COMPARATIVE STUDIES ON ANILINE HYDROXYLATION AND *p*-NITROTOLUENE HYDROXYLATION BY THE LIVER

Masayuki IKEDA, Hatsue OHTSUJI and Toshiko IMAMURA

*Department of Public Health, Faculty of Medicine, Kyoto University,
Sakyo-ku, Kyoto, Japan*

Received for publication December 14, 1971

The question whether or not biotransformation of two drugs, especially hydroxylation, is catalyzed by one "drug-metabolizing enzyme" (1) inevitably arises when one considers possible changes in the effects due to metabolic modification by simultaneous administration of several drugs. Recently, evidence has accumulated toward the view that multiple oxidative enzymes are present in hepatic microsomes (2-9).

It was found in our laboratory (10) that a wide difference exists in the magnitude of induction of hepatic activities between aniline hydroxylation (aryl 4-hydroxylase, EC 1.14.1.1) and *p*-nitrotoluene hydroxylation (EC 1.14.1.0) when rats are pretreated with phenobarbital. This observation led us to speculate that the two compounds of closely related structures are hydroxylated by different enzymes. Although an exact comparison waits further progress in solubilization of hepatic microsomes (11, 12), an attempt is made in the present study to justify our hypothesis by comparative studies on hepatic activities between sexes and species of rodents as well as on the effects of inducers and inhibitors on the kinetics of the two hydroxylation activities.

MATERIALS AND METHODS

Pretreatment of animals

Wistar rats, ICR mice, Hartley guinea pigs, golden hamsters and Japanese albino rabbits were maintained on commercial laboratory chow and water ad libitum. Vegetable leaves were given to the guinea pigs to prevent vitamin C deficiency. The animals were intraperitoneally injected with 75 mg/kg of sodium phenobarbital in saline daily for 4 days (13) or single dose of 20 mg/kg of 3-methylchlanthrene in corn oil (14) and sacrificed for the assays 24 hr following the last injection. Control animals were given either saline or corn oil.

Chemicals

The following compounds were donated: SKF 525-A (2-diethylaminoethyl 2, 2-diphenyl valerate hydrochloride), SKF 8427-A (2-ethylaminoethyl 2, 2-diphenyl valerate hydrochloride)

This work was supported in part by a grant from the Fujiwara Memorial Fund.

Part of this work was presented at the 2nd Symposium on Drug Metabolism and Action in Kyoto on November 21, 1970.

and AEDV (aminoethyl 2, 2-diphenyl valerate) from Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A.; Lilly 18947 (2, 4-dichloro-6-phenylphenoxyethyl diethylamine hydrobromide), Lilly 38704 (2, 4-dichloro-6-phenylphenoxyethyl ethylamine hydrobromide) and DPEA (2, 4-dichloro-phenylphenoxyethylamine) from Lilly Research Laboratories, Indianapolis, Ind., U.S.A.; Metyrapone (2-methyl-1, 2-di-3-dipyridyl-1-porpanone) from Ciba-Geigy Ltd., Basel, Switzerland; aminoantipyrine, 4-methylaminoantipyrine and 4-dimethylaminoantipyrine (aminopyrine) from Nippon Shinyaku Co., Kyoto; heptylamine, N-ethylheptylamine and N-diethylheptylamine from Kissei Pharmaceutical Co., Matsumoto; U-16392-A (*o*-chloro- α -methyl phenethylhydrazine hydrochloride) from the Up-john Co., Kalamazoo, Mich., U.S.A.; MG 3062 (phenyl-(4-chlorophenyl)-4-piridylmethanol) from Maggioni & C.S.p.A., Milano, Italy.

Hydroxylation assays

The activities of aniline hydroxylation and *p*-nitrotoluene hydroxylation were measured by formation of *p*-aminophenol after the methods of Imai and Sato (15) and by formation of *p*-nitrobenzyl alcohol after Gillette (16), respectively, then conducted as previously described (10) with G-6P and 100,000 $\times g$ supernatant fraction as a NADPH generating system. When aniline, aminoantipyrine or monomethylaminoantipyrine (positive to the diazo-coupling reaction for *p*-nitrobenzyl alcohol determination) were added to *p*-nitrotoluene-hydroxylating system, the product in 1 ml of the water layer after heptane-washing was extracted into 15 ml of ether by vigorous shaking for 10 min, the ether layer was washed twice with 5 ml each of 2 N HCl and a 10 ml aliquot of the ether layer was evaporated over 0.5 ml of water in a bath at 70°. The water phase was subjected to *p*-nitrobenzyl alcohol determination.

Km and Ki determination

Unless otherwise specified, preparation from the liver of female phenobarbital-pretreated rats only was used in kinetic studies to avoid possible misunderstanding due to species differences in inhibition kinetics (17, 18). Kinetic parameters were determined after Lineweaver and Burk (19) by double reciprocal plottings of substrate concentration against velocity of reaction with and without inhibitors. Lines were drawn by least-square fitting when necessary.

RESULTS

1) Stability of the activities

Repetition of freezing and thawing of the preparation up to 5 times with dry ice-acetone and running water did not change significantly the activities of either aniline hydroxylation or *p*-nitrotoluene hydroxylation. Both activities decreased in parallel down to less than 70% of the original levels when kept at -20° for 3 weeks (Table 1), therefore, further experiments were conducted within a week following the preparation.

2) Parallel increase in activities after phenobarbital-pretreatment

To investigate whether or not activity is induced at different stages of phenobarbital pretreatment, rats were given intraperitoneal injections up to 4 days and levels of the ac-

TABLE 1. Effect of aging at -20° .

Weeks	No. of determinations	Aniline hydroxylation	<i>p</i> -Nitrotoluene hydroxylation
0	5	100	100
1	5	81 $\pm$ 4	75 $\pm$ 4
2	5	70 $\pm$ 3	67 $\pm$ 3
3	5	63 $\pm$ 2	65 $\pm$ 2

A batch of the preparation from the liver of female rats pretreated with phenobarbital was divided into several test tubes and stored in the dark at -20° . Five tubes of each were taken out for assays. Results are expressed as mean percentages of the original activities and given as means $\pm$ SD. No significant ($p < 0.05$) difference was revealed between any pair when tested using the *t*-test.

TABLE 2. Increase in the hepatic activities after phenobarbital pretreatment.

Duration of pretreatment (day)	No. of determinations	A Aniline hydroxylation	B <i>p</i> -Nitrotoluene hydroxylation	B/A*
0	7	100	100	
1	7	152 $\pm$ 21	553 $\pm$ 85	3.64
2	7	231 $\pm$ 36	787 $\pm$ 94	3.41
3	7	325 $\pm$ 56	954 $\pm$ 98	2.94
4	7	367 $\pm$ 86	1082 $\pm$ 143	2.36

Female rats were given intraperitoneal injections of sodium phenobarbital (75 mg/kg) daily for the period indicated and sacrificed the next day for assays. Results are expressed as mean percentages of the original levels and given as means $\pm$ SD.

* Uniformity of the ratios B/A was tested by χ^2 -test;

$$\chi^2 = 11.27 \quad 0.01 < p < 0.025$$

TABLE 3. Sex and age difference in Michaelis constant of hydroxylation by rat liver.

	No. of determinations	Michaelis constant ($\times 10^{-4}$ M)	
		Aniline hydroxylation	<i>p</i> -Nitrotoluene hydroxylation
Young male*	7	1.26 $\pm$ 0.18	1.39 $\pm$ 0.27
Young female*	7	1.34 $\pm$ 0.19	1.72 $\pm$ 0.36
Adult male**	7	1.33 $\pm$ 0.30	2.43 $\pm$ 0.57
Adult female**	7	1.18 $\pm$ 0.37	1.52 $\pm$ 0.39

No pretreatment of the animals was carried out. Numbers in the table are means $\pm$ SD.

* 5 weeks.

** about 20 weeks.

tivities were determined each day (Table 2). During the course of the experiment, the decrease in the ratio of the activity of *p*-nitrotoluene hydroxylation over that of aniline hydroxylation (B/A in Table 2) was very little, if any, indicating that two activities increased rather in parallel.

3) Sex and age differences in *K_m*'s for the hydroxylation

Comparison of *K_m* values of non-treated rats between the two sexes as well as young

TABLE 4. Changes in kinetic parameters for hepatic hydroxylation by pretreatments with 3-methylcholanthrene and phenobarbital.

Pretreatment with	Species	Substrates	Km ($\times 10^{-4}$ M)		Vmax (m μ oles/g/min)		P/C
			Control	Pretreated	Control	Pretreated	
3-Methylcholanthrene	Rat*	An	1.37 $\pm$ 0.25 (7)	1.47 $\pm$ 0.23 (7)	26.7 $\pm$ 5.4 (7)	41.5 $\pm$ 8.8 (7)	1.6
		<i>p</i> -Nt	1.67 $\pm$ 0.39 (7)	1.86 $\pm$ 0.32 (7)	98 $\pm$ 13 (7)	152 $\pm$ 26 (7)	1.5
Phenobarbital	Rat*	An	1.31 $\pm$ 0.20 (8)	2.35 $\pm$ 0.47 (8)	24.8 $\pm$ 9.3 (8)	83.1 $\pm$ 13.6 (8)	3.4
		<i>p</i> -Nt	1.72 $\pm$ 0.36 (5)	4.01 $\pm$ 0.34 (7)	132 $\pm$ 29 (5)	1170 $\pm$ 96 (7)	8.9
	Rabbit**	An	2.47 $\pm$ 0.80 (7)	3.35 $\pm$ 1.14 (7)	27.2 $\pm$ 7.4 (7)	46.1 $\pm$ 8.4 (7)	1.7
		<i>p</i> -Nt	2.38 $\pm$ 0.41 (7)	2.85 $\pm$ 0.39 (7)	240 $\pm$ 41 (7)	549 $\pm$ 138 (7)	2.3
	Mouse***	An	2.47 $\pm$ 0.47 (8)	4.78 $\pm$ 1.26 (8)	30.1 $\pm$ 5.0 (8)	68.8 $\pm$ 8.4 (8)	2.3
		<i>p</i> -Nt	1.69 $\pm$ 0.30 (8)	1.83 $\pm$ 0.31 (8)	169 $\pm$ 13 (8)	381 $\pm$ 24 (8)	2.3
	Hamster ⁺	An	0.126 $\pm$ 0.017 (6)	0.224 $\pm$ 0.030 (6)	14.2 $\pm$ 1.5 (6)	28.8 $\pm$ 4.7 (6)	2.0
		<i>p</i> -Nt	2.50 $\pm$ 0.66 (6)	1.92 $\pm$ 0.56 (6)	147 $\pm$ 48 (6)	259 $\pm$ 40 (6)	1.8
	Guinea pig ⁼⁼	An	5.93 $\pm$ 1.64 (7)	10.61 $\pm$ 1.67 (7)	30.6 $\pm$ 3.6 (7)	92.3 $\pm$ 19.6 (7)	3.0
		<i>p</i> -Nt	1.64 $\pm$ 0.26 (7)	2.42 $\pm$ 0.39 (9)	312 $\pm$ 54 (7)	621 $\pm$ 89 (9)	2.0

Young female animals were injected with either 3-methylcholanthrene or phenobarbital as described under *Materials and Methods*. Control animals were given vehicles. *Para*-hydroxylation of aniline (An) and hydroxylation at methyl moiety of *p*-nitrotoluene (*p*-Nt) were studied. Numbers in the table are means $\pm$ SD. Together with numbers of determinations in parentheses. P/C stand for the ratio of the pretreated over the control.

* Wistar strain (weighing about 50 g)

** Japanese albino (about 700 g)

*** ICR strain (about 17 g)

⁺ golden (about 70 g)

⁼⁼ Hartley strain (about 300 g)

and adults (Table 3) revealed that there was no difference in K_m values for aniline hydroxylation among the groups studied while the K_m for *p*-nitrotoluene hydroxylation in the liver of adult male rats was somewhat higher than that of adult females ($p < 0.01$, tested with *t*-test) or young males ($p < 0.01$).

4) Species differences in inductive effect of phenobarbital

Pretreatment of rats with 3-methylchlanthrene increased V_{max} 's of both hydroxylations by 1.5 times while K_m 's remained unchanged (Table 4). Due to poor inductivity under the conditions employed, no further experiment was conducted with this compound. With phenobarbital pretreatment, V_{max} for aniline hydroxylation increased about 3–4 times while a 9-fold increase was observed with *p*-nitrotoluene hydroxylation as previously reported (10). In agreement with the observation of Guarino et al. in aniline hydroxylation (20), K_m values were also modified; the ratio of the pretreated over the control (P/C in Table 4) with aniline hydroxylation was 1.8 whereas it was 2.4 with *p*-nitrotoluene hydroxylation. Forming a contrast to these observations with rats, pretreatment of guinea pigs with phenobarbital revealed a greater change in K_m and V_{max} for aniline hydroxylation than those for *p*-nitrotoluene hydroxylation. Changes in kinetic parameters of the hydroxylations in the liver of other rodents (rabbits, mice and hamsters) were between the two extreme cases of rats and guinea pigs. The inductive effect of phenobarbital varied depending

FIG. 1. Relative decrease in the hydroxylation activities in the presence of MG 3062, metyrapone and allylisopropylacetamide.

Assays were conducted as described under *Materials and Methods* with the same concentration (2×10^{-4} M) of the substrates for aniline hydroxylation (○) and *p*-nitrotoluene hydroxylation (●). Acetone concentration was 2% in MG 3062 experiment and 1% in metyrapone and allylisopropylacetamide experiments. The activities are expressed as percentages of the levels obtained in the absence of the inhibitor. Each symbol is a mean of 2 determinations, or a mean of 6 determinations with SD ranges. The difference between any pair of means with SD is statistically significant when tested with *t*-test ($p < 0.01$).

on the species of rodents treated and the ratio of the pretreated over the control (P/C in Table 4) for K_m and V_{max} were unequal between aniline hydroxylation and *p*-nitrotoluene hydroxylation in most of the species studied. While K_m for *p*-nitrotoluene hydroxylation by the control liver was rather constant (i.e., around 2×10^{-4} M) among the species studied, a wide variation was observed in K_m for aniline hydroxylation. Guinea pig liver had the

TABLE 5. Types of inhibition and K_i 's with various inhibitors.

Inhibitors	Substrates			
	Aniline		<i>p</i> -Nitrotoluene*	
AEDV	N	$3.1 \pm 0.6 \times 10^{-6}$ M (4)	N	$1.6 \pm 0.5 \times 10^{-6}$ M (6)
SKF 8742-A	N	$3.4 \pm 0.3 \times 10^{-6}$ M (4)	C	$2.5 \pm 0.4 \times 10^{-6}$ M (4)
SKF 525-A	N	$6.3 \pm 1.6 \times 10^{-6}$ M (4)	C	$9.0 \pm 1.8 \times 10^{-6}$ M (4)
DPEA	N	$7.9 \pm 1.3 \times 10^{-6}$ M (7)	Anti	$2.6 \pm 0.4 \times 10^{-7}$ M (5)
Lilly 38704	N	$1.1 \pm 0.5 \times 10^{-5}$ M (4)	Mix	in the order of 10^{-6} M
Lilly 18947	N	$1.7 \pm 0.6 \times 10^{-5}$ M (4)	C	$9.5 \pm 2.6 \times 10^{-6}$ M (4)
U-16392-A	N	$1.1 \pm 0.4 \times 10^{-5}$ M (4)	C	$3.1 \pm 0.2 \times 10^{-5}$ M (4)
Allylisopropyl-acetamide	N	$8.3 \pm 2.0 \times 10^{-4}$ M (6)	C	$2.0 \pm 0.2 \times 10^{-3}$ M (6)
MG 3062	N	$1.6 \pm 0.2 \times 10^{-5}$ M (4)**	N	$8.7 \pm 0.3 \times 10^{-7}$ M (4)***
Heptylamine	C	$2.4 \pm 0.8 \times 10^{-6}$ M (4)	C	$4.5 \pm 2.5 \times 10^{-5}$ M (4)
N-ethylheptylamine	C	$1.2 \pm 0.4 \times 10^{-2}$ M (3)	C	$2.6 \pm 0.3 \times 10^{-3}$ M (4)
N-diethylheptylamine	C	$3.7 \pm 0.7 \times 10^{-3}$ M (5)	C	$2.6 \pm 1.3 \times 10^{-3}$ M (4)
Aminoantipyrine	N	$4.1 \pm 0.5 \times 10^{-3}$ M (3)	C	$3.0 \pm 0.9 \times 10^{-3}$ M (6)
Monomethyl-aminoantipyrine	N	$1.0 \pm 0.1 \times 10^{-3}$ M (3)	C	$2.1 \pm 0.5 \times 10^{-3}$ M (4)
Aminopyrine	N	$2.3 \pm 0.1 \times 10^{-3}$ M (3)	C	$6.0 \pm 0.4 \times 10^{-3}$ M (5)
Testosterone	N	$7.2 \pm 0.2 \times 10^{-5}$ M (4)***	C	$1.4 \pm 0.6 \times 10^{-4}$ M (6)***
Warfarin	N	$2.2 \pm 0.5 \times 10^{-4}$ M (4)	C	$2.9 \pm 0.7 \times 10^{-3}$ M (4)
Pentobarbital	N	$1.5 \pm 0.6 \times 10^{-4}$ M (3)	C	$6.8 \pm 0.9 \times 10^{-4}$ M (4)
Metirapone	N	$1.9 \pm 0.2 \times 10^{-3}$ M (4)	C	$5.0 \pm 2.6 \times 10^{-6}$ M (6)
Aniline			N	$1.1 \pm 0.3 \times 10^{-3}$ M (7)
<i>p</i> -Nitrotoluene	C	$8.1 \pm 0.7 \times 10^{-5}$ M (3)		
Toluene	C	$3.4 \pm 0.7 \times 10^{-4}$ M (4)	C	$4.0 \pm 0.2 \times 10^{-3}$ M (4)
<i>o</i> -Xylene	C	$1.9 \pm 0.3 \times 10^{-4}$ M (4)	C	$1.2 \pm 0.3 \times 10^{-3}$ M (4)
<i>m</i> -Xylene	C	$2.0 \pm 0.5 \times 10^{-4}$ M (4)	C	$1.3 \pm 0.3 \times 10^{-3}$ M (4)
<i>p</i> -Xylene	C	$1.9 \pm 0.4 \times 10^{-4}$ M (4)	C	$1.6 \pm 0.4 \times 10^{-3}$ M (4)
Ethylbenzene	C	$3.7 \pm 1.0 \times 10^{-4}$ M (3)		
Cumene	C	$7.7 \pm 0.6 \times 10^{-4}$ M (3)		
<i>t</i> -Butylbenzene	N	$8.7 \pm 0.8 \times 10^{-4}$ M (3)		
Acetone	Stimulation		N	$5.1 \pm 0.5 \times 10^{-1}$ M (7)

Experiments were conducted with a liver preparation of young female rats pretreated with phenobarbital as described under *Materials and Methods*. Numbers in the table are means $\pm$ SD together with numbers of determinations in parentheses. C, N, Anti, and Mix stand for competitive, noncompetitive, anti-competitive and mixed type inhibition, respectively.

* *p*-Nitrotoluene hydroxylation system contains 1% acetone to dissolve the substrate.

** Acetone, 4%, was added to dissolve the inhibitor.

*** Final concentration of acetone was 2%.

largest K_m of 6×10^{-4} M as previous reported (9), whereas the constant with hamster liver was in the order of 10^{-5} M.

5) Difference in the effect of inhibitors

Three inhibitors, MG 3062, metyrapone and allylisopropylacetamide, were added to the two hydroxylation systems at various concentrations. The two systems were made exactly comparable, with the same substrate concentration of 2×10^{-4} M and with addition of acetone to aniline hydroxylation system also. Although both activities of hydroxylation were inhibited by the compounds tested, the magnitude of inhibition was significantly different between the two (Fig. 1). MG 3062 and metyrapone inhibited *p*-nitrotoluene hydroxylation more markedly than aniline hydroxylation, while it was reverse with allylisopropylacetamide. Leibman (21) reported the stimulatory effect of metyrapone at the concentration of 10^{-3} M when added to the incubation mixture for acetanilide hydroxylation, but no such effect was observed in the present study with aniline hydroxylation.

6) Type of inhibition with known inhibitors and other compounds

Several inhibitors of microsomal hydroxylation, drugs known to be metabolized by hepatic microsomes and other chemicals were added to the two hydroxylation systems in order to determine types of inhibition and inhibitor constants. A few typical cases of double

FIG. 2. Effect of SKF 525-A and its de-ethylated derivatives on aniline hydroxylation and *p*-nitrotoluene hydroxylation.

Incubation was as described under *Materials and Methods*.

(A) Double reciprocal plots of aniline concentration versus rate of aromatic hydroxylation. Inhibitors added are as follows: A, none (control); B, SKF 525-A at 5×10^{-6} M; C, SKF 8742-A at 5×10^{-6} M; D, AEDV at 5×10^{-6} M.

(B) Double reciprocal plots of *p*-nitrotoluene concentration versus rate of side-chain hydroxylation. Inhibitors are as follows: A, none (control); B, SKF 525-A at 1×10^{-6} M; C, SKF 8742-A at 5×10^{-6} M; D (dotted line), AEDV at 1×10^{-6} M.

reciprocal plottings are shown in Figs. 2-4. The results, as summarized in Table 5, revealed that there is a sharp contrast, as a whole, between the two types of hydroxylation in the manner of inhibition.

Inhibitors: SKF 525-A, Lilly 18947, their de-ethylated derivatives, MG 3062 and U-16392-A were tested. SKF 525-A, Lilly 18947 and U-16392-A inhibited aniline hydroxylation non-competitively while inhibition on *p*-nitrotoluene hydroxylation was in a competitive manner (Figs. 2 and 3). This contrast faded out when ethyl moieties attached to the N-terminal were one by one replaced by hydrogen as observed in the cases of SKF 8472-A and AEDV (Fig. 2), or Lilly 38704 and DPEA (Fig. 3), suggesting the important role of the N-alkylated group in inhibition mechanism. In spite of this, N-ethylated heptyl-amines failed to demonstrate such a contrast as observed with SKF 525-A and Lilly 18947 (Table 5). Allylisopropylacetamide, a non-competitive inhibitor of aniline hydroxylation having an amino-terminal inhibited *p*-nitrotoluene hydroxylation competitively only at a very high concentration. MG 3062, which is a derivative of methanol and has no N-alkylated terminal, was a non-competitive inhibitor for both activities of hydroxylation.

Drugs: Testosterone undergoes microsomal hydroxylation in positions 6 β , 7 α and 16 α of steroid structure (22), warfarin in 6, 7 and 8 of coumarin ring (13) and pentobarbital in position 3 of side chain (23). Aminopyrine is a typical substrate for N-demethylation,

FIG. 3. Effect of Lilly 18947 and its de-ethylated derivatives on aniline hydroxylation and *p*-nitrotoluene hydroxylation.

Incubation was conducted as described under *Materials and Methods*.

A) Double reciprocal plots of aniline concentration versus rate of aromatic hydroxylation. Inhibitors added are follows: A, none (control); B, Lilly 18947 at 1.8×10^{-5} M; C, Lilly 38704 at 1.7×10^{-5} M; DPEA at 2×10^{-5} M.

B) Double reciprocal plots of *p*-nitrotoluene concentration versus rate of side-chain hydroxylation. Inhibitors added are as follows: A, none (control); B, Lilly 18947 at 5×10^{-6} M; C₁, Lilly 38704 at 2×10^{-6} M; C₂, *ibid* at 6×10^{-6} M; D₁, DPEA at 5×10^{-7} M; D₂, *ibid* at 1×10^{-6} M.

FIG. 4. Effect of alkylbenzene on aniline hydroxylation.

Incubation was as described under *Materials and Methods*. Rate of hydroxylation and concentration of substrate (aniline) are double-reciprocally plotted. Alkylbenzenes added and their concentration are as follows: A, none; B, toluene at 5.7×10^{-4} M; C, ethylbenzene at 4.9×10^{-4} M; D, cumene at 4.3×10^{-4} M; E (dotted line), *t*-butylbenzene at 3.9×10^{-4} M.

a possible variation of N-hydroxylation (24). Despite varieties of hydroxylation sites, all drugs tested inhibited aniline hydroxylation non-competitively and *p*-nitrotoluene hydroxylation competitively with only the K_i 's being too high in some cases (Table 5). Removal of methyl moiety from aminopyrine molecule did not change the type of inhibition. Metirapone, a biphasical modifier of acetanilide hydroxylation (21), also inhibited in the same manner as other drugs.

Other chemicals: Aniline was non-competitively inhibitory when added to *p*-nitrotoluene hydroxylation system. Contrary to expectation, *p*-nitrotoluene was a competitive inhibitor of aniline hydroxylation. So were other alkylbenzenes such as toluene, xylene, ethylbenzene and cumene (Fig. 4). However, *t*-butylbenzene with the largest alkyl side-chain thus far studied, was a non-competitive inhibitor, suggesting that alkylbenzene with a smaller side chain might non-specifically bind with the site where aniline hydroxylation is catalyzed.

7) Effects of acetone

The observation of Anders (25) on the stimulatory effect of acetone on aniline hydroxylation was confirmed when the solvent was added to the incubation mixture up to 2% (Fig. 5), the only exception being that the K_m/V_{max} ratio varied depending on the concentration of acetone. On the contrary, a pattern of double reciprocal plottings was obtained to indicate the non-competitive inhibitory effect of acetone on *p*-nitrotoluene hydroxylation when the acetone concentration, 1% in the standard assay system, was elevated to 2 or 4% (Fig. 5).

FIG. 5. Effect of acetone on aniline hydroxylation and *p*-nitrotoluene hydroxylation.

Incubation was as described under *Materials and Methods*.

A) Double reciprocal plots of aniline concentration versus rate of aromatic hydroxylation. Concentrations of acetone are as follows: A, none (control); B, 0.5%; C, 1%; D, 2%. Changes in V_{max} and K_m are shown in terms of acetone concentration in the left-upper quadrant.

B) Double reciprocal plots of *p*-nitrotoluene concentration versus rate of side-chain hydroxylation. Concentrations of acetone are as follows: A, 1%; B, 2%; C, 4%.

DISCUSSION

Commercial toluol, or impure toluene, is often contaminated with an appreciable amount of benzene, and benzene, rather than toluene, is considered responsible for haemopoietic disturbance after the exposure to commercial toluol (26). From several clinical observations (26, 27) it can be presumed that the toxicity of benzene in commercial toluol may be potentiated by the co-presence of toluene. One of the mechanisms of the potentiation is that hydroxylation, the first step of detoxication of benzene is disturbed by the co-existing toluene when aromatic hydroxylation at benzene ring and hydroxylation at the methyl moiety attached to benzene ring are commonly catalyzed by the same enzyme. To examine this possibility, the present study was initiated with aniline and *p*-nitrotoluene as model substrates for these two hydroxylation. The solubilization and purification of hepatic microsomal drug-metabolizing enzyme(s), which catalyze these reactions, are not well established (11, 12), therefore working with unsolubilized preparation is inevitable.

Involvement of a liver microsomal haemoprotein, P-450, in drug oxidation has been sufficiently researched (28), and interaction of a drug with P-450 is represented by the spectral change of the haemoprotein such as type I and II difference spectra. Aniline evokes type II difference spectrum (29, 30) while no such information is available on the binding of *p*-nitrotoluene with P-450. Nevertheless a shift of difference-spectral pattern was reported

between the two types. This depended on the concentration of the drug, hexobarbital, added to liver microsomal preparation from methylcholanthrene-pretreated rats (31) as well as duration of methylcholanthrene pretreatment (32). These observations mean that the pattern of P-450 differential spectrum is not always a determining factor in the classification of hepatic drug-metabolizing enzymes.

The present study revealed sex and age differences in the K_m of *p*-nitrotoluene hydroxylation while no difference was observed in that of aniline hydroxylation. Effect of phenobarbital-pretreatment on kinetic parameters of aniline and *p*-nitrotoluene hydroxylations differs depending on the species of animals studied; the pretreatment induces aniline-hydroxylating activity more markedly than *p*-nitrotoluene hydroxylating activity in the guinea pigs, while it was reverse in rats. If the same enzyme catalyzes the two hydroxylation reactions, the affinities of the enzyme toward aniline and *p*-nitrotoluene should vary depending on sex, age and species. Occurrence is rare. Various compounds inhibited aniline hydroxylation non-competitively and *p*-nitrotoluene competitively. When one enzyme is involved in two reactions, a compound, like SKF 525-A, has to occupy the active site for *p*-nitrotoluene hydroxylation, and should have an inhibitory effect on aniline hydroxylation without attaching to the active site. MG 3062, a non-competitive inhibitor for both reactions requires much higher concentration to inhibit aniline hydroxylation compared with *p*-nitrotoluene hydroxylation. Observation with DPEA indicates that this compound can combine only with *p*-nitrotoluene-enzyme complex as an anti-competitive inhibitor (33), while it attaches to the unbound enzyme for aniline hydroxylation at higher concentration. Stimulatory effect of acetone (25) was observed in aniline hydroxylation but not in *p*-nitrotoluene hydroxylation. After Anders' hypothesis, the finding suggests that acetone modifies the structure of microsomes or the enzyme participating in aniline hydroxylation while the portion for *p*-nitrotoluene hydroxylation remains intact. Therefore, the results obtained are best explained by the assumption that different enzymes are involved in aromatic hydroxylation of aniline and hydroxylation of *p*-nitrotoluene at the methyl moiety.

It is of toxicological interest that aniline inhibits hydroxylation of *p*-nitrotoluene and vice versa. Further projection of this observation to in vivo metabolism of benzene and toluene in connection with potentiation of benzene toxicity is described elsewhere (34).

SUMMARY

Para-hydroxylation of aniline and side-chain hydroxylation at methyl moiety of *p*-nitrotoluene, both dependent on the hepatic microsomal fraction, appear to be catalyzed by different enzymes. This conclusion was arrived at through four experimental approaches: 1) K_m of *p*-nitrotoluene hydroxylation with adult male rat liver was higher than that with the liver of the female or the young while K_m of aniline hydroxylation was essentially constant regardless of sex or age of the rats used. 2) phenobarbital pretreatment of young female guinea pigs resulted in a more marked induction of the activity of aniline hydroxylation than that of *p*-nitrotoluene hydroxylation whereas in rats it was reverse. Results from mice, rabbits and hamsters were between these two extremes. 3) many drugs (including SKF

525-A, Lilly 18947, U-16392-A) inhibited aniline hydroxylation non-competitively and *p*-nitrotoluene hydroxylation competitively. The magnitudes of inhibition in the presence of the given concentration of the inhibitor were significantly different between the two hydroxylations. 4) stimulatory effect of acetone accompanied by an increase in K_m was only observed on aniline hydroxylation. *p*-Nitrotoluene hydroxylation was rather inhibited by acetone.

Acknowledgments: The authors wish to express their appreciation to Professor M. Nishio, Kyoto University, for his support of this work. Thanks are also due to the following companies for their generous gifts of the reagents: Nippon Shinyaku Co.; Kissei Pharmaceutical Co.; Smith, Kline and French Laboratories; Lilly Research Laboratories; Ciba-Geigy Ltd.; The Upjohn Co.; and Maggioni & C.S.p.A.

REFERENCES

- 1) CHA, S.: *Mol. Pharmac.* **4**, 621 (1968)
- 2) SLADEK, N.E. AND MANNERING, G.J.: *Mol. Pharmac.* **5**, 186 (1969)
- 3) NAKAZAWA, K.: *Biochem. Pharmac.* **19**, 1363 (1970)
- 4) GEORGE, W.J. AND TEPHLY, T.R.: *Mol. Pharmac.* **4**, 502 (1968)
- 5) MINEGISHI, K., KUROIWA, Y. AND OKUI, S.: *Chem. Pharm. Bull.* **16**, 1643 (1968)
- 6) DALY, J., GERINA, D., FARNSWORTH, G. AND GUROFF, G.: *Arch. Biochem. Biophys.* **131**, 238 (1969)
- 7) PEDERSON, T.C. AND AUST, S.D.: *Biochem. Pharmac.* **19**, 2221 (1970)
- 8) CONNEY, A.H., LEVIN, W., IKEDA, M. AND KUNTZMAN, R.: *J. biol. Chem.* **243**, 3912 (1968)
- 9) IKEDA, M., TANAKA, S. AND KATAYAMA, T.: *Mol. Pharmac.* **4**, 38 (1968)
- 10) IKEDA, M. AND OHTSUJI, H.: *Toxicol. Appl. Pharmac.* **20**, 30 (1971)
- 11) SHOEMAN, D.W., CHAPLIN, M.D. AND MANNERING, G.J.: *Mol. Pharmac.* **5**, 412 (1969)
- 12) RIKANS, L.E. AND VAN DYKE, R.A.: *Biochem. Pharmac.* **20**, 15 (1971)
- 13) IKEDA, M., CONNEY, A.H. AND BURNS, J.J.: *J. Pharmac. exp. Ther.* **162**, 338 (1968)
- 14) CONNEY, A.H., MILLER, E.C. AND MILLER, J.A.: *Cancer Res.* **16**, 450 (1956)
- 15) IMAI, Y. AND SATO, R.: *Biochim. biophys. Acta* **36**, 571 (1959)
- 16) GILLETTE, J.R.: *J. biol. Chem.* **234**, 139 (1959)
- 17) GILLETTE, J.R.: *Adv. Pharmac.* **4**, 219 (1966)
- 18) SASAME, H.A. AND GILLETTE, J.R.: *Biochem. Pharmac.* **19**, 1025 (1970)
- 19) DIXON, M. AND WEBB, E.C.: *Enzyme*, Ed. 2, p. 327. Academic Press, New York (1964)
- 20) GUARINO, A.M., GRAM, T.E., GIGON, P.L., GREENE, F.E. AND GILLETTE, J.R.: *Mol. Pharmac.* **5**, 131 (1969)
- 21) LEIBMAN, K.C.: *Mol. Pharmac.* **5**, 1 (1969)
- 22) CONNEY, A.H. AND SCHNEIDMAN, K.: *J. Pharmac. exp. Ther.* **146**, 225 (1964)
- 23) KUNTZMAN, R., IKEDA, M., JACOBSON, M. AND CONNEY, A.H.: *J. Pharmac. exp. Ther.* **157**, 220 (1967)
- 24) MCMAHON, R.E.: *J. Pharm. Sci.* **55**, 457 (1966)
- 25) ANDERS, M.W.: *Microsomes and Drug Oxidations*, Ed. by GILLETTE, J.R., CONNEY, H.A., COSMIDES, G.J., ESTABROOK, R.W., FOUTS, J.R. AND MANNERING, G.J., p. 533, Academic Press, New York (1969)
- 26) BROWNING, E.: *Toxicity and Metabolism of Industrial Solvents*, p. 66, Elsevier, Amsterdam and London (1965)
- 27) NOMIYAMA, K., MINAI, M., KITA, H., SHISHIDO, H. AND ONOZAWA, Y.: *Jap. J. industr. Health* **6**, 685 (1964) (*in Japanese*)
- 28) GILLETTE, J.R.: *Adv. Pharmac.* **4**, 219 (1966)
- 29) IMAI, Y. AND SATO, R.: *J. Biochem.* **62**, 239 (1967)
- 30) SCHENKMAN, J.B., REMMER, H. AND ESTABROOK, R.W.: *Mol. Pharmac.* **3**, 113 (1967)
- 31) KITAGAWA, H., MOHRI, T., KAMATAKI, T., FUKAZAWA, H., KITADA, M., KIMURA, T., KIRA,

- T., SHIMOKAWA, M., HIRANO, T. AND YAMAGISHI, M.: *Absorption, Metabolism and Excretion of Drugs*, Ed. by KAKEMI, K., p. 203, Hirokawa Publishing Co., Tokyo (1971) (in Japanese with English abstract)
- 32) ARIYOSHI, T. AND TAKABATAKE, E.: presented at the 3rd Symposium on Drug Metabolism and Action, in Fukuoka, Japan, November 20 (1971,)
- 33) SEGAL, H.L., KACHMAR, J.F. AND BOYER, P.D.: *Enzymologia* **15**, 187 (1952)
- 34) IKEDA, M., OHTSUJI, H. AND IMAMURA, T.: *xenobiotica* **2** 101 (1972)