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MULTIPLE DRUG METABOLISM: *p*-NITROANISOLE REVERSAL OF ACETONE ENHANCED ANILINE HYDROXYLATION

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Abstract—The unique effect of acetone on the *p*-hydroxylation of aniline was evaluated in microsomes prepared from control, phenobarbital- and 3-methylcholanthrene-pretreated animals. The existence of multiple forms of the hydroxylase was evaluated as an explanation of the acetone enhancement. Simultaneous metabolism of aniline in the presence of either *p*-nitroanisole (pNA) or ethylmorphine (EM) was evaluated to probe the participation of different mixed function oxidase systems. Aniline inhibited both *N*- and *O*-demethylation, while pNA and EM both inhibited *p*-hydroxylation of aniline. Acetone decreased the individual demethylation reactions, but enhanced aniline hydroxylation. In multiple drug reactions, acetone decreased *N*-demethylation and proportionately increased aniline *p*-hydroxylation. On the other hand, *p*-nitroanisole blocked the acetone enhancement of aniline metabolism. Kinetic evaluation of the acetone and *p*-nitroanisole effects on aniline metabolism indicated that each agent increased the apparent K_m' by 4- to 5-fold for aniline in the hydroxylation reaction, but only acetone increased the V_{max}'. From the Eadie–Scatchard analysis of the rates of aniline hydroxylation, acetone appeared to produce a biphasic increase in the hydroxylation above 0.75 mM aniline, even in the presence of pNA. Thus, multiple forms of the aniline *p*-hydroxylase are indicated by their altered activities in the presence of other drugs, and acetone seemed to specifically alter a species having a higher K_m' for aniline.

The enhancement of aniline *p*-hydroxylation in the presence of acetone was first reported by Anders [1]. Confirmation of this unique effect has been reported in microsomes prepared from control, phenobarbital- and 3-methylcholanthrene-pretreated animals [2–4]. In addition, the metabolism of several other aromatic amines has also been shown to undergo enhancement in the presence of acetone, including that of acetanilide, *N*-butylaniline, *p*-anisidine and *p*-phenetidine [1, 5, 6]. Interestingly, these compounds are each derivatives of aniline.

The direct interaction of aniline with cytochrome P-450 produced a typical type II binding (difference) spectrum at all concentrations [7]. In the presence of acetone, low concentrations of aniline generated a type I spectrum which reverted to the type II spectrum as the concentration of aniline was increased [3]. These results suggested that acetone either altered the accessibility of aniline to the heme center of cytochrome P-450 or it specifically affected different species of cytochrome P-450 [8].

Chemicals other than acetone have also been reported to enhance aniline hydroxylation. These agents include ethyl isocyanide, metyrapone, paraoxon, 2-pentanone, 2,2-bipyridine, 1,10-phenanthroline, methoxyflurane, halothane and trichloroethane [1, 4, 9–14]. Even though several of these agents are structurally related, there is no common structure or solvent effect existing for them as a group. However, their effects are diminished in microsomal preparations lacking the ability to form type I spectral responses [4]. In addition, these chemicals apparently produce their enhancing effects through mechanisms which differ, at least in part, from that of acetone [1, 5, 8].

Acetone addition inhibits other drug metabolism reactions, including *N*- and *O*-demethylation, as well as hydroxylation of benzo[*a*]pyrene [1, 3, 4, 6]. Thus, an important aspect of acetone enhancement may be the existence of multiple forms of the aniline hydroxylase, utilizing different species of cytochrome P-450. Separation and characterization of multiple cytochrome P-450s have been reported with each differing in substrate specificity and in rates of drug metabolism using reconstituted enzyme systems [15–17]. In fact, the simultaneous metabolism of aniline, *p*-nitroanisole, and other drugs has been reported in isolated hepatocytes and subcellular fractions [18–20].

For these reasons, *p*-nitroanisole was used as an additional probe to evaluate the mechanism of acetone enhancement of aniline metabolism by different species of aniline hydroxylase.

METHODS

Male Sprague–Dawley rats (*ca.* 250 g) were maintained on Purina Rat Chow and allowed to drink water *ad lib*. Phenobarbital-pretreated animals were given sodium phenobarbital (0.15%) in their drinking water for 7 days prior to being killed. Animals pretreated with 3-methylcholanthrene were given

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two intraperitoneal injections of 3-methylcholanthrene (20 mg/kg) in corn oil 24 hr apart and killed on day 4. For each experiment, several livers (three to four) were routinely pooled prior to homogenization and microsomal preparation to minimize animal variance. Microsomes were prepared by a method published previously [21].

The reaction system contained microsomes (1 mg/ml) suspended in 2 ml of Tris (50 mM)–KCl (150 mM) buffer, pH 7.4, containing $MgCl_2$ (10 mM), as well as the appropriate pyridine nucleotides (NADPH, 0.5 mM; NADH, 0.5 mM; or both) and the drug substrates as indicated (aniline, 1 mM; *p*-nitroanisole, 2 mM; ethylmorphine, 3 mM). Acetone (0.08 M or 0.8 M) was added immediately before initiation of the reaction by addition of the microsomes. The reactions were carried out at 37° for 15 min and then terminated by addition of ice-cold trichloroacetic acid (TCA) (7% final). All reactions were linear under these conditions.

Following centrifugation at 2000 *g* for 10 min, aliquots were removed from the clear TCA-supernatant fraction for drug-product analysis. Aniline hydroxylation was determined by the method of Imai *et al.* [22], measuring the formation of *p*-aminophenol. Demethylation of *p*-nitroanisole was determined by quantitating the formation of *p*-nitrophenol as described by Kato and Gillette [23]. The metabolism of ethylmorphine was determined by the release of formaldehyde, using the method of Nash [24].

RESULTS

The effect of acetone on multiple drug metabolism was evaluated in microsomes prepared from control, phenobarbital- (PB) and 3-methylcholanthrene- (3-MC) pretreated animals. The metabolism of aniline and *p*-nitroanisole (pNA) was determined in the absence and presence of acetone (Tables 1 and 2).

In comparison to the hydroxylation of aniline in control microsomes, the *p*-hydroxylase activity was decreased 40% in 3-MC microsomes and increased 30% in PB microsomes. Neither of these changes in activity was as great as reported previously [4, 6, 7]. *p*-Nitroanisole *O*-demethylation was increased in 3-MC (85%) and in PB (139%) microsomes. In the presence of both drugs, aniline inhibited pNA metabolism 40–60%, while pNA inhibited aniline hydroxylation in control (11%) and PB (25%) microsomes and increased aniline metabolism (18%) in 3-MC microsomes.

Addition of acetone increased the metabolism of aniline, but inhibited pNA *O*-demethylation (Tables 1 and 2). The highest concentration of acetone (0.8 M) had the greatest effect on both drug reactions. In the presence of both drugs, acetone enhancement of aniline hydroxylation was lost, suggesting that pNA blocked the acetone effect. Conversely, the inhibition of pNA metabolism in each of the microsomal preparations was increased by acetone.

The effects of acetone on the rates of drug metabolism were also evaluated in PB microsomes using NADPH, NADH or both pyridine nucleotides (Table 2). Acetone enhanced aniline hydroxylation catalyzed by NADPH, NADH or both nucleotides, but inhibited the metabolism of pNA catalyzed by either pyridine nucleotide. When aniline and pNA were metabolized together, pNA slightly increased the NADH hydroxylation of aniline, while aniline inhibited the demethylation of pNA. Acetone decreased the metabolism of pNA even more. Importantly, the enhancement of aniline hydroxy-

Table 1. Effect of acetone on the simultaneous metabolism of aniline and *p*-nitroanisole in microsomes prepared from control or 3-methylcholanthrene-pretreated rats

Animal pretreatment* (microsomes)	Acetone (mM)	Drug metabolism†,‡ (nmoles product/15 min)			
		Aniline (pAP)	*p*-Nitroanisole (pNP)	Aniline + *p*-nitroanisole (pAP)	(pNP)
Control	0	16.2 ± 0.2 (100)	16.3 ± 0.1 (100)	14.4 ± 1.0 (89)	9.9 ± 0.5 (61)
	80	18.9 ± 1.0 (117)	15.4 ± 0.9 (95)	13.6 ± 0.6 (84)	7.8 ± 0.4 (48)
	800	30.5 ± 2.8 (188)	8.4 ± 0.9 (52)	17.5 ± 1.7 (108)	4.6 ± 0.4 (28)
3-Methylcholanthrene	0	10.2 ± 0.2 (100)	30.3 ± 1.7 (100)	12.0 ± 0.1 (118)	12.5 ± 1.6 (41)
	80	12.0 ± 0.9 (118)	26.4 ± 0.9 (87)	11.5 ± 0.1 (113)	11.3 ± 0.9 (37)
	800	17.9 ± 2.0 (175)	8.6 ± 0.3 (28)	10.5 ± 0.9 (103)	4.0 ± 0.3 (13)

* Male Sprague–Dawley rats (*ca.* 250 g) received no pretreatment or 3-methylcholanthrene (20 mg/kg in corn oil) i.p. for 2 days. Each value is the mean ± S.D. of two experiments.

† The reaction system contained microsomes (1 mg/ml) in Tris (50 mM)–KCl (150 mM) buffer, pH 7.4, $MgCl_2$ (10 mM), NADPH (0.5 mM), aniline (1 mM), *p*-nitroanisole (2 mM) or both drugs, and acetone, as indicated. The acetone was added just prior to initiation of the reactions with the microsomes.

‡ Drug metabolism is expressed as nmoles of product formed·(mg protein)$^{-1}$·15 min^{-1}. The rates of metabolism are compared as the percentage of activity determined for the individual drugs (see values in parentheses).

Table 2. Effect of acetone on pyridine nucleotide supported metabolism of aniline and *p*-nitroanisole in microsomes prepared from phenobarbital-pretreated rats*

Pyridine nucleotide (0.5 mM)	Acetone (mM)	Drug metabolism† (nmoles/15 min)			
		Aniline (pAP)	*p*-Nitroanisole (pNP)	Aniline + *p*-nitroanisole (pAP)	(pNP)
NADPH	0	20.9 ± 2.3 (100)	39.0 ± 1.6 (100)	15.6 ± 1.2 (75)	23.5 ± 4.9 (60)
	80	33.0 ± 3.4 (158)	37.8 ± 2.7 (97)	14.8 ± 2.0 (71)	22.2 ± 4.3 (57)
	800	36.2 ± 2.7 (173)	24.6 ± 2.7 (63)	15.5 ± 1.0 (74)	17.3 ± 2.4 (44)
NADH	0	3.0 ± 0.4 (100)	17.9 ± 1.5 (100)	4.6 ± 0.2 (153)	7.7 ± 0.4 (43)
	80	4.9 ± 1.0 (163)	15.9 ± 1.3 (89)	4.1 ± 1.4 (137)	5.2 ± 2.6 (29)
	800	6.6 ± 0.9 (220)	7.7 ± 0.8 (40)	3.6 ± 0.8 (120)	4.3 ± 1.4 (24)
NADPH + NADH	0	19.5 ± 1.6 (100)	42.6 ± 2.7 (100)	18.5 ± 0.7 (95)	27.5 ± 5.9 (65)
	80	34.0 ± 1.4 (174)	42.8 ± 3.0 (100)	19.4 ± 1.7 (99)	26.6 ± 5.4 (62)
	800	47.3 ± 1.3 (243)	30.4 ± 2.3 (71)	23.7 ± 1.0 (122)	23.2 ± 1.0 (54)

* Sodium phenobarbital (0.15%) was given *ad lib.* for 7 days to male Sprague–Dawley rats in place of their drinking water. Each result is the mean ± S.D. of three experiments.

† The conditions of the reaction system are described in Table 1. Drug metabolism is expressed as nmoles of product formed · mg^{-1} · 15 min^{-1}. The rates of metabolism are compared as the percentage (see values in parentheses) of activity determined for the individual drugs. *p*-Aminophenol (pAP) and *p*-nitrophenol (pNP) are the products of aniline and *p*-nitroanisole metabolism respectively.

Table 3. Effect of acetone on pyridine nucleotide supported metabolism of aniline and ethylmorphine in microsomes prepared from phenobarbital-pretreated rats*

Pyridine nucleotide (0.5 mM)	Acetone (mM)	Drug metabolism†,‡ (nmoles/15 min)			
		Aniline (pAP)	Ethylmorphine (HCHO)	Aniline + ethylmorphine (pAP)	(HCHO)
NADPH	0	20.9 ± 2.3 (100)	145.2 ± 22.0 (100)	4.3 ± 1.3 (21)	117.7 ± 18.6 (81)
	80	33.0 ± 3.4 (158)	138.5 ± 20.0 (95)	5.9 ± 2.4 (28)	108.1 ± 14.5 (74)
	800	36.2 ± 2.7 (173)	51.5 ± 9.0 (35)	25.2 ± 2.7 (121)	34.0 ± 1.7 (23)
NADH	0	3.0 ± 0.4 (100)	53.0 ± 10.8 (100)	1.6 ± 1.2 (53)	27.1 ± 11.2 (51)
	80	4.9 ± 1.0 (163)	48.2 ± 11.3 (91)	2.2 ± 0.8 (73)	24.6 ± 7.7 (46)
	800	6.6 ± 0.9 (220)	22.7 ± 7.7 (43)	5.1 ± 1.3 (170)	6.5 ± 1.7 (12)
NADPH + NADH	0	19.5 ± 1.6 (100)	176.3 ± 27.8 (100)	4.4 ± 0.9 (23)	116.2 ± 38.2 (66)
	80	34.0 ± 1.4 (174)	163.9 ± 26.0 (93)	7.7 ± 1.3 (39)	110.1 ± 26.2 (68)
	800	47.3 ± 1.3 (243)	76.7 ± 12.2 (44)	32.5 ± 3.5 (167)	42.5 ± 15.4 (24)

* Animal pretreatment with phenobarbital was described in Table 2. Each value is the mean ± S.D. of three experiments.

† Reaction conditions are described in Table 1, except that ethylmorphine (3 mM) was used.

‡ Drug metabolism is expressed as nmoles of product formed · mg^{-1} · 15 min^{-1}. The rates of metabolism are compared as the percentage (see values in parentheses) of activity determined for the individual drugs using each nucleotide. *p*-Aminophenol (pAP) and formaldehyde (HCHO) are the respective products of aniline and ethylmorphine metabolism.

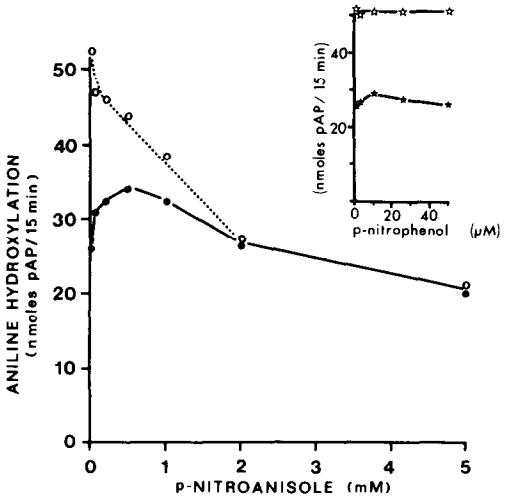

Fig. 1. Effect of *p*-nitroanisole on aniline hydroxylation. Microsomes (1 mg/ml) from phenobarbital-pretreated animals were suspended in Tris (50 mM)–KCl (150 mM) buffer, pH 7.4, containing $MgCl_2$ (10 mM) and NADPH (0.5 mM). The reactions were carried out at 37° for 15 min either in the presence (open symbols) or in the absence (closed symbols) of acetone. Drug concentrations utilized were aniline (2 mM), *p*-nitroanisole (0.05–5.0 mM), or *p*-nitrophenol (2–50 μM), and acetone (0.8 M) as indicated.

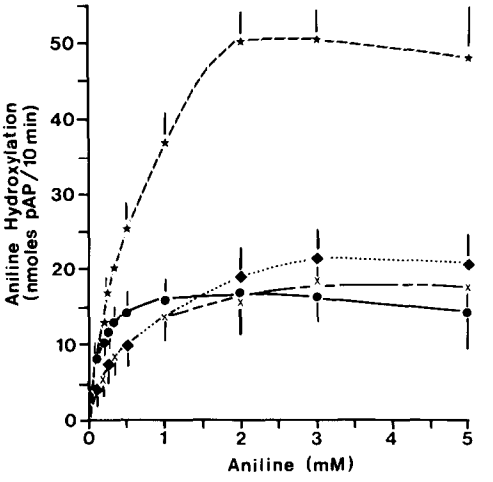

Fig. 2. Effect of acetone and *p*-nitroanisole on aniline hydroxylation at various aniline concentrations. The reaction time was 10 min, while the other experimental conditions were the same as those described in the legend of Fig. 1. The reaction curves represent aniline (●—●), aniline + acetone (★—★), aniline + *p*-nitroanisole (×—×), and aniline + *p*-nitroanisole + acetone (◆—◆). The drug concentrations used were aniline (0.0–5.0 mM), *p*-nitroanisole (2 mM) and acetone (0.8 M). Each data point is the mean ± S.D. of three experiments.

lation produced by 0.8 M acetone was eliminated by only 2 mM pNA.

Ethylmorphine was also evaluated for its effects on acetone enhancement of aniline hydroxylation, since this drug is metabolized by *N*-demethylation, rather than *O*-demethylation (Table 3). Ethylmorphine (EM) strongly inhibited aniline hydroxylation, catalyzed by NADPH and/or NADH. Conversely, aniline inhibited NADPH-dependent metabolism of ethylmorphine 20% and inhibited NADH-dependent *N*-demethylation by 50%. As noted previously, acetone enhanced both NADPH and NADH catalyzed aniline hydroxylation, but severely inhibited EM metabolism. In the presence of both aniline and acetone, the inhibition of EM metabolism was increased, while acetone appeared to relieve the inhibition of aniline hydroxylation produced by EM. Although not included, similar results were obtained for *N*-demethylation of aminopyrine and benzphetamine.

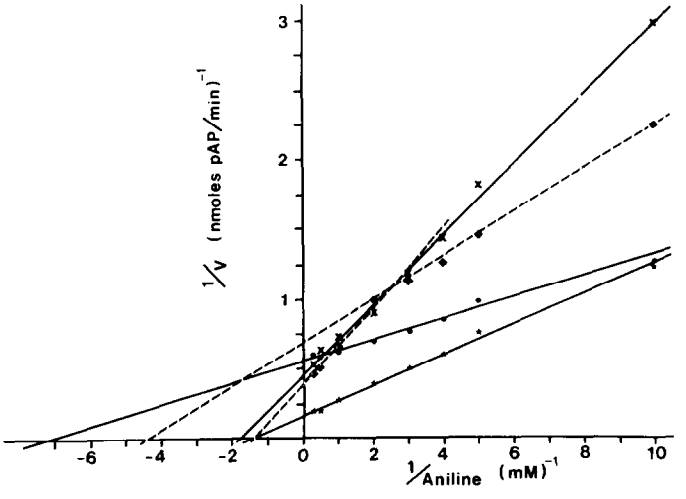

Fig. 3. Lineweaver–Burk plot of aniline hydroxylation in the presence of *p*-nitroanisole and acetone. The reaction conditions are described in the legend of Fig. 1, and the drug concentrations are the same as those used in Fig. 2. The reaction curves represent aniline (●—●), aniline + acetone (★—★), aniline + *p*-nitroanisole (×—×), and aniline + *p*-nitroanisole + acetone (◆—◆).

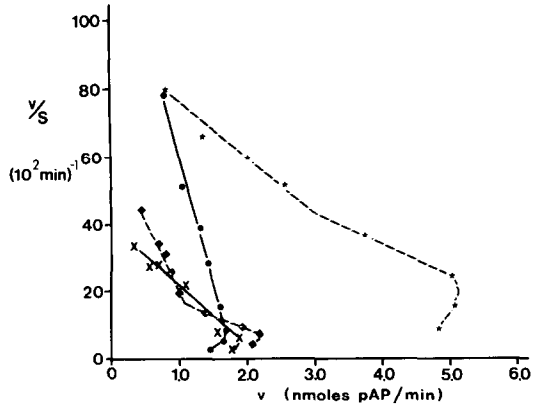

Fig. 4. Eadie–Scatchard plot of aniline hydroxylation in the presence of *p*-nitroanisole and acetone. The reaction conditions and drug concentrations are the same as those described in the legends of Figs. 1 and 2. The reaction curves represent aniline (●—●), aniline + acetone (★—★), aniline + *p*-nitroanisole (×—×), and aniline + *p*-nitroanisole + acetone (◆—◆).

Since *p*-nitroanisole seemed to specifically block the acetone enhancement, it was examined further for its effects on aniline *p*-hydroxylation (Fig. 1). At lower concentrations, pNA actually increased aniline metabolism. However, as the concentration of pNA increased, pNA became a weak inhibitor of aniline metabolism. In these experiments, acetone enhanced aniline hydroxylation 100%, initially. However, when acetone and pNA were both added, the acetone effect was again reversed by pNA. *p*-Nitrophenol was evaluated at concentrations produced during pNA metabolism (Fig. 1, insert) and, as indicated, no major effect on aniline hydroxylation was observed. Thus, the effects of pNA on acetone enhancement of aniline hydroxylation are due to pNA and not its metabolite.

The effects of acetone and pNA on aniline *p*-hydroxylation were examined at different aniline concentrations (Fig. 2). Acetone enhanced aniline hydroxylation from 0.05 mM to 5.0 mM aniline; yet, the maximum rate of metabolism was reached at 2.0 mM. *p*-Nitroanisole (2 mM) inhibited aniline metabolism whenever the concentration of aniline was less than the pNA concentration, but slightly increased the metabolism at higher aniline concentrations. When acetone and pNA were both present, pNA blocked the general acetone enhancement.

The reaction rates were evaluated further using both the Lineweaver–Burk analysis (Fig. 3) and Eadie–Scatchard analysis (Fig. 4) [25]. Both acetone and pNA altered the kinetic parameters of aniline *p*-hydroxylase. Acetone increased the K_m' and V_{max}' of aniline for the reaction (Table 4), while pNA only increased the K_m' for aniline. When both agents were present, a biphasic system was noted by both methods. Uniquely, the Eadie–Scatchard analysis (Fig. 4) indicated that a dual K_m' and V_{max}' existed in each of the reaction systems containing acetone. *p*-Nitroanisole decreased the V_{max}' of the acetone effect. Even though acetone increased the aniline hydroxylation at all concentrations of aniline, the activity was uniquely increased above 0.75 mM aniline even when the reaction was inhibited by pNA. Thus, these results support the contention that multiple forms of aniline *p*-hydroxylase do exist, and are expressed differentially in the presence of acetone.

DISCUSSION

The enhancement of aniline *p*-hydroxylation by acetone remains a complex event. Since the initial report by Anders [1], several hypotheses have developed to explain the unique increase in aniline metabolism. First, an increase in the electron flow between cytochrome P-450 reductase and cytochrome P-450 was considered initially. However, aniline itself was found to decrease the rate of reduction and acetone did not alter this lowered activity nor did it increase the total amount of cytochrome P-450 reduced [3]. In addition, most other drug metabolism reactions were inhibited by acetone (Tables 2 and 3), e.g. pNA *O*-demethylation [1], *N*-demethylation of ethylmorphine [1, 3, 6] and aminopyrine [4], and the aromatic hydroxylation of benzo[*a*]pyrene [3]. Second, a general solvent effect on the membrane was also suggested to explain the enhancement. The previous discussion negates this possibility, as well as the specificity of acetone itself.

Table 4. Kinetic parameters for aniline *p*-hydroxylase in rat liver microsomes*

	Microsomal aniline *p*-hydroxylase activities			
	Lineweaver–Burk method		Eadie–Scatchard method	
Drugs added	K_m' (mM)	V_{max}' (nmoles/min)	K_m' (mM)	V_{max}' (nmoles/min)
(1) Aniline	0.14	1.8	0.13	1.8
			0.75	5.6
(2) Aniline + acetone	0.67	6.5	1.08	2.1
(3) Aniline + pNA	0.55	2.2	0.58	2.3
(4) Aniline + acetone	0.23	1.5	0.30	1.5
+ pNA	0.66	1.7	1.30	1.8

* The reaction conditions were the same as those described in Table 2, using phenobarbital microsomes. These parameters were determined from the hydroxylase activities reported in Figs. 3 and 4.

Anders noted in his original paper that only 2-pentanone similarly enhanced aniline metabolism, even though several other aldehydes and ketones were examined. In addition, agents known to have solvent effects on membranes, i.e. dimethylsulfoxide, dimethylformamide and methyl cellusolve, were determined to be inhibitors of aniline metabolism. Third, an alteration in aniline binding to the terminal cytochrome was substantiated in part. In the absence of acetone, the interactions of aniline with the oxidized cytochrome P-450 produced a typical type II binding spectrum [3, 26]. However, in the presence of acetone, a type I spectrum was formed at lower aniline concentrations, which reverted to a type II spectrum as the concentration of aniline was increased [3]. Even though there is no direct relationship between the intensity of the binding spectrum and the rate of metabolism by the cytochrome P-450, the altered spectrum at least suggested that a change was produced near the heme center of cytochrome P-450. Fourth, similarly, acetone was considered to alter the availability of oxygen for the reaction. Akhrem *et al.* [27] concluded that oxygen was indeed the rate-limiting step in aniline hydroxylation. However, there was less than a 10% change in rate of aniline metabolism as the oxygen concentration was increased [28, 29]. Similarly, in the presence of acetone, only a small increase in rate occurred as the oxygen concentration increased. Fifth, since the K_m/V_{max} ratio remained constant as the acetone concentration was increased, Anders [1] concluded that acetone must be participating in the enzyme–substrate complex, as an activator, decreasing the activation energy for the reaction. Schenkman [30] noted that the Arrhenius plot for aniline metabolism was linear. Since the activation energies for different enzymes, even if they convert the same substrate to the same product, would not be expected to be identical [31] Schenkman concluded that the presence of more than one enzyme in the microsomes to metabolize aniline was unlikely. However, he did not evaluate the effect of acetone. Sixth, multiple forms of the enzymes capable of metabolizing aniline have received some support with the recent characterization of multiple cytochrome P-450 forms, each differing in spectral characteristics and antibody specificity, and to a limited extent substrate specificity [32]. Differences have been noted in the rates of aniline hydroxylation determined in microsomes prepared from animals pretreated with PB, 3-MC, Arochlor and other agents [6, 33–35]. In addition, Kitada *et al.* [6] reported that metabolism of *p*-anisidine and *p*-phenetidine was enhanced by acetone, even though other *O*-dealkylating reactions were inhibited. Since these drugs are aniline analogs, it is at least suggestive that different terminal cytochromes may be involved in their metabolism. Pregnisolone and hydrocortisone altered aniline hydroxylation in both control and PB microsomes, changing the linear reaction kinetics to biphasic reaction rates [34]. In hamsters, aniline *p*-hydroxylase appeared to exist in two forms, having a low affinity and a high affinity for aniline respectively. Chronic ethanol treatment increased the high affinity form [35], supporting the possibility of multiple forms of the hydroxylase in other species.

From the results reported, we support the concept of multiple aniline hydroxylases, since acetone produced a general increase in hydroxylase activity at lower aniline concentrations and a specific increase in activity above 0.75 mM aniline (Fig. 4). In addition, when pNA (2 mM) was used as an additional probe, aniline hydroxylation was inhibited *ca.* 25%, but this concentration of pNA completely blocked the acetone enhancement produced by 800 mM acetone. These results also bring into question the concept of a general solvent effect, since a low concentration of pNA effectively reversed a 40-fold greater acetone concentration. In addition, none of the other drugs examined (ethylmorphine, benzphetamine, or aminopyrine) affected the acetone enhancement, but each was a strong inhibitor of aniline hydroxylation.

Thus, if more than one enzyme form were present and had similar activation energies, then we could explain the biphasic rate of metabolism by a differential effect of acetone on each form. This possibility deserves further attention and may add to our understanding of acetone enhancement in aniline metabolism.

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