Three immunoidentical cytochromes *P*-450 from liver microsomes of phenobarbital-treated rats

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(Received 31 March 1983/Accepted 13 June 1983)

Three forms of cytochrome *P*-450 were purified to homogeneity from liver microsomes of Wistar-strain rats treated with phenobarbital. They had minimum mol.wts. of 52 000, 53 000 and 54 000 as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and are designated as *P*-450(L), *P*-450(M) and *P*-450(H) respectively. They were shown to be immunoidentical by Ouchterlony double-diffusion analysis. Several criteria, such as isoelectric points, substrate specificities and sensitivities to tryptic digestion, however, indicated that these cytochromes are distinct isoenzymes of cytochrome *P*-450. Whereas *P*-450(L) was highly active on various substrates, *P*-450(H) had generally low catalytic activities, except on aminopyrine. The cytochromes purified by immunoaffinity chromatography using anti-*P*-450(L) showed a marked variation in their distribution depending on the strain and colony of rat. Limited tryptic digestion of *P*-450(H) gave one tryptic peptide showing the same mobility as *P*-450(L) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and their primary structures were very similar. The result suggests a possibility that such limited proteolysis is involved in the post-translational modification of the cytochrome or its destruction.

Liver microsomes catalyse the oxidation of various xenobiotics and most of these reactions are catalysed by the cytochrome *P*-450 system (Sato, 1978; Omura, 1978). This diversity in substrate specificity of the system has been shown to be attributed to the presence of multiple forms of cytochrome *P*-450 and thus the problem of how many forms of the cytochrome exist has become an important one. Thus, the term 'cytochrome *P*-450' should be regarded as a collective term for a group of haemoproteins. Recently, multiple forms of cytochrome *P*-450 have been shown in an animal treated with one of the specific inducers such as phenobarbital or 3-methylcholanthrene (Sladek & Mannering, 1966; Ryan *et al.*, 1979; Imai *et al.*, 1980). The studies on the purified cytochromes have increased their importance therefore for elucidation of their molecular multiplicity in conjunction with the investigations at a gene level (Nebert *et al.*, 1981; Fujii-Kuriyama *et al.*, 1982).

In the present paper, we describe the purification to homogeneity of three immunoidentical forms of cytochrome *P*-450 from liver microsomes of Wistar-

strain rats treated with phenobarbital. They can apparently be distinguished on the basis of molecular weight, isoelectric point and substrate specificity, though they are immunochemically indistinguishable. Further, we show apparent variations of phenobarbital-induced cytochromes *P*-450 related to strains and colonies of rats. The results obtained by tryptic digestion of the cytochrome with the highest molecular weight suggest that the multiple forms observed are related to the post-translational modification or the degradation of the cytochrome.

Materials and methods

Animals and preparation of microsomal fraction

Male Wistar rats weighing 250–300 g were obtained from the Kyushu Experimental Animal Center and were used for the purification of cytochromes *P*-450. Rats of Sprague–Dawley, Donryu and Wistar strains weighing 100–150 g and obtained from the Animal Center at Kyushu University were used for immunoaffinity purification of the cytochrome. The animals were injected intraperitoneally with phenobarbital dissolved in 0.9% NaCl (100 mg/kg body wt.) once a day for 1 week. They were starved overnight and killed by bleeding 24 h after the last injection. Microsomal

Abbreviations used: SDS, sodium dodecyl sulphate; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

fraction was prepared and washed with the same medium as described previously (Hino & Minakami, 1981).

Purification of cytochrome P-450 and cytochrome P-450 reductase

Purification of cytochrome *P*-450 was carried out essentially as described by Harada & Omura (1981). A microsomal preparation containing 7 μ mol of cytochrome *P*-450 was extracted with sodium cholate (2.5 mg/mg of microsomal protein) in 100 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1 mM-EDTA, 1 mM-dithiothreitol, 2 μ g of pepstatin/ml and 2 μ g of leupeptin/ml. Cytochrome *P*-450 was purified by columns of amino-octyl-Sepharose 4B and hydroxyapatite successively, and then dialysed.

Separation of cytochrome *P*-450 isoenzymes was done as follows. The sample was applied to a DEAE-Sepharose column (2.0 cm $\times$ 10 cm) equilibrated with 5 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 0.2% (w/v) Emulgen 913, 0.2% sodium cholate and 1 mM-EDTA. After the column was washed with the equilibrating buffer, cytochromes *P*-450 were eluted by increasing the concentration of potassium phosphate stepwise to 10 mM, 20 mM, 30 mM and occasionally to 40 mM, at room temperature. The fractions containing a cytochrome species homogeneous on SDS/PAGE were combined. Three cytochromes *P*-450 were obtained and were designated as *P*-450(H), *P*-450(M) and *P*-450(L), in order of decreasing molecular weight. They were dialysed against 15 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol and 1 mM-EDTA, and freed from the detergents and concentrated by CM-Sephadex chromatography. The final samples were again checked by SDS/PAGE and stored at -70°C . The specific content of three samples was about 13 nmol/mg of protein.

NADPH-cytochrome *P*-450 reductase was eluted from the amino-octyl-Sepharose 4B column by the procedure described by Imai (1976) and further purified by affinity chromatography on 2',5'-ADP-Sepharose (Pharmacia) column as reported by Yasukochi & Masters (1976). One unit of the reductase was defined as the amount catalysing the reduction of 1.0 μ mol of cytochrome *c* per min at 30°C assayed by the method of Dignam & Strobel (1975).

Immunoaffinity purification of cytochromes P-450

The cytochromes were purified on the columns of anti-*P*-450(L) antibody bound to Sepharose 4B. Microsomes, which contained 100–200 nmol of cytochrome *P*-450, were solubilized by suspending in a buffer [20 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 0.9% NaCl,

1 mM-EDTA, 1% (w/v) Emulgen 913, 5 μ g of pepstatin/ml and 5 μ g of leupeptin/ml] at the protein concentration of 2.0 mg/ml. The mixture was stirred in ice for 30 min and centrifuged at 77 000 *g* for 90 min. The antibody-Sepharose 4B (2 g) equilibrated with the buffer was added to the supernatant and was stirred for 1 h at room temperature and overnight at 4°C successively. The gel was washed with the buffer and packed into the column (1.0 cm $\times$ 2.5 cm). The column was thoroughly washed with 300 ml of the buffer containing 100 mM-potassium phosphate and then with 100 ml of the equilibrating buffer. Then, tightly bound proteins were eluted with the buffer containing 3 M-KSCN. The eluates were dialysed against 10 mM-phosphate buffer containing glycerol and EDTA.

Analytical procedures

Metabolic studies with the reconstituted cytochrome *P*-450 system were done essentially as described by Imai (1981). The standard system (final volume, 1.0 ml) contained 0.1 nmol of cytochrome *P*-450, 0.4 unit of NADPH-cytochrome *P*-450 reductase, 8 μ g of (dilauroyl)phosphatidylcholine (didodecylglycerophosphocholine), 0.01% sodium cholate, 50 mM-potassium phosphate buffer, pH 7.25, 0.1 mM-NADPH and a substrate, and the assays were done at 25°C . When the substrate was benzphetamine, aminopyrine or 1-nitropropane, 20 mM-glucose 6-phosphate, 3 mM-MgCl₂ and 1 unit of glucose 6-phosphate dehydrogenase/ml were added for the generation of NADPH. *N*-Demethylation or denitrification activities with these substrates were assayed by the determination of formaldehyde or nitrite with Nash reagent (Nash, 1953) or diazo reagent (Nicholas & Nason, 1957). The formation of *p*-nitrophenol from *p*-nitroanisole or *p*-nitrophenetole was measured spectrophotometrically with a model 556 Hitachi spectrophotometer and the formation of 7-hydroxycoumarin from 7-ethoxycoumarin was determined fluorimetrically with a model 650-40 Hitachi fluorescence spectrophotometer, the initial slopes of the reactions being taken for the calculation of the rates in both cases.

Spectra of cytochromes *P*-450 were taken with a model 340 Hitachi spectrophotometer. They were quantified from CO-reduced difference spectra of samples using an absorption coefficient of 91 mM⁻¹·cm⁻¹ for the difference between the peak (449 or 450 nm) and absorption at 490 nm as described by Omura & Sato (1964). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. SDS/polyacrylamide-slab-gel electrophoresis was done by the procedure of Laemmli & Favre (1973). The separating gel was 2.0 mm thick and 12 cm long. The peptide mappings of *P*-450(H) and *P*-450(L) were done by a reverse-phase high-performance liquid

chromatography essentially as described by Ozols *et al.* (1981).

Materials

Antibodies against *P*-450(L) were raised in a male rabbit by injecting 0.2, 0.1 and 0.1 mg of the cytochrome emulsified with Freund's complete adjuvant, successively at 10-day intervals. At 10 days after the last immunization, 0.05 mg of the cytochrome was injected intravenously as a booster. The immunoglobulin fraction was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose (DE-52) column chromatography. The Sepharose 4B gel (40 g) activated with CNBr was suspended in 40 ml of 0.1 M-carbonate buffer, pH 9.0, at 4°C, mixed with the antibodies (200 mg of protein) in 20 ml of the carbonate buffer and stirred overnight at 4°C. The antibody-Sepharose 4B was stored at 4°C in suspension with 10 mM-potassium phosphate buffer, pH 7.25. Amino-octyl-Sepharose 4B and hydroxyapatite were prepared as described by Cuatrecasas (1970) and Tiselius *et al.* (1956).

Leupeptin and pepstatin were obtained from Protein Research Foundation (Osaka, Japan), NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase from Oriental Yeast Co. Ltd. (Tokyo, Japan), 1-nitropropane, *p*-nitroanisole, *p*-nitrophenetole and *p*-nitrophenol from Nakarai Chemical (Kyoto, Japan), 7-ethoxycoumarin from Aldrich Chemical Company (Milwaukee, WI, U.S.A.), trypsin from Boehringer G.m.b.H. (Mannheim, Germany) and 'TPCK-trypsin' from Millipore Corp. (Freehold, NJ, U.S.A.). Cholic acid was obtained from Nissui Seiyaku Co. (Tokyo, Japan) and was purified as described by Imai & Sato (1974). Benzphetamine was generously given by Dr. Y. Imai, Institute for Protein Research, Osaka University, and Emulgen 913 by Kao-Atlas Co. (Tokyo, Japan). All other chemicals were of the highest purity commercially available.

Results

Separation of P-450 isoenzymes induced with phenobarbital

Three forms of cytochrome *P*-450 from Wistar-strain rats treated with phenobarbital were purified by column chromatography on DEAE-Sepharose at room temperature. We have termed them '*P*-450(H)', '*P*-450(M)' and '*P*-450(L)' according to their order of decreasing molecular weights. *P*-450(L) was eluted from the column with 10 mM-potassium phosphate buffer and *P*-450(M) followed it. *P*-450(H) could only be eluted from the column with the buffer of higher concentrations. The eluate was examined by SDS/PAGE and the fractions containing only one species of the cytochrome was pooled and used for further studies. SDS/PAGE profiles of the purified samples were shown (Fig. 1),

Fig. 1. SDS/PAGE of purified cytochromes *P*-450

The cytochrome isoenzymes were analysed by SDS/PAGE. (7.5%). The gel was stained with Coomassie Brilliant Blue R-250. Lanes 2 and 6, mixture of three isoenzymes; lane 3, *P*-450(H); lane 4, *P*-450(M); lane 5, *P*-450(L). The amount of each isoenzyme was 0.5 µg. The molecular weights of the standard proteins (lanes 1 and 7) were RNA polymerase subunits (Mitsubishi Yuka, Osaka, Japan): β' , M_r 180 000; β , M_r 140 000; X, M_r 100 000; α , M_r 42 000; Z, M_r 39 000.

in which the minimum molecular weights of *P*-450(H), *P*-450(M) and *P*-450(L) were 54 000, 53 000 and 52 000 respectively. They gave single spots in two-dimensional isoelectric focusing combined with SDS/PAGE (profiles not shown), in which *P*-450(H) was most acidic and *P*-450(L) most basic in agreement with the order of the elution from the DEAE-Sepharose column.

Immunochemical properties

The antibodies against *P*-450(L) gave a fused single precipitin line with purified *P*-450(L) and the microsomal extract of phenobarbital-treated Wistar rats by Ouchterlony double-diffusion test as shown in Fig. 2(a), suggesting that the antibodies react only with microsomal proteins having the same immunochemical properties as *P*-450(L). Immunological identity of *P*-450(H) and *P*-450(M) with *P*-450(L) was shown by a single precipitin line in the test (Fig. 2b). The diffusion plates were stained with Co-

Fig. 2. Ouchterlony double-diffusion test with anti-*P*-450(L)

The tests were done in 1.0% agarose containing 100mM-potassium phosphate buffer, pH 7.25, 0.2% Emulgen 913 and 0.02% NaN_3 at room temperature. (a) Centre well (C), anti-*P*-450(L) (100 μg); well 1, *P*-450(L) (3 μg); well 2, *P*-450(H) (3 μg); well 4, mixture of *P*-450(L) and *P*-450(H) (each 2 μg); wells 3 and 5, solubilized microsomes from rats treated with phenobarbital (40 μg). (b) Centre well (C), anti-*P*-450(L) (150 μg); wells 1 and 4, *P*-450(H); wells 2 and 5, *P*-450(M); wells 3 and 6, *P*-450(L). The amount of each isoenzyme was 5 μg .

massie Brilliant Blue R-250 for revealing faint spurs but no spur was observed, indicating that the three isoenzymes have identical immunochemical determinants. The identity was further confirmed by immunoaffinity chromatography.

Spectral properties

Absolute spectra of the three *P*-450 species at room temperature in the presence of the detergent

Emulgen 913 are shown in Fig. 3. The oxidized, reduced and reduced-plus-CO spectra of them were essentially similar but with some minor differences in the peaks. For example, the peak at 568 nm in *P*-450(H) was at 567 nm for *P*-450(M) and at 565 nm for *P*-450(L) in oxidized spectra, and in reduced-plus-CO spectra the Soret peak of *P*-450(H) was at 450 nm, whereas the peaks were at 449 nm for the other two isoenzymes. A broad peak around 416 nm and a small absorption band around 647 nm in the oxidized state indicate that the samples contained a high-spin component at the conditions employed.

Fig. 3. Absolute spectra of *P*-450 isoenzymes. The spectra were recorded in 100mM-potassium phosphate buffer (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913, at room temperature with the reference cuvette containing the same buffer mixture. (a) *P*-450(H) (1.1 nmol/ml); (b) *P*-450(M) (0.5 nmol/ml); (c) *P*-450(L) (1.0 nmol/ml). —, oxidized; ----, reduced; - · - · -, reduced-plus-CO. The right-hand scale refers to the expanded spectra between 500 nm and 700 nm.

Table 1. *Catalytic activities of P-450(H), P-450(M) and P-450(L)*

The assays were performed as described in the Materials and methods section. When benzphetamine, 1-nitropropane and aminopyrine were used as substrates, the reactions were run for 10, 15 and 30 min respectively. Catalytic activities are expressed as nmol of the product formed/min per nmol of the cytochrome. Abbreviation used: n.d., not detectable.

Substrate	<i>P</i> -450(H)	<i>P</i> -450(M)	<i>P</i> -450(L)
Aminopyrine (10 mM)	5.2	n.d.	4.9
Benzphetamine (1 mM)	9.2	16.6	43.8
7-Ethoxycoumarin (0.5 mM)	2.7	11.3	9.2
1-Nitropropane (25 mM)	1.0	28.3	4.0
<i>p</i> -Nitroanisole (1 mM)	n.d.	1.8	2.2
<i>p</i> -Nitrophenetole (2 mM)	2.1	6.8	8.2

Catalytic activities

Table 1 shows the catalytic activities of three isoenzymes toward several substrates. *P*-450(L), the smallest one, showed high catalytic activities with most of the substrates, especially with benzphetamine, which has been known as a good substrate for the cytochrome *P*-450 induced by phenobarbital. *P*-450(H) had low catalytic activities, in general, except that aminopyrine was efficiently metabolized. *P*-450(M) had activities intermediate between the other two isoenzymes but the activity with 1-nitropropane was several times higher than that of *P*-450(H) and the activity with aminopyrine was negligible. Thus, the substrate specificities of these three isoenzymes were distinctly different.

Distribution of the isoenzymes in rats of different strains and colonies

Liver microsomes of rats of three strains (Wistar, Sprague–Dawley and Donryu) were solubilized with Emulgen 913 and applied to anti-*P*-450(L) antibody–Sephacrose 4B. Wistar rats from two colonies were used, one from the Animal Center at Kyushu University (Wistar-KU) and the other from the Kyushu Experimental Animal Center (Wistar-KEA). The latter strain was used for the purification of the isoenzymes. Cytochromes *P*-450 bound to the gels were eluted with 3 M-KSCN and analysed by SDS/PAGE (Fig. 4). Three bands corresponding to *P*-450(H), *P*-450(M) and *P*-450(L) were observed in Wistar-KEA rats (lane 3), whereas the band corresponding to *P*-450(L) was missing in Wistar-KU rats (lane 2). Sprague–Dawley rats gave two bands with the same mobilities as *P*-450(M) and *P*-450(L) with a faint band corresponding to *P*-450(H) in mobility (lane 4). Donryu rats gave only one band corresponding to *P*-450(M) in mobility (lane 5).

Fig. 4. SDS/PAGE (7.5% polyacrylamide) profiles of *P*-450 isoenzymes obtained by the immunoaffinity method from rats of different strains and colonies

Solubilized microsomes from phenobarbital-treated rats were treated with anti-*P*-450(L)–Sephacrose 4B. Tightly bound proteins were eluted with 3 M-KSCN as described in the Materials and methods section. Lane 2, the proteins from Wistar-KU rats; lane 3, proteins from Wistar-KEA rats; lane 4, proteins from Sprague–Dawley rats; lane 5, proteins from Donryu rats; lanes 1 and 6, the mixture of *P*-450(H) and *P*-450(L).

Tryptic digestion of the isoenzymes

Three cytochromes *P*-450 were digested with trypsin and analysed by SDS/PAGE. When *P*-450(H) was digested with a small amount of trypsin, no large fragments were observed by SDS/PAGE (Fig. 5, lanes 2–4) but with a relatively large amount of trypsin, the band with the same mobility as *P*-450(L) appeared (lanes 6 and 7), suggesting that *P*-450(H) was transformed into a fragment with similar size to *P*-450(L) and then rapidly hydrolysed to small peptides. Fig. 6 shows time courses of the tryptic digestion of three isoenzymes. When the mixture of *P*-450(M) and *P*-450(L) was digested (lanes 1–5), *P*-450(L) disappeared rapidly and *P*-450(M) remained (lanes 4 and 5). The digestion of *P*-450(H) gave rise to a band with mobility similar to

Fig. 5. SDS/PAGE. (10% polyacrylamide) profiles of *P-450(H)* digested with various amounts of trypsin. *P-450(H)* ($3\mu\text{g}$) in 100mM-potassium phosphate buffer, pH7.25, containing 20% glycerol and 0.2% Emulgen 913 in a final volume of $50\mu\text{l}$ was treated with various amounts of trypsin for 10 min at 30°C . The reaction was stopped by the addition of $10\mu\text{l}$ of 1mg of leupeptin/ml and $50\mu\text{l}$ of a solubilizing buffer (250mM-Tris/HCl buffer, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol), followed by heating in a boiling-water bath for 5 min. The amount of trypsin was as follows: lane 2, $0.15\mu\text{g}$; lane 3, $0.3\mu\text{g}$; lane 4, $0.75\mu\text{g}$; lane 6, $1.5\mu\text{g}$; lane 7, $3\mu\text{g}$; lane 9, as for lane 7 but leupeptin ($10\mu\text{g}$) was included in the digestion mixture. Lanes 1 and 10, *P-450(H)*; lanes 5 and 8, *P-450(L)*.

Fig. 6. SDS/PAGE. (7.5% polyacrylamide) profiles of *P-450* isoenzymes digested for various time intervals. The isoenzymes were digested with trypsin as in Fig. 5 except that the amount of trypsin was $2\mu\text{g}$ and the incubation times varied. Lanes 1–5 were for the mixture of *P-450(M)* and *P-450(L)* ($3\mu\text{g}$ each): lane 1, 0min; lane 2, 5min; lane 3, 10min; lane 4, 20min; lane 5, 30min. Lanes 7–11 were for *P-450(H)*: lane 11, 0min; lane 10, 5min; lane 9, 10min; lane 8, 20min; lane 7, 30min. Lane 6 contained a mixture of *P-450(H)* and *P-450(L)*.

Fig. 7. High-performance-liquid-chromatography profiles of tryptic peptides from *P-450(H)* and *P-450(L)*. The cytochromes were digested with 'TPCK-trypsin' with a trypsin/cytochrome ratio of 1:24 at room temperature overnight. The peptides (about $50\mu\text{g}$) were applied to a reversed-phase column ($4\text{mm} \times 150\text{mm}$; Merck RP18) and eluted with a gradient of 0–50% acetonitrile in 10mM- KH_2PO_4 . The flow rate was 1 ml/min and the elution of the peptides was monitored by their absorbance at 210nm. Peaks H, present only in *P-450(H)*; peak L, present only in *P-450(L)*; peaks D, different heights in maps of *P-450(H)* and *P-450(L)*.

that of *P-450(L)* as discussed above (lanes 7–11), the observation that prompted us to examine peptide maps of *P-450(H)* and *P-450(L)*. Fig. 7 shows reverse-phase high-performance liquid chromatograms of tryptic peptides from both isoenzymes. The chromatograms are very similar except that four peaks in *P-450(H)* are not observed in *P-450(L)* and a peak absent in *P-450(H)* is present in *P-450(L)*. Thus primary structures of both isoenzymes seemed to be very similar.

Discussion

The presence of phenobarbital-induced cytochrome *P-450* isoenzymes that are dependent on strains and colonies of rats have been demonstrated (Vlasuk *et al.*, 1982). Ryan *et al.* (1982a,b) have purified immunochemically identical variants from rats (Long-Evans and Holtzman strains) treated with phenobarbital and designated them as cytochrome *P-450_{bLE}*, *P-450_{bH}* and *P-450_c* according to the sources. Waxman & Walsh (1982) purified cytochromes *P-450* from phenobarbital-pretreated Sprague-Dawley rats and named them as PB-4 and PB-5, which correspond to cytochromes *P-450_b* and *P-450_c*. We have purified three immunologically identical cytochromes *P-450* from Wistar-strain rats of a single colony and designated them as *P-450(H)*, *P-450(M)* and *P-450(L)* in order of decreasing molecular weight. We consider *P-450(L)* and *P-*

450(M) may correspond to the PB-4 and PB-5 species described by Waxman & Walsh (1982), but P-450(H) corresponds to none of the cytochromes so far reported, because the one corresponding to P-450(H) was scarcely detectable in Sprague-Dawley rats. Donryu-strain rats gave only one band corresponding to P-450(M) and Wistar rats belonging to a different colony did not show a band corresponding to P-450(L) by SDS/PAGE.

Three forms of the cytochrome we have isolated had essentially the same immunochemical properties but the substrate specificities were quite different; the smallest one, P-450(L), had the highest catalytic activities for most of the substrates examined. They were also different in sensitivities to trypsin; P-450(L) seemed to be most sensitive to the proteolysis and P-450(M) most resistant. P-450(H) was at first converted into a fragment with a similar molecular weight to that of P-450(L) and then rapidly degraded into small peptides, which could not be detected by the conditions of SDS/PAGE used. Furthermore, the peptide maps of P-450(H) and P-450(L) indicated that the primary structures of the two isoenzymes were very similar. These observations together with evidence that the phenobarbital-induced cytochromes P-450 were exposed on the outer surface of microsomes (Vlasuk *et al.*, 1982) encouraged us to speculate that P-450(H) is converted into P-450(L) as a way of post-translational modification or degradation of the cytochromes.

The problem of whether there exist post-translational modifications of cytochromes P-450 has not yet been solved. Kumar & Padmanaban (1980) have shown the possibility that the cytochrome P-448 induced by 3-methylcholanthrene is synthesized as a premature form but the work of other investigators, who used the method of cell-free translation (Dubois & Waterman, 1979; Lechner *et al.*, 1979; Bar-Nun *et al.*, 1980), did not support this idea in the cytochrome precursors. Walz *et al.* (1982) have further shown that cytochromes P-450_{BLE}, P-450_{BH} and P-450_c are encoded by different mRNA species. Further studies *in vivo* and *in vitro* are necessary to solve the problem.

We are grateful to Dr. Yukio Yasukochi for his advice during the initial phase of the work and to Dr. Koichiro Takeshige for his critical comments on the manuscript. We also thank Dr. Yoshio Imai for his valuable information on the methods of metabolic studies. The work was supported in part by a grant from the Ministry of Education, Science and Culture.

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