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A HIGH SPIN FORM OF CYTOCHROME P-448 HIGHLY PURIFIED FROM PCB-TREATED RATS—II

CHARACTERISTIC REQUIREMENT OF CYTOCHROME b_5 FOR MAXIMUM ACTIVITY*

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Abstract—A high spin form of cytochrome P-448 (PCB P-448-H), highly purified from microsomes of PCB-treated rats, catalyzed oxidations of several compounds and required cytochrome b_5 for its full activities in all oxidations examined. PCB P-448-H catalyzed the hydroxylation of aniline and *O*-dealkylations of *p*-alkoxy derivatives of aniline and nitrobenzene and 7-alkoxy derivatives of coumarin. Among the activities measured, hydroxylation of aniline and *O*-dealkylation of *p*-alkoxy derivatives of aniline were catalyzed by PCB P-448-H more efficiently than by PCB P-448-L, which was a low spin form of cytochrome P-448 purified from liver microsomes of PCB-treated rats. In all reactions, PCB P-448-H required cytochrome b_5 for maximum activity. Slight requirements were also seen with PCB P-448-L but varied equivocally depending on the substrates. Cytochrome b_5 showed its maximum effects on *p*-propoxyaniline *O*-depropylation activity at a molar ratio of cytochrome b_5 to PCB P-448-H of 1:2. The enhancement by cytochrome b_5 was more pronounced when lower concentrations of either the substrate or NADPH-cytochrome P-450 reductase were added to the reconstituted system. Based on these results, we confirm that PCB P-448-H is a unique form of cytochrome P-448 with respect to the requirements for cytochrome b_5 and is a good probe to study the mechanisms involved in the enhancement of drug oxidations by cytochrome b_5.

Cytochrome P-450‖ in liver microsomes metabolizes a wide variety of xenobiotics as well as endogenous substrates. The metabolism of xenobiotics results in detoxication and intoxication of drugs and toxic compounds, thus leading to modification of the actions of these xenobiotics.

Cytochrome b_5 has been recognized to modify the activity of cytochrome P-450. Hildebrandt and Estabrook [1] reported that the NADH-synergism of NADPH-dependent *N*-demethylation of ethylmorphine could be accounted for by the participation of cytochrome b_5 in the electron flow to cytochrome P-450 for drug oxidations. This was later supported by the results of Correia and Mannering [2]. Further examination of the NADH-synergism clarified that the synergism could be seen depending on the substrate employed: the synergism was seen with type I substrates such as ethylmorphine and benzphetamine, but not with type II substrates such as aniline and *p*-chloro-*N*-methylaniline [3, 4]. Supporting this, Kitada *et al.* [5] reported that no detectable NADH-synergism of NADPH-dependent *p*-hydroxylation of aniline was seen unless known enhancers of aniline *p*-hydroxylation, such as acetone and 2,2′-bipyridine, were added to the microsomal incubation system.

The effects of cytochrome b_5 on the activities of cytochrome P-450 have been examined in the reconstituted system containing purified cytochrome P-450 and NADPH-cytochrome P-450 reductase [6, 7]. Sugiyama *et al.* [8] purified a unique form of cytochrome P-450 from liver microsomes of rabbits by using a cytochrome b_5-immobilized affinity column. The form of cytochrome P-450 obligatorily required cytochrome b_5 for its *p*-methoxynitrobenzene *O*-demethylation activity. In contrtast, cytochrome b_5 did not affect hydroxylations of aniline [9] and benzo[*a*]pyrene [10], or *N*-demethylations of ethylmorphine [9] and benzphetamine [10] when added to a reconstituted system containing a form of cytochrome P-450 purified from microsomes of phenobarbital-treated rats. Making the concept complicated, the addition of cytochrome b_5 to the same

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‖ The term cytochrome P-450 was used to designate any or all forms of liver microsomal cytochrome P-450, and the term cytochrome P-448 to designate the specific forms of cytochrome P-450 which showed peaks at around 448 nm in carbon monoxide difference spectra.

form of cytochrome P-450, however, was obligatory to show its *O*-deethylation activity of *p*-ethoxynitrobenzene [9].

In preceding papers [11–13], we reported on a purification of a high spin form of cytochrome P-448, namely PCB P-448-H,* which showed high activity in the *N*-hydroxylations of mutagenic compounds. During the course of examination of the properties of this form of cytochrome P-448, we found that PCB P-448-H required cytochrome b_5 for its full activity regardless of the substrates examined. In this paper we now report on this unique property of PCB P-448-H.

MATERIALS AND METHODS

Purification of cytochrome P-448, NADPH-cytochrome P-450 reductase and cytochrome b_5. Low and high spin forms of cytochrome P-448, PCB P-448-L and PCB P-448-H, were purified from microsomes of PCB-treated rats by methods reported previously [11]. The specific contents of PCB P-448-L and PCB P-448-H were 16.0 and 15.0 nmole per mg of protein, respectively. NADPH-cytochrome P-450 reductase was purified from microsomes of phenobarbital-treated rats by the method of Yasukochi and Masters [14] with minor modifications. The specific activity of the purified reductase was 50.0 units per mg of protein. Cytochrome b_5 was purified from liver microsomes of untreated male rats as described previously [15]. The specific content of cytochrome b_5 ranged from 45.2 to 47.1 nmole per mg of protein. These purified enzymes did not contain any detectable activities of other undesired enzymes as contaminants.

Assay methods. Protein was determined by the method of Lowry *et al.* [16], with bovine serum albumin as the standard. Cytochrome b_5 was determined using sodium dithionite as a reducing reagent [17]. Cytochrome P-450 was determined according to the method of Omura and Sato [18], except that 20% glycerol and 0.2% Emulgen 911 or 913 were added to all determinations to stabilize the cytochrome. The content of cytochrome P-450 was calculated by an equation described by Imai and Sato [19]. NADPH-cytochrome P-450 reductase activity was measured by the method of Phillips and Langdon [20] with cytochrome *c* as an electron acceptor. Unless otherwise stated, the specific activity of the reductase is expressed as μmole of cytochrome *c* reduced per min (unit).

The incubation mixture for the reconstitution of monooxygenase activities consisted of 0.1 nmole of PCB P-448-H or PCB P-448-L, 0.5 unit of NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl-L-3-phosphatidylcholine, 0.04 mM dithiothreitol, 0.02 μM FMN, 50 μM EDTA, the NADPH-generating system (0.8 mM NADP, 8 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase and 6 mM magnesium chloride), 50 mM HEPES (pH 7.4) and a substrate in a final volume of 1.0 ml. The concentration of the substrate added to the incubation mixture was 5 mM except for the concentrations of *p*-propoxynitrobenzene, coumarin and 7-alkoxy derivatives of coumarin which were 1.0, 0.5 and 0.5 mM, respectively. When necessary, 0.05 nmole or desired amounts of cytochrome b_5 were added. The incubation was started by the addition of the NADPH-generating system and was carried out at 37° for 15 min, aerobically. Activities for *p*-hydroxylation of aniline and *O*-dealkylation of *p*-alkoxy derivatives of aniline were estimated by the determination of *p*-aminophenol [21]. *O*-dealkylase activities for *p*-alkoxy derivatives of nitrobenzene were estimated by the formation of *p*-nitrophenol [22]. Hydoxylase activities for coumarin and *O*-dealkylase activities for 7-alkoxy derivatives of coumarin were estimated by the determination of 7-hydroxycoumarin [23] formed during incubations. All determinations were conducted in duplicate, and the mean values are presented in the tables and figures. The results were confirmed to be reproducible by at least one repeated experiment.

Materials. *p*-Methoxy and *p*-ethoxy derivatives of both aniline and nitrobenzene were purchased from Tokyo Kasei Inc., and *p*-propoxyaniline from Kanto Chemicals (Tokyo, Japan). 7-Alkoxy derivatives of coumarin and *p*-propoxynitrobenzene were synthesized with corresponding alkyl iodides. Other reagents were from commercial sources and were of the highest grade available.

RESULTS AND DISCUSSION

Comparison of PCB P-448-H and PCB P-448-L in catalytic activity and the effects of cytochrome b_5 *on that activity*

In a preceding paper [12], we demonstrated that PCB P-448-H showed higher activity than PCB P-448-L in the *N*-hydroxylation of Trp-P-2, which is highly mutagenic to *Salmonella typhimurium*. Catalytic activities of PCB P-448-H and PCB P-448-L and the effects of cytochrome b_5 on those activities were examined using aniline, coumarin and alkoxy derivatives of aniline, nitrobenzene and coumarin to compare the activities of these cytochrome species systematically. Most of these substrates have been widely used in the examination of the activities of microsomal and purified cytochrome P-450. As seen in Table 1, PCB P-448-H showed higher activity in *p*-hydroxylation of aniline and *O*-dealkylation of *p*-alkoxy derivatives of aniline than did PCB P-448-L. PCB P-448-H and PCB P-448-L showed higher activity in the *O*-dealkylation of aniline and nitrobenzene derivatives with longer alkyl chains. When 7-alkoxy derivatives of coumarin were employed, PCB P-448-H showed its maximum activity in the *O*-depropylation of the 7-isopropoxy derivative while PCB P-448-L showed it in the *O*-deethylation of the 7-ethoxy derivative. These two cytochromes were not capable of hydroxylating coumarin at the 7-position in the presence or absence of cytochrome b_5.

* Abbreviations: Trp-P-2, 3-amino-1-methyl-5*H*-pyrido(3,4-*b*)indole; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfanie acid; PCB, polychlorinated biphenyl mixture (Kaneclor 500).

Table 1. Comparison of PCB P-448-H and PCB P-448-L in the catalytic activities and effects of cytochrome b_5

Substrates	PCB P-448-H			PCB P-448-L		
	$-b_5$*	$+b_5$*	Change (%)	$-b_5$*	$+b_5$*	Change (%)
Aniline and its p-alkoxy derivatives						
Aniline	0.85	2.66	+213	0.33	0.36	+9
p-Methoxy-	0.85	1.29	+52	0.55	0.58	+5
p-Ethoxy-	3.30	4.81	+46	1.37	1.25	−9
p-Propoxy-	3.53	7.15	+103	1.35	1.21	−10
Nitrobenzene p-alkoxy derivatives						
p-Methoxy-	0.17	0.26	+53	1.15	1.55	+35
p-Ethoxy-	3.28	8.40	+156	12.39	15.62	+26
p-Propoxy-	6.87	22.44	+227	34.97	40.10	+15
Coumarin and its 7-alkoxy derivatives						
Coumarin	0.01	0.01	0	0.03	0.03	0
7-Methoxy-	0.34	1.39	+309	0.79	0.79	0
7-Ethoxy-	0.77	2.02	+162	53.25	56.13	+5
7-Propoxy-	0.48	0.97	+102	30.84	33.79	+10
7-Isopropoxy-	1.69	6.19	+266	9.30	9.68	+4
7-Butoxy-	0.16	0.31	+94	10.41	10.25	+2

* Activities are represented as nmole metabolite formed per nmole P-448 per min.

The alkoxy derivatives with *n*-alkyl chains (except for 7-isopropoxycoumarin) and certain other compounds were used as substrates and incubated in the reconstituted system containing purified preparations of PCB P-448-H or PCB P-448-L in the absence or presence of cytochrome b_5. Other experimental conditions are described in Materials and Methods.

Using a purified preparation, we found a unique character of PCB P-448-H: PCB P-448-H required cytochrome b_5 for its maximum activity regardless of the substrates examined. As discussed by Noshiro *et al.* [24], the requirement of cytochrome b_5 for the activities of purified forms of cytochrome P-450 has appeared depending on the substrate and the forms of cytochrome P-450 employed. In fact, Kuwahara and Omura [9] reported that cytochrome P-450 purified from microsomes of phenobarbital-treated rats required cytochrome b_5 for benzphetamine *N*-demethylation but not for aniline hydroxylation. The latter finding was in good agreement with the results that NADH-synergism of NADPH-dependent drug oxidations was not seen in aniline hydroxylation [38]. In contrast with these results, PCB P-448-H required cytochrome b_5 in all metabolic reactions involving aniline hydroxylation. Cytochrome b_5 showed minimum effects on activities of PCB P-448-L. Thus, we propose that PCB P-448-H is a useful enzyme to study the mechanism(s) of enhancement by cytochrome b_5.

Using purified preparations of four forms of cytochrome P-450 and several substrates, Imai [25] demonstrated that the enhancement by cytochrome b_5 was more profound when a substrate metabolized at a lower velocity was employed. However, this was not the case with PCB P-448-H: we did not find any such tendency in our experiments. In the *O*-dealkylation of *p*-alkoxy derivatives of aniline and nitrobenzene, higher enhancement was seen in association with the activities.

It is of interest to note that the effects of cytochrome b_5 on PCB P-448-H tended to be increased with the chain length of *p*-alkoxy derivatives of aniline and nitrobenzene. A reverse order in the effects of cytochrome b_5 was found when 7-alkoxy derivatives of coumarin were used as substrates, except for 7-isopropoxycoumarin: the effect of cytochrome b_5 was similar to that found in the *O*-demethylation. These results probably suggest that the length of the alkyl chain, as well as the ring portions, is the determinant of *O*-dealkylations of these compounds in the presence and absence of cytochrome b_5. Differences in the *O*-dealkylase activities of PCB P-448-H and PCB P-448-L were found between the derivatives of aniline and nitrobenzene, indicating that amino and nitro groups affect the *O*-dealkylations. The order of the enhancement by cytochrome b_5 was, however, essentially the same in these derivatives.

Further examination of the effects of cytochrome b_5 *on* p-*propoxyaniline* O-*depropylase activity of PCB P-448-H*

As shown in Table 1, enhancement by cytochrome b_5 of the activities of PCB P-448-H was found in all substrates examined except for coumarin 7-hydroxylation in which PCB P-448-H did not show any

Table 2. Effects of molar ratio of cytochrome b_5 to PCB P-448-H on *p*-propoxyaniline *O*-depropylation activity

Cyt. b_5/PCB P-448-H molar ratio	*O*-Depropylation activity*	(% of control activity)
0 (control)	4.60	(100)
0.25	9.35	(203)
0.50	10.78	(234)
1.00	9.62	(209)
2.00	6.98	(152)
3.00	4.55	(99)

* nmole *p*-aminophenol/nmole PCB P-448-H per min.

To a reconstituted system containing 0.1 nmole of PCB P-448-H, a desired amount of a purified preparation of cytochrome b_5 was added and assayed for *p*-propoxyaniline *O*-depropylation activity. Details for the experimental conditions are described in Materials and Methods.

significant activity. Thus, to understand the mechanisms by which cytochrome b_5 enhanced the activities of PCB P-448-H, some further experiments were performed using *p*-propoxyaniline as a model substrate. The effects of varying the amounts of cytochrome b_5 on the *O*-depropylation activity are shown in Table 2. The activity was increased then decreased as the amounts of cytochrome b_5 increased. The maximum enhancement was seen when the molar ratio of cytochrome b_5 to PCB P-448-H was 0.5. This result is of interest since cytochrome b_5 has been known to exist in liver microsomes at the ratio to cytochrome P-450 of approximately 0.5. The effects of varying the concentrations of the substrate on the enhancement are shown in Fig. 1. An approximate 200% increase of *O*-depropylation activity was seen when lower concentrations of the substrate were added to the incubation mixture. The enhancement was decreased with the increase in the substrate concentrations to 3.0 mM. The approximate 100% increase of the activity caused by cytochrome b_5, however, was seen even in substrate concentrations higher than 3.0 mM. The apparent K_m and V_{max} in the absence and presence of cytochrome b_5 were calculated to be 0.43 and 0.21 mM, and 8.0 and 15.9 nmole per nmole PCB P-448-H per min, respectively.

Since both NADPH-cytochrome P-450 reductase and cytochrome b_5 transfer electrons to cytochrome P-450, the possibility that the ratio of NADPH-cytochrome P-450 reductase to cytochrome b_5 affects the enhancement of the activity of PCB P-448-H was examined (Fig. 2). As can be seen, remarkable enhancements by cytochrome b_5 were seen when lower concentrations of the reductase were added: the enhancement decreased gradually with the increase in the amounts of the reductase. The approximate 100% increase in this activity, however, still remained in the presence of NADPH-cytochrome P-450 reductase at molar ratios to PCB P-448-H greater than 1: the same level of enhancement was seen even at a ratio of 3.

With respect to the mechansms involved in the enhancement by cytochrome b_5, no clear picture has been proposed. The donation from cytochrome b_5 to cytochrome P-450 of the second of two electrons

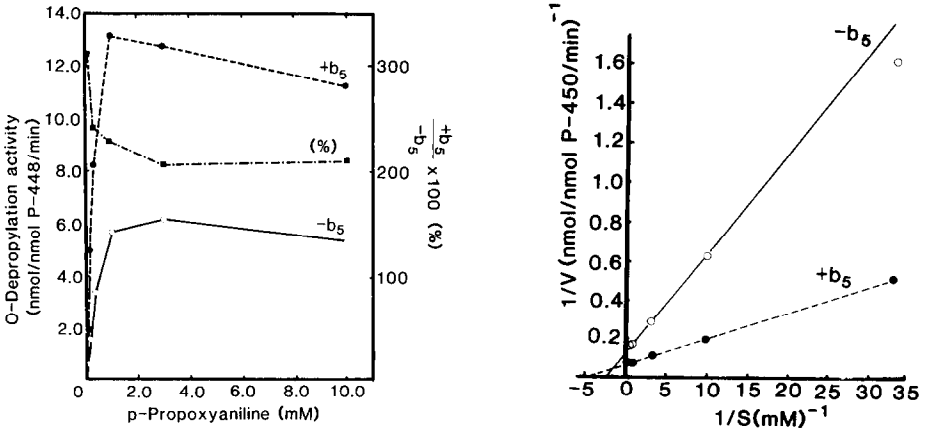

Fig. 1. Effects of varying the concentrations of substrate on the enhancement by cytochrome b_5 of the activity of PCB P-448-H. *p*-Propoxyaniline at indicated concentrations was added to a reconstituted system containing 0.1 nmole of PCB P-448-H, 0.5 unit of NADPH-cytochrome P-450 reductase with or without 0.05 nmole of cytochrome b_5 as described in Materials and Methods. K_m and V_{max} values were calculated from Lineweaver–Burk plots (right).

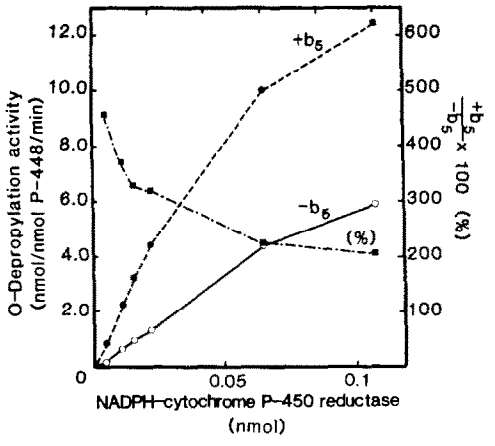

Fig. 2. Effects of varying the amounts of NADPH-cytochrome P-450 reductase on the enhancement by cytochrome b_5 of *p*-propoxyaniline *O*-depropylation activity of PCB P-448-H. The incubation mixture for *p*-propoxycoumarin *O*-depropylation activity consisted of 0.1 nmole of PCB P-448-H and a desired amount of NADPH-cytochrome P-450 reductase with or without 0.05 nmole of cytochrome b_5. Other experimental details are described in Materials and Methods.

required for drug oxidations was first demonstrated by Hildebrandt and Estabrook [1], and this hypothesis was supported later using microsomes [2, 3] and the reconstituted system [7, 25, 26]. In viewing results of experiments with purified enzymes, the involvement of multiple mechanisms for the occurrence of the enhancement has been suggested [7, 24, 25, 27–29]. Analyzing the results presented in this paper, at least two mechanisms by which cytochrome b_5 enhanced drug oxidations appeared to be involved. As shown in Fig. 1, addition of cytochrome b_5 in the incubation mixture increased the availability of the substrate at its lower concentrations, thus resulting in a smaller K_m value. This result indicates that the affinity of PCB P-448-H to the substrate increased by interaction with cytochrome b_5. The affinity between PCB P-448-H and the reductase appears to be increased by cytochrome b_5, since K_d, as calculated from the data shown in Fig. 2, was decreased two to four times by cytochrome b_5. Further, it also seems reasonable to assume that the change in the molar ratio of NADPH-cytochrome P-450 reductase to either cytochrome b_5 or PCB P-448-H showed biphasic effects on the enhancement of *p*-propoxyaniline *O*-depropylation. In this case, the portion of enhancement above 100% can be assumed to be caused by the donation of the first of two electrons from cytochrome b_5 to cytochrome P-450, although no evidence for this has been obtained to date. The varying degrees of the enhancement observed as shown in Table 1 may be explained by the hypothesis that multiple mechanisms are involved in the enhancement to different extents depending on the substrate employed.

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REFERENCES

1. A. Hildebrandt and R. Estabrook, *Archs Biochem. Biophys.* **143**, 66 (1971).
2. M. A. Correia and G. J. Mannering, *Molec. Pharmac.* **9**, 455 (1973).
3. M. A. Correia and G. J. Mannering, *Molec. Pharmac.* **9**, 470 (1973).
4. G. J. Mannering, S. Kuwahara and T. Omura, *Biochem. biophys. Res. Commun.* **57**, 476 (1974).
5. M. Kitada, T. Kamataki and H. Kitagawa, *Archs Biochem. Biophys.* **178**, 151 (1977).
6. A. Y. H. Lu, W. Levin, H. Selander and D. M. Jerina, *Biochem. biophys. Res. Commun.* **61**, 1348 (1974).
7. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **75**, 420 (1977).
8. T. Sugiyama, N. Miki and T. Yamano, *Biochem. biophys. Res. Commun.* **90**, 715 (1979).
9. S. Kuwahara and T. Omura, *Biochem. biophys. Res. Commun.* **96**, 1562 (1980).
10. A. Y. H. Lu, S. West, M. Vore, D. Ryan and W. Levin, *J. biol. Chem.* **249**, 6701 (1974).
11. T. Kamataki, H. Yoshizawa, K. Ishii and R. Kato, in *Microsomes, Drug Oxidations and Drug Toxicity* (Eds. R. Sato and R. Kato), p. 99. Japanese Sci. Soc. Press, Tokyo and Wiley-Interscience, New York (1982).
12. T. Kamataki, Y. Yamazoe, S. Mita and R. Kato, in *Microsomes, Drug Oxidations and Drug Toxicity* (Eds. R. Sato and R. Kato), p. 463. Japanese Sci. Soc. Press, Tokyo and Wiley-Interscience, New York (1982).
13. T. Kamataki, K. Maeda, Y. Yamazoe, N. Matsuda, K. Ishii and R. Kato, *Molec. Pharmac.* in press.
14. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
15. T. Kamataki, K. Maeda, Y. Yamazoe, T. Nagai and R. Kato, *Biochem. biophys. Res. Commun.* **103**, 1 (1981).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. T. Omura and S. Takesue, *J. Biochem.* **67**, 249 (1970).
18. T. Omura and R. Sato, *J. biol. Chem.* **239**, 370 (1964).
19. Y. Imai and R. Sato, *Eur. J. Biochem.* **1**, 419 (1967).
20. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
21. Y. Imai, A. Ito and R. Sato, *J. Biochem.* **60**, 417 (1966).
22. T. Kamataki, M. Kitada, H. Shigematsu and H. Kitagawa, *Jap. J. Pharmac.* **29**, 191 (1971).
23. A. Aitio, *Analyt. Biochem.* **85**, 488 (1978).
24. M. Noshiro, V. Ullrich and T. Omura, *Eur. J. Biochem.* **116**, 521 (1981).
25. Y. Imai, *J. Biochem.* **89**, 351 (1981).
26. M. Ingelman-Sundberg and I. Johnson, *Biochem. biophys. Res. Commun.* **97**, 582 (1980).
27. J. Y. L. Chiang, *Archs biochem. Biophys.* **211**, 662 (1981).
28. C. Bonfils, C. Balny and P. Maurel, *J. biol. Chem.* **256**, 9457 (1981).
29. B. Bosterling, J. R. Trudell, A. J. Trevor and M. Bendix, *J. biol. Chem.* **257**, 4375 (1982).