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Specific activity of hepatic formyltetrahydrofolic acid synthetase and formiminotetrahydrofolic acid transferase after administration of methotrexate to chicks*

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THE ANTINEOPLASTIC action of methotrexate is the result of inhibition by the drug of the enzyme, dihydrofolate reductase.[1] Cellular resistance to the action of methotrexate has already been shown to result from decreased permeability of the cell to the drug,[2,3] and from increased activity of dihydrofolate reductase.[4,5] Increased activity of the tetrahydrofolic acid-dependent enzyme, formyltetrahydrofolic acid synthetase, has been proposed as a third mechanism providing resistance to methotrexate in a mutant bacterial strain of *Streptococcus faecalis*.[6] However, an acquired increased activity of this enzyme in response to methotrexate administration has not been demonstrated. The purpose of this study was to determine if administration of methotrexate to chicks resulted in an acquired increased specific activity of this enzyme in liver tissue. Specific activities of another tetrahydrofolic acid-dependent enzyme, hepatic formiminotetrahydrofolic acid transferase, were also studied, and concentrations of amethopterin of the liver samples were measured. Activities of the target enzyme for methotrexate, dihydrofolic acid reductase, were also assayed.

Forty-eight 1-day-old male White Leghorn chicks were used in the experiment. All chicks were fed Purina Chick Startena (Purina Ralston Co.) *ad lib.* and given free access to water. Twenty-four chicks were injected subcutaneously with methotrexate (Lederle Laboratories), 3·5 mg/kg body weight, twice weekly on the first and fourth days of the week. Control chicks were given saline injections. One control and one treated chick were sacrificed daily by decapitation on the first through the fourth days of each week for 6 weeks, and livers were immediately removed and placed on ice. Thereafter, all procedures, unless indicated otherwise, were performed at 4°. Livers were homogenized in the following buffers with a Teflon-glass hand homogenizer: for the dihydrofolic acid reductase assay, 10 vol. of 0·05 M potassium phosphate, pH 7·5; for the formiminotetrahydrofolic acid transferase assay, 10 vol. of 0·5 M triethanolamine sulfate, pH 7·2; and for the formyltetrahydrofolic acid synthetase assay, 20 vol. of 0·1 M tris, pH 7·0. All samples were centrifuged for 1 hr at 1900 *g*. Enzyme assays were performed within 6 hr using the supernatant fractions. To assure linearity, all assays were performed with more than one concentration of the enzyme. Protein concentrations were determined by the method of Lowry *et al.*,[7] except for those of the formiminotetrahydrofolic acid transferase assay, which were measured by the biuret method.[8] The blank solutions for the biuret determinations were formed by removal of protein from the homogenates by precipitation with trichloroacetic acid. Crystalline serum bovine albumin was used for protein standards. Formyltetrahydrofolic acid synthetase activity was assayed by the method of Albrecht *et al.*[6] Calculation of quantity of product formed in this and the subsequent assay was based upon a molar extinction value for 5,10-methenyltetrahydrofolic acid of 25,000.[9] Formiminotetrahydrofolic acid transferase activity was assayed as described by Tabor and Wyngarden,[10] except that the reaction was allowed to continue for 6 min. Dihydrofolic acid reductase was assayed by the method of Misra *et al.*[5] Dihydrofolate used in the assay was prepared by the method of Futterman.[11] Calculation of results was based upon a combined extinction coefficient of 12,300 for oxidation of NADPH and for conversion of dihydrofolate to tetrahydrofolate.[12] The assay of methotrexate of liver extracts utilized the enzymatic method of Bertino *et al.*[13] with modifications for the direct assay described by Martelli *et al.*[14] Extracts were prepared by heat precipitation and removal of protein from the homogenates prepared for the reductase assay. Inhibition of activity of partially purified dihydrofolic acid reductase by the extract was measured and compared with inhibition produced by known quantities of methotrexate. Enzyme for the assay was obtained from chick liver by purification through the precipitation step with 85% ammonium sulfate as described by Mathews and Huennekens.[15] Precipitated enzyme was resuspended and dialyzed for 12 hr against 1 l. of 0·005 M potassium phosphate, pH 7·5. Concentrations of enzyme were used in the assay which produced a change of absorbance of approximately 0·20 O.D. when no inhibitor was added. The assay was thereafter performed as described, except that change of absorbance was followed over a 15-min period.

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TABLE 1. SPECIFIC ACTIVITIES OF FORMYLTETRAHYDROFOLIC ACID SYNTHETASE, FORMIMINOTETRAHYDROFOLIC ACID TRANSFERASE AND DIHYDROFOLIC ACID REDUCTASE IN CHICKS TREATED WITH METHOTREXATE AND IN UNTREATED CONTROLS*

Chicks	Group I Weeks treated (0–2)	Group II Weeks treated (2–4)	Group III Weeks treated (4–6)	Enzyme assayed
Treated	37 ± 4 (8)	29 ± 3 (7)	20 ± 1 (7)	Formyltetrahydrofolic acid
Untreated	50 ± 4 (9)	28 ± 2 (7)	18 ± 3 (7)	synthetase
Treated	1·12 ± 0·13 (8)	2·25 ± 0·24 (7)	2·84 ± 0·34 (6)	Formiminotetrahydrofolic
Untreated	1·30 ± 0·26 (9)	1·87 ± 0·33 (7)	2·61 ± 0·34 (6)	acid transferase
Treated	0·73 ± 0·16 (8)	0·99 ± 0·25 (7)	0·77 ± 0·14 (7)	Dihydrofolic acid reductase
Untreated	2·73 ± 0·36 (9)	4·32 ± 0·73 (7)	3·98 ± 0·45 (6)	

* The specific activities are expressed in millimicromoles of product formed per minute per milligram of protein. The values are means ± S. E. The number of chicks in each group is given in parentheses.

Results are listed in three groups according to duration of drug administration. Chicks of group I were treated 0–2 weeks and received 1–4 injections of methotrexate. Chicks of group II were treated 2–4 weeks and received 5–8 injections, and group III chicks were treated 4–6 weeks and received 9–11 injections. Assay values of hepatic enzymes were averaged for each group and compared to a comparable number of assays from untreated chicks of the same age. Specific enzyme activities are summarized in Table 1. Concentrations of hepatic methotrexate are summarized in Table 2. As can be seen, specific activities of formyltetrahydrofolic acid synthetase and of formiminotetrahydrofolic acid transferase were no different in liver of chicks treated with methotrexate as compared with controls. These findings are in accordance with results of studies of tetrahydrofolate-dependent enzymes of other tissues and in different species.[16–18] Depressed dihydrofolate reductase activity in treated chicks reflects expected inhibition by the drug. Assay of methotrexate of liver homogenates revealed high concentrations in all treated animals. Levels of methotrexate required to produce saturation of all hepatic drug-binding sites for three different animal species (rat, mouse and guinea pig) were found by Werkheiser[19] to be respectively 1·0, 0·78 and 1·03 mμmoles/g liver. Concentrations measured in liver of treated chicks exceeded these saturation values.

In conclusion, the data of this experiment show that administration of methotrexate, 3·5 mg subcutaneously per kg of body weight, twice weekly for up to 6 weeks resulted in high levels of the drug in liver of chicks and produced demonstrable inhibition of the target enzyme, dihydrofolic

TABLE 2. METHOTREXATE CONCENTRATIONS OF LIVER HOMOGENATES OF CHICKS TREATED WITH THE DRUG*

Chicks	Hepatic methotrexate (mμmoles/g protein)
Group I (treated 0–2 weeks)	2·6 ± 0·4 (8)
Group II (treated 2–4 weeks)	3·2 ± 0·1 (6)
Group III (treated 4–6 weeks)	3·0 ± 0·2 (7)

* The values are expressed as means ± S. E. The number of chicks in each group is given in parentheses.

acid reductase. However, adaptive increased specific activities of two hepatic tetrahydrofolate-dependent enzymes, formyltetrahydrofolic acid synthetase and formiminotetrahydrofolic acid transferase, could not be demonstrated.

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Department of Pediatrics,
The University of Texas,
(Southwestern) Medical School at Dallas,
Dallas, Texas, U.S.A.

LON E. ROGERS

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In vivo effects of carbon tetrachloride and chloroform on liver and kidney glucose-6-phosphatase in mice

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ALTHOUGH biochemical changes in the liver following the administration of carbon tetrachloride and chloroform are wellknown,[1–8] little is known about biochemical changes occurring in the kidney. We have compared the effects of $CHCl_3$ and CCl_4 on glucose-6-phosphatase activity in mouse liver and kidney.