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Diphasic Binding of Carbon Monoxide with Cytochrome P-450 of Rat Liver Microsomes reduced by NADPH

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Periodical change in the binding of carbon monoxide with P-450 reduced by NADPH was found to show diphasic pattern. Effect of concentration of oxygen in the atmosphere and effect of pretreatment of rats with phenobarbital and 3-methylcholanthrene on the first and second phases were investigated. Diphasic binding of carbon monoxide with NADPH-reduced P-450 suggested the existence of multiple P-450 species.

Introduction

There have been many reports on the drug metabolizing enzymes in liver microsomes. Analyzing those data, we may have to admit the existence of so many drug metabolizing enzymes. In addition, it was clarified that cytochrome P-450 (P-450) acts as a terminal oxidase of drugs.[2-4] Recently, discussions on the problem whether multiple P-450 species are present have been made. Estabrook and his associates[5-6] have demonstrated that there would be multiple P-450 species, P-446 and P-450 or P-454, in liver microsomes, while Mannering and his collaborators[7] have reported that P_1-450 (probably the same as P-446) might be induced enzyme by polycyclic hydrocarbons and did not present in microsomes from control rats. 3-Methylcholanthrene and other several polycyclic hydrocarbons are well known to induce synthesis of P_1-450[7-9] or P-446 and to stimulate aniline hydroxylation but to reduce activities of hexobarbital and aminopyrine oxidases. The last fact that activities of hexobarbital and aminopyrine oxidases are decreased by treatment with 3-methylcholanthrene would be inconsistent with other two if P-450 is a drug metabolizing enzyme itself. If there are two P-450 species in microsomes from 3-methylcholanthrene-treated rats, the one P-450 species participating in the oxidation of hexobarbital and aminopyrine should be reduced by pretreatment of rats with 3-methylcholanthrene. However, there have been no evidence showing that 3-methylcholanthrene reduces one species of P-450.

To elucidate substrate specificity of drug metabolizing enzymes, the authors supposed that there might be multiple P-450 species which are participating in drug metabolism.

In the present paper, the authors investigated diphasic binding of carbon monoxide with P-450 reduced by NADPH under aerobic conditions in order to support the existence of two species of P-450 in liver microsomes.

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Experimental

Male rats of Wistar strain weighing 100 to 180 g were used throughout this study. The rats were fasted for 15 to 18 hr prior to sacrifice but water was given *ad libitum*. Livers from two to three rats were pooled and used for each experiment. Phenobarbital sodium (in saline) and 3-methylcholanthrene (in corn oil) were given to the rats 48 hr prior to sacrifice at a dose of 70 mg/kg (*s.c.*) and 40 mg/kg (*i.p.*), respectively. Microsomes were prepared by a method reported previously.[10] Protein was determined by the method of Lowry, *et al.*[11] Bovine serum albumine was employed as the protein standard. Carbon monoxide-induced difference spectra were obtained using HITACHI Two Wave Length recording spectrophotometer, Model 356, with a constant temperature cell compartment. Content of P-450 was determined according to the method of Omura and Sato,[12] and an extinction coefficient of 91 mM^{-1} cm^{-1} was used to convert the data from absorbancy to n moles of P-450. In experiments requiring constant gas phase, Thunberg cell was employed and the atmosphere was replaced by a vacuum-flash method with known gas. The concentration of oxygen in the media was measured by means of Beckman Physiological Gas Analyzer, Model 160, with an oxygen electrode.

Result and Discussion

As shown in Fig. 1, periodical change in absorbance at 450 mμ was found to be diphasic when carbon monoxide was bubbled and NADPH (1 mM) was added into microsomal suspensions. The peaks of carbon monoxide binding spectra were seen at longer wave length than 450 mμ in the first phase (451 or 452 mμ in control, 450 or 451 mμ in 3-methylcholanthrene-treated microsomes) and at the same or shorter wave length in the second phase (450 mμ in control, 448mμ in 3-methylcholanthrene-treated microsomes).

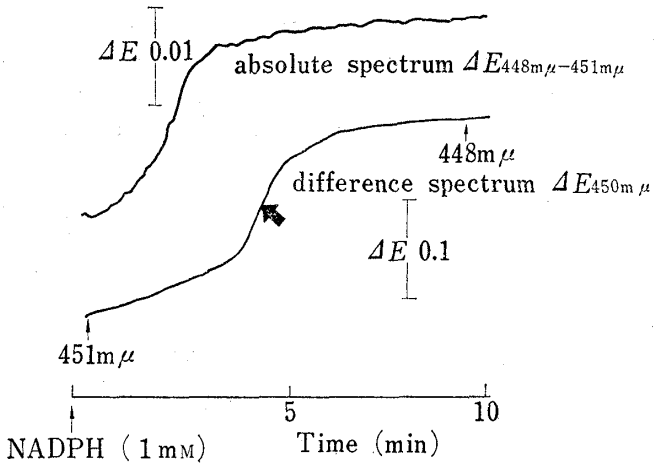

Fig. 1. Periodical Changes in Absorbance at 450 mμ in Carbon Monoxide Difference Spectrum and in the Difference between Absorbances at 448 mμ and 451 mμ in Absolute Spectrum in Microsomes from 3-Methylcholanthrene Induced Rats

microsomal protein; 4.22 mg/ml, P-450; 0.78 mμ mole/mg of microsomal protein. Carbon monoxide was bubbled for 1 min prior to addition of NADPH. The reaction was started with addition of NADPH and the measurements were performed aerobically at 25°. Another microsomal samples from the same livers were used for recording the peak of the P-450-CO binding spectrum.

To elucidate the periodical shift of P-450 peak, two wave length measurement, in which λ_1 and λ_2 waves were set at 448 mμ and 451 mμ respectively, was carried out using 3-methylcholanthrene-induced microsomes. The periodical change in difference of absorbance between at 451 mμ and 448 mμ supported the shift of the peak from 451 mμ to 448 mμ (Fig. 1). Similar results were obtained when control or phenobarbital-induced microsomes were used. On the other hand, the effect of oxygen concentration in the atmosphere was tested, since the second phase was presumed to be reflected by the oxygen concentration in the media. As shown in Fig. 2 and 3, the time required to show sharp slope (the middle point of the transition process, pointed as indicated by a large arrow in Fig. 1) was prolonged with increasing the oxygen concentration, and the absorbance at 450 mμ of the second phase at 20 min after addition of NADPH was reduced. However, the absorbance at the first phase (at 1 min after

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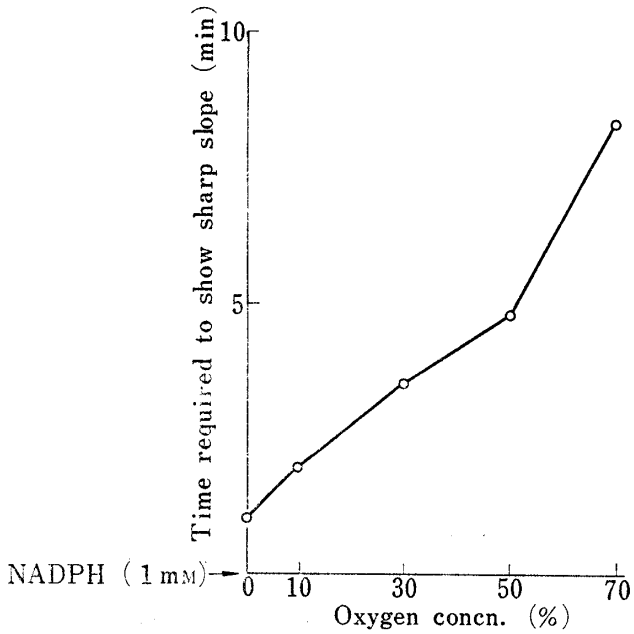

Fig. 2. Effect of Oxygen Concentration on the Time Required to Show Sharp Slope in Diphasic Binding of Carbon Monoxide with P-450 Reduced by NADPH in Microsomes from Non-Treated Rats

microsomal protein; 2.52 mg/ml, P-450; 1.01 mμ mole/mg of microsomal protein. Mixed gas contained CO (30%), O_2 and N_2. Other conditions were the same as Fig. 1.

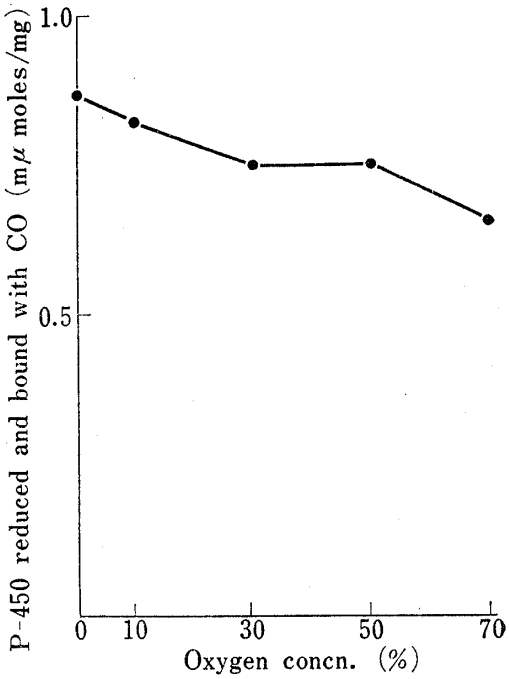

Fig. 3. Effect of Oxygen Concentration on the Second Phase in Diphasic Binding of Carbon Monoxide with P-450 reduced by NADPH in Microsomes from Non-Treated Rats

Experimental conditions were the same as described for Fig. 2.

addition of NADPH) was not changed (data not shown). In this case, when the concentration of oxygen was below 30%, the absorbance at the first phase was unable to be measured since it was impossible to determine the exact level of the first phase.

The diphasic binding of carbon monoxide with P-450 was assumed to be induced by the anaerobicity of the media.[13] As shown in Fig. 4, close relationship between the diphasic pattern and oxygen concentration in the media was found, by which the second phase was confirmed to be caused by anaerobicity of the media. Similar results as shown in Fig. 4 were also obtained when 3-methylcholanthrene or phenobarbital-induced microsomes were used.

NADPH-linked peroxidation of lipids in liver microsomes has been demonstrated to consume considerable amount of oxygen.[14,15] Therefore, the effects of lipid peroxidation on the anaerobicity of the media and the time to show second phase were determined. Addition of EDTA, a potent inhibitor of lipid peroxidation,[14] produced a marked prolongation of the time to show sharp slope (more than 5 times) and a reduction of oxygen concentration with an inverse pattern. Wills[16] has reported that optimum pH of lipid peroxidation was 6 to 7 and that significant reduction of the peroxide formation was observed at higher pH. Fig. 5 indicates that the time to show sharp slope was strongly affected by pH, that is, it was shorter at pH 6.5, prolonged with increasing pH value up to 8.5 (Fig. 5).

From findings described above, it was likely to speculate that there are two P-450 species differing either in the affinity to bind with carbon monoxide or oxygen, or in their steady state, that is, one is stable in reduced form and can bind with carbon monoxide even in aerobic

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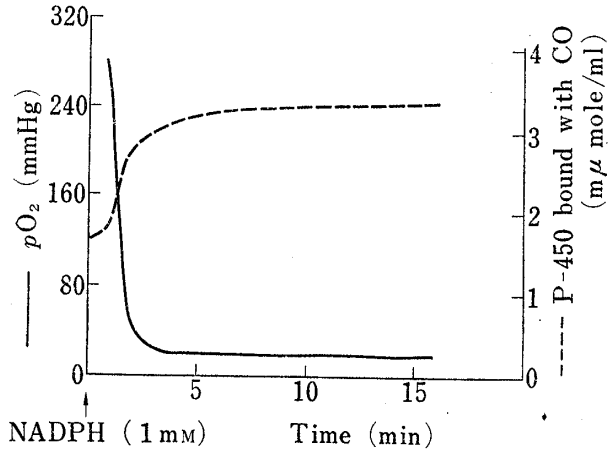

Fig. 4. Relationship between Oxygen Concentration and Binding of P-450 with Carbon Monoxide in Microsomes from Phenobarbital Treated Rats

P-450; 4.26 mμ moles/ml. Other experimental conditions were the same as shown in Fig. 1 except that oxygen electrode was put into upper position in the media in the cell.

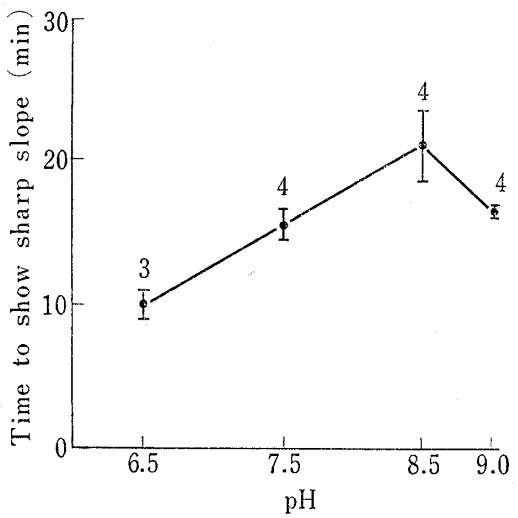

Fig. 5. Effect of pH on the Time to Show Sharp Slope in Diphasic Binding of Carbon Monoxide with P-450 Reduced by NADPH in Microsomes from Non-Treated Rats

Microsomes were suspended in 0.1M phosphate buffer (pH 6.5—9.0). Numbers in parentheses and each bar represent number of experiments and standard error, respectively, Gas phase was replaced with known gas containing CO (50%), N_2 (30%) and O_2 (20%).

P-450; 2.35 mμ moles/ml, microsomal protein; 2.28—2.74 mg/ml. Other experimental conditions were the same as described for Fig. 1.

environment, while the another is stable in oxidized form in an aerobic environment, and this may be rapidly oxidized with oxygen if it is reduced. The former has a peak at 451 or 452 mμ when it is reduced then bound with carbon monoxide, and the latter has a peak at shorter wave length than 450 mμ. Moreover, it may be able to suppose that the former catalyzes metabolism of type I compounds, such as hexobarbital and aminopyrine, while the latter does the oxidation of type II compounds since 3-methylcholanthrene is thought to induce the latter P-450 species. 3-Methylcholanthrene has been demonstrated to induce total P-450 or P-446 synthesis but to reduce metabolic rate of hexobarbital and aminopyrine. This apparent discrepancy between changes in content of P-450 and drug oxidation has yet been dissolved since there have been no evidence revealing that 3-methylcholanthrene reduces synthesis of a P-450 species catalyzing oxidation of hexobarbital and aminopyrine.

Table I. Effects of Phenobarbital and 3-Methylcholanthrene Treatments on the First Phase Binding of Carbon Monoxide with P-450 Reduced by NADPH

Microsomes	P-450-CO binding in the first phase (mμ mole/ml)
Saline-treated	0.551±0.055[a)]
Phenobarbital-treated	0.657±0.031
Corn oil-treated	0.552±0.038
3-Methylcholanthrene-treated	0.380±0.047[b)]

Microsomes were diluted with 0.1M phosphate buffer (pH 7.5), and adjusted P-450 concentrations of all samples up to 2.74 mμ moles/ml. Gas phase was replaced with the gas containing CO (30%), N_2 (20%) and O_2 (50%).

a) standard error of five experiments

b) statistically significant ($p < 0.05$)

To account for this discrepancy with present hypothesis, effect of phenobarbital and 3-methylcholanthrene treatments on the first phase binding of carbon monoxide with P-450 were tested (Table I). As was expected, the binding in the first phase was slightly increased by phenobarbital treatment but significantly reduced by 3-methylcholanthrene treatment. Accordingly, it may be possible to confirm that there are two P-450 species, one having a peak at about 451 mμ in carbon monoxide binding spectrum catalyzes oxidation of hexobarbital and aminopyrine, and the another having a peak at shorter wave length than 450 mμ, probably P-446, catalyzes aniline hydroxylation.

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