Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 320-327

# PURIFICATION OF CYTOCHROME P-450$_{D1\alpha}$ (25-HYDROXYVITAMIN $D_3$-1α-HYDROXYLASE) OF BOVINE KIDNEY MITOCHONDRIA

Atsuo HIWATASHI, Yasuho NISHII*, and Yoshiyuki ICHIKAWA

Department of Biochemistry, Kagawa Medical School, Miki-cho, Kita-gun, Kagawa 761-07, Japan, and * Research Laboratories of Chugai Pharmaceutical Co., Takada, Toshimaku, Tokyo 171, Japan

Received December 22, 1981

SUMMARY: Cytochrome P-450$_{D1\alpha}$ purified from bovine renal mitochondria was electrophoretically homogeneous and gave a single protein band. The cytochrome P-450$_{D1\alpha}$ is an immunochemical possessing properties in common with the mitochondrial cytochrome P-450's of other tissues. The 1α-hydroxylase system of 25-hydroxyvitamin $D_3$ was reconstituted with the cytochrome P-450$_{D1\alpha}$, NADPH-renoredoxin reductase and renoredoxin, each component being essential to the 1α-hydroxylase system. The substrate specificity of the cytochrome P-450$_{D1\alpha}$ was investigated.

Two types of cytochrome P-450-linked mixed function oxidase (monooxygenase) system exist, the microsomal and the mitochondrial types (1,2). One α- and 24-hydroxylations of 25-hydroxyvitamin $D_3$(25-OH-$D_3$) of the mitochondrial type are thought to occur in kidneys (3-7).

We purified a 1α-hydroxylase system of 25-OH-$D_3$ from bovine renal mitochondria, although only a very low amount was present in comparison with the cytochrome P-450 of hepatic mitochondria (8), and tentatively named the cytochrome P-450 of 1α-hydroxylase cytochrome P-450$_{D1\alpha}$. The purified cytochrome P-450$_{D1\alpha}$ was electrophoretically a single protein band. The cytochrome P-450$_{D1\alpha}$ was an immunochemical similar to other mitochondrial cytochrome P-450's of adrenocortices and hepatocytes, which have already been purified (1). The 1α-hydroxylase system was reconstituted with NADPH-renoredoxin reductase, renoredoxin (renal ferredoxin) and the cytochrome P-450$_{D1\alpha}$, and the substrate

Abbreviations used: $D_3$, vitamin $D_3$; 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; 1α,25-$(OH)_2$-$D_3$, 1α,25-dihydroxyvitamin $D_3$; 24*R*,25-$(OH)_2$-$D_3$, 24*R*,25-dihydroxyvitamin $D_3$; cytochrome P-450$_{D1\alpha}$, cytochrome P-450 catalyzing 25-OH-$D_3$-1α-hydroxylation; cytochrome P-450$_{scc}$, cytochrome P-450 catalyzing the side-chain cleavage (lyase; desmolase) of cholesterol; cytochrome P-450$_{s21}$, cytochrome P-450 catalyzing steroid 21-hydroxylation; SDS, sodium dodecyl sulfate.
Renoredoxin and adrenodoxin are a kind of redox component, redoxin. The chemical and immunochemical properties of the ferredoxins differ even among bovine tissues. Therefore, we named the non-heme iron protein (renal ferredoxin) from renal inner mitochondrial membranes renoredoxin.

0006-291X/82/050320-08$01.00/0

specificity was investigated. This study confirmed that 24-hydroxylase and 1α-hydroxylase do not coexist in a single cytochrome P-450 molecule.

## MATERIALS AND METHODS

*Preparation of bovine kidney mitochondria*: Bovine kidneys were obtained from a local slaughterhouse within 2 h after sacrifice of the animals. The surrounding connective and fat tissues of the kidneys were removed with scissors. Next, the kidney cortices were separated and collected from the kidney medulla. The kidney cortices were homogenized with 5-fold volumes of 0.25 M sucrose solution (adjusted with Tris to pH 7.4) and the mitochondrial fraction was obtained by the method of Johnson and Lardy (9).

*Enzyme purifications*: NADPH-renoredoxin reductase was purified from bovine kidney mitochondria by the method of Ichikawa *et al.*(2). Renoredoxin was purified by the method of Ichikawa (10).

*Spectral analyses*: Optical absorption spectra were taken with Cary spectrophotometers, Models 219 and 17D, equipped with thermostatically controlled cell holders and cuvettes of 1-cm light path. A neodynium glass filter was used to obtain a standard wavelength. The cytochrome P-450 content was determined by the method of Ichikawa *et al.*(11). The cytochrome P-450 content was estimated by using $115.0 \times 10^3$ $M^{-1}cm^{-1}$ as the molar extinction coefficient between 450 and 500 nm in the difference spectrum of the sodium dithionite-reduced CO complex and the oxidized forms to exclude the complication of interference from the optical absorption spectra of any contaminating hemoglobin CO complex present.

*Electrophoresis*: To determine the purity and molecular weight of the cytochrome $P\text{-}450_{D1\alpha}$, SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (12).

*Reconstitution of the 1α-hydroxylase system of 25-OH-D3*: 25-OH-$D_3$-1α-hydroxylase activity was assayed with a 1.21-ml test system containing 0.25 μM NADPH-renoredoxin reductase, 2 μM renoredoxin, 0.1 μM cytochrome P-450, 50 nM 25-OH-$D_3$, 0.5 unit of isocitrate dehydrogenase, 0.5 mM sodium isocitrate and 1 mM $MgCl_2$, in 10 mM potassium phosphate buffer, pH 7.4. 25-OH-$D_3$ was solubilized in 95%(v/v) ethanol and used as the substrate. The test system was preincubated for 2 min at 37°C and the reaction was started by adding 0.1 mM NADPH. The reaction was carried out for 20 min with shaking in air. The reaction was stopped by the addition of 10 ml of methanol-trichloromethane (3:1 by volume) and the extraction of products was performed according to the procedure of Bligh and Dyer (13).

*Measurement of 25-OH-D3-1α-hydroxylase activity:* Chromatography of the extracted reaction product was carried out with a high performance liquid chromatograph (Hitachi HPLC, Model 635, equipped a Lichrosorb SI-100 column 4 x 250 mm). The vitamin $D_3$ ($D_3$) metabolites were monitored spectrophotometrically by the height of absorbance at 264 nm. The solvent system was 2%(v/v) methanol in dichloromethane. The reaction product was identified by high performance liquid chromatography with authentic 25-OH-$D_3$, 1α,25-dihydroxyvitamin $D_3$ [1α,25-$(OH)_2$-$D_3$] and 24*R*,25-dihydroxyvitamin $D_3$ [24*R*,25-$(OH)_2$-$D_3$] under the same conditions.

*Preparation of antibody:* Antibodies to cytochrome $P\text{-}450_{scc}$ and cytochrome $P\text{-}450_{s21}$ of bovine tissues were prepared from the blood serum of a rabbit given injections of 5 mg protein of the cytochrome in 5 ml of Freund's complete adjuvant into the foot pads. After two weeks, a booster injection of 5 mg of protein of the cytochrome was administrated. The rabbits were bled from the *Arteria carotis*, after the antibody in the rabbit blood had been confirmed by double diffusion test. The serum was separated from the blood cells and the antibody was precipitated with 50% saturated ammonium sulfate. The precipitate was dialyzed against 10 mM potassium phosphate buffer, pH 7.4, at 0°C and used for the experiments. The precipitin reaction in double diffusion agar test was performed by the method of Ouchterlony (14).

*Other analytical procedures:* The concentrations of $D_3$, 25-OH-$D_3$, 1α,25-$(OH)_2$-$D_3$ and 24*R*,25-$(OH)_2$-$D_3$ were estimated by their molar extinction coefficients at 264 nm, 18.0 x $10^3$, 18.0 x $10^3$, 18.1 x $10^3$ and 18.0 x $10^3$ $M^{-1}cm^{-1}$, respectively. The concentrations of NADPH and NADH were determined by taking the molar extinction coefficient at 340 nm as 6.30 x $10^3$ $M^{-1}cm^{-1}$. The molar extinction coefficient of cytochrome c in the reduced minus oxidized forms was 21.0 x $10^3$ $M^{-1}cm^{-1}$ at 550 nm. Renoredoxin-linked NADPH-cytochrome c reductase activity of NADPH-renoredoxin reductase was determined as described previously (2). Protein content was determined by the biuret reaction and the method of Lowry *et al.*, using bovine serum albumin as a standard protein.

*Chemicals*: DEAE-Sepharose CL-6B and Sepharose 4B were obtained from Pharmacia Fine Chemical Co., and NADPH and cytochrome c (type III) from Sigma Chemical Co. Emulgen 913 was a kind gift from Kao-Atlas Co. 25-OH-$D_3$, 1α-25-$(OH)_2$-$D_3$ and 24*R*, 25-$(OH)_2$-$D_3$ were kind gifts from Chugai Pharmaceutical Co. All other reagents were of the highest commercially available grade and were used without further purification.

## RESULTS AND DISCUSSION

*Purification of cytochrome P-450$_{D1\alpha}$*: The mitochondrial fraction of bovine kidney cortices (wet weight, about 1 Kg) was suspended at a protein concentration of about 15 mg/ml in 10 mM sodium phosphate buffer, pH 7.4, containing 20%(v/v) glycerol and 1 mM EDTA (15). This solution (2500 ml) was mixed with 100 ml of 15% (w/v) sodium cholate and stirred gently for 30 min at 4°C. After incubation of the solution, a cold 50%(w/v) polyethylene glycol (#6000) solution was added dropwise, and stirring was continued for 15 min at -10°C. The precipitate between 6 and 15% polyethylene glycol was fractionated by centrifugation at 7000 x *g* for 20 min. The supernatant, which contained most of the contaminating hemoglobin, was discarded. The precipitate was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.4% sodium cholate, then the solution was centrifiged at 105000 x *g* for 90 min, in order to discard the insoluble substances. The red-brown supernatant was collected and subjected to chromatography on DEAE-cellulose column (5 x 10 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.5% sodium cholate. The column was washed with 200 ml of the equilibrating buffer. The effluent solution was fractionated into 2-ml samples. The fractions of an absorbance higher than 0.3 at 417 nm were collected as the material source of cytochrome P-450$_{D1\alpha}$. The brown pigment, renoredoxin, remained at the top of the column. The collected solution containing cytochrome P-450$_{D1\alpha}$ was fractionated between 7.5 and 12.5% polyethylene glycol by centrifugation at 7000 x *g* for 20 min. The resulting pellet was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.24%(v/v) Emulgen 913 and the solution was centrifuged at 105000 x *g* for 90 min, in order to remove the insoluble substances. The supernatant was diluted 4-fold with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The solution was subjected to chromatography on an ω-amino *n*-octyl Sepharose column (0.8 x 10 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.06% Emulgen 913. The column

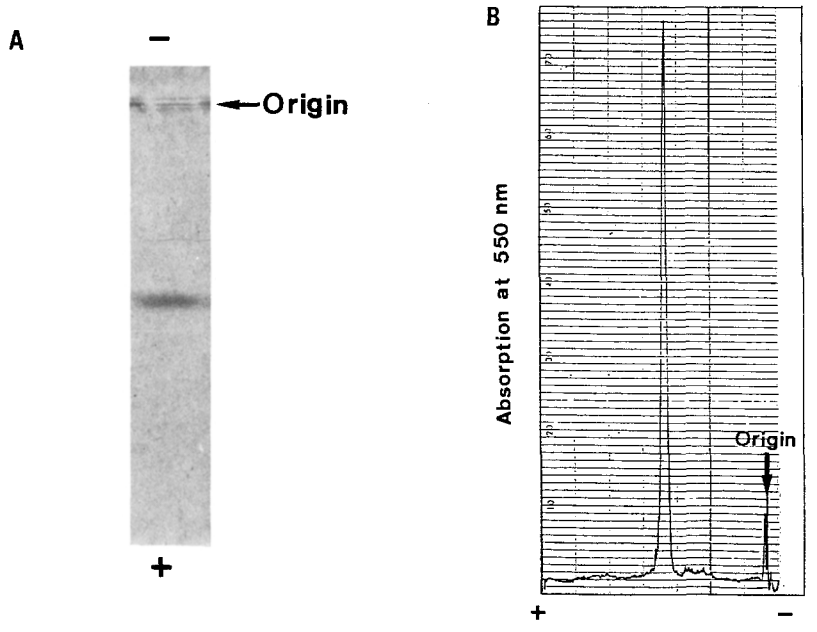


Figure 1: (A) Electrophoretic pattern of cytochrome P-450$_{D1\alpha}$ of bovine kidney mitochondria on SDS-polyacrylamide gel. A 7-μg protein sample of the cytochrome P-450$_{D1\alpha}$ was used on the gel.
(B) Densitometric scanning pattern of the gel of Figure 1(A).

was washed extensively with 100 ml of the equilibrating buffer, and then the fraction containing cytochrome P-450$_{D1\alpha}$ was eluted with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.2% Emulgen 913. The eluted cytochrome P-450$_{D1\alpha}$ solution was diluted 5-fold with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The solution was applied to DEAE-Sepharose 6B column (0.8 x 4 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.04% Emulgen 913. The column was washed with 50 ml of the equilibrating buffer, then the cytochrome P-450$_{D1\alpha}$ was eluted with 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.2% Emulgen 913. The purified cytochrome P-450$_{D1\alpha}$ was electrophoretically homogeneous and appeared as a single protein band (Figure 1). The purified cytochrome P-450 preparation could be stored at -80°C in the dark for at least 3 months without any loss of its cytochrome P-450$_{D1\alpha}$ content and 1α-hydroxylase activity of 25-OH-$D_3$. A typical purification procedures of cytochrome P-450$_{D1\alpha}$ is summarized in Table 1. The specific content of the purified cytochrome P-450$_{D1\alpha}$ was 13 nmoles per mg protein. The yield of the cytochrome P-450$_{D1\alpha}$ was about 3% on the basis of the total original content of cytochrome P-450's (multiple molecular cytochrome P-450's).

*Properties of cytochrome P-450$_{D1\alpha}$*: The optical absorption spectra of cytochrome P-450$_{D1\alpha}$ in the oxidized, reduced and CO complex forms are shown in Figure 2. The optical absorption peaks of cytochrome P-450$_{D1\alpha}$ were at 560, 534 and 414 nm in the

Table 1. Purification of cytochrome P-450$_{D1\alpha}$ from bovine kidney mitochondria

| Fraction | Total volume (ml) | Total protein (mg) | Cytochrome P-450 | | Purification (-fold) | Yield (%) | Specific activity[c] |
|---|---|---|---|---|---|---|---|
| | | | Total content[a] | Specific content[b] | | | |
| Mitochondria | 2500 | 37500 | 2625 | 0.07 | 1.0 | 100 | 0.24 |
| Cholate extract | 2170 | 20500 | 2255 | 0.11 | 1.6 | 86 | 0.41 |
| Polyethylene glycol fractionation(6-15%) | 1350 | 10140 | 1927 | 0.19 | 2.7 | 73 | 0.83 |
| DEAE-cellulose column eluate | 320 | 1866 | 896 | 0.48 | 6.9 | 34 | 1.86 |
| Aminooctyl Sepharose column eluate | 8 | 7 | 85 | 12.2 | 174.3 | 3 | 89.1 |
| DEAE-Sepharose column eluate | 4 | 6 | 79 | 13.1 | 187.1 | 3 | 99.7 |

[a] nmoles; [b] nmoles/mg protein; [c] 25-OH-$D_3$-1$\alpha$-hydroxylase activity, pmoles/min/mg protein.

oxidized form. The peaks in the reduced form were at 550 and 417 nm. The CO complex form had peaks at 553, 450 and 420 nm. The heme-iron of the oxidized cytochrome

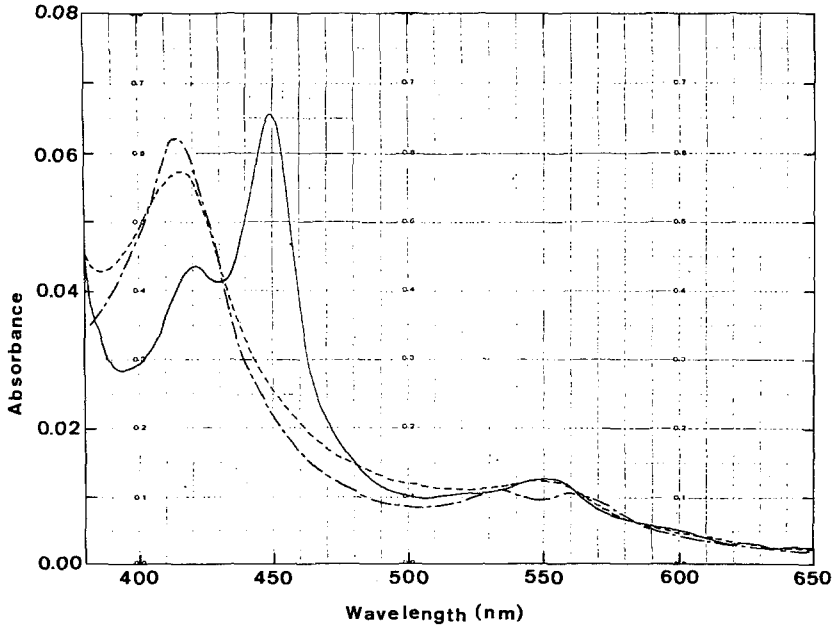


Figure 2: Absolute optical absorption spectra of cytochrome P-450$_{D1\alpha}$ in the oxidized, reduced and CO-complex forms. 0.61 μM Cytochrome P-450$_{D1\alpha}$ in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol at 25°C. —·—, Oxidized form; - - - - -, reduced form; ————, CO-complex form.

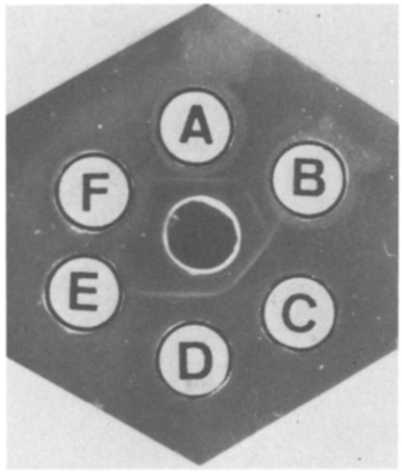


Figure 3: Immunochemical reactions in the Ouchterlony double diffusion agar test. The center well contained anti-cytochrome P-450$_{scc}$ (15 μg) of bovine adrenocortical mitochondria and each surrounding well contained the following cytochrome P-450 (300 pmoles): A,B, cytochrome P-450$_{D1\alpha}$ of bovine kidney mitochondria; C,D, cytochrome P-450$_{scc}$ of bovine adrenocortical mitochondria; E,F, cytochrome P-450$_{s21}$ of bovine adrenocortical microsomes.

P-450$_{D1\alpha}$ was a typical low spin form but it was a high spin form in the reduced form. A small amount of cytochrome P-420 was detectable in the purified cytochrome P-450$_{D1\alpha}$. The optical absorption peaks in the CO difference spectrum were a weak one at 552 nm and 450 nm of the Soret band. The molecular weight of cytochrome P-450$_{D1\alpha}$ was estimated from the standard proteins of various molecular weights by SDS-polyacrylamide gel electrophoresis. The molecular weight of the cytochrome P-450$_{D1\alpha}$ was calculated to be 49000. The amino acid composition of cytochrome P-450$_{D1\alpha}$ was analyzed (data not given). The immunochemical property of cytochrome P-450$_{D1\alpha}$ was investigated using antibodies to cytochrome P-450$_{scc}$ of bovine adrenocortical mitochondria and cytochrome P-450$_{s21}$ of bovine adrenocortical microsomes in the Ouchterlony double diffusion agar test. The microsomal anti-cytochrome P-450$_{s21}$ did not react with the cytochrome P-450$_{D1\alpha}$. However, mitochondrial anti-cytochrome P-450$_{scc}$ reacted with cytochrome P-450$_{D1\alpha}$ and the precipitin line was observed (Figure 3). In addition, spur formation was observed between cytochrome P-450$_{D1\alpha}$ and cytochrome P-450$_{scc}$ against anti-cytochrome P-450$_{scc}$. This fact shows that the mitochondrial cytochrome P-450$_{D1\alpha}$ is similar immunochemically to the other mitochondrial cytochrome P-450's of different organs, although it is not identical to them.

*Reconstitution of the 1α-hydroxylase system of 25-OH-D$_3$:* The 1α-hydroxylase system of 25-OH-D$_3$ could be reconstituted with the three components of cytochrome P-450$_{D1\alpha}$, NADPH-renoredoxin reductase and renoredoxin, which were purified from bovine kidney mitochondria, as shown in Table 2. NADPH-renoredoxin reductase, renoredoxin and cytochrome P-450$_{D1\alpha}$ were essential for the reconstitution and the amount of 1α,25-(OH)$_2$-D$_3$ produced was proportional to the content of cytochrome

Table 2. Reconstitution of the 1α-hydroxylase system of 25-OH-$D_3$

| Reaction system | No. of expt. | 1α-Hydroxylase activity of 25-OH-$D_3$ |
|---|---|---|
| | | pmoles 1α,25-$(OH)_2$-$D_3$/ min/nmole of cyt.P-$450_{D1\alpha}$ |
| Complete system | 7 | 8.15 ± 1.04 |
| *minus* NADPH | 3 | <0.05 |
| *minus* NADPH-renoredoxin reductase | 3 | 0.14 ± 0.04 |
| *minus* Renoredoxin | 3 | 0.18 ± 0.04 |
| *minus* Cytochrome P-$450_{D1\alpha}$ | 3 | <0.05 |

The complete system included 0.25 μM NADPH-renoredoxin reductase, 2 μM renoredoxin, 0.1 μM cytochrome P-$450_{D1\alpha}$, 50nM 25-OH-$D_3$, 0.1 mM NADPH, 0.5 unit of isocitrate dehydrogenase, 0.5 mM sodium isocitrate and 1 mM $MgCl_2$, in 10 mM potassium phosphate buffer, pH 7.4. The reaction conditions were as described in "METHODS". Values are the means and their standard deviations.

P-$450_{D1\alpha}$ employed and the reaction time. The respective components, except cytochrome P-$450_{D1\alpha}$ of the 1α-hydroxylase system of 25-OH-$D_3$, could be substituted for their counterparts such as NADPH-adrenodoxin reductase, NADPH-hepatoredoxin reductase, adrenodoxin and hepatoredoxin of bovine adrenocortical and hepatic mitochondria. However, optimum activity of the 1α-hydroxylase system was obtained with NADPH-renoredoxin reductase and renoredoxin of the kidney mitochondria. The substrate specificity of cytochrome P-$450_{D1\alpha}$ was examined with various substrates with the reconstituted 1α-hydroxylase system. The experiments were carried out with a rate-limiting concentration of the cytochrome P-$450_{D1\alpha}$. 24-Hydroxylation of 25-OH-$D_3$ could not be catalyzed by the reconstituted 1α-hydroxylase system. 24-Hydroxylation of 25-OH-$D_3$ may have been performed by another cytochrome P-450. The result showed that the cytochrome P-$450_{D1\alpha}$ was specific for 1α-hydroxylation of 25-OH-$D_3$. However, *p*-hydroxylation of aniline and *N*-demethylation of (+)-benzphetamine were catalyzed by the 1α-hydroxylase system. This result can be expected from the facts that hemoglobin, myoglobin or peroxidase can catalyze the hydroxylation and demethylation of xenobiotics in the presence of their reductase systems.

ACKNOWLEDGEMENTS: This research was supported in part by a grant from the Shimabara Science Promotion Foundation and by grants 56570103 and 56770170 from the Scientific Research Fund of the Ministry Education of Japan.

REFERENCES

1. Ichikawa,Y., Hiwatashi,A., and Yamano,T.(1980) in "Microsomes, Drug Oxidations, and Chemical Cartinogenesis" (Coon,M.J., Conney,A.H., Estabrook,R.W., Gelboin,H.V., Gillete,J.R., and O'Brien,P.J., eds.), Vol.I, pp.221-224, Academic Press, New York.
2. Ichikawa,Y., Hiwatashi,A., Kim,H.-J., Maruya,N., and Yamano,T.(1980) in "Flavins and Flavoproteins" (Yagi,K., and Yamano,T., eds.), pp.677-691, Japan Scientific Societies Press, Tokyo.
3. Ghazarian,J.G., Schnoes,H.K., and DeLuca,H,F.(1973) Biochemistry 12, 2555-2558.
4. Ghazarian,J.G., Jefcoate,C.R., Knutson,J.C., Orme-Johnson,W.H., and DeLuca, H.F.(1974) J. Biol. Chem. 249, 3026-3033.
5. Pedersen,J.I., Ghazarian,J.G., Orme-Johnson,N.R., and DeLuca,H.F.(1976) J. Biol. Chem. 251, 3933-3941.
6. Vieth,R., and Fraser,D.(1979) J. Biol. Chem. 254, 12455-12460.
7. Yoon,P.S., Rawling,J., Orme-Johnson,W.H., and DeLuca,H.F.(1980) Biochemistry 19, 2172-2176.
8. Ichikawa,Y., and Mason,H.S.(1973) in "Oxidases and Related Redox Systems" (King,T.E., Mason,H.S., and Morrison,M., eds.), pp.605-625, University Park Press, Baltimore.
9. Johnson,D., and Lardy,H.(1967) Methods Enzymol. 10, 94-96.
10. Ichikawa,Y.(1971) in "Mitochondria" (Hagihara,B., ed.), pp.266-291, Asakura Publishing Co., Tokyo.
11. Ichikawa,Y., Kuroda,M., and Yamano,T.(1970) J. Cell Biol. 45, 640-643.
12. Weber,K., and Osborn,M.(1969) J. Biol. Chem. 244, 4406-4412.
13. Bligh,E.G., and Dyer,W.J.(1959) Can. J. Biochem. Physiol. 37, 911-917.
14. Ouchterlony,Ö.(1949) Acta Pathl. Microbial. Scand. 26, 507-515.
15. Ichikawa,Y., and Yamano,T.(1967) Biochim. Biophys. Acta 131, 490-497