ENHANCEMENT IN VIVO OF DRUG OXIDATIONS FOLLOWING ADMINISTRATION OF BENZPHETAMINE, ACETONE, METYRAPONE AND DIMETHYLSULFOXIDE

Mitsukazu KITADA, Tetsuya KAMATAKI and Haruo KITAGAWA
Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba-shi, Chiba 280, Japan
Accepted October 20, 1977

Abstract—Effects of benzphetamine, acetone, metyrapone and dimethylsulfoxide administration to rats on the metabolism of drugs by liver 9,000 × g supernatant fraction were studied herein. Activities for aniline hydroxylation and phenacetin O-deethylation were increased while ethylmorphine and benzphetamine N-demethylations were unchanged by the single administration of acetone, metyrapone or dimethylsulfoxide. Increase in aniline hydroxylase activity by about 53.4% and in phenacetin O-deethylase activity by about 44.4% were observed at 30 min after the single administration of benzphetamine whereas ethylmorphine N-demethylase activity was slightly decreased. NADPH-cytochrome P-450 reductase activity and cytochrome P-450 content were unaltered until 12 hr after the single administration of benzphetamine. Aniline hydroxylation was increased by the addition of benzphetamine to the incubation mixture and the increase in aniline hydroxylation caused by benzphetamine could be reversed by washing the microsomes.

Inhibition of liver microsomal drug oxidations by the addition of drugs or chemicals such as chlorpromazine, acetanilide and SKF 525-A is well known (1–3). Rubin et al. (1) have shown that hexobarbital, phenylbutazone and acetanilide competitively inhibited N-demethylation of ethylmorphine. In addition, it has been shown that ethylmorphine, hexobarbital and chlorpromazine are mutually inhibitory. Therefore, it has been considered that one drug inhibited the oxidation of another by serving as an alternative substrate. On the contrary, there are some reports regarding the activation of drug oxidations caused by drugs or chemicals. Imai and Sato (4) have shown that the addition of ethylisocyanide to the incubation mixture resulted in the marked increase in aniline hydroxylation, thereafter, Anders (5, 6) demonstrated the stimulatory effects of acetone and benzphetamine on aniline and acetanilide hydroxylase activities. Furthermore, Leibman (7), Stevens and Greene (8) and Korten and Van Dyke (9) demonstrated a stimulation of microsomal drug oxidations by the addition of metyrapone, paraaxon or volatile anesthetics to the incubation mixture. However, these studies have shown in vitro enhancement of drug oxidations and only a few experiments are conducted in vivo. It is the purpose of this paper to demonstrate the enhancement of drug oxidations following the single administration of benzphetamine, metyrapone, acetone or dimethylsulfoxide.

MATERIALS AND METHODS
Male Sprague-Dawley rats weighing 250–300 g were maintained on commercial rat
chow, CE-2, Nippon Clea Co. Ltd., Japan. The animals were deprived of food about 18 hr prior to sacrifice but were allowed tap water ad libitum. Liver 9,000 x g supernatant fraction and microsomes were prepared essentially the same as described previously (10). Commercial aniline was redistilled under vacuum and the distillate was stored at about -10°C under an atmosphere of nitrogen. Other drugs and chemicals were purchased from commercial sources and used without further purification. A typical reaction mixture for drug oxidation assay consisted of Na,K-phosphate (100 mM, pH 7.4), EDTA (0.1 mM), NADP (0.33 mM), glucose 6-phosphate (8 mM), MgCl₂ (6 mM), glucose 6-phosphate dehydrogenase (0.045 unit, EC 1.1.1.49, Boehringer Mannheim, Grade I), 9,000 x g supernatant fraction and a substrate (benzphetamine, aniline, aminopyrine, ethylmorphine: 2 mM, phenacetin: 1 mM) in a final volume of 1.0 ml. Semicarbazide (5 mM) was added for the demethylation assay. Incubation was carried out aerobically at 37°C. Ethylmorphine, aminopyrine and benzphetamine N-demethylations were assayed by determining formaldehyde formed according to the method of Nash (11). p-Aminophenol formed was determined according to the method of Brodie and Axelrod (12) as modified by Kato and Gillette (13), and N-acetyl-p-aminophenol formed was determined according to the method of Shimazu (14) as modified by Anders (6). Cytochrome P-450 was determined by the method of Omura and Sato (15) using Aminco DW-2 recording spectrophotometer. NADPH-cytochrome P-450 reductase activity was measured by the method of Phillips and Langdon (16) using cytochrome c as electron acceptor. Protein was determined by the method of Gornall et al. (17) using bovine serum albumin as a standard.

RESULTS

Effects of the single administration of metyrapone, acetone, dimethylsulfoxide and benzphetamine on drug oxidations

To determine whether or not stimulation of drug oxidations by the administration of drugs or chemicals which have been shown to activate microsomal drug oxidations, can be observed in vivo, the effects of administration of metyrapone, acetone, dimethylsulfoxide and benzphetamine, on oxidations of various substrates were investigated using isolated 9,000 x g supernatant fraction. As shown in Figs. 1 and 2, the administration of metyrapone, dimethylsulfoxide or benzphetamine resulted in a significant increase in the activity of aniline hydroxylation and the administration of acetone, dimethylsulfoxide or benzphetamine resulted in a significant increase in the activity of phenacetin O-deethylation whereas ethylmorphine, aminopyrine and benzphetamine N-demethylase activities were virtually unaffected by the administration of acetone, metyrapone or dimethylsulfoxide except that aminopyrine N-demethylase activity was significantly increased by dimethylsulfoxide. From these results, it may be assumed that oxidation of type II compounds such as aniline and phenacetin are more preferentially affected than that of type I compounds such as benzphetamine, ethylmorphine and aminopyrine by the administration of acetone, metyrapone or dimethylsulfoxide.
Dose-response relationship between the activities for aniline hydroxylation, phenacetin O-deethylation and ethylmorphine N-denethylation and the dose of benzphetamine administration

Table I shows the relationship between the dose of benzphetamine and changes in the activities of aniline hydroxylation, phenacetin O-deethylation and ethylmorphine N-demethylation. The N-demethyase activity was decreased with increase of the dose of benzphetamine and was significantly inhibited when rats were given 100 mg/kg of benzphetamine, whereas aniline hydroxylase activity was increased with the dose of benzphetamine given.
| Dose (mg/kg) | Aniline | Phenacetin | Ethylmorphine |
|-------------|---------|------------|---------------|
|             | p-Aminophenol | p-Acetyl-p-aminophenol | Total (A + B) | HCHO |
| 0           | 310.83 ± 18.80 | 318.10 ± 41.19 | 26.64 ± 1.89 | 344.73 ± 42.67 | 1.487 ± 0.001 |
| 5           | 402.75 ± 15.92 | 380.69 ± 49.55 | 40.05 ± 5.90 | 420.73 ± 50.99 | 1.427 ± 0.042 |
| 25          | 430.78 ± 17.91$^a$ | 497.11 ± 26.40$^a$ | 40.17 ± 1.78$^a$ | 537.28 ± 27.29$^a$ | 1.296 ± 0.095 |
| 50          | 491.86 ± 19.93$^c$ | 381.00 ± 37.74 | 58.36 ± 5.71$^c$ | 439.35 ± 40.76 | 1.272 ± 0.016 |
| 100         | 480.17 ± 17.99$^c$ | 218.76 ± 18.62 | 56.97 ± 3.24$^c$ | 275.73 ± 20.01 | 1.127 ± 0.088$^c$ |

Rats were administrated various doses of benzphetamine as indicated 30 min before sacrifice. Values represent means ± S.E. of three or four experiments. a) nmole p-aminophenol or N-acetyl-p-aminophenol formed/g of liver/15 min ($n=4$). b) pmole formaldehyde formed/g of liver/15 min ($n=3$). c) $p<0.05$. 
and significantly increased when rats were administrated more than 25 mg/kg of benzphetamine. The administration of 50 mg/kg of benzphetamine resulted in the maximal increment of aniline hydroxylation. On the other hand, the activity of phenacetin O-deethyl as measured by the formation of p-aminophenol and N-acetyl-p-aminophenol was also increased by benzphetamine administration. The formation of N-acetyl-p-aminophenol was maximally increased when rats were given 25 mg/kg of benzphetamine and was gradually decreased with increase of the dose of benzphetamine. N-Acetyl-p-aminophenol formation was inhibited by about 31.3% by the administration of 100 mg/kg of benzphetamine. On the other hand, the formation of p-aminophenol was maximally increased by the administration of 50 mg/kg of benzphetamine and the stimulation of p-aminophenol formation was observed even when rats were given 100 mg/kg of benzphetamine. Dose-response relationship between the dose of benzphetamine administration and the activity of aniline hydroxylation was somewhat different from that between the dose of benzphetamine and the activity of phenacetin O-deethyl asation.

Activities of aniline hydroxylase, phenacetin O-deethyl as, ethylmorphine N-demethylase and NADPH-cytochrome P-450 reductase and the content of cytochrome P-450 at various times after the single administration of benzphetamine.

The stimulation of drug oxidations following administration of drugs and chemicals such as phenobarbital and 3-methylcholanthrene is accompanied by increase in cytochrome P-450 content or NADPH-cytochrome P-450 reductase activity, component of microsomal monooxygenase system (18). Therefore, rats were pretreated with 25 mg/kg of benzphetamine either 0.5, 6 or 12 hr prior to sacrifice and activities of aniline hydroxylation, phenacetin O-deethyl asation, ethylmorphine N-demethylase and NADPH-cytochrome P-450 reductase and content of cytochrome P-450 were measured. As can be seen in Table 2, the administration of benzphetamine resulted in a slight decrease in ethylmorphine N-demethylase and in a significant increase in aniline hydroxylation and phenacetin O-deethyl asation. Maximal increase in aniline hydroxylation was observed at 30 min after benzphetamine administration and that in phenacetin O-deethyl asation was observed at 6 hr.

Table 3 shows the effects of benzphetamine administration on cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity. Content of cytochrome P-450 and

### Table 2. Activities for aniline hydroxylation, phenacetin O-deethyl asation and ethylmorphine N-demethylase at various times after the single administration of benzphetamine.

| Time after administration (hr) | Aniline(a) | Phenacetin(b) | Ethylmorphine(c) |
|-------------------------------|------------|---------------|------------------|
| 0                             | 222.9±7.3  | 367.1±42.7    | 1.723±0.079     |
| 0.5                           | 342.1±28.4c| 561.8±34.1c   | 1.517±0.046     |
| 6                             | 309.1±26.3c| 577.4±38.8c   | 1.613±0.035     |
| 12                            | 241.8±27.3c| 563.5±31.5c   | 1.583±0.052     |

Rats were pretreated with 25 mg/kg of benzphetamine. Values represent means±S.E. of three experiments. a) nmole p-aminophenol or N-acetyl-p-aminophenol formed/g of liver/15 min. b) µmole formaldehyde formed/g of liver/15 min. c) p<0.05.
activity of NADPH-cytochrome P-450 reductase were not affected significantly by benzphetamine administration. Therefore, it can be concluded that the stimulatory effects of benzphetamine on aniline hydroxylase activity and phenacetin O-deethylase activity are not due to the increase in the content of cytochrome P-450 or the activity of NADPH-cytochrome P-450 reductase.

### Table 3. NADPH-Cytochrome P-450 reductase activity and cytochrome P-450 content at various times after a single administration of benzphetamine

| Time after administration (hr) | NADPH-cytochrome P-450 reductase<sup>a</sup> | Cytochrome P-450<sup>b</sup> |
|-------------------------------|---------------------------------------------|-----------------------------|
| 0                             | 0.161±0.036                                 | 0.940±0.121                 |
| 0.5                           | 0.118±0.002                                 | 0.829±0.036                 |
| 6                             | 0.136±0.009                                 | 0.859±0.094                 |
| 12                            | 0.126±0.006                                 | 0.954±0.065                 |

Experimental details were the same as described in Table 2. Each value represents mean±S.E. of three experiments.  

a) nmole cytochrome e reduced/mg of protein/min.  
b) nmole/mg of protein.

Effect of washing the microsomes on the stimulatory effect of benzphetamine on aniline hydroxylation

To determine whether or not the stimulatory effect of benzphetamine on aniline hydroxylation is reversible, the effect of washing the microsomes on the stimulation of aniline hydroxylation by benzphetamine was studied. As can be seen in Fig. 3, the enhancement in aniline hydroxylation due to benzphetamine was decreased with increase of the washings and the further addition of benzphetamine partially recovered the stimulatory effect. Therefore, it may be assumed that the stimulatory effect of benzphetamine on aniline hydroxylation is reversible and may involve a direct interaction of benzphetamine with the microsomal

![Fig. 3. Effects of washing the microsomes on the stimulation of aniline hydroxylation by the addition of benzphetamine. Concentration of benzphetamine added to the incubation mixture was 0.1 mM. Initial activity of aniline hydroxylation in the absence of benzphetamine was 10.13 nmole p-aminophenol formed/mg of protein/15 min. Results are expressed as a percentage of enhancement with [ ] or without (——) further addition of benzphetamine and represent means of duplicate determinations.](image-url)
aniline hydroxylase.

Enhancing effect of acetone on aniline hydroxylation was also completely removed by washing the microsomes. (data not included)

**DISCUSSION**

We carried out studies to determine whether or not the stimulative effects of benzyphenetamine, acetone, dimethylsulfoxide and metyrapone on drug oxidations could be observed in vivo. We used a 9,000 × g supernatant fraction rather than isolated microsomes as enzyme source because it could be expected, from the results that the enhancing effects of acetone and benzyphenetamine on aniline hydroxylation were decreased by washing the microsomes and that the stimulatory effects due to these compounds in 9,000 × g supernatant fraction might be greater than those in microsomes.

The administration of benzyphenetamine, acetone, dimethylsulfoxide or metyrapone increased the oxidation activities of aniline and phenacetin (Fig. 1), in addition, dimethylsulfoxide administration significantly increased the N-demethylase activity of aminopyrine but not the N-demethylase activities of ethylmorphine and benzyphenetamine (Fig. 2). Aust and coworkers (19, 20) reported that there are two Km values obtainable in the Lineweaver-Burk Plots of aminopyrine N-demethylation. In addition, recent studies from several laboratories indicated that there are multiple forms of cytochrome P-450 in liver microsomes of rats (21), mice (22) and rabbits (23), therefore, it can be assumed that forms of cytochrome P-450 catalyzing the oxidations of aniline, phenacetin and aminopyrine may be different from those of cytochrome P-450 catalyzing the oxidations of benzyphenetamine and ethylmorphine, and may be preferentially influenced by benzyphenetamine, acetone, metyrapone and dimethylsulfoxide.

The increase in drug oxidations following administration of substrates such as steroid hormones, drugs and pollutants is generally accompanied by higher levels of cytochrome P-450 and NADPH-cytochrome P-450 reductase, components of the mixed-function oxidase system. Therefore, cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity were measured in order to determine whether or not the stimulatory effects of benzyphenetamine on aniline hydroxylation and phenacetin O-deethylation were due to the increase in the activities of these enzymes. The changes in the activities of drug oxidations following the administration of benzyphenetamine were not accompanied by any significant changes in the activity of NADPH-cytochrome P-450 reductase and the content of cytochrome P-450 (Table 3). However, the possibility cannot be excluded that cytochrome P-450 catalyzing aniline hydroxylation and phenacetin O-deethylation is preferentially induced and composition of cytochrome P-450s in microsomes from benzyphenetamine pre-treated rats is altered.

The marked increase in aniline hydroxylation was also observed when benzyphenetamine was added to the incubation mixture. The activation of aniline hydroxylation caused by the addition of benzyphenetamine was decreased by washing the microsomes and could be reversed by the further addition of benzyphenetamine (Fig. 3). These results suggest that the
enhancement of aniline hydroxylation by the addition of benzphetamine may be reversible
and may be due to direct interaction of benzphetamine with aniline hydroxylase. Moreover,
it is reasonable to assume that the stimulatory effects of benzphetamine on aniline hydroxy-
lation and phenacetin O-deethylation might be caused by benzphetamine itself incorporated
in the liver cells but not in the blood since livers were perfused thoroughly with 1.15% KCl
solution in order to remove the blood. Further studies are needed to clarify the mechanism(s)
of enhancing effect of benzphetamine on drug oxidations.

Acknowledgement: We are indebted to Mr. Kinzo Matsumoto for skillful technical
assistance.

REFERENCES

1) Rubin, A., Tephly, T.R. and Mannering, G.J.: Inhibition of hexobarbital metabolism by
ethylmorphine and codeine in the intact rat. Biochem. Pharmacol. 13, 1053-1057 (1964)
2) McMahon, R.E.: Competitive inhibition of the N-demethylation of butyramine by 2,4-
dichloro-6-phenylphenoxymethylanine. J. Pharmacol. exp. Ther. 138, 382-386 (1962)
3) Cooper, J.R., Axelrod, J. and Brodie, B.B.: Inhibitory effects of diethylaminoethyl 2,2-
diphenylvalerate (SKF 525-A) on a variety of drug metabolic pathways in vitro.
J. Pharmacol. exp. Ther. 112, 55-63 (1954)
4) Imai, Y. and Sato, R.: Activation and inhibition of microsomal hydroxylation by ethyl
isocyanide. Biochem. biophys. Res. Commun. 25, 80-86 (1966)
5) Anders, M.W.: Acetone enhancement of microsomal aniline para-hydroxylase activity.
Arch. Biochem. Biophys. 126, 269-275 (1968)
6) Anders, M.W.: Effect of phenobarbital and 3-methylcholanthrene administration on the
in vitro enhancement of microsomal aromatic hydroxylation. Arch. Biochem. Biophys.
153, 502-507 (1972)
7) Leibman, K.C.: Effects of metyrapone on liver microsomal drug oxidations. Mol. Pharmacol.
5, 1-9 (1969)
8) Stevens, J.T. and Greene, F.E.: Response of the mixed function oxidase system of rat
hepatic microsomes to parathion and malathion and their oxygenated analogs. Life
Sci. 13, 1677-1691 (1973)
9) Korten, K. and van Dyke, R.A.: Acute interaction of drugs I: The effect of volatile anes-
thesics on the kinetics of aniline hydroxylase and aminopyrine demethylase in rat
hepatic microsomes. Biochem. Pharmacol. 22, 2105-2112 (1973)
10) Kamataki, T. and Kitagawa, H.: Effects of lyophilization and storage of rat liver micro-
osomes on activity of aniline hydroxylase, contents of cytochrome b; and cytochrome
P-450 and aniline-induced P-450 difference spectrum. Japan. J. Pharmacol. 24, 195-
203 (1974)
11) Nash, T.: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction.
Biochem. J. 55, 416-421 (1953)
12) Brodie, B.B. and Axelrod, J.: The estimation of acetylcholine and its metabolic products,
aniline, N-acetyl p-aminophenol and p-aminophenol (free and total conjugated) in
biological fluids and tissues. J. Pharmacol. exp. Ther. 94, 22-28 (1948)
13) Kato, R. and Gillette, J.R.: Effect of starvation on NADPH-dependent enzymes in liver
microsomes of male and female rats. J. Pharmacol. exp. Ther. 150, 279-284 (1965)
14) Shimazu, T.: Response to 20-methylcholanthrene of hepatic aniline- and acetanilide 4-
hydroxylase of rats with hypothalamic lesions. Biochim. Biophys. Acta 105, 377-380
(1965)
15) Omura, T. and Sato, R.: The carbon monoxide-binding pigment of liver microsomes
I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378 (1964)
16) Phillips, A.H. and Langdon, R.G.: Hepatic triphosphopyridine nucleotide-cytochrome c
reductase: Isolation, characterization, and kinetic studies. J. Biol. Chem. 237, 2652-
2660 (1962)
17) Gornall, A.G., Badawill, C.S. and David, M.M.: Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751–766 (1948)

18) Cooney, A.H.: Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**, 317–366 (1967)

19) Perderson, T.C. and Aust, S.D.: Aminopyrine demethylase; Kinetic evidence for multiple microsomal activities. *Biochem. Pharmacol.* **19**, 2221–2230 (1970)

20) Aust, S.D. and Stevens, J.B.: Aminopyrine demethylase; Multiplicity shown by dieldrin and DDT inhibition. *Biochem. Pharmacol.* **20**, 1061–1069 (1971)

21) Welton, A.F. and Aust, S.D.: Multiplicity of cytochrome P-450 hemoproteins in rat liver microsomes. *Biochem. biophys. Res. Commun.* **56**, 898–906 (1974)

22) Huang, M.-T., West, S.B. and Lu, A.Y.H.: Separation, purification and properties of multiple forms of cytochrome P-450 from the liver microsomes of phenobarbital-treated mice. *J. biol. Chem.* **251**, 4659–4665 (1976)

23) Philpot, R.M. and Arinc, E.: Separation and purification of two forms of hepatic cytochrome P-450 from untreated rabbits. *Mol. Pharmacol.* **12**, 483–493 (1976)