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TIGHT BINDING INHIBITORS—VIII

STUDIES OF THE INTERACTIONS OF 2′-DEOXYCOFORMYCIN AND TRANSPORT INHIBITORS WITH THE ERYTHROCYTIC NUCLEOSIDE TRANSPORT SYSTEM*

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Abstract—Studies were performed to extend earlier observations that the rate-limiting step in the inactivation of intraerythrocytic human adenosine deaminase (ADA) by 2′-deoxycoformycin (dCF) is the nucleoside transport system (NTS). The NTS inhibitors 2-amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine (HNBTGR), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine (NBMPR), 2-amino-6-[(4-nitrobenzyl)seleno]-9-β-D-ribofuranosyl purine (NBSeGR), dipyridamole and the competitive permeant, uridine, all decreased the rate of ADA inactivation by dCF in a concentration-dependent manner. Lineweaver–Burk plots of $1/k_\lambda$ (where k_λ is the pseudo first-order rate constant for the inactivation of ADA) 1/dCF concentrations were linear, giving a K_m for dCF for the NTS of 6×10^{-7} M. The maximal k_λ calculated by extrapolation to infinite dCF concentrations was 6×10^{-3} per sec which corresponds to a $T_{\frac{1}{2}}$ of about 115 sec. Similar plots for experiments with the NTS inhibitors and uridine yielded classic patterns of competitive inhibition for NBMPR, HNBTGR, NBSeGR and uridine, whereas with dipyridamole a pattern of non-competitive inhibition was obtained. Dissociation or inhibition constants have been reported for several of these compounds (determined by other methods) and values similar to these were obtained. Inhibition by dipyridamole was non-competitive ($K_I = 2.5 \times 10^{-7}$ M) and was of a bi-phasic nature with respect to time. Dipyridamole caused rapid and irreversible inhibition for the first 7–15 min with slow and progressive but reversible inhibition thereafter. These observations are consistent with the hypothesis that NBMPR, HNBTGR, NBSeGR and uridine interact with the same site on a macromolecular component of the NTS that forms ligands with dCF. The behavior of dipyridamole appears more complex and will require more extensive study.

Interest has been directed to the enzyme adenosine deaminase (ADA)† as a target for inhibitors that might potentiate the action of chemotherapeutic adenosine analogs and also serve as immunosuppressive agents [1–9]. A number of ADA inhibitors of varying potency have been identified, and several have been studied intensively in this laboratory and elsewhere during the past few years. Because of its unusually high potency and specificity for ADA, the antibiotic 2′-deoxycoformycin (dCF, pentostatin, Covidarabine) has received special attention and has shown promise in initial clinical trials [5].

Earlier studies with purified human erythrocytic ADA indicated that dCF has a K_I of about 2.5×10^{-12} M [8]. Direct measurements of the rate of dissociation of the ADA-dCF complex demonstrated a $T_{\frac{1}{2}}$ value of 25–30 hr with a first-order velocity constant of about 6.6×10^{-6} sec^{-1}. Recent studies of the effects of dCF on ADA in erythrocytes and Sarcoma 180 cells, however, gave very different results [9]. The rate of inactivation in intact cells was 300 to 500-fold slower than with purified enzyme, and it was not possible to demonstrate significant reactivation of dCF-inhibited ADA in isolated intact cells by a variety of techniques, e.g. dialysis, repeated washing, and suspension of the cells in hemoglobin-coated charcoal. When dCF-treated erythrocytes were hemolyzed and stirred with charcoal, however, reactivation of the ADA occurred in a manner essentially identical to that found with the purified enzyme. These observations indicated that the intact cellular membrane plays a crucial role in the behavior of dCF. Studies that employed uridine (not metabolizable in erythrocytes) and the nucleoside transport inhibitor, HNBTGR, also offered support for the concept that the nucleoside transport system of the erythrocytic membrane is intimately involved in the transport of dCF into the cell [9]. To test this hypothesis further, experimental approaches and analytical methods were used that are similar to those often employed for the study of enzymes and their interactions with substrates and inhibitors. The results of these investigations are described below. The structures of dCF and some of the nucleoside transport inhibitors studied are shown in Fig. 1. Preliminary reports of these investigations have been presented elsewhere [10–12].

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† Abbreviations used: ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); 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-ribofuranosyl purine; HNBTGR, 2-amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine; NBSeGR, 2-amino-6-[(2-hydroxy-4-nitrobenzyl)seleno]-9-β-D-ribofuranosyl purine; and NTS, facilitated nucleoside transport system of human erythrocytes.

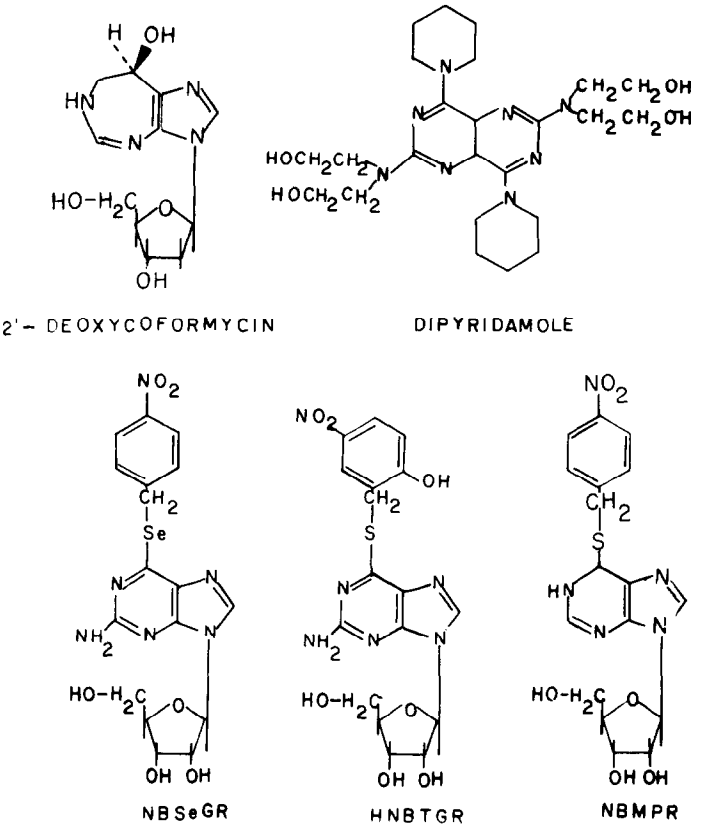

Fig. 1. Structures of the adenosine deaminase inhibitor 2'-deoxycoformycin (dCF), the nucleoside transport inhibitors NBMPR, HNBTGR and NBSeGR, and the vasodilator dipyridamole (Persantine).

MATERIALS AND METHODS

Samples of 2'-deoxycoformycin (dCF, pentostatin, Covidarabine) were provided by Dr. H. W. Dion of Parke, Davis & Co., Detroit, MI [13], and Dr. John Douros of the Drug Development Branch of the National Cancer Institute, Bethesda, MD. The concentrations of solutions of dCF were determined spectrophotometrically (ε_{282} in H_2O = 8 × 10^3) [13]. NBMPR (ε_{290} in H_2O = 2.5 × 10^4) and HNBTGR(ε_{314} in H_2O = 1.9 × 10^4) were gifts from Dr. A. R. P. Paterson of the University of Alberta, Edmonton, Alberta [14]. NBSeGR was synthesized by Dr. Shih-Hsi Chu of Brown University, and dipyridamole ([2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-*d*)pyrimidine] Persantine) was a gift of Dr. Ralph P. Miech. Human erythrocytes were obtained through the Division of Hematological Research, Memorial Hospital, Pawtucket, RI.

Preparation of cells. Human erythrocytes were separated from plasma and buffy coat by washing twice in 0.9% NaCl and were suspended in 1 vol. of Standard Medium (potassium phosphate buffer, 50 mM, pH 7.4; NaCl, 75 mM; $MgCl_2$, 2 mM; glucose, 10 mM; penicillin, 10,000 units/l; and streptomycin, 10 mg/l). After determination of the hematocrit, the cells were diluted to a final suspension of 20% (v/v) by addition of Standard Medium.

Determination of k_λ *with 2'-deoxycoformycin and adenosine deaminase in intact erythrocytes.* As described in an earlier publication, k_λ values were determined from the $T_{\frac{1}{2}}$ of the pseudo first-order decay curves of the inactivation of intraerythrocytic ADA by dCF, through the relationship:

$$k_\lambda = 0.693/T_{\frac{1}{2}}. \quad (1)$$

The units of k_λ are reciprocal time, i.e. sec^{-1}. Because prior studies of NTS inhibitors uncovered several compounds with unusually low dissociation constants [15], it was suspected that they might display the characteristic behavior of semi-tight-binding enzyme inhibitors [8]. Therefore, erythrocytes were preincubated with prospective NTS inhibitors before addition of dCF. Suspensions of human erythrocytes (20%, v/v) were preincubated at 30° in a Dubnoff metabolic incubator for the following time periods: uridine and dipyridamole, 15 min; NBMPR, HNBTGR and NBSeGR, 30 min. After preincubation with the appropriate transport inhibitors or permeants, dCF (usual range 0.5–2.0 μM) was added to initiate the inactivation of intraerythrocytic ADA. At zero time and periodically thereafter, aliquots (0.5 ml) were withdrawn and centrifuged in a clinical centrifuge at 1350 *g* for 30 sec. The supernatant fluids were discarded and the erythrocytic pellets were resuspended to a final volume of 10 ml in cold Standard Medium. The suspensions were centrifuged at 1350 *g* for at least 2 min, the supernatant fluids were removed, and cold distilled water was added to a final volume of 3 ml with stirring on a vortex mixer

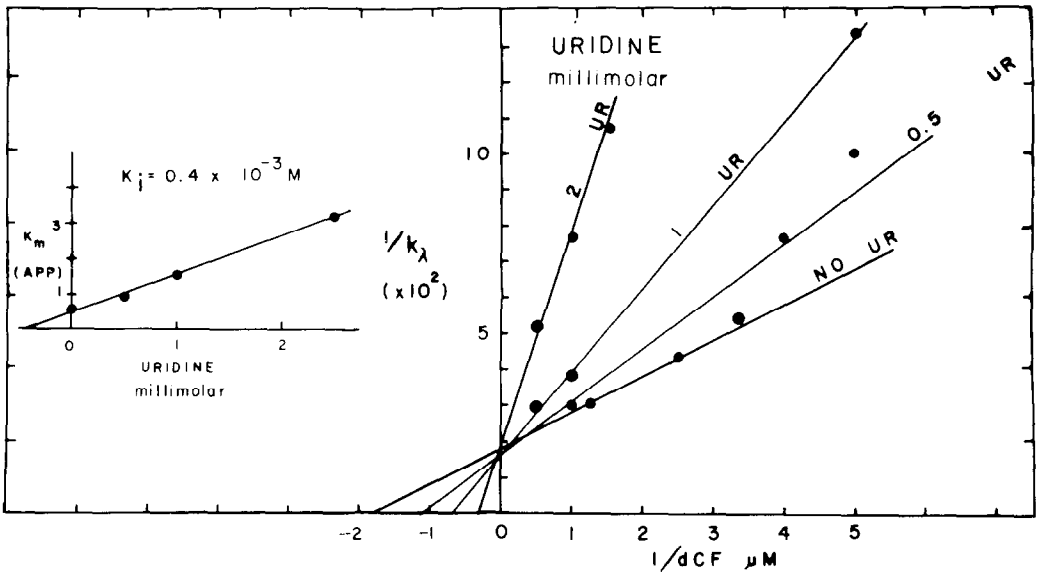

Fig. 2. Determination of the transport inhibition constant K_I for uridine transport in human erythrocytes, utilizing 2'-deoxycoformycin (dCF) as a molecular probe. A 20%(v/v) suspension of human erythrocytes was incubated with uridine for about 15 min at 30°. Transport activity was measured by adding dCF to the suspension at varying concentrations and assaying the intraerythrocytic inactivation of ADA by dCF. Utilizing $1/k_\lambda$ values vs 1/dCF concentration, K_m (App) values were determined. A replot of K_m (App) values gave an inhibition constant (K_I) for the nucleoside permeant uridine of 4×10^{-4} M.

for about 15 sec to promote hemolysis. Hemolysates rather than intact cells were used to assay ADA in studies of the nucleoside transport inhibitors because compounds such as NBMPR interfere with ADA assays in intact erythrocytes [16]. The ADA activities of these hemolysates agreed with those in which isotonicity was restored. The ADA activity of intact cells and hemolysates was measured by the ammonia liberation procedure described previously [9].

RESULTS

Competitive nucleoside transport inhibitors and permeants. Earlier evidence showed that the rate of formation of the dCF–ADA complex in intact erythrocytes is decreased by HNBTGR and uridine [9]. We now have found that the association reaction between dCF and intraerythrocytic ADA can be used as a tool to evaluate the interactions of permeants and inhibitors with the NTS. When human erythrocytes were preincubated with several concentrations of uridine, a know permeant of the NTS [17], the rate of ADA inactivation was decreased in a concentration-dependent manner. Lineweaver–Burk plots of $1/k_\lambda$ (the reciprocals of the pseudo first-order velocity constants) vs 1/dCF gave graphs characteristic of competitive inhibition (Fig. 2). The K_m for dCF binding to the NTS was found to be 6×10^{-7} M. The maximal k_λ value estimated by extrapolation to infinite concentrations of dCF is 6.0×10^{-3} sec^{-1}. This corresponds to a maximal $T_{\frac{1}{2}}$ of 115 sec. As seen in the inset of Fig. 2, the replot of the apparent K_m values [K_m (App)] gave an inhibition constant for uridine of 4×10^{-4} M, a value consistent with the dissociation constant of uridine for the NTS (7×10^{-4} M) as determined by Oliver and Paterson [17] who used different experimental techniques (see Table 1).

As shown in Figs. 3–5, the nucleoside transport inhibitors, NBMPR, HNBTGR and NBSeGR, also

Table 1. Kinetic values of transport inhibitors and permeants

Transport inhibitors	K_I (nM)	Type of interaction	K_d (nM)	Ref.
NBMPR	1.6	Competitive	1	15
HNBTGR	15	Competitive		
NBSeGR	10	Competitive		
Dipyridamole	250	Non-competitive	230	18
Transport permeants	**K_m* (μM)**	**Type of interaction**	**K_d (μM)**	**Ref.**
Uridine	400	Competitive	700	17
2'-Deoxycoformycin	0.6	Competitive		

* For uridine: K_I.

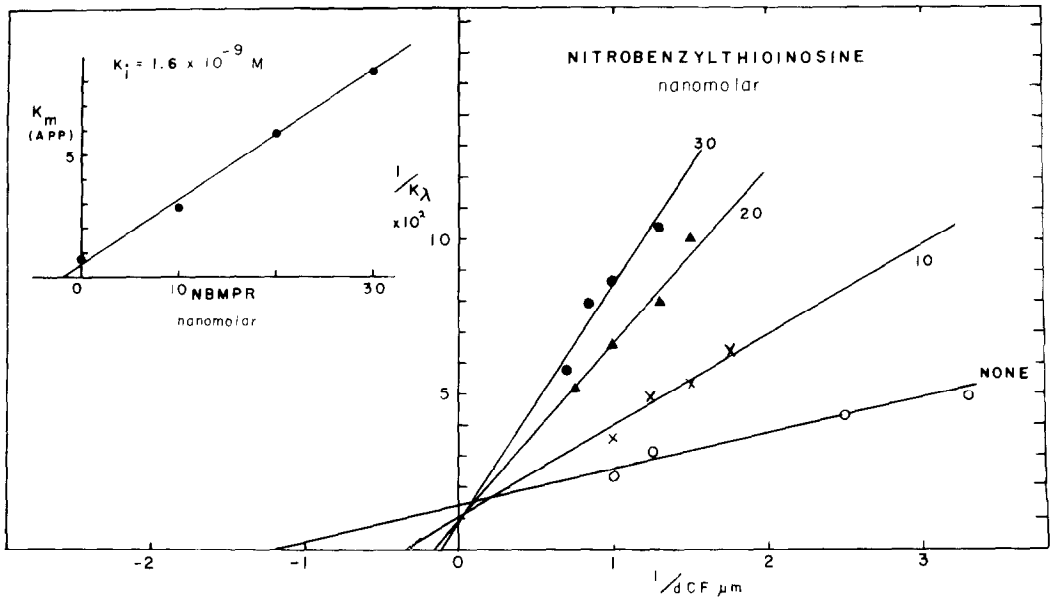

Fig. 3. Determination of the transport inhibition constant K_I for the nucleoside transport inhibitor NBMPR. The experiment was performed as described for Fig. 2, except that NBMPR was incubated with erythrocytes for 30 min in the absence of uridine. A replot of the K_m (App) values gave a K_I value of 1.6×10^{-9} M.

block the inactivation of ADA in intact erythrocytes competitively with dCF. Table 1 presents the K_I values of these three compounds determined by replotting the data as shown in the insets to Figs. 3–5. These values compare favorably with the K_d values determined by the use of other techniques [14, 15, 17, 18]. When uridine or the transport inhibitors were incubated with hemolysate or purified ADA, the rates of inactivation by dCF were unaffected.

Dipyridamole, a non-competitive nucleoside transport inhibitor. When human erythrocytes were preincubated with varying concentrations of dipyridamole for 15 min and the rate of inactivation of ADA was measured at varying concentrations of dCF, Lineweaver–Burk plots gave patterns characteristic of non-competitive inhibition, as shown in Fig. 6. The replot of this data (see inset Fig. 6) gave a K_I value of 2.5×10^{-7} M. It must be emphasized that these results were obtained under specified conditions, i.e. a 15-min preincubation with dipyridamole in Standard Medium at 30°. In experiments in which the time of incubation was varied from 7 min to 6 hr the degree of inhibition of the NTS increased progressively, e.g. with 2.5×10^{-7} M dipyridamole from 60 per cent inhibition to 80 per cent after 6 hr. When

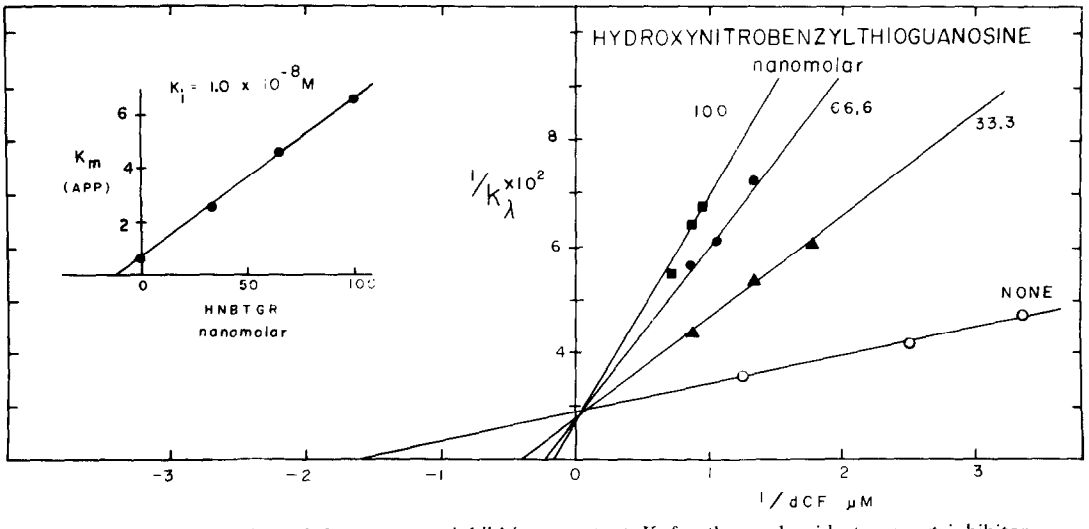

Fig. 4. Determination of the transport inhibition constant K_I for the nucleoside transport inhibitor HNBTGR. The experimental details were as described in Fig. 3. A replot of the K_m (App) values gave a K_I value of 1.0×10^{-8} M.

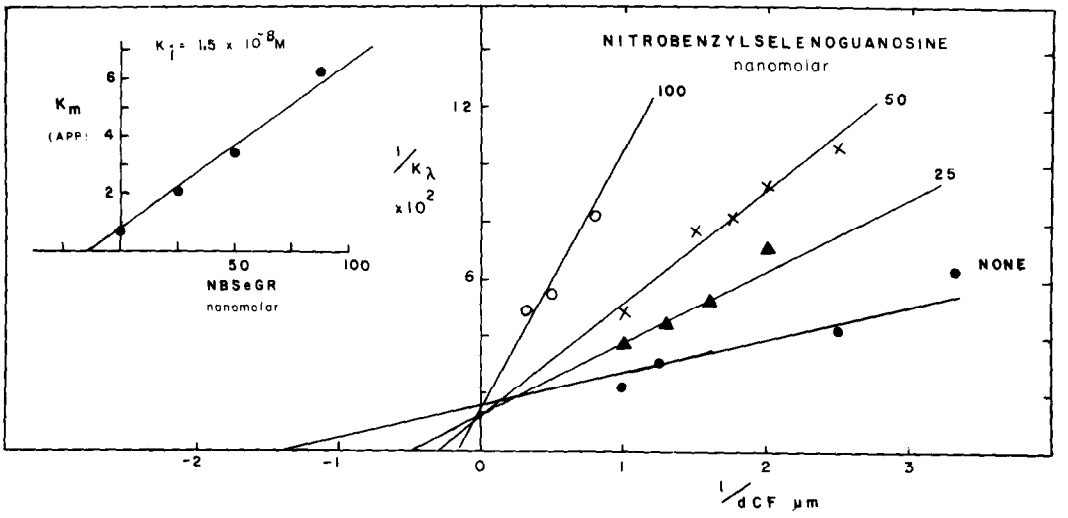

Fig. 5. Determination of the transport inhibition constant K_I for the nucleoside transport inhibitor, NBSeGR. The experiment details were as described in Fig. 3. A replot of the K_m (App) values gave a K_I value of 1.5×10^{-8} M.

cells were preincubated for 6 hr and then washed repeatedly with Standard Medium, the degree of inactivation of the NTS decreased from 80 to 60 per cent, whereas repeated washing did not increase the NTS activity of cells preincubated for 7 min.

DISCUSSION

These studies confirm and extend earlier observations that identified a role for the NTS of the plasma membrane in the inactivation of ADA by dCF in intact erythrocytes [9, 10]. The NTS is relatively non-specific in these cells and interacts with 2'-deoxyribonucleosides and ribonucleosides of both purine or pyrimidine bases [19]. Since the rate of inactivation of intraerythrocytic ADA is 300–500 times slower than with hemolysates or the isolated enzyme, it was proposed that the interaction of dCF with the NTS is the rate-limiting step in the intact cell [9]. Also, the rate of reactivation of dCF-inhibited ADA is so slow, i.e. $T_{\frac{1}{2}} = 25$–30 hr [8] that it may be considered negligible. Furthermore, reac-

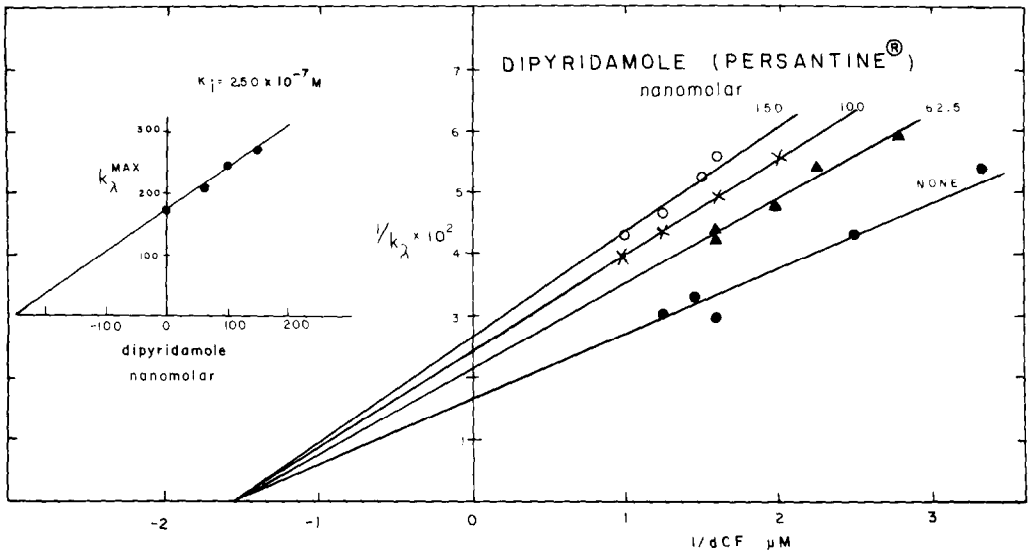

Fig. 6. Determinations of the inhibition constant K_I, by the use of 2'-deoxycoformycin (dCF), for dipyridamole (Persantine) interacting with the nucleoside transport system (NTS) in human erythrocytes. Dipyridamole was added to a 20% (v/v) suspension of human erythrocytes and incubated for 15 min at 30° in a Dubnoff metabolic incubator. Transport activity was measured by adding the appropriate concentration of dCF and measuring the inactivation of intraerythrocytic adenosine deaminase by dCF. These data are plotted as a Lineweaver–Burk plot of $1/k_\lambda$ versus 1/dCF concentration. A replot of the k_λ (max) values versus dipyridamole concentration gave a K_I for dipyridamole transport inhibition of 2.5×10^{-7} M.

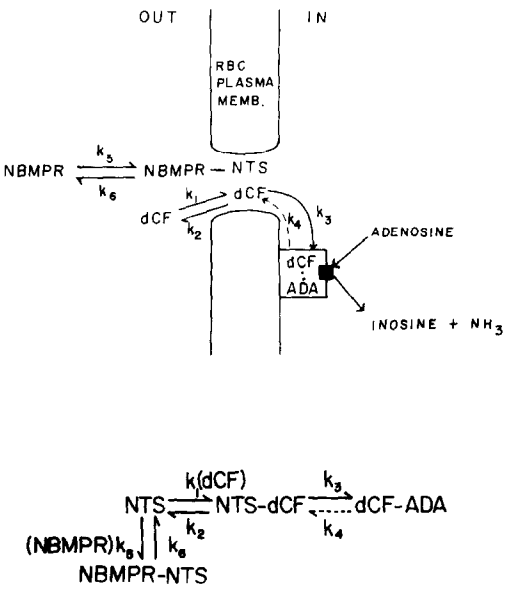

Fig. 7. A molecular and kinetic model depicting the membrane association of the nucleoside transport system and adenosine deaminase, and their interactions with the nucleoside permeant, 2′-deoxycoformycin, and specific nucleoside transport inhibitors (example shown is NBMPR). Broken line represents negligible activity.

tivation has not been detected in intact erythrocytes [9]. Recent studies with rhesus monkeys given dCF indicate that recovery of erythrocytic ADA occurs with a $T_{\frac{1}{2}}$ of about 35 days and probably results from the introduction of new cells into the peripheral blood from the bone marrow [20]. Studies on the incorporation of radiolabeled adenosine into intra-erythrocytic nucleotides in the presence and absence of an NTS inhibitor indicated that ADA is physically associated with the inner surface of the plasma membrane close to the transport site [16]. These observations suggest the working model shown in Fig. 7.

This model assumes a specific interaction of dCF with the plasma membrane-associated NTS, followed by a much faster and irreversible binding to the catalytic center of the membrane-associated ADA. These assumptions are similar to those of the original Michaelis–Menten equation that attempted to explain interactions between substrates and enzymes [21]. The K_m value based on this model closely approximates the dissociation constant (K_d) of the dCF–NTS complex:

$$K_m \simeq K_d = k_2/k_1. \quad (2)$$

In addition, the K_I values of competitive inhibitors such as NBMPR are equal to the K_d of the inhibitor-NTS complex:

$$K_I = K_d = k_6/k_5. \quad (3)$$

In accord with this proposal are the linear Lineweaver–Burk plots of Figs. 2–6. The K_m (or K_d) of dCF is 6×10^{-7} M in contrast to the K_I of dCF with human erythrocytic ADA of 2.5×10^{-12} M [8]. Also consistent is the close approximation of the K_I values of uridine, NBMPR and dipyridamole reported here and the K_d values determined elsewhere by the use of entirely different techniques (Table 1).

On the basis of binding studies with radiolabeled NBMPR, Paterson *et al.* [19] have estimated that there are 1×10^4 nucleoside transport sites per human erythrocyte. Since it has been proposed that ADA is physically associated with the NTS in erythrocytes, it is of interest to estimate the number of molecules of this enzyme per cell. Earlier evidence indicated that this enzyme is monovalent with a molecular weight of 33,000 [1]. The molar equivalency has been estimated to be 1×10^{-10} moles/unit of enzymatic activity [7]. The mean activity of ADA is about 0.3 units/ml of packed cells and 1 ml contains about 1×10^{10} erythrocytes. With the use of these assumptions and Avogadro's number, one may calculate that one unit of ADA consists of 6×10^{13} molecules and that each erythrocyte contains 1.8×10^3 molecules of ADA and 1×10^{-16} g of ADA protein. If these estimations and those of Paterson *et al.* are valid, they indicate a ratio of transporter sites to ADA molecules in the order of 6:1. It is appreciated that phenomena such as the non-specific binding of NBMPR to sites other than the NTS or the degradation of ADA molecules in ageing erythrocytes would result in estimates of a higher number of transport sites or lower activity of ADA per cell than actually occurs. Both factors would bring this ratio closer to unity. In any case it is of interest that these estimated values fall well within one order of magnitude.

Although the NTS has been examined in some detail in human erythrocytes, information is incomplete for other mammalian tissues. Recent studies with HeLa cells indicate the existence of four separate mechanisms for the uptake of nucelosides which increase in activity during G_1 and S phases of the cell cycle [22]. When adequate supplies of the ribonucleoside coformycin become available, it will be of interest to compare its activity with that of dCF as a permeant of various mammalian cells. If differences in NTS specificity occur from one tissue to the next in the interactions of ribonucleosides and 2′-deoxyribonucleosides, this may be detected and analyzed by the use of these tight-binding ADA inhibitors. It may also become possible to design specific inhibitors for the different NTS mechanisms. If such differences occur, they might offer attractive therapeutic opportunities. Thus, it may be possible to inhibit selectively the ADA of particular tissues by the use of either dCF or coformycin or protect specific tissues by use of the appropriate NTS inhibitor.

The results obtained here indicate that dipyridamole behaves differently to the other inhibitors of the NTS, e.g. NBMPR. Studies with various cell systems indicate that dipyridamole can inhibit the transport of anions [23], purine bases, 2-deoxy-D-glucose and D-glucosamine [24]. This indicates a relatively non-specific mechanism of action, perhaps on a component of the plasma membrane that is common to several specific transport mechanisms. The finding of non-competitive inhibition of the NTS by dipyridamole is in accord with this suggestion. On the other hand, it has been reported that dipyridamole can displace ^{14}C-labeled NBMPR from bind-

ing sites on HeLa cells [19], suggesting a more specific mechanism of action. It is possible that dipyridamole is a tight-binding competitive inhibitor of the NTS, but yields Lineweaver–Burk plots characteristic of non-competitive inhibition because of the preincubation periods used in these studies as has been described for the tight-binding competitive inhibitor of ADA, coformycin [7]. Obviously, the action of dipyridamole is complex and will require further study for its elucidation. A topic of immediate interest is the use of methods employed in the above studies to monitor behavior of dipyridamole during clinical use. It may be possible to assess directly the degree of inhibition of the NTS by measuring the rates of inactivation of ADA by dCF in erythrocytes drawn before and during treatment with dipyridamole.

REFERENCES

1. R. P. Agarwal, S. M. Sagar and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 693 (1975).
2. E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet* **2**, 1067 (1972).
3. R. P. Agarwal, S. Cha, G. W. Crabtree and R. E. Parks, Jr., in *Chemistry and Biology of Nucleosides and Nucleotides* (American Chemical Society, Advances in Chemistry Series) (Eds. R. E. Harmon, R. K. Robins and L. B. Townsend), p. 159. Academic Press, New York (1978).
4. M. M. Chassin, R. H. Adamson, D. W. Zaharevitz and D. G. Johns, *Biochem. Pharmac.* **28**, 1849 (1979).
5. J. F. Smyth, M. M. Chassin, K. R. Harrap, R. H. Adamson and D. G. Johns, *Proc. Am. Ass. Cancer Res.* **20**, 47 (1979).
6. W. Plunkett and S. S. Cohen, *Ann. N. Y. Acad. Sci.* **284**, 91 (1977).
7. S. Cha, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 2187 (1975).
8. R. P. Agarwal, T. Spector and R. E. Parks, Jr., *Biochem. Pharmac.* **26**, 359 (1977).
9. T. Rogler-Brown, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **27**, 2289 (1978).
10. T. Rogler-Brown and R. E. Parks, Jr., *Pharmacologist* **20**, 183 (1978).
11. R. P. Agarwal, R. E. Parks, Jr. and T. Rogler-Brown, *Birth Defects Symposium* IX*: Inborn Errors of Specific Immunity*. Academic Press, New York, in press.
12. R. E. Parks, Jr., K. C. Agarwal, R. P. Agarwal, G. W. Crabtree, T. Rogler-Brown, T. M. Savarese and J. D. Stoeckler, in *Biochemical Factors that Influence the Therapeutic Potential of Purine Analogs. Third International Round Table: Nucleosides, Nucleotides and Their Biological Applications* (Eds. J. L. Barascut and J. L. Imbach), INSERM Symposia Series, Vol. 81, pp. 209–238. National Institute of Health and Medical Research, Paris (1978).
13. P. W. K. Woo, H. W. Dion, S. M. Lange, L. F. Dahl and L. J. Durham, *J. heterocyclic Chem.* **11**, 641 (1974).
14. B. Paul, M. Chen and A. R. P. Paterson, *J. med. Chem.* **18**, 968 (1975).
15. A. R. P. Paterson, S. R. Naik and C. E. Cass, *Molec. Pharmac.* **13**, 1014 (1977).
16. R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 547 (1975).
17. J. M. Oliver and A. R. P. Paterson, *Can. J. Biochem.* **49**, 262 (1971).
18. K. Turnheim, B. Plank and N. Kolassa, *Biochem. Pharmac.* **27**, 2191 (1978).
19. A. R. P. Paterson, in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Eds. H. P. Baer and G. I. Drummond), p. 305. Raven Press, New York (1979).
20. T. Rogler-Brown, J. A. Widness, K. C. Agarwal and R. Schwartz, *Proc. Am. Ass. Cancer Res.* **20**, 85 (1979).
21. L. Michaelis and L. Menten, *Biochem. Z.* **49**, 333 (1913).
22. C. E. Cass, E. Dahlig, E. Y. Lau, T. P. Lynch and A. R. P. Paterson, *Cancer Res.* **39**, 1245 (1979).
23. E. Gerlach, B. Deuticke and J. Duhm, *Pflügers Arch. ges. Physiol.* **280**, 243 (1964).
24. P. G. W. Plagemann and D. P. Richey, *Biochim. biophys. Acta* **344**, 263 (1974).