Biochemical Pharmacology, Vol. 29, pp. 187–193.

0006-2952/80/0115-0187 $02.00/0

IN VIVO INHIBITION OF ADENOSINE DEAMINASE BY 2′-DEOXYCOFORMYCIN IN MOUSE BLOOD AND LEUKEMIA L1210 CELLS*

RAM P. AGARWAL

Section of Medical Oncology, The Evans Memorial Department of Clinical Research and the Departments of Medicine and Pharmacology, Boston University Medical Center, Boston, MA 02118, U.S.A.

(*Received* 18 *May* 1979; *accepted* 29 *June* 1979)

Abstract— Adenosine deaminase (ADA) activities in mouse whole blood, washed erythrocytes and L1210 cells were 0.48, 0.93 and 4.76 units/ml respectively. Methods were developed to determine the second-order association rate constant (k_1) of a tight-binding ADA inhibitor, deoxycoformycin (DCF), and ADA in mouse blood and L1210 cells *in vivo*. After i.v. injection of DCF, the inhibition of the enzyme was of a monophasic pseudo-first-order nature in blood and biphasic (with an initial lag of 3–5 min) in L1210 cells. In contrast, i.p. injection of DCF produced the opposite pattern, monophasic in L1210 cells and biphasic in blood. The apparent k_1 values determined from the linear portions of these curves were compared with the k_1 values obtained *in vitro*. The mean k_1 values *in vivo* were: 4.2×10^4 and 1.4×10^4 M^{-1} sec^{-1} in blood after i.v. and i.p. injections, respectively, and 2.6×10^3 and 2.2×10^4 M^{-1} sec^{-1} in L1210 cells after i.v. and i.p. injections respectively. The k_1 values with either whole blood or L1210 *in vitro* (3.1×10^4 and 5.5×10^3 M^{-1} sec^{-1}, respectively) were of the same order of magnitude as those obtained with these tissues *in vivo*. In contrast, the k_1 values were about 150 to 1400-fold higher when either blood hemolysates (4.8×10^6 M^{-1} sec^{-1}) or homogenized L1210 cells (7.5×10^6 M^{-1} sec^{-1}) were used. The 150 to 1400-fold higher k_1 values for blood hemolysates and homogenized L1210 cells than for intact cell samples (whole blood or whole L1210 ascitic fluid) suggest that the cell membrane plays a role in the interaction of DCF and ADA in these cell lines. The similarity of the rates of association of DCF and ADA *in vivo* and *in vitro* for mouse blood and ascites L1210 cells suggests that data obtained *in vitro* may be used to estimate the k_1 values in *in vivo* conditions.

Deoxycoformycin (DCF)† is a tight-binding inhibitor of adenosine deaminase (ADA) with a K_i value of 2.5×10^{-12} M [1]. The theoretical approaches of Cha and our studies [1–4] with partially purified human erythrocytic ADA suggested that the association of a tight-binding inhibitor and the enzyme occurs slowly and that it is therefore possible to measure directly the second-order velocity constant of the association reaction (k_1) between ADA and DCF. The k_1 value determined with partially purified human erythrocytic ADA was 2.5×10^6 M^{-1} sec^{-1} [1]. Similar values (1.4×10^6 M^{-1} sec^{-1}) were found with hemolyzed human erythrocytes [5]. However, k_1 values were much lower with intact human erythrocytes *in vitro*, suggesting that the human erythrocytic membrane interferes with the inhibition of ADA by DCF. Studies in which a nucleoside transport-inhibitor was used further supported this hypothesis and provided evidence that DCF shares the transport system used by other nucleosides in this tissue [5, 6].

Reports from several laboratories have demonstrated that the presence of DCF or another ADA inhibitor markedly increased the incorporation of adenosine analogs into the intracellular nucleotide pools and enhances their chemotherapeutic effectiveness [7–13]. These findings have generated a surge of interest in DCF as a chemotherapeutic tool and the drug may soon be available for Phase I clinical trials. Therefore, it is important to examine the effects of DCF on ADA *in vivo*. This paper describes a study of this nature and examines the association rate constants of DCF and ADA in mouse blood and L1210 cells *in vivo*. The k_1 values determined *in vivo* are compared with those obtained *in vitro*. Preliminary reports of these studies have been presented [14, 15].

MATERIALS AND METHODS

Deoxycoformycin was provided by the Drug Development Branch, Developmental Therapeutic Program, Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD.

Collection and preparation of cells

Blood was collected in heparinized (sodium salt) capillary tubes by retro-orbital puncture of CDF mice. The blood either was used directly or the plasma and erythrocytes were separated by centrifugation at 600 *g* for 5 min. The plasma was collected and the buffy coat was removed carefully and discarded, then the erythrocytes were suspended in an equal volume of the 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; streptomycin, 10 mg/l; and heparin, 50

* This work was supported by American Cancer Society Grant IN-97B, and National Cancer Institute Grant CA 18837.

† Abbreviations used are: ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); and DCF, 2′-deoxycoformycin (Covidarabin or NSC 218321).

units/ml) and the hematocrit was determined. These cell suspensions were used either directly or after hemolysate preparation. The hemolysates were prepared by adding 4 vol. of 5 mM potassium phosphate buffer (pH 7.4) to the erythrocytic pellet obtained by centrifugation. The pH and salt concentrations of the mixtures were adjusted to conditions identical with those used for intact erythrocytes by adding 1 vol of 10-fold concentrated standard medium to 9 vol. of hemolysate. The hemolysate was centrifuged at 18,000 *g* for 30 min and the supernatant fluid was used for ADA activity measurement.

L1210 leukemia cells were maintained in CDF mice by i.p. injection of 10^5 cells. On day 6, ascitic fluid withdrawn directly from the peritoneal cavity was used for cell counts and for determination of ADA activity in whole ascitic fluid and in cell-free ascitic fluid. Where washed intact L1210 cells and their homogenates were used, the cells were harvested by rinsing the peritoneal cavity with Hanks' balanced salt solution and then washed twice with normal saline. Contaminating erythrocytes, when present, were removed by hypotonic shock as described earlier [16]. The cells were suspended in about 1 vol. of normal saline, and the hematocrit and the cell number (by hemacytometer) were determined. These cell suspensions were used either directly or after homogenization for determination of ADA activity as described below. L1210 homogenates were prepared by grinding cells for 2 min. in a glass homogenizer. The supernatant fluid obtained after centrifugation at 12,000 r.p.m. for 30 min was used for determination of ADA.

All preparations were maintained on ice during processing, unless mentioned otherwise.

Determination of adenosine deaminase activity

Adenosine deaminase activity in whole blood, washed intact erythrocytes, hemolysates, blood plasma, whole ascitic fluid, washed L1210 cells, cell-free ascitic fluid and cell homogenates was determined by an ammonia liberation method essentially as described earlier [5] except that the reactions were carried out at 37°. The activity in the L1210 homogenates was also determined spectrophotometrically at 265 nm [17]. Protein concentrations were determined by the method of Lowry *et al.* [18].

One unit of the enzyme activity is defined as the amount of enzyme catalyzing the deamination of 1 μmole adenosine/min.

Determination of the association rate constant (k_1) *of deoxycoformycin and adenosine deaminase*

Studies in vitro. For the determination of k_1 values in blood hemolysates and L1210 cell homogenates, blood from ten to fifteen mice was collected in heparinized tubes and was pooled. In experiments where washed erythrocytic hemolysates were used, the erythrocytes were collected by centrifugation and were hemolyzed by adding 24 vol. of 5 mM potassium phosphate (pH 7.4) to 1 vol. of packed cells. Whole blood hemolysate was prepared by adding 12 vol. of 5 mM potassium phosphate buffer to 1 vol. of whole blood. The cell debris was removed by centrifugation at 18,000 *g* for 30 min and the supernatant fluid (corresponding to a 4 per cent concentration of packed cells) was used for k_1 determination.

Hemolysates (4.3 ml) were incubated at 37°. An aliquot of 0.5 ml was withdrawn for determination of ADA activity (0 time, 100 per cent). To the remaining hemolysate (3.8 ml), deoxycoformycin solution (10–20 μl), was added to obtain final concentrations of DCF of 1.34 to 5.40 nM. After incubation with DCF for various time intervals at 37°, aliquots were withdrawn for determinations of remaining ADA activity by the NH_3 liberation method.

Supernatant fluid from L1210 cell homogenates (10 μl) and DCF (0 to 0.82 nM) were incubated in 50 mM phosphate buffer, pH 7.4 (total volume 0.99 ml), in a spectrophotometric cuvette. After incubation at 37° for various time intervals, the activity was determined by measuring the decrease in absorbance at 265 nm after the addition of 10 μl of 10 mM adenosine (final concentration, 0.1 mM), as described earlier [17].

For the determination of k_1 values in whole blood and whole ascitic fluid heparinized mouse whole blood was collected by retro-orbital puncture, and L1210 ascitic fluid (from a day 6 mouse) was collected by making a small peritoneal incision. Whole blood or ascitic fluid (180 μl) was incubated at 37° with DCF solution (20 μl) to obtain a final DCF concentrations of 0–130 nM. Twenty μl samples were withdrawn at various time intervals after incubation at 37°, and the remaining ADA activity was determined by adding the aliquots to the standard medium (total volume 1.5 ml) containing 1 mM adenosine. The k_1 values were calculated from $T_{\frac{1}{2}}$ values (time taken for 50 per cent inhibition) from the following relationship [2]:

$$k_1 = \frac{0.693}{(T_{\frac{1}{2}})\,(I)}$$

where I is the DCF concentration.

Studies in vivo. Various concentrations of DCF solution in isotonic saline (0.2 ml) were injected either i.v. in the tail vein or i.p. into day 6 mice. The k_1 values in blood were determined in non-tumor-bearing mice. The tissue concentration of DCF was estimated by assuming uniform distribution to a volume equivalent to 60 per cent of the body weight.

Blood and ascitic fluid were collected at time intervals after DCF injection, and the remaining ADA activity was determined. The blood or ascitic fluid taken before DCF injection served as controls. The blood (20 μl) was collected in heparinized graduated tubes by retro-orbital puncture, whereas ascitic fluid (10 μl) was collected directly from the peritoneal cavity using a 22-gauge needle.

RESULTS

Adenosine deaminase activity in mouse blood and L1210 *ascitic fluid*

Tables 1 and 2 present ADA activities in various components of mouse blood and L1210 ascitic fluid. The ADA activity in whole blood (hematocrit 51 per cent) was 0.48 units/ml of whole blood, while in the washed erythrocytes it was 0.95 units/ml of packed cells. Plasma had very low activity (0.005 units/ml

Table 1. Adenosine deaminase activity in mouse blood

Tissues	Activity
Blood	
Washed erythrocytes	0.93* ± 0.15† (4)
Washed erythrocytic hemolysate	0.88* ± 0.10 (15)
Whole blood	0.48† ± 0.07 (15)
Whole blood-hemolyzed	0.48† ± 0.12 (23)
Blood plasma	0.005§ ± 0.006 (2)

* Units/ml of packed cells.
† Mean ± S.D.; number of estimations is given in parentheses.
‡ Units/ml of whole blood.
§ Units/ml.

of plasma). The activity in the hemolyzed blood was similar to that of the non-hemolyzed blood (Table 1).

Table 2 presents the ADA activity and L1210 cell concentration in day 6 ascitic fluid. The mean ADA activity in washed L1210 cells was 4.8 units/ml of packed cells, while that in cell-free ascitic fluid was 0.06 units/ml. Based on these values and a packed cell volume of 18.77 per cent, the calculated ADA activity in whole ascitic fluid should have been 0.95 units/ml of ascitic fluid. This value is within one standard deviation of the observed mean value of 1.08 units/ml of whole ascitic fluid (Table 2). The data above indicate that the ADA activity in whole blood and whole ascitic fluid represented activity due principally to erythrocytes and L1210 cells, respectively, and that the presence of plasma or cell-free ascitic fluid made an insignificant contribution (less than 5 per cent) to the total activity. Therefore, whole blood or whole ascitic fluid was used in the following studies, unless mentioned otherwise.

Association rate constant (k_1) *of deoxycoformycin and adenosine deaminase in mouse blood and L1210 ascitic fluid*

Studies in vitro. Figure 1 presents semilogarithmic plots of the remaining ADA activity after incubation of mouse blood with DCF at various concentrations. The patterns were similar with hemolysates, whole

Table 2. Adenosine deaminase activity and the concentration of L1210 cells in day 6 ascitic fluid

Packed cell volume (%)	18.77 ± 1.37* (9)
L1210 cells/ml whole ascitic fluid	2.49×10^8 ± 0.36 (13)
Number of cells/ml packed cells†	1.32×10^9
ADA activity	
Whole ascitic fluid	1.08‡ ± 0.18 (25)
Washed L1210 cells	4.76§ ± 0.59 (3)
Cell-free ascitic fluid	0.063‡ ± 0.004 (3)
L1210 cell homogenate	4.87§ ± 0.23 (2)

* Mean ± S.D.; number of determinations is given in parentheses.
† Calculated value.
‡ Units/ml.
§ Units/ml of packed cells.

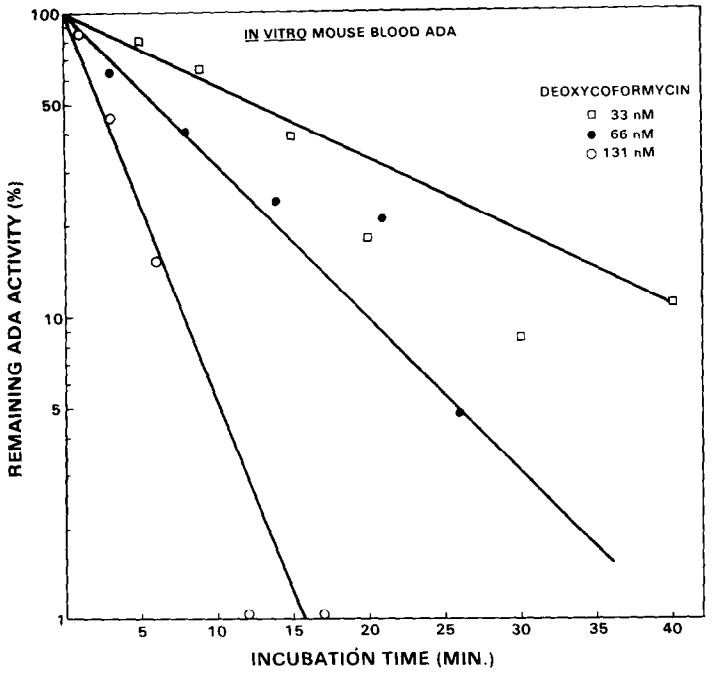

Fig. 1. *In vitro* determination of the association velocity constant (k_1) of deoxycoformycin and adenosine deaminase in mouse blood. See Materials and Methods for details.

Table 3. Association rate constants (k_1) of deoxycoformycin and adenosine deaminase in mouse blood and L1210 cells

Tissues	k_1 (M^{-1} sec^{-1})
Blood	
In vitro whole blood	$3.1 \times 10^4 \pm 0.7$* (3)
hemolysate	$4.8 \times 10^6 \pm 1.4$ (4)
In vivo whole blood (DCF, i.v.)	$4.2 \times 10^4 \pm 3.4$ (4)
whole blood (DCF, i.p)	$1.4 \times 10^4 \pm 0.7$ (3)
L1210 cells	
In vitro whole ascitic fluid	$5.5 \times 10^3 \pm 0.6$ (4)
L1210 cell homogenate	$7.5 \times 10^6 \pm 1.3$ (4)
In vivo whole ascitic fluid (DCF, i.v.)	$2.6 \times 10^3 \pm 0.4$ (5)
whole ascitic fluid (DCF, i.p.)	$2.2 \times 10^4 \pm 0.5$ (4)

* Mean ± S.D.; number of determinations is given in parentheses.

ascitic fluid, and L1210 cell homogenates. From $T_{\frac{1}{2}}$ values, the k_1 values were estimated as presented in Table 3. The k_1 values for whole blood and whole ascitic fluid, *in vitro*, were similar, i.e. 3.1×10^4 and 5.5×10^3 M^{-1} sec^{-1}, respectively. In contrast, the k_1 values were much higher for hemolysate (4.8×10^6 M^{-1} sec^{-1}) and L1210 cell homogenates (7.5×10^6 M^{-1} sec^{-1}). The 150–1400-fold increase in k_1 values with hemolysates and L1210 cell homogenates above that found in the samples containing intact cells (whole blood and whole ascitic fluid) suggests that the cell membrane interferes in the interaction of DCF and ADA in these tissues.

Studies in vivo. *In vivo* inhibition patterns of mouse blood ADA after i.v. and i.p. injections of DCF are presented in Fig. 2 and 3. After i.v. injections of DCF, the inhibition was linear, with a mean apparent k_1 value of 4.2×10^4 M^{-1} sec^{-1}. On the other hand, i.p. DCF injection produced a biphasic inhibition curve (Fig. 3) in blood. After an initial lag of about 2–5 min, inhibition was linear. The mean k_1 value estimated from the linear portions of these curves was 1.4×10^4 M^{-1} sec^{-1}. It appears from these data that the drug distribution was rapid in blood after i.v. injection, whereas 2–5 min were required for distribution to erythrocytes after i.p. injection. In contrast, the inhibition patterns were reversed with L1210 ascitic fluid, i.e. biphasic after i.v. injections (Fig. 4) and monophasic after i.p. injection (Fig. 5), suggesting that drug distribution to ascitic fluid was more rapid after i.p. injection than after i.v. injection. The mean k_1 values were estimated to be 2.6×10^3 and 2.2×10^4 M^{-1} sec^{-1} respectively. It is interesting to note that, with intact cells of either tissue type, the k_1 values obtained *in vitro* and *in vivo* did not differ by more than a factor of 10.

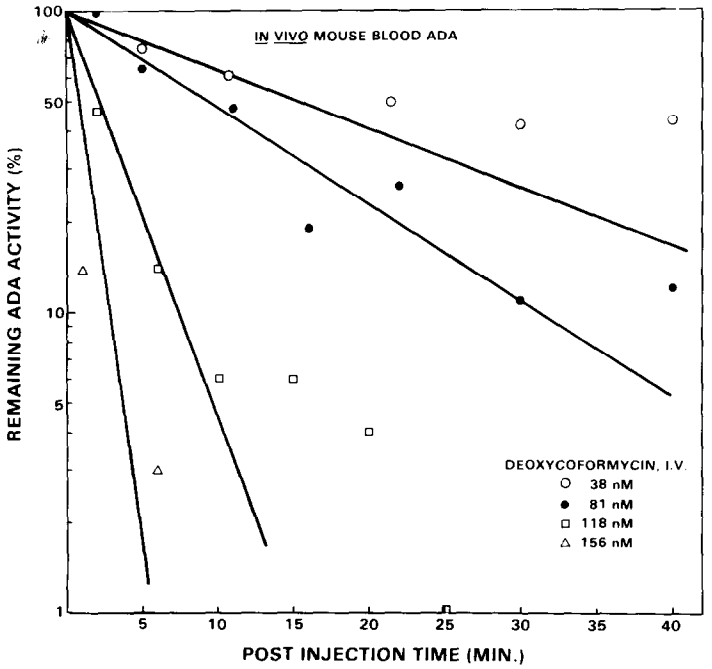

Fig. 2. *In vivo* determination of the association velocity constant (k_1) of adenosine deaminase in mouse blood and deoxycoformycin given by i.v. injection. One animal was used for each concentration of DCF in all *in vivo* experiments. See Materials and Methods for details.

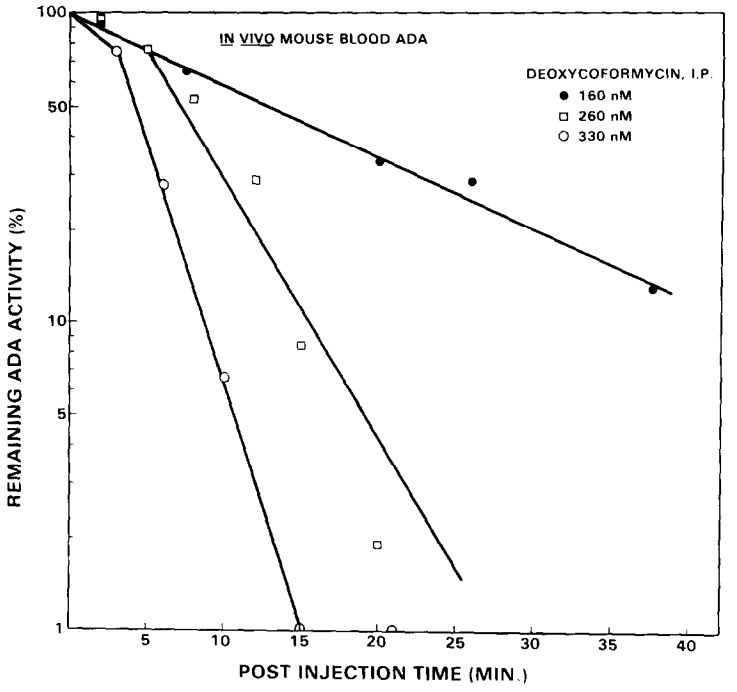

Fig. 3. *In vivo* determination of the association velocity constant (k_1) of adenosine deaminase in mouse blood and deoxycoformycin given by i.p. injection. See Materials and Methods for details.

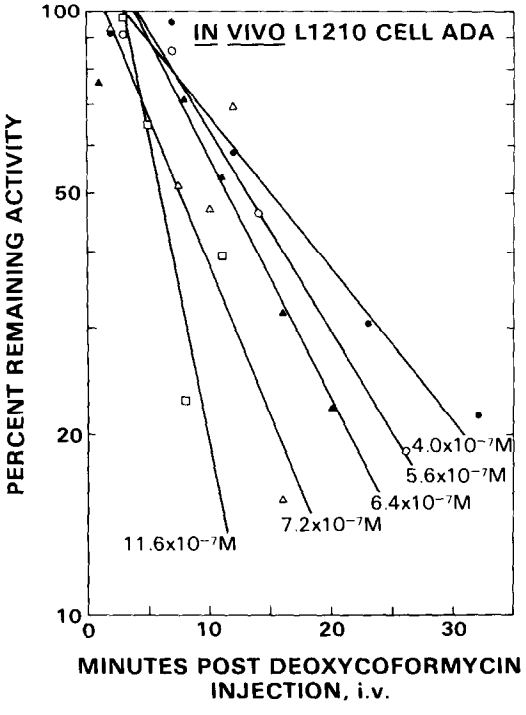

Fig. 4. *In vivo* determination of the association velocity constant (k_1) of adenosine deaminase in L1210 ascitic fluid and deoxycoformycin given by i.v. injection. See Materials and Methods for details.

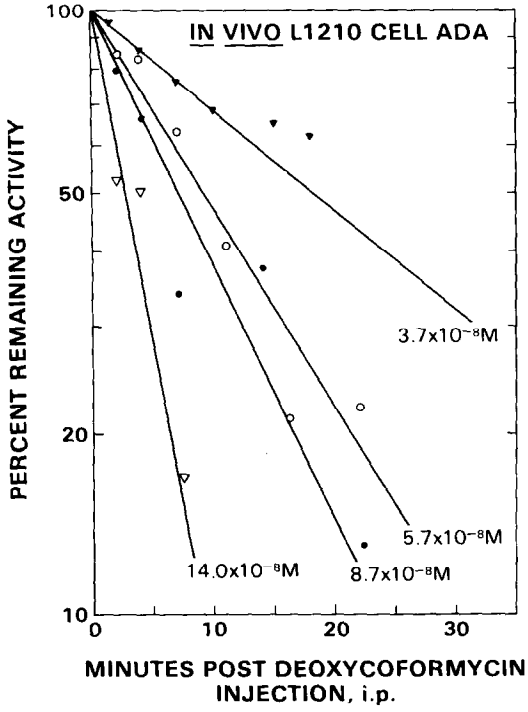

Fig. 5. *In vivo* determination of the association velocity constant (k_1) of adenosine deaminase in L1210 ascitic fluid and deoxycoformycin given by i.p. injection. See Materials and Methods for details.

DISCUSSION

Earlier we reported *in vitro* determination of the second-order association rate constant (k_1) of DCF and ADA in intact and hemolyzed human erythrocytes [5]. This paper describes methods for *in vivo* determination of k_1 values in mouse blood and L1210 ascitic fluid. In this method, the blood or L1210 cell ADA activity was monitored serially before and after DCF injection. Thus, each animal served as its own control. Comparisons of k_1 values estimated with hemolysates, homogenates, and intact cells revealed that the cell membrane plays a role in the interaction of DCF and ADA in these tissues.

Several unique properties of the animal model used made these studies possible. These are: relatively high ADA activity in erythrocytes and L1210 cells, compared to low activity and lack of interference from the plasma and cell-free ascitic fluid; ease with which the blood and ascitic fluid could be withdrawn in small volumes for serial activity measurement from a single animal; and an assay method that allowed use of whole blood for whole ascitic fluid for the determination of ADA.

In both tissue types, the k_1 values obtained *in vitro* and *in vivo* were similar. However, the inhibition patterns *in vivo* differed for different routes of administration. As expected, the distribution of DCF was rapid in blood after i.v. injection and in L1210 ascitic fluid after i.p. injection, producing linear inhibition patterns (Fig. 2 and 5). On the other hand, 2–5 min were required for distribution of DCF to erythrocytes after i.p. injection and to L1210 cells after i.v. injection, as is evident from the lag period prior to linear inhibition (Fig. 3 and 4). These differences in drug distribution time are also reflected in small differences between the k_1 values estimated after i.v. and i.p. injection. For example, the k_1 values in blood were about 3-fold higher after i.v. injection ($4.2 \times 10^4\ M^{-1}\ sec^{-1}$) than after i.p. injection ($1.4 \times 10^4\ M^{-1}\ sec^{-1}$), whereas in L1210 cells the values after i.p. injection were 10-fold higher ($2.2 \times 10^4\ M^{-1}\ sec^{-1}$) than after i.v. injection ($2.6 \times 10^3\ M^{-1}\ sec^{-1}$). The k_1 values presented here should be regarded as approximate rather than exact values. These values were estimated assuming rapid distribution of DCF to a volume equivalent to 60 per cent of the body weight. This assumed volume of distribution falls within the range of values (54–74 per cent) reported recently for deoxycoformycin in BDF mice [19]. The $T_{\frac{1}{2}}$ values for the α- and β-phase of elimination of DCF from mouse plasma after i.v. injection are 10.4 and 33.2 min respectively [19]. Therefore, the deviation from linearity seen in Fig. 2 may be attributable to changes in plasma DCF concentrations at longer time points. However, the similarity of the *in vivo* estimated k_1 values to those determined *in vitro* indicates that one can estimate these values *in vivo* with a reasonable degree of accuracy.

In hemolysates and L1210 cell homogenates, the k_1 values were, respectively, 140- and 1500-fold higher than those found in the corresponding intact cells. These observations are similar to those obtained with hemolyzed and intact human erythrocytes *in vitro* [5] and further emphasize the role of the cell membrane in the interaction of DCF and ADA in all tissues studied. In the studies with human erythrocytes, it was demonstrated that DCF shares the transport system used by other nucleosides and that its entry into cells can be affected by specific transport inhibitors [5, 6]. The lower k_1 values obtained with intact mouse erythrocytes and L1210 cells may be due to the involvement of such a transport mechanism. This transport system has been demonstrated in many cell types including mouse erythrocytes and L1210 cells [20, 21]. Another possible hypothesis has yet to be explored with mouse erythrocytes and L1210 cells: ADA might be physically associated with the membrane in intact cells, as was postulated for human erythrocytes [22]. If this is true, the associated enzyme might have a conformation different from the free enzyme. Such a conformational difference might affect the interaction with DCF.

It is interesting to note that all the tissues examined—mouse blood, L1210 cells (presented here) and Sarcoma-180 cells and human erythrocytes [5]—have similar k_1 values. This indicates that the enzyme in these various tissues may be similar. If this were the case, then one would expect the rates of dissociation of the ADA–DCF (enzyme–inhibitor) complex also to be similar. However, we have reported recently that the *in vivo* recovery of the DCF-inhibited enzyme in mouse blood and L1210 cells is markedly different [16]. The *in vivo* recovery of DCF-inhibited enzyme also differs in other mouse tissues, such as spleen, liver and intestine (unpublished results). The differences in recovery observed suggest that the apparent recovery of the enzymic activity in individual tissues will depend on factors other than the enzyme dissociation constant, such as cell proliferation rate and the rate of new enzyme synthesis. Differences among tissues in the rate of recovery of the enzyme from this tight-binding inhibitor is a factor to be considered in the future chemotherapeutic trials of DCF. Of important clinical application is the similarity of the k_1 values determined *in vivo* and *in vitro* for both tissues (blood and L1210 cells). This suggests that data obtained *in vitro* may be used to estimate the k_1 values in *in vivo* conditions.

Acknowledgements—I wish to thank Michael Tranfaglia and Ruth Craig for their excellent technical assistance.

REFERENCES

1. R. P. Agarwal, T. Spector and R. E. Parks, Jr., *Biochem. Pharmac.* **26**, 359 (1977).
2. S. Cha, *Biochem. Pharmac.* **24**, 2177 (1975).
3. S. Cha, *Biochem. Pharmac.* **25**, 2695 (1976).
4. S. Cha, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 2187 (1975).
5. T. Rogler-Brown, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **27**, 2289 (1978).
6. T. Rogler-Brown and R. E. Parks, Jr., *Pharmacologist* **20**, 200 (1978).
7. R. P. Agarwal, G. W. Crabtree, R. E. Parks, Jr., J. A. Nelson, R. Keightley, R. Parkman, F. S. Rosen, R. C. Stern and S. H. Polmar, *J. clin. Invest.* **57**, 1025 (1976).

8. R. P. Agarwal, S. Cha, G. W. Crabtree and R. E. Parks, Jr., in *Chemistry and Biology of Nucleosides and Nucleotides* (Eds. R. E. Harmon, R. K. Robins and L. B. Townsend) p. 159. Academic Press, New York (1978).
9. N. Caron, S. H. Lee and A. P. Kimball, *Cancer Res.* **37**, 3274 (1977).
10. C. E. Cass and T. H. Au-Yeung, *Cancer Res.* **36**, 1486 (1976).
11. S. S. Cohen, *Med. Biol.* **54**, 299 (1976).
12. D. Johns and R. H. Adamson, *Biochem. Pharmac.* **25**, 1441 (1976).
13. G. A. LePage, L. W. Worth and A. P. Kimball, *Cancer Res.* **36**, 1481 (1976).
14. R. P. Agarwal, *Fedn. Proc.* **37**, 1541 (1978).
15. R. P. Agarwal, *Proc. Am. Ass. Cancer Res.* **20**, 12 (1979).
16. R. P. Agarwal, *Cancer Res.* **39**, 1425 (1979).
17. R. P. Agarwal, S. M. Sagar and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 693 (1975).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. W. R. McConnell, R. L. Furner and D. L. Hill, *Drug Metab. Dispos.* **7**, 11 (1979).
20. C. E. Cass and A. R. P. Paterson, *Biochem. Pharmac.* **24**, 1989 (1975).
21. C. E. Cass, T. H. Tan and M. Selner, *Cancer Res.* **39**, 1563 (1979).
22. R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 547 (1975).