Biochemical Pharmacology, 1966, Vol. 15, pp. 555–561. Pergamon Press Ltd., Printed in Great Britain.

THE RELATIVE TOXICITIES OF METHOTREXATE AND AMINOPTERIN*

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(*Received* 10 *October* 1965; *accepted* 12 *November* 1965)

Abstract—Hepatic aldehyde oxidases from rabbit, mouse, rat, and guinea pig have been purified 10-fold to 15-fold and shown to catalyze the oxidation of methotrexate to 7-hydroxymethotrexate. The mouse and rat liver enzymes, however, were unable to catalyze the oxidation of the related compound aminopterin, and the aminopterin-oxidizing ability of the rabbit liver enzyme was much inferior to its methoxtrexate-oxidizing ability. The oxidase from all four species was stimulated by ammonium ion and inhibited by menadione; species differences were noted, however, in pH optima and susceptibility to inhibition by Triton X-100. The results support the hypothesis that, in some species, the greater toxicity of aminopterin (as compared with that of methotrexate) is attributable to the lesser ability of aminopterin to serve as a substrate for hepatic aldehyde oxidase.

THE folic acid antagonists methotrexate (MTX) (4-amino-4-deoxy-10-methylpteroylglutamate) and aminopterin (AM) (4-amino-4-deoxypteroylglutamate) differ significantly in toxicity; in mice, rats, and dogs, for example, the toxicity of AM is severalfold that of MTX.[1–3] To date, no explanation has been available for this difference; the compounds appear to have the same mode of action—inhibition of the enzyme dihydrofolate reductase[4–6]—and differ little in their respective affinities for this enzyme. Similarly, available evidence suggests that the absorption and distribution of MTX and AM do not differ in any important respect.[7] It is the purpose of this communication to present evidence obtained from four mammalian species supporting the contention that the difference in the toxicity of MTX and AM is determined by their relative rates of oxidative alteration by the enzyme hepatic aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1).

In preliminary communications,[8, 9] we reported that MTX is rapidly converted by rabbit liver aldehyde oxidase to its relatively inactive 7-hydroxy analog, whereas AM is only rather slowly converted to 7-hydroxyAM; evidence was insufficient, however, to permit the suggestion that this relationship applied to species other than rabbit. It now appears, however, that MTX can indeed serve as a substrate for hepatic aldehyde oxidase from a number of mammalian species, whereas AM is almost inactive as a substrate for this enzyme. An exception to this general rule is provided by the guinea pig: liver aldehyde oxidase from this species can metabolize AM readily,

* This work was supported by Research Grants CA-02817 and CA-08010 from the U.S. Public Health Service.

† Recipient of a Career Development Award from the U.S. Public Health Service.

a circumstance that may account for the unusual resistance of the guinea pig to AM.[10–12]

METHODS

Methotrexate, aminopterin, and dichloromethotrexate (DCM) were kindly provided by Dr. J. M. Ruegsegger of Lederle Laboratories. The compounds were chromatographed on DEAE-cellulose before use.[13] After chromatography, MTX solutions were stable for several weeks when stored at 4°; it was necessary, however, to prepare and chromatograph new AM and DCM solutions daily, since the ability of these compounds to act as substrates declined rapidly on storage.

In early studies, fresh-frozen livers from commercial sources were used as a source of aldehyde oxidase, but it was found that the aldehyde oxidase content of frozen liver was considerably lower than that of fresh liver, and that there were also variations in enzyme content from one batch of livers to another. In the experiments to be described below, therefore, livers from freshly killed animals were used. Experimental animals were male white New Zealand rabbits, 1·5–2·9 kg; female C3H mice, 20–25 g; male Wistar rats, 150–200 g; and male English short-hair guinea pigs, 450–550 g. The following standardized procedure was used for the preparation of enzyme. Livers were homogenized in 2 volumes of cold distilled water and the heavier cellular debris was removed by centrifuging the homogenate for 10 min at 27,000 *g*. The crude supernatant fraction was immersed in a water bath at 60° for 10 min, with continuous stirring, and then transferred immediately to an ice-bath. Heat-precipitated protein was removed by centrifugation, and the supernatant subjected to fractionation with an ammoniacal ammonium sulfate solution prepared by adding 60 ml ammonium hydroxide solution (29%) to 940 ml saturated ammonium sulfate solution. For each 100 ml supernatant, 59 ml ammoniacal ammonium sulfate solution was added with stirring, and the precipitate was removed by centrifuging. An additional 23 ml ammoniacal ammonium sulfate solution was then added, and the precipitate was recovered. The precipitate was redissolved in ice-cold distilled water and the concentration adjusted to give about 30 mg protein/ml. This procedure resulted in a 10-fold to 15-fold concentration of enzyme activity, as compared to that of the crude supernatant, and the solution thus obtained retained its aldehyde oxidase activity for several days when stored at 4°. Since the enzyme appeared to be more stable at an alkaline pH and in the presence of ammonium ion, one-tenth volume of ammoniacal ammonium sulfate solution was routinely added to the enzyme solution before storage. In the case of aldehyde oxidase from rabbit liver, further purification could be obtained by acetone fractionation, DEAE-cellulose chromatography, and fractionation with calcium phosphate gel;[8] the enzyme from the other three species studied was considerably less stable, however, and little or no further increase in specific activity could be obtained by these methods. For the studies to be described below, therefore, purification of rabbit-liver aldehyde oxidase was not carried beyond the final stage employed for the enzyme from the other species.

As previously described,[9] enzyme activities were determined by measuring the rates of conversion of DCM to 7-hydroxyDCM, of MTX to 7-hydroxyMTX, and of AM to 7-hydroxyAM; reaction rates were followed by determining the rate of increase in absorption at 340 mμ in a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbance recorder. Experimental details regarding the individual

enzyme assays are shown in the legends for the figures and tables; preliminary studies confirmed the early observation of Carpenter[14] that the activity of the enzyme was stimulated by ammonium ion, and 250 μmoles ammonium sulfate was therefore added to the reaction cuvettes. The aldehyde oxidase preparations also possessed the ability to catalyze the oxidation of acetaldehyde and other aliphatic aldehydes, with dichlorophenolindophenol as an electron acceptor.[15] As noted by previous workers, however, this method was not suitable for routine determination of enzyme specific activity because of the high blank rates obtained at the early stages of purification.[16]

Protein concentrations were determined by the biuret method.[17]

RESULTS

Substrate specificity of hepatic aldehyde oxidase

Hepatic aldehyde oxidase from all four species examined catalyzed the oxidation of MTX, but the ratio of MTX-oxidizing ability to DCM-oxidizing ability varied

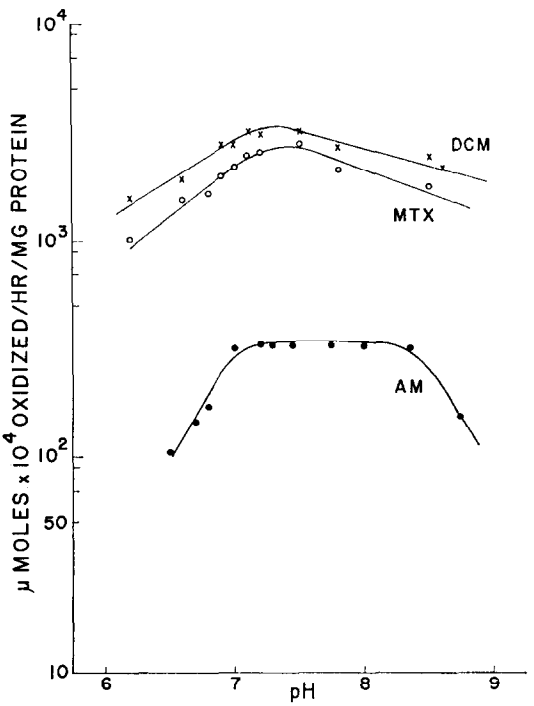

FIG. 1. Effect of pH on the rate of oxidation of 4-amino analogs of pteroylglutamate by rabbit liver aldehyde oxidase. Reaction rates were determined with aldehyde oxidase puried 10-fold to 15-fold as described in Methods; in order to facilitate comparison between species, measured rates have been converted to rates per milligram total protein in the soluble liver supernatant before purification. Each cuvette contained substrate, 0·20 μmole; potassium phosphate buffer, 300 μmoles; Versene Fe-3, 15 μg; ammonium sulfate, 250 μmoles; and from 0·2 to 2·0 mg partially purified enzyme. Total volume was 1 ml.

greatly from one species to another (Table 1); thus, the MTX-oxidizing ability of a given species cannot be predicted directly from its DCM-oxidizing ability. Rabbit was the most efficient of the species tested in its ability to oxidize MTX; when substrate levels sufficient to saturate the enzyme were used, the rate of MTX oxidation was about 80% of that of DCM oxidation (Fig. 1). Mouse (Fig. 2) and rat (Fig. 3)

TABLE 1. RATES OF ENZYMATIC OXIDATION OF 4-AMINO ANALOGS OF PTEROYLGLUTAMATE

Species	DCM	MTX	AM	Rate MTX / Rate DCM
	(μmoles × 10^4 oxidized/hr/mg protein)			
Rabbit	2740	2182	316	1/1·25
Mouse	21	2	<0·1	1/10
Rat	83	4	<0·1	1/21
Guinea pig	17,000	49	490	1/347

Reaction rates were determined with liver aldehyde oxidase purified 10-fold to 15-fold, as described in Methods; in order to facilitate comparison between species, measured rates have been converted to rates per milligram total protein in the soluble liver supernatant before purification. Reaction cuvettes contained substrate, 0·20 μmole; potassium phosphate buffer, pH 7·0, 300 μmoles; Versene Fe-3, 15 μg, ammonium sulfate, 250 μmoles; and from 0·2 to 2·0 mg partially purified enzyme. Total volume was 1 ml.

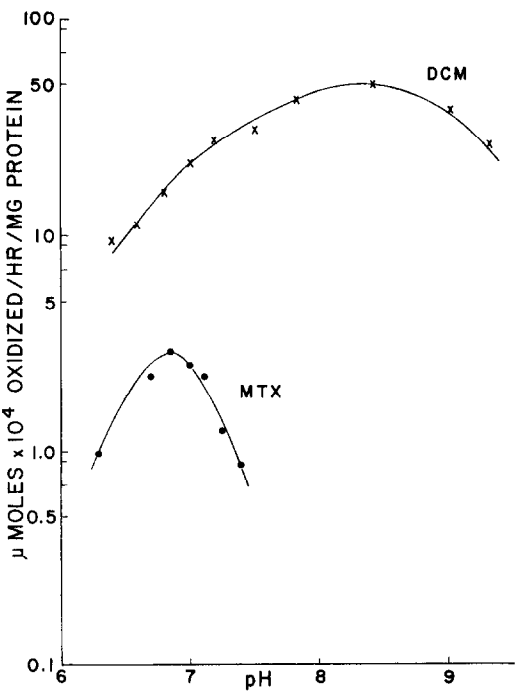

FIG. 2. Effect of pH on the rate of oxidation of 4-amino analogs of pteroylglutamate by the aldehyde oxidase of mouse liver. Reaction rates were determined as described in the legend for Fig. 1. No rate was detectable with AM as substrate.

oxidized MTX at a much slower rate, with the optimal rate for MTX being only 5–10 per cent of the rate for DCM when both rates were determined at pH 7. With the rat and the mouse, no rate was detectable with AM; guinea pig differed from the other species examined in that AM was a more readily oxidized substrate than was MTX (Fig. 4).

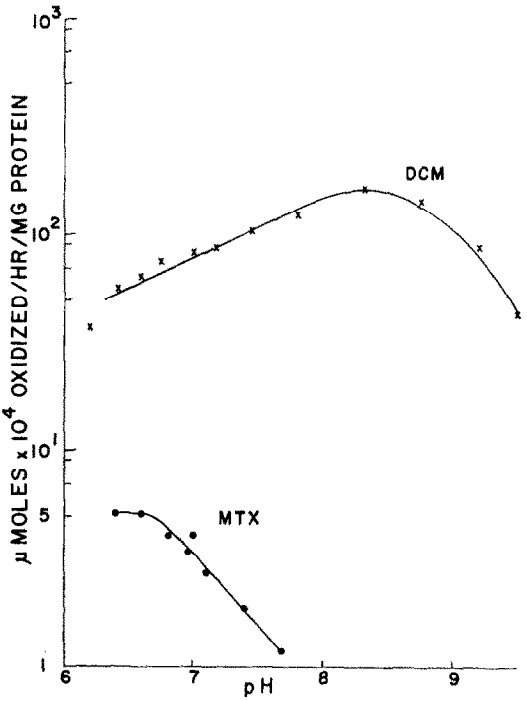

FIG. 3. Effect of pH on the rate of oxidation of 4-amino analogs of pteroylglutamate by the aldehyde oxidase of rat liver. Reaction rates were determined as described in the legend for Fig. 1. No rate was detectable with AM as substrate.

In rat, mouse, and guinea pig, the pH optimum for the oxidation of MTX was lower than that for the oxidation of DCM and appeared to lie between pH 6 and 7; because of precipitation of the enzyme below pH 6, the lower limb of the pH curve could not be determined. With the guinea pig enzyme, pH optima for the oxidation of all three substrates appeared to lie below pH 7 (Fig. 4).

Inhibition of aldehyde oxidase

It was noted in a previous communication[8] that in the rabbit, MTX oxidation was highly sensitive to the aldehyde oxidase inhibitors menadione and Triton X-100;[18, 19] menadione produced 50 per cent inhibition of the rabbit liver enzyme at a concentration of 2×10^{-6} M, and Triton X-100 produced 50 per cent inhibition at a concentration of 1×10^{-4} per cent. With the rat, mouse, and guinea pig enzymes (Table 2), oxidation of MTX and DCM was also strongly inhibited by menadine; the inhibition by Triton X-100, however, was less than was the case with the rabbit enzyme.

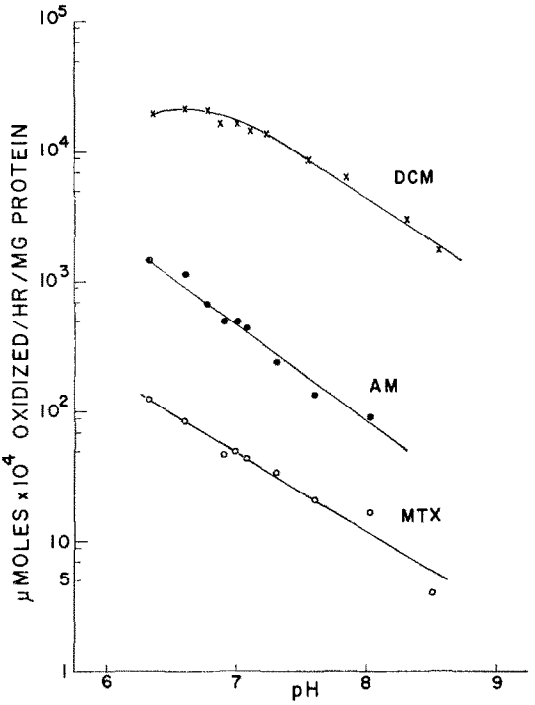

FIG. 4. Effect of pH on the rate of oxidation of 4-amino analogs of pteroylglutamate by the aldehyde oxidase of guinea pig liver. Reaction rates were determined as described in the legend for Fig. 1.

TABLE 2. INHIBITION OF ENZYMIC OXIDATION OF DCM

Inhibitor	Rate of oxidation (% of control)		
	Mouse	Rat	Guinea pig
Menadione, 1×10^{-7} M	87	90	95
Menadione, 1×10^{-6} M	41	40	77
Menadione, 1×10^{-5} M	10	7	50
Triton X-100, 1×10^{-5}%	100	100	100
Triton X-100, 1×10^{-4}%	92	93	77
Triton X-100, 1×10^{-3}%	87	86	64

Reaction conditions are described in the legend for Table 1. Inhibitors were incubated with enzyme for 1 min before the addition of substrate.

DISCUSSION

Hepatic aldehyde oxidase from the four mammalian species examined shows certain similarities; the enzyme can oxidize both aldehydes and heterocyclic compounds such as the 4-aminofolates and N-methylnicotinamide.[8] Similarly, the enzyme from all four species is inhibited by menadione and stimulated by ammonium ion. Nevertheless, considerable difference exists between the enzymes from these species; aldehyde oxidase from mouse and rat, for instance, appears to be unable to oxidize AM, whereas the enzyme from guinea pig can oxidize AM readily. Similarly, the ratio of MTX-oxidizing activity to DCM-oxidizing activity appears to differ with

enzyme from different species. The rabbit liver enzyme is inhibited strongly by Triton X-100, while the enzyme from mouse and rat is only weakly inhibited by this agent. Finally, the pH optimum for the oxidation of MTX, AM, and DCM differs from one species to another. It would appear, therefore, that aldehyde oxidases obtained even from relatively closely related species, such as the guinea pig, rat, mouse, and rabbit, are not identical; only in the enzyme from rat and mouse was it not possible to detect significant species differences.

Good inverse correlation was seen between the relative rates of oxidation of MTX and AM and their toxicity *in vivo*. The rabbit and guinea pig, for example, are both highly resistant to AM,[10] and aldehyde oxidase from both species is able to oxidize AM readily. Mouse and rat, on the other hand, are sensitive to AM,[1] and enzyme from these species has no detectable AM-oxidizing ability. The rat and mouse, however, can tolerate considerably higher doses of MTX than of AM, and the oxidation of MTX by hepatic aldehyde oxidase from these species proceeds at a slow but significant rate at physiologic pH. With respect to the significance of these observations in the intact animal, the recent report of Redetzki and his co-workers[20] is of considerable interest. These workers have shown that, in the rabbit, MTX is excreted in the urine in an altered form; the ultraviolet absorption spectrum and other properties of the MTX metabolite appear to be identical with the properties of the product of oxidation of MTX by rabbit-liver aldehyde oxidase.[21]

REFERENCES

1. F. S. Philips, J. B. Thiersch and F. C. Ferguson, *Ann. N.Y. Acad. Sci.* **52**, 1349 (1950).
2. A. Goldin, J. M. Venditti, S. R. Humphreys, D. Dennis, N. Mantel and S. W. Greenhouse, *J. nat. Cancer Inst.* **15**, 1657 (1955).
3. A. E. Sloboda, *J. Pharmac. exp. Ther.* **128**, 419 (1960).
4. M. J. Osborn, M. Freeman and F. M. Huennekens, *Proc. Soc. exp. Biol. (N.Y.)* **97**, 429 (1958).
5. W. C. Werkheiser, *J. biol. Chem.* **236**, 888 (1961).
6. J. R. Bertino, B. W. Gabrio and F. M. Huennekens, *Biochem. biophys. Res. Commun.* **3**, 461 (1960).
7. L. Delmonte and T. H. Jukes, *Pharmac. Rev.* **14**, 91 (1962).
8. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Biochim. biophys. Acta* **105**, 300 (1965).
9. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Life Sci.* **3**, 1383 (1964).
10. V. Minnich, C. V. Moore, D. E. Smith and G. V. Elliott, *Archs Path.* **50**, 787 (1950).
11. R. H. Girdwood, *Br. J. Nutr.* **5**, 1 (1951).
12. J. J. Vitale, S. N. Gershoff, L. Sinisterra, D. M. Hegsted and N. Zamchek, *J. biol. Chem.* **220**, 363 (1956).
13. V. Oliverio, *Analyt. Chem.* **33**, 263 (1961).
14. F. H. Carpenter, *Acta chem. scand.* **5**, 406 (1951).
15. H. R. Mahler, B. Mackler, D. E. Green and R. M. Bock, *J. biol. Chem.* **210**, 465 (1954).
16. G. Palmer, *Biochim. biophys. Acta* **56**, 444 (1962).
17. H. W. Robinson and C. G. Hogden, *J. biol. Chem.* **135**, 707 (1940).
18. K. V. Rajagopalan, I. Fridovich and P. Handler, *J. biol. Chem.* **237**, 922 (1962).
19. K. V. Rajagopalan and P. Handler, *J. biol. Chem.* **239**, 2022 (1964).
20. H. M. Redetzki, J. E. Redetzki and A. L. Elias, *Pharmacologist* **7**, 180 (1965).
21. H. M. Redetzki. Personal communication.