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STUDIES OF THE METABOLISM OF METHOTREXATE BY INTESTINAL FLORA—I

IDENTIFICATION AND STUDY OF BIOLOGICAL PROPERTIES OF THE METABOLITE 4-AMINO-4-DEOXY-N^{10}-METHYLPTEROIC ACID

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Abstract—Incubation of tritium-labeled methotrexate (MTX) with caecal contents obtained from CDF_1 mice results in the production of at least three labeled metabolites separable from MTX by chromatography on DEAE-cellulose. The principal metabolite has been isolated and identified by means of its chromatographic properties and its ultraviolet and infra-red absorption spectra as 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA). In studies *in vivo*, APA has been recovered from the urine and feces of CDF_1 mice in the 6 and 12-hr period after the intraperitoneal administration of tritium-labeled MTX (3 mg/kg). While APA is the major metabolite present in the feces, only small amounts are detectable in the urine of CDF_1 mice previously injected with labeled MTX. The major portion of the radioactivity associated with MTX metabolites in urine migrated prior to APA on DEAE-cellulose.

Some of the biological properties of APA were determined. The compound was found to be a moderately effective inhibitor of dihydrofolate reductase (apparent $K_i = 1{\cdot}3 \times 10^{-9}$ M) and a substrate for aldehyde oxidase (apparent $K_m = 2{\cdot}2 \times 10^{-4}$ M); the product of oxidation of APA by the latter enzyme was 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroic acid. In studies *in vivo*, APA was found to be considerably less toxic than MTX in CDF_1 mice.

These studies provide direct experimental support for the hypothesis of Zaharko *et al.** that in the mouse MTX is subject to extensive metabolism by intestinal bacteria.

METHOTREXATE (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid; MTX) is a folic acid antagonist with significant anti-tumor activity in human neoplastic disease[1] and in experimental tumor systems.[2] A few species (e.g. rabbit and guinea-pig) can metabolize MTX to 7-hydroxy MTX;[3] in most mammalian species, however, this conversion does not occur and early studies in man and also the mouse, rat, monkey, and dog indicate that the drug was excreted unchanged[4,5] or that only traces of non-MTX metabolites could be detected.[5,6]

Recently, however, Zaharko *et al.*[7,8] have reported that in the mouse and rat significant amounts of previously undetected MTX metabolites are present in urine and feces after intraperitoneal administration of the tritium-labeled compound. The production of these metabolites was significantly less in germ-free mice and also in mice which had been pretreated with antibiotics; it was suggested, therefore, that intestinal bacteria were responsible for the conversion observed. In addition, a number

* D. S. Zaharko, H. Bruckner and V. T. Oliverio, *Science, N.Y.* **166,** 887 (1969).

of workers have reported that strains of pseudomonas and of other bacterial species have the ability to catabolize and inactivate MTX.[9–12]

Because of the importance of a complete knowledge of the pharmacology of MTX for both clinical and experimental cancer chemotherapy, the studies which are described below were initiated to determine whether MTX metabolites indeed arise from cleavage of the drug by the intestinal flora. Preliminary accounts of some of these studies have appeared.[13,14]

MATERIALS AND METHODS

Metabolism of MTX by mouse caecal contents in vitro. Male CDF_1 mice averaging 21 g in weight were used in these studies. Caeca from 10 mice were excised, cut into small sections (ca. 26 mm), and added to 40 ml of isotonic, sodium phosphate-buffered saline, pH 7·4. To minimize diurnal variations in the bacterial population of the caecal contents, the excision procedure for all experiments was carried out at the same time of day. To the filtrate obtained from the caecal extract was added chromatographically pure 3′,5′-ditritiated MTX (1·0 mg, $4{\cdot}8 \times 10^7$ dis./min; obtained from Dhom, North Hollywood, Calif.). This mixture was incubated for 6 hr at 37°; the pH at the end of the incubation period was 6·6. In order to determine the relative amounts of MTX metabolites and of unchanged MTX, an aliquot of the filtrate was applied to a column of DEAE-cellulose, 1 × 16 cm, and elution carried out with a linear gradient (0·1–0·4 M) of ammonium bicarbonate (pH 8·3). In some studies, the incubation time, as well as the amount of MTX, was increased to obtain more of the metabolite for analytical purposes.

To determine the effects of antibiotic treatment on the bacterial metabolism of MTX, mice were given neomycin, 4 mg/ml, and sulfathiazole, 1 mg/ml, in their drinking water for 5 days. The caeca were then removed and treated as described above.

Isolation of the major MTX metabolite from the caecal filtrate. The filtered mixture obtained from incubation of tritium-labeled MTX with caecal contents was frozen and thawed, heated to 90° for 5 min, and the precipitate removed by centrifugation. The supernatant solution was acidified by the dropwise addition of hydrochloric acid, 6 N, 20 ml of water was added, and the solution then extracted three times with 35 ml of ether. The ether extracts were discarded and the aqueous layer was then brought to neutral pH with sodium hydroxide, 1 N, and lyophilized. The lyophilized material was then dissolved in a small volume of ammonium bicarbonate, 0·1 N, applied to a column of DEAE-cellulose, 22 × 250 cm, and eluted with a linear gradient of ammonium bicarbonate, 0·01–0·25 M. Tubes containing the metabolite(s) were located by determining the level of tritium radioactivity of the individual collected fractions. Identification of the major MTX metabolite was carried out by the spectrophotometric and chromatographic procedures described below.

Metabolism of MTX in vivo. To study the metabolism of MTX in the mouse and to produce the metabolite in sufficient quantity for chromatographic and spectrophotometric studies, 15 CDF_1 mice were injected intraperitoneally with chromatographically pure tritium-labeled MTX (3 mg/kg; $5{\cdot}22 \times 10^7$ dis./min/mouse). The animals were then sacrificed either 6 or 12 hr after MTX administration. During the 6 or 12-hr period, urine and feces were collected separately under ice and minimal light to prevent photodecomposition of the metabolite and MTX.

The quantitative estimation of the amount of metabolite(s) appearing in the urine

and feces was performed by chromatography of the mixture on DEAE-cellulose using an ammonium bicarbonate linear gradient of 0·1–0·4 M as described by Zaharko *et al.*[7] Paper chromatography of the metabolite fraction after lyophilization revealed that most of the radioactivity could be attributed to the metabolite but a portion (< 13 per cent) could also be attributed to a second component.

Isolation of the metabolite from urine and feces. To isolate the metabolite from the feces, the contents of the caecum and the rectum were added to the collected feces. Approximately 30 ml of ammonium bicarbonate, 0·1 M, was then added, and the resulting fecal suspension subjected to the same freezing and thawing, heating, extraction and chromatographic procedures that were described for the caecal filtrates.

To isolate the metabolite from the urine, urine from mice previously injected with ^{3}H-MTX was collected for 12 hr, pooled, lyophilized and chromatographed on DEAE-cellulose as described above.

Spectrophotometric and chromatographic studies of MTX metabolites. Ultra-violet and visible absorption spectra were obtained with a Cary 15 recording spectrophotometer. Samples were prepared for spectroscopic studies as follows: fractions of maximum radioactivity eluted from DEAE-cellulose as described above were lyophilized and redissolved in a minimum amount of ammonium bicarbonate solution, 0·1 M. These latter samples were then applied to Whatman No. 1 chromatography paper and subjected to ascending paper chromatography with sodium phosphate buffer, pH 7·0, 0·1 M as solvent. MTX metabolite spots (R_f *ca.* 0·35) were located visually under u.v. light (quenching spot), cut out and extracted with either hydrochloric acid, 0·1 N; ammonium bicarbonate, 0·1 M; or sodium hydroxide, 0·1 N, and their absorption spectra determined.

For the determination of the infra-red spectrum potassium bromide pellets were prepared of the lyophilized material, isolated and purified as previously described. A Perkin–Elmer 421 Infra-red Spectrophotometer was used.

To locate radioactive spots on paper chromatograms, the latter were cut into 5 mm sections, and the individual sections then cut into smaller pieces and added to a counting vial containing NCS solubilizer (Amersham/Searle). After 10 hr, 18 ml of scintillation fluid prepared by the method of Gjone *et al.*[15] was added, and radioactivity was measured with a Packard TriCarb liquid scintillation counter. In all studies, corrections for quenching were made by the channels ratio method.

Thin-layer chromatography (TLC) was carried out with DEAE-cellulose coated thin-layer plates (Analtech, Inc.). To locate radioactive spots on the thin-layer plates, the autoradiographic procedure of Randerath[16] was employed. The TLC plates were coated with an ether solution of the organic scintillator, 2,5-diphenyloxazole, 9 g/100 ml, placed in a casette with Kodak Blue Sensitive X-ray film, stored in a shielded box at dry ice temperature for 5 days, and the film was then removed and developed. In a few experiments in which the radioactivity was below 500 counts/min, the spots were located by scraping off sections (5 mm) of DEAE-cellulose from the plate and adding them to a counting vial. Radioactivity was determined as described above.

Enzyme studies. Aldehyde oxidase was purified *ca.* 30-fold from rabbit liver by the procedure of Johns *et al.*[3] Specific activity was 0·4 μmoles of *N*-methylnicotinamide oxidized/min/mg. Dihydrofolate reductase purified *ca.* 100-fold from L1210 mouse leukemia cells was kindly supplied by Dr. J. R. Bertino of Yale University, New Haven, Conn.; specific activity was 5·4 μmoles of dihydrofolate reduced/min/mg. Bovine

mice xanthine oxidase was obtained from Boehringer; specific activity was 0·4 μmoles of hypoxanthine oxidized/min/mg. Serial spectral changes occurring during enzyme-catalyzed reactions were followed by means of a Cary 15 recording spectrophotometer, and reaction rates at a single wavelength were determined with a Gilford multiple sample absorbance recorder equipped with a thermostated sample compartment. Protein concentrations in the enzyme studies were determined by the biuret method.

Oxidative degradation of the metabolite. The MTX metabolite (1 mg) was dissolved in 5 ml of sodium hydroxide, 0·1 N, and 1 ml of potassium permanganate solution, 5%, was added dropwise with vigorous stirring at 90° for 6 min. The manganese dioxide was then removed by centrifugation. To isolate the degradation product for spectroscopic studies, the mixture was applied as a continuous streak onto Whatman No. 1 chromatography paper; following development, it was eluted with either sodium hydroxide, 0·1 N, or hydrochloric acid, 0·1 N.

Zakrzewski[17] has recently reported that MTX labeled with tritium in the 3′,5′-positions by reductive dehalogenation of 3′,5′-dichloro MTX also contains tritium at the C-9 position. To facilitate identification of the MTX metabolites, a knowledge of the distribution of the tritium label in the parent compound was required; therefore, the labeled MTX used in this study was subjected to the degradation procedure described by the latter author. The cleavage products were separated by chromatography on DEAE-cellulose and the distribution of label determined. Approximately one-half (52 per cent) of the total radioactivity was found to be associated with the pteridine portion of the molecule.

Synthesis of possible MTX metabolites. A number of compounds that could result from the bacterial cleavage of MTX were prepared synthetically, to aid in the chromatographic and spectrophotometric identification of the MTX metabolites recovered from the *in vivo* and the caecal incubation studies. The selection of most of the compounds which were synthesized was based on data from previous studies of the metabolism and degradation of conjugated pteridines. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (APA) and N^{10}-methylpteroic acid were synthesized by the method of Seeger *et al.*;[18] final purification of these compounds was carried out by DEAE-cellulose chromatography. Pteroic acid was synthesized by the method of Waller *et al.*[19] 2,4-2,4-Diamino-6-pteridinecarboxylic acid was prepared by the permanganate oxidation of MTX; the procedure was identical with that described by Dion and Loo[20] for the oxidative cleavage of 3′,5′-dichloro MTX. 2-Amino-4-hydroxy-6-hydroxymethylpteridine and 2,4-diamino-6-hydroxymethylpteridine were synthesized by the method of Baugh and Shaw.[21] 7-Hydroxy MTX was prepared enzymically from MTX by the procedure of Johns and Loo,[22] utilizing rabbit liver aldehyde oxidase. 7-Hydroxy APA was synthesized enzymically from APA by the same method.

Other possible metabolites were obtained commercially or through the courtesy of other investigators. *p*-Methylaminobenzoylglutamate was a gift of Prof. Ti Li Loo, M.D., Anderson Hospital and Tumor Institute, University of Texas, Houston, Texas. *p*-Methylaminobenzoic acid and 2-amino-4-hydroxy-6-pteridinecarboxylic acid were obtained from the Aldrich Chemical Co. *p*-Aminobenzoylglutamic acid was purchased from CalBiochem. MTX was obtained from CCNSC, NCI, Bethesda, Md. Aminopterin was obtained from the Pierce Chemical Co.

Relative toxicity and anti-tumor activity of APA. The relative toxicity of APA and MTX was determined using male CDF_1 mice weighing approximately 25 g (range

24·1–25·9). Both APA and MTX were purified on DEAE-cellulose prior to toxicity studies, since commercial preparations of the latter compound are known to contain significant quantities of impurities.[23] A large amount of APA was synthesized by the microbial degradation of MTX according to the method of Levy and Goldman[10] using a strain of pseudomonas supplied by Dr. Carl Levy, NCI, Baltimore, Md. Prior to the initiation of the toxicity study, the mice were observed for 10 days for any sign of infection, segregated from the rest of the colony and fasted for 12 hr prior to injection of the compounds. Ten mice were used in each experimental group. Compounds were dissolved in 2% sodium bicarbonate and administered intraperitoneally. Following the injection of the compounds, the animals were allowed food and water *ad lib.* The animals were observed for mortality for 24 days following administration of the compounds. The calculation of the LD_{50} was performed according to the method of Litchfield and Wilcoxon.[24] The effectiveness of APA in inhibiting the growth of L1210 cells in culture was performed according to a procedure described by Adamson *et al.*[25]

RESULTS

Metabolism of MTX during incubation with mouse caecal filtrates. When tritium-labeled MTX was incubated, as described above, with filtrates of the caecal contents of mice, and the resulting products chromatographed on DEAE-cellulose, elution patterns such as that shown in Fig. 1 were obtained. In the latter experiment, 76 per cent of the recovered radioactivity was found to migrate more rapidly than MTX. On pretreatment of the mice for 5 days with neomycin and sulfathiazole, this pre-MTX radioactivity was reduced to 10·7 per cent of the recovered radioactivity (Fig. 1). APA was not produced when ^{3}H-MTX was incubated for 6 hr with homogenates of caecal tissue which had been washed free of all contents.

TABLE 1. PAPER AND THIN-LAYER CHROMATOGRAPHIC CHARACTERISTICS OF APA AND MTX, AND OF THE MTX-METABOLITE OBTAINED FROM URINE AND FECES

Material	R_f (system)*		
	1	2	3
Metabolite from urine	0·48	0·31	0·11
Metabolite from feces (*in vivo*)	0·49	0·33	0·11
Metabolite from caecal mixture (*in vitro*)	0·50	0·35	0·12
4-Amino-4-deoxy-N^{10}-methylpteroic acid (APA)	0·52	0·34	0·12
Methotrexate (MTX)	0·72	0·56	0·05

* The R_f of the spot containing greater than 85 per cent of the radioactivity on the chromatogram. The systems employed are as follows: (1) paper chromatography, 5% acetic acid; (2) paper chromatography, 0·1 M (pH 7·0) phosphate buffer; and (3) thin-layer chromatography, 0·1 M ammonium bicarbonate (pH 8·3).

Chromatographic properties of the major MTX metabolite from mouse caecal filtrates (in vitro) *and fecal extracts* (in vivo). The fractions collected from DEAE-cellulose column chromatography which contained the highest levels of pre-MTX radioactivity

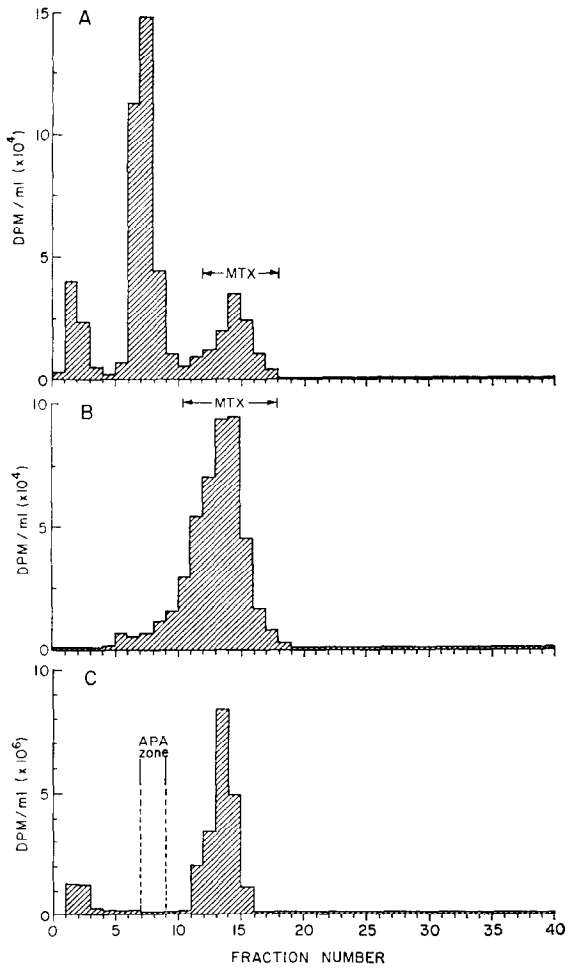

FIG. 1. DEAE-cellulose elution profiles of the mixtures *in vitro* (fecal) and *in vivo* (urine). (A) *In vitro* caecal incubation mixture (6-hr incubation); (B) *in vitro* caecal incubation mixture from antibiotic-pretreated mice (6-hr incubation); (C) elution profile of urine collected 6 hr after the i.p. administration of ^{3}H-MTX. Each fraction number corresponds with 10 ml of eluate. The ones to which carrier APA and MTX migrate are indicated on these illustrations.

were pooled, lyophilized, and chromatographed in the thin-layer and paper chromatographic systems described in the legend for Table 1. At least two zones of radioactivity were found on each paper chromatogram, one of which contained the greater part > 85 per cent of the total radioactivity. Comparison of the R_f of the latter zone with the R_f of synthetic 4-amino-4-deoxy-N^{10}-methylpteroic acid revealed that they were identical. Paper chromatography of the fractions migrating before APA revealed the presence of at least two other metabolites in both urine and feces.

Metabolism of MTX in vivo. To study the metabolism of MTX in the mouse, an experimental procedure identical with that described by Zaharko *et al.*[7] was employed. The DEAE-cellulose elution profile of urine and feces was similar to that found by Zaharko *et al.*[7] Approximately 40 per cent of the recovered fecal radioactivity could

be attributed to APA; however less than 5 per cent of the recovered urinary radioactivity could be attributed to APA (6 hr after the administration of ^{3}H-MTX). The DEAE-cellulose elution profile of urine in a typical experiment is illustrated in Fig. 1. A number of studies were also performed to determine the extent of urinary excretion of APA 12 hr after the administration of ^{3}H-MTX. APA accounted for 11·2 per cent of the recovered radioactivity (range 10·3–11·7 per cent). In no experiment was this greater than 5 per cent of the original dose administered.

Spectral properties of the major MTX (fecal) metabolite. Comparison of the absorption spectra of the MTX metabolite and of authentic APA showed that the two compounds exhibited identical absorption maxima in the ultraviolet and visible regions [λ_{max} = 259, 286 and 373 nm in sodium hydroxide, 0·1 N, (Fig. 2) and 312 nm

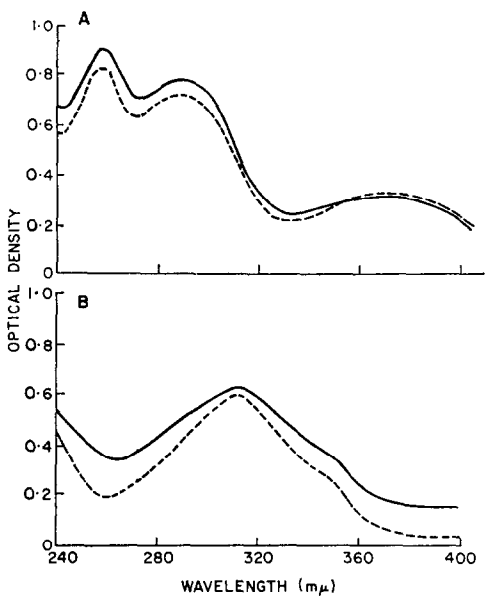

FIG. 2. Ultra-violet absorption spectra of the major caecal metabolite (*in vitro*). (A) 0·1 N NaOH; (B) 0·1 N HCl. The solid line in each diagram represents the metabolite. The dashed line represents 4-amino-4-deoxy-N^{10}-methylpteroic acid.

in hydrochloric acid, 0·1 N (Fig. 2)]. Identical u.v. spectra were obtained with the metabolite isolated from urine and feces obtained *in vivo*. As shown in Fig. 3, the infra-red spectrum obtained for the metabolite was identical to that of 4-amino-4-deoxy-N^{10}-methylpteroic acid.

Oxidation of APA and the metabolite. Potassium permanganate oxidation of both synthetic 4-amino-4-deoxy-N^{10}-methylpteroic acid and the metabolite (*in vitro*) produced 2,4-diamino-6-pteridine carboxylic acid. The ultra-violet spectra of the oxidation product revealed absorption maxima at 267 and 370 nm (sodium hydroxide, 0·1 N) and at 257 and 335 nm (hydrochloric acid, 0·1 N). These values were identical with the maxima for synthetic 2,4-diamino-6-pteridine carboxylic acid and also with the values reported by Dion and Loo.[20] The R_f of the oxidation product (0·26) in 0·1 M phosphate buffer (pH 7·0) was identical with that of synthetic 2,4-diamino-6-pteridine carboxylic acid.

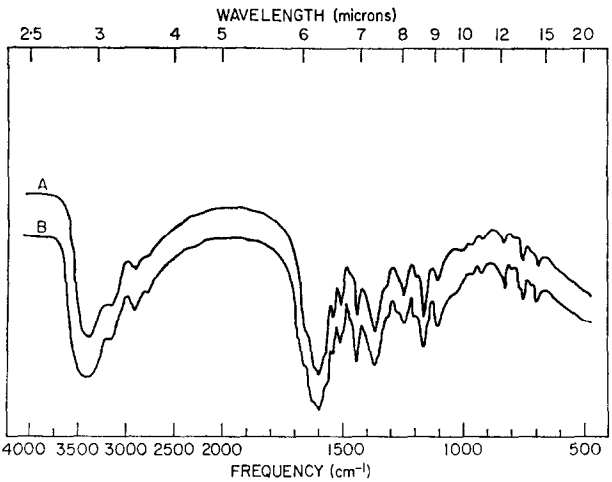

FIG. 3. Infra-red spectrum of the major caecal metabolite (*in vitro*). (A) Authentic 4-amino-4-deoxy-N^{10}-methylpteroic acid; (B) metabolite isolated from the DEAE-cellulose eluate of the caecal mixture.

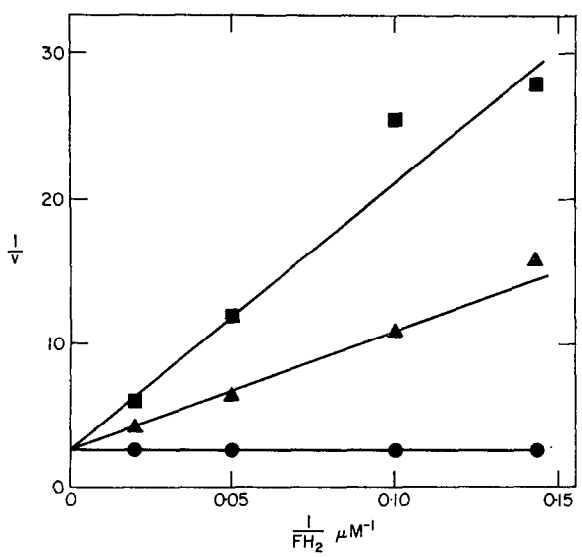

FIG. 4. Lineweaver–Burk plot of the inhibition of dihydrofolate reductase by 4-amino-4-deoxy-N^{10}-methylpteroic acid. Dihydrofolate reductase activity was assayed by the method of Bertino *et al.*[26] Cuvettes contained tris–HCl buffer, pH 7·5, 100 mM; potassium chloride, 150 mM; 2-mercaptoethanol, 1 mM; NADPH, 0·060 mM; FH_2 as indicated, and enzyme. Total volume was 1 ml. Assays were performed at 37°. ●: no inhibitor; ▲: APA, 0·125 μM; ■: APA, 0·375 μM.

APA as an inhibitor of dihydrofolate reductase. Since APA appears from these studies to be a major metabolite of MTX in the mouse, it was of interest to determine its activity as an inhibitor of dihydrofolate reductase, the enzyme generally considered to be the site of action of MTX and the other 4-aminofolate antagonists. The activity of the isolated metabolite, and also of synthetic APA, as inhibitors of dihydrofolate reductase from mouse L1210 leukemia cells is illustrated in Fig. 4; enzyme activity

was assayed by the procedure of Bertino *et al.*[26] No significant difference was seen between the inhibitory properties of the synthetic and the isolated metabolite; from the change in slope of the reciprocal plots shown in Fig. 4 and utilizing the K_m value of 0·3 μM for dihydrofolate reported by McCullough *et al.*,[12,27] an apparent K_i of $1{\cdot}3 \times 10^{-9}$ M can be calculated.* The activity of APA as an inhibitor of dihydrofolate reductase is thus significantly less than that of the parent compound MTX ($K_i < 3 \times 10^{-11}$ M).[29]

When incubated with dihydrofolate reductase and NADPH in the absence of dihydrofolate, APA showed no detectable substrate activity for the enzyme.

APA as a substrate for aldehyde oxidase. In certain species (e.g. rabbit and guinea pig) MTX, 3′,5′-dichloro MTX, aminopterin and other 4-amino analogs of pteroylglutamate are subject to oxidation at the C-7 position by the enzyme hepatic aldehyde oxidase;[3] the corresponding 2-amino-4-hydroxy compounds, however, lack substrate activity for this enzyme.[30] It was felt, therefore, that it would be of interest to determine the activity of APA as a substrate for aldehyde oxidase, both to act as supporting evidence that this compound retains the 2,4-diamino structure, and also to determine whether further metabolism would be anticipated in those species known to possess high levels of this enzyme. The isolated metabolite and the synthetic compound were both tested as substrates; substrate activity was determined from the direct measurement of the rate of formation of the 7-hydroxy compound at 340 mμ, and also from the rate of reduction of the artificial electron acceptor 2,6-dichlorophenol-indophenol.[31] The K_m of APA as a substrate for aldehyde oxidase was $2{\cdot}2 \times 10^{-4}$ M; no significant difference was seen between the isolated and the synthetic metabolite in their properties as substrates for this enzyme. It is of interest that APA is a more rapidly oxidized substrate for aldehyde oxidase than is its parent compound, MTX; the V_{max} was 2·3-fold that for the latter compound.

APA as a substrate for xanthine oxidase. A number of non-conjugated pteridines can act as substrates for the enzyme xanthine oxidase.[32] When bovine milk xanthine oxidase, 5 units, was incubated under aerobic conditions with APA (0·1 μmoles) for periods of up to 5 hr, no changes were noted in the absorption spectrum of the compound, suggesting that APA does not undergo oxidation by this enzyme.

Relative toxicity of APA. The LD_{50} for MTX in CDF_1 mice, injected i.p. daily on 2 consecutive days, was found to be 58 mg/kg. The LD_{50} for APA, administered on the same dose schedule, was 260 mg/kg.

APA as an inhibitor of the growth of L1210 cells in vitro. APA was compared with MTX as an inhibitor of the growth of L1210 cells *in vitro*. At a dose of 0·1 μg/ml, growth inhibition was 70 per cent for MTX and 11 per cent for APA, i.e. APA was approximately 6-fold less active than MTX in inhibiting the growth of L1210 cells in culture.

DISCUSSION

This study lends experimental support to the hypothesis of Zaharko *et al.*[7,8] that the rapidly migrating or "pre-MTX" faction ,which is seen on DEAE-cellulose chromatography of feces from mice which have received tritium-labeled MTX, is due to

* Kessel[28] has reported a K_i value of 5×10^{-8} M for APA as an inhibitor of dihydrofolate reductase. The differences between our value and that reported by the latter author may reflect the use of different values for the K_m of the substrate, dihydrofolate.

the metabolism of the drug by the intestinal flora, and furthermore, identifies the major intestinal metabolite as APA (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid). That this metabolic process occurs in the gastrointestinal tract is shown by the observation that caecal contents from the mouse can cleave MTX to APA *in vitro*, and this cleavage reaction is largely prevented by pretreatment of mice with antibiotics.

Several investigators have reported that methotrexate can be cleaved at the amide bond by various bacterial species.[10,33–35] The enzyme responsible for some of these reactions has been isolated and identified as a carboxypeptidase.[10,33] It would appear from the results of the present study that bacteria of the mouse gastrointestinal tract produce a similar enzyme. Other examples are known of pharmacological agents which undergo hydrolysis at an amide bond; for example, the intestinal antiseptics, phthalylsulfathiazole and succinylsulfathiazole, owe their activity to the liberation of sulfathiazole as a result of bacterial hydrolysis.

Metabolic alteration of MTX in some mammalian species has been reported by several investigators. MTX is oxidized to 7-hydroxy MTX by the rabbit and guinea pig, although this reaction does not occur to a significant extent in man or in other mammalian species.[3] In man, the presence in urine of small amounts of non-MTX radioactivity after the parenteral administration of the tritium-labeled drug has been described by Johns *et al.*[6] and by Henderson *et al.*[5] The latter group suggested that these possible trace metabolites originate from bacterial cleavage of MTX during enterohepatic circulation of the drug. The quantitative importance of biliary excretion and intestinal reabsorption of MTX in man does not appear to have been determined; the related compound 3′,5′-dichloro MTX, however, is known to undergo extremely active biliary secretion.[36]

It is of interest that, while APA represents a major metabolite of MTX in the feces of the mouse, it is present in only small amounts in the urine; the major urinary pre-MTX metabolites seen in this species are due to compounds with chromatographic properties different from those of APA. The present studies do not establish whether this difference is due to inability of the mouse to absorb APA from its site of production in the gastrointestinal tract or to further metabolism of the compound *in vivo*.

These studies would indicate that the bacterial cleavage of MTX in the gastrointestinal tract can be regarded as a variety of detoxication reaction. The metabolite, APA, is less effective than MTX as an inhibitor of dihydrofolate reductase (Fig. 4 and Ref. 28), and is less toxic than MTX *in vivo*. Since the major site of toxicity of MTX in the mouse and in other mammalian species appears to be the gastrointestinal tract, such a conversion, therefore, could lessen the toxicity of the drug, and the experiments of Bruckner[37] do in fact indicate that blocking of this metabolic pathway by means of antibiotics results in increased MTX toxicity.

REFERENCES

1. J. R. BERTINO and D. G. JOHNS, *A. Rev. Med.* **18,** 27 (1967).
2. J. A. R. MEAD, H. B. WOOD and A. GOLDIN, *Cancer Chemother. Rep.* **1,** 273 (1968).
3. D. G. JOHNS, A. T. IANNOTTI, A. C. SARTORELLI and J. R. BERTINO, *Biochem. Pharmac.* **15,** 555 (1966).
4. E. S. HENDERSON, R. H. ADAMSON, C. DENHAM and V. T. OLIVERIO *Cancer Res.* **25,** 1008 (1965).
5. E. S. HENDERSON, R. H. ADAMSON and V. T. OLIVERIO, *Cancer Res.* **25,** 1018 (1964).
6. D. G. JOHNS, J. W. HOLLINGSWORTH, A. R. CASHMORE, I. H. PLENDERLEITH and J. R. BERTINO, *J. clin. Invest.* **43,** 621 (1964).
7. D. S. ZAHARKO, H. BRUCKNER and V. T. OLIVERIO, *Science, N.Y.* **166,** 887 (1969).

8. D. S. Zaharko and V. T. Oliverio, *Biochem. Pharmac.* **19,** 2923 (1970).
9. M. Webb, *Biochim. biophys. Acta* **17,** 212 (1955).
10. C. C. Levy and P. Goldman, *J. biol. Chem.* **242,** 2933 (1967).
11. P. Goldman and C. C. Levy, *Biochem. Pharmac.* **17,** 2265 (1968).
12. J. McCullough, Ph.D. Thesis, Yale University (1971).
13. D. M. Valerino, D. S. Zaharko, D. G. Johns and V. T. Oliverio, *Fedn Proc.* **30,** 388 (1971).
14. D. G. Johns and D. M. Valerino, *Ann. N.Y. Acad. Sci.* **186,** 378 (1971).
15. E. Gjone, H. G. Vance and D. A. Turner, *Int. J. appl. Radiat. Isotopes* **8,** 95 (1960).
16. K. Randerath, *Analyt. Chem.* **41,** 991 (1969).
17. S. F. Zakrzewski, *Analyt. Biochem.* **36,** 197 (1970).
18. D. R. Seeger, D. B. Cosulich, J. M. Smith and M. E. Hultquist, *J. Am. chem. Soc.* **71,** 1753 (1949).
19. C. W. Waller, B. L. Hutchings, J. H. Mowat, E. L. R. Stokstad, J. H. Boothe, R. B. Anger, J. Semb, Y. Subbarow, D. B. Cosulich, M. J. Fahrenbach, M. E. Hultquist, E. Kuh, E. H. Northey, D. R. Seeger, J. P. Sickels and J. M. Smith, *J. Am. chem. Soc.* **70,** 19 (1948).
20. R. L. Dion and T. L. Loo, *J. org. Chem.* **26** 1857 (1961).
21. C. M. Baugh and E. Shaw, *J. chem. Soc.* 3610 (1964).
22. D. G. Johns and T. L. Loo, *J. pharm. Sci.* **56,** 356 (1967).
23. V. T. Oliverio, *Analyt. Chem.* **33,** 263 (1961).
24. J. T. Litchfield and F. Wilcoxon, *J. Pharmac. exp. Ther.* **96,** 99 (1949).
25. R. H. Adamson, L. G. Hart, V. T. DeVita and V. T. Oliverio, *Cancer Res.* **28,** 343 (1968).
26. J. R. Bertino, J. P. Perkins and D. G. Johns, *Biochemistry, N.Y.* **4,** 839 (1965).
27. J. L. McCullough, P. F. Nixon and J. R. Bertino, *Fedn Proc.* **28,** 878 (1969).
28. D. Kessel, *Molec. Pharmac.* **5,** 21 (1969).
29. W. C. Werkheiser, *J. biol. Chem.* **236,** 888 (1961).
30. D. G. Johns, A. Iannotti, A. Sartorelli, B. Booth and J. Bertino, *Life Sci.* **3,** 1383 (1964).
31. D. G. Johns, *J. clin. Invest.* **46,** 1492 (1967).
32. D. M. Valerino and J. J. McCormack, *Biochim. biophys. Acta* **184,** 154 (1969).
33. J. R. Bertino, P. O'Brien and J. L. McCullough, *Science, N.Y.* **172,** 161 (1971).
34. W. S. McNutt, *Archs Biochem. Biophys.* **101,** 1 (1963).
35. A. G. Pratt, E. J. Crawford and J. J. Friedkin, *J. biol. Chem.* **243,** 6367 (1968).
36. J. D. Davidson and V. T. Oliverio, *Clin. Pharmac. Ther.* **6,** 321 (1965).
37. H. Bruckner, *Fedn Proc.* **28,** 263 (1969).