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LIGAND BINDING TO HUMAN PLACENTAL CYTOCHROME P-450: INTERACTION OF STEROIDS AND HEME-BINDING LIGANDS

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SUMMARY

Type II ligands such as *n*-octylamine, 1-(2-isopropylphenyl)-imidazole, cyanophenylimidazole, aniline, ethylisocyanide, metyrapone, nicotinamide and aminoglutethimide bind to microsomal cytochrome P-450 from human placenta to give rise to optical difference spectra which are similar in form to those observed with rat hepatic microsomal cytochrome P-450 but which are more intense relative to the CO-spectrum. Addition of the type I ligands androstenedione or 19-norandrostenedione cause changes in both magnitude and λ_{max} of the difference spectra produced by type II ligands. These changes appear to be due to the displacement of the type II ligand and are not shown with rat liver cytochrome P-450. Differences between the two steroids are related to their binding constants. Recent studies with π-acceptor ligands such as tin and germanium dihalides are extended to placental cytochrome P-450 with which CO-like spectra may be formed. Type II ligands, which bind to the heme iron, are inhibitors of placental aromatase activity while inhibitors that require metabolic activation, such as the methylenedioxyphenyl compounds, are not.

INTRODUCTION

Optical difference spectroscopy has been the single most useful tool in the characterization of microsomal cytochrome P-450. The well-known carbon monoxide complex [1, 2] with the reduced cytochrome gives rise to a difference spectrum with a prominent peak at or about 450 nm, from which the name is derived. Interactions between numerous organic ligands and the oxidized cytochrome P-450 from various tissues give rise to difference spectra which fall predominantly into two classes [3–5]: type I, with a peak at 385 nm and a trough at 420 nm, believed to be due to binding to a lipophilic site removed from the heme iron, and type II, with a peak at 430 nm and a trough at 390–410 nm, believed to be due to binding to the heme iron. Variations in the type II spectrum of *n*-octylamine have been related to high and low spin forms of the cytochrome and used to characterize different forms of mammalian hepatic cytochrome P-450 [6]. Type III spectra are the result of interactions with the reduced form of the cytochrome and consist of two peaks, in pH dependent equilibrium, in the Soret region. The best known is that formed by ethyl isocyanide, which has also been used to characterize different forms of hepatic cytochrome P-450 [7] but it is also characteristic of such compounds as the methylenedioxyphenyl synergists [8].

Characterization of microsomal cytochrome P-450 of human placenta is less complete than that of liver and other organs but it is apparent that the above spectral changes all can be detected [9–15]. Significant differences do exist, however, particularly in the inability of the placental cytochrome to bind [14] a vast array of xenobiotics known to interact with hepatic microsomal cytochrome P-450 [5] and in the high affinity for certain steroids [15]. This may be related to the inability of this cytochrome to oxidize drugs and to its apparent adaptation to carry out steroid atomatization [14, 15]. Even in those placentae in which aryl hydrocarbon hydroxylase has been very strongly induced there appears to be little change in the physical characteristics of the cytochrome P-450 [10].

The further characterization of human placental cytochrome P-450 is important, therefore, not only because of the importance of the placenta in human reproduction, but also because of the insight that may be gained into the important problems of cytochrome P-450 specificity by comparison of this highly specific cytochrome P-450 with cytochrome P-450s exhibiting less specificity such as that of mammalian liver microsomes.

MATERIALS AND METHODS

Human placentae were obtained at term from the delivery room of the University of Washington or Group Health Hospitals of Seattle, Washington. Microsomes sedimented at 105,000 *g* were used in all experiments except those involving partially purified cytochrome P-450. Following removal of fetal membranes the placental tissue was minced with scissors

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and rinsed thoroughly with ice-cold 1.15% KCl. Following homogenization with an equal vol. of 1.15% KCl in a Waring blender (low speed for 3 min followed by high speed for 1 min) the mitochondria and heavier particles were removed by centrifugation at 14,500 *g* for 20 min. The postmitochondrial supernatant was centrifuged at 104,000 *g* for 1 h, the microsomal pellet was resuspended in 1.15% KCl and the microsomes were resedimented at 104,000 *g* for 1 h. All operations were carried out at 0–4°. For spectral or metabolic measurements the microsomes were resuspended in 0.1 M potassium phosphate buffer at pH 7.35.

Partial purification of the placental microsomal P-450 cytochrome(s) was accomplished by a modification [15] of procedures described by Symms and Juchau[10]. Calcium chloride precipitated microsomes were used as the starting material and were prepared as follows: 4.0 ml of a 1.0 M solution of $CaCl_2$, previously adjusted to pH 7.5 with NaOH, were added slowly to each 100 ml of 14,500 *g* supernatant prepared as above, the solution stirred for an additional 5 min and the microsomes sedimented by centrifugation at 22,000 *g* for 30 min. Microsomal pellets were resuspended by gently homogenizing in a 0.16 M solution of sucrose such that the final protein concentration was 15–25 mg/ml. For each ml of the solution, 0.44 ml glycerol, 0.20 ml potassium phosphate buffer (1.0 M, pH 7.7), 0.03 ml of 0.1 M EDTA and 0.02 ml of freshly prepared dithiothreitol were added. The microsomal suspension then was sonicated three times at full output (150 W) for 20 s each with a Branson Sonifier, model W-185 D. To each ml of the sonicated mixture, 0.16 ml of a 10% sodium cholate solution containing 10^{-3} M butylated hydroxytoluene was added and stirred under anaerobic conditions for 30 min. The mixture then was centrifuged at 22,000 *g* for 30 min and the supernatant placed in a prechilled graduated cylinder. To the sonicated, solubilized preparation, sufficient ammonium sulfate (240 mg/ml) to produce a 42% saturation was added slowly, with stirring. The pH was maintained at 7.7 by adding a few drops of 2 N NH_4OH. The mixture then was stirred for 20 min and centrifuged at 20,000 *g* for 10 min. To the supernatant, sufficient ammonium sulfate (70 mg/ml) to produce a 50% saturation was added slowly with stirring. The pH was maintained at 7.7 with 2 N NH_4OH. The mixture was stirred for 20 min and centrifuged at 20,000 *g* for 15 min. The supernatant fraction was carefully removed as completely as possible, and the precipitate was redissolved in a small vol. of 0.01 M potassium phosphate buffer (pH 7.7) containing 20% glycerol, 10^{-4} M dithiothrietol, 5×10^{-4} M EDTA and 0.05% sodium cholate. This resuspended preparation was either analyzed immediately, or divided into small vol. and stored anaerobically at −85° A 4-fold purification was obtained with this procedure.

[4-^{14}C]-Androstenedione was obtained from New England Nuclear Corporation (Boston, MA), other steroids from Steraloids, Inc. (Pawling, NY). 1-(2-Isopropylphenyl) imidazole and 1-(2-cyanophenyl) imidazole were gifts from Dr. E. Stenenson, Du Pont de Nemours, Inc. (Wilmington, DE) and ethyl isocyanide was synthesized by Moses Namkung of the Department of Pharmacology, University of Washington Medical School. All other chemicals were of the highest purity commercially available and were used without further purification.

Spectral analyses were carried out on an American Instrument Company DW-2 UV-VIS Spectrophotometer as previously described [15]. Each spectrum was calibrated with a holmium oxide filter. Specific experimental details for various experiments in which spectral analyses were performed are given in the legends to figures and tables. All spectra were recorded at 6°. Concentrations of cytochrome P-450 were determined by utilizing the absorbance difference between 450 and 500 nm according to the method of Greim[16]. Ligands were added in from one to twenty μl of ethanol, with an equivalent vol. of ethanol added to the reference cuvette when appropriate. The vol. of ethanol used had no apparent effect on the spectral parameters of microsomal cytochrome P-450 from the placenta nor did it affect the difference spectra caused by other ligands. Protein was determined by the method of Lowry *et al.* [17].

Estimation of rates of conversion of androstenedione to estrogens (aromatase activity) in placental microsomes were determined according to the following procedure [15]: Microsomes equivalent to 20–25 mg protein were incubated with shaking (50–60 rev./min) in a Dubnoff metabolic incubator at 37° for 30 min under a 100% oxygen atmosphere. Typical incubation flasks contained [4-^{14}C]-androstenedione (0.5 μCi, 58.8 mCi/mmol) unlabeled androstenedione (1.9×10^{-4} M, final concentration), 50 μmol glucose 6-phosphate, 5 units of glucose 6-phosphate dehydrogenase, 5 umol NADPH and sufficient potassium phosphate buffer (0.05 M, pH 7.35) to provide a total vol. of 3.7 ml. The reaction was stopped by adding 25 ml dichloromethane to the reaction vessels. The mixture was shaken vigorously for 10 min. After transferring 20 ml dichloromethane, 25 ml ethyl acetate was added and the mixture was shaken again and centrifuged. Twenty ml of the ethyl acetate layer was added to the dichloromethane previously transferred, mixed and evaporated to dryness. The residue was taken up into 0.1 ml of 95% ethanol. The extracts were spotted in 2.0 μl quantities on top of known steroid standards for accurate visualization of substrate and metabolites on the chromatograms. Activated Silica gel (Baker, 100 nm) plates were employed with a chloroform–toluene–ethyl acetate (4:8:3 by vol.) developing system. After development, the plates were air-dried, sprayed with 50% phosphoric acid and charred to visualize the spots. The migration of steroids is illustrated in Fig. 1 of Zachariah *et al.* [15].

The spots were cut out and placed directly into

counting vials. Scintillation liquid (4 g PPO and 0.1 g POPOP/l. of toluene) then was added to the vials and these were counted in a Nuclear-Chicago Mark I liquid scintillation system. Rates of estrogen formation under the described conditions were linear for 1 h and increased linearly with increasing protein concentrations.

The rates of hydroxylation of 3,4-benzpyrene in incubation flasks containing placental microsomes were determined by measuring the appearance of fluorescent hydroxylated metabolites according to slight modifications of the method of Wattenberg *et al.* [18] as previously described [12]. Typical reaction vessels contained 0.5 ml of the resuspended placental microsomes (5–10 mg of protein), 0.1 ml of 3,4-benzpyrene in acetone (10^{-4} M, final concentration), 0.2 ml of NADPH in potassium phsophate buffer (1.2×10^{-3} M, final concentration), and sufficient potassium phosphate buffer (0.1 M, pH 7.35) to yield a total vol. of 2.0 ml. Reaction rates were not increased by additions of higher concentrations of NADPH or NADPH-generating systems. The mixtures were incubated with shaking in a Dubnoff metabolic incubator (50–60 rev./min) under a saturated oxygen gas phase for 15 min at 37°. Flasks were incubated in triplicate. Extracts of various control reaction mixtures containing heat-inactivated (100°, 5 min) microsomes or of those from which NADPH or 3,4-benzpyrene had been omitted, did not demonstrate measurable fluorescence. Product formation was linear with respect to time and protein concentrations under the reaction conditions utilized. Specific activities were expressed as nmol of substrate hydroxylated per g of protein per 15 min. Quinine sulfate in 0.1 N sulfuric acid was employed as the standard, and specific activities were calculated on the basis of the observation that 0.036 nmol/ml in 1 N NaOH of an authentic 8-hydroxybenzpyrene standard emits the same fluorescence as 0.3 μg of quinine sulfate per ml in 0.1 N sulfuric acid at an excitation wavelength of 400 nm and an emission wavelength of 522 nm [19].

RESULTS AND DISCUSSION

1-(2-Isopropylphenyl)imidazole is known to be type II ligand with a high affinity for rat hepatic cytochromes P-450 [4, 20]. Similar results were obtained with human placental microsomes (Fig. 1A). The type

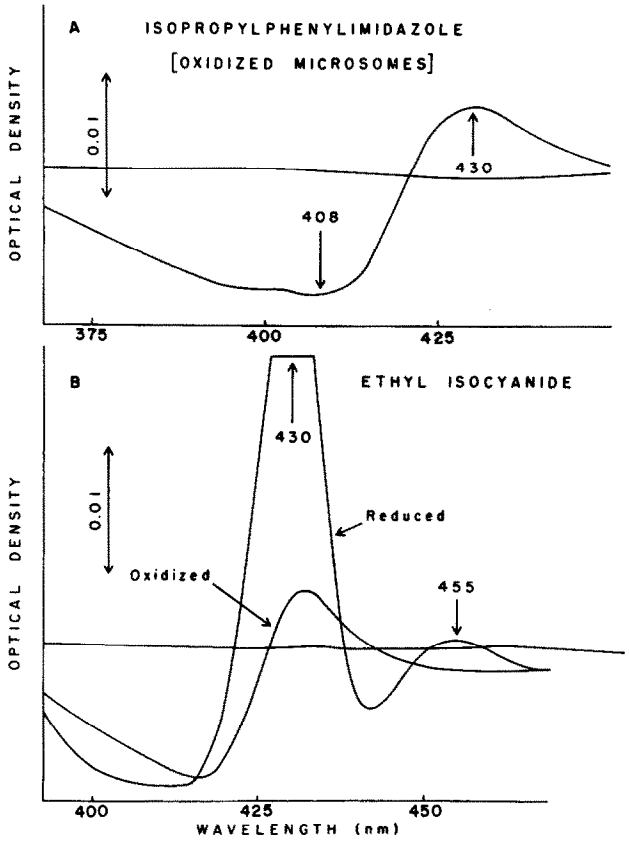

Fig. 1A. Type II optical difference spectrum of 1-(2-isopropylphenyl) imidazole with human placental microsomes. Each cuvette contained 2.0 ml microsomal suspension (4 mg protein/ml). 2.0 μl 1-(2-Isopropylphenyl) imidazole in ethanol was added to the sample cuvette (final concentration 1×10^{-7} M) and 2.0 μl ethanol to the reference.

Fig. 1B. Ethyl isocyanide optical difference spectrum of human placental microsomes. Each cuvette contained 2.0 ml microsomal suspension (4.0 mg protein/ml) and 1.0 μl ethyl isocyanide was added to the sample cuvette. Following the recording of the oxidized spectrum both cuvettes were reduced by the addition of 2–3 mg of sodium dithionite and the reduced spectrum recorded.

II spectrum exhibited a peak at 430 nm and a trough at 408 nm. Essentially identical spectra also were given by the following compounds and oxidized placental microsomes: 1-(2-cyanophenyl) imidazole; ethyl isocyanide; *n*-octylamine; aniline; aminogluteth-imide; nicotinamide; metyrapone. Spectral size was unusually large, being 0.88 of the CO spectrum for the same preparation. This compares with 0.45–0.91 for mouse liver microsomes [4] in which only *n*-octylamine gives a spectrum of similar size. Unlike mouse liver these spectra are essentially identical in size and form regardless of the ligand used.

Previous studies [10, 12] have demonstrated type II binding with *n*-octyalmine and aniline, the K_s for aniline being 4.0×10^{-4} M. In the present study the K_s for 1-(2-isopropylphenyl)-imidazole was 5×10^{-8} M indicating a high affinity for this ligand by placental cytochrome P-450 similar to or greater than that of hepatic cytochrome P-450 for similar imidazole derivatives [20].

Although previous studies from this laboratory had failed to demonstrate a 455 nm peak in the ethyl isocyanide-reduced cytochrome P-450 spectrum of placental microsomes, a finding which could be interpreted to mean that the cytochrome exists almost entirely in the low spin form [6], under the most favorable circumstances such a peak can be resolved (Fig. 1B). This peak was demonstrated using microsomes with an unusually high arylhydrocarbon hydroxylase activity and low hemoglobin contamination and could not be demonstrated with microsomes from any other placenta tested.

The type II ligand of highest affinity, 1-(2-isopropylphenyl) imidazole, was tested as a potential inhibitor of placental aryl-hydrocarbon hydroxylase and aromatase activity. At high levels, (2×10^{-4} M) twice that of the substrate, benzo(a)pyrene (1×10^{-4} M), 1-(2-isopropylphenyl) imidazole effected a 70% inhibition of arylhydrocarbon hydroxylase activity and was also effective as an inhibitor of aromatase. Eighty per cent inhibition was effected at a concentration (5×10^{-4} M) 2.5 times that of the substrate, androstenedione (2×10^{-4} M). The methylenedioxy compounds piperonyl butoxide, piperonyl nitrile and methylenedioxycyclohexane, all potent inhibitors of hepatic cytochrome P-450 [8], each failed to inhibit either enzyme activity at any concentration tested. This latter finding is in agreement with earlier studies which indicated that placental microsomes failed either to produce difference spectra or to metabolize most xenobiotics [15] since the methylenedioxy inhibitors must be metabolized to an active intermediate in order to exert their inhibitory effects [8].

The effect of steroids on type II binding. The addition of 19-norandrostenedione to human placental microsomes caused a dramatic change in the type II

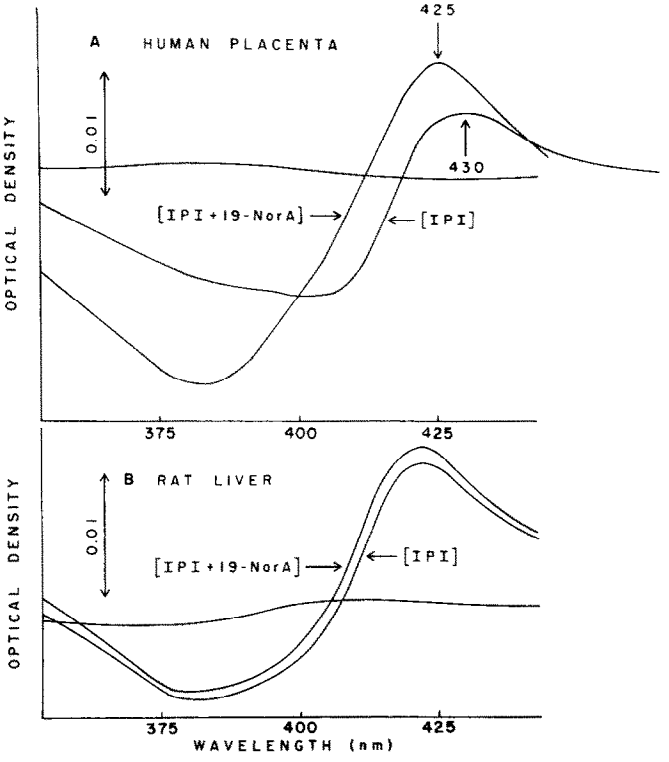

Fig. 2. Effect of 19-norandrostenedione on the type II optical difference spectrum of 1-(2-isopropylphenyl) imidazole with human placental microsomes and rat hepatic microsomes. (A) Human placental microsomes as in Fig. 1. 2.0 μl isopropylphenyl-imidazole in ethanol were added to the sample cuvette (final concentration 1×10^{-7} M) and 2.0 μl ethanol of the references cuvette. Following the recording of the 1-(2-isopropylphenyl) imidazole spectrum, 2.0 μl 19-norandrostenedione in ethanol were added to both cuvettes (final concentration 1×10^{-5} M). (B) Rat hepatic microsomes. As in 2A except that the suspension of rat hepatic microsomes contained 1.0 mg protein/ml.

spectrum of isopropylphenylimidazole (Fig. 2A). This involved a shift in the λ_{max} of approximately 5 nm toward a shorter wavelength and an increase in spectral size of approximately 2-fold. This effect was not seen with rat hepatic microsomes (Fig. 2B) but was readily apparent with a partially purified preparation of cytochrome P-450 from human placenta (Fig. 3).

The effect of the steroid was initially believed to be related to the effect of steroids on CO-binding to the reduced cytochrome since it had been shown [15, 21] that 19-norandrostenedione facilitated CO-binding and androstenedione inhibited it. This hypothesis was reinforced by the observation that, at the same concentration, androstenedione caused a reduction in the magnitude of type II spectra.

However, titration of the type II spectrum caused by 1-(2-isopropylphenyl) imidazole with either 19-norandrostenedione or androstenedione (Figs. 4 and 5) showed that both steroids first caused an increase in spectral size followed, at high concentrations, by a decrease. Since the point of maximum increase caused by androstenedione is at a lower concentration than that caused by 19-norandrostenedione, there are concentrations at which one steroid will cause an increase and the other a decrease in spectral size. The difference in concentration is related to the effectiveness of binding of the two steroids since androstenedione exhibited a K_s value of 1.3×10^{-8} M and 19-norandrostenedione 1.5×10^{-7} M in partially purified preparations [15]. These relationships suggested that mutual displacement between type II ligands and type I steroids would be the most appropriate hypothesis for this phenomenon.

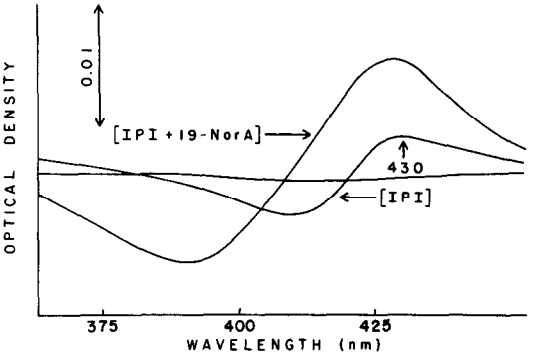

Fig. 3. Effect of 19-norandrostenedione on the type II optical difference spectrum of 1-(2-isopropylphenyl) imidazole and a partially purified preparation of human placental microsomes. Each cuvette contained 2.0 ml of cytochrome P-450 solution. 1-(2-Isopropylphenyl)-imidazole and 19-norandrostenedione added as in Fig. 2.

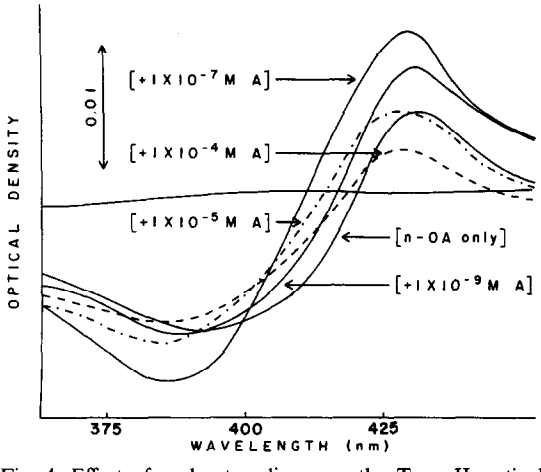

Fig. 4. Effect of androstenedione on the Type II optical difference spectrum of *n*-octylamine and human placental microsomes. Microsomes as in Fig. 1 and 1.0 μl *n*-octylamine was added to the sample cuvette. Androstenedione in ethanol was added sequentially to the concentration shown. Total ethanol added to each cuvette was 8.0 μl.

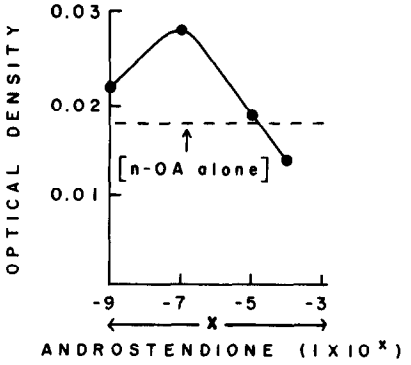

Fig. 5. Effect of androstenedione on spectral size of the type II spectrum of *n*-octylamine and human placental microsomes. Additions as in Fig. 4.

If this hypothesis is correct, the addition of a low concentration of steroid to both cuvettes after the addition of a type II ligand to the sample cuvette, would result, due to the displacement of the steroid in the sample cuvette, in an excess of type I binding in the reference cuvette. Due to the nature of optical difference spectroscopy [22] a type I spectrum in the reference cuvette is indistinguishable from an inverse type I spectrum in the sample cuvette. Since an inverse type I spectrum is similar in form to a type II spectrum the excess type I and the type II would be additive and the net result would appear as an increase in size of the type II spectrum with a slight spectral shift toward the trough position of a normal type I spectrum i.e., toward a shorter wavelength. At higher steroid concentrations the mutual displacement hypothesis would provide that actual displacement of the type II ligand occur and the total spectrum decrease in size.

Although Figs. 4 and 5 clearly illustrate these phenomena, a further test was made by determining the effect of a type II ligand on the type I spectrum caused by a steroid. If the hypothesis is correct there should be a decrease in spectral size and a shift in λ_{max} toward longer wavelengths. This did in fact occur and is illustrated in Fig. 6.

Mutual displacement by type I and II ligands appears to represent a clear difference between placental cytochrome P-450 and hepatic cytochrome P-450 and may be related to the specificity and high

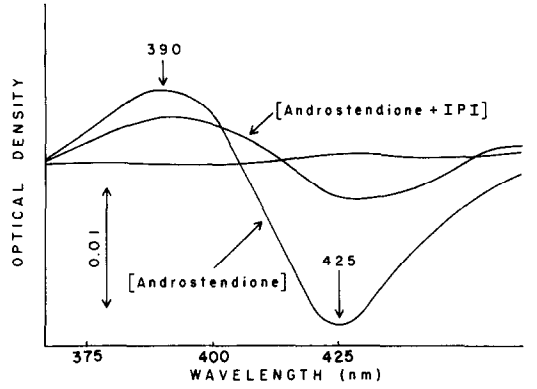

Fig. 6. Effect of 1-(2-isopropylphenyl) imidazole on the type I spectrum of androstenedione and human placental microsomes. Microsomes as in Fig. 1. 2.0 μl Androstenedione in ethanol was added to the sample cuvette (final concentration—1×10^{-7} M) and 2.0 μl ethanol to the reference. After the type I spectrum was recorded 2.0 μl of 1-(2-isopropylphenyl) imidazole in ethanol (final concentration—1×10^{-5} M) was added to both sample and reference cuvettes.

affinity of the cytochrome for its steroid substrates. One possible explanation is that steroid binding causes an allosteric change in the cytochrome P-450 molecule which affects the environment of the heme and thereby, type II binding to the heme iron.

The high contamination of placental microsome preparations by hemoglobin and the resultant difficulty in measuring cytochrome P-450 as its carboxy derivative is well documented. The recent demonstration [23] that certain inorganic ligands, group IVA dihalides, gave spectra with cytochrome P-450 identical to that of CO and the relationship of this to π-bonding between the ligand and the heme iron prompted the examination of the spectra of these ligands with human placental cytochrome P-450. Although the search for a ligand that would interact with cytochrome P-450 but not hemoglobin was unsuccessful, the results were still germane to the hemoglobin contamination problem. As illustrated in Fig. 7, $SnCl_2$ gave rise to a spectrum identical to that of CO with a peak at 430 nm due to hemoglobin, cytochrome b_5 and cytochrome P-420 and a peak at 450 nm due to cytochrome P-450. Germanium difluoride, on the other hand, reacted with hemoglobin but not with cytochrome P-450. This last finding was put to use by adding GeF_2 to both cuvettes, thus balancing the hemoglobin spectra, and permitting the CO spectrum to be determined by the method of Omura and Sato[24]. Since, in this method, both cuvettes are reduced, no cytochrome b_5 spectrum is apparent. This allows for a far more accurate quantitation of the placental microsomal cytochromes.

In conclusion, further spectral characterization of human placental cytochrome has been carried out and it is apparent that differences exist between it and hepatic cytochrome P-450. These differences appear to be related to the binding of steroids and may be of importance in further studies of the specificity of cytochrome P-450 from different tissues. Further insight into the multiplicity of cytochrome P-450 in both placenta and liver may require re-examination

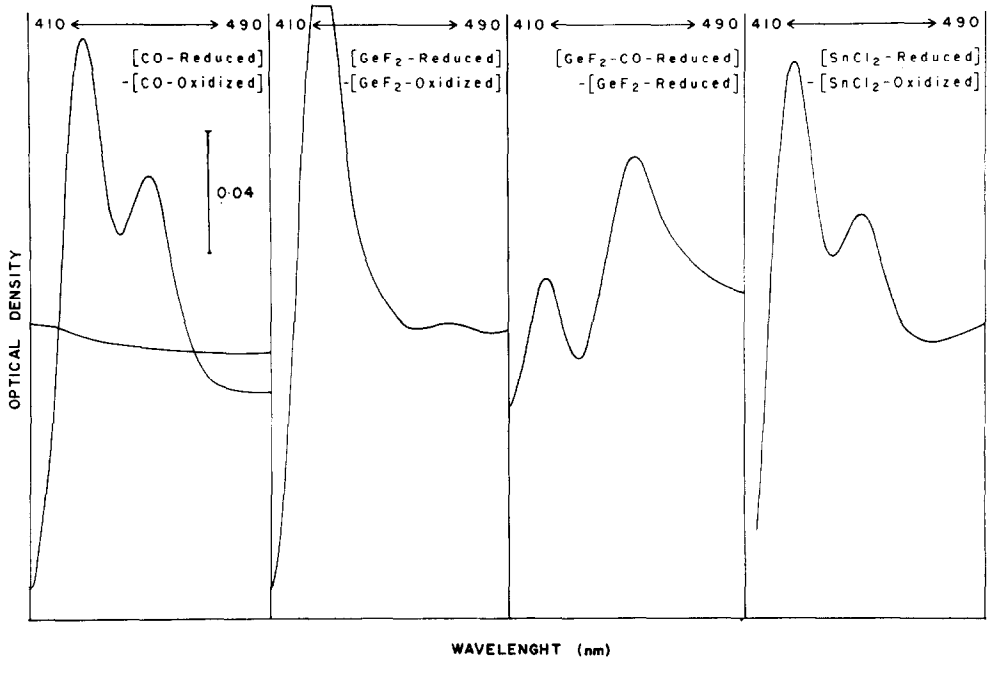

Fig. 7. Difference spectra of group IVA dihalides and human placental microsomes. Microsomes as in Fig. 1. 2–3 mg of GeF_2 or $SnCl_2$ added directly to the sample cuvette. Where indicated, reduction was by the addition of 2–3 mg sodium dithionite to the appropriate cuvette and carbon monoxide was added by bubbling for 60 s.

of these phenomena with single purified cytochrome P-450s from each source.

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REFERENCES

1. Klingenburg M.: *Archs Biochem. Biophys.* **75** (1958) 376–386.
2. Garfinkel D.: *Archs Biochem. Biophys.* **77** (1958) 493–509.
3. Schenkman J. B., Remmer H. and Estabrook R. W.: *Mol. Pharmac.* **3** (1967) 113–123.
4. Mailman R. B., Kulkarni A. P., Baker R. C. and Hodgson E.: *Drug Metab. Disp.* **2** (1974) 301–308.
5. Kulkarni A. P., Mailman R. B., Baker R. C. and Hodgson E.: *Drug Metabol. Disp.* **2** (1974) 309–320.
6. Jefcoate C. R. E., Gaylor J. L. and Calabrese R.: *Biochemistry* **8** (1969) 3455–3463.
7. Sladek N. E. and Mannering G. J.: *Biochem. biophys. Res. Commun.* **24** (1966) 668–674.
8. Hodgson E. and Philpot R. M.: *Drug Metab. Revs.* **3** (1974) 231–301.
9. Bergheim P., Rothger G. H. and Netter K. J.: *Biochem. Pharmac.* **22** (1973) 1633–1642.
10. Symms K. G. and Juchau M. R.: *Life Sci.* **13** (1973) 1221–1230.
11. Thompson E. A. and Siiteri P. K.: *Ann. N.Y. Acad. Sci., U.S.A.* **212** (1973) 378–389.
12. Juchau M. R., Zachariah P. K., Colson J., Symms K. G., Krasner J. and Yaffe S. J.: *Drug metabl. Disp.* **2** (1974) 79–86.
13. Thompson E. A. and Siiteri P. K.: *J. biol. Chem.* **249** (1974) 5373–5379.
14. Juchau M. R. and Zachariah P. K.: *Biochem. Pharmac.* **24** (1975) 227–233.
15. Zachariah P. K., Lee Q. P., Symms K. G. and Juchau M. R.: *Biochem. Pharmac.* **25** (1976) 793–800.
16. Greim H.: *Naunyn-Schmiedebergs Arch. Exp. Pharmakol. Pathol.* **266** (1970) 261–265.
17. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1957) 265–275.
18. Wattenberg L. W., Leong J. L. and Strand P. J.: *Cancer Res.* **22** (1962) 1120–1125.
19. Morrison G., Meigs R. A. and Ryan K. J.: *Steroids* **6** (Suppl. 2) (1965) 177–188.
20. Wilkinson C. F., Hetnarski K. and Yellin T. O.: *Biochem. Pharmac.* **21** (1972) 3187–3192.
21. Zachariah P. K. and Juchau M. R.: *Life Sci.* **16** (1975) 1689–1695.
22. Estabrook R. W., Peterson J., Baron J. and Hildebrandt A.: *Meth. Pharmac.* **2** (1972) 303–337.
23. Dahl A. R. and Hodgson E.: *Fedn Proc.* **35** (1976) 379.
24. Omura T. and Sato R.: *J. biol. Chem.* **239** (1964) 2370–2379.