however, show the considerable differences between the individual animals, based on the difficulty in obtainning the reproducible results by treatment with the cellophane tape.

As shown in Table II, the total amont of absorption from the damaged skin was approximately 80 times that from the intact skin. The recoveries of radioactivity were 36.26% in the urine and 1.83% in the bile 30 hr after application. The distribution of radioactivity in the main organs was much larger than that of the intact skin.

Since a large amount of ^{14}C–AOS was absorbed from the damaged skin, the distribution of radioactivity in the liver was in excess compared with other organs. The significance of the high accumulation of radioactivity in the lung was obscure, because the values varied remarkably in each animal. The total recovery of radioactivity in the urine, bile and main organs of damaged skin rat was about 50% dose. Such a high absorption from the damaged skin shows that the *stratum corneum* functions as a kind of barrier controlling the percutaneous absorption.

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NADH-dependent O-Demethylation of *p*-Nitroanisole with Rabbit Liver Microsomes

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Following the previous study on deethylation of *p*-nitrophenetole with rabbit liver microsomes, demethylation of *p*-nitroanisole was analogously investigated, using reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADH plus NADPH as the cofactor. It was found that this reaction proceeded well with NADH as well as NADPH, and this NADH-dependent demethylation was different from those of other two systems. For example, optimum pH was 6.0 in the NADH system, but was 7.4 in the NADPH or NADH plus NADPH system, and the reaction was inhibited by CO in the NADPH or NADH plus NADPH system, but not in the NADH system. Furthermore, KCN did not inhibit the demethylation in any one of three systems at 10^{-4}M. These results were just the same as obtained in the deethylation of *p*-nitrophenetole and suggested also a possible involvement of a new type of NADH-dependent oxygenase which was different from cytochrome P-450 and cyanide-sensitive factor, in the demethylation of *p*-nitroanisole with rabbit liver microsomes. The NADPH- and NADH plus NADPH-dependent demethylations, on the other hand, were assumed to be catalyzed by the enzyme system involving cytochrome P-450.

Keywords——*p*-nitroanisole; microsomal O-demethylation; rabbit liver; NADH-dependent oxygenase; NADPH-dependent oxygenase; cytochrome P-450

Most of oxidative metabolisms of foreign compounds are known to be catalyzed by the mixed-function oxidase system in the liver microsomes. In these reactions, the cytochrome P–450 generally participates as a terminal oxidase and reduced nicotinamide adenine di-

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nucleotide phosphate (NADPH) is usually the most effective cofactor although its role is not specific and can be partially replaced by reduced nicotinamide adenine dinucleotide (NADH). The O-dealkylation of alkylaryl ethers is one of the extensively documented reactions catalyzed by liver microsomes in the presence of NADPH and molecular oxygen. This reaction proceeds through a hydroxylated intermediate with unstable hemiacetal structure which is immediately decomposed to the corresponding phenol and aldehyde.[2,3]

On the other hand, NADH-dependent electron transport system also exists in liver microsomes. This system implicates cyanide-sensitive factor as a terminal oxidase which catalyzes oxidative desaturation of fatty acid.[4,5] In the previous work on O-demethylation of brucine and 4-substituted veratroles with rabbit liver microsomes, we also noticed considerable effectiveness of NADH as a cofactor in the place of NADPH.[6] The subsequent study further suggeted, together with participation of the usual NADPH-dependent system, the posible involvement of a new type of NADH-dependent oxygenase in the deethylation of *p*-nitrophenetole with rabbit liver microsomes.[7] In this NADH-dependent reaction, neither cyanide-sensitive factor nor cytochrome P-450 were involved.

It was also found that a synergistic effect of NADH on the deethylation of *p*-nitrophenetole using NADPH as a cofactor was occured. This phenomenon was similar to that reported on N-demethylation of aminopyrine and ethylmorphine by Hildebrandt, *et al.*[8] In that report, they insisted to interact NADH- and NADPH-linked electron trasport systems with each others.

The present investigation has been undertaken in order to learn whether such NADH-dependent system as described above is also concerned in demethylation of *p*-nitroanisole with rabbit liver microsomes or not. For this purpose, the study is carried out analogously with that of the previously reported *p*-nitrophenetole deethylation, in which the effect of NADH on this *in vitro* demethylation is compared with those of NADPH and of NADH plus NADPH under various conditions.

Experimental

Materials——NADH, NADPH and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Ltd., Chicago. *p*-Nitroanisole, mp 53.0—53.5°, was purchased from Yoneyama Chemical Ind. Ltd., Tokyo.

Preparation of Microsomes——The liver microsomes were prepared as described previously, using male albino rabbit (2.0—3.0 kg).[7] The washed microsomes thus obtained were finely suspended in 0.1M phosphate buffer (pH 7.4), usually at a concentration of about 15 mg protein per ml. The resultant microsomal suspensions could be stored at −20° without any appreciable loss of the demethylation activity for a week.

Assay for Demethylation of *p*-Nitroanisole——Demethylation activity of *p*-nitroanisole was determined similarly as deethylation activity of *p*-nitrophenetole.[7] The incubation mixture contained, in a final volume of 4.0 ml, a suitable amount of microsomes (0.43—1.3 mg protein per ml), cofactor (0.25 mM NADH, 0.25 mM NADPH or 0.25 mM NADH plus 0.25 mM NADPH), 0.1M phosphate buffer (pH 6.0 for NADH system or 7.4 for NADPH and NADH plus NADPH systems) and 2.0 mM *p*-nitroanisole. *p*-Nitroanisole was dissolved in 0.1 ml acetone and added to the incubation mixture. Before the reaction was initiated by the addition of cofactor, preincubation had been performed for 5 min at 37° without any cofactor, in order to eliminate a short lag time. Incubation was carried out in open test-tube for 10 min (for NADPH or NADH plus NADPH system) or 30 min (for NADH system) with continuous shaking. The reaction was terminated by addition of 1.0 ml of a 20% trichloroacetic acid solution. After 20 min, the reaction mixture was centrifuged for 20 min at 2000 rpm. To 4.0 ml of the supernatant fraction was added 1.0 ml of 4N KOH solution and 2.0 ml

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7) H. Shigematsu, S. Yamano, and H. Yoshimura, *Arch. Biochem. Biophys.*, **173**, 178 (1976).
8) A. Hildebrandt and R.W. Estabrook, *Arch. Biochem. Biophys.*, **143**, 66 (1971).

of chloroform. This mixture was shaken for 20 min and centrifuged for 30 min at 2000 rpm. The reaction product, *p*-nitrophenol, in the clear aqueous layer was determined spectrophotometrically at 400 nm. The activity was expressed in terms of nmoles of *p*-nitrophenol formed per mg of microsomal protein. The formation of *p*-nitrophenol from *p*-nitroanisole was confirmed by thin-layer chromatography using a silica gel plates (Silica gel G, Merck, 0.25 mm thick, activated at 105° for 30 min) and a solvent system of hexane–benzene–acetone (5: 4: 1). In all systems (NADH, NADPH and NADH plus NADPH systems), *p*-nitrophenol was detected at *Rf* of 0.22 as a yellow spot when the plate was sprayed with 5% KOH solution.

Other Experimental Procedures——Assay method for codeine demethylation was the same as described in the previous paper.[7] Inhibition experiment with CO in demethylation of *p*-nitroanisole and codeine were also conducted similarly as described in the previous paper.[7] Protein concentration was measured by the method of Lowry, *et al.*[9]

Results

First of all, time course study of *p*-nitroanisole demethylation with rabbit liver microsomes was conducted by using NADH, NADPH or NADH plus NADPH as the cofactor. The results are illustrated in Fig. 1. As can be seen from this figure, the rate of the NADH-dependent demethylation incresed linearly for 40 min, whereas in the NADPH and NADH plus NADPH systems the linear increase of the reaction rate ceased after 10 min and 20 min, respectively. Based on these results, the incubation time was set to 30 min for the NADH-dependent system or 10 min for the NADPH- and NADH plus NADPH-dependent systems. In these incubation times, demethylation rates were found to increase proportionally to microsomal concentration up to 1.2 mg, 0.65 mg or 1.3 mg protein per ml, respectively, in the NADH, NADPH or NADH plus NADPH system.

Next, the effect of substrate concentration on demethylation of *p*-nitroanisole was investigated. As shown in Fig. 2, the enzyme activity of two systems using NADH and

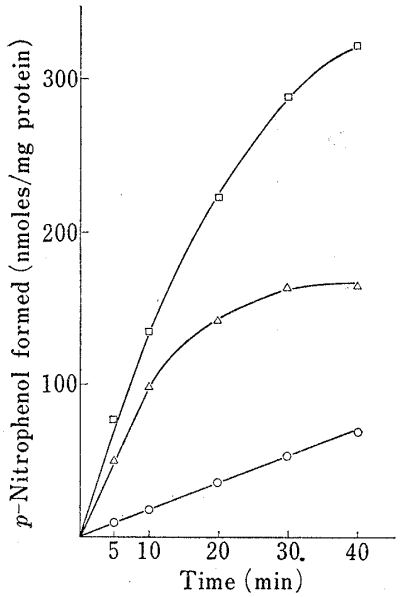

Fig. 1. Time Course of *p*-Nitroanisole Demethylation with Liver Microsomes

The rabbit liver microsomes were suspended at a concentration of 0.43 mg protein per ml. Incubation was permed at pH 6.0 (NADH system) or at pH 7.4 (NADPH and NADH plus NADPH systems). Other experimental conditions were described in Experimental.
—○—: NADH-, —△—: NADPH-, —□—: NADH plus NADPH-dependent reactions

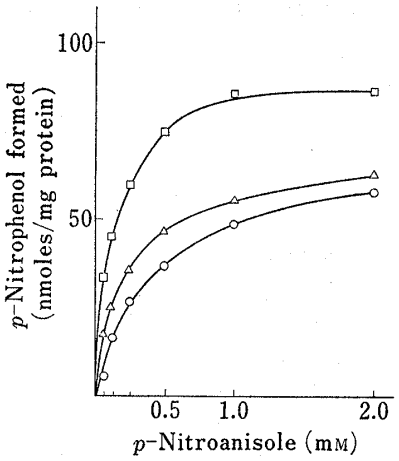

Fig. 2. Effect of Substrate Concentration on *p*-Nitroanisole Demethylation with Liver Microsomes

The incubation mixtures contained 1.0 mg microsomal protein per ml and incubation was performed for 30 min (NADH system) or 10 min (NADH and NADH plus NADHP systems). Other experimental conditions were described in Expreimental.
—○—: NADH-, —△—: NADPH-, —□—: NADH plus NADPH-dependent reactions

9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

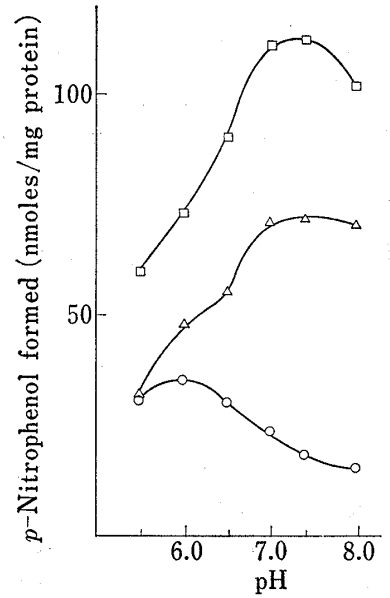

Fig. 3. Effect of pH on *p*-Nitroanisole Demethylation with Liver Microsomes

Microsomes were suspended at a concentration of 0.97 mg protein per ml (NADH system) or 0.49 mg protein per ml (NADPH and NADH plus NADPH systems) and pH was varied from 5.5 to 8.0. Other experimental conditions were described in Experimental.
—○—: NADH-, —△—: NADPH-, —□—: NADH plus NADPH-dependent reactions

NADPH reached a maximum at the concentration of about 2.0 mM *p*-nitroanisole, whereas that of the NADH plus NADPH system did so at lower substrate concentration (1 mM).

Fig. 3 shows that the optimum pH in demethylation of *p*-nitroanisole with liver microsomes was 6.0 in the NADH system and 7.4 in the two other systems. Although every optimum pH is rather broad, a significant difference can be seen between the values of the NADH system and of the NADPH or NADH plus NADPH systems.

It has been well documented that CO inhibits the NADPH-dependent oxidative demethylation of codeine with liver microsomes, as well as many other cytochrome P-450-dependent monooxygenations.[10,11] As indicated in Fig. 4, of the three systems, the NADPH- and NADH plus NADPH-dependent demethylations of *p*-nitroanisole with liver microsomes were also inhibited by CO in the atmospheres of 20% oxygen and 20, 40, 60 or 80% CO in nitrogen. With increasing ratios of CO to O_2, the inhibition became greater. This inhibitory effect of CO on *p*-nitroanisole demethylation was just the same as observed on codeine demethylation which was used as the positive

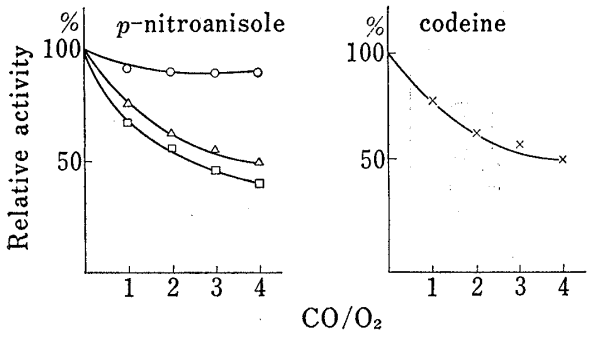

Fig. 4. Effect of CO on *p*-Nitroanisole Demethylation and Codeine Demethylation with Liver Microsomes

Warburg vessels were used in these reactions. Mixtures of O_2, CO, and N_2 in various ratios were used as gas phases. Microsomes were suspended at a concentration of 1.10 mg protein per ml (NADH and NADH plus NADPH systems) or 0.60 mg protein per ml(NADPH system). Other experimental conditions were described in Experimental. The relative activities are expressed as percentages of the activity observed for the gas mixture of O_2 (20%) and N_2 (80%).
—○—: NADH-, —△—: NADPH-, —□—: NADH plus NADPH-dependent reactions, —×—: codeine demethylation in NADPH-generating system

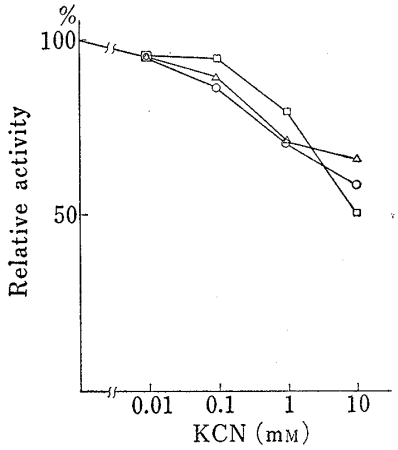

Fig. 5. Effect of KCN on *p*-Nitroanisole Demethylation with Liver Microsomes

Concentration fo KCN was varied from 10^{-5} to 10^{-2} M. Microsomes were suspended at a concentration of 1.10 mg (NADH system), 0.60 mg (NADPH system) or 0.83 mg protein per ml (NADH plus NADPH system). The relative activities are expressed as percentages of the activity observed in the absence of KCN. Other experimental conditions were described in Experimental.
—○—: NADH-, —△—: NADPH-, —□—: NADH plus NADPH-dependent reactions.

10) T. Omura, R. Sato, D.Y. Cooper, O. Rosenthal, and R.W. Estabrook, *Federation Proc.*, **24**, 1181 (1965).
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control. On the contrary, NADH-dependent demethylation was not inhibited significantly by CO. The similar results were also obtained in the atmospheres of 10% oxygen and 10, 20, 30 and 40% CO in nitrogen.

It was assumed at the begining of this study that cyanide-sensitive factor, which participates as a terminal oxidase in fatty acid desatutation system of liver microsomes and utilizes NADH as a more effective cofactor than NADPH,[4,5] might also function in the NADH-dependent demethylation of *p*-nitroanisole. This possibility was then examined by addition of potassium cyanide, because the desaturation was known to be highly sensitive to cyanide (50% inhibition with 10^{-4} M CN^-).[4] As could be seen in Fig. 5, all the reactions using three systems did not show any significant inhibition with 10^{-4} M caynide.

Discussion

Various drugs are oxidized with liver microsomes in the presence of both oxygen and NADPH. These reactions are, in general, catalyzed by the cytochrome P–450 which is involved as terminal component of following electron transfer pathway.[2,3] In addition,

NADPH → NADPH cytochrome c reductase → cytochrome P–450

another electron transport system which was correlated to the oxidative conversion of stearoyl CoA to oleyl CoA in liver microsomes was reported by Oshino, *et al.*[4]

NADH → cytochrome b_5 reductase → cytochrome b_5 → cyanide-sensitive factor

Recently, Hildebrandt, *et al.* found that a synergistic effect of NADH on the N-demethylation of aminopyrine and ethylmorphine using NADPH as a cofactor. To explain this phenomenone, they considered the possible participation of NADH in transfer of one of two electrons, *via* cytochrome b_5 to cytochrome P–450. Another electron was transfered through NADPH-cytochrome c reductase to cytochrome P–450.[8]

Quite recently we reported that deethylation of *p*-nitrophenetole with rabbit liver microsomes proceeded well with NADH as a cofactor and this NADH-dependent deethylation was not inhibited by CO.[7] On the other hand, the NADPH-dependent deethylation was inhibited significantly by CO. When both NADH and NADPH were used, the deethylation increased markedly and was also inhibited by CO. These observations and others suggested that the mechanism of NADH-dependent deethylation was different from those of the NADPH- and NADH plus NADPH-dependent deethylation, which were thought to be cytochrome P-450-mediated monooxgenation. Furthermore, potassium cyanide was found not to inhibit the NADH-dependent deethylation of *p*-nitrophenetole as well as the NADPH- and NADH plus NADPH-dependent reaction. This indicated clearly that the cyanide-sensitive factor is not concerned with the deethylation of *p*-nitrophenetole.[7]

In the present study, the demethylation of *p*-nitroanisole with rabbit liver microsomes were performed analogously to above deethylation of *p*-nitrophenetole, using NADH, NADPH or NADH plus NADPH as the electron donor. The results showed that both deethylation of *p*-nitrophenetole and demethylation of *p*-nitroanisole were almost same in nature. For example, any one of NADH, NADPH or NADH plus NADPH could serve as effective cofactor in either demethylation or deethylation, although the enzyme activity was little less in the NADH system than in the others. Optimum pH of NADH-dependent system and of the other two systems was 6.0 and 7.4, respectively, in the demethylation, same as in the deethylation. Moreover, both dealkylations were inhibited by CO in the NADPH and NADH plus NADPH systems, but not in the NADH system. Potassium cyanide (10^{-4} M) did not inhibit significantly either deethylation or demethylation in all of the three systems.

These results suggested that, different from both NADPH- and NADH plus NADPH-dependent demethylation which may be catalyzed by the enzyme system involving cytochrome P–450, the NADH-dependent demethylation of *p*-nitroanisole is perhaps independent

on cytochrome P-450 or cyanide-sensitive factor, and associated with a new oxygen-activating enzyme, as well as the NADH-dependent deethylation of *p*-nitrophenetole. This new type of NADH-dependent oxygenase is, therefore, very likely to be also involved in common in other O-dealkylations with rabbit liver microsomes.

NADH plus NADPH-dependent demethylation activity of *p*-nitroanisole was increased more than NADH-dependent activity plus NADPH-dependent (Fig. 3). This activity was inhibited little more by CO than NADPH-dependent activity, although this mechanism was not known at the present.

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Metabolism of Budralazine, a New Antihypertensive Agent. II.[1] Metabolic Pathways of Budralazine in Rats[2]

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The metabolic pathways of budralazine were studied *in vivo* and *in vitro* experiments and characterized as follows; 1) 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (M-1), one of the major metabolites of budralazine, was mainly biotransformed *via* 1-hydrazinophthalazine. 2) *s*-triazolo[3,4-*a*]phthalazine (M-3) was also biotransformed *via* 1-hydrazinophthalazine. 3) Experiments using liver preparations of rat were also discussed briefly.

Keywords——metabolism; budralazine; antihypertensive agent; metabolic pathway; *in vivo*; *in vitro*

Budralazine (1-[2-(1,3-dimethyl-2-butenylidene)hydrazino]phthalazine, DJ-1461) is an orally active antihypertensive agent.[4] In our previous work,[1] ten metabolites of the drug were isolated from rat urine and these structures were characterized by several physical methods. However, the metabolic pathway has not been established because two of the metabolites, 3-methyl-s-triazolo[3,4-*a*]phthalazine (M-1) and s-triazolo[3,4-*a*]phthalazine (M-3) can be presumably produced through several metabolic pathways as shown in Chart I and II, respectively.

In this report, we wish to present the metabolic pathways of budrazine by the confirmation of the formation routes of M-1 and M-3.

Experimental

Materials——Deuterated budralazine (budralazine-d_{10}) was synthesized by using mesityl oxide-d_{10} by the method of Ueno, *et al.*[5] The existence ratio of budralazine-d_{10} in the deuterated budralazine was

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2) A part of this work was presented at the 95th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, April 1975.
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