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SHORT COMMUNICATIONS

Reinvestigation of methotrexate metabolism in rodents

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IN RECENT studies we observed that host bacteria of mice are probably responsible for the metabolites of methotrexate (MTX) found in the urine and feces.[1] It has also been observed that MTX is more toxic to rats than mice.[2, 3] Approximately four times as much MTX is needed to effect an LD_{10} in mice than in rats when doses are compared on the basis of body surface area (mg/m^2). An even greater difference is observed when the doses are compared on the basis of body weight (mg/kg).[2] These observations suggest the hypothesis that the difference in toxicity of MTX in these two species is related to the inability of host bacteria of the rat to metabolize MTX. For these reasons we re-examined by chromatographic techniques the urine and feces of rats (Sprague–Dawley, NIH colony) and mice (CDF_1) at carefully chosen appropriate time intervals after the administration of similar dose levels of MTX.

Materials and methods

Chromatographically purified 3′,5′-tritiated MTX was used for all experiments.[4] All doses were given intraperitoneally. The dose was 21 mg/m^2 to both species. If this dose was given to mice on a QD 1–5 day schedule, it would approximate two times the LD_{10}.[2] The reasoning was that if this quantity was toxic to mice, based on past data, it would be expected to be four times as toxic to rats. Therefore, if metabolism by bacteria did, indeed, contribute to the cause of this toxicity difference, then the difference in metabolism at this dose should be apparent between the two species. The quantity of radioactivity given was 25 μc/rat whose body weights ranged 300 $\pm$ 50 g and 16 μc/mouse (24 $\pm$ 2 g body weight). The times for collection of urine and feces were based on previous findings that the plasma behavior of MTX suggest that its kidney and biliary removal is twice as fast in mice than in rats.[5] Thus, 0–3 hr and 3–6 hr in mice might be expected to be equivalent to 0–6 hr and 6–12 hr, respectively, in rats.

The urine and feces were collected separately for the above stated times in each species and then the animals were killed. The contents of the bladder were added to the last collected urine, and the contents of the cecum and rectum were included with the collected feces. The urine was lyophilized immediately and the dried residue was dissolved in 0·1 M NH_4HCO_3 buffer, pH 8. Approximately 1 mg of unlabeled carrier MTX was added, and the solution was chromatographed on diethylaminoethyl ion-exchange cellulose column for separation into its various components.[4] Feces were frozen and thawed to disrupt cells and then boiled for 5 min to free any bound MTX. The fecal mixture was centrifuged and the supernatant was treated in the same way as the urine. In control experiments, over 94 per cent of the MTX was recovered chromatographically when added to frozen urine or feces and then subjected to the above procedure.

Results and discussion

Where, in fact, we expected to find few metabolites in the rat urine and feces, if the hypothesis held, we found as many as, if not more, metabolites (see Table 1). In the urine over the second timed period of collection, 10 per cent of the radioactive dose is excreted in both species.

In the rat, only 13 per cent of this radioactivity is associated with MTX and in the mouse, 40 per cent is associated with MTX. In the feces of the rat, 44 per cent of the radioactivity given is excreted and only 33 per cent of this radioactivity is associated with MTX. In mouse feces, 32 per cent of the radioactive dose is excreted and 42 per cent of this radioactivity is associated with MTX. In the urine collected during the initial time interval, 0–3 hr in mice and 0–6 hr in rats, the amount of total radioactivity excreted is 39 and 38 per cent respectively. MTX dominates during this time interval. Over 90 per cent of the urine radioactivity is associated with the parent compound. These findings suggest that the majority of the radioactivity in the blood perfusing the kidney during the first time interval was associated with MTX, whereas during the second time interval it was not. A likely explanation is that a significant amount of the drug had not yet moved sufficiently far down the intestinal lumen, after biliary excretion, to be at sites of bacterial metabolism during the early time period.

TABLE 1. RELATIVE EXCRETION AND CHROMATOGRAPHIC FRACTIONATION OF RADIOACTIVITY IN URINE AND FECES*

Sample	Time (hr)	Percentage of radioactivity excreted	Percentage of radioactivity associated with MTX	Pre MTX	Post MTX
Rats					
Urine	0–6	38 (33–42)	97 (95–98)	3 (2–4)	0·4 (0·3–0·8)
Urine	6–12	10 (4–17)	13 (7–23)	87 (77–91)	0
Feces	0–12	44 (38–51)	33 (22–40)	64 (57–77)	3 (1–6)
Mice					
Urine	0–3	39	90	8	2
Urine	3–6	10	40	56	4
Feces	0–6	32	42	51	7

* Date represent average of three rats (range in parentheses) and pooled samples for three mice.

Even though the hypothesis that the difference in toxicity of MTX between rats and mice is because of its lack of metabolism in rats was not substantiated, the results are interesting from another point of view. They emphasize the role that intestinal bacteria may play in the metabolism of MTX. Significant bacterial metabolism has now been detected in two species in which previously metabolism was thought to be insignificant. This phenomenon may also play a significant role in the other species, including man.

Long term studies *in vivo* using radioactive labeled MTX must be interpreted with care. The critical time period is, of course, dependent upon the species being studied. A recent paper describing kinetic studies in mice[6] reiterates the fact that a time delay of approximately 3 hr exist before MTX which is excreted in the bile and moves down the gut lumen reaches the cecum in significant quantities after intravenous injection. In larger species, this time period is no doubt increased. Reported data in the literature for small intestine transit times are 6 hr in rats.[7] In man, gastrointestinal transit time varies from 24 hr to 9 days.[8]

In light of the findings of similar degrees of metabolism of MTX in rats and mice, we are again faced with the problem of providing an explanation for the difference in toxicity between rats and mice. There are many literature reports which allude to possible mechanisms. Species differences in cellular permeability,[9] dissociation constants of the drug from dihydrofolate reductase,[10] quantity of drug needed to deplete dihydrofolate reductase,[11] cells ability to synthesize new dihydrofolate,[12] all may be possible. We feel that species differences in critical exposure time of the sensitive tissues to MTX based on the kinetics of distribution in that species provide yet another explanation which has not yet been thoroughly investigated.

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Enzymic acetylation of the stereoisomers of α- and β-methyl choline*

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A NUMBER of workers have investigated the *N*-alkyl group substrate specificity of choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase; EC 2.3.1.6) (ChAc),[1–4] and in a recent study we reported on the enzymic acetylation of a group of choline analogues where the methyl groups on the quaternary nitrogen atom were replaced successively by ethyl groups.[5] The results suggested that replacement of methyl by ethyl groups resulted in a lower affinity of the substrate to the active site of the enzyme and that the rate of acetylation was proportional to the binding force of the quaternary nitrogen to a negative charge at the active site of the enzyme.

In the present work we have attempted to elucidate further the mode of interaction of choline and some choline analogues with ChAc. Choline analogues were used with methyl groups substituted in the methylene chain. These compounds possess an asymmetric carbon atom and the stereoisomers of both α- and β-methyl choline were employed.

Partially purified ChAc was prepared from bovine caudate nucleus as described previously.[5] ChAc activity was determined using ^{14}C acetyl-CoA as substrate by a modification of the method of McCaman and Hunt[6] and the incubation conditions were as described by Hemsworth and Smith.[5]

The choline analogues D-(+)-α-methyl choline, L-(−)-α-methyl choline, D-(−)-β-methyl choline and L-(+)-β-methyl choline were prepared by Prof. A. H. Beckett and Dr. J. W. Clitherow, Chelsea School of Pharmacy, London University, England. The absolute configurations of these compounds have been established by Beckett *et al.*[7] (+)-α-Methylcholine was shown to be related to D-(−)-alanine hydrochloride and (+)-β- methylcholine to L-(+)-lactic acid. The stereospecificity of these compounds can be confirmed by the use of Cahn's "sequence rule"[8] with the aid of CPK and Prentice-Hall framework molecular models.

Concentrations of substrate from 10^{-5} to 2×10^{-2} M were employed. Time studies demonstrated that the rates of acetylation of choline and the choline analogues were linear for at least 15 min at the concentrations and under the incubation conditions employed. In this study, 15-min incubations were used to obtain the presented data. L-β-Methyl choline was not a substrate for ChAc. The other compounds were acetylated at a lower rate than choline.

Choline showed substrate inhibition as reported previously,[4, 5] but up to 2×10^{-2} M none of the other substrates exhibited substrate inhibition. It has been shown previously that ChAc is a two substrate enzyme and each substrate affects the affinity of the other for the enzyme.[9] At a constant concentration of acetyl-CoA, apparent Michaelis–Menten constants were determined for each substrate from the graphs (Fig. 1) according to the method of Lineweaver and Burk,[10] and the apparent V_{max} for each substrate was also determined (Table 1). Choline has a much lower apparent K_m than the other choline analogues, indicating that choline has a greater affinity for ChAc.

Models of choline and the choline analogues are shown in Fig. 2. If the choline molecule is assumed to adopt the conformation shown when bound to the catalytic site of ChAc, then the relative rates of acetylation of the other substrates could be explained in terms of interference by the added methyl groups with binding of the substrate molecule to the active site of the enzyme or with transfer of the acetyl group to the substrate hydroxyl group. D-α-Methylcholine was a better substrate for ChAc than

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