INHIBITION BY SKF 525-A OF 7-ETHOXYCOUMARIN O-DEETHYLATION IN MICROSONAL AND THE RECONSTITUTED MONOOXYGENASE SYSTEMS FROM PCB-TREATED RAT LIVERS

Tetsuya KAMATAKI, Mebae ANDO, Kenji ISHII and Ryuichi KATO
Department of Pharmacology, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160, Japan
Accepted June 20, 1980

Abstract—Addition of diethylaminoethyl 2,2-diphenylvalerate-HCl (SKF 525-A) to the incubation mixture containing liver microsomes or purified cytochrome P-450 (PCB P-450) from PCB (KC-500)-treated rats resulted in non-competitive inhibition of 7-ethoxycoumarin O-deethylation activity whereas the addition to the incubation mixture containing purified cytochrome P-448 (PCB P-448) showed a competitive inhibition. Fortification of PCB-induced microsomes with purified NADPH-cytochrome P-450 reductase enhanced the O-deethylation activity. With the reductase-fortified microsomes, SKF 525-A inhibited the O-deethylation in a competitive manner. Based on these results, we confirmed that SKF 525-A inhibits non-competitively and competitively, depending on the species of cytochrome P-450. Our results also support the view that in microsomes from PCB-treated rats, PCB P-450 rather than PCB P-448 is mainly involved in the O-deethylation reaction, presumably due to the presence of a limited amount of NADPH—cytochrome P-450 reductase in microsomes.

Diethylaminoethyl 2,2-diphenylvalerate-HCl (SKF 525-A) potentiates the pharmacological actions of a number of drugs including hypnotics (1-3). The mechanism by which SKF 525-A potentiates the actions of drugs is apparently by inhibiting the biotransformation of drugs (4, 5), and the catalyzation by cytochrome P-450 in hepatic microsomes has been reported (6).

Microsomal cytochrome P-450 plays the major role in drug oxidations (6-8). Schenkman et al. (9) demonstrated that SKF 525-A had a high binding affinity to microsomal cytochrome P-450. The binding of SKF 525-A was, therefore, assumed to be the cause of inhibition of drug oxidations. However, in view of studies on the inhibitory pattern by SKF 525-A of drug oxidations, SKF 525-A does not necessarily inhibit drug oxidations in a uniform pattern. Competitive and non-competitive inhibitions apparently depend on the animal species and the substrate employed (10-12). To account for such variation in the inhibitory pattern, Schenkman et al. (13) reported that SKF 525-A formed a complex with cytochrome P-450 to inactivate the cytochrome when the inhibitor underwent oxidative metabolism, thus resulting in a non-competitive inhibition of drug oxidations. In further spectral examinations of the complex formation with SKF 525-A, Buening and Franklin (14, 15) reported that the extent of the binding of the reactive intermediate of SKF 525-A to cytochrome P-450 as measured by the formation of the spectral complex having a peak at about 455 nm was greater in microsomes from phenobarbital-treated and untreated rat livers than
in microsomes from 3-methylcholanthrene-treated rat livers. These results suggest that SKF 525-A inhibits drug oxidations competitively or non-competitively, depending on the cytochrome P-450 species present in the microsomes and due to the ability of each cytochrome P-450 species to metabolize SKF 525-A.

Franklin and Estabrook (16) and Peterson et al. (17) demonstrated that in liver microsomes there was about 10- to 20-fold larger amounts of cytochrome P-450 than NADPH-cytochrome P-450 reductase, when calculated on a molecular number basis. Thereafter, it is possible that the amount of reductase limits the activity of cytochrome P-450 (18–22). Kitada et al. (21, 22) recently proposed that depending on the substrate employed there was an order among the multiple species of cytochrome P-450 to receive electrons from the limited reductase. Thus, it may be that the inhibitory pattern is changed when a reconstituted cytochrome P-450 system is used instead of microsomes, since the competition among cytochrome P-450 species may be abolished in the presence of an excess amount of NADPH-cytochrome P-450 reductase.

This report concerns a comparison of the inhibitory pattern by SKF 525-A of 7-ethoxy-coumarin O-deethylation between the microsomal and the reconstituted cytochrome P-450 systems using microsomes and purified cytochrome P-450 prepared from PCB-treated rat livers. Since PCB reportedly induces multiple species of cytochrome P-450 (23, 24), the PCB-induced microsomes were assumed to be a useful tool for the purpose of these experiments.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 120 to 130 g, maintained on a commercial rat chow, CE-2, Nippon Clea, Tokyo, were fasted for about 18 hr prior to sacrifice, but were allowed tap water ad libitum. The animal was given PCB (KC-500) i.p. dissolved in corn oil, at a dose of 500 mg/kg 5 days before sacrifice. Liver microsomes were prepared by the method of Kamataki and Kitagawa (25).

A typical incubation mixture for the assay of microsomal drug oxidation activities contained liver microsomes (0.5 mg), an NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 0.5 unit of glucose 6-phosphate dehydrogenase and 10 mM MgCl2), 50 mM HEPES (N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid) (pH 7.4) and a substrate in a final volume of 0.5 ml. Substrate concentrations were 1 mM, 5 mM and 0.5 mM for the O-alkyl derivatives of p-nitrophenol, p-aminophenol and 7-hydroxycoumarin, respectively, unless otherwise stated. The incubation mixture for the assay of the activities of purified cytochrome P-450 preparations consisted of 0.05 nmole of purified cytochrome P-450, 0.5 unit of NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl-L-3-phosphatidylcholine and other necessary components as described for the microsomal activities. Incubations were carried out at 37° for 10 min, during which the reaction was linear with time. Fortification of microsomes with purified NADPH-cytochrome P-450 reductase was performed after treatment of the microsomes with cholic acid. To a tube containing microsomes (0.1 mg), were added 50 mM HEPES (pH 7.4), glycerol (25%) and sodium cholate
(1%) in a final volume of 20 µl. The mixture was treated at 20˚C for 10 min, then to this mixture was added a desired amount of a purified preparation of NADPH-cytochrome P-450 reductase. The mixture was diluted with 50 mM HEPES (pH 7.4) containing 0.1 mM EDTA. The microsomes thus obtained were used for the assay of 7-ethoxycoumarin O-deethylase activity. NADPH-cytochrome P-450 reductase was purified from phenobarbital-treated rats by the method of Yasukochi and Masters (26) with some modifications. The specific content of the purified reductase was 42.8 units/mg protein. Cytochrome P-450 was purified from liver microsomes of PCB-treated rats essentially by the method described by Imai and Sato (27, 28). Liver microsomes were solubilized by cholic acid at a concentration of 0.8% (18) and these microsomes were applied on a column of ω-amino-n-octyl Sepharose 4B. Cytochrome P-450 was eluted into two broad peaks from the column, as monitored by the absorption at 416 nm. The fractions eluted in the earlier fractions exhibited a peak at 450 nm in a carbon monoxide difference spectrum while fractions eluted in the latter fractions at 449 nm. Thus, the earlier (Fraction I) and the latter fractions (Fraction II) were collected in separate flasks. The combined fractions were diluted 3-fold with 20% glycerol and were applied on hydroxylapatite columns. The columns were washed successively with 35 mM, 100 mM and 150 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol. Cytochrome P-450 (PCB P-450) having a peak at 450-450.5 nm in the carbon monoxide difference spectrum was eluted by washing the column with the buffer containing 100 mM potassium phosphate (pH 7.25). A wash of the column with the buffer containing 150 mM potassium phosphate (pH 7.25) resulted in elution of cytochrome P-448 (PCB P-448) with a peak at 448 nm in the carbon monoxide difference spectrum. PCB P-450 from Fraction I and PCB P-448 from Fraction II were subjected to further purification. PCB P-450 preparation was diluted 5-fold with 0.2% Emulgen 913 containing 20% glycerol and applied on a CM-Sephadex (C-50) column which had been equilibrated with 20 mM potassium phosphate (pH 7.25) containing 20% glycerol. The column was washed with 20 mM potassium phosphate (pH 7.25) containing 20% glycerol, then PCB P-450 was eluted with 70 mM potassium phosphate (pH 7.25) containing 0.4% sodium cholate and 20% glycerol. PCB P-448 fractions eluted from the hydroxylapatite column were diluted 3-fold with 0.2% Emulgen 913 containing 20% glycerol and were applied on a CM-Sephadex (C-50) column previously equilibrated with 50 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol. The column was washed with 50 mM potassium phosphate (pH 7.25) containing 20% glycerol, then successively with 50 mM and 150 mM potassium phosphate (pH 7.25) containing 0.4% sodium cholate and 20% glycerol. Major PCB P-448 was eluted by washing the column with the latter buffer. Carbon monoxide difference spectra of the final preparations of PCB P-450 and PCB P-448 showed peaks at 450.2 and 447.5 nm and the specific contents of these preparations were 10.9 and 11.9 nmoles per mg of protein, respectively. Both PCB P-450 and PCB P-448 were assumed to be in low spin states, as determined by absolute spectra. The activity of NADPH-cytochrome P-450 reductase was assessed using cytochrome c as an electron acceptor, by the method of Phillips and Langdon (29). The activity of the reductase was
defined as a unit which reduced one μmole of cytochrome c per min. Cytochrome P-450 was determined by the method of Omura and Sato (30) except that 0.2% Emulgen 913 and 20% glycerol were added to all determinations. The content of cytochrome P-450 was calculated, as described by Imai and Sato (31). Oxidative O-dealkylation activities of O-methyl-, O-ethyl- and O-propyl-derivatives of p-aminophenol, p-nitrophenol and 7-hydroxycoumarin were estimated by determination of p-aminophenol (32), p-nitrophenol (33) and 7-hydroxycoumarin (34), respectively. Protein was determined according to the method of Lowry et al. (35). The O-alkyl derivatives of 7-hydroxycoumarin and the O-propyl derivative of p-nitrophenol were synthesized using corresponding alkyl iodides. ω-Amino-n-octyl Sepharose 4B was synthesized from cyanogen bromide-activated Sepharose 4B and 1,8-diaminooctane by the method described by Cuatrecasas (36). SKF 525-A and Emulgen 913 were generous gifts from Smith-Klein & French Lab. Philadelphia, Pa., U.S.A. and Kao-Atlas Co., Tokyo, respectively. Commercial cholic acid was recrystallized from 50% ethanol and was used as the sodium salt. NADP, NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Tokyo. Sepharose 4B and CM-Sephadex (C-50) were obtained from Pharmacia, Uppsala, Sweden, hydroxylapatite from Bio-Rad Lab., Richmond, CA, U.S.A. The O-methyl- and the O-ethyl-derivatives of p-aminophenol and p-nitrophenol were purchased from Tokyo Kasei, Tokyo, the O-propyl derivative of p-aminophenol was from Kanto Chemicals, Tokyo, and cyanogen bromide from Wako Pure chemicals, Osaka. Other chemical reagents were from commercial sources but were of the highest grade available.

RESULTS

To determine the substrate specificity of purified cytochrome P-450 preparations, the O-dealkylation activities of the O-methyl-, O-ethyl- and O-propyl-derivatives of p-aminophenol, p-nitrophenol and 7-hydroxycoumarin were compared (Table 1). In all activities measured, except in the O-demethylation activity of 7-methoxycoumarin, PCB P-448 showed higher activities than PCB P-450. The greatest difference in the activities between PCB P-448 and PCB P-450 was found in the O-dealkylation of 7-propoxycoumarin. The inhibition by SKF 525-A of the activity of 7-ethoxycoumarin O-deethylation in liver microsomes from PCB-treated rats is shown in Fig. 1. The Lineweaver-Burk plots gave a Vmax value for the O-deethylation activity of 5.35 nmoles/nmole P-450/min in the absence of SKF 525-A. The addition of SKF 525-A produced a non-competitive inhibition of the O-deethylase activity. The inhibition constant (Ki) was about 505 μM. The inhibition by SKF 525-A of the reconstituted O-deethylase activity of PCB P-450 is shown in Fig. 2. The Vmax value for the O-deethylation was 11.76 nmoles/nmole P-450/min which was higher than that seen with PCB-treated microsomes. The inhibitory pattern by SKF 525-A was non-competitive, whereas the inhibition constant (Ki) was considerably lower than that seen in the microsomes. The inhibition constant was 39.5 μM. The inhibition kinetics were also examined using purified PCB P-448 (Fig. 3). The Vmax was 47.62 nmoles/nmole P-450/min. In contrast to the results with microsomes and the purified PCB P-450, SKF
525-A inhibited the activity competitively with the inhibition constant of 40.8 μM. Thus, it was clarified from the inhibitory pattern that microsomal activity apparently reflects the activity due to PCB P-450 rather than PCB P-448, although the activity of PCB P-450 as calculated on the nmole of cytochrome P-450 basis was higher than the activity in the microsomes. Recent work by Kitada et al. (21, 22) suggested the possibility that there is an order

| Table 1. Comparison of O-dealkylation activities between PCB P-450 and PCB P-448 purified from PCB-treated rat liver microsomes |
|---------------------------------------------------------------|
| Substrate | PCB P-450 (nmole product formed/nmole P-450/min) | PCB P-448 (nmole product formed/nmole P-450/min) | P-448/P-450 |
|-----------|----------------------------------|----------------------------------|--------------|
| p-Aminophenol O-alkyl derivatives | | | |
| Methyl | 0.87 | 1.41 | 1.6 |
| Ethyl | 0.98 | 3.02 | 3.1 |
| Propyl | 1.73 | 2.40 | 1.4 |
| p-Nitrophenol O-alkyl derivatives | | | |
| Methyl | 2.12 | 3.96 | 1.9 |
| Ethyl | 15.18 | 50.48 | 3.3 |
| Propyl | 8.75 | 26.87 | 3.1 |
| 7-Hydroxycoumarin O-alkyl derivatives | | | |
| Methyl | 0.43 | 0.34 | 0.8 |
| Ethyl | 8.82 | 31.26 | 3.5 |
| Propyl | 5.35 | 29.14 | 5.4 |

An O-alkyl derivative of p-aminophenol, p-nitrophenol or 7-hydroxycoumarin was incubated with a reconstituted monoxygenase system containing PCB P-450 or PCB P-448. For experimental details, see MATERIALS AND METHODS. Values are the mean of duplicate determinations.

**Fig. 1.** Inhibition by SKF 525-A of 7-ethoxycoumarin O-deethylation in liver microsomes from PCB-treated rats. An incubation mixture containing liver microsomes from PCB-treated rats, varying concentrations of 7-ethoxycoumarin and other necessary components was incubated in the presence or absence of 500 μM SKF 525-A. Other experimental details are described in MATERIALS AND METHODS. Each point represents the mean of duplicate determinations. 7-EtO-Cou, 7-ethoxycoumarin; 7-OH-Cou, 7-hydroxycoumarin.
among various cytochrome P-450 species to accept election by NADPH-cytochrome P-450 reductase to receive electrons because of the presence of a limited amount of the reductase compared to the amount of cytochrome P-450 in liver microsomes (16, 17). In fact, the ratio of cytochrome P-450 to NADPH-cytochrome P-450 reductase was about 24 on a molecular number basis in microsomes from PCB-treated rat livers (not shown), assuming that the molecular weight of the reductase is 78,000 and there is a specific activity of the homogeneous preparation of 60 units per mg of protein (26). To determine whether or not microsomal activity is due mainly to the activity of PCB P-450, the effects of fortification of microsomes with purified NADPH-cytochrome P-450 reductase on the O-deethylation activity were examined. Microsomes were treated with cholic acid in the presence of glycerol which prevents the conversion to cytochrome P-420 of cytochrome P-450 due to

![Graph](image1)

**Fig. 2.** Inhibition by SKF 525-A of 7-ethoxycoumarin O-deethylation catalyzed by PCB P-450. The reaction mixture containing 0.05 nmole of PCB P-450, excess amount (0.5 unit) of purified NADPH-cytochrome P-450 reductase, varying concentrations of 7-ethoxycoumarin and other necessary components was incubated in the presence of 100 μM SKF 525-A. Other experimental conditions were as described in MATERIALS AND METHODS. Each point represents the mean of duplicate determinations. 7-EtO-Cou, 7-ethoxycoumarin; 7-OH-Cou, 7-hydroxycoumarin.

![Graph](image2)

**Fig. 3.** Inhibition by SKF 525-A of 7-ethoxycoumarin O-deethylation catalyzed by PCB P-448. Experimental details were as described for Fig. 2, except that PCB P-448 was used instead of PCB P-450 and 200 μM rather than 100 μM SKF 525-A was added. Each point represents the mean of duplicate determinations. 7-Eto-Cou, 7-ethoxycoumarin; 7-OH-Cou, 7-hydroxycoumarin.
detergents (37), a desired amount of the purified reductase was added, and the preparation was diluted with the buffer containing other necessary components for the enzyme assay. As shown in Fig. 4, the microsomal O-deethylase activity was enhanced according to the amount of purified reductase added. The fortification of microsomes in the absence of cholic acid increased the O-deethylase activity but the extent of increase was considerably less than that obtained in the presence of cholic acid. The effect of fortification of microsomes in the presence of cholic acid with purified reductase on the Vmax of ethoxy-coumarin O-deethylation activity is shown in Table 2. The Vmax value increased with increase in the amount of reductase added. Thus, the inhibition kinetics of SKF 525-A

![Graph](image)

**Fig. 4.** Effects of fortification of microsomes with purified NADPH-cytochrome P-450 reductase (fpT) on 7-ethoxycoumarin O-deethylation. Liver microsomes from PCB-treated rats were treated at 20° for 10 min in the presence of cholic acid. The indicated amount of fpT was then added and the mixture was diluted. The fpT-fortified microsomes thus obtained were used for the assay of the O-deethylation activity. Other experimental details were as described in MATERIALS AND METHODS. Each point represents the mean of duplicate determinations.

| fpT added (unit) | Vmax (nmole 7-hydroxycoumarin formed/mg/min) |
|-----------------|---------------------------------------------|
| 0               | 15.4                                       |
| 0.5             | 41.2                                        |
| 2.0             | 64.1                                        |

Liver microsomes (0.1 mg) were fortified with an indicated amount of purified NADPH-cytochrome P-450 reductase (fpT) in the presence of cholic acid, as described in MATERIALS AND METHODS. The Vmax values were calculated from Lineweaver-Burk plots, and were represented as the nmole product formed per min per mg protein of intact microsomes.
was examined using microsomes to which had been added 2.0 units of purified reductase per 0.1 mg of microsomes. The results shown in Fig. 5 show that SKF 525-A inhibits the O-deethylase activity competitively after fortification with the reductase with an inhibition constant of 1,500 /\mu M. The activities were expressed as nmole 7-hydroxycoumarin formed per mg protein of intact microsomes per min. Each point represents the mean of duplicate determinations. Other experimental details are described in MATERIALS AND METHODS.

DISCUSSION

As shown in Figs. 1, 2 and 3, SKF 525-A inhibited 7-ethoxycoumarin O-deethylation less sensitively when liver microsomes rather than a reconstituted cytochrome P-450 system were utilized as the enzyme source. The mechanism by which the difference occurred is not known at present, however, the difference in the sensitivity to SKF 525-A may be due to the effect of microsomal membranes. In support of our idea, a marked difference in the sensitivity to carbon monoxide between microsomal and the reconstituted systems has been reported. Cooper et al. (38) observed a striking increase in the sensitivity of benzo(a)-pyrene hydroxylase activity to carbon monoxide in the reconstituted system containing cytochrome P-448. They confirmed that the difference in the sensitivity to carbon monoxide between microsomal and the reconstituted systems is not due to change in the heme protein cytochrome P-448 per se but to the composition and/or structural organization of the microenvironment of cytochrome P-448 in endoplasmic membranes.

Buening and Franklin (14, 15) demonstrated that the rate of metabolism as measured by the formation of a complex spectrally detectable after incubation with SKF 525-A in the presence of NADPH and molecular oxygen is enhanced by treatment of rats with phenobarbital but not with 3-methylcholanthrene. The complex of cytochrome P-450 with a reactive intermediate of SKF 525-A formed during prolonged incubation may cause a non-competitive inhibition of drug oxidations, since the complex formation results in an
inactivation of cytochrome P-450. Thus, in our experiments, the incubation lasted for 10 min to minimize the possible formation of such a reactive intermediate. Preliminary experiments showed that incubation of PCB P-450 with SKF 525-A for 1 hr in the presence of NADPH produces a moderate acceleration in decrease of the amount of the hemoprotein remaining in the incubation mixture, as determined by carbon monoxide difference spectrum, while PCB P-448 is not degraded, rather is even stabilized by the presence of SKF 525-A. Therefore, the possibility that the non-competitive inhibition seen with PCB P-450 is due to a partial degradation of the hemoprotein cannot be ruled out. In these incubation conditions, the inhibitory pattern by SKF 525-A of 7-ethoxycoumarin O-deethylation was also found to be non-competitive when microsomes were used. If both PCB P-450 and PCB P-448 are equally participating in the O-deethylation, the inhibitory pattern can be expected to be of a mixed type or of a nearly competitive type because of the higher activity of PCB P-448. The non-competitive inhibition thus seems to be due to PCB P-450 rather than to PCB P-448 regarding the O-deethylation in liver microsomes. This finding probably supports the previous view that there is an order among cytochrome P-450 species to receive electrons from a limited amount of NADPH-cytochrome P-450 reductase. In microsomes from PCB-treated rats, therefore, PCB P-450 may accept election by NADPH-cytochrome P-450 reductase more preferentially than PCB P-448. In support of our idea, fortification of microsomes with purified NADPH-cytochrome P-450 reductase to make the reductase not a rate limiting enzyme, enhanced the O-deethylation activity, and in the presence of excess NADPH-cytochrome P-450 reductase, the inhibitory pattern by SKF 525-A was of a competitive type. The apparent competitive inhibition can be accounted for by the larger amount of activity due to PCB P-448, as discussed above.

Treatment of microsomes with cholic acid in the presence of purified NADPH-cytochrome P-450 reductase resulted in a marked stimulation of the O-deethylation activity. The exact cause of the enhancement is unknown at present, however, it seems reasonable to assume that treatment with cholic acid improved the incorporation of purified reductase into microsomal membranes. Further studies on the difference between the intact and the solubilized-reorganized microsomes are in progress. This system should be a useful tool in studies on the nature of the mixed function oxidase enzymes since one can reconstitute the enzyme system without changing the components surrounding cytochrome P-450.

Acknowledgement: This work was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1) COOK, L., TONER, J.J. AND FELLOWS, E.J.: The effect of β-diethylaminoethylidiphenylpropylacetate hydrochloride (SKF No. 525-A) on hexobarbital. J. Pharmacol. exp. Ther. 111, 131-141 (1954)
2) COOK, L., MACKO, E. AND FELLOWS, E.J.: The effect of β-diethylaminoethylidiphenylpropylacetate hydrochloride on the action of a series of barbiturates and C. N. S. depressants. J. Pharmacol. exp. Ther. 112, 382-386 (1954)
3) COOK, L., NAVIS, G. AND FELLOWS, E.J.: Enhancement of the action of certain analgesic drugs by β-diethylaminoethylidiphenylpropylacetate hydrochloride. J. Pharmacol. exp.
4) AXELROD, J., REICHENTHAL, J. AND BRODIE, B.B.: Mechanism of the potentiating action of \( \alpha \)-diethylaminoethyldiphenylpropylacetate. *J. Pharmacol. exp. Ther.* **112**, 49–54 (1954)

5) COOPER, J.R., AXELROD, J. AND BRODIE, B.B.: Inhibitory effects of \( \alpha \)-diethylaminoethyldiphenylpropylacetate on a variety of drug metabolic pathways in vitro. *J. Pharmacol. exp. Ther.* **112**, 55–63 (1954)

6) COON, M.J., VERMILLION, J.L., VATHIS, K.P., FRENCH, J.S., DEAN, W.L. AND HAUGEN, D.A.: *Drug Metabolism Concept*, Edited by JERINA, D.M., ACS Symp. Series 44, p. 46–71, American Chemical Society, Washington (1977)

7) COOPER, D.Y., LEVIN, S., NARASIMHULU, S., ROSENTHAL, O. AND ESTABROOK, R.W.: Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science* **147**, 400–402 (1965)

8) OMURA, T., SATO, R., COOPER, D.Y., ROSENTHAL, O. AND ESTABROOK, R.W.: Function of cytochrome P-450 of microsomes. *Fedn. Proc.* **24**, 1181–1189 (1965)

9) SCHENKMAN, J.B., REMMER, H. AND ESTABROOK, R.W.: Spectral studies of drug interaction with hepatic microsomal cytochrome. *Mol. Pharmacol.* **3**, 113–123 (1967)

10) KATO, R., ONODA, K. AND TAKAYANAGI, M.: Species difference in the inhibition of drug metabolism by liver microsomes by different inhibitors. *Japan. J. Pharmacol.* **19**, 438–450 (1969)

11) ANDERS, M.W.: Enhancement and inhibition of drug metabolism. *Annu. Rev. Pharmacol.* **11**, 37–56 (1971)

12) MANNERING, G.J.: *Concept in Biochemical Pharmacology*, part 2, Handbuch der experimentellen Pharmakologie, Bd. 28, Edited by BRODIE, B.B. AND GILLETTE, J.R., p. 452–476, Springer-Verlag, New York (1971)

13) SCHENKMAN, J.B., WILSON, B.J. AND CINTI, D.L.: Diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A)-In vivo and in vitro effects of metabolism by rat liver microsomes. Formation of an oxygenated complex. *Biochem. Pharmacol.* **21**, 2373–2383 (1972)

14) BUENING, M.K. AND FRANKLIN, M.R.: The formation of complexes absorbing at 455 nm from cytochrome P-450 and metabolites of compounds related to SKF 525-A. *Drug Metab. Dispo.* **2**, 386–390 (1974)

15) BUENING, M.K. AND FRANKLIN, M.R.: The formation of cytochrome P-450-metabolic intermediate complexes in microsomal fractions from extrahepatic tissues of the rabbit. *Drug Metab. Dispo.* **4**, 556–561 (1976)

16) FRANKLIN, M.R. AND ESTABROOK, R.W.: On the inhibitory action of mersalyl on microsomal drug oxidation: A rigid organization of the electron transport chain. *Arch. Biochem. Biophys.* **143**, 318–329 (1971)

17) PETERSON, J.A., EBEL, R.E., O’KEEFFE, D.H., MATSUBARA, T. AND ESTABROOK, R.W.: Temperature dependence of cytochrome P-450 reduction. A model for NADPH-cytochrome P-450 reductase: cytochrome P-450 interaction. *J. biol. Chem.* **251**, 4010–4016 (1976)

18) KAMATAKI, T., BELCHER, D.H. AND NEAL, R.A.: Studies of the metabolism of diethyl p-nitrophenylphosphorothionate (parathion) and benzphetamine using an apparently homogeneous preparation of rat liver cytochrome P-450: Effect of a cytochrome P-450 antibody preparation. *Mol. Pharmacol.* **12**, 921–932 (1976)

19) MIWA, G.T. AND CHO, A.K.: Stimulation of microsomal N-demethylation by solubilized NADPH-cytochrome c reductase. *Life Sci.* **18**, 983–988 (1976)

20) MIWA, G.T., WEST, S.B. AND LU, A.Y.H.: Studies on the rate-limiting enzyme component in the microsomal monoxygenase system. Incorporation of purified NADPH-cytochrome c reductase and cyochrome P-450 into rat liver microsomes. *J. biol. Chem.* **253**, 1921–1929 (1978)

21) KITADA, M., KITAGAWA, H. AND KAMATAKI, T.: The effects of incorporation of purified NADPH-cytochrome c (P-450) reductase on drug oxidations. *Biochem. Pharmacol.* **28**, 2670–2673 (1979)

22) KITADA, M., KUBOTA, K., KITAGAWA, H. AND KAMATAKI, T.: Stimulation of microsomal drug oxidation activities by incorporation into microsomes of purified NADPH-cytochrome c (P-450) reductase. *Japan. J. Pharmacol.* **29**, 877–887 (1979)
23) ALVARES, A.P., BICKERS, D.R. AND KAPPAS, A.: Polychlorinated biphenyls: A new type of inducer of cytochrome P-448 in the liver. Proc. natn. Acad. Sci. U.S.A. 5, 1321-1325 (1973)

24) SHIMADA, T., NUNOURA, Y., KITANAKA, E., IWAGAMI, S. AND MIZUTA, Y.: Induction of mouse and rat liver microsomal enzymes by polychlorinated biphenyls in relation to the PCB levels in tissue. Folia Pharmacol. japon. 72, 955–967 (1976) (Abs. in English)

25) KAMATAKI, T. AND KITAGAWA, H.: Effects of lyophilization and storage of rat liver microsomes on activity of aniline hydroxylase, contents of cytochrome b_{5} and cytochrome P-450 and aniline-induced P-450 difference spectrum. Japan. J. Pharmacol. 24, 195–203 (1974)

26) YASUKOCHI, Y. AND MASTERS, B.S.S.: Some properties of a detergent solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. J. biol. Chem. 251, 5337–5344 (1976)

27) IMAI, Y. AND SATO, R.: An affinity column method for partial purification of cytochrome P-450 from phenobarbital-induced rabbit liver microsomes. J. Biochem. 75, 689–697 (1974)

28) IMAI, Y. AND SATO, R.: A gel-electrophoretically homogeneous preparation of cytochrome P-450 from liver microsomes of phenobarbital-pretreated rabbits. Biochem. biophys. Res. Commun. 60, 8–14 (1974)

29) PHILLIPS, A.H. AND LANGDON, R.G.: Hepatic triphosphopyridine nucleotide-cytochrome c reductase: Isolation, characterization, and kinetic studies. J. biol. Chem. 237, 2652–2660 (1962)

30) OMURA, T. AND SATO, R.: Carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. J. biol. Chem. 239, 2379–2385 (1964)

31) IMAI, Y. AND SATO, R.: Conversion of P-450 to P-420 by neutral salts and some other reagents. Europ. J. Biochem. 1, 419–426 (1967)

32) IMAI, Y., ITO, A. AND SATO, R.: Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. J. Biochem. 60, 417–428 (1966)

33) KAMATAKI, T., KITADA, M., SHIGEMATSU, H. AND KITAGAWA, H.: The involvement of cytochrome P-448 and P-450 in NADH-dependent O-demethylation of p-nitroanisole in rat liver microsomes. Japan. J. Pharmacol. 29, 191–201 (1979)

34) AITIO, A.: A simple and sensitive assay of 7-ethoxycoumarin deethylation. Annal. Biochem. 85, 488–491 (1978)

35) LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J.: Protein measurement with the folin phenol reagent. J. biol. Chem. 193, 265–275 (1951)

36) CUATRECASAS, P.: Protein purification by affinity chromatography: Derivatizations of agarose and polyacrylamide beads. J. biol. Chem. 245, 3059–3065 (1970)

37) ICHIKAWA, Y. AND YAMANO, T.: Reconversion of detergent and sulfhydryl reagent-produced P-420 to P-450 by polyols and glutathione. Biochim. Biophys. Acta 131, 490–497 (1967)

38) COOPER, D.Y., SCHLEYER, H., ROSENTHAL, O., LEVIN, W., LU, A.Y.H., KUNTZMAN, R. AND CONNEY, A.H.: Inhibition by CO of hepatic benzo(a)pyrene hydroxylation and its reversal by monochromatic light. Europ. J. Biochem. 74, 69–75 (1977)