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EFFECT OF ACETONE ON ANILINE HYDROXYLATION BY A RECONSTITUTED SYSTEM

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Abstract—Acetone stimulated NADPH-dependent aniline hydroxylation catalysed by cytochrome P-450 purified from phenobarbital-treated rats. No activation by acetone occurred in a reconstituted system containing cytochrome P-448 purified from 3-methylcholanthrene-treated rats. Cumene hydroperoxide-supported aniline hydroxylation catalysed by cytochrome P-450 was not increased by the addition of acetone at the concentration which stimulated NADPH-dependent aniline hydroxylation in the reconstituted system. The NADPH-dependent cytochrome P-450 reduction was stimulated by acetone in the presence or absence of aniline. On the other hand, acetone did not exhibit any stimulatory effect on NADPH-dependent reduction of cytochrome P-448. The stimulatory effect of acetone on aniline hydroxylation was decreased with increasing pH of the reaction media. Furthermore, in the system containing cytochrome P-450, the ratio of product to hydrogen peroxide formation was virtually unchanged by the addition of acetone.

In view of drug interaction the enhancement of drug oxidation due to another chemical or drug is considered to be a pharmacologically and toxicologically important problem. It has already been shown that hydroxylation of aniline and acetanilide in liver microsomes is enhanced by the addition of various compounds such as ethyl isocyanide [1], acetone [2, 3], 2,2′-bipyridine [4], metyrapone [5] and cyanide [6] to the reaction mixture. Imai and Sato [7] have demonstrated that ethyl isocyanide stimulates aniline hydroxylation in rabbit liver microsomes but not in guinea pig liver microsomes. Anders [3] has reported that the stimulatory effect of 2,2′-bipyridine on acetanilide hydroxylation in microsomes derives from phenobarbital-treated rats whereas 2,2′-bipyridine inhibits acetanilide hydroxylation activity in microsomes from 3-methylcholanthrene-treated rats. Powis and Boobis [8] have obtained essentially the same results using various compounds which are known to enhance aniline hydroxylation.

On the other hand, it has been confirmed by several investigators [9–12] that multiple species of cytochrome P-450 are present in microsomes, and the main cytochrome P-450 induced by phenobarbital treatment has been shown to have different properties from cytochrome P-448 preferentially induced by polycyclic hydrocarbon. These results suggest that the stimulatory effects of 2,2′-bipyridine and other reagents on aniline hydroxylation may be dependent upon the cytochrome P-450 species.

In order to study whether or not the stimulatory effect of acetone on aniline hydroxylation is dependent on the species of cytochrome P-450, we investigated the effect of this compound on aniline hydroxylation catalysed by a reconstituted system.

MATERIALS AND METHODS

Male rats of Crl;CD (SD) strain weighing 100–120 g were maintained on a commercial rat chow (CE-2 Nippon Clea Co., Japan). Rats were starved for about 20 hr prior to sacrifice but were given tap water *ad libitum*. They received either an intraperitoneal injection of 3-methylcholanthrene (25 mg/kg) dissolved in corn oil once a day for 8 successive days, or 0.1% sodium phenobarbital in drinking water for 3 days. Microsomes were prepared as described previously [13].

Cytochrome P-450 was purified from phenobarbital-treated rat liver microsomes by a method described by Kamataki *et al.* [14] which is a modification of the method of Imai and Sato [15]. Cytochrome P-448 was purified from 3-methylcholanthrene-treated rat liver microsomes by a modification of the method of Hashimoto and Imai [16]. Cytochrome P-450 was determined according to the method of Omura and Sato [17] using a molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ except that 20% glycerol and 0.2% Emulgen 913 were added in order to stabilize reduced cytochrome P-450. The specific contents of purified cytochrome P-450 and P-448 were 13.7 and 18.7 nmole/mg, respectively. NADPH-cytochrome *c* (P-450) reductase was purified from phenobarbital-treated rats by a slight modification of the method of Yasukochi and Masters [18]. The activity of NADPH-cytochrome *c* (P-450) reductase was measured according to the method of Phillips and Langdon [19] using a cytochrome *c* (horse heart) as an electron acceptor. The specific activity of purified NADPH-cytochrome *c* (P-450) reductase was 21.1 units/mg.

The reconstituted NADPH-dependent aniline hydroxylation system contained (in a final volume of 0.5 ml) purified cytochrome P-450 or P-448, purified NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl L-3-phosphatidylcholine, 2 mM aniline, NADPH-generating system (0.33 mM NADPH, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-

phosphate dehydrogenase and 6 mM $MgCl_2$) and 100 mM potassium phosphate (pH 7.25). When necessary, various concentrations of acetone or 2,2'-bipyridine were added to the reaction mixture. Incubation was carried out at 37° for 15 min aerobically, and the formation of *p*-aminophenol from aniline was measured by the method of Imai *et al.* [20]. The cumene hydroperoxide-supported aniline hydroxylation system contained purified cytochrome P-450 (1.64 μM) or P-448 (1.94 μM), 15 μg of dilauroy L-3-phosphatidylcholine, 2 mM aniline and 1.5 mM cumene hydroperoxide in a final volume of 0.5 ml. Reaction was initiated by the addition of cumene hydroperoxide. Hydrogen peroxide production was measured in the presence of catalase by the method of Hildebrandt *et al.* [21]. Reduction of cytochrome P-450 by NADPH was followed anaerobically in the presence of carbon monoxide by recording the change in the absorbance between 450 and 490 nm in a Hitachi Dual Wavelength Spectrophotometer, model 356. The method was similar to that of Diehl *et al.* [22]. The reduction rate was measured in the presence of 10 mM glucose, 6 mM $MgCl_2$, 130 units of catalase and 15 units glucose oxidase in a special anaerobic cuvette (Aminco). The reaction was started by quickly depressing a stop-cock plunger containing 30 μmoles of NADPH in 10 μl. Protein was determined by the method of Lowry *et al.* [23] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The acetone enhancement of aniline hydroxylation by microsomal mixed-function oxidase *in vitro* has been described earlier [2]. Powis and Boobis [8] have shown that the stimulatory effect of acetone on aniline hydroxylation is abolished by 3-methylcholanthrene pretreatment. In addition, aniline hydroxylation has been demonstrated to be catalysed by several species of cytochrome P-450, both in microsomes and the reconstituted system [6, 24, 25]. Therefore, it seemed reasonable to assume that the acetone enhancement of aniline hydroxylation may be dependent on the species of cytochrome P-450. Figure 1 shows the effects of various acetone concentrations on the reconstituted aniline hydroxylation system containing cytochrome P-450 purified from phenobarbital-treated rats and cytochrome P-448 purified from 3-methylcholanthrene-treated rats. Both cytochromes were active towards the hydroxylation of aniline. Aniline hydroxylation activity catalysed by cytochrome P-450 was increased by acetone and the maximal enhancing effect of acetone on aniline hydroxylation was observed at a concentration of 0.5 M, whereas aniline hydroxylation catalysed by cytochrome P-448 was decreased with increasing concentrations of acetone. As can be seen in Fig. 2, similar results were noted when 2,2'-bipyridine was used in place of acetone. The stimulation of aniline hydroxylation activity by 2,2'-bipyridine occurred in the system containing cytochrome P-450 but not in that containing cytochrome P-448. The maximal stimulatory effect of 2,2'-bipyridine observed in the system containing cytochrome P-450 was obtained when 0.5 mM 2,2'-bipyridine was added to the reaction mixture. From these results, it is suggested that the different cytochromes have different sensitivities towards acetone or 2,2'-bipyridine. Recently the activating and inhibiting effects of flavones on benzo[*a*]pyrene metabolism have also been shown to be dependent on the type of cytochrome P-450 used [26, 27]. Benzphetamine and metyrapone did not exhibit any stimulatory effects on aniline hydroxylation catalysed by either cytochrome P-450 or cytochrome P-448 (data not shown).

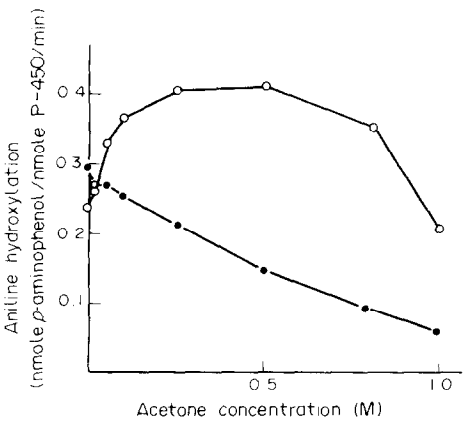

Fig. 1. Effect of acetone on the aniline hydroxylation activity in the reconstituted system. The cytochrome P-450 system (○) consisted of 0.113 nmole of cytochrome P-450 (13.7 nmole/mg), 0.23 unit of NADPH-cytochrome *c* (P-450) reductase (21.1 units/mg) and other components, as described in Materials and Methods, in a final volume of 0.5 ml. The cytochrome P-448 system (●) contained the same components as in the case of the cytochrome P-450 system except that 0.10 nmole of cytochrome P-448 (18.7 nmole/mg) was used in place of cytochrome P-450.

In addition, from the result that acetone also enhanced cumene hydroperoxide-supported aniline hydroxylation, Anders and Gander [28] have pointed out that the stimulatory effect of acetone on aniline hydroxylation may be due to it facilitating either the

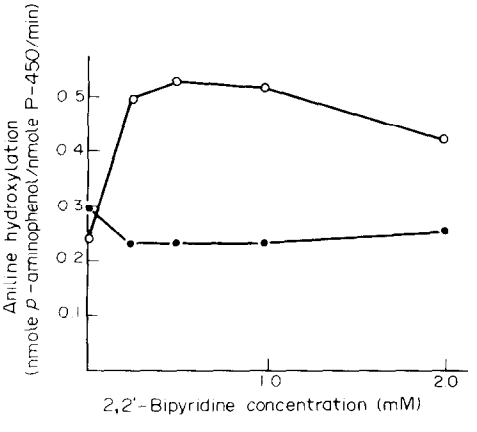

Fig. 2. Effect of 2,2'-bipyridine on the aniline hydroxylation activity in the reconstituted system. The cytochrome P-450 system (○) and cytochrome P-448 system (●) were the same as those described in Fig. 1.

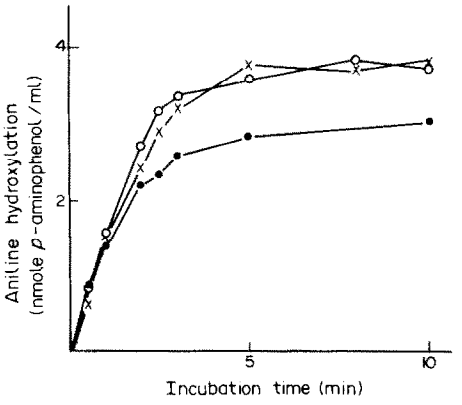

Fig. 3. Effects of acetone and 2,2′-bipyridine on cumene hydroperoxide-supported aniline hydroxylation catalysed by cytochrome P-450. Aniline hydroxylation was measured in the presence of acetone (●) or 2,2′-bipyridine (×) or absence of these compounds (○). The final concentrations of acetone, 2,2′-bipyridine and aniline used were 0.5 M, 0.5 mM and 2 mM, respectively.

formation of an active oxygen species or the insertion of oxygen into the substrate. Therefore, the effect of acetone on cumene hydroperoxide-supported aniline hydroxylation by a purified cytochrome P-450 was investigated. As shown in Fig. 3, although the linearity of the reaction in the presence of acetone was somewhat different from that obtained in the absence of acetone, neither acetone nor 2,2′-bipyridine stimulated aniline hydroxylation supported by cumene hydroperoxide. Under our experimental conditions, acetone (0.1, 0.25, 0.5 M) and 2,2′-bipyridine (0.25, 0.5, 1.0 mM) did not exhibit any stimulatory effect on cumene hydroperoxide (0.05, 0.1, 0.15, 0.2 mM)-supported aniline hydroxylation (data not shown). The reason for the discrepancy between the results reported by Anders and Gander [28] and our results cannot be entirely explained at present. However, as shown in Fig. 1, the effect of acetone on the cytochrome P-450 catalysing reaction was different from that on the cytochrome P-448 catalysing reaction. In addition, Anders and Gander used control microsomes while we used cytochrome P-450 purified from phenobarbital-treated rats. Therefore, the cytochromes present in the control microsomes may exhibit a different response to acetone than the cytochrome P-450 forms employed in this paper.

Table 1 shows the effects of acetone on the reduction of cytochrome P-450 and cytochrome P-448 by NADPH. The addition of aniline to the reaction mixture resulted in a decrease in the rate of cytochrome P-450 reduction by NADPH. In addition, acetone at the concentration employed in the studies of reconstituted aniline hydroxylation virtually abolished the inhibitory effect of aniline on the cytochrome reduction. In contrast, neither aniline nor acetone produced any significant effect on NADPH-dependent cytochrome P-448 reduction.

Hydrogen peroxide has been considered to be formed by the autoxidation of oxygenated reduced cytochrome P-450 [29–31]. Therefore, the formation of hydrogen peroxide during aniline hydroxylation catalysed by cytochrome P-450 or P-448 was measured in the presence of aniline and/or acetone in order to clarify whether or not acetone affects the stability of the oxy complex. As can be seen in Table 2, aniline inhibited the formation of hydrogen peroxide in the presence or absence of acetone. On the other hand, acetone stimulated the formation in the reconstituted system containing cytochrome P-450. Although acetone increased the formation of hydrogen peroxide in the system containing cytochrome P-450, the ratio of *p*-aminophenol formation to hydrogen peroxide production was essentially independent of the presence of acetone. Similar results were obtained when the hydrogen peroxide produced was measured by the ferrithiocyanate method [32]. From these results, it was suggested that the autoxidation rate of oxycytochrome P-450 might be unaffected by acetone, and the increase in hydrogen peroxide formation observed in the presence of acetone might be due to an increase in the rate of cytochrome P-450 reduction. In addition, the stimulatory effect of acetone on aniline hydroxylation in the system containing cytochrome P-450 was not affected by the addition of catalase to the reaction mixture (data not shown).

According to Werringloer and Kawano [33], NADPH-dependent donation of the first electron to cytochrome P-450 is slowed down at lower pH values due to an impaired function of NADPH-cytochrome P-450 reductase. They have also suggested that the rate-limiting step shifts from the reduction of the ferric cytochrome P-450–substrate complex to the reduction of oxycytochrome P-450 with increasing pH. Figure 4 shows the effect of pH on aniline hydroxylation in the presence or absence of acetone. Although aniline hydroxylation in the presence or

Table 1.

Addition	Cytochrome P-450 reduction	
	Cytochrome P-450 system (nmole P-450 or P-448 reduced/min)	Cytochrome P-448 system
None	5.9	3.5
Aniline	3.2	3.6
Acetone	8.1	3.8
Aniline + acetone	7.7	3.2

Table 2.

Groups	Formation of * p-aminophenol (nmole/nmole cytochrome/min)	Formation of * H_2O_2 (nmole/nmole cytochrome/min)	Ratio of product/H_2O_2	Difference**
Cytochrome P-450 system				
None		2.40 ± 0.06		
Aniline	0.29 ± 0.01	1.83 ± 0.06	0.16	0.57
Acetone		3.83 ± 0.01		
Aniline + acetone	0.47 ± 0.03	2.97 ± 0.05	0.17	1.05
2,2'-Bipyridine		4.88 ± 0.05		
Aniline + 2,2'-bipyridine	0.41 ± 0.01	2.83 ± 0.12	0.15	2.05
Cytochrome P-448 system				
None		1.99 ± 0.02		
Aniline	0.26 ± 0.02	1.71 ± 0.03	0.15	0.28
Acetone		1.80 ± 0.06		
Aniline + acetone	0.24 ± 0.05	1.58 ± 0.03	0.15	0.22
2,2'-Bipyridine		2.36 ± 0.18		
Aniline + 2,2'-bipyridine	0.27 ± 0.02	2.03 ± 0.02	0.13	0.33

absence of acetone was increased with increasing pH of reaction media, the stimulatory effect of acetone or aniline hydroxylation was decreased as the pH increased. The same results were obtained when 2,2'-bipyridine was added to the system containing cytochrome P-450 in place of acetone. From these results, it seems possible to assume that the reversal effect of acetone on the inhibition of cytochrome P-450 reduction due to aniline may be responsible for the activating effect of acetone. At present, the reason why acetone stimulated cytochrome P-450 reduction by NADPH is unknown. In the preliminary experiments, acetone decreased the type II spectral change induced by aniline, suggesting that the addition of acetone in the presence of aniline results in an increase in a higher spin state of cytochrome P-450. Thus it seems possible that a shift to a higher spin state of the cytochrome may be related to acetone stimulation of aniline hydroxylation. Further studies will be required to establish the exact mechanism(s) of the enhancement of aniline hydroxylation by acetone.

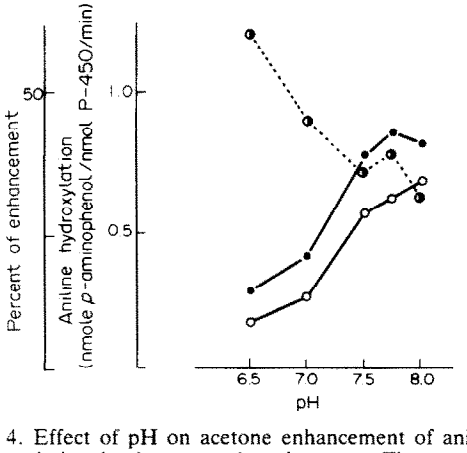

Fig. 4. Effect of pH on acetone enhancement of aniline hydroxylation in the reconstituted system. The reaction mixture consisted of 0.139 nmole of cytochrome P-450, 0.29 unit of NADPH-cytochrome *c* (P-450) reductase and other components as described in Fig. 1, except that 50 mM Tris/Hepes of the indicated pH was used in place of phosphate (pH 7.25) in a final volume of 0.5 ml. The aniline hydroxylation activity was measured in the presence (●) or absence (○) of acetone. The activating effect of acetone (◑) on aniline hydroxylation was represented as per cent of enhancement.

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