Metabolite of 4-Amino-4-deoxy-N^{10}-methylpteroylglutamic Acid (Methotrexate)

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4-Amino-4-deoxy-N^{10}-methylpteroylglutamic acid (methotrexate; MTX) is rapidly metabolized by rabbit liver aldehyde oxidase. The MTX metabolite, on oxidative cleavage with permanganate, yields a simple pteridine having the spectrophotometric and chromatographic properties of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid. Alkaline hydrolysis of the latter at 70° slowly converts it to 2-amino-4,7-dihydroxy-6-pteridinecarboxylic acid. The nonpteridine moiety of the MTX metabolite is identical with the nonpteridine moiety of MTX. The biological properties of the MTX metabolite are compatible with those of a 2,4-diamino derivative of pteroylglutamate. In view of these findings, the structure, 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid (7-hydroxy-MTX), is proposed for the MTX metabolite.

THE FOLIC ACID analog 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (methotrexate; MTX), a compound of considerable clinical interest in the treatment of neoplastic diseases and of psoriasis, undergoes rapid biological inactivation by a flavin enzyme present in the supernatant fraction of rabbit liver homogenates (1, 2). A similar, but slower conversion occurs in the supernatant fraction of liver homogenates from guinea pig and several other species (3). Johns *et al.* (1) have identified the MTX-inactivating enzyme as aldehyde oxidase (aldehyde:O_2 oxidoreductase, EC 1.2.3.1) and have shown it to be identical with the enzyme catalyzing the inactivation of 3′,5′-dichloro-MTX and a number of other halogenated 4-amino analogs of pteroylglutamate (4). The structure of the MTX metabolite has not been established, although Redetzki and his co-workers (2), on the basis of elementary analyses, suggested that the compound is 4,7-dihydroxy-MTX (7-hydroxy-N^{10}-methylpteroylglutamic acid). Earlier studies by Loo and Adamson (5) with 3′,5′-dichloro-MTX, however, have shown that, in the latter compound, the 4-amino group remains intact during enzymic inactivation and that the 3′,5′-dichloro-MTX metabolite differs from the parent compound only in the presence of a hydroxyl group at the 7-position. Since the same enzyme system is responsible for the inactivation of both MTX and its 3′,5′-dichloro derivative, it appears unlikely that the 4-amino group of MTX would undergo hydrolysis while the 4-amino group of 3′,5′-dichloro-MTX would not. Furthermore, it is well known that elementary analytical data alone are rarely conclusive in establishing the structure of pteridines (5, 6). The studies described below strongly suggest that, with MTX, as with 3′,5′-dichloro-MTX, enzymic inactivation involves only 7-hydroxylation, and that the 4-amino group remains intact.

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EXPERIMENTAL

Preparation of the MTX Metabolite—Commercial grade MTX[1] was twice precipitated from aqueous solution with glacial acetic acid, washed with ice-cold acetone, and dried over phosphorus pentoxide; of the MTX powder thus obtained, 150 mg. was dissolved in 60 ml. of water with the aid of 1 *N* potassium hydroxide added dropwise. The pH of the solution was adjusted to 7.5 with 2 *N* phosphoric acid.

Frozen mature rabbit liver (600 Gm.; Pel-Freez Biologicals, Inc.) was allowed to thaw at 5° and homogenized in 2 vol. of distilled water. After centrifuging for 10 min. at 27,000 × g, the crude supernatant fraction was collected and immersed in a water bath at 60° for 10 min. Heat-precipitated protein was removed by centrifugation, and the supernatant fractionated with an ammoniacal solution of ammonium sulfate prepared by adding 60 ml. ammonium hydroxide solution (29%) to 940 ml. saturated solution of ammonium sulfate. For each 100 ml. of the supernatant, 59 ml. of the solution of ammoniacal ammonium sulfate was added with stirring, and the precipitate was removed by centrifuging. An additional 23 ml. of ammoniacal solution of ammonium sulfate was then added, and the precipitate was recovered. The precipitate was redissolved in sufficient ammoniacal ammonium sulfate solution of 10% saturation to give a protein concentration of about 30 mg./ml. A typical purification resulted in 28-fold concentration of aldehyde oxidase activity, as compared with that

[1] Kindly provided by Dr. J. M. Ruegsegger, Lederle Laboratories.

of the crude supernatant, and yielded 36 ml. of enzyme solution with a protein concentration of 31 mg./ml. Aldehyde oxidase activity was determined with *N*-methylnicotinamide as substrate (7), and protein concentration was determined by the method of Waddell (8).

To the enzyme solution were added 10,000 units of catalase[2] (Worthington Biochemical Corp.), 6 ml. of 0.4 *M* sodium phosphate buffer, pH 7.8, containing 0.005% iron chelating agent,[3] and 100 ml. of distilled water. The pH was adjusted to 8.4 by the addition of ammoniacal ammonium sulfate, and the MTX solution (prepared as described above) was added dropwise with stirring at room temperature, at a rate of 8 ml./hr. (20 mg. of MTX per hour). The solution was then allowed to stand at 5° overnight, and the yellowish-white flocculent precipitate was collected by centrifuging. The precipitate was suspended in 16 ml. of distilled water, and a 0.2-ml. aliquot was removed for chromatographic examination; the remainder was heated in a boiling water bath for 4 min., centrifuged, and the insoluble protein precipitate discarded. The supernatant fraction was cooled in an ice-bath, 0.8 vol. of glacial acetic acid was added, and the solution was allowed to stand overnight at 5°. The lemon-yellow precipitate was washed twice with cold acetone and dried at room temperature over phosphorus pentoxide. The yield in a typical preparation was 94 mg. Chromatography on DEAE-cellulose, as previously described (9), showed the product to migrate as a single peak, and to be free of MTX. The ultraviolet absorption characteristics of the product are: in 0.1 *N* hydrochloric acid, $\lambda_{max.}$ 218 mμ (log ε 4.48), 300 mμ (log ε 4.26), and 334 mμ (infl.) (log ε 4.07); at pH 7.6 in 0.05 *M* Tris buffer, $\lambda_{max.}$ 224 mμ (infl.) (log ε 4.23), 261 mμ (log ε 4.04), 303 mμ (log ε 4.29), and 340 mμ (infl.) (log ε 4.08); in 0.1 *N* sodium hydroxide solution, $\lambda_{max.}$ 223 mμ (log ε 4.54), 260 mμ (log ε 4.07), 308 mμ (log ε 4.31), and 337 mμ (infl.) (log ε 4.13).

Activity of the MTX Metabolite as an Inhibitor of Dihydrofolate Reductase—The MTX metabolite was tested for ability to inhibit the enzymatic conversion of dihydrofolate to tetrahydrofolate, using a partially purified preparation of dihydrofolate reductase from L1210 mouse leukemia cells (10). Data were plotted by the method of Zakrzewski (11). The MTX metabolite was of intermediate potency as an inhibitor of dihydrofolate reductase; the K_i was 6×10^{-9} *M* at pH 5.5. For comparison, the parent compound, MTX, a strong inhibitor of dihydrofolate reductase, has a K_i of $<3 \times 10^{-11}$ *M* (12), while pteroylglutamate, a weak inhibitor of the enzyme when dihydrofolate is used as substrate, has a K_i of 3×10^{-6} *M* (10).

Oxidative Degradation of the MTX Metabolite—The procedure was similar to that used for 3′,5′-dichloro-MTX (5). The MTX metabolite (50 mg.), dissolved in 10 ml. of 0.1 *N* sodium hydroxide, was oxidized with 1.5 ml. of 5% potassium permanganate added dropwise with vigorous stirring at 90° for 6 min. After all the potassium permanganate had been added, the reaction mixture was kept at 90° for an additional minute. The brown precipitate was removed by centrifugation, washed with water, and centrifuged again. The combined supernatant fractions were acidified with 1 ml. of glacial acetic acid and centrifuged. The weakly acidic supernatant from which the greenish-yellow gelatinous precipitate separated was saved for later investigation (see below). The precipitate was washed with water, redissolved in 6 ml. of 1 *N* sodium hydroxide, warmed with activated charcoal, and filtered. The filtrate was added slowly, dropwise, to hot dilute acetic acid (1 ml. of glacial acetic acid diluted to 20 ml. with water). On cooling, the pale yellow precipitate was collected by centrifugation and washed with water; this treatment was repeated. The product was then washed with acetone and dried in a vacuum desiccator. The pteridine acid (X) so obtained weighed 9 mg.

Anal.—Calcd. for $C_7H_6N_6O_3$ (2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid; the diamino acid): N, 37.84; for $C_7H_5N_5O_4$ (2-amino-4,7-dihydroxy-6-pteridinecarboxylic acid; the dihydroxy acid): N, 31.39. Found: N, 38.15; 38.26.

The pteridine acid (X) showed ultraviolet absorption spectra identical to those of an authentic sample of the diamino acid (13) in 14.7 *N* sulfuric acid, in 0.1 *N* sodium hydroxide solution, and in 0.05 *M* Tris buffer, pH 8.0. Except in alkaline solution, the spectra of acid X bear little resemblance to those of the dihydroxy acid. The spectra of the three acids in 0.05 *M* Tris buffer, pH 8.0, are shown in Fig. 1.

When chromatographed on Whatman No. 1 paper, and developed with 0.5% sodium carbonate solution, ascending flow, the R_f values were 0.46–0.49 for pteridine acid (X), 0.46–0.49 for the diamino acid, and 0.71–0.73 for the dihydroxy acid. With 3% ammonium chloride solution as solvent, the R_f values were 0.19–0.20 for pteridine acid (X), 0.20–0.21 for the diamino acid, and 0.40–0.42 for the dihydroxy acid. All paper chromatograms were viewed under ultraviolet light of 254 mμ.

Alkaline Hydrolysis of Pteridine Acid (X)—Pteridine acid (X) was hydrolyzed at 70° in 2 *N* sodium hydroxide solution. Aliquots were removed at the times indicated in Table I, and ultraviolet absorption spectra determined in 0.05 *M* Tris buffer, pH 8.0. The maximum at 262 mμ displayed a gradual decrease in intensity, with a concomitant increase in intensity at 285 mμ, a change compatible with the conversion of the diamino acid to the dihydroxy acid (Table I).

Nonpteridine Moiety Derived from Permanganate Oxidation of the MTX Metabolite—The weakly acidic supernatant from which the pteridine acid (X) had been removed was further acidified with 3 ml. of concentrated hydrochloric acid, saturated with sodium chloride, and extracted first with 15 ml. of ethyl acetate, and then twice with 10 ml. of the same solvent. The combined extract was washed with 25 ml. of water and dried over anhydrous sodium sulfate. The ethyl acetate extract was then concentrated at 100° in an atmosphere of nitrogen. The reddish oil thus obtained defied all attempts at crystallization; however, its ultraviolet absorption spectra closely resembled that of *p*-methylaminobenzoylglutamic acid (14). When compared with the product obtained from a similar oxidative degradation of MTX, it showed identical *Rf* values on Whatman No. 1 paper, developed by

[2] The addition of catalase is necessary in order to prevent inactivation of the enzyme by hydrogen peroxide formed during the reaction.

[3] Marketed as Versene Fe-3 by the Dow Chemical Co., Midland, Mich.

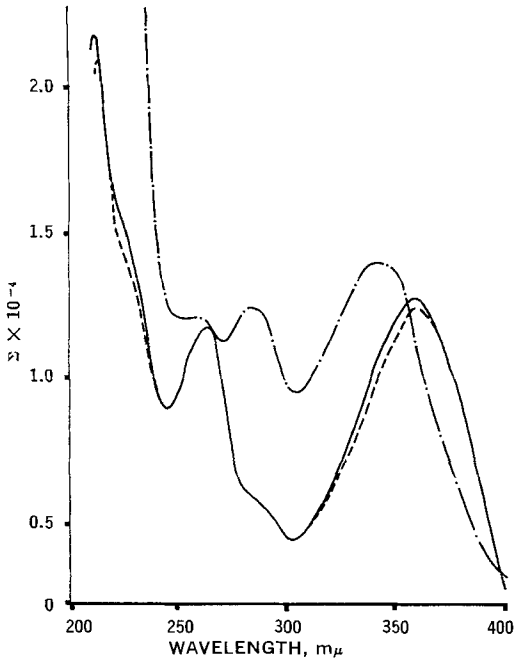

Fig. 1—Ultraviolet absorption spectra of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid (————), 2-amino - 4,7 - dihydroxy - 6 - pteridinecarboxylic acid (- · - · - · -), and pteridine acid (X) derived from permanganate oxidation of the MTX metabolite (- - - - - - - -). Solvent: 0.05 M Tris buffer, pH 8.0.

TABLE I—CHANGES IN ABSORBANCE OF PTERIDINE ACID (X) ON ALKALINE HYDROLYSIS[a]

Time, hr.	$A_{263m\mu}/A_{358m\mu}$	$A_{285m\mu}/A_{358m\mu}$
0	0.808	0.306
2.5	0.749	0.309
13.5	0.746	0.359
21.5	0.696	0.437

[a] Hydrolysis was carried out at 70° in 2 *N* sodium hydroxide solution. Ultraviolet absorption spectra were determined in 0.05 *M* Tris buffer, pH 8.0.

ascending flow, in three solvent systems, as indicated in Table II. All paper chromatograms were viewed under ultraviolet light of 366 mμ.

RESULTS AND DISCUSSION

The demonstration by ultraviolet spectroscopy, by chromatography, and by elementary analysis, that the MTX metabolite gives rise to 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid on permanganate oxidation is compatible with the structure 7-hydroxy-MTX for the parent compound. It would appear, therefore, that the enzyme-catalyzed hydroxylation involves only position-7 of MTX, and that the 4-amino group is unaltered. While 7-hydroxylation is compatible with the known catalytic properties of rabbit liver aldehyde oxidase, this enzyme possesses no known deaminase activity. For the 2-amino-4,7-dihydroxy structure, therefore, one would have to postulate the presence of an MTX-4-deaminase in the reaction mixture, a remote possibility, since the reaction proceeds equally well

TABLE II—R_f VALUES OF THE NONPTERIDINE MOIETY OF MTX (*A*) AND OF MTX METABOLITE (*B*)

Chromatographic System	R_f Value *A*	*B*	Mixture of *A* + *B*
0.1 *M* acetate buffer, pH 4.4	0.83	0.82	0.82
0.5% sodium carbonate soln.	0.92	0.89	0.91
1:1 mixture of 5% ammonium sulfate soln. and isopropanol	0.59	0.63	0.58

with highly purified aldehyde oxidase. Alternatively, nonenzymic hydrolytic deamination might occur at the 4-position. This appears equally unlikely, since lengthy heating at 70° in 2 *N* sodium hydroxide solution is required to bring about the conversion of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid to its 2-amino-4,7-dihydroxy analog.

Supporting evidence for the 2,4-diamino structure is the moderate antifolate activity of the MTX metabolite when tested in an *in vivo* system (2), or *in vitro*, against partially purified dihydrofolate reductase from L1210 mouse leukemia cells; barring alteration of the *p*-methylaminobenzoylglutamic acid moiety, such activity is compatible with a 2,4-diamino structure but not with a 2-amino-4-hydroxy structure.

The suggestion by other workers that the MTX metabolite is 2-amino-4,7-dihydroxy-MTX was based largely on elementary analyses. The difficulties encountered in elementary analysis, however, and particularly nitrogen analysis of pteridines (6), make additional criteria obligatory before identity can be established. The additional data presented here would favor the 7-hydroxy-MTX structure over the 4,7-dihydroxy-MTX structure.

The rapid hydroxylation of MTX and other 4-amino analogs of pteroylglutamate by the hepatic aldehyde oxidase of either rabbit or guinea pig probably accounts for the very high tolerance of these species for these drugs. The resistance of the hamster to the toxicity of MTX recently demonstrated by Freireich and his co-workers (15) would suggest that the latter species also belongs to this group. On the other hand, the extremely low level of MTX-oxidizing activity of the rat and mouse hepatic aldehyde oxidases (3) correlates well with the sensitivity of the two latter species to the antifolates. The human enzyme has not been studied; however, chromatographic studies of human urine after the administration of MTX show that most of the drug is excreted unchanged (16–18);[4] in addition, clinical data suggest that man is relatively sensitive to both MTX and aminopterin. These observations support the contention that the MTX-hydroxylating properties of human hepatic aldehyde oxidase must be rather low.

SUMMARY

1. Methotrexate (MTX) is readily metabolized by rabbit liver aldehyde oxidase.

[4] Chromatographic examination of human urine 2 weeks after the administration of tritium-labeled MTX has shown the presence of a small amount of non-MTX radioactivity (17); the latter migrated more rapidly on DEAE-cellulose than did MTX, however, and thus cannot be attributed to 7-hydroxy-MTX.

2. Permanganate oxidation of the metabolite of MTX yields a simple pteridine with the properties of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid.

3. Heating of the latter compound in 2 *N* sodium hydroxide solution at 70° leads to slow hydrolysis of the 4-amino group.

4. The nonpteridine moiety of the metabolite of MTX is identical with the nonpteridine moiety of MTX.

5. The structure 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid (7-hydroxy-MTX) is proposed for the MTX metabolite.

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Statistical Analysis of Log-Dose Response Bioassay Experiments with Unequal Dose Ratios for the Standard and Unknown Preparations

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Well-known statistical analyses are available for the analysis of log-dose response assays when successive doses are in the same ratio for both the standard and unknown preparations. It is, however, sometimes convenient and advantageous in practice to use unequal dose ratios. Appropriate analyses are offered for such cases, analyses which reduce to the usual ones when the ratios are equal. It is seen that the operational flexibility thus permitted is obtained in return for only slightly increased computation. Four and six-point assays are discussed in detail together with a numerical example of the former. An improved method for calculating confidence interval estimates in log-dose response assays is also given.

STATISTICAL ANALYSES for log-dose response parallel line assays in which the ratios of the successively higher doses are the same for both the standard and unknown preparations are well known. (For example, see *Reference 1.*) The case to be considered here is that in which the ratios of successive doses are constant within each preparation but the constant ratio differs from one preparation to the other. Such a relaxation of the usual single constant ratio condition is occasionally desirable in practice (2) and, for example, may permit the linear response log-dose range of the standard preparation to be exploited in the presence of more uncertainty about the linear range for the unknown preparation.

It will be shown that such flexibility can be achieved with only small changes from the single ratio analyses and, in fact, the latter can be regarded as special cases of the more general analysis proposed here. This analysis has some computational advantages over the alternative based on a single log-dose transformation which gives integral dose-metameter values for one preparation but nonintegral values for the other.

SPECIFICATION AND DOSE TRANSFORMATIONS

It will be supposed that, in an $(h + k)$-point assay, the h and k doses (concentrations) of the standard and unknown preparations, respectively, are chosen so that,

$$\left.\begin{aligned} \frac{z_{Si}}{z_{Si-1}} &= D_S,\ i = 1, 2, \ldots, h \\ \text{and} \\ \frac{z_{Uj}}{z_{Uj-1}} &= D_U,\ j = 1, 2, \ldots, k \end{aligned}\right\} \quad \text{(Eq. 1)}$$

where z_{Si} and z_{Uj} are, respectively, the ith dose of the standard and the jth dose of the unknown preparation.

As usual, it will be assumed that the observed

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