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N-Hydroxylation of Carcinogenic and Mutagenic Aromatic Amines

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N-Hydroxylation and mutagenic activation of heterocyclic aromatic amines from protein pyrolysis products were studied in rat liver microsomes and nuclei, rat hepatocytes and various species of purified cytochrome P-450. These mutagenic amines include Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole), Glu-P-1 (2-amino-6-methyldipyrido-[1,2-*a*:3',2'-*d*]imidazole), Glu-P-2 (2-amino-dipyrido[1,2-*a*:-3',2'-d]imidazole) and IQ (2-amino-3-methyl-3*H*-imidazo-[4,5-*f*]quinoline).

The number of revertants of ***Salmonella typhimurium*** TA 98 was always correlated to the amount of each of the *N*-hydroxylated metabolites in various experimental conditions. The N-hydroxylated amines covalently bound to DNA directly or after being acylated with amino acids by aminoacyl-tRNA synthetase. Various species of cytochrome P-450 preparations showed markedly different activity in *N*-hydroxylation and mutagenic activation of Trp-P-2, Glu-P-1 and IQ. A high spin form of cytochrome P-450, isolated from the liver of PCB-treated rats, showed very high activity in *N*-hydroxylation of Trp-P-2, Glu-P-1 and 2-aminofluorene, although its activity was very low in benzo(a)pyrene hydroxylation. The present results indicate that different species of cytochrome P-450 are involved in the *N*-hydroxylation and mutagenic activation of aromatic amines.

Numerous *N*-substituted aryl compounds are reported to be carcinogens and mutagens in experimental animals and man. The *N*-hydroxylations of arylamines and arylamides are considered as common pathways for the metabolic activations to ultimate carcinogens. The metabolic activations in various tissues are mainly catalyzed by microsomal cytochrome P-450, but *N*-hydroxylations of some amines also catalyzed by a microsomal flavin enzyme (mixed-function amine oxidase).

The substrate specificities of these microsomal monooxygenases have not been precisely defined, but in general, as suggested by Gorrod *(1)*, basic amines are preferred substrates for the amine oxidase and the less basic amines are preferred substrates for the cytochrome P-450 system. However, there are many exceptions and the substrate specificities for these two microsomal monooxygenases based on pKa are only a very approximate guide.

Recently Sugimura et al. *(2)* found that various pyrolysis products from amino acids, proteins and proteinaceous foods were highly mutagenic to *Salmonella typhimurium* TA 98 and TA 100 when the liver S-9 fraction was used as the activating system. Subsequently, the chemical structures of these mutagens were elucidated to be new heterocyclic aromatic amines (Fig. 1) *(2, 3)*. Thereafter, some of them have been proven to be carcinogenic to mice and rats *(4, 5)*. *N*-Hydroxylations by hepatic cytochrome P-450 systems have been demonstrated as metabolic activation processes of these amines *(6, 7)*. We report herein the characteristics of *N*-hydroxylations of these heterocyclic aromatic amines by comparison with those of other aromatic amines.

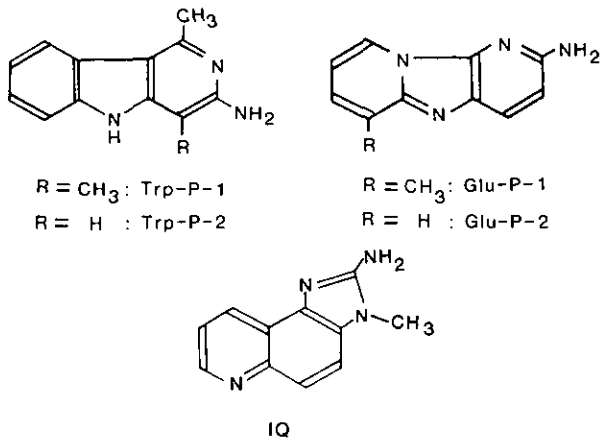

FIGURE 1. Structures of mutagenic heterocyclic aromatic amines from food pyrolyzates.

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Metabolic Activation of Mutagenic Heterocyclic Aromatic Amines by Hepatic Microsomes

Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*] indole) and Trp-P-2 (3-amino-1-methyl-5*H*-pyrido [4,3-*b*] indole), mutagenic pyrolysis products from tryptophan, and Glu-P-1 (2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole and Glu-P-2 (2-amino-dipyrido[1,2-*a*:3′,2′*d*]imidazole) from glutamic acid, IQ (2-amino-3-methyl-3*H*-imidazo[4,5-*f*]-quinoline) from broiled fish were hydroxylated by rat liver microsomes to direct mutagens to the Salmonella *(3, 6, 7)*. The formations of *N*-hydroxylated metabolites by rat liver microsomes were stimulated 30- to 200-fold by pretreatment with methylcholanthrene (MC) or polychlorinated biphenyl (PCB), whereas the pretreatment with phenobarbital (PB) stimulated only slightly.

As shown in Table 1, the formation of active metabolites by liver microsomes required NADPH and was markedly inhibited by 7,8-benzoflavone *(8)*. These results indicate the involvement of cytochrome P-450 in the metabolic activation of Trp-P-1 and Trp-P-2 by hepatic microsomes.

Table 1. Requirement of cofactor and effects of inhibitors on microsomal activation of Trp-P-1 and Trp-P-2.

Treatment	Revertants/plate	
	Trp-P-1 (1 μg)	Trp-P-2 (1 μg)
Complete[a]	30,000	54,700
− NADPH	126	544
− NADPH-generating system	79	201
+ Carbon monoxide	5,100	13,200
+ 7,8-Benzoflavone (10μ*M*)	288	1,049
+ *n*-Octylamine (100μ*M*)	4,900	30,900

[a]Liver microsomes (50 μg protein) from PCB-treated rats were used.

N-Hydroxylation and Mutagenic Activation of Heterocyclic Aromatic Amines by Purified Cytochrome P-450

Subsequently, we demonstrated that the reconstituted system of purified cytochrome P-450 converted Trp-P-1 and Trp-P-2 to mutagenic metabolites. The P-448 type cytochrome isolated from MC- or PCB-treated rats (MC P-448 or PCB P-448) was more active in inducing the Salmonella revertants than was the P-450-type cytochrome isolated from phenobarbital-treated rats (Fig. 2) *(9)*. The formation of *N*-hydroxylated metabolites of Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2 as mutagenic activation process was confirmed: the number of revertants was always parallel to the formation of *N*-hydroxylated metabolites (Table 2).

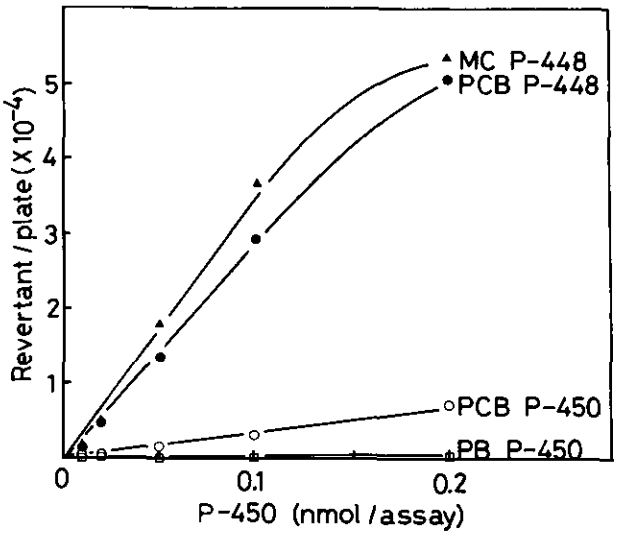

FIGURE 2. Mutagenic activation of Trp-P-2 by purified preparations of cytochrome P-450. PCB P-448, PCB P-450, MC P-448, and PB P-450 were used. The reaction mixture was incubated for 5 min. Each point represents the mean number of revertants from duplicate determinations *(10)*.

Table 2. Mutagenic activation and *N*-hydroxylation of Trp-P-2 by purified cytochrome P-450 preparations.

Preparation	Mutagenicity, rev./pmole P-450/10 min[a]	*N*-Hydroxy-Tr-P-2 formation, pmole/pmole P-450/10 min
PB P-450	10	<0.18
MC P-448	1172	9.60
PCB P-450	60	0.57
PCB P-448	1029	7.94

[a]*Salmonella typhimurium* TA 98 was used as the tester strain.

Covalent Binding of [^{14}C]Trp-P-2 and [^{3}H]Trp-P-2 to Calf Thymus and Hepatic Nuclear DNA

The incubation of [^{14}C]Trp-P-2 or [^{3}H]Trp-P-2 with the reconstituted cytochrome P-450 system and NADPH-generating system caused covalent binding of the radioactivity to added calf thymus DNA *(10)*. The amount of radioactivity bound to DNA was the highest when PCB P-448 was used and the lowest when PB P-450 was used (Fig. 3). The amount of radioactivity bound to DNA was closely correlated with that of *N*-hydroxy-Trp-P-2 formed *(10)*. These results suggest that *N*-hydroxy-Trp-P-2 formed by the cytochromes can bind to DNA of the Salmonella, thereby causing mutation.

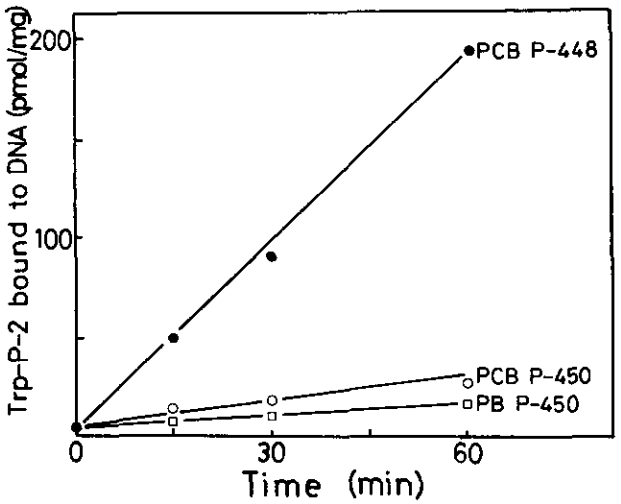

FIGURE 3. Cytochrome P-450-mediated covalent binding of [^{14}C]Trp-P-2 to DNA. DNA (1 mg) from calf thymus was incubated with [^{14}C]Trp-P-2 (50 nmole) and the reconstituted system containing PCB P-448, PCB P-450, or PB P-450 for the indicated length of time. Each point represents the mean of duplicate determinations *(10)*.

To confirm the direct covalent binding of the *N*-hydroxy-Trp-P-2 to DNA, we isolated [^{3}H]*N*-hydroxy-Trp-P-2 by high performance liquid chromatography after incubation of [^{3}H]-Trp-P-2 with rat liver microsomes and cofactors. Isolated [^{3}H] *N*-hydroxy-Trp-P-2 was mixed with calf thymus DNA and incubated for 30 min. About 1% of added radioactivity bound covalently to DNA (25 pmole/mg DNA) *(10)*. It was reported that the binding of *N*-hydroxy-β-naphthylamine to DNA was pH-dependent; at pH 7.4 the binding was negligible and increased with lowering pH *(11)*. However, the binding of *N*-hydroxy-Trp-P-2 to DNA was relatively high at pH 7.4 and slightly decreased at lower pH *(12)*. The binding to DNA was inhibited by addition of dithiothreitol and stimulated by ascorbic acid.

On the other hand, we observed that the covalent binding of *N*-hydroxy-Trp-P-2 to DNA was markedly enhanced by seryl-tRNA synthetase isolated from yeast in the presence of serine and ATP *(13)*.

Moreover, we found that the addition of amino acids and ATP to hepatic supernatant fraction enhanced the covalent binding of *N*-hydroxy-Trp-P-2 to calf thymus DNA; proline was the best amino acid to increase binding so far examined. These results indicate the presence of other metabolic pathways for the covalent binding to DNA in addition to the direct binding.

The covalent binding of [^{14}C]Trp-P-2 to DNA occurred by the incubation with hepatocytes. The amount of radioactivity bound to RNA and protein was higher than that which was bound to DNA (Table 3). The capacity of hepatocytes to cause the binding to RNA, DNA and protein was markedly induced by pretreatment with PCB, but only slightly with phenobarbital. The amounts of binding were clearly decreased when hepatocytes were incubated in the presence of cysteine.

Table 3. Covalent binding of [^{3}H]Trp-P-2 to macromolecules in hepatocytes.[a]

Treatment	DNA	RNA	Protein
	(pmole bound/mg macromolecule/30 min)		
None	22	50	77
3-MC	258	537	401

[a]Reaction mixture consisted of 30υ*M* ^{3}H-Trp-P-2 and 2.5 × 10^{6} cells/mL of hepatocytes obtained from untreated or 3-MC-treated (15 mg/kg, 3 days) rats.

Species Difference in *N*-Hydroxylation of Trp-P-2

Rat liver microsomes showed very weak activity in *N*-hydroxylating Trp-P-2, whereas hamster and guinea pig liver microsomes were very active (Table 4) *(14)*. However, pretreatment with PCB resulted in the stimulation of the *N*-hydroxylation activity by about 260-fold in the rat and only about 1.5- and 4-fold in the guinea pig and hamster, respectively. The capabilities of microsomes isolated from different species of experimental animals in causing the mutation of the Salmonella with Trp-P-2 were closely associated with the capabilities of their activities in *N*-hydroxylating Trp-P-2 (Fig. 4). These results also indicate that in all species examined *N*-hydroxylation is an essential metabolic step for mutagenic activation of Trp-P-2. Razzouk et al. *(15)* recently reported that guinea pig liver microsomes were very active in *N*-hydroxylating 2-aminofluorene; the activity was about 5 and 25 times higher than the

Table 4. *N*-Hydroxylation of Trp-P-2 by liver microsomes from various species.[a]

Species	*N*-Hydroxylase activity, pmole/mg protein/min		Ratio, (PCB/untreated)
	Untreated	PCB-treated	
Rat (male, 6)	9 ± 3	2315 ± 629	257.2
Rat (female, 6)	5 ± 1	—[b]	
Mouse (4)	57 ± 37	700 ± 145	12.3
Hamster (5)	127 ± 39	516 ± 59	4.1
Guinea pig (5)	88 ± 41	134 ± 31	1.5
Rabbit (3)	41 ± 17	569 ± 158	13.9

[a]Male animals of each species were used, except that both sexes of rats were used. Numbers in parentheses indicate the number of animals used. Activities are represented as means ± S.D.

[b]Not determined.

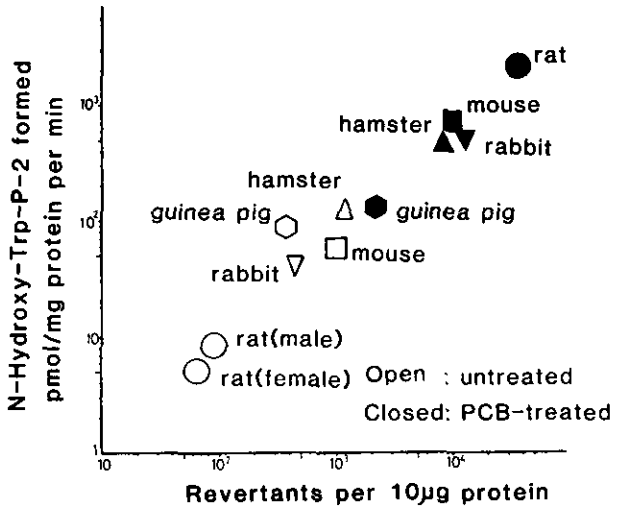

FIGURE 4. Relationship between the formation of *N*-hydroxy-Trp-P-2 and the number of revertants. Rates of formation of *N*-hydroxy-Trp-P-2 and the number of revertants induced by liver microsomes from rats, mice, hamsters, guinea pigs and rabbits were plotted on the same logarithmic scales (*13*, *14*).

levels observed in hamsters and rats, respectively. Analyzing the data obtained with MC-responsive and nonresponsive mice, we previously suggested that Trp-P-2 *N*-hydroxylase is the same enzyme as, or is closely related to, benzo(a)pyrene hydroxylase. However, we showed here that both enzymes are clearly different in rabbits, since Trp-P-2 hydroxylase, but not benzo(a)pyrene hydroxylase, was enhanced 14-fold by PCB treatment.

Comparison of *N*-Hydroxylation of Aromatic Amines by Various Species of Purified Cytochrome P-450

Recently we purified a high-spin form of cytochrome P-450, having a peak at 447.1 nm in reduced-CO difference spectrum, from liver microsomes of rats treated with PCB (PCB P-448 II-a) (*16*). PCB P-448 II-a showed a high activity in *N*-hydroxylating Trp-P-2. Therefore, we made a comparison of *N*-hydroxylation activities of various species of purified cytochrome P-450 with mutagenic aromatic amines.

The specific contents of the purified cytochromes were 11-15 nmole/mg protein; detailed procedures for purification will be published elsewhere. Benzo(a)pyrene hydroxylation activities of cytochrome P-450 preparations are given in Table 5. PCB P-448 II-a showed very low activity of benzo(a)pyrene hydroxylation, but it was very active in *N*-hydroxylating Glu-P-1. The ratios of PCB P-448 II-a to PCB P-448 II-d in the hydroxylation of benzo(a)pyrene, Trp-P-2 and Glu-P-1 were 0.016, 3.00 and 18.3, respectively. PCB P-448 II-a also showed high activity in *N*-hydroxylating 2-aminofluorene and 4-aminobiphenyl.

Moreover, rabbit PB P-448 showed very low activity in benzo(a)pyrene hydroxylation (C. Hashimoto-Yutsudo, Y. Imai, and R. Sato, personal communication), but it was very active in *N*-hydroxylating Trp-P-2 and Glu-P-1. These results suggest that the high-spin form of cytochrome P-450 has high activity in *N*-hydroxylating aromatic amines.

The capabilities of various species of cytochromes P-450 to convert aromatic amines to direct mutagens are shown in Table 6. Concerning Trp-P-2 and Glu-P-1, the number of revertants induced by the purified cytochromes closely correlated with *N*-hydroxylation activities.

PCB P-448 II-a and rabbit PB P-448 showed high activities in converting IQ to a direct mutagen. The ratios in the capabilities of PCB P-448 II-a to PCB P-448 II-d to convert Glu-P-1, IQ and Trp-P-2 to direct mutagens were 73.6, 11.9 and 2.5, respectively.

Effect of *N*-Acetylation on Mutagenic Activation of Aromatic Amines

Aromatic arylamides are *N*-hydroxylated by hepatic microsomes and converted to mutagens which

Table 5. Activities of Trp-P-2 and Glu-P-1 *N*-hydroxylases and benzo(a)pyrene hydroxylase of several cytochrome P-450 preparations.

Preparation	Activity, nmole formed/nmole P-450 (P-448)/min		
	Trp-P-2 *N*-hydroxylase	Glu-P-1 *N*-hydroxylase	Benzo(a)pyrene hydroxylase
Rat PB P-450	0.05	0.004	1.80
MC P-448	1.49	0.946	4.61
PCB P-450 I-c	0.05	0.011	2.59
PCB P-450 I-d	0.05	0.011	0.88
PCB P-448 II-a	2.09	3.208	0.07
PCB P-448 II-d	0.70	0.175	4.33
Rabbit PB P-448	0.57	0.918	0.03

showed direct mutagenesis to the Salmonella.

As shown in Table 7, the mutagenic activations of Trp-P-2 and AF by various species of cytochrome P-450 were decreased by *N*-acetylation. The activity

Table 6. Mutagenic activation of pyrolyzate mutagen by purified cytochrome P-450 preparations.

Preparation	Activation, revertants X 10^{-3}/nmole P-450		
	Trp-P-2	Glu-P-1	IQ
Rat PB P-450	128	44	69
MC P-448	7,206	2,030	1,030
PCB P-450 I-c	207	52	41
PCB P-450 I-d	188	45	30
PCB P-448 II-a	7,018	7,068	2,410
PCB P-448 II-d	2,826	96	202
Rabbit PB P-448	1,494	2,534	5,003

Table 7. Effect of acetylation of arylamines on the mutagenic activation by purified cytochrome P-450 preparations.

P-450 species	Activation, revertants/10^{-3}/nmole P-450 (P-448)			
	Trp-P-2	Trp-P-2-Ac	2-AF	2-AAF
Rat (PCB)				
P-450 I-c	640	5.3	31.0	0.25
P-450 I-d	464	4.0	25.4	0.22
P-448 II-d	3,648	10.8	63.1	1.43
P-448 II-a	7,810	31.0	211.4	5.05
Rabbit (PB)				
P-448	2,152	17.8	—[a]	36.30

[a]Not determined.

profiles of various species of cytochrome P-450 for the aromatic amines and amides were similar. These results suggest that similar species of cytochrome P-450 may be involved in the *N*-hydroxylation of both aromatic amines and amides.

Our preliminary results indicate that the low mutagenic activities of *N*-acetyl-Trp-P-2 and *N*-acetyl-AF in the presence of various species of cytochrome P-450 may be accounted for by the low mutagenic activities of *N*-hydroxylated metabolites rather than the low formation of these metabolites.

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