.

† ADA activities of 4–6 samples are expressed as units/ml of packed cells.

‡ The significance of the difference between the means, $P < 0.002$, was calculated by Student's t-test.

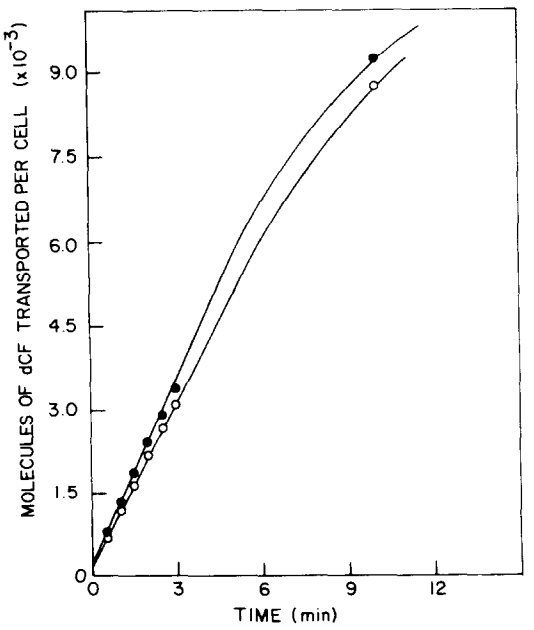

Fig. 4. Comparison of the rates of dCF transport using the NBMPR-stop and adenosine-stop methods. Human erythrocytes (20%) were incubated at 30° with 2 μM [^{3}H] dCF. At indicated time intervals, samples (0.5 ml) were withdrawn, and transport was terminated by the NBMPR-stop method (●) and the adenosine-stop method (○). After thorough washing as described in Methods, the intra-erythrocytic [^{3}H]dCF was determined.

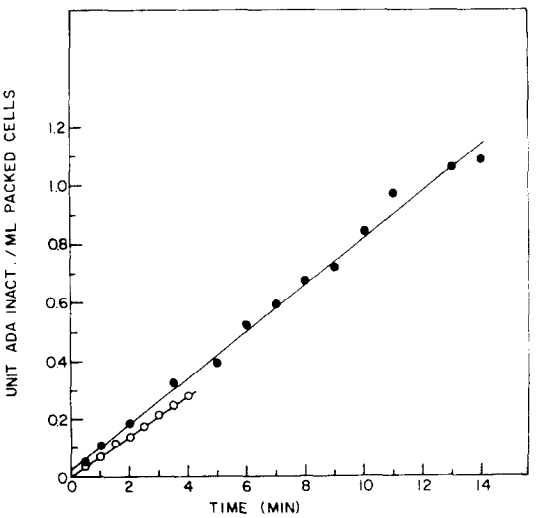

Fig. 5. Comparison of the rates of ADA inactivation in control erythrocytes and in erythrocytes preloaded with calf intestinal ADA. Control (○) and preloaded (●) 20% erythrocyte suspensions in Standard Medium were incubated with 0.8 μM dCF at 30°. The ADA activities of the control and preloaded erythrocytes were 0.32 and 1.69 units/ml of packed cells respectively.

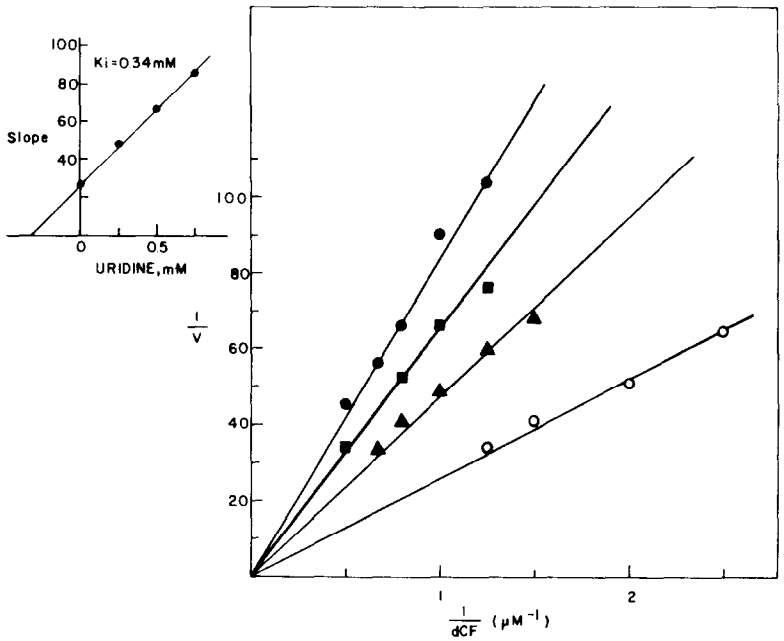

Fig. 6. Determination of the K_i of uridine for nucleoside transport in human erythrocytes. Double-reciprocal plots of v vs dCF concentration at various fixed concentrations of uridine: 0 mM (○), 0.25 mM (▲), 0.50 mM (■) and 0.75 mM (●). Velocity (v) is the percentage of ADA inactivated per min. The inset is a replot of the slopes vs the uridine concentrations and gives a K_i value of 3.4×10^{-4} M.

stop procedure was only slightly slower than that determined with the NBMPR-stop method.

The slightly slower apparent uptake of radioactive dCF by the adenosine-stop procedure was also illustrated by the direct measurement of residual ADA activities. As shown in Table 2, the percentage of ADA inactivated by 2 μM dCF at 90 sec was about 9% lower when the adenosine-stop procedure was used. A P value <0.002 calculated from Student's *t*-test indicates that the difference between the two means was significant. This indicates that, under these conditions, the interaction between dCF and intraerythrocytic ADA is rapid.

Comparison of the rate of ADA inactivation in control erythrocytes and erythrocytes preloaded with calf intestinal ADA. Linear rates of ADA inactivation were also seen when erythrocytes were preloaded with calf intestinal ADA to elevate their

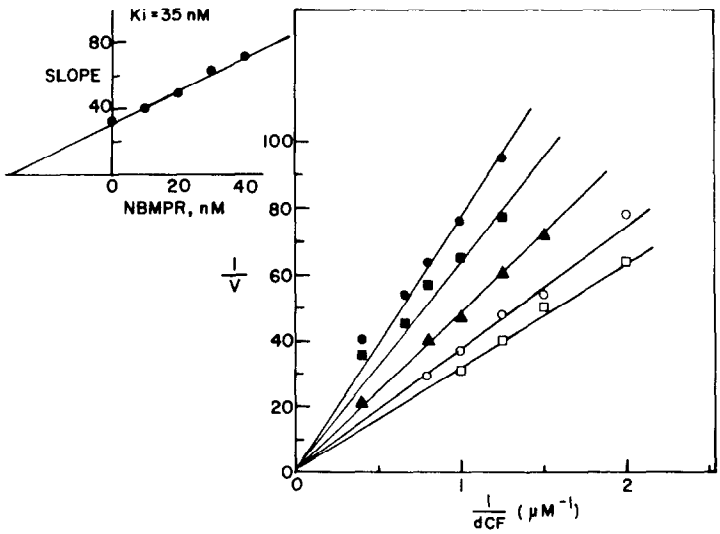

Fig. 7. Determination of the K_i of NBMPR for nucleoside transport in human erythrocytes. Double-reciprocal plots of v vs dCF concentration at various fixed concentrations of NBMPR: 0 nM (□), 10 nM (○), 20 nM (▲), 30 nM (■) and 40 nM (●). Velocity (v) is the percentage of ADA inactivated per min. The inset is a replot of the slopes vs the NBMPR concentrations and gives a K_i value of 3.5×10^{-8} M.

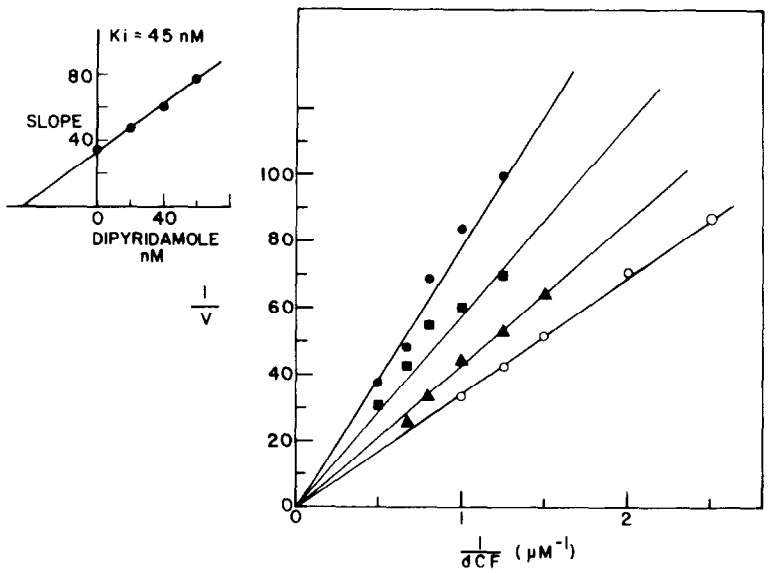

Fig. 8. Determination of the K_i of dipyridamole for nucleoside transport in human erythrocytes. Double-reciprocal plots of v vs dCF concentration at various fixed concentrations of dipyridamole: 0 nM (○), 20 nM (▲), 40 nM (■), and 60 nM (●). Velocity (v) is the percentage of ADA inactivated per min. The replot of the slopes vs dipyridamole concentrations gives a K_i value of 4.5×10^{-8} M.

ADA content by 5.3-fold. As shown in Fig. 5, the rates of ADA inactivation by 0.8 μM dCF were 0.07 and 0.08 units/min/ml of packed cells for the control and calf intestinal ADA-loaded erythrocytes respectively.

Use of the ADA titration assay to determine inhibition constants (K_i) *of uridine, NBMPR, and dipyridamole for dCF transport in human erythrocytes.* As shown in Figs. 6–8, uridine, NBMPR and dipyridamole blocked the inactivation of ADA by dCF in intact erythrocytes. In all cases, the double-reciprocal plots of the rate of ADA inactivation versus dCF concentration at various fixed inhibitor concentrations gave a family of straight lines that intersected near the origin. Therefore, it could not be determined from the graphs whether inhibition was competitive or noncompetitive. Replots of the slopes versus inhibitor concentrations gave K_i values as follows: uridine, 0.34 mM; NBMPR, 35 nM; and dipyridamole, 45 nM.

Estimation of the affinity constant of dCF for human erythrocytic nucleoside transporter. The double-reciprocal plots of Figs. 6–8 gave lines that intersected near the origin, indicating a very high K_d value. Also, as noted above, at high dCF concentrations, i.e. 100 μM, the ADA activity was abolished within seconds. Thus, the K_m of dCF for the nucleoside transporter could not be determined directly by the ADA titration assay.

When direct measurement of radioactive dCF uptake was examined over a concentration range of 25–100 μM, the double-reciprocal plot of the rate of radioactive dCF uptake versus dCF concentrations gave a straight line which also intersected the axes near the origin (results not shown). This indicates that the K_m value is much higher than 100 μM. Although it is feasible to measure the uptake of radioactive dCF at concentrations higher than 100 μM, further attempts were abandoned as too costly.

Nevertheless, the degree of affinity of dCF for the nucleoside transporter was estimated by studying the inhibition of the transport of [^{14}C]adenosine at high concentrations of dCF. The concentration of [^{14}C] adenosine selected (2 μM) was near its reported K_m value for the nucleoside transporter [20, 21]. Over

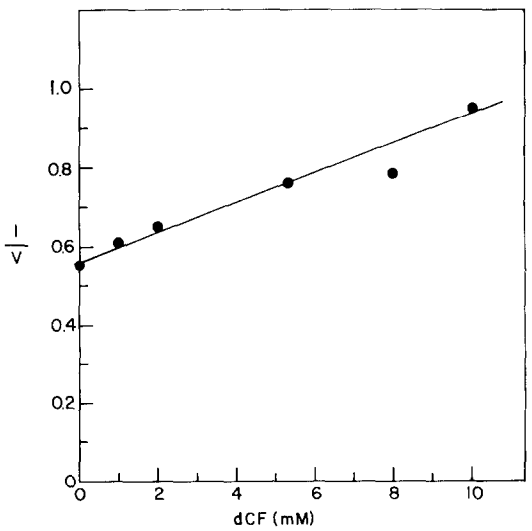

Fig. 9. Dixon plot of the effect of the dCF concentration on the rate of transport 2 μM [^{14}C]adenosine. $1/v$ = (pmoles/min per 10^6 cells)$^{-1}$. The apparent K_i value was estimated from the intersection point with the X-axis.

a range of dCF concentrations from 1 to 10 mM, progressive inhibition of adenosine transport was seen with 30% inhibition at 10 mM dCF. From a Dixon plot, i.e. the plot of 1/rate of adenosine transport versus dCF concentration (Fig. 9), the intersection point with the X-axis allowed estimation by linear regression of an apparent K_i value of 19 ± 3 mM. Since K_m of adenosine is 2.2 ± 0.4 μM [21], assuming that dCF and adenosine are competitive, K_i = apparent $K_i/(1 + S/K_m) = 10.0 \pm 1.8$ mM.

DISCUSSION

This report reevaluates and extends earlier studies on the use of the ADA titration assay as a measure of dCF transport in intact cells. Inactivation of intraerythrocytic ADA by dCF is 300- to 500-fold slower than inactivation of the enzyme in hemolysates and is subject to inhibition by permeants or inhibitors of the nucleoside transporter [18, 19]. As shown here, pretreatment of erythrocytes with 5 or 10 μM concentrations of the transport inhibitors NBMPR, dilazep or dipyridamole caused a 98% reduction in the rate of inactivation of ADA. The residual dCF influx probably represents passive diffusion. This confirms the postulate that interaction with the nucleoside transporter is rate-limiting for the inactivation of intraerythrocytic ADA [18]. Figure 10 presents a model of our present concept of the interaction of dCF with the nucleoside transporter and intracellular ADA. This scheme assumes that the K_i of dCF for intraerythrocytic ADA is the same as that for the isolated enzyme and that the affinity of intracellular dCF for the nucleoside transporter is low as seen with external dCF. The failure to detect reactivation of the intraerythrocytic ADA·dCF complex may result from the very low K_i of dCF with ADA (2.5×10^{-12} M) coupled with its relatively high K_m for nucleoside efflux.

The validity of the ADA titration assay for dCF transport has been established by parallel studies with radiolabeled dCF. An approximate 1:1 stoichiometry was shown between the molecules of ADA inactivated and the molecules of dCF entering the cells. The uptake of dCF was linear with respect to time when measured by either the ADA titration or the radioisotope assay at low concentrations of labeled dCF. As noted below, the intracellular ADA concentration is about 50 nM and the lowest concentration of dCF used in these studies was 400 nM. Thus, the extracellular concentration of dCF would not decrease more than 2.5% during the ADA titration assay, which can account for the linear reactions. The slow rates of dCF transport at concentrations in the range of 10^{-6} to 10^{-7} M permit initial velocity conditions to prevail over a time span of 2–15 min.

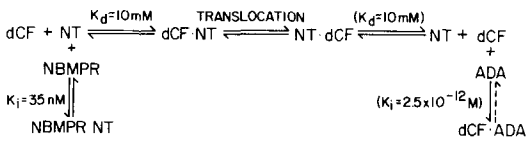

Fig. 10. Proposed interaction of dCF with the nucleoside transporter (NT) and intracellular ADA. dCF enters the erythrocyte via a nucleoside transporter with a low affinity ($K_d \cong 10$ mM). The transport of dCF can be blocked by nucleoside transport inhibitors, NBMPR and dipyridamole, and a permeant, uridine. After translocation, the dCF is released from the NT·dCF complex and associates with intracellular ADA. The free ADA in the cells is measured by the ammonia liberation assay. The dissociation of intracellular ADA is assumed to be extremely slow. The kinetic parameters in parentheses have not been determined experimentally.

The finding of linear dCF uptake rates in this study contradicts the earlier reports [18, 19] of pseudo first-order kinetics for the inactivation of ADA in intact cells. Those reports resulted from the misinterpretations of data derived from measurements made with an imprecise influx stop procedure. The protocol was modified by including a rapid 5-fold dilution of the incubation mixture in ice-cold Standard Medium containing NBMPR at a concentration 100 times higher than its K_i. This procedure not only stops nucleoside transport almost instantly but also permits many more aliquots to be taken during the transport assay. The laboratory of Paterson [32, 33] has shown that NBMPR effectively stops the transport of nucleosides and yields linear initial velocities. The present study shows that a double-reciprocal plot of the rate of ADA inactivation versus dCF concentration gives a straight line that intersects the axes near the origin. These results, and the observation that the erythrocytic ADA was inactivated within 2 sec by 100 μM dCF, demonstrate that the maximal $T_{1/2}$ of 115 sec for ADA inactivation calculated from the earlier studies [19] is invalid.

The studies with uridine and the transport inhibitors also give families of lines on plots of $1/v$ vs 1/dCF that intersect near the origin, suggesting a very high K_m for the association of dCF with the nucleoside transporter. The affinity of dCF for the transporter was estimated by measuring the inhibition of adenosine transport at high concentrations of dCF. The K_i value of 10 mM corrects the previously reported K_m of 0.6 μM [19] and explains why other investigators [22–24] found no inhibition of nucleoside transport by dCF in various cell lines. We have also observed similar low affinities of dCF for the nucleoside transporter in murine Sarcoma 180 cells and in HL-60 and other human leukemic cell lines (unpublished data). Since Lum *et al.* also reported the slow inactivation of ADA in P388 cells after treatment with 1 μM dCF [23], these cells may also have a low affinity transport for dCF.

The NBMPR-stop method entails a prolonged time-lapse before residual ADA activity is measured and provides no information on the rate at which dCF associates with ADA after entering the cell. When the rates of dCF transport measured with the NBMPR-stop and adenosine-stop methods were compared, the rate was about 9% slower with the adenosine-stop method. Since adenosine transport is extremely rapid [20, 21], an intracellular adenosine concentration greater than 400 μM should be achieved in less than 10 sec after adding the adenosine-stop solution. This concentration (about twenty times the K_m of adenosine for ADA) should be sufficient to saturate the catalytic site of ADA and to slow down further inactivation by dCF. Therefore,

the association between intracellular dCF and ADA must not be rate-limiting. The intracellular ADA concentration, based on a cellular volume of 1×10^{-13} liter/cell, a water content of 60%, and other assumptions stated in Results is about 5×10^{-8} M when the ADA activity is 0.3 units/ml packed cells. This ADA concentration is more than 100-fold greater than the concentrations commonly used in kinetic studies with the free enzyme [2] and can account for the very rapid intracellular association with dCF.

Since transport activity can vary considerably among individuals, time points should be selected to give inactivation values in the range of 10–80%. As seen in Table 1, although most individuals had similar transport rates, two donors showed several-fold higher values. Also of interest is the observation that the erythrocytic nucleoside transport activities remained relatively constant in blood samples of the same person taken at different times (Table 1). More detailed studies of a number of donors over a longer time range would be needed before the conclusion could be drawn that nucleoside transport rates are an individual characteristic. In view of the importance of nucleoside transport in the actions of many drugs, this point is worth further study. ADA activities per ml of packed cells also differed among the donors, but no correlation was seen between ADA content and the dCF transport rate. This agrees with the finding that the rate of ADA inactivation was similar in control erythrocytes and in erythrocytes preloaded with a 5-fold excess of calf intestinal ADA (Fig. 5). Thus, it may be concluded that, although the ADA molecules trap the entering dCF molecules, the amount of ADA within the cell does not affect the initial rate of dCF transport.

The low affinity of dCF for the human erythrocytic nucleoside transporter could be a major determinant of the behavior of the drug in animals and humans. In these studies the K_i value of 10 mM for the inhibition of adenosine influx by dCF was about 5×10^3 times higher than the K_m of adenosine (2 μM) and about 2×10^5 times greater than the K_i values of NBMPR and dipyridamole (35 and 45 nM respectively). Also, 98% of the influx of 10 μM dCF was via the nucleoside transporter. Therefore, co-infusion of a competitive permeant or nucleoside transport inhibitor would markedly decrease the rate of inactivation of erythrocytic ADA by dCF. In several experiments with monkeys,* the rates of inactivation of erythrocytic ADA by dCF were decreased markedly by infusions of NBMPR at doses designed to maintain plasma concentrations of $1–10 \times 10^{-5}$ M, i.e. a 20 mg loading dose followed by continuous infusion at a rate of 10 mg/hr. Another consideration is the marked and rapid increases to the micromolar level in plasma 2′-deoxyadenosine concentrations seen in patients treated with dCF [16]. It is possible that the elevated levels of natural endogenous nucleosides in extracellular fluids would inhibit dCF influx into cells.

Although a limited number of tissues and organs have been examined, substantial differences have been observed in nucleoside transport capacity (unpublished observations). Thus, differences in the rate and degree of ADA inhibition by dCF may occur in different tissues [34]. It is possible that local infusion of an inhibitor or a competitive permeant might prevent ADA inactivation in a specific organ. This could be of value in organs such as the liver where the rate of restoration of dCF-inactivated ADA is very slow [35] and which is subject to toxicity from certain adenosine analogs, i.e. formycin and 8-azaadenosine, that are degraded to less toxic inosine analogs by adenosine deaminase.

* Unpublished experiments, this laboratory.

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