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DEMETHYLATION OF TRIMETHADIONE AND METHARBITAL BY RAT LIVER MICROSOMAL ENZYMES: SUBSTRATE CONCENTRATION–YIELD RELATIONSHIPS AND COMPETITION BETWEEN SUBSTRATES*

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Abstract—A study has been made of the relationship between substrate concentration and yield and of competition between substances in the demethylation of trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione) and metharbital (5,5-diethyl-1-methyl barbituric acid) by microsomal enzymes of rat liver. The syntheses of trimethadione-^{14}C and metharbital-^{14}C for use as substrates are described. Trimethadione has a much lower affinity for the demethylating enzyme or enzymes than has metharbital. Each of the two substrates has an inhibitory effect on the demethylation of the other. 5,5-Dimethyl-2,4-oxazolidinedione (DMO), the product of dimethylation of trimethadione, has an inhibitory effect about equal to that of trimethadione on the demethylation of metharbital. DMO also inhibits the demethylation of trimethadione.

MANY N-methyl derivatives of the closely related heterocyclic structures, barbituric acid, hydantoin, and 2,4-oxazolidinedione, have recently been shown to be demethylated by enzymes localized in the microsomal fraction of homogenates of rat liver.[1–3] Although the demethylation of most of the compounds of this type proceeds more slowly than that of the N-methyl compounds of other chemical types that had previously been studied, all the microsomal N-demethylation reactions that have been investigated have in common the production of formaldehyde and the requirement for NADPH and oxygen. Pretreatment of animals with a number of different drugs has been found to cause large increases of microsomal enzyme activity.

The present report concerns a study of the relationship between substrate concentration and yield and of competition between substrates in the demethylation of two N-methylated heterocyclic compounds by rat liver microsomal enzymes. The two compounds chosen for study are trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione) and metharbital (5,5-diethyl-1-methyl barbituric acid). The products of demethylation are, respectively, 5,5-dimethyl-2,4-oxazolidinedione (DMO) and 5,5-diethyl barbituric acid (barbital). Trimethadione and metharbital were selected as the substrates for this study because they represent two different heterocyclic structures, because they have widely different affinities for the demethylating enzymes, and because the products of demethylation undergo no further metabolic change. In the present experiments microsomal enzyme activity was increased by pretreatment of the rats with

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nikethamide. Because of difficulty in developing u.v. spectrophotometric methods of adequate sensitivity and accuracy for determination of the demethylated products, ^{14}C-labeled substrates were used.

METHODS AND MATERIALS

Synthesis of radioactive compounds. Barbital-^{14}C was synthesized from urea-^{14}C of specific activity 0·05 mc/mmole. In a glass-stoppered tube 600 mg (10 mmoles) of urea-^{14}C was dissolved in 20 ml butanol containing 30 mmoles sodium butylate. Diethyl diethylmalonate in an amount of 2·2 ml (10 mmoles) was added, and the tube was heated at 110° for 3 hr. It was then cooled in an ice bath and 10 ml water was added. The tube was shaken, and the aqueous phase was transferred to another tube. The butanol was extracted with 5 ml of 1 N NaOH, which was added to the first aqueous phase. The combined aqueous extracts were washed with 10 ml ethyl ether. They were then acidified with HCl and extracted with five 10-ml portions of ether. The combined ether extracts were washed with 10 ml water and evaporated. Weight of residue: 1·26 g (69 per cent yield); m.p. 185–190° corr.

Metharbital-^{14}C was prepared from the barbital-^{14}C described above. Barbital-^{14}C in an amount of 920 mg (5 mmoles) was dissolved in 5·1 ml of 1 N NaOH. At intervals over a period of an hour a total of 0·48 ml (5 mmoles) dimethyl sulfate was added in several portions. The preparation stood for an hour after the last addition. It was then acidified with HCl and extracted with 5 ml methylene chloride. The methylene chloride was washed with four 5-ml portions of water and then evaporated. The residue was crystallized twice from ethanol–water. Yield: 406 mg (41 per cent); m.p. 155·5–156·0° corr.

Trimethadione-^{14}C was synthesized from DMO-^{14}C which had been prepared by the method of Butler and Davidson.[4] To 1·0 g (7·75 mmoles)of DMO-^{14}C of specific activity 0·05 mc/mmole in a glass-stoppered test tube, was added 10 ml ethanol containing 1 equivalent of sodium ethylate. Dimethyl sulfate in an amount of 0·73 ml (7·75 mmoles) was added and the tube was heated at 70° for 2 hr. The ethanol was evaporated by drawing over a stream of air with an impinger. The residue was extracted with two 10-ml portions of ethyl ether. The combined ether extracts were evaporated to a volume of 10 ml and washed with two 2·5-ml portions of a 1 M phosphate buffer (pH 8·0) and then with two 2·5-ml portions of water. The ether solution was dried with anhydrous Na_2SO_4 and evaporated. Weight of residue: 595 mg (45 per cent yield); m.p. 45·5–47·2°.

Animals. Male Wistar rats weighing 300 to 500 g were used. On each of the 3 days preceding the day on which a rat was to be killed, it received an i.p. injection of 200 mg nikethamide/kg.

Microsome preparation. The rats were decapitated and the livers removed immediately. The liver was homogenized in a glass homogenizer, 4 ml of a cold 0·2 M potassium phosphate buffer (pH 7·4) being used for each gram of liver. The homogenate was centrifuged at 9,000 *g* for 30 min in a refrigerated centrifuge. The supernatant, containing microsomes and the soluble fraction, was used in the incubations.

Incubations. In a 25-ml flask 2·5 ml of the microsome preparation was added to 1·5 ml of a solution containing 100 μmoles nicotinamide, 1·4 μmoles NADP, 10 μmoles glucose-6-phosphate, 50 μmoles $MgCl_2$, 140 μmoles K_2HPO_4, and 35 μmoles KH_2PO_4. Solutions of the substrate or substrates in saline in a total volume of 2·0

ml were then added. The flask was flushed with O_2 and shaken at 38° for 30 min. Incubations containing both metharbital and trimethadione were made in duplicate, one containing metharbital-^{14}C and unlabeled trimethadione and the other trimethadione-^{14}C and unlabeled metharbital. In each experiment there was a flask to which 100 μg barbital-^{14}C of specific activity 0·05 mc/mmole was added and one to which 50 μg DMO-^{14}C of specific activity 0·05 mc/mmole was added. These served as standards.

Extractions. After completion of an incubation, 2 ml of 5 M NaH_2PO_4 was added to the contents of the flask. Extraction was carried out with two 10-ml portions of ethyl ether. The combined ether extracts in a glass-stoppered tube were washed with 5 ml of 1 N HCl and then evaporated almost to dryness by drawing over air with an impinger. To the tube was added 10 ml benzene, which was then extracted with two 5-ml portions of a 0·1 M borate buffer (pH 9·0). The combined buffer extracts were washed with 5 ml benzene and then acidified with 0·5 ml concentrated HCl. This acidified solution was washed with 1 ml methylene chloride and then extracted with 20 ml ethyl acetate. The ethyl acetate was washed with 4 ml water. It was then evaporated to a small volume with an impinger and spotted on paper for chromatography.

This extraction procedure eliminates all the nicotinamide and reduces the amounts of the unreacted methylated substrates sufficiently so that they do not interfere with chromatography. The percentages of the various compounds originally in the incubation mixture that will remain in the final extract are: barbital 78 per cent, DMO 64 per cent, metharbital 17 per cent, trimethadione 2 per cent.

Chromatography was carried out on Whatman 2 paper in descending systems. In most of the experiments benzene was the solvent. The R_F values in this system are: barbital 0·2, DMO 0·3, metharbital 0·9, trimethadione 0·9. In the experiments in which DMO in high concentrations was studied as an inhibitor of the demethylation of metharbital, DMO interfered with chromatography in this system. In these experiments paper was used that had previously been dipped in 0·5 N NaOH and dried. The solvent was ethyl acetate. In this system DMO is retained at the origin. The R_F of barbital is 0·5 and that of metharbital 0·9.

Measurement of radioactivity. Chromatograms were scanned with a Scanogram II automatic windowless paper chromatogram scanner with associated ratemeter and recorder. Amounts of barbital or DMO were calculated from the areas of the peaks in comparison with the standards in the same experiment. The methylated compounds have sufficient vapor pressure that some radioactivity is transferred to adjacent turns of paper on the spool of the scanner. Since it was desired only to measure the demethylated products, this difficulty was obviated by removing the part of the chromatogram containing the methylated substrates and scanning only the portion containing the demethylated products. Chromatograms in the benzene system were cut at a point 0·6 of the distance from origin to front, and only the upper part was scanned. On the alkali-soaked paper barbital can be visualized under an ultraviolet lamp and that region cut out for scanning.

RESULTS

Yield as a function of substrate concentration. Figure 1 shows the results of an experiment in which the same rat liver microsome preparation was used in incubations with different amounts of trimethadione and of metharbital. There is a striking difference

between the two substrates. Enzymatic saturation is approached at 10 μmoles of metharbital, and there is only a small increase in the velocity of the reaction as the amount is increased from half that level. With trimethadione the reaction velocity is still increasing when the amount is 200 μmoles, and this velocity is less than was obtained with 1/20 that amount of metharbital.

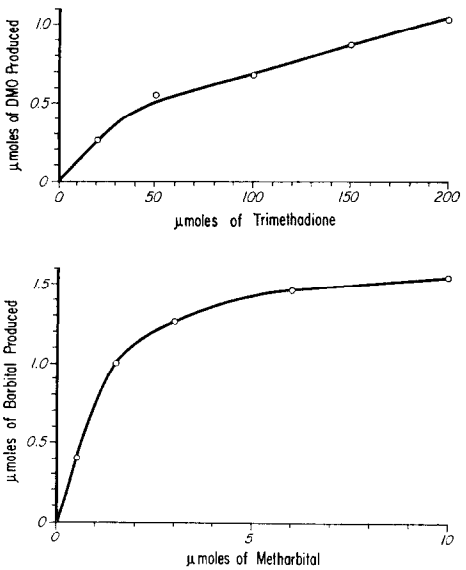

FIG. 1. Yields of demethylated products from different amounts of trimethadione and metharbital. Portions of the same microsomal preparation were used in all the incubations with both substrates. The total amount of the substrate added to an incubation is indicated on the abscissa.

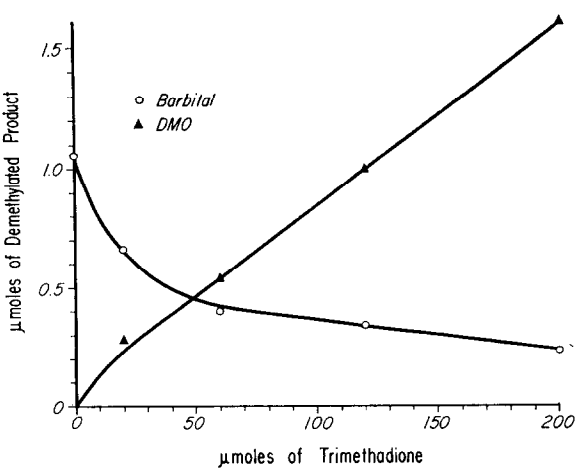

FIG. 2. Yields of demethylated products when metharbital and trimethadione are present together. Portions of the same microsomal preparation were used in all the incubations. The total amount of metharbital in each incubation was 5 μmoles. The total amount of trimethadione added to an incubation is indicated on the abscissa.

Competition between trimethadione and metharbital. A series of incubations with the same microsome preparation was carried out in which the amount of metharbital was held constant at 5 μmoles and the amount of trimethadione varied. The results are shown in Fig. 2. As the concentration of trimethadione is increased, the yield of demethylated metharbital decreases and the yield of demethylated trimethadione increases.

The results of a similar experiment in which the amount of trimethadione was held constant at 20 μmoles and the amount of metharbital was varied are shown in Fig. 3.

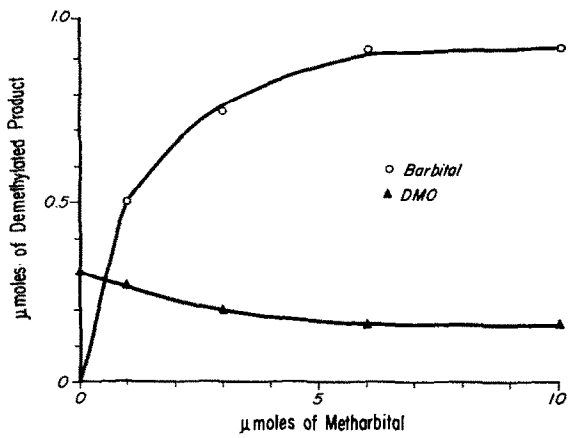

FIG. 3. Yields of demethylated products when metharbital and trimethadione are present together. Portions of the same microsomal preparation were used in all the incubations. The total amount of trimethadione in each incubation was 20 μmoles. The total amount of metharbital added to an incubation is indicated on the abscissa.

As the concentration of metharbital is increased, the yield of demethylated trimethadione decreases and the yield of demethylated metharbital increases.

Inhibitory effect of DMO. In an experiment similar to that of Fig. 2, metharbital-^{14}C in an amount of 5 μmoles was incubated with different amounts of trimethadione and of DMO. At equal concentrations trimethadione and DMO had approximately equal inhibitory effects on the demethylation of metharbital. In another experiment, 20 μmoles trimethadione-^{14}C was incubated with different amounts of unlabeled DMO. The same concentrations of DMO that had been found inhibitory to the demethylation of the 5 μmoles of metharbital also inhibited the demethylation of the 20 μmoles of trimethadione to approximately the same proportion.

DISCUSSION

Because of the limited solubilities of the substrates and the low yields of products obtainable in short incubation times, the present experiments were not carried out in such a way as to furnish complete information about the relationship of substrate concentration to reaction velocity for these demethylation reactions. Nevertheless, from experiments as they were performed in this study (Fig. 1), it is possible to infer that the Michaelis constant is much higher for trimethadione than for metharbital. The low reaction velocity at high substrate concentrations is indicative that the high

Michaelis constant reflects a lower affinity of trimethadione for the demethylating enzyme or enzymes.

Although it is not conclusively established from the present experiments, the mutual inhibitory effects of trimethadione and metharbital on the demethylation of the other are most plausibly interpreted on the assumption that the two substrates are acted upon by the same enzyme or enzymes and that they are competitive for the same active site. This assumption can presumably be extended to include other N-methyl compounds of related structures.

The approximate equality of the inhibitory effects of DMO and trimethadione is indicative that the complex between trimethadione and the enzyme is formed through interactions with parts of the molecule other than the N-methyl group. It would also appear to indicate that these interactions are little if at all influenced by the state of ionization of the molecule. Trimethadione is an undissociable molecule, whereas DMO is about 95 per cent ionized at pH 7·4. However, it is conceivable that in its complex with the enzyme the degree of ionization of DMO might be quite different.

Whether an inhibitory effect that one drug may exert on the metabolism of another drug administered simultaneously to a patient is ever a factor of any practical importance in therapeutics is a question about which there is little knowledge and a question that has attracted little attention. Patients receiving therapeutic doses of trimethadione in chronic schedule may have concentrations of DMO in the plasma as high as 10 μmoles/ml.[5] In these *in vitro* experiments such concentrations of DMO were found definitely inhibitory to the demethylation of trimethadione and metharbital. In patients having high levels of DMO in the plasma, it might be expected that the demethylation of trimethadione would be inhibited to some extent. It is also conceivable that high concentrations of DMO might inhibit drug-metabolizing reactions other than demethylations.

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