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RELATIONSHIP BETWEEN THE INTERCONVERSION OF CYTOCHROME P-450 AND P-420 AND ITS ACTIVITIES IN HYDROXYLATIONS AND DEMETHYLATIONS BY P-450 OXIDASE SYSTEMS

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SUMMARY

The relationship between the interconversion of microsomal cytochrome P-450 and P-420 and the hydroxylation and demethylation activities of the P-450 oxidase system was studied. The hydroxylation and demethylation activities could only be partly restored by complete reconversion of P-420 to P-450 by addition of polyols, by dilution or by washing. The P-420 produced by detergents, monohydric alcohols or anilines did not retain hydroxylation and demethylation activities. Cytochrome P-450 is not rate limiting in the overall reaction for hydroxylation and demethylation of compounds such as anilines, nitroanisoles and aminopyrine.

The substrate specificities of the hydroxylations and demethylations by the P-450 oxidase systems of different tissues differ.

The rate of hydroxylation of anilines at the *p*-position was influenced by not only the hydrophobic character, but also by electronic and steric effects of the substrate.

The magnitude of the spectral changes of the microsomes induced by the substrate corresponded to the ESR signal height at $g_m = 2.25$ of microsomal Fe_x rather than to the absorption peak of the CO complex of P-450 at 450 mμ. The hydroxylation and demethylation activities were observed with either NADPH or NADH. Evidence is presented that microsomal NADPH–cytochrome *c* reductase is reduced by NADH and the K_m for the reductase with NADH is greater than the K_m for NADPH.

INTRODUCTION

It is known that the hydroxylations and demethylations of drugs and steroids are mediated by mixed function oxidase systems in microsomes and mitochondria of several animal tissues[1–11]. These systems generally contain cytochrome P-450 as the oxygen-activating enzyme[12–14]. Cytochrome P-450 is very readily converted to P-420 by organic compounds and under various conditions[15–19].

We found that the P-420 produced by various mild treatments can be recon-

verted to P-450 by polyols and reduced glutathione under appropriate conditions[20,21]. On the basis of this, the relationship between the interconversion of P-450 and P-420 and the hydroxylation and demethylation activities was studied to elucidate the mechanism of the metabolic reactions of the cytochrome P-450 oxidase system.

MATERIALS AND METHODS

Preparation of microsomes

Adult male albino rabbits weighing about 2.5 kg were sacrificed by injecting air into the ear vein. The livers, lungs and kidneys were immediately removed and carefully perfused through the main blood vessels with ice-cold 1.15% KCl solution. Then these organs were each cut with a pair of scissors, and microsomes were obtained from various tissues by the method as described previously[22]. Cow adrenal glands obtained at a local slaughterhouse, carried to the laboratory on ice and homogenized within 30 min by the same procedure except that tissues were washed several times by centrifugation to remove as much extraneous hemoglobin as possible. It was noticed that microsomes from frozen tissues bound hemoglobin more tightly than those from fresh tissues.

The microsomes obtained as firmly packed pellets were stored at 0° under anaerobic conditions and used within 2 h after preparation. They retained full activity for hydroxylation of aniline and demethylations of aminopyrine and nitroanisole for 15 h. However, storage of microsomal suspension (5 mg microsomal protein per ml of 0.1 M Tris–maleate at pH 7.5) at 0° led to the loss of 20% of these activities within 15 h.

Assay of enzymes

Hydroxylation of anilines at the *p*-position was assayed by the method of BRODIE AND AXELROD[23] with slight modifications. The standard reaction mixture contained, in a final vol. of 1.0 ml, 2–5 mg of microsomal protein per ml, 0.1 M Tris–maleate buffer (pH 8.0, 37°), a suitable amount of anilines (usually 7 mM) and an NADPH-generating system consisting of 0.5 mM $NADP^+$, 2.5 mM $MgCl_2$, 5.0 mM sodium isocitrate and isocitrate dehydrogenase sufficient to reduce oxidized $NADP^+$ and 0.5 mM nicotinamide was added to prevent destruction of the oxidized nucleotide by NAD(P) nucleosidase (EC 3.2.2.6). It was confirmed that the concentration of $NADP^+$ used was sufficient to obtain maximal hydroxylation and demethylation activities even though some $NADP^+$ was destroyed by the inorganic pyrophosphatase (EC 3.6.1.1) in the microsomes[24].

The demethylations of nitroanisoles and aminopyrine were measured by the method of NETTER AND SEIDEL[25,26] and NASH[27], respectively. The amount of nitrophenol produced by the demethylation of nitroanisoles was determined spectrophotometrically by the absorbance at 420 mμ to exclude the interference from the absorption of the nitroanisoles used as the substrates as far as possible. The reaction mixture was the same as that used for measuring aniline hydroxylation except that 1.0 mM nitroanisoles or 8 mM aminopyrine was used as substrate for oxidative demethylation.

Reactions were carried out at 37° for 10 min with mechanical shaking (120 times per min) in air unless otherwise stated and stopped by the addition of 0.5 ml of 7.5% trichloroacetic acid. After centrifugation to remove denatured protein, aliquots of the supernatant were taken for measurement by the methods described above.

No reduction of nitrophenol, the demethylated product, by nitroreductase[28] to aminophenol, occurred under the experimental conditions used for estimation of demethylation of nitroanisoles.

The hydroxylation of 17-hydroxyprogesterone to 17,21-dihydroxyprogesterone was measured by the method of PORTER AND SILBER[29].

Transhydrogenase activity, which catalyzes the transfer of hydrogen from NADPH to NAD^+, was measured by the method of COLOWICK, KAPLAN, NEUFELD AND CIOTTI[30]. Another transhydrogenase activity which catalyzes the transfer of hydrogen from NADH to $NADP^+$ was assayed by a similar method, except that an NADH-generating system of yeast alcohol dehydrogenase (EC 1.1.1.1) and NAD^+ was used instead of an NADPH-generating system.

NADPH– or NADH–cytochrome *c* reductase activity was followed as the change in absorbance at 550 mμ by the method of ERNSTER[31]. The extinction coefficient at 550 mμ for reduced *minus* oxidized cytochrome *c* was used in calculations by the method of MARGOLIASH[32].

NADPH– or NADH–ferricyanide reductase activity was measured as decrease in absorbance at 420 mμ following the method of SCHELLENBERG AND HELLERMAN[33]. The molar absorption coefficient of ferricyanide was taken as $1.02 \cdot 10^3 M^{-1} \cdot cm^{-1}$ at 420 mμ.

NADPH– or NADH–neotetrazolium reductase activity was measured by the method of LESTER AND SMITH[34] and DALLNER, SIEKEVITZ AND PALADE[35].

Measurements of difference spectra

Difference spectra were recorded at room temperature in a Cary Model 14 spectrophotometer equipped with a light-scattering transmission attachment, using cuvettes of 1-cm optical path.

Measurements of ESR spectra

ESR spectroscopy was performed with a Varian V-4500-10A spectrometer with a 100 kcycles/sec field modulation unit and spectra were generally obtained at a sample temperature of −170°.

Analytical procedures

The contents of P-450 and P-420 were determined from the CO-difference spectra after reduction of samples with dithionite following the method of OMURA AND SATO[36] with some modification.

The content of microsomal Fe_x was estimated with the cupric sulfate–EDTA complex as described previously[17].

The concentrations of $NADP^+$, NAD^+ and their reduced form were estimated taking $17.8 \cdot 10^3 M^{-1} \cdot cm^{-1}$ at 260 mμ in the oxidized form and $6.22 \cdot 10^3 M^{-1} \cdot cm^{-1}$ at 340 mμ in the reduced form as the molecular extinction coefficient[37,38].

The concentrations of adrenaline and noradrenaline were measured by fluorimeter determination by the method of EULER AND FLODING[39].

The amount of ascorbic acid was measured by the method of FUJITA[40]. After reduction with dithionite, the amount of reduced cytochrome *c* was measured spectrophotometrically at room temperature and at the temperature of liquid nitrogen by the

appearance of absorptions at 550 mμ and 547 mμ, respectively. This method can be used even if only a small amount of cytochrome *c* is present.

The protein content of microsomes was determined by the biuret method with some modifications[41,20].

The π values of organic compounds were determined by the method of FUJITA, IWASA AND HANSCH[42].

Enzyme preparations

NADPH–cytochrome *c* reductase from rabbit liver microsomes was purified by the method of WILLIAMS AND KAMIN[43].

Isocitrate dehydrogenase (EC 1.1.1.42) was prepared from pig heart as described by COLOWICK AND KAPLAN[44]. These enzyme preparations were carried out using a barrel rotor of a Hitachi preparative ultracentrifuge (Model 55 PA).

Chemicals

Nicotinamide–adenine dinucleotide and its reduced form, and nicotinamide–adenine dinucleotide phosphate and its reduced form were purchased from Sigma Chemical Co.

tert.-Butyl isocyanide was synthesized by the method of UGI *et al.*[45]. It was distilled twice just before use.

Anilines and aminophenols were purchased from Nakarai Chemical Co. and Wako Pure Chemical Co. The anilines which were liquid at room temperature were distilled twice and solid anilines were recrystallized twice from methanol just before use. Aminophenols were used as standards in measurement of hydroxylations of anilines and were purified in the same way as anilines. All other reagents were of the highest grade available and were used without further purification.

RESULTS

Fig. 1 (a, b, c, d) shows the relationship between the microsomal hydroxylation or demethylation activities by the P-450 oxidase system and the conversion of P-450 to P-420 by organic compounds or under conditions at lower and higher pH values. Unexpectedly, the initial rapid conversion of P-450 to P-420 with these organic compounds occurred instantaneously and the steady state was little affected by the experimental conditions over a period of 30 min. During longer periods of incubation, the P-420 was unstable and disappeared[15]. This may be due to the oxidative decomposition of P-420, because the disappearance of P-420 could be prevented by anaerobic conditions or by addition of antioxidants of lipids. Fig. 1 shows that the hydroxylation and demethylation activities of the P-450 oxidase system are not directly proportional to the amount of P-450 or P-420 added. To see whether organic compounds-produced P-420 can preserve the hydroxylation and demethylation activities, activities were measured after destruction of the P-420 produced by these organic compounds by incubation for 40 min at 37° under aerobic conditions. The activities were little affected by the disappearance of P-420. The same result was obtained by treatment at a very high or low pH instead of by organic compounds. Therefore, organic compound-produced P-420 can not preserve the activities for hydroxylation or demethylation of anilines, aminopyrine and nitroanisoles. The activities of enzymes of the P-450

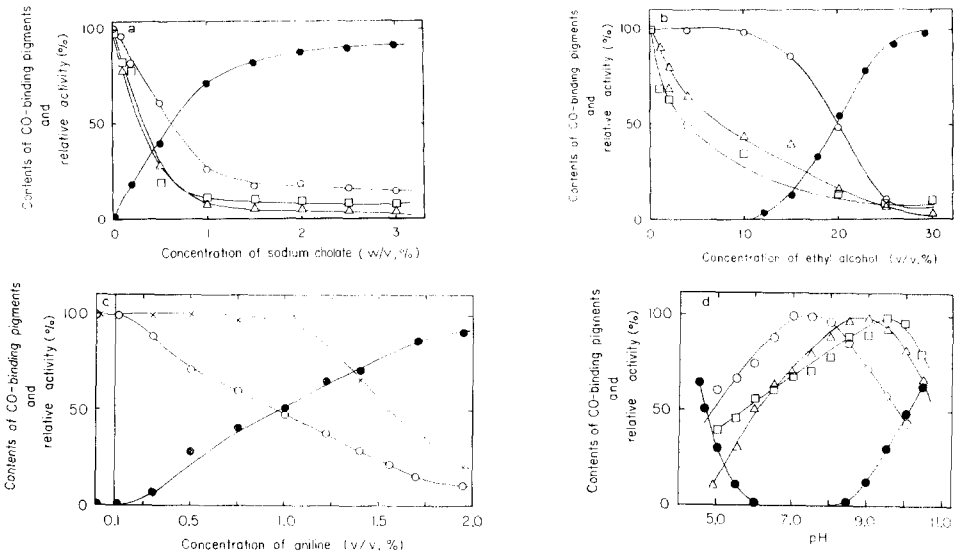


Fig. 1. Relationship between CO-binding pigments and the hydroxylation and demethylation activities of rabbit liver microsomes after various treatments. Various concentrations of sodium cholate(a), ethanol (b), or aniline (c) were incubated with microsomal suspension at 37° for 5 min to convert P-450 to P-420. After the incubation, the microsomal suspensions were used for enzyme assays. (d) The P-450 and P-420 contents and hydroxylation and demethylation activities after incubation at various pH values (37° and 5 min). Enzyme activities were assayed as described in the text except that the liver microsomes were treated with organic compounds or incubated at various pH values. The buffers used are 0.1 M Tris–maleate at below pH 8.5 and 0.1 M glycine–NaOH buffer at above pH 8.6. The ordinate shows the content of P-450 as a percentage of that in untreated microsomes or the P-420 content after complete conversion. The *p*-hydroxylation of *o*-chloroaniline and demethylation of *p*-nitroanisole are indicated on the ordinate as percentages of the amount of product formed on incubation at 37° for 10 min with untreated microsomes. Microsomal protein, 2.5 mg/ml; 0.1 M Tris–maleate at pH 8.0 (37°). ○—○, P-450; ●—●, P-420; □—□, demethylation of *p*-nitroanisole; △—△, *p*-hydroxylation of *o*-chloroaniline; ×—×, *p*-hydroxylation of aniline.

oxidase system other than P-450 were not decreased by these treatments. A detailed explanation of this will be given later in this paper. Accordingly, the unique properties of P-450 are indispensable for these metabolic reactions and P-450 is not a rate-limiting component of the reactions.

Fig. 2 shows the relationship between the hydroxylation and demethylation activities of the P-450 oxidase system and the reconversion of P-420, formed by organic compounds, to P-450 by polyols or by dilution. This figure shows that the complete reconversion of P-420 formed by sodium cholate or monohydric alcohols to P-450 by polyols or by dilution only partially restores the hydroxylation and demethylation activities. Namely, the activities were not always parallel with the content of P-450. Although this might be because the activities were inhibited by the detergents or monohydric alcohols, this seems unlikely because the same result was obtained when aniline as was used to form P-420 and as substrate in the *p*-hydroxylation, instead of detergents or monohydric alcohols. The results in Figs.1 and 2 are in disagreement with the finding of parallel change between benzpyrene hydroxylation activity and the P-450 content[46]. However, the results in Figs. 1 and 2 do not mean that NADPH-

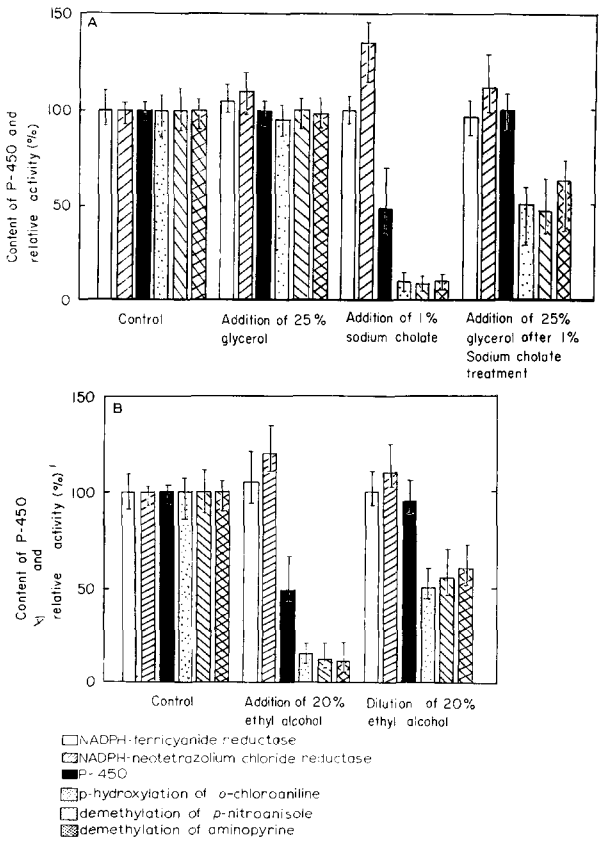


Fig. 2. Relationship between reconversion of P-420 to P-450 and activities of the P-450 oxidase system of rabbit liver microsomes. A. Reconversion of P-420 formed with 1% sodium cholate to P-450 by 25% glycerol and the activities of the P-450 oxidase system. B. Reconversion of P-420 formed with 20% ethyl alcohol to P-450 by 2-fold dilution and the activities of the P-450 oxidase system. The 100% value corresponds to the mean of 10 determinations of the content of P-450 and activities of the P-450 oxidase system obtained with different preparation of rabbit liver microsomes. The vertical bars correspond to the maximal deviations below and above the mean. The liver microsomes were incubated in the presence of 1% (w/v) sodium cholate or 20% (v/v) ethanol for 10 min at 20°. After incubation, the sodium cholate or ethanol-treated microsomal solutions were diluted 2-fold with 50% (v/v) glycerol Tris–maleate buffer (0.05 M, pH 8.0) or 0.05 M Tris–maleate buffer (pH 8.0), respectively. They were used as a source of enzymes. The reactions were as described in the text except that they were carried out at 20° for 20 min. The ordinates show values as percentages of the content of P-450 or activities of the P-450 oxidase system of untreated microsomes on a protein basis. The *p*-hydroxylation and demethylation activities were apparently 1/2.5 of those at 37°. Microsomal protein, 2.5 mg/ml; 0.1 M Tris–maleate buffer at pH 8.0.

dependent hydroxylations of anilines and demethylation of aminopyrine or nitroanisoles are not carried out by the P-450 oxidase system. Evidence that P-450 is an essential component of the hydroxylation and demethylation reactions has already been given by ORRENIUS[47] and ESTABROOK and co-workers[12–15]. To confirm this, the experiment was made using *tert.*-butyl isocyanide instead of carbon monoxide. It is well known that a *b*-type hemoprotein generally has a strong affinity for CO, whereas the reduced form of P-450 is much more autoxidizable than other hemoproteins with

protoheme prosthetic groups[22], and the reduced P-450–CO complex is rapidly converted to oxidized P-450 even on brief exposure to a very low concentration of oxygen. Alkylisocyanides did not convert P-450 to P-420 even when added at relatively high concentrations[48]. The 90% of *p*-hydroxylation and demethylation activities of anilines, aminopyrine and *p*-nitroanisole are inhibited by $5 \cdot 10^{-3}$ M *tert.*-butyl isocyanide and these activities can be completely restored by removal of the isocyanide by washing and centrifugation at 5° or by dialysis at 0° for 10 h under anaerobic conditions. It was shown magnetically and spectrophotometrically that these procedures released isocyanide from the oxidized P-450 isocyanide complex. However, alkyl isocyanides had no spectrophotometric and magnetic influence on adrenodoxin, ferredoxin, rubredoxin, cytochrome b_5 and the ESR signal at $g_m = 4.3$ of rabbit liver microsomes due to nonheme rhombic iron in the oxidized and high spin form at temperature of liquid nitrogen. Furthermore, the activities of NADPH–ferricyanide reductase, NADPH–cytochrome *c* reductase and NADPH–neotetrazium chloride reductase which are considered to be components of the P-450 oxidase system, were not inhibited by isocyanide at the concentration used in this experiment. Therefore, these results show that the inhibition of the hydroxylation or demethylation activities is due to a combination of isocyanide with P-450, and that oxygen cannot be activated by P-450. Thus, like previous reports, these results substantiate the conclusion that P-450 is an essential component of systems for the hydroxylation and demethylation of anilines, aminopyrine and nitroanisoles, and the amount of P-450 present does not limit the rates of the hydroxylation and demethylation reactions.

As reported previously[17,49], P-450 is found in various tissues. However, the rates of hydroxylation and demethylation of drugs and steroids were not directly correlated with the amount of P-450 in the tissues, as described above. Fig. 3 shows the relationship between the hydroxylation and demethylation activities and the amount of P-450 in the microsomes of rabbit liver, lung and kidney and the adrenal glands of cow. Hydroxylation or demethylation of anilines, aminopyrine and *p*-nitroanisole were observed with liver, lung and kidney microsomes, but not with adrenocortical and medullary microsomes. Conversely, 21-hydroxylation of 17-hydroxyprogesterone occurred only with adrenocortical microsomes, and not with liver, lung, kidney or

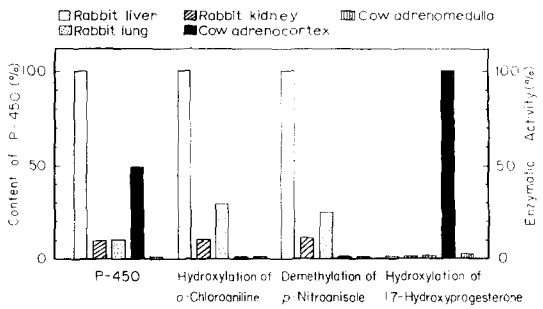


Fig. 3. Comparison of P-450 content and hydroxylation and demethylation activities of microsomes of various tissues. Values are averages of 10 determinations of the P-450 content or enzymatic activities. The amount of P-450 and the *p*-hydroxylation activity of *o*-chloroaniline and demethylation activities of *p*-nitroanisole and aminopyrine are shown as percentages of those of rabbit liver microsomes. The 21-hydroxylation activity of 17-hydroxyprogesterone is shown as a percentage of that of adrenocortical microsomes of cow. Microsomal protein, 5.0 mg/ml.

TABLE I

EFFECTS OF VARIOUS INHIBITORS ON THE *p*-HYDROXYLATION ACTIVITY OF RABBIT LIVER MICROSOMES ON *o*-CHLOROANILINE

Enzyme was assayed by incubation at 37° for 10 min as described in the text except for the additions of various inhibitors. Inhibition is expressed as percentage inhibition of hydroxylation activity calculated from the value without inhibitor. Values show the averages of 5 determinations of hydroxylation activity. Microsomal protein, 2.5 mg/ml; 0.1 M Tris–maleate buffer at pH 8.0.

Inhibitor	*Concn. (M)*	*Inhibition (%)*	*Inhibitor*	*Concn. (M)*	*Inhibition (%)*
2,6-Dichlorophenolindophenol	$6 \cdot 10^{-5}$	35	1,2,4-Triazole	$1 \cdot 10^{-2}$	69
FAD	$6 \cdot 10^{-5}$	40	Adrenaline	$5 \cdot 10^{-4}$	28
Janus green B	0.002%	85	2′-AMP	$1 \cdot 10^{-3}$	45
Methylene blue	$6 \cdot 10^{-5}$	17	Ascorbic acid	$1 \cdot 10^{-3}$	12
Methyl viologen	$5 \cdot 10^{-5}$	65	Nicotinamide	$1 \cdot 10^{-2}$	0
Neotetrazolium chloride	0.002%	35	Noradrenaline	$1 \cdot 10^{-5}$	54
Phenosafranine	$6 \cdot 10^{-5}$	20	Phenobarbital	$1 \cdot 10^{-2}$	53
Potassium ferricyanide	$6 \cdot 10^{-5}$	15	L-Phenylaniline	$1 \cdot 10^{-3}$	20
Safranine T	0.002%	11	L-Tyrosine	$1 \cdot 10^{-3}$	8
EDTA	$2 \cdot 10^{-3}$	12	Bovine heart myoglobin	$1 \cdot 10^{-3}$	4
Imidazole	$1 \cdot 10^{-3}$	85	Cytochrome b_5	$1 \cdot 10^{-4}$	0
KCN	$2 \cdot 10^{-3}$	69	Cytochrome *c*	$1 \cdot 10^{-6}$	50
NaF	$1 \cdot 10^{-2}$	9	Oxygenated hemoglobin (adult rabbit)	$1 \cdot 10^{-3}$	0
NaN_3	$1 \cdot 10^{-2}$	65	Adrenodoxin (pig adrenocortex)	$2 \cdot 10^{-4}$	0

adrenomedullary microsomes. Tests should be made to determine whether these hydroxylation and demethylation activities are inhibited by physiological substances such as ascorbic acid, adrenaline, noradrenaline and cytochrome *c*. Judging from Table I, the concentrations of substances were not sufficiently high to inhibit the hydroxylation and demethylation activities. In addition, the hydroxylations and demethylations by liver microsomes were never affected by the addition of cow adrenocortical microsomes and *vice versa*. Although there was no difference in the optical and magnetic properties of P-450 in liver and adrenocortical microsomes, the P-450 oxidase system in adrenocortical microsomes is different from that of liver, lung and kidney. Logically, the next problem is to study where the substrate binding site is in the P-450 oxidase system. There have been a number of reports that the spectral change of microsomes induced by substrates of the P-450 oxidase system may be due to the binding of the substrates with P-450[50–57]. However, as reported in the previous paper[48], purified P-450 is readily reduced by a ferredoxin–$NADP^+$ reductase system. Similar results were obtained with xanthine oxidase, hypoxanthine and pigments such as methylviologen, benzyl viologen, safranine T, FMN or FAD[58]. However, no hydroxylation of anilines or demethylation of aminopyrine or *p*-nitroanisole by these enzyme systems was observed. It was shown that this was not because the hydroxylation and demethylation activities were inhibited by the EDTA used during purification of P-450 from rabbit liver microsomes or by oxygen consumption in the test solution by a ferredoxin–$NADP^+$ reductase system or xanthine oxidase system. This result suggests that the substrate binding site may be independent of the P-450 molecule or that the structural relation of the components of the P-450 oxidase system may be important for the hydroxylation and demethylation activities. However, it is

uncertain whether the purified P-450 molecule is still intact and considerable further study is required to resolve these questions.

Table I shows the effect of inhibitors on the hydroxylation of *o*-chloroaniline. Various dyes were used to study the electron transport between P-450 and related flavoproteins. The inhibitory effects of physiological substances are also shown. These results are significant in studies on the substrate specificities and the components involved in electron transport of the P-450 oxidase system. It was found that the hydroxylations and demethylations of drugs and steroids by microsomes were greatly inhibited by cytochrome *c* like the 21-hydroxylation of 17-hydroxyprogresterone[59], although the potassium cyanide-sensitive demethylation of sarcosine was activated by the addition of cytochrome *c*[60].

The hydroxylations and demethylations of anilines, aminopyrine and nitroanisoles are well known to require NADPH as reductant. However, these enzymatic reactions could occur with NADH instead of NADPH. Fig. 4 shows the time courses

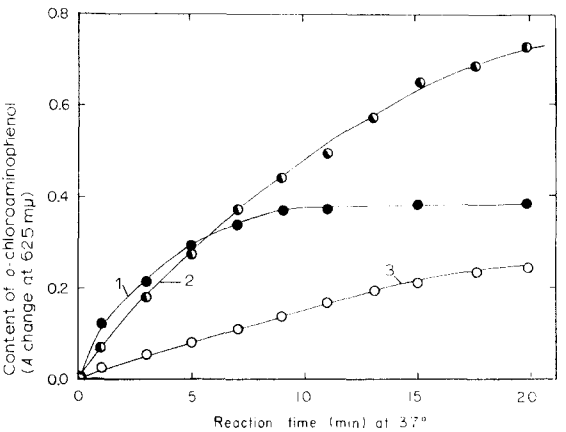


Fig. 4. Effects of NADPH, NADH and both together on the *p*-hydroxylation of *o*-chloroaniline by rabbit liver microsomes as function of reaction time. The enzyme was assayed as described in the text except that $1.0 \cdot 10^{-5}$ M NADPH, $5 \cdot 10^{-3}$ M NADH and their generating systems were used. Microsomal protein, 2.5 mg/ml. Curve 1, NADPH; Curve 2, NADPH + NADH; Curve 3, NADH.

of *p*-hydroxylation of *o*-chloroaniline in the presence of NADPH, NADH and both together. When NADPH and NADH were both present, the initial hydroxylation activity was somewhat less than with NADPH alone. Although the inhibition is very small, it is observable. A similar result was obtained on hydroxylation of position 21 of 17-hydroxyprogesterone in cow adrenocortical microsomes. The inhibition of the initial activities of NADPH-dependent hydroxylations by NADH could not be explained by decomposition of NADPH by NADase, pyrophosphatase or the effect of the NADH-dependent electron transport system in microsomes. This result is considered to be due to the difference in the affinities of NADPH and NADH for NADPH–cytochrome *c* reductase. This was demonstrated with purified NADPH–cytochrome *c* reductase from rabbit liver microsomes. According to the kinetic studies of binary reaction[61], the Michaelis constant for NADPH was $3.2 \cdot 10^{-6}$ M and for NADH was

$1.3 \cdot 10^{-3}$ M. Results suggest that NADPH and NADH are attached to the same site on the reductase. In addition, when the hydroxylation activities with NADPH, NADH and both together are compared, the activity with both together was greater than the sum of the activities with each separately. Similar results were obtained by CONNEY *et al.*[62] and NILSSON AND JOHNSON[63]. They could be interpreted as due to the activity of the transhydrogenase between NADH and $NADP^+$. The transhydrogenase activity was associated with the NADH–cytochrome b_5 reductase (EC 1.6.2.2) rather than the NADPH–cytochrome *c* reductase.

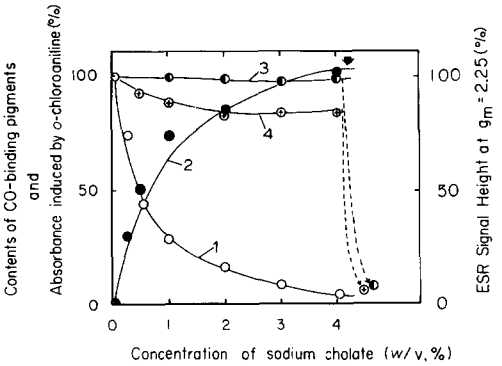


Fig. 5. Relationship between spectral change induced by *o*-chloroaniline and the P-450 or P-420 produced by sodium cholate treatment of rabbit liver microsomes. Rabbit liver microsomes were incubated for 10 min at 20° in the presence of various concentrations of sodium cholate. After incubation, spectra were measured immediately. $7 \cdot 10^{-3}$ M *o*-chloroaniline was used as substrate to induce spectral changes. The ordinate shows the percentage contents of P-450 and P-420 and of the ESR signal height at $g_m = 2.25$ of microsomal Fe_x of untreated microsomes. The content of P-420 is expressed as 100% when P-450 was completely converted to P-420. The induced spectra are shown as percentages of the absorption between 428 mμ and 490 mμ. The arrow indicates the point when 0.1% snake venom was added to the microsomal suspension to produce P-420 without the ESR signal of microsomal Fe_x. The dotted lines show that the spectral change induced by the substrate and the content of microsomal Fe_x decrease. Microsomal protein, 2.0 mg/ml; 0.1 M Tris–maleate, pH 8.0. Curve 1, P-450; Curve 2, P-420; Curve 3, microsomal Fe_x; Curve 4, spectral change by substrate.

Fig. 5 shows the interrelationship between the microsomal CO-binding species, microsomal $Fe_x(g_m = 2.25)$ and the spectral changes induced by hydroxylation substrates. According to many reports[50–57], the spectral changes induced by substrate are due to the interaction between the P-450 molecule and the substrate. This figure shows that the spectral changes of microsomes induced by substrates are not proportional to the amount of P-450 or P-420, but to the amount of microsomal Fe_x. This suggests that the substrate combines not only with P-450, but also with P-420 which has the ESR signal of microsomal Fe_x due to octahedral heme iron in the oxidized and low spin form at the temperature of liquid nitrogen. However, as described above, P-420 could not preserve the hydroxylation and demethylation activities. When microsomal Fe_x was destroyed by treatment with snake venom or proteinase, the spectral changes of microsomes induced by substrate could no longer be observed. It is considered that the conditions around the ligands of the heme-iron of P-450 are important for the binding of the substrate to the P-450 oxidase system.

TABLE II

COMPARISON OF THE *p*-HYDROXYLATIONS OF ANILINES BY RABBIT LIVER MICROSOMES

Values are averages of 5 determinations of hydroxylation activities. Specific activity is expressed as nmoles/min per mg microsomal protein. Relative activity is expressed as a percentage of the *p*-hydroxylation of *o*-chloroaniline. Stars indicate the π values obtained by FUJITA, IWASA AND HANSCH[13]. Microsomal protein, 2.5 mg/ml; 0.1 M Tris–maleate buffer at pH 8.0.

Anilines	*Specific activity*	*Relative activity*	π *Value*	*Anilines*	*Specific activity*	*Relative activity*	π *Values*
H—	0.72	52.8	0.00*	*p*-Cl	0.05	3.7	0.94 ± 0.02
N-CH_3—	0.79	58.0	0.92 ± 0.02	*o*-Br	1.32	97.0	1.39 ± 0.02
N-CH_3CH_2—	1.87	137.0	1.36 ± 0.02	*m*-Br	0.59	43.4	1.20 ± 0.02
N-$CH_3CH_2CH_2$—	2.22	163.0	1.55 ± 0.02	*p*-Br	0.05	3.7	1.30 ± 0.02
N-$CH_3CH_2CH_2CH_2$—	2.75	202.0	2.68 ± 0.02	*o*-I	1.74	128.0	2.44 ± 0.02
o-CH_3O—	0.03	2.2	0.05 ± 0.02	*m*-I	0.52	38.3	2.08 ± 0.02
m-CH_3O—	0.05	3.7	0.03 ± 0.02	*p*-I	0.01	0.7	2.44 ± 0.02
p-CH_3O—	0.04	3.0	0.05 ± 0.02	*o*-NO_2	0.50	36.8	0.54 ± 0.02
o-F—	1.59	117.0	0.36 ± 0.02	*m*-NO_2	0.10	7.5	0.47 ± 0.02
m-F—	0.66	49.0	0.40 ± 0.02	*p*-NO_2	0.01	0.7	0.49 ± 0.02
p-F—	0.31	22.8	0.25 ± 0.02*	*o*-CH_3	1.01	74.5	0.39 ± 0.02
o-Cl—	1.36	100.0	0.91 ± 0.02*	*m*-CH_3	0.43	31.7	0.50 ± 0.02*
m-Cl—	0.69	50.7	0.98 ± 0.02*	*p*-CH_3	0.00	0.0	0.49 ± 0.02*

Table II shows the relationship between the relative activities for hydroxylation of the *p*-position of anilines and their π values; the logarithms of the distribution constants of anilines between water and 1-octanol[42]. GAUDETTE AND BRODIE have already pointed out that the dealkylation reaction increases with increase in hydrophobicity of the substrate[64]. Although the conversion of P-450 to P-420 by anilines was directly related to the π values, there was no close relationship between the hydroxylation activity and the π values[18]. As a corollary to this result, it is considered that the rate of hydroxylation is effected not only by the hydrophobic character of anilines, but also by electronic and steric effects of substituents on them. This table shows that anilines substituted in the *m*- or *p*-position by halogens are not readily hydroxylated to the aminophenol derivatives. Among those substituted in the *p*-position, *p*-fluoroaniline was more easily hydroxylated to *p*-aminophenol than the other *p*-halogen substituted anilines. In the hydroxylation of halogen substituted compounds, the NIH shift recently reported by UDENFRIEND and co-workers[65,66] should be taken into consideration in elucidation of the mechanism of hydroxylation. The activities in demethylation of anisoles of nitro groups in the *o*-, *m*- and *p*-positions are summarized in Table III. Table III shows the high percentage of demethylation of *m*-nitroanisole. The molar extinction coefficient of absorption at 420 mμ of *m*-nitrophenol is about 1/11 of that of *p*-nitrophenol. Using these values, the *m*/*p* and *m*/*o* ratios of demethylation of nitroanisoles were both found to be about 2.

Fig. 6 shows that *p*-nitroanisole inhibits the *p*-hydroxylation of *o*-chloroaniline, and the demethylation of *p*-nitroanisole is also coincidently inhibited by *o*-chloroaniline. In Fig. 6, the lines meeting at the ordinate indicate competitive inhibition. The apparent K_m for hydroxylation of *o*-chloroaniline is $9.7 \cdot 10^{-5}$ M, the value for demethylation of *p*-nitroanisole is $3.9 \cdot 10^{-4}$ M, the K_i for *p*-nitroanisole with respect

TABLE III

COMPARISON OF THE DEMETHYLATION OF NITROANISOLES BY RABBIT LIVER MICROSOMES

Values are averages of 5 determinations of demethylation activities. Specific activity is expressed as nmoles/min per mg microsomal protein. Relative activity is expressed as a percentage of the demethylation of *p*-nitroanisole. Microsomal protein, 2.5 mg/ml; 0.1 M Tris–maleate buffer at pH 8.0.

Nitroanisoles	*Specific activity*	*Relative activity*	*π Values*
Anisole	—	—	0.00
o-NO_2—	0.79	89	−1.24 ± 0.02
m-NO_2—	1.96	220	−1.16 ± 0.02
p-NO_2—	0.89	100	−1.22 ± 0.02

to the hydroxylation of *o*-chloroaniline was graphically determined to be $4.1 \cdot 10^{-3}$ M and the K_i for *o*-chloroaniline with respect to demethylation of *p*-nitroanisole was $6.4 \cdot 10^{-6}$ M. The results show that the catalytic sites of hydroxylation and demethylation of *o*-chloroaniline and *p*-nitroanisole are identical.

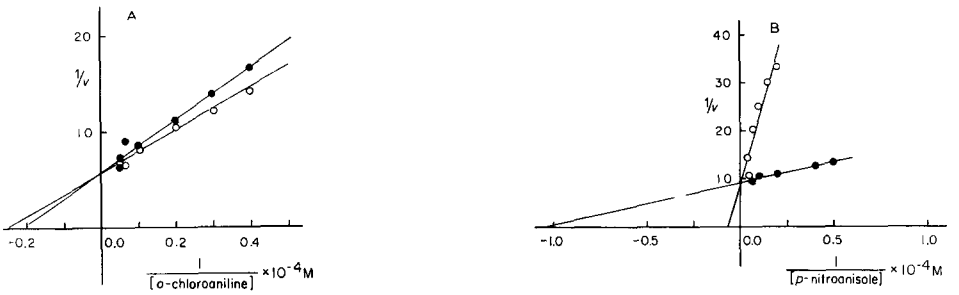


Fig. 6. Kinetics of *p*-hydroxylation of *o*-chloroaniline and demethylation of *p*-nitroanisole by rabbit liver microsomes. A. Double reciprocal plots of *p*-hydroxylation activity *versus* *o*-chloroaniline concentration. ○—○, activity with *o*-chloroaniline; ●—●, activity when $1 \cdot 10^{-3}$ M *p*-nitroanisole is present as inhibitor. B. Double reciprocal plots of demethylation activity *versus* *p*-nitroanisole concentration. ●—●, activity with *p*-nitroanisole; ○—○, activity when $1 \cdot 10^{-4}$ M *o*-chloroaniline is present as inhibitor. The reaction mixture was as described in the text except that inhibitor was added. Microsomal protein, 2.5 mg/ml. Units on the ordinate are (μmoles/ml per 50 min)$^{-1}$.

DISCUSSION

Cytochrome P-450 is mainly present in the microsomes of liver, lung, adrenocortex, kidney, small intestine, ovary and pituitary gland[17], and P-450 is also involved in the mitochondrial fraction of endocrine glands such as the adrenocortex[67], ovary[68] and pituitary glands (Y. ICHIKAWA, unpublished data). Although the demethylation activity of lung microsomes has already been investigated by CONNEY and co-workers[62,69], the contents of cytochrome P-450 and microsomal Fe_x have not been reported. Table IV summarizes the contents of microsomal hemoproteins in the lungs of various animals. Consequently, hydroxylation and demethylation of drugs and steroids occur in the microsomes and mitochondrial fraction of these tissues.

TABLE IV

COMPOSITIONS OF CYTOCHROMES OF LUNG MICROSOMES OF VARIOUS ANIMALS

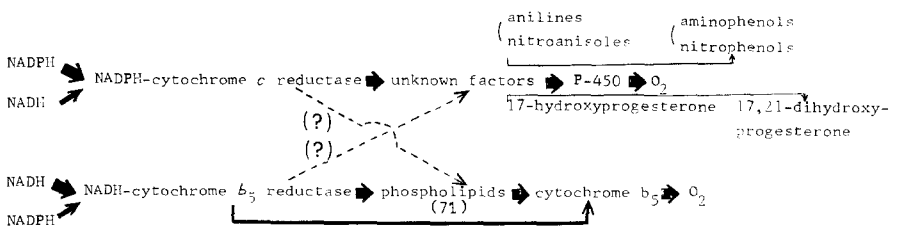
Values show averages of 10 determinations of the cytochrome contents of lungs of different animals of each species. Microsomal cytochrome contents are expressed as nmoles per mg of microsomal protein.

Animals	*Cytochrome* b_5	*P-450*	*Microsomal* Fe_X
Male rabbits	0.05	0.17	0.17
Phenobarbital-treated rabbits*	0.06	0.18	0.19
Male guinea pigs	0.12	0.16	0.15
Male pigs	0.08	0.04	0.06
Male rats	0.07	0.06	Trace
Oxen	0.08	0.21	0.24
Cocks	0.06	0.03	Trace

* Male rabbits were injected intraperitoneally with 100 mg of phenobarbital per kg body wt. once daily for 5 days using the method of ORRENIUS AND ERNSTER[9]. 20 h after the last injection the rabbits were sacrificed.

Although it seems that the substrate specificity of the P-450 oxidase system is rather broader than that of other enzymes, no 21-hydroxylase activity could be observed in the liver, lung or kidney microsomes. The hydroxylations and demethylations of anilines, aminopyrine and *p*-nitroanisole did not occur in adrenocortical microsomes of pig or cow. From our present knowledge, we consider that a single cytochrome P-450 molecule is in multiform (high or low spin forms) rather than that there are various species of P-450. Therefore, in addition to having a general function in the cytochrome P-450 oxidase system as an electron transport carrier, there is an individual active center giving substrate specificity somewhere in the P-450 oxidase system in various tissues, because the hydroxylation of anilines and 17-hydroxyprogesterone in the liver and adrenocortical microsomes are both inhibited by CO or isocyanides.

On the other hand, some flavoproteins are possible to require either NADPH or NADH as carrier substrate, like ferredoxin–$NADP^+$ reductase (EC 1.6.99.4), and have in common a secondary activity as a diaphorase and transhydrogenase[70]. Microsomal NADPH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase (EC 1.6.2.2) were also found to be of this type. Accordingly, the cytochrome P-450 oxidase system in microsomes can be reduced by either NADPH or NADH. Thus hydroxylation and demethylation may occur in the presence of NADH instead of NADPH. Although it might be considered that the reduction of the P-450 oxidase system of microsomes



Scheme 1. Scheme of components on the two electron transport systems of mammalian microsomes. The arrows indicate the direction of the electron transport.

by NADH would be catalyzed by NADH–cytochrome b_5 reductase, the competition between NADPH and NADH for the *p*-hydroxylation activities of anilines suggests that at a high concentration of NADH, the cytochrome P-450 oxidase system can be reduced by NADH directly, but not by a transhydrogenation from NADH to $NADP^+$ nor by electron flow from the NADH-dependent cytochrome b_5 system. This is summarized in Scheme I.

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