EFFECT OF TYPE I COMPOUNDS ON *p*-HYDROXYLATION OF ANILINE IN VITRO

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During the past fifteen years, it has become evident that a variety of lipid soluble drugs such as aniline, aminopyrine and hexobarbital are oxidized by NADPH-dependent enzymes in hepatic microsomes (1-4). Cytochrome(s) P-450 plays a critical role as the terminal oxidase in the metabolism of drugs, steroids and heme (5-7). It is generally accepted that the hepatic drug-metabolizing enzyme system has few specificities for substrates but recent studies have suggested the existence of more than two microsomal enzymes which metabolize the drugs (8, 9).

On the other hand, the addition of various substrates of microsomal mixed-function oxidase system to aerobic liver microsomes causes two types of spectral change. One class of spectral change (termed type I) is characterized by the appearance of a trough at 420 $m\mu$ and an absorption peak at 385 $m\mu$. Aminopyrine, hexobarbital, chlorpromazine, SKF 525-A and DDT cause type I spectral change. Another class of spectral change (termed type II) is characterized by the appearance of a trough at 392 $m\mu$ and an absorption peak at 430 $m\mu$. Aniline, nicotinamide, pyridine and *p*-aminophenol cause type II spectral change (10).

Drug interaction with endoplasmic reticulum occurs not only to constitute enzyme-substrate complex but also to modify the physical properties, amounts, composition and turnover of constitutive membrane components (11). At present aniline is thought to bind to the CO-binding site of cytochrome P-450, whereas aminopyrine or hexobarbital is thought to bind to the lipids of the endoplasmic reticulum or hydrophobic region cytochrome P-450 (12).

The present paper concerns the effects of type I binders such as aminopyrine, hexobarbital and chlorpromazine on *p*-hydroxylation of aniline in vitro.

METHODS

Male Wistar rats weighing approx. 250 g were used. The animals were deprived of food 12 hr before sacrifice except for tap water given ad libitum. The animals were exsanguinated and the livers were perfused with ice-cold isotonic KCl before removal. All operations were performed at 4°C. Livers of three or four animals were pooled. The minced livers were homogenized in ice-cold isotonic 2 vol of KCl containing 0.02 M Tris-HCl buffer,

pH 7.4, in a Potter homogenizer fitted with a loose Teflon pestle. Homogenates were passed through a gauze layer, then centrifuged at 700 g for 10 min and at 9,000 g for 20 min in succession to remove nuclear and mitochondrial fractions. In the present experiments 9,000 g supernatant fractions were used as the enzyme source. The activity of the drug-metabolizing enzyme was expressed in terms of the metabolized drug per 100 mg protein of microsomes.

The reaction mixtures of 5.0 ml total volume were incubated in 30 ml Erlenmeyer flasks for 10 min in an atmosphere of air with a Dubnoff metabolic shaker at 37°C. Each incubation flask contained the following constituents: NADP, 2 μ moles; glucose-6-phosphate disodium salt, 25 μ moles; magnesium chloride, 20 μ moles; aniline hydrochloride, 0.02 to 4.0 mM; one ml of the 9,000 g supernatant fraction (7 mg microsomal protein). Aminopyrine, hexobarbital or chlorpromazine was added to the incubation medium, at 4 to 10 mM, at 1 to 2 mM and at 0.4 to 1.0 mM respectively. pH of the incubation mixtures was adjusted to 7.4 with 0.3 M Tris-HCl buffer.

The *p*-hydroxylation of aniline was determined by measuring the *p*-aminophenol production as described previously (13). The protein contents in 9,000 g supernatant fractions and microsomes were determined by the method of Lowry et al. (14). For the study of kinetics of aniline hydroxylase, the graphical procedures of Lineweaver and Burk and of Dixon were used (15, 16).

RESULTS

Wada et al. (17) reported that a hyperbolic curve was obtained by plotting the reciprocal of the velocity against the reciprocal of the substrate concentration in the aniline hydroxylase of mice and rats. On our experiments on aniline hydroxylase during the past four years the Lineweaver-Burk plots for aniline hydroxylase were 90% linear, irrespective of the use of 9,000 g supernatant fractions or washed microsomes as enzyme source.

1. Effect of aminopyrine

When aminopyrine was added to the incubation mixtures with final concentrations at 5 to 10 mM the aniline hydroxylase was inhibited significantly. However, the Lineweaver-Burk plots of the activities of aniline hydroxylase in the presence of aminopyrine were not linear (Fig. 1). The inhibition by aminopyrine was pronounced at concentrations or 0.1-0.4 mM of aniline.

2. Effect of hexobarbital

In the presence of hexobarbital at 1 to 2 mM the *p*-hydroxylation of aniline was inhibited significantly. The Lineweaver-Burk plots were not linear (Fig. 2). The

FIG. 1. Effect of aminopyrine on *p*-hydroxylation of aniline in vitro.

V : μ moles *p*-aminophenol formed/100 mg microsomal protein/10 min
S : aniline concentration mM $\times$ 10

FIG. 2. Effect of hexobarbital on *p*-hydroxylation of aniline in vitro.

V : μ moles *p*-aminophenol formed/100 mg microsomal protein/10 min
S : aniline concentration mM $\times 10$

FIG. 3. Effect of chlorpromazine on *p*-hydroxylation of aniline in vitro.

V : μ moles *p*-aminophenol formed/100 mg microsomal protein/10 min
S : aniline concentration mM $\times 10$

type of inhibition was rather competitive.

3. Effect of chlorpromazine

Addition of chlorpromazine to the incubation mixtures caused inhibition of the activity of aniline hydroxylase. In contrast to the cases of aminopyrine and hexobarbital, the inhibition was observed at high concentrations of the substrate (Fig. 3).

DISCUSSION

The hydroxylation of drugs by hepatic microsomes is thought to proceed by mixed-function oxidase (1-4). If this concept is correct, it can be predicted that numerous drugs would inhibit competitively the metabolism of another drug employed as a substrate for the microsomal system. Rubin et al. (18) reported that the N-demethylase of ethylmorphine was competitively inhibited by hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone and acetanilide.

In the present studies it was revealed that additions of various type I compounds such as aminopyrine, hexobarbital and chlorpromazine to the incubation mixtures for the assay of the aniline hydroxylase caused significant inhibition of the *p*-hydroxylation of aniline. The types of inhibition, however, were not always equal. When aminopyrine was added to the incubation mixtures the *p*-hydroxylation of aniline was inhibited but the Lineweaver-Burk plots of aniline hydroxylase were not linear.

On the other hand, addition of hexobarbital to the incubation mixtures caused inhibition of *p*-hydroxylation of aniline but the type of inhibition was rather competitive although Lineweaver-Burk plots were not linear in the presence of hexobarbital. When chlorpromazine was added to the incubation mixtures, the Lineweaver-Burk plots became much more complicated.

Wada et al. (17) reported the existence of more than one enzyme for the hydroxylation

of aniline. In rats, though nearly linear Lineweaver-Burk plots were found in the hydroxylation of aniline, nonlinearity was evident when the inhibitor prednisolone was included in the incubation mixtures. In spite of apparent K_m similarity of the two enzymes, inhibition by prednisolone shown by kinetic evaluation was different.

Aust and Stevens (19) described that Lineweaver-Burk plots were not useful for a number of reasons. For example, there is difficulty in obtaining a linear or useful Lineweaver-Burk plot in the absence of an inhibitor. The curves become much more complex in the presence of inhibitors.

At present, the binding site of type I compounds is thought to be either phospholipids of endoplasmic reticulum or the hydrophobic region of cytochrome P-450 (12). Furthermore, the physical and biochemical properties of cytochrome P-450 are thought to depend to a large degree on the association of the hemoprotein with the lipid components of microsomal membrane (20, 21). Chaplin and Mannerling (12) also suggested that the ligand state of hemoprotein is affected by its association with phospholipids in the membrane.

Thus, a possible mechanism of the different types of inhibition of aniline hydroxylase by aminopyrine, chlorpromazine and hexobarbital may be in the relation between the effects of these on the endoplasmic reticulum membrane.

The drug-metabolizing system of hepatic microsomes is very complicated. Recent studies have suggested the existence of more than two enzymes which metabolize the drugs (8, 9). With the mixed-function oxidase for hydroxylation, the difference of the terminal oxidase or the variety of binding sites of type I compounds may also cause a complicated inhibition of the hydroxylation of type II compounds.

Experiments are now in process with phospholipase C-treated microsomes.

SUMMARY

The effects of type I compounds on *p*-hydroxylation of aniline in vitro were studied.

Aminopyrine, hexobarbital and chlorpromazine inhibited significantly the activities of aniline hydroxylase but each drug inhibited the activities of aniline hydroxylase in a different fashion.

Possible mechanisms of difference in inhibition by each drug were discussed.

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