HYDROXYLATION OF THE AROMATIC AND HETEROCYCLIC RINGS OF THE PHENAZEPAM MOLECULE IN THE ENDOPLASMIC RETICULUM OF ALBINO RATS AND MICE

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In previous investigations [3-5] the distribution of [^{14}C]phenazepam among the organs and tissues of albino rats and excretion of metabolites of the drug after administration by single and repeated doses were studied and the pharmacokinetic parameters of these processes were calculated.

This paper gives the results of a study of the structural selectivity of action of the hydroxylating complex of the liver, which depends on the species of the experimental animal, on the phenazepam molecule.

EXPERIMENTAL METHOD

Male Wistar rats weighing 180-200 g and BALB/c mice weighing 18-20 g were used. The animals were kept on a restricted diet for 24 h before decapitation. To obtain microsomes the liver of the animals were perfused in 1.15% KCl solution, then homogenized in 3 volumes of this same solution containing 40 mM Tris-HCl buffer, pH 7.4, and 100 mM EDTA, in a Waring blender and centrifuged at 9000g for 20 min. The supernatant was centrifuged at 105,000g for 60 min in a Beckman centrifuge. The resulting microsomes were suspended in the isolation medium. The protein concentration was determined by Lowry's method and cytochrome P-450 in the turbid samples was measured on a Hitachi-356 spectrophotometer by a differential scheme [6].

Liver microsomes, in an amount of 1-1.5 mg protein, were added to the incubation medium in a total volume of 2 ml, containing 50 mM Tris-HCl buffer, pH 7.4, 16 mM $MgCl_2$, and a series of concentrations of [^{14}C]phenazepam or a definite concentration of [^{3}H]phenazepam. The reaction was started by the addition of 1 mM NADPH to the medium. At the end of incubation at 37°C, which was accompanied by oxygenation for 30 min, the samples were treated with 4 volumes of chloroform. The phenazepam and its metabolites were extracted by intensive shaking. To obtain better separation of the phases the samples were centrifuged at 6000g for 15 min. The chloroform extract was collected and the aqueous protein phase was reextracted. The chloroform phases were pooled and concentrated. Phenazepam and its metabolites were fractionated by thin-layer chromatography in a chloroform–acetone–ammonia (3:1:0.01) system on Silufol UF-254 plates. In some cases a radiochromatographic analysis was made of the chloroform extracts, using the method in [3].

The chloroform extracts obtained from the incubation medium to which [^{3}H]phenazepam was added were evaporated and hydrolyzed in 6N HCl on a boiling water bath for 30 min. The digests were neutralized with 1N NaOH and extracted with chloroform. The benzophenones thus formed were chromatographed in a chloroform–carbon tetrachloride (2:1) system. The metabolites were identified by the method described previously [1]. A type SL-30 scintillation photometer (France) was used for the quantitative analysis of the substances.

The results took into account the degree of extractability of the metabolites from the incubation medium and also the fact that, during acid hydrolysis of [^{3}H]phenazepam, 60% of the radioactivity was preserved in the benzophenone molecule.

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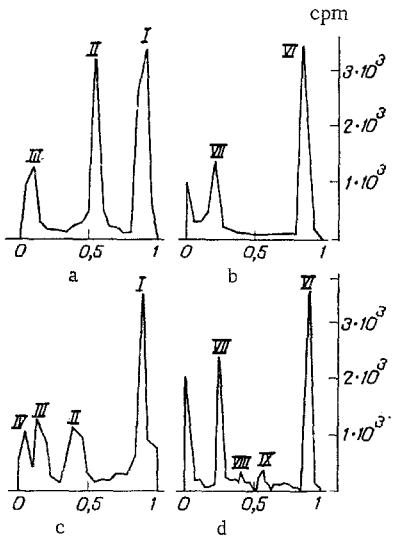


Fig. 1. Radiochromatograms of chloroform extracts of metabolites of [^{3}H]phenazepam after its incubation with liver microsomes of mice (a) and rats (b), and also of their acid hydrolysis products (c and d , respectively).

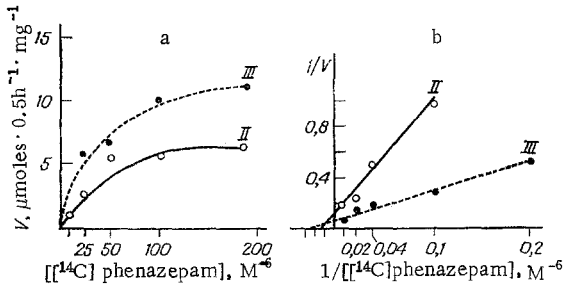


Fig. 2

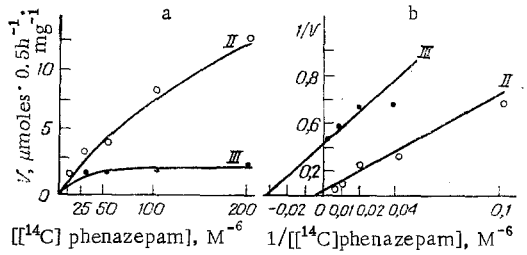


Fig. 3

Fig. 2. Velocity of aromatic hydroxylation (III) and oxidation of the heterocyclic ring (II) in the phenazepam molecule as a function of its concentration in rat liver microsomes, plotted between direct coordinates (a) and Lineweaver–Burk coordinates (b).

Fig. 3. Velocity of aromatic hydroxylation (III) and of oxidation of the heterocyclic ring (II) in the phenazepam molecule as a function of its concentration in mouse liver microsomes, plotted between direct coordinates (a) and Lineweaver–Burk coordinates (b).

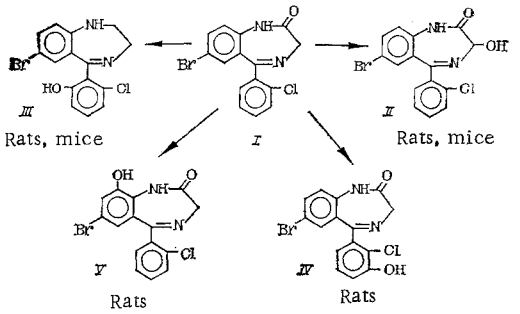
EXPERIMENTAL RESULTS

Analysis of the chloroform extracts by the radiochromatographic method showed (Fig. 1) that after addition of [^{3}H]phenazepam to the incubation medium three peaks of radioactivity are found in mouse liver microsomes (Fig. 1a), and four peaks in albino rat liver microsomes (Fig. 1b). It was shown by physicochemical methods that radioactivity peak I corresponds to the original compound [^{3}H]phenazepam, peak II to the 3-hydroxy derivative, and peaks III and IV in this case were not identified. The radiochromatograms of the digests of the chloroform extracts showed that an identical benzophenone – VI (5-bromo-2-chloro-2-aminobenzophenone) was formed from compounds I and II, and this was characteristic of liver microsomes of both mice and rats (Fig. 1c, d). In both cases, peaks of radioactivity also were observed at the start of the chromatograms. Since, after acid hydrolysis of [^{3}H]phenazepam directly, part of the radioactivity remains at the start of the chromatographic plates, it was concluded that this peak is of similar origin. Radioactivity observed on the chromatograms as peaks VII and VIII belonged to the corresponding benzophenones with an oxidized chlorobenzene ring. It is evident from the color in UV light and the R_f value that metabolite VIII is a benzophenone with a hydroxyl group in the bromine-containing aromatic ring, as the writers proved previously by various physicochemical methods [2].

TABLE 1. Kinetic Parameters of Reactions of Aromatic Hydroxylation (III) and Oxidation of the Heterocyclic Ring (II) of the Phenazepam Molecule in Liver Microsomes of Rats and Mice

Oxidation reaction	K_m, M		V_{max}, moles · min^{-1}, mg^{-1}	
	rat	mouse	rat	mouse
III	$3{,}75 \cdot 10^{-4}$	$3{,}03 \cdot 10^{-4}$	$5{,}5 \cdot 10^{-6}$	$7{,}9 \cdot 10^{-7}$
II	$7{,}7 \cdot 10^{-4}$	$2{,}5 \cdot 10^{-3}$	$3{,}03 \cdot 10^{-6}$	$8{,}3 \cdot 10^{-6}$

On the basis of these results, the metabolism of [^{3}H]phenazepam in vitro can be represented as follows:



Meanwhile, the principal metabolites of [^{3}H]phenazepam in experiments in vitro are the 3-hydroxy derivative (II) and the aromatic hydroxylation products of phenazepam (III).

The addition of [^{14}C]phenazepam to the incubation medium led to similar results. Since [^{14}C]phenazepam has higher specific radioactivity (4 Ci/mole), subsequent investigations were carried out with this preparation.

Addition of various concentrations of [^{14}C]phenazepam to incubation media containing mouse liver microsomes led to predominance of hydroxylation of the heterocyclic ring (Fig. 2a), whereas for rat liver microsomes, selective oxidation of aromatic rings was observed to a greater degree (Fig. 3a).

Plotting the experimental data between Lineweaver–Burk coordinates (Figs. 2b and 3b; Table 1) revealed differences in the affinity of the substrate for enzymes catalyzing oxidation and isolated from the liver of different animals. One of the factors explaining this phenomenon may be the cytochrome P-450 level in the liver of the experimental animals. The cytochrome P-450 content in mouse liver microsomes is 1.1 ± 0.3 nmole/mg protein and in rat liver, 0.64 ± 0.05 nmole/mg protein. This hypothesis was tested in additional experiments. The cytochrome P-450 content in rat liver microsomes increased to 1.38 ± 0.03 nmole/mg protein after induction by phenobarbital (100 mg/kg for 4 days). There was a parallel increase in the kinetic parameters of these reactions. For instance, for C_3-hydroxylation (II) the values of K_m and V_{max} in this case were $1{,}42 \cdot 10^{-3}$M and $5{,}0 \cdot 10^{-3}$ mole $\cdot$ $min^{-1} \cdot mg^{-1}$, respectively. For aromatic hydroxylation (III), these values were $3{,}03 \cdot 10^{-6}$M and $1{,}25 \cdot 10^{-4}$ mole $\cdot$ $min^{-1} \cdot mg^{-1}$, respectively. An increase in the cytochrome P-450 content in rat liver microsomes thus leads to a change in the direction of hydroxylation of phenazepam. This fact, together with the different kinetic parameters in the formation of products II and III in the same animal, are evidence that the two processes are catalyzed not by one, but by at least two enzymes.

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