Biochemical Pharmacology, Vol. 35, No. 8, pp. 1325–1330, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Press Ltd.

HYDROXYLATION OF 4-AMINO-ANTIFOLATES BY PARTIALLY PURIFIED ALDEHYDE OXIDASE FROM RABBIT LIVER*

GERARD FABRE†, RICHARD SEITHER‡ and I. DAVID GOLDMAN§

Departments of Medicine and Pharmacology, Medical College of Virginia, Richmond, VA 23298, U.S.A.

(*Received* 19 *November* 1984; *accepted* 26 *September* 1985)

Abstract—This paper explores the interaction between 4-amino-antifolates and aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1) that was purified 60- to 120-fold from rabbit liver with yields of 5–15%. The purification procedure consisted of one heat and two ammonium sulfate precipitations followed by chromatography on hydroxylapatite and then Sephacryl S-200. Analysis of initial rates of hydroxylation of methotrexate, aminopterin and dichloromethotrexate indicated an order of affinities of dichloromethotrexate (10 μM) > methotrexate (35 μM) > aminopterin (272 μM). There was no difference in the V_{max} of methotrexate and dichloromethotrexate (248 and 231 nmoles/min/mg protein respectively); aminopterin (130 nmoles/min/mg protein) was less than that of the other two. The V_{max}/K_m ratios were 24.1, 7.20 and 0.48 for dichloromethotrexate, methotrexate and aminopterin respectively. This enzyme preparation also mediated the hydroxylation of methotrexate polyglutamyl derivatives with a decrease in the rates of hydroxylation, as the total number of glutamyl residues was increased to four, a consequence of a marked increase in K_m values and/or decrease in V_{max}; the ratios of the V_{max}/K_m for the di-, tri-, and tetraglutamates were 0.94, 0.31 and 0.21 respectively. This low activity of the polyglutamyl derivatives of methotrexate for aldehyde oxidase is consistent with the observations that the predominant forms of 4-amino-antifolate polyglutamates found in human liver after administration of methotrexate are the polyglutamyl derivatives of the parent compound. Finally, substrate inhibition for methotrexate and dichloromethotrexate was observed at concentrations in excess of 150 and 30 μM, respectively, about 5- and 3-fold higher than their respective K_m values. Hence, while dichloromethotrexate had the lowest K_m for aldehyde oxidase amongst the 4-amino-antifolates studied, the actual rates of hydroxylation depended upon the concentration employed because of substrate inhibition. Aminopterin was a very poor substrate for this enzyme at low and saturating concentrations. These properties of the hydroxylation of 4-amino-antifolates may be of importance in the design of clinical regimens with these agents—in particular, regimens that employ infusion of these drugs into the hepatic artery. However, the relevance of these observations to the hydroxylation of 4-amino-antifolates by human liver remains to be established.

The folate antagonists, methotrexate‖, aminopterin and dichloromethotrexate, are converted to their 7-hydroxyl derivatives by aldehyde oxidase, an enzyme found in the liver of many species [1–7]. The affinities of these different compounds for a crude preparation of the rabbit liver enzyme were partially described by Johns *et al.* [4, 5] with an order of substrate specificity of dichloromethotrexate > methotrexate > aminopterin. Recently, methotrexate polyglutamyl derivatives with one to three additional glutamyl residues were also reported to be substrates for this enzyme [8–11].

In this paper we provide a comprehensive analysis of the kinetics of the 7-hydroxylation of methotrexate, its polyglutamyl derivatives, aminopterin and dichloromethotrexate, using a partially purified aldehyde oxidase preparation from rabbit liver. This follows the characterization of the 7-hydroxylation of methotrexate in freshly isolated hepatocytes *in vitro*, a very rapid process that dominates the metabolic disposition of methotrexate in this cell [12].

MATERIALS AND METHODS

Materials. [3′,5′,7-^{3}H]Methotrexate and [7,9-^{3}H] aminopterin were synthesized by Amersham (Arlington Heights, IL) and purified by HPLC as described previously [13]. [7-^{3}H]Dichloromethotrexate was synthesized by Moravek (Brea, CA) and was also purified by HPLC [14]. Unlabeled methotrexate, aminopterin and dichloromethotrexate, obtained from the National Cancer Institute, were purified by DEAE cellulose chromatography [15] or by HPLC [13]. 4-NH_2-10-CH_3-PteGlu$_2$ to -Glu$_4$ were supplied by Dr. C. M. Baugh (University of South

* This investigation was supported by the PHS Grant CA-16906 awarded by the National Cancer Institute, National Institutes of Health.

† Exchange Scientist under the Clinical Cancer Research Program Area of the U.S.-France (NCI-INSERM) Cancer Program (No. G50111). Present address: INSERM SC 16, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5, France.

‡ Supported by PHS Training Grant CA-09340 from the National Cancer Institute, National Institutes of Health.

§ To whom reprint requests should be sent.

‖ Abbreviations: methotrexate, 4-NH_2-10-CH_3-PteGlu; aminopterin, 4-NH_2-PteGlu; dichloromethotrexate, 3′,5′-Cl-4-NH_2-10-CH_3-PteGlu; and HPLC, high performance liquid chromatography.

Alabama, Mobile, AL) and used without further purification. Unlabeled 7-hydroxymethotrexate was obtained by direct 7-hydroxylation from methotrexate after incubation with a crude preparation of fresh mature rabbit liver and was purified by DEAE cellulose as described previously [8, 16].

As specified for 7-hydroxymethotrexate [12], the data for 7-hydroxyaminopterin and 7-hydroxydichloromethotrexate were also corrected for the loss of [^{3}H] at the 7-position of the pteridine molecule that occurs with the hydroxylation of aminopterin or dichloromethotrexate (Amersham and Moravek reports).

Partial purification of rabbit liver aldehyde oxidase. Livers of white male New Zealand rabbits (2–3 kg) were perfused via the portal vein with calcium-free Krebs–Henseleit buffer (pH 7.4, 37°) to completely eliminate blood from tissues. The livers were then removed, homogenized for 3 min in 2 vol. of double-distilled water at room temperature, and then centrifuged for 30 min at 15,000 rpm at 4°. The supernatant fraction was then stirred continuously for 10 min in a water bath at 60°, and heat-precipitated protein was removed by centrifugation at 15,000 rpm for 30 min. The supernatant fraction was collected, and saturated ammoniacal ammonium sulfate solution, prepared as described previously [5], was added to obtain a 37% solution. The supernatant solution was stirred slowly at room temperature for 30 min. After centrifugation at 15,000 rpm for 30 min, the precipitate was discarded, the same amount of the ammoniacal solution was added to the supernatant fraction, and the solution was centrifuged again at 15,000 rpm for 30 min. The precipitate was dissolved in 0.05 M potassium phosphate buffer (K_2HPO_4, pH 7.4) containing 0.005% Versene Fe-3 (Sigma Chemical Co.). The enzyme solution was then applied to an hydroxylapatite column previously washed for several hours with 0.05 M potassium phosphate buffer (pH 7.4) containing 0.005% Versene Fe-3. The fractions containing enzyme activity (determined spectrophotometrically at 340 nm with 50 μM methotrexate as substrate) were combined and concentrated osmotically to 0.1 vol. by adding powdered sucrose to the external surface of a dialysis bag containing the protein solution. The enzyme solution was then applied to a Sephacryl S-200 column previously washed with potassium phosphate buffer (0.2 M, pH 7.4). The fractions containing the enzyme were pooled, divided into small portions, and stored at −20° for several days.

Aldehyde oxidase assay. All assays were performed under conditions in which hydroxylation was linear with time (i.e. 15–180 sec after addition of the substrate) at 27°. Aldehyde oxidase activity was assayed on a Beckman model 24 spectrophotometer in a buffer consisting of 0.2 M potassium phosphate and ammonium bicarbonate (0.1 M), at pH 7.4, containing 0.005% Versene Fe-3, by measuring the increase in optical density at 340 nm. The same wavelength was used for methotrexate, methotrexate polyglutamyl derivatives, aminopterin and dichloromethotrexate oxidation products. At this wavelength, the oxidation products had strong absorption while the parent compounds showed little absorption [5]. Because of the lack of availability of purified 7-hydroxy derivatives, extinction coefficients for the different antifolates and their respective 7-hydroxy derivatives were obtained after complete hydroxylation of the antifolates in an ultraviolet cuvette in the presence of partially purified aldehyde oxidase (Fig. 1, panels A, B, and C). Complete conversion was documented by spectral analyses identical to those reported in the literature [14], and by HPLC analysis of the products of the hydroxylation of tritiated aminopterin or methotrexate. Hydroxylation of dichloromethotrexate was confirmed by disappearance of the [7-^{3}H] moiety with a corresponding increase in an early eluting peak, presumably ^3H_2O, by HPLC. The extinction coefficients for the different compounds at 340 nm and pH 7.4, are reported in Table 1. Because the extinction coefficients for methotrexate and its polyglutamyl derivatives are identical [8], the extinction coefficients for 7-hydroxymethotrexate polyglutamyl derivatives were assumed to be the same as that of 7-hydroxymethotrexate. In all determinations, the data were calculated on the basis of the change in optical den-

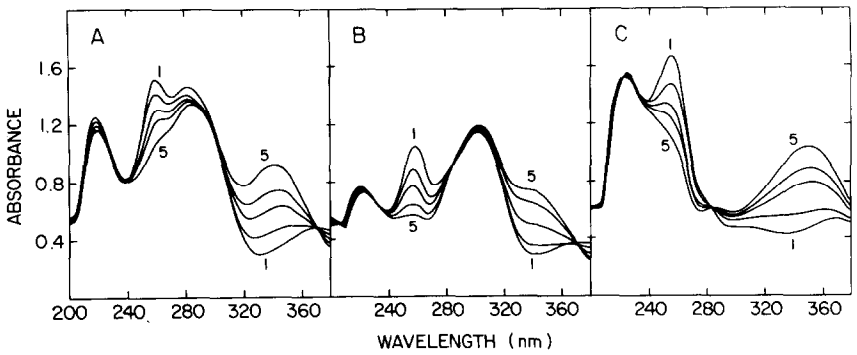

Fig. 1. Enzymatic conversion of different antifolates to their 7-hydroxy derivatives by rabbit liver aldehyde oxidase. The reference cuvette contained potassium phosphate buffer (50 mM, pH 7.4), 0.005% Versene Fe-3 and rabbit liver aldehyde oxidase (50–100 μg) in a total volume of 1 ml. The sample cuvettes contained the same constituents plus 50 μM aminopterin (A), 60 μM methotrexate (B) or 70 μM dichloromethotrexate (C). Line 1 in each panel represents unchanged antifolates and Line 5 represents the 7-hydroxy derivatives.

Table 1. Extinction coefficients for methotrexate, aminopterin, dichloromethotrexate and their hydroxylated derivatives

	Extinction coefficients
Methotrexate	4,830
7-Hydroxymethotrexate	12,000
Aminopterin	5,620
7-Hydroxyaminopterin	15,400
Dichloromethotrexate	6,230
7-Hydroxydichloromethotrexate	14,200

The reference cuvette contained potassium phosphate buffer (50 mM, pH 7.4), Versene Fe-3 (0.005%) and rabbit liver aldehyde oxidase (50–100 μg). The sample cuvettes contained the same constituents, plus the different antifolates at six different concentrations from 5 to 80 μM. The extinction coefficients for the hydroxy derivatives were determined at 340 nm as described in Materials and Methods after the complete conversion of the antifolates to their 7-hydroxy derivatives as described in the text.

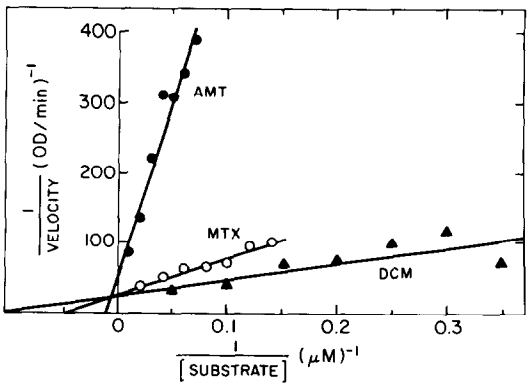

Fig. 2. Relationship between reaction velocity and substrate concentration for methotrexate (MTX), aminopterin (AMT), or dichloromethotrexate (DCM). A representative experiment is shown in which each symbol is the mean of two separate determinations.

sity due to the appearance of the product and the disappearance of the substrate [17].

Proteins were measured by the Bio-Rad protein assay method as described previously [18].

RESULTS

Purification of rabbit liver aldehyde oxidase. The details of a typical preparation of the enzyme are shown in Table 2. This procedure permits a 60 to 120-fold purification with an overall recovery of 5–15%. The enzyme solution was divided into equal portions and stored at −20° for several days with only a small loss of activity (10–15% in a week). Because of the appreciable loss of activity during dialysis of the final enzyme solution (40% in 2 days), this procedure was omitted. Ammonium ions (final concentration, 0.1 M), which were found to stabilize the enzyme, were added to the final enzyme solution.

Menadione (vitamin K_3; 2-methyl-1,4-naphtoquinone) is a potent inhibitor of oxygen reduction by hepatic aldehyde oxidase, with 85% inhibition at a menadione concentration as low as 10^{-7} M [7]. Likewise, methotrexate (final concentration, 50 μM) hydroxylation was inhibited by 90% in the presence of 2×10^{-6} M menadione in this preparation.

Substrate activities of different antifolates and the polyglutamyl derivatives of methotrexate. Rates of hydroxylation were measured over concentration ranges of 3–20 μM, 7–50 μM and 14–100 μM for dichloromethotrexate, methotrexate and aminopterin, respectively, and over an interval up to 3 min, when the rates of appearance of the 7-hydroxy derivatives were constant. A direct comparison of aminopterin, dichloromethotrexate, and methotrexate hydroxylation by the enzyme preparation revealed that aminopterin was a poor substrate, methotrexate intermediate and dichloromethotrexate the best substrate for this enzyme. Based upon Lineweaver–Burk analyses, the K_m values for methotrexate, aminopterin and dichloromethotrexate hydroxylation were 35, 272 and 10 μM respectively (Fig. 2, Table 3). There was no difference between the methotrexate and dichloromethotrexate, V_{max} 248 and 231 nmoles/min/mg protein, respectively, but the V_{max} for aminopterin was lower than that of the other two antifolates (130 nmoles/min/mg protein). For aminopterin, the differences in both K_m and V_{max}

Table 2. Purification of rabbit liver aldehyde oxidase

Fractions	Total volume (ml)	Total protein (mg)	Total activity (units)*	Specific activity (nmoles/min/mg)	Yield (%)	Purification (factor)
1. Crude preparation	136	6503	13.52	2.08	100	0
2. Supernatant from homogenate heated at 60°	88	1864	10.61	5.69	76.8	2.7
3. First ammonium sulfate fractionation (37%)	136	1114	8.67	7.78	62.3	3.7
4. Second ammonium sulfate fractionation (54%)	15	725	8.46	11.7	60.8	5.6
5. Hydroxylapatite column	60	41	1.79	43.7	12.8	20.9
6. Sephacryl S-200 column	12	6	1.5	250	11.1	120

* One unit is the amount of enzyme necessary to hydroxylate 1 μmole of methotrexate per min with 50 μM methotrexate as substrate.

Table 3. Comparison of kinetic constants of 4-amino-antifolates as substrates for rabbit liver aldehyde oxidase

Compound	N*	K_m† (μM)	V_{max}† (nmoles/min/mg)	V_{max}/K_m
Methotrexate	10	34.5 ± 5.5	248 ± 31	7.20
Dichloromethotrexate	6	9.6 ± 3.0‡	231 ± 41	24.1
Aminopterin	8	272.1 ± 85.6‡	130 ± 36§	0.48

* N is the number of separate experiments.
† Values are means ± standard error.
‡ Statistically significant difference ($P \leq 0.01$) compared to methotrexate, by Student's t-test.
§ Statistically significant difference ($P < 0.05$) compared to methotrexate or dichloromethotrexate.

values contribute to making this a very poor substrate at both low and high concentrations.

Because of the extensive metabolism of methotrexate to polyglutamyl derivatives in tumor cells [13, 16], and to a much lesser extent in rabbit liver [12], and the earlier observation [8] that 4-NH_2-10-CH_3-$PteGlu_4$ is a substrate for a crude preparation of aldehyde oxidase, the kinetics of hydroxylation of the different polyglutamyl derivatives of methotrexate, containing up to four total glutamyl moieties, were assessed. There was a marked decrease in the rates of hydroxylation as the number of glutamyl residues increased (Fig. 3), associated with an increase in the K_m values. There was, in addition, a decrease in the V_{max} values for the di- and triglutamates (Table 4). Hence, the absolute rates of this reaction at concentrations below the K_m should be far lower than those observed for methotrexate and are in the range observed for aminopterin.

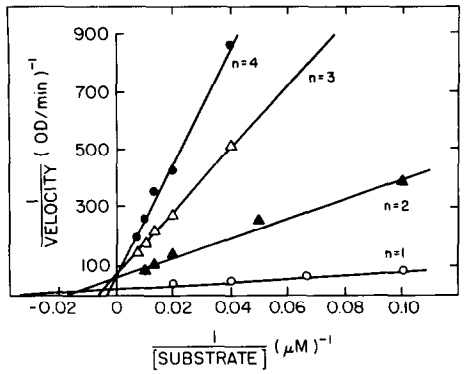

Fig. 3. Relationship between reaction velocity and substrate concentration for methotrexate and its polyglutamyl derivatives, 4-NH_2-10-CH_3-$PteGlu_n$ with n equal to 1, 2, 3, and 4. Each symbol is the mean of two determinations from a representative experiment.

Of particular interest is evidence for substrate inhibition seen on the double-reciprocal plot as the concentrations of methotrexate or dichloromethotrexate were increased to the range of 150 and 30 μM, respectively, levels about 5- and 3-fold greater than their respective K_m values (Fig. 4). Hanes and Woolf analyses [19] were used to more accurately detect the lowest concentration of methotrexate or dichloromethotrexate at which substrate inhibition was significant; this suggested negligible inhibition at concentrations below 150 and 30 μM respectively. When the rates of hydroxylation were predicted based upon the kinetic constants in Table 3 for 150 μM methotrexate and dichloromethotrexate, comparable values were predicted. However, actual measurement of hydroxylation at this concentration indicated that the rate of this reaction was 1.74 ± 0.43 (N = 3)-fold faster for methotrexate than for dichloromethotrexate—consistent with substrate inhibition for dichloromethotrexate under these conditions. Substrate inhibition for aminopterin was not detected. However, because of the low affinity of aminopterin for aldehyde oxidase and technical limitations due to the spectrophotometric determinations, the rate of hydroxylation at concentrations above 200 μM could not be evaluated.

Table 4. Comparison of kinetic constants of methotrexate and methotrexate polyglutamates for rabbit liver aldehyde oxidase

Compound	N*	K_m† (μM)	V_{max}† (nmoles/min/mg)	V_{max}/K_m
Methotrexate	9	49.3 ± 4.0	324.4 ± 40.0	6.58
4-NH_2-10-CH_3-$PteGlu_2$	4	62.5 ± 9.4‡	58.5 ± 4.8§	0.94
4-NH_2-10-CH_3-$PteGlu_3$	3	297.5 ± 66.6§	93.5 ± 20.4§	0.31
4-NH_2-10-CH_3-$PteGlu_4$	2	1627 ± 38.5§	339.3 ± 30.1	0.21

* N is the number of separate experiments.
† Values are the means ± standard error.
‡ Statistically significant difference ($P < 0.05$) compared to methotrexate, by Student's t-test.
§ Statistically significant difference ($P \leq 0.01$) compared to methotrexate.

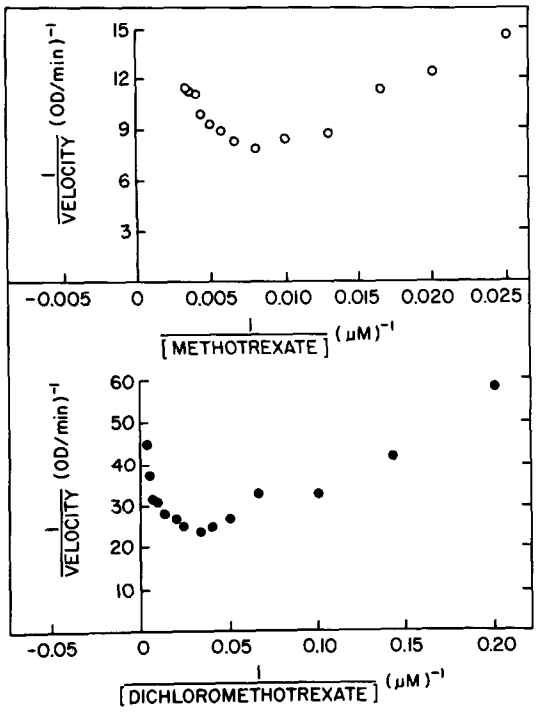

Fig. 4. Substrate inhibition by methotrexate (upper panel) and dichloromethotrexate (lower panel) of 7-hydroxylation.

DISCUSSION

Hepatic aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1) was first described by Gordon *et al.* [2] in hog liver. Since that time, enzyme activity has also been observed in livers of rabbit [4–7], rat [5], guinea pig [5], horse [1], and human [3]. Aldehyde oxidase catalyzes the oxidation of a variety of aliphatic and aromatic aldehydes as well as a number of non-aldehyde heterocyclic compounds such as the 4-amino-antifolates. Since the rabbit liver has much greater catalytic activity than does aldehyde oxidase from other species, most studies have utilized this source. Purification procedures and some properties of the aldehyde oxidase from rabbit liver, including the ability of the enzyme to transfer electrons to a variety of acceptors and its susceptibility to a variety of inhibitors, have been reported previously [1–3, 7, 20–23].

Hydroxylation of 4-amino-antifolates by this enzyme was described by Johns *et al.* [5] using a 10- to 15-fold purified aldehyde oxidase from several species. Based upon relative rates of enzymatic oxidations at a single drug concentration (200 μM), rabbit enzyme preparations demonstrated higher activity than preparations from other species with an order of substrate specificity of dichloromethotrexate > methotrexate > aminopterin.

In this paper we report the kinetics of methotrexate, aminopterin and dichloromethotrexate hydroxylation by rabbit liver aldehyde oxidase purified 60- to 120-fold with yields of 5–15%. The first steps (heat and ammonium sulfate treatments) of the purification procedure are similar to those reported previously [5–7, 21]. However, the combination of the hydroxylapatite and Sephacryl S-200 chromatographies permits the removal of additional protein impurities. The final enzyme preparation was similar to the most pure preparation obtained previously [3, 7, 21] and demonstrated a comparable degree of inhibition by the specific aldehyde oxidase inhibitor, menadione [7].

Kinetic analyses for the three different 4-amino-antifolates demonstrated that dichloromethotrexate had a 4- and 28-fold higher affinity for the enzyme than methotrexate or aminopterin respectively. These results are consistent with those obtained with partially purified enzyme [4, 5] and with intact freshly isolated rabbit hepatocytes in which the rate of hydroxylation of methotrexate was faster than that for aminopterin*.

Recently, studies from this and other laboratories demonstrated the direct hydroxylation of the polyglutamyl derivatives of methotrexate to the corresponding 7-hydroxymethotrexate polyglutamates by a crude preparation of rabbit liver aldehyde oxidase [8–11]. The present study indicates that, while the polyglutamyl derivatives of methotrexate were substrates for this enzyme, there was an inverse relationship between the affinity of these compounds for the aldehyde oxidase and their glutamyl chain lengths, consistent with another report [11]. There was a smaller decrease in the V_{max} for the di- and triglutamates with the ratios of the V_{max}/K_m for the three polyglutamyl derivative 1/6 to 1/30 that of the monoglutamate. Hence this study suggests that the benzoylglutamyl portion of the molecule is an important element in oxidase activity. Indeed, Valerino *et al.* [24] showed that addition of a glutamyl moiety to 4-NH_2-10-CH_3-pteroic acid to form methotrexate results in a decrease in V_{max} in this aldehyde oxidase mediated reaction. Johns *et al.* [25] also showed that esterification of the carboxyl group of methotrexate markedly increases its properties as a substrate for aldehyde oxidase. The observation that methotrexate polyglutamyl derivatives are poor substrates for aldehyde oxidase could account for the persistence of these derivatives in human liver for long intervals after high-dose methotrexate therapy, although this could also be related to the association of polyglutamates of methotrexate with dihydrofolate reductase which may prevent their hydroxylation [26].

Of particular interest is the observation that, at concentrations above the K_m values, there is substrate inhibition of aldehyde oxidase activity for both methotrexate and dichloromethotrexate. This is consistent with substrate inhibition of aldehyde oxidase reported for other substrates including purines, salicylaldehyde, acetaldehyde, and quinoline [23]. This may account for the earlier observations by Johns *et al.* [4, 5] that the ratio of dichloromethotrexate to methotrexate oxidation decreases when the substrate concentrations are increased by a factor of three. The importance of substrate inhibition of 7-hydroxylation during high-dose methotrexate therapy is uncertain. It might, however, contribute to the low levels of 7-

* G. Fabre and I. D. Goldman, unpublished observations.

hydroxymethotrexate formed during the interval of methotrexate infusion, when methotrexate levels are high, which is followed by an increase in the 7-hydroxymethotrexate level as the methotrexate plasma levels decline [14].

Finally, the use of dichloromethotrexate for the treatment of hepatic neoplasms by hepatic arterial infusion, based upon extensive hydroxylation due to its lower K_m, with expected removal of the major portion of drug during the first pass through the liver, must be considered within the context of the actual plasma level of drug achieved and the point at which substrate inhibition may limit hydroxylation in the human liver. However, the relevance of these and other findings for rabbit liver enzymes to the characteristics of the 7-hydroxylation of these compounds in the human liver is uncertain. Hence, while the relative substrate specificities among the 4-amino-antifolates are qualitatively similar between the species, there are large differences in the absolute ratios of hydroxylation and sensitivities to inhibitors [3, 4, 7].

REFERENCES

1. F. H. Carpenter, *Acta chem. scand.* **5**, 406 (1951).
2. A. H. Gordon, D. E. Green and V. Subrahmanyan, *Biochem. J.* **34**, 764 (1940).
3. D. G. Johns, *J. clin. Invest.* **46**, 1492 (1967).
4. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Life Sci.* **3**, 1383 (1964).
5. D. G. Johns, A. T. Iannotti, A. C. Sartorelli and J. R. Bertino, *Biochem. Pharmac.* **15**, 555 (1966).
6. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Biochim. biophys. Acta* **105**, 380 (1965).
7. K. V. Rajagopalan, I. Fridovich and P. Handler, *J. biol. Chem.* **237**, 922 (1962).
8. G. Fabre, I. Fabre, L. H. Matherly, J-P. Cano and I. D. Goldman, *J. biol. Chem.* **259**, 5066 (1984).
9. G. Fabre, L. H. Matherly and I. D. Goldman, *Fedn Proc.* **43**, 698 (1984).
10. P. A. Newton and R. L. Blakley, *Biochem. biophys. Res. Commun.* **122**, 1212 (1984).
11. J. J. McGuire, P. Hsieh and J. R. Bertino, *Biochem. Pharmac.* **33**, 1355 (1984).
12. G. Fabre, I. Fabre, D. A. Gewirtz and I. D. Goldman, *Cancer Res.* **45**, 1086 (1985).
13. D. W. Fry, J. C. Yalowich and I. D. Goldman, *J. biol. Chem.* **25**, 1890 (1982).
14. W. P. Tong, J. L. Wisnicki, J. Horton and D. B. Ludlum, *Clinica chim. Acta* **107**, 67 (1980).
15. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, *J. biol. Chem.* **213**, 5007 (1968).
16. G. Fabre, L. H. Matherly, R. Favre, J. Catalin and J-P. Cano, *Cancer Res.* **43**, 4648 (1983).
17. D. G. Johns and D. M. Valerino, *Ann. N.Y. Acad. Sci.* **186**, 378 (1971).
18. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
19. M. Dixon and E. C. Webb, *Enzymes* pp. 55–166. Academic Press, New York (1964).
20. J. Hurwitz, *J. biol. Chem.* **212**, 757 (1955).
21. G. Palmer, *Biochim. biophys. Acta* **56**, 444 (1962).
22. K. V. Rajagopalan and P. Handler, *J. biol. Chem.* **239**, 2022 (1964).
23. K. V. Rajagopalan and P. Handler, *J. biol. Chem.* **239**, 2027 (1964).
24. D. M. Valerino, D. G. Johns, D. S. Zaharko and V. T. Oliverio, *Biochem. Pharmac.* **21**, 821 (1972).
25. D. G. Johns, D. Farquhar, B. A. Chabner and J. J. McCormack, *Biochem. Soc. Trans.* **2**, 602 (1974).
26. S. A. Jacobs, C. J. Derr and D. G. Johns, *Biochem. Pharmac.* **26**, 2310 (1977).