STIMULATORY EFFECT OF CYANIDE ON ANILINE *p*-HYDROXYLATION IN RAT LIVER MICROSOMES

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Enhancement of aniline hydroxylation was observed by the addition of cyanide in rat liver microsomes. The enhancing effect of cyanide on aniline hydroxylation activity was also observed in rabbit liver microsomes, but not in guinea pig liver microsomes. Aniline stimulated the reoxidation of reduced nicotinamide adenine dinucleotide (NADH)-reduced cytochrome *b₅*. The stimulation of cytochrome *b₅* reoxidation by aniline was most remarkable in liver microsomes from desaturase-induced rats followed by that from fed and fasted rats. The increment in cytochrome *b₅* reoxidation by aniline in all groups of rats was inhibited by the simultaneous addition of cyanide. However, the stimulatory effect of cyanide on aniline hydroxylation in fasted rat was not different from that observed in desaturase-induced rat. In addition, the effect of cyanide on aniline hydroxylation was suggested to be reversible.

Keywords——aniline hydroxylation; enhancement; cyanide; liver microsomes; rat.

Liver microsomal cytochrome P-450 has been implicated as oxygen-activating¹⁾ and the substrate-binding enzyme.^{2,3)} Recent reports have shown that cyanide binds to cytochrome P-450 and produces a spectral change in cytochrome P-450.^{4,5)} On the other hand, the microsomal monooxygenase system associated with drug hydroxylation has been shown to be insensitive to cyanide.^{6,7)} Recently, Matsubara and Tochino^{8,9)} have demonstrated that the activity of aniline hydroxylation was inhibited by cyanide. In the previous report¹⁰⁾ we have shown that cyanide also inhibited the activities of aminopyrine, ethylmorphine and codeine demethylations, and the inhibitory effect of cyanide was partially avoided by increasing the oxygen tension. The result obtained in the previous study led to a conclusion that competition of cyanide with oxygen for the heme of reduced cytochrome P-450 as in the case of ethylisocyanide is responsible for the inhibitory effect. Imai and Sato¹¹⁾ have shown that ethylisocyanide enhanced rabbit liver microsomal aniline hydroxylation, therefore, it seemed of in-

terest to study whether cyanide enhance aniline hydroxylation or not. The purpose of this paper is to report that under appropriate conditions aniline hydroxylation in rat liver microsomes is stimulated by cyanide. Species difference in the stimulatory effect of cyanide was also described.

MATERIALS AND METHODS

Male rats of Wistar strain weighing 70 to 150 g, male guinea pigs weighing 320 to 340 g and male New Zealand white rabbits weighing 1.7 to 2.0 kg were maintained on a standard diet (Nippon CLEA, Japan).

After being fed on a standard diet for at least 3 days, the rats were fasted for one day and then refed on the fat-free, high carbohydrate diet prepared as described by Oshino *et al.*¹²⁾ for 3 days. The rats were fasted for 2 days and then refed on the fat-free, high carbohydrate diet again. The rats were killed within 18 to 20 hr after refeeding. These animals were referred to as "induced" rats. Microsomes obtained from these animals, desaturated stearyl CoA at a rate of 5.17 to 7.43 nmol of oleate per

min per mg of protein at 30°. The “fed” rats were allowed free access to a standard diet and microsomes prepared from these animals produced 0.25 to 0.98 nmol of oleate per min per mg of protein. The “fasted” rats were deprived of food about 18 to 20 hr prior to sacrifice. Microsomes obtained from “fasted” rats produced 0.01 to 0.19 nmol of oleate per min per mg of protein. Microsomes were prepared as reported previously.¹³⁾

NADH, nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate and glucose 6-phosphate dehydrogenase (EC 1.1.1.49), Grade I, were purchased from Boehringer Mannheim Co., and stearyl CoA from Sigma Chemical Co. Commercial aniline was redistilled under vacuum and the distillate was stored at -10° under atmosphere of nitrogen. All other chemicals were obtained from commercial sources and used without further purification. Standard incubation mixture consisted of microsomes (1 mg of protein), 0.1 M Na, K-phosphate (pH 7.4), 0.1 mM ethylenediaminetetraacetic acid (EDTA), an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 6 mM MgCl₂ and 0.045 unit glucose 6-phosphate dehydrogenase), aniline and, if necessary, cyanide in a final volume of 1.0 ml. The cyanide solution was prepared before use and the pH of the solution was adjusted to 7.4 with 1 N HCl. The formation of *p*-aminophenol from aniline was measured by the method of Imai *et al.*¹⁴⁾ Cytochrome *b*₅ reoxidation was measured by the method of Oshino and Sato.¹⁵⁾ The redox behavior of cytochrome *b*₅ was followed by recording the changes in absorbance between 424 nm and 409 nm using HITACHI Two-Wave Length Double Beam Spectrophotometer (model 356). The reaction mixture contained microsomes (0.6 to 0.9 mg protein), 80 mM Na, K-phosphate (pH 7.4) 0.1 mM EDTA and, if necessary, 5 mM aniline and/or 1 mM cyanide in a final volume of 3.0 ml. After preincubation of the mixture for 3 min in a cuvette of spectrophotometer maintained at 25°, 5 µl of NADH (final concentration, 0.83 µM to 1.67 µM) was added and the reoxidation of cytochrome *b*₅ was recorded. The amount of NADH added was adjusted so that reoxidation could occur within 2

min after establishment of steady state reduction level of cytochrome *b*₅. Protein was determined by the method of Lowry *et al.*¹⁶⁾ using bovine serum albumin as a standard.

RESULTS

Effect of Cyanide on Microsomal Aniline Hydroxylation

Cyanide has been shown to bind to both the reduced and oxidized forms of cytochrome P-450¹⁷⁾ and to inhibit aniline hydroxylation in rat liver microsomes.⁸⁻¹⁰⁾ It was, however, found that cyanide activated rather than inhibited aniline hydroxylation when higher concentration of aniline was used (Table I). Aniline hydroxylase activity in the presence or absence of cyanide was not observed when NADPH-generating system was not added and when boiled microsomes were used. Moreover, no enhancement of aniline hydroxylation by cyanide was observed when cyanide was added at the end of the reaction.

Enhancement of Cyanide on Aniline Hydroxylation in Desaturase-Induced Rat Liver Microsomes

Table II shows the enhancing effects of cyanide on aniline hydroxylation with “induced” rat liver microsomes in the presence or absence of stearyl CoA. Cyanide enhanced not only NADPH-dependent aniline hydroxylation but NADH-dependent aniline hydroxylation.

Aniline hydroxylase activity in the presence of NADH and/or NADPH was virtually unchanged when stearyl CoA, substrate for desaturase system, was added to the reaction mixture. Moreover, the stimulatory effect of cyanide on aniline hydroxylation in the presence of NADH and/or NADPH was not increased by stearyl CoA.

The addition of aniline to the reaction mixture resulted in the stimulation of NADH-reduced cytochrome *b*₅ reoxidation (Table III).

Most remarkable stimulation of cytochrome *b*₅ reoxidation was observed in “induced” rat liver microsomes, followed by “fed” and “fasted” rats. In addition, the stimulation of cytochrome *b*₅ reoxidation by aniline in all groups of rats was largely decreased by the simultaneous addition of cyanide to the reaction mixture. Similar results

TABLE I. *Inhibitory and Stimulatory Effects of Cyanide on Aniline Hydroxylation in Rat Liver Microsomes*

Aniline (mM)	Aniline hydroxylation		% of control
	–KCN (nmol <i>p</i> -aminophenol formed/mg protein/20 min)	+KCN	
1	20.66±0.05	15.23±0.07	73.7
2	20.39±0.09	19.14±0.21	93.8
4	20.84±0.05	23.63±0.11	113.4
6	20.84±0.05	26.46±0.16	127.0
8	21.10±0.04	28.51±0.13	135.7
10	21.37±0.05	30.17±0.17	141.2
15	22.24±0.75	31.83±0.15	143.1
20	21.72±0.05	32.61±0.15	150.1

The concentration of cyanide was 2 mM and aniline concentrations employed were shown in Table. Other experimental details are as described in Materials and methods. Each value represents mean ±S.D. of duplicate determinations.

TABLE II. *Effects of Stearyl CoA and Cyanide on Aniline Hydroxylase Activity in Microsomes from "Induced" Rats*

Cofactor Addition	Aniline hydroxylation		
	NADH (nmol <i>p</i> -aminophenol formed/mg protein/15 min)	NADPH	NADPH+NADH
None	2.41±0.10	5.63±0.17	7.26±0.00
Stearyl CoA (50 μM)	2.11±0.16	5.59±0.00	7.92±0.09
Cyanide (10 mM)	3.57±0.00	11.24±0.00	12.24±0.00
Stearyl CoA +cyanide	3.57±0.06	9.50±0.10	11.01±0.20

The concentration of aniline employed was 20 mM and other experimental conditions are as described in Materials and methods. Each value represents mean ±S.D. of duplicate determinations.

TABLE III. *Effect of Aniline on Cytochrome *b*₅ Reoxidation in Microsomes from "Fasted", "Fed" and "Induced" Rats*

Microsomes Addition	Cytochrome <i>b</i> ₅ reoxidation		
	"Fasted"	"Fed"	"Induced"
	(nmol cyt. <i>b</i> ₅ oxidized/mg protein/min)		
None	0.134±0.016	0.182±0.051	0.353±0.024
Cyanide	0.107±0.003	0.161±0.036	0.221±0.032
Aniline	0.181±0.041	0.466±0.064	1.745±0.357
Aniline +cyanide	0.120±0.007	0.198±0.015	0.525±0.015

Spectrophotometric measurements were conducted as described in Materials and methods. Each value represents mean ±S.D. of duplicate determinations.

were observed when NADH was replaced by NADPH (data not shown). From the results that the effect of aniline on cytochrome *b*₅ reoxidation

with "induced" rat liver microsomes was considerably different from that observed with "fasted" rat, it could be considered that the elec-

TABLE IV. *Enhancement of Aniline Hydroxylation by Cyanide in Microsomes from "Fasted", "Fed" and "Induced" Rats*

Cyanide (mM)	Aniline hydroxylation (nmol <i>p</i> -aminophenol formed/mg protein/20 min)		
	"Fasted"	"Fed"	"Induced"
0	16.93 ± 1.22	11.10 ± 1.14	9.31 ± 0.35
1	20.04 ± 0.91 (18.4)	13.66 ± 2.26 (23.1)	11.24 ± 2.51 (20.7)
2	21.49 ± 1.52 (26.9)	13.84 ± 2.57 (24.7)	11.29 ± 2.50 (21.3)
5	21.70 ± 0.67 (28.2)	13.43 ± 3.68 (21.0)	11.88 ± 2.11 (27.6)

The concentration of aniline employed was 5 mM and cyanide concentrations added to the incubation mixture were shown in Table. Each value represents mean $\pm$ S.D. of duplicate determinations. Numbers in parentheses represent per cent of enhancement. Other experimental details are as described in Materials and methods.

tron flow from cytochrome b_5 to desaturase was stimulated by aniline. Therefore, the effects of cyanide on aniline hydroxylation were investigated using microsomes prepared from "fasted", "fed" and "induced" rats. As shown in Table IV, although the highest activity of aniline hydroxylation was demonstrated using the microsomes from "fasted" rat, the stimulatory effect of cyanide on aniline hydroxylation in "fasted" rat liver microsomes was similar to that observed in "induced" rat liver microsomes. From these results described above, it was suggested that electron drain to desaturase due to stearyl CoA or aniline itself, did not affect the activity of aniline hydroxylation. Therefore, it seemed reasonable to assume that inhibitory effect of cyanide on electron shunt to desaturase by way of cytochrome b_5 was not responsible for the enhancing effect of cyanide on aniline hydroxylation.

Species Difference in Stimulatory Effect of Cyanide on Aniline Hydroxylation

Imai and Sato¹⁸⁾ and Anders¹⁹⁾ have shown the species difference in the stimulatory effects of ethylisocyanide and acetone on aniline hydroxylation. Therefore, we investigated the effect of cyanide on aniline hydroxylation with rat, guinea pig and rabbit liver microsomes. As shown in Fig. 1, the effect of cyanide on aniline hydroxylation catalyzed with guinea pig liver microsomes was different from that observed with rat liver microsomes. Only inhibition was observed in the guinea pig liver microsomes and the effect of

cyanide on aniline hydroxylation in rabbit liver microsomes was shown as intermediate between rat and guinea pig.

FIG. 1. *Species Differences in the Effect of Cyanide on Aniline Hydroxylase Activity*

The concentration of aniline employed was 20 mM and cyanide concentrations added to the incubation mixture were shown in the Figure. Aniline hydroxylase activities in the absence of cyanide in microsomes from rat ($-\circ-$), rabbit ($-\bullet-$) and guinea pig ($-\square-$) were 25.37 ± 0.24 nmol *p*-aminophenol formed/mg protein/20 min, 12.72 ± 0.05 nmol *p*-aminophenol formed/mg protein/20 min and 26.22 ± 0.19 nmol *p*-aminophenol formed/mg protein/20 min, respectively. Other experimental conditions are as described in Materials and methods. Each point indicates mean $\pm$ S.D. of duplicate determinations.

Effects of Washing the Microsomes on the Stimulatory Effect of Cyanide in Rat Liver Microsomes

As shown in Table V, cyanide enhanced aniline hydroxylation by about 68% before washing the microsomes and the stimulatory effect of cyanide was largely obliterated by washing the microsomes.

However, further addition of the respective cyanide caused a restoration of the stimulatory effect on aniline hydroxylation. From these results, it can be concluded that the effect of cyanide on aniline hydroxylase activity is reversible and does not result in irreversible conversion of cytochrome P-450 or microsomal membrane.

TABLE V. *Effects of Washing the Microsomes on the Stimulation of Aniline Hydroxylation by the Addition of Cyanide*

Washing times	Aniline hydroxylation		
	A	B	C
	(nmol <i>p</i> -aminophenol formed/mg protein/15 min)		
0	12.39±0.41	20.84±0.24	
1	13.55±0.15	15.77±0.46	20.99±0.80
2	12.93±0.06	14.77±0.25	23.61±0.11
3	13.57±0.30	12.96±0.49	22.51±0.66

Microsomes was washed by the centrifugation at 105000×g for 30 min. The resulting pellet was re-suspended with 0.1 M Na, K-phosphate (pH 7.4) and was used for the assay of aniline hydroxylase activity. Numbers in column A represent the activity of aniline hydroxylation in the absence of cyanide and numbers in column B and C represent the activities of aniline hydroxylation in cyanide treated microsomes without or with further addition of cyanide after washing. Each value represents mean ±S.D. of duplicate determinations. The concentration of aniline employed was 20 mM and cyanide concentration added to the incubation mixture was 10 mM. Other experimental conditions are as described in Materials and methods.

DISCUSSION

It has been shown by several investigators that aniline hydroxylation was inhibited by cyanide.⁸⁻¹⁰⁾ As shown in this paper, cyanide enhanced aniline hydroxylation under appropriate conditions in rat and rabbit liver microsomes, therefore, it is suggested that cyanide may exert simultaneously both a stimulatory effect and an inhibitory effect on aniline hydroxylation and apparent effect caused by the addition of cyanide may be determined by a balance of these two effects. Cyanide enhanced not only aniline hydroxylation but also *p*-anisidine and *p*-phenetidine O-dealkylation when higher concentrations of substrates together with higher concentrations of cyanide were employed (data not shown).

Oshino *et al.*¹²⁾ have demonstrated that the enzyme system catalyzing the oxidative desaturation of stearyl CoA in rat liver microsomes contains a

desaturase as the terminal enzyme induced by feeding a fat-free, high-carbohydrate diet and reducing equivalents required for the function of the enzyme system can be supplied from NADH or NADPH *via* cytochrome *b*₅. In addition, it has been shown that microsomal NADH-dependent stearyl CoA desaturation was strongly inhibited by cyanide and reoxidation of NADH-reduced cytochrome *b*₅ was stimulated by the addition of stearyl CoA.⁷⁻¹⁵⁾ If the stimulatory effect of cyanide on aniline hydroxylation was due to the inhibition of electron shunt to desaturase by way of cytochrome *b*₅, it was expected that aniline hydroxylation in "fasted" rat liver microsomes is virtually unaffected by cyanide and that aniline hydroxylation in "induced" rat liver microsomes is remarkably increased by cyanide. More recently, Correia and Mannering²⁰⁾ have shown that ethylmorphine N-demethylation was inhibited by

the addition of stearyl CoA and the inhibitory effect of stearyl CoA on ethylmorphine N-demethylation was reversed by cyanide. The results shown in Table II that aniline hydroxylation activity in "induced" rat liver microsomes was unaffected by the addition of stearyl CoA led us to consideration that the electron shunt from NADPH-cytochrome *c* reductase to desaturase via cytochrome *b₅* did not affect aniline hydroxylation. However, in view of the report by Schenkman and Jansson²¹⁾ the possibility that stearyl CoA desaturase activity is inhibited by aniline cannot be excluded. As shown in Tables III and IV, the most remarkable stimulation of cytochrome *b₅* reoxidation by aniline was observed in "induced" rat, followed by "fed" and "fasted" rat liver microsomes. On the other hand, the stimulatory effect of cyanide on aniline hydroxylation with "fasted" rat liver microsomes was similar extent to that observed with "induced" rat liver microsomes. These results suggest that electron channeling to desaturase *via* cytochrome *b₅* due to aniline itself does not affect the activity of aniline hydroxylation catalyzed by cytochrome P-450. Therefore, it seems possible to assume that enhancement of aniline hydroxylation by cyanide is not due to the inhibition of desaturase.

We have previously shown²⁾ that NADH-synergism of NADPH-dependent aniline hydroxylation was observed in the presence of enhancing agents of aniline hydroxylation such as acetone¹⁹⁾ and 2,2'-bipyridine.^{23, 24)} As shown in Table II, cyanide enhanced both NADH- and NADPH-dependent aniline hydroxylation, but synergistic effect of NADH on NADPH-dependent aniline hydroxylation was not observed in the presence of cyanide. These results suggest that cyanide affect aniline hydroxylation in a different manner from acetone and 2,2'-bipyridine.

Enhancement of microsomal hydroxylase activity *in vitro* has been reported by several investigators. Imai and Sato,¹¹⁾ Anders,¹⁹⁾ Stevens and Greene²⁵⁾ and Korten and Van Dyke²⁶⁾ demonstrated a stimulation of microsomal hydroxylation by the addition of ethylisocyanide, acetone, metyrapone, paraoxon and volatile anesthetics in the reaction mixture, respectively. Imai and

Sato¹⁸⁾ have shown the species difference in the stimulatory effects of ethylisocyanide on aniline hydroxylation: the highest enhancement was observed in rabbits followed by rats liver microsomes, and inhibition was observed in guinea pigs liver microsomes. Moreover, Anders¹⁹⁾ has demonstrated the species difference in the enhancing effects of acetone on aniline hydroxylation. As shown in Fig. 1, the highest enhancement of aniline hydroxylation by cyanide was observed in microsomes from rat followed by rabbit, but not from guinea pig. Recently, Powis and Boobis²⁷⁾ have demonstrated that pretreating rats with 3-methylcholanthrene markedly reduced the enhancing effects of acetone, 2,2'-bipyridine and metyrapone on aniline hydroxylation. In our experiments, the stimulatory effect of cyanide on aniline hydroxylation was partly abolished by pretreatment of rats with either phenobarbital or 3-methylcholanthrene and was slightly increased by pretreatment with ethanol (data not shown). In recent years, several groups of workers²⁸⁻³²⁾ have shown the existence of multiple forms of cytochrome P-450 in several animal species and the species difference in catalytic activity of cytochrome P-450. Therefore, it seems possible to assume that a difference in the proportion of each form in a microsomal preparation may be able to contribute to the species difference observed in stimulatory effect of cyanide on aniline hydroxylation.

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