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Reduced Nicotinamide Adenine Dinucleotide-dependent O-Deethylation of *p*-Nitrophenetole with Rabbit Liver Microsomes. II.[1] Involvement of Cytochrome b_5

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Following the previous studies, the present investigation was undertaken to obtain definite evidence suporting the participation of cytochrome b_5 in reduced nicotinamide adenine dinucleotide (NADH)-dependent deethylation of *p*-nitrophenetole with rabbit liver microsomes *in vitro*. The results obtained are as follows: 1) Treatment of liver microsomes with trypsin resulted in decrease of cytochrome b_5 concentration in parallel with decrease of the activity of NADH-dependent deethylation of *p*-nitrophenetole with rabbit liver microsomes. 2) The oxidation of reduced cytochrome b_5 was stimulated by the presence of *p*-nitrophenetole. 3) The NADH-dependent deethylation of *p*-nitrophenetole with rabbit liver microsomes was strongly inhibited by a rabbit antiserum which was prepared against rat liver microsomal cytochrome b_5.

From these observations, it was concluded that cytochrome b_5 was an obligatory component in the NADH-dependent deethylation of *p*-nitrophenetole.

Keywords——cytochrome b_5; microsomes; O-deethylation; electron-transport; *p*-nitrophenetole

In the preceding paper,[1] it was reported that the deethylation of *p*-nitrophenetole with rabbit liver microsomes proceeded quite well using reduced nicotinamide adenine dinucleotide (NADH) as a cofactor as well as reduced nicotinamide adenine dinucleotide phosphate (NADPH), and that different mechanisms were involved in these NADH- and NADPH-dependent deethylations. The latter reaction (NADPH-dependent) was considered to be catalyzed by the enzyme system involving cytochrome P-450 as a terminal oxidase,[3] same as many other drug hydroxylations. In the former reaction (NADH-dependent), however, neither this cytochrome nor cyanide-sensitive factor which is known to catalyze oxidative desaturation of fatty acid in the presence of NADH[4,5] seemed to participate as a terminal oxidase, and a new oxygen activating enzyme was suggested to be involved.

Contrary to the abundant reports describing NADPH-linked monooxygenation systems, only a few reports has been published concerning with NADH-linked monooxygenation system. For instance, Ichikawa and Loehr[6] found that cytochrome P-450 could be reduced with NADH in subparticles containing no cytochrome b_5 obtained from trypsin-treated rabbit liver microsomes. More recently, West, *et al.*[7] reported the reconstitution of an enzyme system from rat liver microsomes which catalyzed NADH-dependent hydroxylation of benzo(*a*)pyrene with a reaction rate similar to that in microsomes from 3-methylcholan-

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threne-treated rats. This NADH-dependent cytochrome P-448 mediated hydroxylation of benzo(*a*)pyrene required both cytochrome b_5 and cytochrome b_5 reductase.

The present study was undertaken to obtain more definite evidence supporting the presence of new oxygenation system which includes cytochrome b_5 as an obligatory component in NADH-dependent deethylation of *p*-nitrophenetole using liver microsomes of rabbits.

Experimental

Materials——NADH, NADPH, sodium *p*-chloromercuribenzenesulfonate (PCMS), trypsin, trypsin inhibitor and cytochrome *c* were purchased from Sigma Chemical Co. Ltd. *p*-Nitrophenetole, mp 58.5°, was synthesized as same as reported previously.[1]

Preparation of Microsomes——Liver microsomes of adult male albino rabbits weighing 2.0—3.0 kg were prepared by the method previously described[1] and stored at 0—4° with a concentration of 15 mg protein per ml. All procedures were completed within 48 hrs after sacrifice of the animal. Protein concentration was determined by the method of Lowry, *et al.*[8] using bovine serum albumin as the standard. Contents of cytochrome b_5 and cytochrome P-450 were determined by the method of Omura and Sato.[9]

Assay for Enzyme Activity——Deethylation activity of *p*-nitrophenetole was determined according to the method previously described[1] or on a quarter scale. NADPH-cytochrome *c* reductase activity was determined by the method of Philips and Langdon.[10]

Time Course Study of Oxidation-Reduction of Cytochrome b_5——A Hitachi digital dual wavelength spectrophotometer, Model 156, was used for this experiment. The reduction level of cytochrome b_5 was determined by measuring the absorbance change between 424 and 410 nm. This experiment was conducted by use of 0.1 M phosphate buffer (pH 6.0), liver microsomes containing 2.1 mg protein and with or without *p*-nitrophenetole. Detailed experimental conditions are given in figure legends.

Digestion of Microsomes with Trypsin——Liver microsomal suspension (4.0 ml) which contained 10 mg protein per ml were incubated with various concentrations of trypsin in 0.1 M phosphate buffer, pH 7.4 (final volume, 4.5 ml) at 37° for 10 min. The reaction was stopped by addition of 3-fold excess amount of trypsin inhibitor. To these microsomal suspensions were added about 5.0 ml of 0.1 M phosphate buffer (pH 7.4), and 10 ml of the resulting suspension was centrifuged at 105000 ***g*** for 60 min in a Hitachi preparative ultracentrifuge, Model 40. The packed pellets were suspended in 0.1 M phosphate buffer (final volume 4.0 ml). These subparticles of microsomes were stored at 0° and enzyme assays were carried out within a few hr.

Experiment of Antiserum——A rabbit antiserum against rat liver microsomal cytochrome b_5 was a generous gift from Dr. T. Omura. The immunoglobulin fraction was prepared by the method of Oshino and Omura.[11] Control immunoglobulin was similarly prepared from serum of the untreated rabbit. The activity of NADH-cytochrome *c* reductase was determined using cytochrome *c* as the electron acceptor.[12]

Results

Effect of Trypsin Digestion on Liver Microsomal Components and Deethylation of *p*-Nitrophenetole

Although NADPH cytochrome *c* reductase, cytochrome P-450, and cytochrome b_5 are all associated with the liver microsomal vesicle membranes, the association seems not to be equally firm for the individual enzymes. Applying graded digestion with trypsin, Omura, *et al.*[13] and Orrenius, *et al.*[14] have been able to achieve a stepwise solubilization of NADPH cytochrome *c* reductase and cytochrome b_5. Fig. 1 shows the effect of mild digestion with lower concentration of trypsin on microsomal enzymes (less than 10 μg trypsin per mg microsomal protein).

A release of NADPH-cytochrome *c* reductase resulted in parallel decrease of NADPH-dependent deethylation, but did not give any significant influence on NADH-dependent

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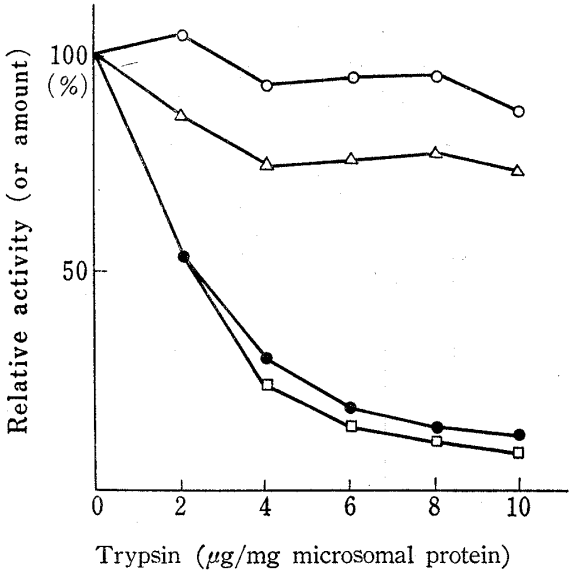

Fig. 1. Effect of Digestion of Liver Microsomes with Lower Concentration of Trypsin on Microsomal Enzyme Activities

Each point is expressed as relative enzyme activity to the corresponding activity in untreated microsomes or relative protein concentration. Control activities were 1.02, 1.75 and 160 nmoles per min per mg of microsomal protein for NADH-, NADPH-dependent deethylase and NADPH-cytochrome *c* reductase, respectively. See Experimental for other experimental conditions. —○—, activity of NADH-dependent deethylation of *p*-nitrophenetole; —●—, activity of NADPH-dependent deethylation of *p*-nitrophenetole; —□—, activity of NADPH-cytochrome *c* reductase; —△—, protein concentration.

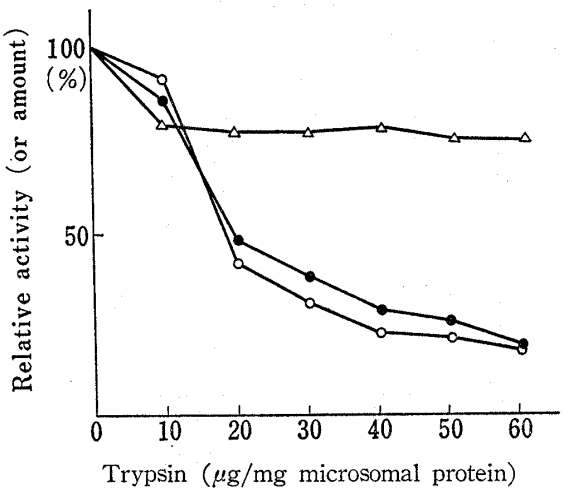

Fig. 2. Effect of Digestion of Liver Microsomes with Higher Concentration of Trypsin on the Activity of NADH-dependent Deethylation of *p*-Nitrophenetole and the Content of Cytochrome b_5

Each point is expressed as relative enzyme activity to the coresponding activity in untreated microsomes or relative cytochrome b_5 content. Control activity was 0.99 nmoles per min per mg of microsomal protein. Control concentration was 0.50 nmoles per mg microsomal protein for cytochrome b_5. See Experimental for other experimental conditions. —○—, activity of NADH-dependent deethylation of *p*-nitrophenetole; —●—, cytochrome b_5 content; —△—, protein concentration.

deethylation activity. In addition, microsomal protein concentration did not decrease more than 40% and cytochrome b_5 was scarecely solubilized from liver microsomes by this treatment.

On the other hand, as shown in Fig. 2, the digestion with higher concentration of trypsin (20—60 μg trypsin per mg microsomal protein) caused a release of cytochrome b_5 from liver microsomes, which was related closely with inactivation of NADH-dependent deethylation of *p*-nitrophenetole.

Effect of *p*-Nitrophenetole on the Oxidation of Reduced Cytochrome b_5

It was reported that cytochrome b_5 retained a steady-state reduction level as a result of NADH addition to liver microsomal suspensions, since reduction rate of cytochrome b_5 with NADH was much faster than that of autooxidation.[15] On the other hand, NADH-cytochrome *c* reductase is known to be strongly inhibited by PCMS.[16] In agreement with these findings, when PCMS was added to microsomal suspension, this steady-state reduction level of cytochrome b_5 was lowered and the reduction of cytochrome b_5 with NADH was inhibited completely by the addition of 0.1 mM PCMS. In this experiment, cytochrome b_5 was firstly reduced by an addition of NADH. After 30 seconds, 0.1 mM PCMS was added to stop the electron transport to cytochrome b_5 from NADH. And just then, cytochrome b_5 started to be slowly autooxidized (Fig. 3). In this mixture, the presence of *p*-nitrophenetole oxidized cytochrome b_5 more rapidly than in the case of addition of PCMS alone. This sug-

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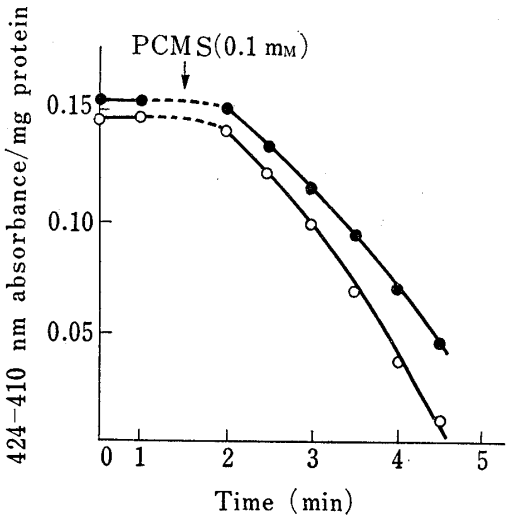

Fig. 3. Effect of *p*-Nitrophenetole on the Oxidation of Reduced Cytochrome b_5

The reaction mixture contained 2.1 mg of liver microsomal protein and 1.0 ml of saturated *p*-nitrophenetole solution and 0.1 M phosphate buffer (pH 6.0) to make final volume 2.8 ml. After preincubation at 37° for 5 min, 0.1 ml of NADH (141 nmoles) was added in the reaction mixture. To this, 0.1 ml of PCMS (final 0.1 mM) was added at the time indicated. Redox behavior of cytochrome b_5 was followed by the difference of absorbance between 424 nm and 410 nm with the Hitachi digital dual-wavelength spectrophotometer, Model 156. —●—, the difference of absorbance between 424 nm and 410 nm in the absence of *p*-nitrophenetole; —○—, the difference of absorbance between 424 nm and 410 nm in the presence of *p*-nitrophenetole.

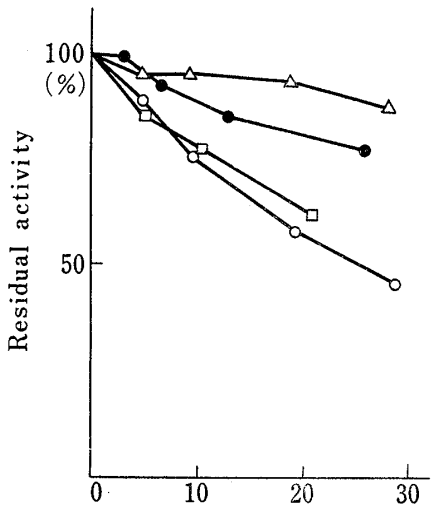

Fig. 4. Effect of Anti-b_5 IG on the NADH- and NADPH-dependent Deethylase Activities of *p*-Nitrophenetole and on the NADH-Cytochrome *c* Reductase Activity in Rabbit Liver Microsomes

Rabbit liver microsomes were preincubated with anti-b_5 IG or control IG in 0.1 M phosphate buffer (pH 6.0 or 7.4) for 5 min at 37° and then the activities of NADH- and NADPH-dependent deethylation of *p*-nitrophenetole were measured after addition of NADH or NADPH. NADH-cytochrome *c* reductase activity was measured after preincubation of rabbit liver microsomes with anti-b_5 IG in 0.1 M phosphate buffer (pH 7.4). The final concentration of microsomes in the reaction mixture were 1.09 mg protein per ml (NADH-dependent deethylase) and 0.55 mg protein (NADPH-dependent) in all determinations. The NADH- and NADPH-dependent deethylase activities or NADH-cytochrome *c* reductase activity in the presence of anti-b_5 IG or control IG are expressed as percentage to the corresponding activities measured without the addition of the immunoglobulines. Control activities were 0.54, 1.90 and 1940 nmoles per min per mg of microsomal protein for NADH-, NADPH-dependent deethylase and NADH-cychrome *c* reductase, respectively. —□—, NADH-cytochrome *c* reductase activity in the presence of anti-b_5 IG; —○—, NADH-dependent deethylase activity of *p*-nitrophenetole in the presence of anti-b_5 IG; —△—, NADPH-dependent deethylase activity of *p*-nitrophenetole in the presence of anti-b_5 IG; —●—, NADH-dependent deethylase activity of *p*-nitrophenetole in the presence of control IG.

gested that oxidation of NADH-reduced cytochrome b_5 was accompanied by the deethylation of *p*-nitrophenetole.

Inhibition of NADH-dependent Deethylase Activity by Antibody to Cytochrome b_5

The effects of antibody prepared against rat liver microsomal cytochrome b_5 on NADH-dependent deethylase activity in rabbit liver microsomes are illustrated in Fig. 4. The inhibition of NADH-dependent deethylase activity by anti-cytochrome b_5 antibody was dependent on the ratio of the added antibody to microsomes and about 55% of the NADH-dependent deethylase activity was inhibited when 28 mg of anti-b_5 IG was added per mg of microsomal protein. NADH-cytochrome *c* reductase was inhibited almost similarly to NADH-dependent deethylase, but NADPH-dependent deethylase was not inhibited significantly.

Discussion

We have previously shown that either NADH or NADPH is an effective cofactor for deethylation of *p*-nitrophenetole with rabbit liver microsomes, and that among these two

reactions, NADH-dependent deethylation was not inhibited by carbon monoxide. This observation suggested that NADH-dependent deethylation system was different from NADPH-dependent system involving cytochrome P-450 which catalyzes many other monooxygenase reactions.[1] Recently several reports have been published one after another concerning with the reactions which are associated with NADH-linked electron transport systems in liver microsomes. These are desatutation reaction of fatty acids,[4,5] NADH-dependent hydroxylation of benzo(*a*)pyrene,[7] reduction of hydroxylamine,[17] reduction of N-oxides[18] and NADH-dependent decomposition of hydroperoxides.[19] In all these reactions, cytochrome b_5 is an obligatory component. The present investigation also provided the definite evidence that cytochrome b_5 was involved in NADH-dependent deethylation of *p*-nitrophenetole in liver microsomes.

Based on the results of the present and preceding studies,[1] together with the well known finding that electron could be transfered from NADPH-cytochrome *c* reductase to cytochrome b_5,[11,16] the microsomal electron-transport systems associated with deethylation of *p*-nitrophenetole could be described as shown in Chart 1.

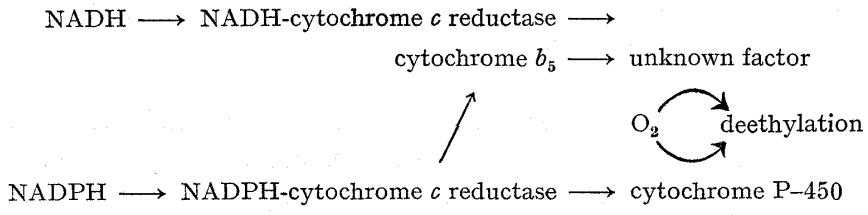

Chart 1. Proposed Scheme for NADH- and NADPH-linked Electron-transport Systems in Liver Microsomes Associated with Deethylation of *p*-Nitrophenetole

Among these reactions, NADH-dependent deethylation of *p*-nitrophenetole will be profound interest from the view point of drug metabolism. Concerning with this reaction, West, *et al.* reported that an electron-transport system to cytochrome P-448 from cytochrome b_5 was involved in NADH-dependent hydroxylation of benzo(*a*)pyrene by 3-methylcholanthrene-induced rat liver microsomes.[7] This would suggest also the possibility that unknown factor might be cytochrome P-448, if this cytochrome P-448 mediated reaction is not inhibited with carbon monoxide. However, further studies are necessary to establish unequivocally the presence of this new mechanism.

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