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PARTIAL PURIFICATION AND PROPERTIES OF CYTOCHROME P450 FROM HOMOGENATES OF HUMAN FETAL LIVERS

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Abstract—Using ω-amino-n-octyl Sepharose 4B and hydroxylapatite columns, cytochrome P450 was purified to approx. 6.7 nmoles per mg of protein from the 105,000 *g* precipitate of homogenates of human fetal livers. The partially purified preparation of cytochrome P450 was free of detectable amounts of cytochrome b_5, NADPH–cytochrome P450 reductase and NADH–cytochrome b_5 reductase. The absolute spectrum of the preparation exhibited a peak at 417 nm in the Soret region, indicating that this cytochrome P450 is a low spin species. Binding of aniline and SKF 525-A to this partially purified preparation of cytochrome P450 produced a modified type II and a type I difference spectra, respectively. As judged by Ouchterlony double diffusion analysis, the cytochrome P450 preparation did not cross react with antibody against cytochrome P450 isolated from the livers of phenobarbital-pretreated rats. In reconstituted systems, the cytochrome exhibited considerable activity for aniline hydroxylation but only a low ethlymorphine *N*-demethylation activity compared to cytochrome P450 isolated from the livers of phenobarbital-pretreated rats.

Unlike experimental animals, except for a strain of the monkey [1], human fetal liver even at early mid gestational age has been demonstrated to possess a considerable amount of cytochrome P450. The presence of cytochrome P450 in humal fetal liver microsomes was first reported by Yaffe *et al.* [2]. Thereafter, the presence of the cytochrome in liver 200 *g* pellet [3], or mitochondrial fraction [4] as well as in liver microsomes [3–11] has been reported. In spite of these reports, the chemical properties of the cytochrome still remains to be elucidated, perhaps because of the diffeculty in removing hemoglobin from the microsomal and mitochondrial preparations. In addition, the isolation and purification of the cytochrome from human fetal liver has not yet been accomplished.

In this communication, we would like to report on some properties of cytochrome P450 isolated from 105,000 g precipitate of human fetal livers using the ω-amino-n-octyl Sepharose 4B affinity column method of Imai *et al.* [12–14].

MATERIALS AND METHODS

Partial purification of cytochrome P450. Livers from fetuses of gestational age of 3 to 7 months prematurely delivered for medical reasons (such as danger of the life of the mother due to traffic accident or epilepsy) were homogenized in 10 volumes of 1.15% KCl and centrifuged at 105,000 *g* for 1 hr. The resulting pellet was washed once by homogenization with 1.15% KCl and centrifuged at 105,000 *g* for 30 min. The washed 105,000 *g* precipitates were combined and dissolved by stirring for 30 min at 0° with a buffer containing 100 mM potassium phosphate (pH 7.25), 0.8% sodium cholate, 1 mM EDTA, 1 mM dithiothreitol and 20% glycerol. The protein concentration was adjusted with this same buffer to 2 mg per ml. The solubilized sample was centrifuged at 105,000 *g* for 1 hr, and the supernatant applied to a column of ω-amino-n-octyl Sepharose 4B (1.7 × 7.5 cm) which had been equilibrated with the same buffer used for solubilization of the 105,000 *g* precipitates. The column was washed with three to four column volumes of 100 mM potassium phosphate (pH 7.25) containing 0.5% sodium cholate, 1 mM EDTA, 1 mM dithiothreitol and 20% glycerol. To elute cytochrome P450 the column was washed successively with 100 mM potassium phosphate (pH 7.25) containing 0.08% Emulgen 913, 0.4% sodium cholate and 20% glycerol, followed by 100 mM potassium phosphate (pH 7.25) containing 0.5% Emulgen 913 and 20% glycerol. No additional cytochrome P450 was eluted with the latter buffer. The fractions having an optical density at 417 nm greater than 0.100 were combined and the combined sample was diluted five-fold with 20% glycerol. The diluted sample was applied to a column of hydroxylapatite (0.9 × 3.3 cm) which had been equilibrated with 20 mM potassium phosphate (pH 7.25) containing 20% glycerol. The column was washed with 80 ml each of 30 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol, 50 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol and 50 mM potassium phosphate (pH 7.25) containing 20% glycerol. The column was further washed with 40 ml of 200 mM potassium phosphate (pH 7.25) containing 20% glycerol. Cytochrome P450 was eluted by washing the column with 500 mM potassium phosphate (pH 7.25) containing 20% glycerol. The cytochrome P450 preparation

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eluted in this manner was used in examining the properties of the cytochrome P450. Cytochrome P450 was determined by the method described by Omura and Sato [15], except that 0.2% Emulgen 913 and 20% glycerol were added in all determinations unless otherwise stated. A molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ was used for calculation of the amount of cytochrome P450. Measurements of the spectra of cytochrome P450 were carried out using an Aminco recording spectrophotometer, Model DW-2. Protein was determined by the method of Lowry *et al.* [16], using bovine serum albumin as a standard.

Partial purification of NADPH–cytochrome P450 reductase from phenobarbital-pretreated rat liver microsomes. NADPH–cytochrome P450 reductase was purified from phenobarbital-pretreated rat liver microsomes as reported by Kamataki *et al.* [17]. The specific activity of the purified preparation was 21.4 unit per mg of protein and was free from detectable amounts of cytochrome P450, cytochrome b_5 and NADH–cytochrome b_5 reductase. The enzyme preparation thus obtained was used for reconstituting the drug oxidation system. The determination of cytochrome b_5 was carried out using the magnitude of dithionite-reduced minus oxidized difference spectrum. NADH–cytochrome b_5 reductase activity was measured using ferricyanide as an electron acceptor as described by Takesue and Omura [18]. NADPH–cytochrome P450 reductase activity was measured using cytochrome *c* as an electron acceptor according to the method of Phillips and Langdon [19]. A unit of NADPH–cytochrome P450 reductase is defined as one μmole cytochrome *c* reduced per min.

Assay of aniline hydroxylation and ethylmorphine N-demethylation activities. A typical incubation mixture for the assay of ethylmorphine *N*-demethylation activity consisted of 0.122 nmole of human fetal liver cytochrome P450, 0.70 unit of NADPH–cytochrome P450 reductase isolated from rat liver, 30μg of dilauroyl-L-3-phosphatidylcholine, 100μg of sodium deoxycholate, 0.1 mM EDTA, 1 mM ethylmorphine and an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.045 unit of glucose 6-phosphate dehydrogenase and 30 mM $MgCl_2$) in a final volume of 1.0 ml. The incubation was started by addition of the NADPH-generating system. The assay mixture for aniline hydroxylation contained the same concentrations of components except that 0.488 nmole of cytochrome P450, 2.80 unit of NADPH–cytochrome P450 reductase and 20 mM aniline were used. The incubations were carried out at 37° for 30 min aerobically. Oxidative *N*-demethylation activity of ethylmorphine was estimated by determining formaldehyde production using the method of Nash [20]. Aniline hydroxylation activity was measured by determining *p*-aminophenol by the method described by Imai *et al.* [21].

Immunological property of cytochrome P450 from human fetal liver. Antiserum to cytochrome P450 was obtained using adult female New Zealand rabbits which had been immunized with cytochrome P450 purified from phenobarbital-pretreated rat liver microsomes, according to the method previously described [17]. The γ-globulin fraction was obtained by ammonium sulfate fractionation (20 to 30 per cent saturation) and DEAE–cellulose column chromatography. The immunoreactivity of human fetal liver cytochrome P450 to the partially purified anti-cytochrome P450 antibody was examined using Ouchterlony double diffusion analysis [17].

Materials. Emulgen 913, a non-ionic detergent, was kindly supplied by Kao–Atlas Co., Japan. SKF 525-A was a generous gift from Smith, Kline and French Laboratories. Dilauroyl-L-3-phosphatidylcholine was purchased from Serdary Research Laboratories, Canada. NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and cytochrome *c* (horse heart) were purchased from Boehringer Mannheim. Commer-

Table 1. Partial purification of cytochrome P450 from humal fetal livers

	Total protein (mg)	Total P450 content (nmole)	Specific P450 content (nmole/mg protein)
Experiment 1			
105,000 *g* ppt.	420	76.2	0.18
ω-Aminooctyl column eluate	10.2	43.9	4.3
Hydroxylapatite column eluate	2.9	19.8	6.7
Experiment 2			
ω-Aminooctyl column eluate	8.0	19.4	2.4
Hydroxylapatite column eluate	2.7	10.3	3.8
Experiment 3			
ω-Aminooctyl column eluate	10.9	7.6	0.7
Hydroxylapatite column eluate	0.3	1.4	4.7

Whole or parts of fetal livers from one (experiment 1, about 7 months of gestation), two (experiment 2, about 7 months of gestation) and five fetuses (experiment 3, about 3 to 4 months of gestation) were used. The wet weight of the livers employed for each experiment ranged from 13.0 to 15.0 g.

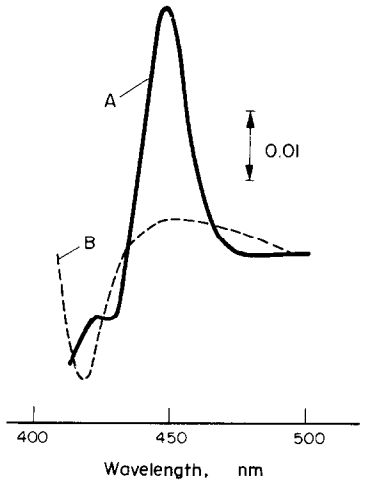

Fig. 1. Carbon monoxide-reduced minus reduced (A) and reduced minus oxidized (B) difference spectra of purified human fetal liver cytochrome P450. The difference spectra of the purified cytochrome P450 (0.407 μM) was measured in the presence of 100 mM potassium phosphate (pH 7.25), 20% glycerol and 0.2% Emulgen 913.

cial aniline was twice distilled under vacuum and the distillate was stored under an atmosphere of nitrogen at −20° until use. Other chemical reagents were purchased from commercial sources and were used without further purification. Sepharose 4B was purchased from Pharmacia Fine Chemicals and hydroxylapatite (Bio-Gel HT) from Bio-Rad. The ω-amino-n-octyl derivative of Sepharose 4B was prepared from cyanogen bromide-activated Sepharose 4B and 1, 8-diaminooctane by a method described by Cuatrecasas [22].

RESULTS

Partial purification of cytochrome P450 from human fetal liver. The carbon monoxide difference spectrum of washed 105,000 *g* precipitate of human fetal liver homogenate indicated that a considerable amount of hemoglobin was still present in the preparation (data not shown). The application of the solubilized sample to ω-amino-n-octyl Sepharose 4B column resulted in a complete separation of hemoglobin and other cytochromes from cytochrome P450. Hashimoto and Imai [14] reported that a species of cytochrome P450 induced by 3-methylcholanthrene was successfully separated from the other cytochrome P450 species by washing the affinity column with an increased concentration of Emulgen 913. Therefore, we washed the column with a buffer containing 0.5% Emulgen 913 after elution of cytochrome P450 with the buffer containing 0.08% Emulgen 913. However, no detectable cytochrome P450 was eluted by this procedure. The sample of cytochrome P450 eluted from the ω-amino-n-octyl Sepharose 4B column was applied to a column of hydroxylapatite. The column was washed with 200 mM potassium phosphate (pH 7.25) containing 20% glycerol. This procedure eluted a trace amount of cytochrome P450 but the majority remained bound to the hydroxylapatite column. Cytochrome P450 was eluted from the column by washing with 500 mM potassium phosphate (pH 7.25) containing 20% glycerol. The results of three experiments are shown in Table 1. The specific content of cytochrome P450 isolated in this manner ranged from 3.8 to 6.7 nmoles per mg of protein. The purified samples did not contain detectable amounts of NADH–cytochrome b_5 reductase, NADPH–cytochrome P450 reductase or cytochrome b_5.

Spectral properties of partially purified cytochrome P450 from human fetal liver. Reduced minus oxidized

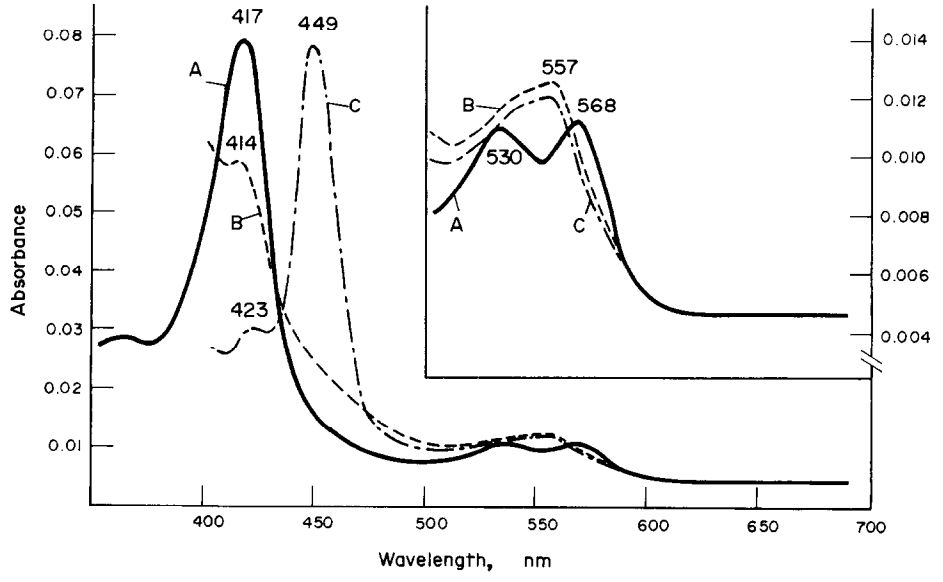

Fig. 2. The absolute spectra of cytochrome P450 isolated from humal fetal livers. The absolute spectra of the purified cytochrome P450 (2.44 μM) was recorded in the presence of 100 mM potassium phosphate (pH 7.25), 20% glycerol and 0.4% Emulgen. Curve A: oxidized form; curve B: reduced form; and curve C: carbon monoxide-bound reduced form.

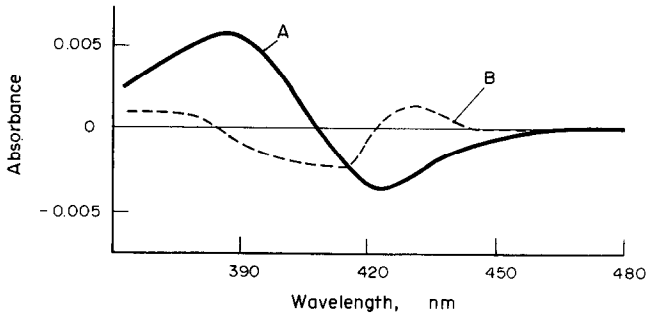

Fig. 3. Substrate-induced difference spectra of purified human fetal liver cytochrome P450. The difference spectrum of the purified cytochrome P450 (0.488 μM) was recorded after addition of 0.25 mM SKF 525-A or 5 mM aniline (curve B) in the presence of 100 mM potassium phosphate (pH 7.25) and glycerol.

difference spectrum of cytochrome P450 and the carbon monoxide difference spectrum of reduced cytochrome P450 are shown in Fig. 1. The reduced minus oxidized difference spectrum exhibited a broad peak from 440 to 450 nm which is comparable to those observed using cytochrome P450 isolated from experimental animals [12]. The carbon monoxide-reduced minus reduced difference spectrum of the cytochrome exhibited a peak at 449.5 to 450 nm. The partially purified preparation of cytochrome P450 was unstable after the addition of dithionite. The addition of 0.2% Emulgen 913 resulted in a partial stabilization of the cytochrome P450. The unstable nature and the stabilization by Emulgen 913 appeared to be similar to that reported by Imai and Sato [13] using purified cytochrome P450 isolated from phenobarbital-pretreated rabbit liver microsomes. The absolute spectra of cytochrome P450 in oxidized, reduced and carbon monoxide-bound reduced forms are shown in Fig. 2. The oxidized form had peaks at 417, 530 and 568 nm. The presence of a peak at 417 nm may suggest that this cytochrome P450 is a low spin species. The reduced form exhibited peaks at 414 and 557 nm and the carbon monoxide-bound reduced form peaks at 449 and 557 nm.

It is known that a characteristic difference spectrum is seen when a substrate binds to cytochrome P450 [23, 24]. The difference spectra induced by SKF 525-A and aniline are shown in Fig. 3. The addition of SKF 525-A produced a typical type I difference spectrum having a peak at 387 nm and a trough at 422 nm. Aniline induced a type II difference spectrum, which had a peak at 430 nm and a trough at 414 nm. Chemical compounds including drugs have been classified into two or three groups depending upon the types of difference spectra when bound to cytochrome P450 [23]. According to the reported classification, aminopyrine, ethylmorphine, dimethylaniline and SKF 525-A are type I compounds and aniline a type II compound. However, Yaffe *et al.* [2] have reported that aminopyrine exhibited a type II difference spectrum when added to human fetal liver microsomes. Rane *et al.* [8] also demonstrated that aminopyrine exhibited a modified type II difference spectrum and desmethylimipramine did not show a measurable difference spectrum using human fetal liver microsomes. Pelkonen [5] also reported a type II difference spectrum using aminopyrine and hexobarbital. Further, Rane and Ackerman [25] indicated that ethylmorphine exhibited a reverse type I difference spectrum. No type I difference spectra have been reported using humal fetal liver microsomes except with endogeneous substrates such as testosterone and laurate [2]. As shown in Fig. 3, SKF 525-A induced a type I difference spectrum when added to the partially purified human liver cytochrome P450. This result may suggest that human cytochrome P450 contains a type I binding site(s) for foreign compounds as well as for endogeneous substrates.

Catalytic properties of partially purified cytochrome P450 from human fetal liver. The catalytic activity of the cytochrome was also examined using the isolated cytochrome P450, NADPH–cytochrome P450 reductase purified from phenobarbital-pretreated rat liver microsomes and dilauroyl-L-3-phosphatidylcholine. The isolated cytochrome P450 catalyzed aniline hydroxylation at a faster rate than ethylmorphine *N*-demethylation (Table 2). The requirement for lipid for maximal activity of drug oxidations has been reported using cytochrome P450 purified from experimental animals [26–29]. Our results also indicated that lipid

Table 2. Activity of cytochrome P450 isolated from human fetal livers for aniline hydroxylation and ethylmorphine *N*-demethylation

Conditions	Aniline hydroxylation (nmole/nmole cytochrome P450/min)	Ethylmorphine *N*-demethylation (nmole/nmole cytochrome P450/min)
Complete system	0.69	0.49
–Cytochrome P450	0.09	0.05
–Reductase	0.01	0.01
–NADPH	0.00	0.00
–Lipid	0.34	0.10

was required for reconstitution of the drug oxidation system using human fetal cytochrome P450. Of the activities measured, aniline hydroxylation activity has been reproducibly detectable using human fetal liver [4–6, 25, 30–33], whereas the activities for other substrates including aminopyrine [2, 4–6, 31–33] and ethylmorphine [25, 31] varied markedly. These findings suggest that a species of cytochrome P450 catalyzing aniline hydroxylation exists in higher amounts in human fetal liver than other species. As shown in Table 2, we found higher activity for aniline hydroxylation with our preparation than for ethylmorphine *N*-demethylation.

The immunological properties of the partially purified cytochrome P450 was examined using antibody to cytochrome P450 purified from the livers of phenobarbital-pretreated rats. The cytochrome P450 of human fetal liver did not cross-react with the antibody as judged using Ouchterlony double diffusion analysis (not shown).

DISCUSSION

It has been reported that the physical properties of the fetal liver endoplasmic reticulum are different from the adult. Homogenization of fetal liver caused the endoplasmic reticulum to form long, slender cistanae that sedimented at lower *g* values [34]. Consequently, most of the cytochrome P450 is found in the 200*g* pellet [3]. This evidence led us to use the 105,000*g* pellet of human fetal liver homogenates rather than microsomal precipitates for purification of cytochrome P450.

Fetal livers used in these experiments were isolated from mothers for medical reasons. The mothers had ingested some drugs including phenobarbital and diphenylhydantoin which are known to induce drug metabolizing enzymes of experimental animals [35]. Rane *et al.* [36] reported that pretreatment of pregnant rabbits near term with phenobarbital induced the fetal liver cytochrome P450. In addition, Pecile *et al.*[37] reported that pretreatment of rats and rabbits with phenobarbital resulted in increased activity for strychine hydroxylation. Hexobarbital hydroxylation activity has been reported to be detectable in fetal liver of rabbits if the mothers had been treated with phenobarbital [38]. On the contrary, Gunthner and Mannering quite recently reported that phenobarbital administered to pregnant rats did not induce cytochrome P450 and ethylmorphine *N*-demethylation activity of fetal livers [39]. Experiments designed to evaluate the inducibility of drug oxidizing activity in human fetal liver by phenobarbital, other drugs, and cigarette smoking have yielded equivocal [40] or negative results [41]. However, the possibility that our partially purified preparation of cytochrome P450 contains a specific species induced by these drugs cannot be excluded.

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REFERENCES

1. B. H. Dvorchick. V. G. Stenger and S. L. Quattropane, *Drug Metab. Dispos.* **2**, 539 (1974).
2. S. J. Yaffe, A. Rane, F. Sjöqvist, L.-O. Boreus and S. Orrenius, *Life Sci.* **9**, 1189 (1970).
3. E. Ackerman, A. Rane and J. L. E. Ericsson, *Clin. Pharmac. Ther.* **13**, 652 (1972).
4. M. R. Juchau and M. G. Pedersen, *Life Sci.* **12**, pt.II, 193 (1973).
5. O. Pelkonen, *Biochem. Pharmac.* **22**, 2357 (1973).
6. O. Pelkonen, E. H. Kaltiala, T. K. I. Larmi and N. T. Kärki, *Clin. Pharmac. Ther.* **14**, 840 (1973).
7. A. Rane, F. Sjöqvist and S. Orrenius, *Clin. Pharmac. Ther.* **14**, 666 (1973).
8. A. Rane, F. Sjöqvist and S. Orrenius, *Chem.-Biol. Interact.* **3**, 305 (1971).
9. C. Bahr, A. Rane, S. Orrenius and Sjöqvist, *Acta pharmac. Tox.* **34**, 58 (1974).
10. A. B. Rifkind, S. Bennett, E. S. Forster and M. I. New, *Biochem. Pharmac.* **24**, 839 (1975).
11. O. Pelkonen and N. T. Kärki, *Acta Pharmac. Tox.* **30**, 158 (1971).
12. Y. Imai and R. Sato, *J. Biochem., Tokyo* **75**, 689 (1974).
13. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **60**, 8 (1974).
14. C. Hashimoto and Y. Imai, *Biochem. biophys. Res. Commun.* **68**, 821 (1976).
15. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. T. Kamataki, D. H. Belcher and R. A. Neal, *Molec. Pharmac.* **12**, 921 (1976).
18. S. Takesue and T. Omura, *J. Biochem., Tokyo* **67**, 259 (1970).
19. A. H. Phillips and R. G. Langdon, *J. biol Chem.* **237**, 2652 (1962).
20. T. Nash, *Biochem. J.* **55**, 416 (1953).
21. Y. Imai, A. Ito and R. Sato, *J. Biochem., Tokyo* **60**, 417 (1966).
22. P. Cuatrecasas, *J. biol. Chem.* **245**, 3059 (1970).
23. J. B. Shenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
24. Y. Imai and R. Sato, *J. Biochem., Tokyo* **62**, 238 (1967).
25. A. Rane and E. Ackerman, *Clin. Pharmac. Ther.* **13**, 663 (1972).
26. A. Y. H. Lu, K. W. Junk and M. J. Coon, *J. biol Chem.* **244**, 3714 (1969).
27. A. Y. H. Lu, H. W. Strobel and M. J. Coon, *Biochem. biophys. Res. Commun.* **36**, 545 (1969).
28. H. W. Strobel, A. Y. H. Lu, J. Heidma and M. J. Coon, *J. biol Chem.* **245**, 4851 (1970).
29. M. J. Coon, A. P. Autor and H. W. Strobel, *Chem.-Biol. Interact,* **3**, 248 (1971).
30. E. Ackerman and A. Rane, *Chem. -Biol. Interact* **3**, 233 (1971).
31. T. Kamataki, M. Kitada and H. Kitagawa, *Chem. pharm. Bull., Tokyo* **21**, 2329 (1973).
32. O. Pelkonen and N. T. Kärki, *Biochem. Pharmac.* **22**, 1538 (1973).
33. O. Pelkonen, *Res. Commun. Chem. Path. Pharmac.* **10**, 293 (1975).
34. I. B. Chatterjee, Z. H. Price and P. W. Mckee, *Nature Lond.* **207**, 1168 (1965).
35. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
36. A. Rane, M. Berggreen, S. Yaffe and J. L. E. Ericsson, *Xenobiotica* **3**, 37 (1973).
37. A. Pecile, E. Chiesara, C. Frinzi and F. Conti, in *The Foeto-Placental Unit* (Eds. A. Pecile and C. Finzi) p.255. Excerpta Med. Found. Amsterdam (1969).
38. L. G. Hart, R. H. Adamson, R. L. Dixon and J. R. Fouts, *J. Pharmac. exp. Ther.* **137**, 103 (1962).
39. T. M. Guenthner and G. J. Mannering, *Biochem. Pharmac.* **26**, 567 (1977).
40. O. Pelkonen, P. Jouppila and N. T. Kärki, *Archs. int. Pharmacodyn. Thér.* **202**, 288 (1973).
41. O. Pelkonen, P. Jouppila and N. T. Kärki, *Toxic. appl. Pharmac.* **23**, 399 (1972).