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Possible Role of Cytochrome P-448 in the O-Deethylation of *p*-Nitrophenetole by Rat Liver Microsomes

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In order to obtain definite evidence for the participation of cytochrome P-448 in the reduced nicotinamide adenine dinucleotide (NADH)-dependent O-deethylation of *p*-nitrophenetole and to elucidate the role of the NADH-coupled electron transport system in drug hydroxylation systems, *in vitro* studies were conducted using rat liver microsomes, and the following results were obtained. 1) The NADH-dependent O-deethylation activities in liver microsomes of rats pretreated with 3-methylcholanthrene (3-MC), benzo[*a*]pyrene (BP) and 3,4,5,3′,4′,5′-hexachlorobiphenyl (HCB) were markedly increased (about 20 times), but phenobarbital (PB) pretreatment had a much smaller effect (about 1.5-fold increase). 2) When cytochrome P-448 highly purified from HCB-pretreated rat liver microsomes was added to an incubation mixture for NADH-dependent O-deethylation of *p*-nitrophenetole, the activity was increased. 3) The synergistic effect of NADH on NADPH-dependent O-deethylation of *p*-nitrophenetole occurred at the initial stage with untreated microsomes, but not with 3-MC- and HCB-pretreated liver microsomes. On the basis of these results, the role of cytochrome P-448 in NADH-dependent oxidation by liver microsomes and the mechanism of the synergistic effect of NADH are discussed.

Keywords——cytochrome P-448; O-deethylation; *p*-nitrophenetole; liver microsomes; NADH-coupled electron transport system; 3,4,5,3′,4′,5′-hexachlorobiphenyl; synergistic effect of NADH

Liver microsomes contain at least two electron transport systems. Of these, the NADPH-dependent mixed function oxidase system consisting of NADPH-cytochrome P-450 reductase and cytochrome P-450 (448) catalyzes the oxidative metabolism of many foreign compounds.[2,3] The other NADH-linked electron transport system participates in the desaturation of fatty acids[4] and the reduction of hydroxylamines.[5] Concerning the pathway of electron flow from NADH to cytochrome P-450, Ichikawa and Loehr[6] reported that NADH-cytochrome b_5 reductase can transfer electrons to cytochrome P-450. Cytochrome P-450 also accepts electrons from cytochrome b_5 in the decomposition of hydroperoxides[7] and in the reduction of N-oxides.[8] Recently, West *et al.*[9] showed that NADH-dependent hydroxylation of benzo[*a*]pyrene required cytochrome P-448, NADH-cytochrome b_5 reductase and cytochrome b_5. Noshiro and Omura[10] reported that NADPH-cytochrome c reductase can receive electrons from NADH in several drug oxidations by liver microsomes. On the other hand,

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Estabrook *et al.*,[11,12] and Correia and Mannering[13] observed a cooperative interaction between NADH- and NADPH-linked electron transport systems and a function of cytochrome b_5 in supplying reducing equivalents to cytochrome P-450 for drug oxidation by liver microsomes.

In the preceding paper,[14,15] it was reported that cytochrome b_5 is involved in the NADH-dependent O-deethylation of *p*-nitrophenetole in rabbit liver microsomes, and it was suggested that cytochrome P-448 might function in this O-deethylation as the terminal oxidase, accepting electrons from cytochrome b_5. The present investigation was undertaken to investigate this possibility, using rat liver microsomes.

Experimental

Materials——NADH, NADPH, benzo[*a*]pyrene (BP) and 3-methylcholanthrene (3-MC) were purchased from Sigma Chemical Co. Ltd. Sodium phenobarbital (PB) was purchased from Fujinaga Pharmaceutical Co. Ltd. 3,4,5,3′,4′,5′-Hexachlorobiphenyl (HCB), mp 211°, was synthesized by the method of Yoshimura *et al.*[16] and confirmed to be pure by gas–liquid chromatography using an electron capture detector (column, 4 mm × 1.7 m; column packing, 1.5% OV-1 on Chromosorb W; carrier gas, N_2). *p*-Nitrophenetole, mp 58.5°, was purchased from Nakarai Chemicals, Ltd. and recrystallized from ethanol.

Preparation of Microsomes——The rats used were adult males of the Wistar strain. They were starved for 12 hours and killed by cervical dislocation. Other procedures were performed as described previously.[14]

Pretreatment of Animals——PB (80 mg/kg, in 1.0 ml of saline solution) and 3-MC (20 mg/kg, in 2.0 ml of corn oil) were injected intraperitoneally into rats once a day for three days. HCB was injected intraperitoneally at a single dose of 5 or 10 mg/kg (in 1.0 ml of corn oil). These animals were killed on the fourth day after the initial injection.

Purification of Cytochrome P-448 from HCB-pretreated Rat Liver Microsomes——Liver microsomes were prepared from the HCB-pretreated rats as described previously.[14] Cytochrome P-448 was purified by the method of Hashimoto and Imai.[17] This cytochrome P-448 had a peak of the CO difference spectrum at 448 nm, and showed a peak at around 396 nm and a shoulder near 410 nm in the absolute spectrum. The specific content of the cytochrome P-448 was 17.0 nmol per mg protein.

Other Experimental Procedure——O-Deethylation activity towards *p*-nitrophenetole was determined by the method described previously.[15] The effect of partial pressure of oxygen and the inhibitory effect of CO were examined by the methods described previously.[14] Contents of cytochrome P-450(448) and b_5 were determined according to the method of Omura and Sato[18] using extinction coeficients of 91 cm^{-1} mM^{-1} and 185 cm^{-1} mM^{-1}, respectively. Protein was determined by the method of Lowry *et al.*[19] with bovine serum albumin as a standard.

Results

Effects of Pretreatment of Rats with Phenobarbital (PB), 3-Methylcholanthrene (3-MC), 3,4,5,-3′,4′,5′-Hexachlorobiphenyl (HCB) and Benzo(*a*)pyrene (BP) on the O-Deethylation of *p*-Nitrophenetole with Liver Microsomes

Pretreatment of rats with PB, 3-MC, HCB and BP resulted in increase in the amount of cytochrome P-450 (448) and b_5 of about 2.07, 1.89, 3.36 and 1.65 times and about 1.10, 1.38, 1.40 and 1.69 times, in accord with data already reported.[16,20,21] The NADPH-dependent activities with these liver microsomes were activated about 1.87, 4.56, 9.22 and 6.08 times by pretreatment with PB, 3-MC, HCB and BP, respectively. It was interesting that

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NADH-dependent O-deethylation activities were increased about 20.93, 23.23 and 24.30 times in 3-MC-, HCB- and BP-induced microsomes, in contrast to 1.50 times in PB-induced microsomes.

Table I. O-Deethylase Activities and Contents of Cytochromes in Liver Microsomes after Pretreatment of Rats with Phenobarbital (PB), 3-Methylcholanthrene (3-MC), 3,4,5,3′,4′,5′-Hexachlorobiphenyl (HCB) and Benzo[*a*]pyrene (BP)

	Control	PB[a)]	3-MC[a)]	HCB[a)]	BP[a)]
	O-Deethylase activities (nmol/mg protein/minute)[b)]				
NADH	0.30±0.07(4)	0.45±0.16(4)	6.28±2.09(5)	6.97±0.73(3)	7.29±1.53(4)
NADPH	3.40±0.65(4)	6.93±1.64(4)	15.52±4.52(5)	31.36±3.97(3)	20.67±3.37(4)
	Contents of cytochromes (nmol/mg protein)[c)]				
Cytochrome P-450 (448)	0.85±0.13(7)	1.76±0.35(5)	1.61±0.23(4)	2.86±0.27(3)	1.65±0.35(4)
Cytochrome b_5	0.42±0.07(7)	0.46±0.06(5)	0.58±0.07(4)	0.59±0.06(3)	0.71±0.09(4)

a) PB (sodium phenobarbital 80 mg/kg), 3-MC (20 mg/kg) and BP (20 mg/kg) were injected intraperitoneally into rats once a day for three days. HCB was injected intraperitoneally at a single dose of 5 mg/kg.
b) The assay methods are described in "Experimental."
c) Determined by the methods of Omura and Sato.[18]
b,c) Values are expressed as means±S.D. and numbers of experiments are shown in parentheses.

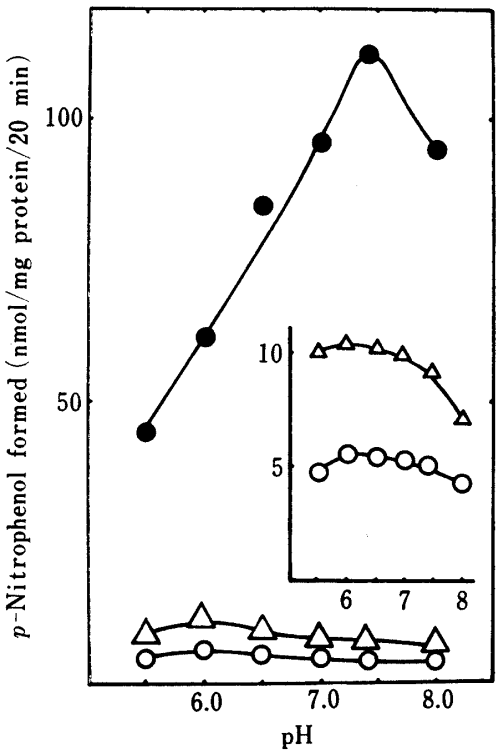

Fig. 1. Effect of pH on NADH-dependent O-Deethylation of *p*-Nitrophenetole

The concentration of *p*-nitrophenetole was 10.0 mM, and rat liver microsomes were suspended at a concentration of 2.0 (control), 1.12 (PB-pretreated) or 0.64 (3-MC-pretreated) mg protein per ml in 0.1 M phosphate buffer. Incubation was performed at 37° for 20 minutes. ○—○, microsomes from control rats; ●—●, microsomes from 3-MC-pretreated rats; △—△, microsomes from PB-pretreated rats.

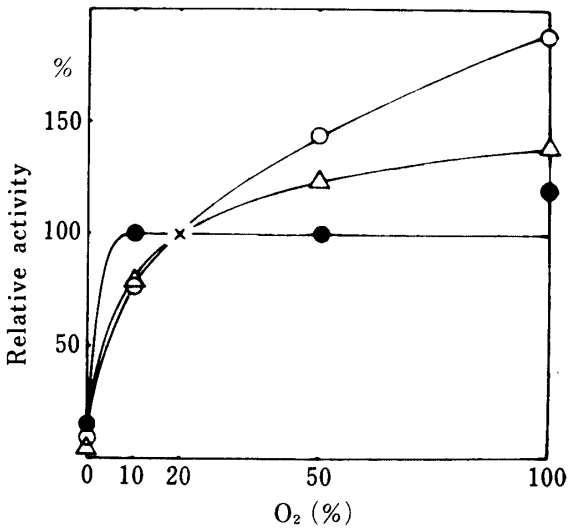

Fig. 2. Effect of Partial Pressure of Oxygen on the NADH-dependent O-Deethylation of *p*-Nitrophenetole

Rat liver microsomes were suspended at a concentration of 2.33 (control), 1.87 (PB-pretreated) or 1.00 (3-MC-pretreated) mg of protein per ml, and various mixtures of O_2 and N_2 were used as gas phases. Incubation was performed for 20 minutes. The relative activities are expressed as percentages of the activity observed for the gas mixture of O_2 (20%) and N_2 (80%). ○—○, microsomes from control rats; △—△, microsomes from PB-pretreated rats; ●—●, 3-MC-pretreated rats.

Effect of pH on the NADH-dependent O-Deethylation of *p*-Nitrophenetole

As shown in Fig. 1, the optimum pH was 6.0 in the O-deethylation reaction with control and PB-pretreated microsomes, which is the same as that with rabbit liver microsomes.[14] However, the optimum pH was 7.4 for the 3-MC-induced microsomal system. Maximum activities in NADPH-dependent reactions were seen at pH 7.4 in all three systems (not shown).

Effect of Partial Pressure of Oxygen

Incubation of *p*-nitrophenetole with rat liver microsomes under increasing concentrations of oxygen in a nitrogen atmosphere affected the rate of O-deethylation (Fig. 2). Under anaerobic conditions O-deethylation did not proceed in the three systems, indicating a requirement for oxygen. Under aerobic conditions, the O-deethylation activities in control and PB-induced microsomes rose markedly with increasing concentration of oxygen, but in 3-MC-induced microsomes the activity increased rapidly up to 10% oxygen and failed to increase further when the oxygen content was increased above 10%. These results show that 3-MC-induced microsomes had a higher affinity for oxygen than the other microsomes in NADH-dependent O-deethylation of *p*-nitrophenetole.

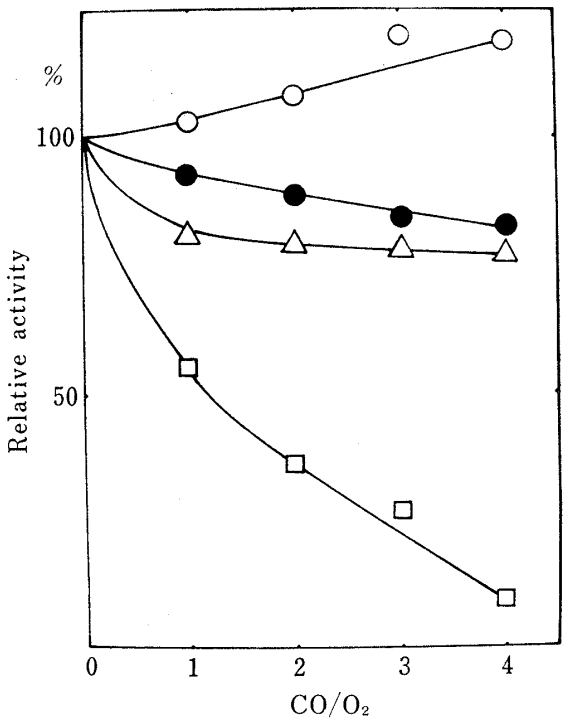

Fig. 3. Effect of CO on the NADH-dependent O-Deethylation of *p*-Nitrophenetole

Warburg vessels were used in this experiment. Rat liver microsomes were suspended at a concentration of 2.0 (control), 1.4 (PB-pretreated) or 0.84 (3-MC-pretreated) mg in the NADH system, and 0.65 mg protein (control) in the NADPH system per ml. Incubation was performed for 20 minutes (NADH system) or 10 minutes (NADPH system). See "Experimental" for other reaction conditions. The relative activities are expressed as percentages of the activity observed for the gas mixture of O_2 (20%) and N_2 (80%). ○—○, control; ●—●, 3-MC-pretreated; △—△, PB-pretreated; □—□, NADPH-dependent O-deethylation of *p*-nitrophenetol (control).

Effect of CO

In many monooxygenase reactions, including drug hydroxylations with liver microsomes, it has been established that cytochrome P-450 acts as the oxygen-activating enzyme. CO has already been shown to combine with this cytochrome with high affinity[18] and to inhibit the monooxygenase reactions.[22] Such inhibition was observed in the NADPH-dependent O-deethylation of *p*-nitrophenetole in rat liver microsomes, but the NADH-dependent reaction with untreated microsomes was not inhibited by CO, like that with rabbit liver microsomes[14] (Fig. 3). The reactions with PB- and 3-MC-induced rat liver microsomes were slightly inhibited by CO.

Effect of Addition of Purified Cytochrome P-448 on NADH-dependent O-Deethylation Activity towards *p*-Nitrophenetole

In order to investigate the participation of cytochrome P-448 in NADH-dependent O-deethylation, 2.1 and 4.2 nmol of this cytochrome were added to incubation mixtures containing 2.7 mg protein of untreated microsomes (2.2 nmol of cytochrome P-450). The O-deethylation activities were increased 1.87 and 2.22 times, respectively (Table II). However, the increases of activity were not as high as that of microsomes from HCB-pretreated rats (Table I).

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Table II. Effect of Addition of Purified Cytochrome P-448 on NADH-dependent O-Deethylation Activity in Liver Microsomes

Addition	O-Deethylation activity (nmol/20 min)	% of control
Control system[a]	12.76	100
+ Cytochrome P-448[b] (2.1 nmol)	22.70	187
+ Cytochrome P-448[b] (4.2 nmol)	27.00	222

a) The incubation mixture contained 2.7 mg protein of liver microsomes from untreated rats and all necessary components.
b) Untreated microsomes and purified cytochrome P-448 were incubated for 30 minutes at 37° and then O-deethylation activity was determined.

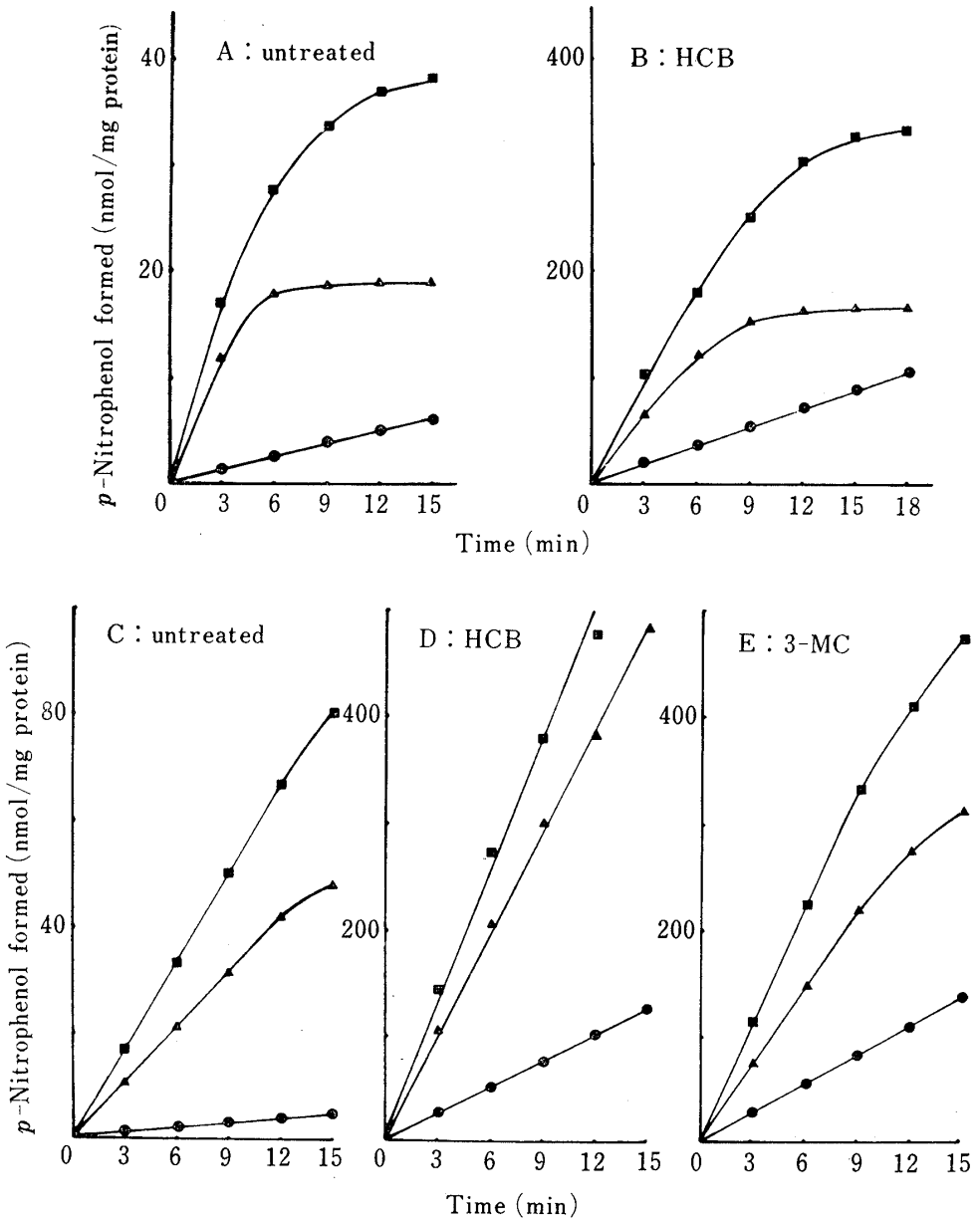

Fig. 4. Time Course of NADH-, NADPH- and NADH plus NADPH-dependent O-Deethylation of *p*-Nitrophenetole

A and C, with untreated microsomes; B and D, with HCB-pretreated microsomes; E, with 3-MC-pretreated microsomes. The rat liver microsomes were suspended at a concentration of 2.53(A), 0.95(B), 0.62(C), 0.23(D), or 0.45(E) mg protein per ml in 0.1 M phosphate buffer (pH 7.4). Other experimental conditions are described in "Experimental." ●—●, NADH; ▲—▲, NADPH; ■—■, NADH plus NADPH systems.

Effects of Cofactors on the O-Deethylation of *p*-Nitrophenetole by Various Microsomes

The O-deethylation activities of untreated, and HCB- and 3-MC-pretreated microsomes were measured using three kinds of electron donors, NADH, NADPH, and NADH plus NADPH (Fig. 4). As shown in Fig. 4A, control microsomes at a concentration of 2.53 mg protein per ml formed 5.91, 18.78 and 38.14 nmol of *p*-nitrophenol per mg protein per 15 minutes in NADH, NADPH, NADH plus NADPH systems, respectively. With HCB-pretreated microsomes (Fig. 4B), 92.03 (NADH system), 157.64 (NADPH system) and 325.76 (NADH plus NADPH system) nmol of *p*-nitrophenol per mg protein per 18 minutes were released. A synergistic effect of NADH on the NADPH-dependent O-deethylation of *p*-nitrophenetole occurred in both the untreated and HCB-pretreated microsomes. Fig. 4C, D and E shows the initial rate of these O-deethylation reactions. The activities in the systems with NADH, NADPH, NADH plus NADPH were measured using smaller amounts of microsomes than those in Fig. 4A and B. The specific activities thus obtained were 0.33 (NADH system), 3.40 (NADPH system), and 5.22 (NADH plus NADPH system) nmol per mg microsomal protein per minute with untreated microsomes. Activities with HCB- and 3-MC-pretreated microsomes were found to be 8.57 and 9.15 (NADH system), 33.43 and 24.37 (NADPH system), and 42.97 and 37.41 (NADH plus NADPH system) nmol of *p*-nitrophenol per mg microsomal protein per minute, respectively. NADH exerted a synergistic effect on the initial rate of the O-deethylation with untreated microsomes, but the effect seemed rather additive with HCB- and 3-MC-pretreated microsomes.

Discussion

We have previously shown that either NADH or NADPH is an effective cofactor for the O-deethylation of *p*-nitrophenetole by rabbit liver microsomes[14] and that cytochrome b_5 is involved in the NADH-dependent O-deethylation.[15] This NADH-dependent reaction was not inhibited by CO and in this respect was different from the NADPH-dependent system involving cytochrome P-450, which catalyzes many other monooxygenase reactions. As shown in Fig. 3, NADH-dependent O-deethylation with liver microsomes of untreated rats was not inhibited at all by CO, and was only slightly inhibited with 3-MC- and PB-pretreated microsomes. Recently, Cooper *et al.*[23] reported that the CO sensitivity of microsomal benzo[*a*]pyrene hydroxylase was reduced in liver microsomes of 3-MC- or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated rats. It can be concluded that changes in the composition and/or the structural organization of the microenvironment of cytochrome P-448 in the endoplasmic reticulum take place in response to the inducing action of polycyclic aromatic hydrocarbons and related agents, although the nature of these changes is not yet clear. The results of Cooper *et al.* suggest that CO inhibition is not a necessary event for the participation of cytochrome P-448 in NADH-dependent O-deethylation by liver microsomes. This NADH-dependent reaction was greatly increased by pretreatment of rats with 3-MC, which is a potent inducer of cytochrome P-448 in liver microsomes.[20] Furthermore, NADH-dependent O-deethylation with HCB- and BP-pretreated microsomes was also increased. HCB[16] and BP[20,21] are known to induce cytochrome P-448, which is involved in this NADH-dependent O-deethylation. Addition of cytochrome P-448 purified from HCB-pretreated rats to untreated microsomes activated the NADH-dependent reaction, although the activity was not as high as that of HCB-pretreated microsomes. In this study, although 2.1 and 4.2 nmol of cytochrome P-448 were added, the amount of this cytochrome bound to microsomes after the preincubation was not determined. Therefore, it is very likely that marked difference between the activities is due to a difference in the amount of cytochrome P-448 actually utilizable

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in microsomes. The series of results in this paper thus supports the view that the terminal oxidase for the NADH-dependent O-deethylation of *p*-nitrophenetole by liver microsomes is cytochrome P-448. West *et al.*[9] recently showed that cytochrome P-448 was required for the reconstitution of an NADH-supported benzo[*a*]pyrene hydroxylation system. Kamataki *et al.*[24] reported that cytochrome P-450 and P-448 were required for NADH-dependent O-demethylation of *p*-nitroanisole by rat liver microsomes.

As shown in Figs. 4A and C, a synergistic effect of NADH on the NADPH-dependent O-deethylation of *p*-nitrophenetole by untreated microsomes was observed. Such a synergistic action of NADH on the hydroxylation of drugs and endogeneous substances by liver microsomes has already been reported by other groups of workers.[11-13,25,26] Two hypotheses for the cooperative interaction of NADH and NADPH have been proposed. Estabrook *et al.*[11,12] suggested that two electrons are required in these oxidations; the first electron is transferred to cytochrome P-450 only *via* NADPH-cytochrome c reductase, whereas the second electron is supplied by either NADPH-cytochrome c reductase or cytochrome b_5 (Fig. 5). Staudt *et al.*[26] suggested that the electrons were transferred from NADPH to cytochrome P-450 and to cytochrome b_5 through NADPH-cytochrome c reductase, and that the former was used to activate an oxygen molecule which subsequently hydroxylated the substrate, whereas the latter was consumed to produce water by reducing active oxygen which was not used for hydroxylation. In this case, NADH reduces cytochrome b_5 at a faster rate and provides electrons with a higher efficiency for the reduction of the uncoupled active oxygen than NADPH, so more NADPH electrons are available for oxygen-activating reactions. They concluded that this effect could be the major mechanism of the NADH synergism. In our study of the O-deethylation of *p*-nitrophenetole, no synergistic action of NADH was observed when the NADH-dependent reaction was strongly activated by pretreatment with HCB and 3-MC (Fig. 4D and E). However, as the activity with HCB-induced microsomes was synergistically activated when the reactions were almost completed (Fig. 4B), NADH may provide electrons to replace those from NADPH which are consumed for O-deethylation or other reaction(s), although the mechanism remains to be determined.

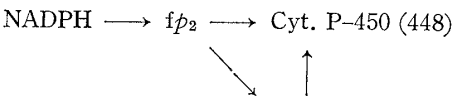

Fig. 5. Proposed Electron Pathways in the Microsomal Transfer System

This NADH-dependent monooxygenation reaction may be more important in detoxicating reactions when animals are exposed by environmental polutants such as polychlorinated biphenyls or BP. The present system should be useful for studying the cooperative interaction of NADH and NADPH in various drug oxidation reactions by liver microsomes.

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