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ENHANCEMENT BY CYANIDE OF ANILINE *p*-HYDROXYLATION ACTIVITY IN RAT LIVER MICROSOMES

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Abstract—Aniline *p*-hydroxylation activity of rat liver microsomes was found to be enhanced, rather than inhibited, at aniline concentrations higher than about 3 mM. The cyanide-induced enhancement increased as the oxygen tension was increased. The activation by cyanide was, however, significantly diminished with liver microsomes from phenobarbital- and 3-methylcholanthrene-pretreated rats. The enhancement was also decreased when liver microsomes were fortified with NADPH-cytochrome P-450 reductase. Aniline hydroxylation by reconstituted systems consisting of partially purified preparations of several species of cytochrome P-450 and the reductase was inhibited by cyanide, though the degree of inhibition was dependent on the species of cytochrome P-450 used for reconstitution. In several respects, the cyanide-induced enhancement of aniline hydroxylation is different from the enhancement caused by acetone and 2,2′-bipyridine, but is similar to the activation by ethylisocyanide.

Imai and Sato [1, 2] first demonstrated that ethylisocyanide exerts both stimulatory and inhibitory effects on aniline hydroxylation by rabbit liver microsomes, depending on the assay conditions. They concluded that ethylisocyanide inhibits the hydroxylation by competing with molecular oxygen for the reduced heme of cytochrome P-450 and activates by altering the reactivity of the cytochrome, probably through binding to a non-heme site on the cytochrome molecule. Since then, the addition of acetone [3–7], 2,2′-bipyridine [4, 7], benzphetamine [5, 8], metyrapone [9, 10] and paraoxon [11, 12] *in vitro* to liver microsomes has been reported to enhance markedly the hydroxylation of aniline and some other aromatic amines. However, the mechanisms of activation by these chemicals have not yet been fully elucidated.

Previously, it was widely accepted that drug oxidations by liver microsomes are insensitive to cyanide [13–15]. The current view, however, indicates that the terminal oxidase of the microsomal drug metabolizing system, cytochrome P-450, is a heme protein [16] and therefore is capable of binding cyanide to produce a characteristic difference spectrum [17, 18]. Actually, recent studies have demonstrated that microsomal oxidations of several drugs, including aniline hydroxylation, are sensitive to cyanide [19–21].

In this communication, we report that cyanide activates aniline hydroxylation by rat liver microsomes under appropriate conditions, as in the case of ethylisocyanide [1]. Studies conducted which attempt to elucidate the mechanism of this activation are also reported and discussed.

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MATERIALS AND METHODS

Animals and chemicals. Male rats of Wistar strain, weighing 90–130 g, were used unless otherwise stated. The animals were fasted for about 48 hr prior to being killed, but were given tap water *ad lib.* When necessary, rats received intraperitoneal injection of 3-methylcholanthrene (MC) dissolved in olive oil at a daily dose of 25 mg per kg body wt for 8 consecutive days, and then were starved for 18 hr prior to being killed. Pretreatment of the animals with sodium phenobarbital (PB) was performed by adding 0.1% PB in drinking water for 3 days. Liver microsomes were prepared as described previously [22]. Emulgen 913, a non-ionic detergent, was supplied by Kao-Atlas Co. Dilauroyl-L-3-phosphatidylcholine was purchased from Serdary Co. $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and cytochrome *c* (horse heart) were obtained from Boehringer, Mannheim. Commercially available aniline was redistilled under vacuum and stored at −20° under nitrogen until use. Sepharose 4B and CM-Sephadex C-50 were purchase from Pharmacia and hydroxylapatite (Bio-Gel HT) from Bio-Rad. ω-Amino-*n*-octyl Sepharose 4B was prepared as described by Cuatrecasas [23]. KCN solutions were prepared freshly and adjusted to pH 7.4 with 1 N HCl.

Purification of cytochrome P-450*'s and cytochrome* P-450 *reductase.* Three species of cytochrome P-450 were partially purified from liver microsomes of drug-untreated rats according to the method of Imai and Sato [24], as modified by Kamataki *et al.* [25].

The results of the purification experiment are summarized in Table 1. The molecular weights of cytochrome P-450's in fractions I-1-a, I-1-b and I-2 were estimated to be 47,000, 52,000 and 47,000, respectively, from polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [26]. All these cytochrome P-450's appeared to be low-spin species as judged from their absorption spectra, and were free from cytochrome b_5 detectable by dithionite-reduced minus oxidized difference spectrophotometry.

A cytochrome P-448 was purified from liver microsomes of MC-pretreated rats by the method of Hashimoto and Imai [27]. The purified cytochrome showed a Soret peak at 448 nm in the CO-difference spectrum and its molecular weight was estimated to be 52,500. Its specific content and recovery from microsomes were 18.4 nmoles per mg of protein and 1.2 per cent, respectively. This preparation appeared to contain both high- and low-spin species, because its oxidized absorption spectrum showed peaks at both 398 and 414 nm.

NADPH-cytochrome P-450 (cytochrome *c*) reductase was partially purified from liver microsomes of male CR:CD (SD) rats pretreated with PB. The liver microsomes were washed with 0.1 M pyrophosphate, pH 7.4, and NADPH-cytochrome P-450 reductase was purified to a specific activity of 21.1 unit per mg of protein by the previously described method [28]. The partially purified preparation was devoid of cytochrome b_5 and P-450.

Analytical and assay methods. Protein was determined by the method of Lowry *et al.* [29] using bovine serum albumin as standard. Cytochrome P-450 (and P-448) was estimated as described by Omura and Sato [30]; for purified preparations, 0.2% Emulgen 913 and 20% glycerol were included in all determinations. NADPH-cytochrome *c* reductase activity as described by Phillips and Langdon [31], and one unit is defined as the amount catalyzing the reduction of 1 μmole of cytochrome *c* per min.

The standard reaction mixture for assay of microsomal aniline hydroxylation activity contained, in a final volume of 1.0 ml, microsomes (about 1 mg of protein), 20 mM aniline, 80 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, and an NADPH-generating system consisting of 0·33 mM $NADP^+$, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$ and 0·045 unit of glucose 6-phosphate dehydrogenase. When necessary, 10 mM KCN was also included. The reaction was carried out aerobically at 37° for 20 min. The reaction mixture for reconstitution of aniline hydroxylation activity contained, in a final volume of 0·5 ml, a cytochrome P-450 preparation (0.272 nmole of I-1-a, 0.277 nmole of I-1.b, 0.251 nmole of I-2 or 0.266 nmole of cytochrome P-448), 0.892 unit of NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl-L-3-phosphatidylcholine, 50 μg of sodium deoxycholate, 2 or 20 mM aniline, the NADPH-generating system mentioned above, and 150 mM potassium phosphate buffer, pH 7.25. When necessary, 10 mM KCN, 0.8 M acetone or 1 mM 2,2'-bipyridine was also added. The reaction was run aerobically at 37° for 30 min. In both the microsomal and reconstituted system, *p*-aminophenol formation was determined as described by Imai *et al.* [32].

Binding of NADPH-cytochrome P-450 *reductase*

Table 1. Partial purification of three species of cytochrome P-450 from liver microsomes of intact rats*

		Protein (mg)	Total content (nmoles)	Specific content (nmoles/mg protein)	Recovery (%)
Microsomes		696	594	0.85	100
Cholate-solubilized microsomes		626	575	0.92	96.8
Aminooctyl column	(I)	99.0	417.8	4.22	70.3
eluate	(II)	41.4	81.0	1.96	13.6
Hydroxylapatite column-(I)	(I-1)	17.4	145.0	8.33	24.4
eluate	(I-2)	19.1	80.5	4.21	13.6
CM-Sephadex column-(I-1)	(I-1-a')	8.1	78.1	9.64	13.1
eluate	(I-1-b)	2.3	16.8	7.30	2.8
CM-Sephadex column-(I-2) eluate	(I-2)	8.7	36.6	4.21	6.2
CM-Sephadex column-(I-1-a) eluate	(I-1-a)	6.0	41.4	6.90	7.0

* Fraction I: Aminooctyl eluate with 100 mM buffer containing 0.4% sodium cholate and 0.08% Emulgen 913; Fraction I-1: Hydroxylapatite eluate with 100 mM buffer containing 0.2% Emulgen 913; Fraction I-2: Hydroxylapatite eluate with 300 mM buffer containing 0.2% Emulgen 913; Fraction I-1-a': 1st CM eluate with 100 mM buffer containing 0.2% Emulgen 913; Fraction I-1-b: CM eluate with 500 mM buffer; Fraction I-2: CM eluate with 500 mM buffer; Fraction I-1-a: 2nd CM eluate with 300 mM buffer. Fractions I-1-a, I-1-b and I-2 exhibited Soret peak at 450, 451 and 450 nm, respectively. Fraction II eluted with 100 mM buffer containing 0.5% Emulgen 913 showed a Soret peak at 488.5–449 nm in the CO-difference spectrum but its further purification was not attempted.

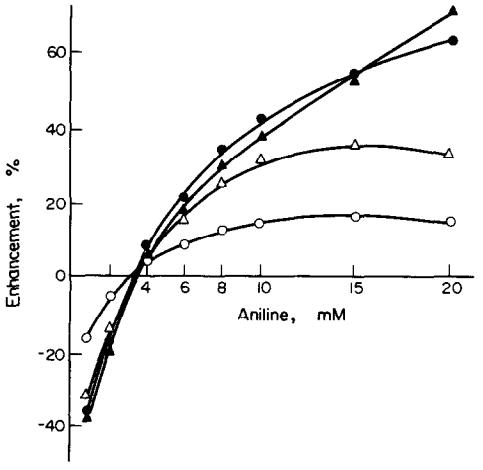

Fig. 1. Inhibition and enhancement of aniline hydroxylation activity by cyanide. Experimental details are as described in Materials and Methods except that 1–20 mM aniline and 0 mM, 0.25 mM (—○—), 2 mM (—△—), 8 mM (—●—), and 15 mM (—▲—) KCN were used. The control activities (without cyanide) at 1, 2, 4, 6, 10, 15 and 20 mM concentrations of aniline were 18.94, 18.32, 18.44, 18.57, 18.69, 18.94, 19.56 and 20.05 nmoles per mg protein per 20 min, respectively.

to microsomes. A mixture (4.5 ml) containing rat liver microsomes (18 mg of protein), a desired amount of partially purified NADPH-cytochrome P-450 reductase, and 140 mM potassium phosphate buffer, pH 7.4, was incubated at 30° for 20 min and then diluted 4-fold with ice-cold 100 mM potassium phosphate buffer, pH 7.4. The diluted sample was centrifuged at 105,000 *g* for 60 min. The precipitated microsomes were resuspended in 100 mM potassium phosphate buffer, pH 7.4, and subjected to enzyme assays.

RESULTS

As shown in Fig. 1, cyanide inhibited aniline hydroxylation by liver microsomes when the substrate concentration was lower than about 3 mM, in confirmation of our previous finding [21]. However, when the aniline concentration was higher than 3 mM, cyanide was found to stimulate the activity. The enhancement was greater as the concentrations of aniline and cyanide were increased. With 20 mM aniline and 15 mM cyanide, the activation reached about 70 per cent. Cyanide did not interfere with the assay method under these conditions. This stimulation was reversible; it disappeared on washing the microsomes by centrifugation and reappeared on adding cyanide again to the washed microsomes. Since these effects of cyanide on microsomal aniline hydroxylation seemed to resemble those of ethylisocyanide reported previously [1, 2], it was expected that cyanide-induced enhancement would increase at higher oxygen tensions, as in the case of the ethylisocyanide effect [1]. The data shown in Table 2 indicate that this was actually the case, suggesting that the mechanism of cyanide-induced enhancement is similar to that operating in the ethylisocyanide-induced activation. As reported by Anders [3], high oxygen tensions did not alter the stimulatory effect of acetone but rather decreased the enhancement caused by 2,2′-bipyridine (data not shown).

Acetone-induced activation of aniline hydroxylation by rat liver microsomes has been shown to be decreased by pretreatment of animals with MC [5]. As shown in Table 3, pretreatment of rats not only with MC but also with PB resulted in partial abolition of the enhancing effect of cyanide on aniline hydroxylation by liver microsomes.

To study further the mechanism of cyanide-induced enhancement, the effects of cyanide on aniline hydroxylation by reconstituted systems containing either one of the three partially purified cytochrome P-450 preparations (fractions I-1-a, I-1-b and I-2) from control liver microsomes or a highly purified cytochrome P-448 preparation from MC-induced liver microsomes were examined. As can be seen in Table 4, all the cytochrome P-450 and P-448 preparations tested catalyzed aniline hydroxylation when reconstituted with NADPH-cytochrome P-450 reductase; the activity was by far the highest with cytochrome P-448, followed by fractions I-1-a, I-1-b and I-2 in this order. In contrast to the case of liver microsomes, however, 10 mM cyanide inhibited rather than activated the activity in all the reconstituted systems. Thus, the activities with cytochrome P-448 and fraction I-2 were very strongly inhibited (about 70 per cent), whereas those with fractions I-1-a and I-1-b were inhibited 17 and 37 per cent, respectively. In contrast, acetone and 2,2′-bipyridine enhanced aniline hydroxylation catalyzed by all the reconstituted systems, with the single exception that the activity supported by fraction I-2 was inhibited by acetone (Table 5).

Table 2. Effect of oxygen tension on aniline hydroxylation activity in the absence and presence of cyanide*

Oxygen tension (%)	Aniline hydroxylation (nmoles/mg protein/20 min)		Enhancement (%)
	−KCN	+KCN	
20	18.99	23.88	25.8
40	19.53	26.62	36.3
100	19.93	38.37	92.5

* Experimental details are as described in Materials and Methods except that concentration of KCN added is 2.0 mM. Values are the means of two separate determinations.

Table 3. Effects of pretreatment of rats with phenobarbital or 3-methylcholanthrene on cyanide enhancement of aniline hydroxylation*

	Cyt. P-450 (nmoles/mg protein)	Aniline hydroxylation (nmoles/mg protein/20 min)		Enhancement (%)
		−KCN	+KCN	
Control (saline)	0.83 ± 0.10	24.09 ± 0.69	41.04 ± 1.20	70.40 ± 1.60
Phenobarbital-treated	2.74 ± 0.29	34.62 ± 1.42	43.15 ± 2.69	24.19 ± 3.03
Control (corn oil)	0.86 ± 0.06	25.72 ± 1.11	49.04 ± 2.18	90.67 ± 2.19
3-Methyl cholanthrene-treated	1.63 ± 0.04	30.00 ± 2.79	38.05 ± 3.02	27.46 ± 3.27

* Sodium phenobarbital, dissolved in saline, and 3-methylcholanthrene, dissolved in corn oil, were injected intraperitoneally for three consecutive days at daily doses of 80 mg/kg and 40 mg/kg, respectively. Other experimental details are as described in Materials and Methods. Values represent the means ± S.E. from five determinations.

Table 4. Effects of cyanide on aniline hydroxylation activity of various species of cytochrome P-450 isolated from intact and 3-methylcholanthrene-treated rat liver microsomes

	Cytochrome P-450			
Addition	I-1-a*	I-1-b*	I-2*	Cyt. P-448†
None	0.425‡	0.417	0.368	0.720
Cyanide	0.352	0.262	0.118	0.223
Inhibition (%)	17.2	37.2	67.9	69.0

* Cytochrome P-450 species contained in I-1-a, I-1-b and I-2 fractions as described in Materials and Methods.

† Cytochrome P-448 isolated from 3-methylcholanthrene-treated rat liver microsomes.

‡ Aniline hydroxylation activity, nmoles *p*-aminophenol formed per nmole cytochrome P-450 per min.

Kamataki *et al.* [25] have shown that the rate of cytochrome P-450 reduction by NADPH in microsomes is limited by the amount of NADPH-cytochrome P-450 reductase. In support of this, it has been reported that drug oxidations by liver microsomes are enhanced by extra binding of purified NADPH-cytochrome P-450 reductase to the microsomes [33–35]. As shown in Table 6, incorporation *in vitro* of partially purified NADPH-cytochrome P-450 reductase to liver microsomes actually increased aniline hydroxylation activity, in confirmation of the previous finding [33]. The hydroxylation activity in the presence of 10 mM cyanide also increased with the amount of the reductase incorporated, but to lesser extents than in the absence of cyanide. Consequently, the enhancement of aniline hydroxylation was markedly decreased by the fortification of microsomes with NADPH-cytochrome P-450 reductase. On the other hand, the enhancement of aniline hydroxylation caused by acetone or 2,2′-bipyridine was not significantly affected by extra binding of the reductase to the microsomes (Table 7).

Table 5. Effects of acetone and 2,2′-bipyridine on aniline hydroxylation activity of various species of cytochrome P-450 isolated from intact rat liver microsomes

	Cytochrome P-450		
Additions	I-1-a*	I-1-b*	I-2*
None	0.498†	0.286	0.237
Acetone	0.753	0.417	0.145
change (%)	+51.2	+45.8	−38.8
2,2′-Bipyridine	0.668	0.500	0.368
change (%)	+34.1	+74.8	+55.3

* Cytochrome P-450 species contained in I-1-a, I-1-b and I-2 fractions isolated from intact rat liver microsomes as described in Materials and Methods.

† Aniline hydroxylation activity, nmoles *p*-aminophenol formed per nmole cytochrome P-450 per min.

DISCUSSION

The possibility that the cyanide-induced enhancement of aniline hydroxylation reported in this paper is due to inhibition of lipid peroxidation, which impairs drug-metabolizing enzymes, can be ruled out, because 0.1 mM EDTA and the high concentration of aniline included in the reaction mixture should have completely abolished the peroxidation reaction [36]. It has been shown that the activation of aniline hydroxylation by 2,2′-bipyridine is not due to inhibition of lipid peroxidation [4]. Inhibition by cyanide of the respiratory chain of contaminating

Table 6. Effect of fortification of microsomes with NADPH-cytochrome P-450 reductase on the enhancement of aniline hydroxylation activity by cyanide*

Added fp_2†	Fortified fp_2‡	Aniline hydroxylation (nmoles/mg protein/10 min) −KCN	Aniline hydroxylation (nmoles/mg protein/10 min) +KCN	Enhancement (%)
		9.83	13.63	38.7
3.0	0.67	12.67	17.13	35.2
7.00	1.20	14.36	18.44	28.4
13.33	1.96	17.43	20.78	19.2

* Incubations were carried out at 37° for 10 min aerobically in the presence of 20 mM aniline.
† NADPH-cytochrome P-450 reductase (unit) added to microsomes.
‡ NADPH-cytochrome P-450 reductase activity (unit) incorporated into microsomal membrane.

mitochondria is also an unlikely cause of the enhancement, because the cyanide effect becomes more pronounced at concentrations much higher than that required for complete inhibition of the respiratory chain (about 1 mM).

Correia and Mannering [37] have reported that appropriate concentrations of cyanide stimulate ethylmorphine N-demethylation by rat liver microsomes in the presence of both NADPH and NADH and interpreted this stimulation as due to the inhibition of cyanide-sensitive factor (CSF), the terminal enzyme of microsomal fatty acid desaturase system. This interpretation is worth consideration, since the electrons transferred to cytochrome b_5 from NADH (via NADH-cytochrome b_5 reductase) and NADPH (via NADPH-cytochrome P-450 reductase) can be utilized for supplying the second electron required for drug hydroxylation catalyzed by cytochrome P-450 [38–41], as well as for fatty acid desaturation by CSF [42]. In our hands, however, no stimulation of ethylmorphine N-demethylation by cyanide was observed even when liver microsomes from desaturase-induced rats were used (data not shown). In addition, as described in this paper, cyanide-induced enhancement of aniline hydroxylation can be observed with liver microsomes prepared from starved rats, in which practically no desaturase activity is detectable [43]. It can, therefore, be concluded that inhibition of CSF is not responsible for the observed enhancement of aniline hydroxylation.

Acetone [3–7] and 2,2′-bipyridine [4, 7] have been reported to stimulate aniline hydroxylation by rat liver microsomes, but the enhancements caused by these chemicals are clearly different from that induced by cyanide in the following respects. First, the cyanide-induced effect becomes more pronounced as the oxygen tension is increased (Table 2), whereas the effects caused by acetone and 2,2′-bipyridine are not influenced by the oxygen tension. Secondly, cyanide inhibits, rather than stimulates, aniline hydroxylation by reconstituted systems containing purified cytochrome P-450 preparations (Table 4). In contrast, both acetone and 2,2′-bipyridine stimulate the hydroxylation reaction catalyzed by both intact microsomes and reconstituted systems (except that acetone inhibits the reconstituted system containing fraction I-2) (Table 5). Finally, binding of partially purified NADPH-cytochrome P-450 reductase to liver microsomes leads to significant decreases in the extent of cyanide-induced enhancement (Table 6), whereas this treatment does not decrease the enhancement caused by acetone or 2,2′-bipyridine (Table 7). Furthermore, Kitada *et al.* [44] and Powis *et al.* [45] have reported that NADH synergism of NADPH-dependent aniline hydroxylation can be elicited in the presence, but not in the

Table 7. Enhancement of aniline hydroxylation activity by acetone and 2,2′-bipyridine in NADPH-cytochrome P-450 reductase-fortified microsomes

Additions	Aniline hydroxylation (nmoles/mg protein/10 min) Cont.	Enhancement (%)	fp_2-Fortified Ms†	Enhancement (%)
None	9.17		15.46	
Acetone (0.8 M)	16.06	75.1	26.59	72.0
2,2′-Bipyridine (1.0 mM)	15.15	65.2	26.65	65.9

* Incubations were carried out at 37° for 10 min in the presence of 2 mM aniline.
† NADPH-cytochrome P-450 reductase-fortified microsomes were prepared by addition of 13.3 unit of partially purified NADPH-cytochrome P-450 reductase. The amount of the activity incorporated into microsomal membranes was 1.96 units per mg of protein.

absence, of acetone and 2,2′-bipyridine. Our preliminary experiments, however, showed that no NADH synergism of aniline hydroxylation occurred in the presence of cyanide. All these findings indicate that the mechanism of cyanide effect is clearly different from those of acetone- and 2,2′-bipyridine-induced enhancements of aniline hydroxylation.

The enhancement of aniline hydroxylation by cyanide is similar to that caused by ethylisocyanide in that both become more pronounced as the oxygen tension is increased (Table 2 and ref. 1). Imai and Sato [2] have reported that ethylisocyanide-induced enhancement of liver microsomal aniline hydroxylation is most pronounced in rabbits, followed by rats, but that no enhancement is observable in guinea pigs. We have also observed that cyanide caused no stimulation of aniline hydroxylation by guinea pig liver microsomes (data not shown), suggesting that the same species difference exists in both cyanide- and ethylisocyanide-induced enhancements. Moreover, cyanide and ethylisocyanide are also similar in that they exert both inhibitory and stimulatory effects on aniline hydroxylation depending on experimental conditions (Fig. 1 and ref. 1). As mentioned earlier, Imai and Sato [2] have concluded that ethylisocyanide inhibits the hydroxylation by competing with molecular oxygen for the reduced heme of cytochrome P-450 and activates by changing the reactivity of the cytochrome through binding to the non-heme site. The similarities between the cyanide- and ethylisocyanide-induced enhancements of aniline hydroxylation suggest that cyanide stimulates the hydroxylation reaction by a mechanism similar to that functional in the ethylisocyanide-induced enhancement. Further work is, however, needed to reach a definite conclusion on this point, since cyanide usually has higher affinities for the oxidized heme rather than the reduced heme of various hemoproteins. Furthermore, it is necessary to propose a mechanism which can explain the results reported on the effects on induction (Table 3), the activities of reconstituted systems (Table 4), and the effect of extra binding of NADPH-cytochrome P-450 reductase (Table 6). Unfortunately, however, no satisfactory explanations of these results are at present available.

Nevertheless, it seems to be possible to present a likely, but not yet proven, possibility accounting for the results. This possibility is conceivable on the following bases. First, multiple species of cytochrome P-450 exist in liver microsomes [46–48]. Secondly, these species of cytochrome P-450 differ from one another in specific activity of aniline hydroxylation and sensitivity to cyanide (*cf.* Table 4). It is to be noted that, with the exception of cytochrome P-448, the cyanide sensitivity of the cytochrome P-450 species listed in Table 4 increases as the specific aniline hydroxylation activity decreases. Finally, since NADPH-cytochrome P-450 reductase has been shown to be the rate-limiting enzyme in the NADPH-linked electron transport chain [25, 33, 35], the various species of cytochrome P-450 in microsomes should compete with one another for the reductase. It is now likely that cyanide activates aniline hydroxylation by inhibiting preferentially the species of cytochrome P-450 with low aniline hydroxylation activity and thus improving the efficiency of interaction of the reductase with the cytochrome having high aniline hydroxylation activity. If this is actually the case, then incorporation of extra reductase into microsomes is expected to diminish the competition, leading to a decrease in cyanide-induced enhancement. Since pretreatment of animals with phenobarbital increases not only cytochrome P-450 but also NADPH-cytochrome P-450 reductase [49], this treatment leads to a decrease in the extent of the cyanide effect, probably by changing the ratio of the reductase to a particular species of cytochrome P-450. However, pretreatment with 3-methylcholanthrene, which does not induce the reductase [5], also causes a significant decrease in the cyanide effect (Table 3). This may be explained by the fact that the 3-methylcholanthrene-inducible species of cytochrome P-450 (cytochrome P-448) is very strongly inhibited by cyanide, though it has a very high aniline hydroxylase activity. The mechanism of cyanide-induced enhancement of aniline hydroxylation just discussed is still tentative and should be refined further by future studies.

Finally, it should be mentioned that the reconstituted hydroxylase systems are all inhibited by cyanide and not activated (Table 4), in contrast to the case of intact microsomes. This may be due to the difference in cyanide sensitivity of cytochrome P-450 between the solubilized and membrane-bound forms. In fact, Cooper *et al.* [51] have reported that a species of cytochrome P-450 is strongly inhibited by carbon monoxide in its purified form but is slightly sensitive when it is bound to microsomes.

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