differences in methodology. Also, serotonin binding in human platelets may differ from that in rat platelets.

There are several possibilities why this study did not demonstrate the binding of serotonin to the receptor site responsible for shape change and aggregation: (1) the specific activity of the ligand might not have been sufficient to detect extremely small numbers of receptors for shape change and aggregation; (2) leakage of minute amounts of endogenous serotonin may have occurred during the incubation conditions which diluted low concentrations of labeled serotonin or occupied serotonin receptor sites, and (3) the incubation of platelets at 4° altered the binding site for shape change. This possibility is not likely because serotonin receptor binding is easily identified in nerve tissue when incubated at 4°.

This study did identify binding to the serotonin uptake site which will enable us to identify the role of specific membrane components in platelet serotonin receptor binding.

Acknowledgements—The authors would like to thank Drs. Paula Goldberg and Alan Frazer for their helpful advice. This work was supported by Grant HL 18249 from the National Institute of Health and the Medical Research Service at the Philadelphia Veterans Administration Hospital.

Division of Hematology and Oncology,
Temple University School of Medicine,
3401 North Broad Street,
Philadelphia, PA, 19140,
U.S.A.

PAUL K. SCHICK
MAR-LEE MCKEAN

REFERENCES

1. G. V. R. Born, K. Juengjaroen and F. Michal, *Br. J. Pharmac.* **44**, 117 (1972).
2. H. J. Haigler and G. K. Aghajanian, *Fed. Proc.* **36**, 2159 (1977).
3. L. Edvinsson, J. E. Hardebo and C. Owman, *Circulation Res.* **42**, 143 (1978).
4. A. H. Drummond and J. L. Gordon, *Biochem. J.* **150**, 129 (1975).
5. P. K. Schick and B. P. Yu, *J. Lab. clin. Med.* **82**, 546 (1973).
6. P. K. Schick, K. B. Kurica and G. K. Chacko, *J. clin. Invest.* **57**, 1221 (1976).
7. G. V. R. Born and J. Bricknell, *J. Physiol., Lond.* **147**, 153 (1959).

Biochemical Pharmacology, Vol. 28, pp. 2670–2673
© Pergamon Press Ltd. 1979. Printed in Great Britain.

0006-2952/79/0901-2670 $02.00/0

The effects of incorporation into microsomes of purified NADPH-cytochrome *c* (P-450) reductase on drug oxidations

(*Received* 20 *September* 1978; *accepted* 19 *March* 1979)

The current view of the liver microsomal monooxygenase enzyme system is that in the microsomal membranes cytochrome P-450 catalyzes the biotransformation of a variety of drugs, toxic compounds including carcinogens, and endogeneous substrates such as steroids and fatty acids utilizing molecular oxygen and electrons [1–5]. Recent efforts made by several laboratories have realized the almost complete purification of cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase [6–11]. The determination of molecular weight and specific activity of these purified enzymes has led to the conclusion that cytochrome P-450 is present in 10 to 25 times larger amounts than NADPH-cytochrome *c* (P-450) reductase in microsomal membranes, and that at least six species of cytochrome P-450 exist in microsomes from phenobarbital- and 3-methylcholanthrene-treated rats [12]. As regards the possibility of whether or not NADPH-cytochrome *c* (P-450) reductase is the rate limiting enzyme in drug oxidations in whole microsomes, not many reports have appeared. From results obtained using reconstituted mixed function oxidase system, Kamataki *et al.* [10] proposed that in whole microsomes NADPH-cytochrome *c* (P-450) reductase rather than cytochrome P-450 is the rate limiting enzyme for benzphetamine *N*-demethylation. In support of this hypothesis, quite recent reports by Miwa and Cho [13] and Miwa *et al.* [14] demonstrated that a detergent solubilized NADPH-cytochrome *c* (P-450) reductase was incorporated into microsomes to enhance drug oxidation activities. If cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase are not rigidly organized in the membranes as mentioned by Yang and Strickhart [15] and Yang [16], then it seems reasonable to hypothesize that multiple species of cytochrome P-450 compete with each other in functional binding to the limited amount of NADPH-cytochrome *c* (P-450) reductase in microsomal membranes.

Thus, in this paper we would like to report the results of the experiments showing the effects of fortification of rat liver microsomes with purified NADPH-cytochrome *c* (P-450) reductase to further support the idea that there is an order in the cytochrome P-450 species for receiving electrons from limited NADPH-cytochrome *c* (P-450) reductase in microsomal membranes.

Materials and methods. Male rats of Cr1:CD(SD) strain weighing 120 to 150 g were used throughout this study. The animals, which were maintained on a commercial rat chow, CE-2 Nippon Clea Co., Japan, were starved for about 20 hr prior to sacrifice, but were given tap water *ad lib.* When necessary, intraperitoneal (i.p.) injection of 3-methylcholanthrene (25 mg/kg) dissolved in olive oil and subcutaneous (s.c.) injection of phenobarbital (80 mg/kg) dissolved in saline were conducted simultaneously once a day for three days.

DEAE-Sephadex (A-50) and 2′,5′ ADP-Sepharose 4B were purchased from Pharmacia Fine Chemicals Co., and hydroxylapatite from Bio-Rad. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Grade I) and cytochrome *c* (horse heart) were purchased from Boehringer Mannheim. Emulgen 913, a non-ionic detergent, was kindly provided by Kao-Atlas Co. Commercial aniline was redistilled under vacuum and the distillate was stored at about −10° under an atmosphere of nitrogen. Other chemicals were of highest purity commercially available. Microsomes were prepared as described previously [17]. Protein was determined by the method of Lowry *et al.* [18] using bovine serum

albumin as a standard. Cytochrome P-450 was measured by the method of Omura and Sato [19] using an Aminco DW-2 recording spectrophotometer, and the content was calculated from absorbance difference between 450 nm and 490 nm using the extinction coefficient of 91 $mM^{-1}cm^{-1}$ [20]. NADPH-cytochrome *c* (P-450) reductase (EC 1.6.99.2) activity was measured by the method of Phillips and Langdon [21] using cytochrome *c* as an electron acceptor.

NADPH-cytochrome *c* (P-450) reductase was purified from phenobarbital-treated rat liver microsomes by a minor modification of the method of Yasukochi and Masters [11]. Eluate from 2′,5′ ADP-Sepharose 4B column was adsorbed on a hydroxylapatite column equilibrated with 0.01M-K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The column was washed with 0.01M-K-phosphate (pH 7.25) containing 20% glycerol until the absorbance at 276 nm was less than 0.03 and then NADPH-cytochrome *c* (P-450) reductase was eluted with 0.15M-K-phosphate (pH 7.25) containing 20% glycerol. The final NADPH-cytochrome *c* (P-450) reductase preparation had a specific activity greater than 50 units per mg of protein. One unit of NADPH-cytochrome *c* (P-450) reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome *c* at an initial rate of 1 μmole per min at 25°.

A typical reaction mixture for drug oxidation assay consisted of Na,K-phosphate (0.1 M, pH 7.4), NADP (0.33 mM), glucose 6-phosphate (8 mM), $MgCl_2$ (6 mM), glucose 6-phosphate dehydrogenase (0.045 unit), EDTA (0.1 mM), microsomes (approx. 1.0 mg of protein) and a substrate (5 mM) in a final volume of 1.0 ml. *N*-Demethylation of ethylmorphine or aminopyrine and hydroxylation of aniline or *O*-deethylation of *p*-phenetidine were carried out at 37° for 10 min and 20 min, respectively. Drug oxidation reactions were linear during the incubation period under these incubation conditions. *N*-Demethylase activities of ethylmorphine and aminopyrine were measured by determining formaldehyde formed by the method of Nash [22]. Aniline hydroxylation and *p*-phenetidine *O*-deethylation were assayed by determing *p*-aminophenol formed according to the method of Imai *et al.* [23].

Fortification of rat liver microsomes with purified NADPH-cytochrome *c* (P-450) reductase was carried out essentially by the method described by Miwa and Cho [13]. Microsomes, phosphate (0.1 M, pH 7.4) and EDTA (0.1 mM) were incubated at a concentration of about 10 mg of protein per ml for 20 min at 30° with desired amounts of purified NADPH-cytochrome *c* (P-450) reductase. Control samples were incubated with buffer in place of the reductase. After the incubation period, the mixtures were diluted about 5- to 10-fold with ice cold 0.1M-phosphate (pH 7.4) and centrifuged at 105,000 *g* for 60 min. The microsomal pellets were resuspended in 0.1M-phosphate (pH 7.4) and centrifuged at 105,000 *g* for 30 min. The washed microsomal pellets were resuspended in 0.1M-phosphate (ph 7.4) and used for the assay.

Results and discussion. The effects of varying amounts of added NADPH-cytochrome *c* (P-450) reductase on drug oxidations activities were examined. The amount of the reductase incorporated into the intact liver microsomes was increased with the amount of the purified reductase added; roughly 20 to 25 per cent of the added reductase was incorporated (data not shown). The possibility that not all of the reductase recovered in the microsomes, after washing with the buffer as described by Miwa and Cho [13], was functionally involved in drug oxidations, remains to be elucidated; however, at least a portion of the 'fortified' reductase is assumed to be functionally acting in the microsomal membranes since the rate of cytochrome P-450 reduction by NADPH as well as drug oxidation activities was enhanced by the fortification in accordance with the finding by Miwa *et al.* [14] (data not shown). Since the reductase was assumed to limit the rate of drug oxidations in whole microsomes, the double reciprocal plots of the activities were drawn towards the amounts of the reductase presented in microsomes. As shown in Fig. 1, the amounts of the reductase required for giving a half maximal velocity of drug oxidations were found to vary with the substrates employed. The amounts of the reductase in the fortified microsomes to give a half maximal activities of *p*-phenetidine *O*-deethylation, aniline hydroxylation and aminopyrine and ethylmorphine *N*-demethylation were 0.177, 0.132, 0.108 and 0.056 unit per mg of microsomal protein, respectively. The most pronounced enhancement was also seen in *p*-phenetidine *O*-deethylation, followed by aniline hydroxylation and aminopyrine and ethylmorphine *N*-demethylations when microsomes from rats treated with 3-methylcholanthrene or phenobarbital was used (data not

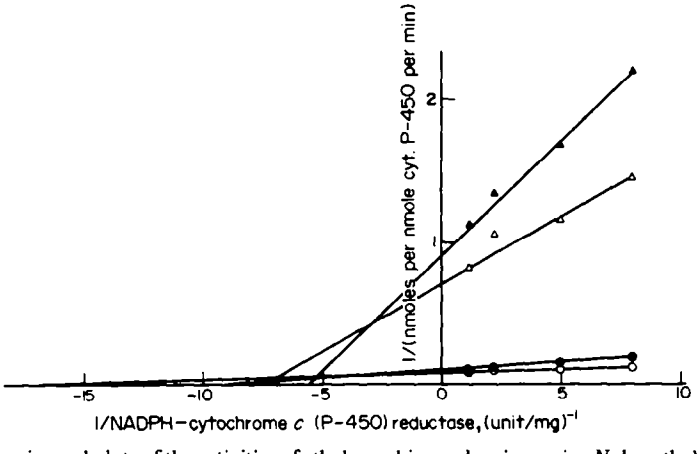


Fig. 1. Double reciprocal plots of the activities of ethylmorphine and aminopyrine *N*-demethylations, aniline hydroxylation and *p*-phenetidine *O*-deethylation against the NADPH-cytochrome *c* (P-450) reductase activity. Microsomes (80 mg of protein) isolated from untreated rats were divided into four flasks. The flasks were incubated after addition of 0, 10.4, 34.6 and 114.2 units of purified NADPH-cytochrome *c* (P-450) reductase in the presence of 0.1 M-Na, K-phosphate (pH 7.4) Specific activity of microsomal NADPH-cytochrome *c* (P-450) reductase before incubation was 0.123 unit per mg of protein. Activities of ethylmorphine (○) and aminopyrine (●) *N*-demethylations, aniline (△) hydroxylation and *p*-phenetidine (▲) *O*-deethylation were measured using microsomes fortified with various amounts of reductase. Other experimental details are as described in Materials and Methods.

Table 1. Inhibition by α-naphthoflavone of aniline hydroxylation activity in microsomes fortified with purified NADPH-cytochrome *c* (P-450) reductase

	Aniline hydroxylation *			
Addition (μM)	Control	(Inhibition, %)	Reductase-fortified	(Inhibition, %)
None	0.254		0.586	
α-Naphthoflavone (0.2)	0.254	(0.0)	0.547	(6.7)
α-Naphthoflavone (1.0)	0.223	(12.2)	0.422	(28.0)
α-Naphthoflavone (10.0)	0.225	(11.4)	0.354	(39.6)

Liver microsomes (40 mg of protein) from rats treated with phenobarbital and 3-methylcholanthrene were divided into two flasks. Purified NADPH-cytochrome *c* (P-450) reductase (216.4 units) was added to one of the two flasks and corresponding amounts of the buffer to the other as a control. The specific activity of NADPH-cytochrome *c* (P-450) reductase and the specific content of cytochrome P-450 in the control and the fortified microsomes were 0.174 and 0.714 unit per mg of protein and 2.11 and 1.92 nmoles per mg of protein, respectively. Other experimental details are as described in Materials and Methods.

* nmoles *p*-Aminophenol formed per nmole cytochrome P-450 per min.

shown). These observations probably suggest that the cytochrome P-450 species catalyzing ethylmorphine *N*-demethylation receives election by limited NADPH-cytochrome *c* (P-450) reductase for accepting electrons after binding with the substrate ethylmorphine, while in the case of *p*-phenetidine, cytochrome P-450 species concerned in the *O*-deethylation can not receive electrons even after binding with the substrate, probably because of the interference by other species of cytochrome P-450.

Aniline hydroxylation has been assumed to be catalyzed by more than one drug metabolizing enzyme [24]. Recently, Lu *et al.* [25] have demonstrated that aniline hydroxylation is catalyzed by different forms of cytochrome P-450 purified from rat liver microsomes. Furthermore, in accordance with the previous results, we* also observed that all species of cytochrome P-450 partially purified from intact, phenobarbital- and 3-methylcholanthrene-treated rat liver microsomes had the activity for aniline hydroxylation. In addition, α-naphthoflavone is known to be a specific inhibitor of one of the cytochrome P-450 species, cytochrome P-448, which is induced by polycyclic aromatic hydrocarbons. Therefore, assuming that aniline is oxidized by a variety of cytochrome P-450 species, the effect of α-naphthoflavone on aniline hydroxylation was determined using microsomes from rats treated with both phenobarbital and 3-methylcholanthrene in the presence of incorporated NADPH-cytochrome *c* (P 450) reductase to know whether or not there is a preferential species of cytochrome P-450 involved in aniline hydroxylation in whole microsomes (Table 1). The aniline hydroxylation activities seen in both control microsomes and the microsomes fortified with the reductase were inhibited by α-naphthoflavone, however, the inhibition was greater in the fortified microsomes than in the control. At the 10 μM concentration of α-naphthoflavone, aniline hydroxylation in the fortified microsomes was inhibited by about 40 per cent whereas that in the control microsomes was inhibited by only about 11 per cent. This result indicates that a cytochrome P-450 species other than a species sensitive to α-naphthoflavone is a preferential species for aniline hydroxylation in the absence of the fortified NADPH-cytochrome *c* (P-450) reductase. The results similar to those observed for aniline hydroxylation were also obtained when *p*-phenetidine was used as a substrate (data not shown).

Although immunochemical studies [26] have suggested that cytochrome P-450 is located on the surface of endoplasmic reticulum, the possibility that the effects of NADPH-cytochrome *c* (P-450) reductase might be due to failure of the reductase to reach cytochrome P-450 molecules which are not on the surface of microsomal membrane cannot be excluded.

Clark and Powis [27] have demonstrated that acetone administration *in vivo* enhances the activities of aniline hydroxylation and NADPH-cytochrome *c* (P-450) reductase but not the content of cytochrome P-450. From the results shown in the present study, it seems possible to assume that the stimulation of aniline hydroxylation activity might be due to increase in the activity of reductase rather than the qualitative change in cytochrome P-450.

Acknowledgement– We wish to express our appreciation to Miss Kikue Kubota for her skillful technical assistance.

Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences, Chiba University, 1–33 Yayoi-cho, Chiba-shi
MITSUKAZU KITADA
HARUO KITAGAWA

Department of Pharmacology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-Ku, Japan
TETSUYA KAMATAKI

* T. Kamataki, M. Kitada, K. Chiba, H. Kitagawa, Y. Imai and R. Sato, unpublished data.

REFERENCES

1. A. Y. H. Lu, R. Kuntzman, S. West, M. Jacobson and A. H. Conney, *J. biol. Chem.* **247**, 1727 (1972).
2. J. E. Tomaszewski, D. M. Jerina, W. Levin and A. H. Conney, *Archs Biochem. Biophys.* **176**, 788 (1976).
3. A. W. Wood, W. Levin, A. Y. H. Lu, H. Yagi, O. Hernandez, D. M. Jerina and A. H. Conney, *J. biol. Chem.* **251**, 4882 (1976).
4. H. W. Strobel, A. Y. H. Lu, J. Heidema and M. J. Coon, *J. biol. Chem.* **245**, 4851 (1970).
5. T. A. Van der Hoeven, D. A. Haugen and M. J. Coon, *Biochem. Biophys. Res. Commun.* **60**, 569 (1974).
6. Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.* **60**, 8 (1974).
7. C. Hashimoto and Y. Imai, *Biochem. Biophys. Res. Commun.* **68**, 821 (1976).
8. D. A. Haugen and M. J. Coon, *J. biol. Chem.* **251**, 7929 (1976).
9. D. Ryan, A. Y. H. Lu, J. Kawalek, S. B. West and W. Levin, *Biochem. Biophys. Res. Commun.* **64**, 1134 (1975).
10. T. Kamataki, M. C. Lee, D. H. Belcher and R. A. Neal, *Drug Metab. Dispos.* **4**, 180 (1976).

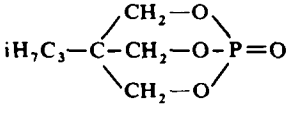
11. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
12. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin, *Molec. Pharmac.* **12**, 746 (1976).
13. G. T. Miwa and A. K. Cho, *Life Sci.* **18**, 983 (1976).
14. G. T. Miwa, S. B. West and A. Y. H. Lu, *J. biol. Chem.* **253**, 1921 (1978).
15. C. S. Yang and F. S. Strickhart, *J. biol. Chem.* **250**, 7968 (1975).
16. C. S. Yang, *J. biol. Chem.* **252**, 293 (1977).
17. M. Kitada, T. Igarashi, T. Kamataki and H. Kitagawa, *Jap. J. Pharmac.* **27**, 481 (1977).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
20. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
21. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
22. T. Nash, *Biochem. J.* **55**, 416 (1953).
23. Y. Imai, A. Ito and R. Sato, *J. Biochem., Tokyo* **60**, 417 (1966).
24. F. Wada, H. Shimakata, M. Takasugi, T. Kotake and Y. Sakamoto, *J. Biochem, Tokyo* **64**, 109 (1968).
25. A. Y. H. Lu, W. Levin, S. B. West, M. Jacobson, D. Ryan, R. Kuntzman and A. H. Conney, *J. biol. Chem.* **248**, 456 (1973).
26. W. L. Dean and M. J. Coon, *J. biol. Chem.* **252**, 3255 (1977).
27. H. Clark and G. Powis, *Biochem. Pharmac.* **23**, 1015 (1974).

Biochemical Pharmacology, Vol. 28, pp. 2673–2675
0006-2952/79/0901-2673 $02.00/0

Adenylate and guanylate cyclases in the cerebellum

(*Received* 1 *February* 1978; *accepted* 19 *March* 1979)

Intracerebroventricular injection of 4-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2,2,2]octane (IPTBO), of caffeine, and of *O*-3,3-dimethylbut-2-yl methylphosphonofluoridate (soman) led to changes in the levels of cyclic AMP and cyclic GMP in the mouse brain [1].



IPTBO

A speculative scheme, in which cyclic AMP was implicated in the inhibitory action of GABA and cyclic GMP was implicated in the excitatory action of ACh, was used to explain the observations and to provide a possible basis for further study. The scheme would suggest that the adenylate cyclase (EC 4.6.1.1) activity and the guanylate cyclase (EC 4.6.1.2) activity in the cerebellum, where the changes were most marked, should be activated by GABA and ACh, respectively. Thus, the effects of GABA and ACh on the adenylate and guanylate cyclase activities in cerebellar homogenates are now reported, together with the effects of some putative GABA antagonists and some anticonvulsants on the modified activities.

Table 1. Enhancement of the basal rates of adenylate cyclase and guanylate cyclase by GABA and ACh respectively

Adenylate cyclase		Guanylate cyclase	
Concentration of GABA (M)	Enzyme activity† (%)	Concentration of ACh (M)	Enzyme activity† (%)
0	100* (5)	0	100
10^{-7}	100 ± 3 (5)	10^{-7}	100 ± 3 (5)
$5\cdot10^{-7}$	110 ± 3 (5)	$5\cdot10^{-7}$	110 ± 3 (5)
10^{-6}	120 ± 4 (5)	10^{-6}	115 ± 3 (5)
$5\cdot10^{-6}$	135 ± 4 (5)	$5\cdot10^{-6}$	130 ± 4 (5)
10^{-5}	145 ± 4 (5)	10^{-5}	140 ± 4 (5)
$5\cdot10^{-5}$	150 ± 5 (5)	$5\cdot10^{-5}$	150 ± 4 (5)
10^{-4}	150 ± 5 (5)	10^{-4}	150 ± 4 (5)
$5\cdot10^{-4}$	140 ± 7 (5)	$5\cdot10^{-4}$	—
10^{-3}	130 ± 8 (5)	10^{-3}	140 ± 6 (5)

* Normal levels (100 per cent) — adenylate cyclase 10–20 pmoles cAMP/assay
≡ 0.5–1.0 μmoles cAMP/mg cerebellum/3 min.
— guanylate cyclase 0.5–1.0 pmoles cGMP/assay
≡ 25–50 nmoles cGMP/mg cerebellum/3 min.

† Percentage of unactivated rate ± S.E.M. Figures in parentheses are number of determinations.