POLYGAMMAGLUTAMYL METABOLITES OF METHOTREXATE

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Summary. Heretofore unrecognized metabolites of methotrexate (MTX) have been detected in human red blood cells and isolated from rat liver and viscera. The metabolites from the rat were identified as 2,4-diamino-N^{10}-methylpteroylglutamyl-γ-glutamic acid [MTX(G_1)] and 2,4-diamino-N^{10}-methylpteroylglutamyl-γ-glutamyl-γ-glutamic acid [MTX(G_2)] by comparison with authentic synthetic compounds.

Methotrexate has been in clinical use for more than two decades,[1] becoming an important chemotherapeutic agent. Presently it is used either alone or in combination with other drugs in treating leukemia,[2] Burkitt's lymphoma,[3] and choriocarcinoma[4] as well as psoriasis,[5] and for suppression of the immune response.[6] Studies on the mechanism of action of methotrexate (MTX) and its metabolism in a variety of species have resulted in a very large literature. Although the rabbit[7] and the guinea pig[8] possess the ability to metabolize MTX and its 3',5'-dichloro analog by hydroxylation at the seven position on the pteridine nucleus, no other species studied has been found capable of performing this or any other metabolic alteration of these drugs. Studies in man, monkeys, mice, rats, and dogs indicate that MTX may, in large part, be recovered from the urine and feces as unaltered drug within 24 hours.[9,10] However, a portion of the drug is retained in the tissues for long periods; the major fraction of this material (half-life about three months) may be recovered from the liver. Significant portions of the long half-life MTX may also be recovered from the intestine, stomach, and kidneys.[9,11] One theory which has been offered to explain the retention of MTX for long periods involves its irreversible binding to intracellular dihydrofolate reductase.[12]

Studies in our laboratories on the natural folates and particularly their poly-γ-glutamyl derivatives have made it apparent that these derivatives coexist with enzymes which can carry out their degradation, the pteroylglutamyl-γ-gluta-

myl carboxypeptidases (conjugases). It became clear that the isolation of folyl-polyglutamates from natural sources required special conditions to prevent their degradation. Suitable conditions have been developed.[13] These involve the homogenization of the tissue in 6 or 8M urea and chloroform. The urea serves to unfold protein and release the folates while the chloroform denatures and precipitates the protein, facilitating its removal. These techniques have been applied to the isolation of intracellular MTX and other pteroylpolyglutamates.[13]

Preliminary studies were carried out by paper chromatography of a urea extract from the red blood cells of a patient with acute leukemia being maintained in remission by MTX therapy.[14] The patient had received 150 mg of MTX intravenously at two-week intervals for several months, but had not received MTX for two weeks prior to drawing the blood sample. About 50 ml of packed, saline-washed red cells were homogenized with 50 ml of 8M urea and 250 ml chloroform in the Waring Blendor. The top urea and bottom chloroform layers were strained off from the middle layer of gelled protein, separated, and the chloroform discarded. The urea layer was diluted 1:4 with water and applied to a 2 x 8 cm column of DEAE in the chloride form. The column was washed with water, then stripped with 0.5N NH_4OH, and the ammonium eluate concentrated to about 10 ml *in vacuo*. This solution was spotted on paper along with authentic MTX and the chromatograms developed in isopropyl alcohol/NH_4OH/H_2O (7/1/2) and n-butyl alcohol/acetic acid/H_2O (4/1/5) by volumes. After drying, the chromatograms were analyzed by incubation on agar plates containing complete medium, folic acid, and seeded with *Streptococcus faecium* ATCC 8043. Two zones of inhibition were seen. When the inhibition assay was repeated after the chromatograms were either sprayed or dipped with hog kidney conjugase at pH 4.5, two additional spots of inhibition were seen. The R_f values of the inhibitory materials suggested regularly increasing anionic properties, such as previously shown to be characteristic of the poly-γ-glutamates of folic acid. The appearance of new inhibitory spots after conjugase treatment gave impetus to the design of more definitive experiments aimed at identifying these metabolites, and provided a working hypothesis for their structures.

An attempt to determine the molecular weights of these metabolites was conducted in two adult male rats following three daily injections of 3',5'-H^3MTX, specific activity 100 c/μmole, totaling 148 μc of labeled compound. Two days after the last injection the animals were sacrificed, the livers excised, frozen immediately in liquid freon, homogenized in cold 6M urea, and dialyzed. A charcoal eluate prepared from the dialysates was then subjected to chromatography on a molecular sieve column (Corning porous glass-10, 75 Å) coated with polyethylene glycol.

Two radioactive peaks which behaved as compounds of molecular weight higher than authentic methotrexate were clearly resolved. The heavier compound was inhibitory for *Lactobacillus casei* ATCC 7469, but inhibited *S. faecium* only after conjugase treatment, suggesting a triglutamate structure. Because of adsorption phenomena of MTX and its derivatives, it was impossible to estimate the molecular weights of the new metabolites using the coated porous glass bead columns, and further refinements in technique became necessary.

Two additional rats were injected intraperitonealy on two occasions four days apart with 0.12 μmoles MTX uniformly labeled with C^{14} in the glutamyl moiety,[15] specific activity 39 x 10^6 cpm/μmole. Two days after the final injection, the animals were anesthesized with ether and the liver, kidneys, spleen, and small intestine were removed. The intestine was cleaned and all tissues washed in ice cold isotonic saline, blotted dry, and their combined weight rapidly determined. They were next added to a volume of 6M urea in milliliters equal to five times their combined wet weight in grams in a 1 liter Waring Blendor. A volume of chloroform equal to five times the urea volume was added, and the Blendor run for four minutes. The homogenate was centrifuged for 310,000 g-minutes at room temperature. The middle layer of gelled protein was pushed aside with a spatula and the urea and chloroform layers transferred to a separatory funnel. The chloroform layer was drawn off, determined to have no radioactivity, and discarded. More than 90% of the total radioactivity of the homogenate (5% of the amount injected) was recovered in the urea. The urea extract was diluted 1:4 with water and applied to a 1.2 x 30 cm column of DEAE

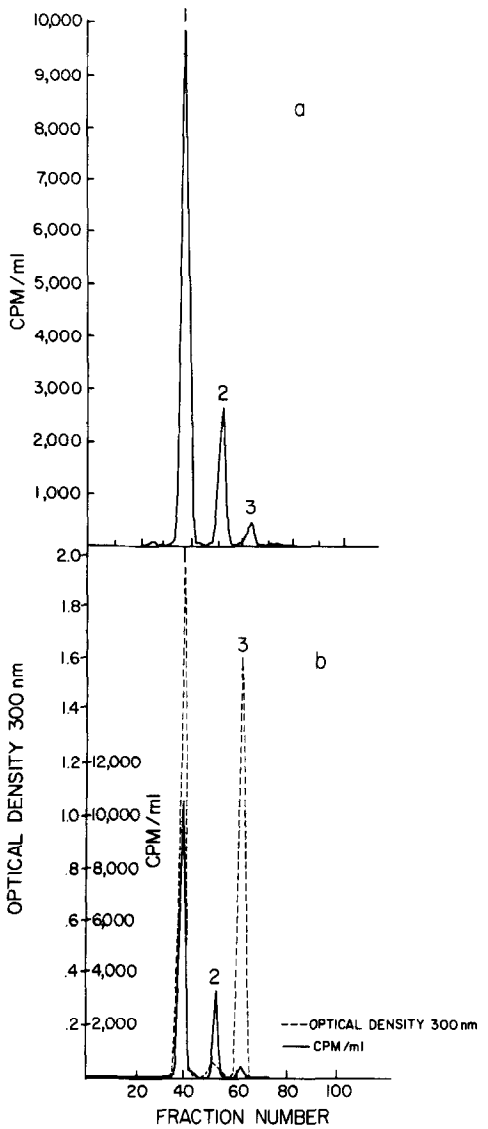

FIGURE I

Figure Ia shows the radioactive elution profile after DEAE cellulose chromatography of a urea extract of rat viscera. The rat had previously been injected with methotrexate labeled in the glutamyl moiety with C^{14}. See text for details.

Figure Ib shows the rechromatography of the pooled peaks 1, 2, and 3 from Ia, after the addition of non-radioactive carrier MTX and MTX(G_2).

cellulose chloride. The column was washed with 100 ml water, then eluted with a linear gradient of 1 liter 0.005M phosphate buffer pH 7.0 in the mixing chamber and 1 liter 0.5M NaCl in the same buffer in the reservoir. The radio-

active elution profile may be seen in Figure Ia. Assuming that these metabolites are poly-γ-glutamyl derivatives of MTX, and hoping to establish the range of the number of glutamyl groups involved, non-radioactive carrier MTX and MTX(G_2) were added to the pooled peaks 1, 2, and 3 of radioactivity seen in Figure Ia. The mixture was diluted 1:5 with water and rechromatographed on DEAE cellulose as before. As may be seen in Figure Ib, a precise correlation between the carrier compounds monitored in the ultraviolet at 300 mμ and radioactivity was obtained. Peaks 1 and 3 (Figure I) were thus provisionally identified as MTX and MTX(G_2), respectively. The MTX (peak 1) was not examined further. The column fractions in peaks 2 and 3 in Figure Ib were pooled and 5 μmoles non-radioactive carrier MTX(G_1) was added. The pool was diluted 1:5 with water and rechromatographed on DEAE as before. The elution pattern may be seen in Figure II. On the basis of the close correspondence between the absorption of UV light and radioactivity, these metabolites were assigned the structures of the mono- and di-γ-glutamyl derivatives of methotrexate.

Further evidence on the structure of these metabolites was obtained by performing an enzymatic degradation and identifying the product. Hog kidney conjugase was prepared by the procedure of Iwai.[16] The ability of polyglutamates of MTX to serve as substrate for this enzyme was determined by incubating 5 μmoles of MTX(G_2) with 3.0 ml of the conjugase preparation at pH 4.5 for 2 hours at 37°. The reaction was terminated with 10% CCl_3COOH and protein centrifuged out. A trace (0.3 OD units @ 300 mμ) of high specific activity H^3MTX marker was added and the supernatant neutralized and repurified by DEAE chromatography as described. Figure IIIa shows that the MTX(G_2) serves as substrate for hog kidney conjugase. Most of the MTX(G_2) was hydrolyzed to MTX as evidenced by the elution of UV absorbing material along with the H^3 marker. The fractions from peaks 2 and 3 of Figure II were combined. adjusted to pH 4.5, and incubated with the hog kidney conjugase for 2 hours. The reaction was terminated and chromatographed along with a trace of H^3MTX marker as above. The UV absorption at 300 mμ and the counts from both H^3 and C^{14} are clearly superimposible

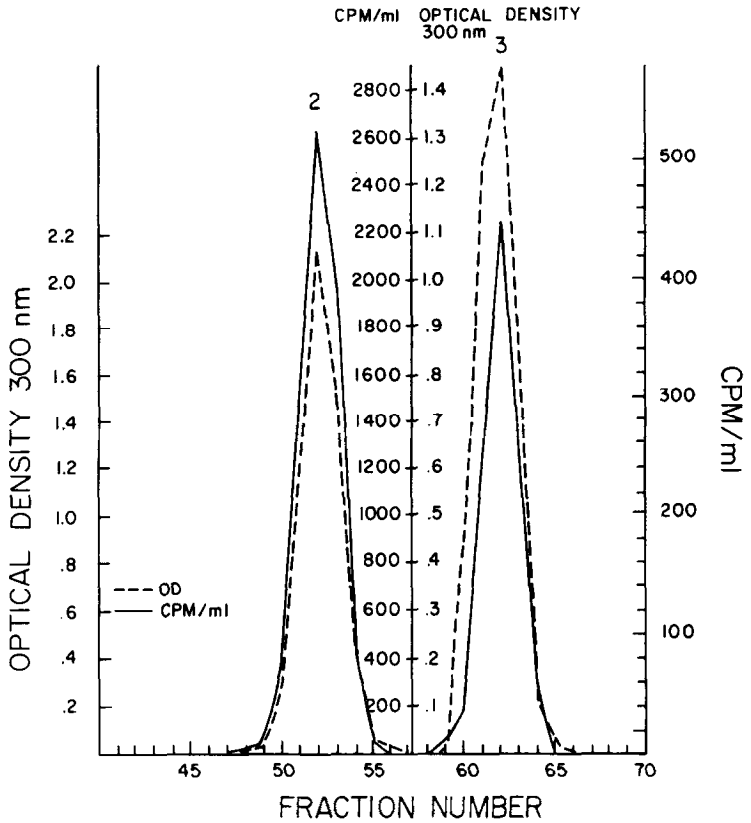

Figure II depicts the agreement of absorption of UV light at 300 mμ with radioactivity (C^{14}) upon rechromatography on DEAE cellulose columns of peaks 2 and 3 from Figure I after the addition of non-radioactive carrier MTX(G_1) and MTX(G_2).

(Figure IIIb) and provide additional evidence of the poly-γ-glutamyl nature of these metabolites of methotrexate.

Discussion. Chromatographic studies and enzymatic degradations carried out with metabolites of MTX isolated from the rat permit the assignment of structure to these derivatives. These compounds are the mono- and di-γ-glutamyl derivatives of methotrexate. Experiments not reported here suggest that higher poly-γ-glutamyl derivatives are also formed.

It is not practical, based on the data presently available, to speculate on the role that these derivatives might play in MTX chemotherapy. Clearly these newly discovered metabolites must now be considered in any hypothesis concerning the mechanism of action of this drug. An alternate explanation for the reten-

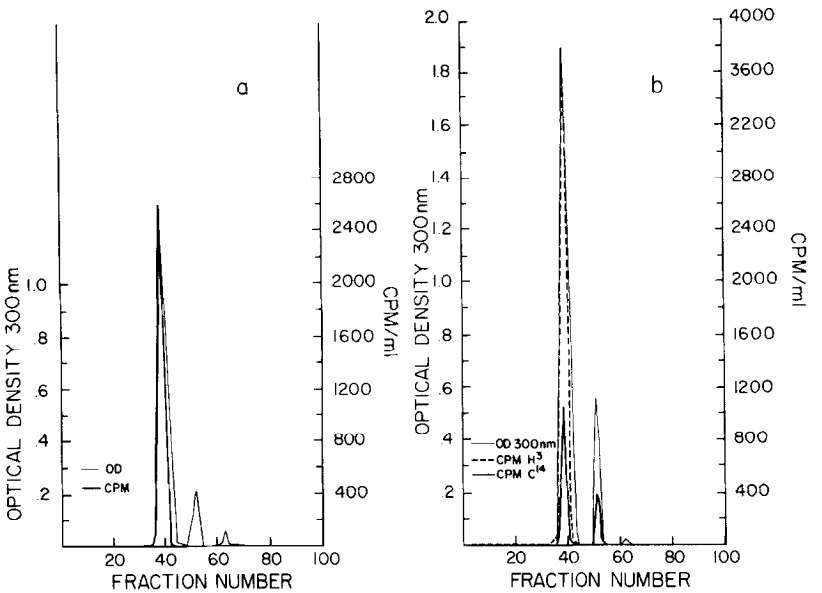

Figure IIIa shows the conversion of MTX(G_2) to MTX by a crude preparation of hog kidney conjugase. A trace of H^3MTX was used as a marker for the MTX. A small amount of what is presumed to be MTX(G_1) may be seen around fraction #50.

Figure IIIb shows the rechromatography of the pooled peaks 2 and 3 from Figure II after treatment with hog kidney conjugase. Again a trace of H^3MTX was used as marker.

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tion of MTX inside cells is now at hand. Methotrexate appears to follow the biosynthetic routes of the vitamin folic acid in being raised to poly-γ-glutamyl derivatives. If the mammalian biosynthetic system for pteroylpoly-γ-glutamates is similar to the one found in bacteria[17] it is likely that reduction of the pteridine nucleus must precede these biosynthetic reactions.

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