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FURTHER STUDIES ON THE PROPERTIES OF HUMAN PLACENTAL MICROSOMAL CYTOCHROME P-450*

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Abstract—Spectral studies of human placental microsomal cytochromes provided evidence that androstenedione will bind either to two separate cytochromes or to two sites on the same cytochrome. Scatchard and Lineweaver–Burk plots indicated the presence of a binding site with high affinity and low capacity and a second site with a lower affinity but higher capacity. Both sites were discernible in the presence of high concentrations of NADPH, and similar binding constants were obtained. The absorption maximum (450 nm) of the carbon monoxide complex of NADPH-reduced placental cytochrome P-450 could be abolished completely by additions of low concentrations of androstenedione, 19-hydroxyandrostenedione or 19-oxoandrostenedione, but not by high concentrations of 19-norandrostenedione, 19-nortestosterone, pregnenolone or benzo[a]pyrene. Compounds capable of binding significantly to placental microsomal cytochrome P-450 appeared to fall into three categories: C-19 or C-18 steroids, with structures similar to that of androstenedione or 19-norandrostenedione; C-18 steroids with structures very similar to that of β-estradiol; or substances capable of forming ferrihemochromes. A large number of foreign organic compounds which produced type-I binding spectra in rat liver microsomes exhibited either no or very minimal binding to placental cytochrome P-450. Most compounds that exhibited significant binding to placental cytochrome P-450 also were inhibitors of rates of androstenedione aromatization (aromatase activity). Important exceptions were carbon monoxide, metyrapone and nicotinamide. Diethylstilbestrol acted as a relatively potent inhibitor of aromatase activity but displayed no discernible binding. Results of the study strongly supported a functional role for placental cytochrome P-450 in the aromatization reaction.

In very recent years studies [1–6] have appeared in the literature that have verified and extended the original observations of Meigs and Ryan [7] concerning the presence of cytochrome P-450 in the endoplasmic reticulum of human placental cells. Various properties of this cytochrome(s) have been described in the same reports, and solubilization and partial purification also have been achieved [2]. As yet, however, a definitive biochemical role for placental microsomal cytochrome(s) P-450 has not been demonstrated convincingly, although several possible functions have been postulated. On the basis of binding specificity, Thompson and Siiteri [3, 5] have proposed that the cytochrome may serve as the terminal oxidase in the conversion of androstenedione to estrone, a mixed-function oxidative reaction which is not inhibited by high concentrations of carbon monoxide (CO). (In earlier studies, Meigs and Ryan [7] also proposed this function for the cytochrome.) The placental microsomal mixed-function oxidation of foreign organic compounds including benzo[a]pyrene [8, 9] and 19-norsteroids [3, 5, 7] can be inhibited readily by CO, indicating a possible role for the cytochrome in these drug-metabolic reactions. Other investigators [10, 11] have suggested that placental cytochrome P-450 may be functional in the facilitated transport of oxygen from the maternal to the fetal circulation. One group of workers [12] has postulated that cytochrome P-450 of the endoplasmic reticulum may facilitate transport of oxygen from the cell surface to the mitochondria. The possibility that cytochrome P-450 may be observable in human placental microsomal fractions solely as a result of mitochondrial cross-contamination recently was ruled out [4].

The purpose of the present investigation was to provide further information on the nature and possible functions of this highly interesting cytochrome. Evidence is presented for the presence of two or more CO-binding pigments in placental microsomal fractions. A large series of drugs and steroids was studied to provide further insights into the binding specificity, apparent relative affinity, maximal spectral changes producible and numbers of apparent binding sites. Additional evidence was found to support our earlier theory [6] that the lack of observable drug-metabolic activities in placental microsomes is due, at least in part, to extremely low affinities of drug substrates for placental cytochrome P-450. This contrasts with extremely high affinities for certain endogenous steroids. Evidence is presented that androstenedione and related steroids are capable of preventing CO from complexing with NADH-reduced placental microsomal cytochrome P-450.

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MATERIALS AND METHODS

Tissues. Human placentas were obtained at term from the delivery rooms of the University and Group Health Hospitals, Seattle, Wash. Homogenates were prepared as described previously [2]. Microsomes were prepared by first centrifuging the homogenate at 14,500 *g* for 25 min in an International (model B-20) refrigerated centrifuge in order to minimize mitochondrial cross-contamination [4]. The microsomal fraction then was sedimented by centrifuging at 104,000 *g* for 1 hr or by precipitation with calcium chloride as follows: a 1·0 M solution of $CaCl_2$ was adjusted to pH 7·5 with NaOH, and 4 ml of the $CaCl_2$ solution/100 ml of supernatant was added to the 14,500 *g* supernatant fraction slowly with stirring. The solution was stirred for an additional 5 min at 4°. This then was centrifuged at 22,000 *g* for 30 min and as much of the soluble fraction was removed as possible. The pellets were resuspended in a solution containing 10^{-2} M Tris HCl (pH 7·7), 10^{-4} M dithiothreitol, 1·15% KCl and 5×10^{-4} M $CaCl_2$ in approximately one-third the original volume. This solution was centrifuged at 22,000 *g* for 30 min and the pellet was resuspended in the same solution a second time and recentrifuged. Procedures for precipitation also were employed in which potassium sulfate, magnesium sulfate or sodium chloride was substituted for $CaCl_2$. Specific activities of the aromatase system were highest with calcium chloride-precipitated microsomes; therefore this preparation frequently was utilized in enzyme assays and purification procedures. Microsomes precipitated with salts, however, were less suitable for spectral analyses because of rapid settling in the cuvettes and also were obtained in a somewhat lower yield per g of tissue (wet weight). For these reasons, microsomes prepared by ultracentrifugation also were utilized in many of the analyses. Hepatic microsomes were prepared from adult, male, Sprague Dawley rat livers according to the method described by Mazel [13].

Chemicals. Radioactive chemicals, including [4-^{14}C] androstenedione, [4-^{14}C]testosterone, [4-^{14}C]estrone and [4-^{14}C]β-estradiol and the corresponding tritiated steroids were obtained from New England Nuclear Corp. (Boston, Mass.). 19-Oxoandrostenedione was prepared according to methods described by Meigs and Ryan [14]. Other steroids were obtained from Steraloids, Inc., Pawling, N.Y. $NADP^+$, NADPH, NADH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, sodium cholate, reduced glutathione, sodium succinate and diethylstilbestrol were obtained from Sigma Chemical Co., Rochester, N.Y. Aminoglutethimide and metyrapone were obtained as gifts from Ciba Pharmaceutical Co., Summit, N.J. Hexobarbital was a gift from the Sterling Winthrop Research Institute, New York, N.Y. All other chemicals and solvents utilized were reagent grade and of the highest purity commercially available.

Enzyme assays and spectral analyses. Analyses of rates of formation of 3-hydroxybenzo[a]pyrene from benzo[a]pyrene in placental microsomes were performed as previously described [4]. Estimations of rates of conversion of androstenedione to estrogens (aromatase activity) in placental microsomes were determined according to the following procedure: 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/mmole), unlabeled androstenedione ($1·9 \times 10^{-4}$ M, final concentration), 50 μmoles glucose 6-phosphate, 5 units of glucose 6-phosphate dehydrogenase, 5 μmoles NADPH and sufficient potassium phosphate buffer (0·05 M, pH 7·35) to provide a total volume 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) 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.

The spots were cut out and placed directly into counting vials. Scintillation liquid (4 g PPO and 0·1 g POPOP/liter of toluene) then was added to the vials and these were counted in a Nuclear-Chicago Mark I liquid scintillation system.

Recoveries of the labeled individual compounds following direct application of radiochemicals to thin-layer sheets or following their extraction from incubation flasks were fully sufficient (85–97 per cent) to allow calculations of total aromatase activity within the system. Variability in aromatase activity between placentas was considerable, but replication of values for a given placenta was within 5 per cent. Radiochemical purities of the metabolites recovered were

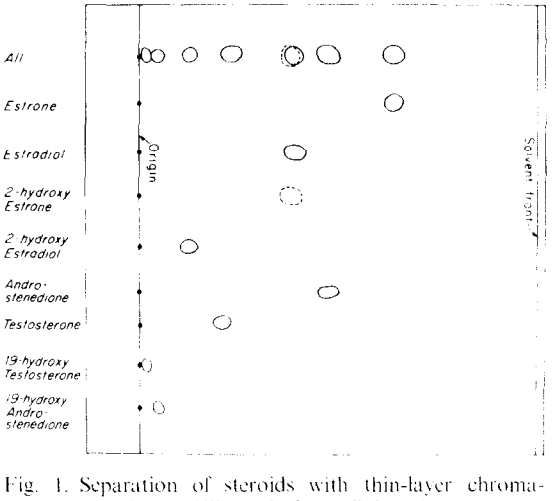

Fig. 1. Separation of steroids with thin-layer chromatography. Activated Silica gel plates (Baker, 100 nm) were employed with a chloroform toluene ethyl acetate (4:8:3) developing system.

confirmed by establishing constant $^3H/^{14}C$ ratios in three consecutive recrystallizations. It is important to note that the testosterone spot was radiochemically pure, since 2-hydroxyestrone ran very close to the testosterone spot in this system. Several spots were pooled in order to accumulate sufficient counts to provide for a 95 per cent statistical confidence interval for each steroid. Rates of estrogen formation under the described conditions were linear for 1 hr and increased linearly with increasing protein concentrations.

To determine the effect of varying CO concentrations on rates of aromatization of androstenedione and hydroxylation of benzo[a]pyrene, appropriate amounts of N_2, O_2 and CO were mixed, and a continuous stream of the gas of the desired composition was passed directly over the reaction mixture in the incubation flask. This flask was sealed except for inlet and outlet gassing needles. Microsomes, buffer and cofactors were equilibrated at 37° with the gas mixture for 15 min prior to initiation of the reaction by addition of the appropriate substrate. N_2 and CO were deoxygenated prior to entry into the gas mixing flask by bubbling the gases through a sintered disc in a tall (60 cm) column of a solution containing 0·5% sodium hydrosulfite and 0·05% anthraquinone-2-sulfonate in 0·1 N sodium hydroxide. All incubations and preincubations were carried out with shaking in the absence of light.

Analyses of difference spectra were performed with a model DW-2 recording spectrophotometer (American Instrument Co). 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 and calculating with an extinction coefficient of 100 mM^{-1} cm^{-1} according to the method of Greim [15]. Protein concentrations were determined according to the method of Lowry *et al.* [16]. Maximal spectral changes inducible (E_{max}) and spectral dissociation constants (K_s) were determined by utilization of linear transforms of the data. Double reciprocal (Lineweaver–Burk) plots and plots of ΔA/concentration vs concentration (Scatchard) were both employed.

Partial purification of placental cytochrome P-450. Partial purification of the placental microsomal P-450 cytochrome(s) was accomplished by modifying the procedures described by Symms and Juchau [2] and by affinity chromatography. Calcium chloride-precipitated microsomes (prepared as described above) were used as the starting material in these procedures. 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 sec each with a Branson Sonifier, model W-185D. 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 volume of 0·01 M potassium phosphate buffer (pH 7·7) containing 20% glycerol, 10^{-4} M dithiothreitol, 5×10^{-4} M EDTA and 0·05% sodium cholate. This resuspended preparation was either analyzed immediately, further processed or divided into small volumes and stored anaerobically at −85°. A 4-fold purification was obtainable with this procedure

RESULTS

Studies on the binding of several steroids as well as a large number of nonendogenous compounds to human placental microsomal cytochrome P-450 indicated a high degree of structural specificity (Table 1). None of the nonsteroidal compounds investigated produced clearly observable type-I difference spectra in most preparations, although hexobarbital, aminopyrine and ethylmorphine did produce very weak type-I spectral changes in one partially purified preparation. In several subsequent preparations, however, no definitive type-I spectral changes could be detected at concentrations exceeding 10^{-3} M. Desmethylimipramine, benzo[a]pyrene, diethylstilbestrol, ethanol and benzphetamine at similar concentrations also failed to produce spectral changes in placental microsomes, whereas the same compounds produced readily observable spectral changes in rat hepatic microsomes. Several steroids also failed to produce discernible spectral changes in any of the preparations studied even when present in high concentrations ($>10^{-4}$ M). Included in this group were cortisol, deoxycorticosterone, pregnenolone, progesterone, cholesterol, estrone, estriol and sodium taurocholate. Steroids which produced very weak type-I binding spectra included dehydroepiandrosterone ($K_s = 3{\cdot}8 \times 10^{-4}$ M) and sodium deoxycholate ($K_s > 10^{-2}$ M). Compounds exhibiting intense type-I binding spectra were close structural congeners of androstenedione for the most part. An important exception to this rule, however, was β-estradiol and two of its hemisuccinate derivatives (Table 1). Binding constants obtained with these compounds were highly variable from preparation to preparation and in some instances, no binding could be observed. Hemisuccinate derivatives of androstenedione (substituted at the 6 and 17 positions) also exhibited relatively pronounced difference spectra with comparably high apparent affinities but were not studied in the same preparation and are therefore not included in Table

Table 1. Binding parameters observed with solubilized, partially purified human placental microsomal cytochrome P-450*

Compound	Human placenta					Rat liver				
	λ_{max} (nm)	λ_{min} (nm)	K_s (M)	E_{max} mg protein ($\times 10^{-3}$)	E_{max} nmole P-450 ($\times 10^{-4}$)	λ_{max} (nm)	λ_{min} (nm)	K_s (M)	E_{max} mg protein ($\times 10^{-3}$)	E_{max} nmole P-450 ($\times 10^{-4}$)
Androstenedione	390	425	$1{\cdot}3 \times 10^{-8}$	18	11·3	389	421	$0{\cdot}9 \times 10^{-7}$	39	32
19-Hydroxyandrostenedione	385	424	$7{\cdot}4 \times 10^{-8}$	16	10·9	385	419	$2{\cdot}5 \times 10^{-7}$	15	16
19-Oxoandrostenedione	388	423	$5{\cdot}3 \times 10^{-8}$	17	11·0	385	420	$2{\cdot}4 \times 10^{-7}$	17	18
19-Norandrostenedione	385	420	$1{\cdot}5 \times 10^{-7}$	9	5·4	386	420	$1{\cdot}1 \times 10^{-7}$	12	13
1,4,6-Androstatrien-3,17-dione	386	420	3×10^{-8}	17	11·1	385	420	$8{\cdot}3 \times 10^{-8}$	17	19
6-Oxoandrostenedione	384	421	$2{\cdot}1 \times 10^{-7}$	10	6·3	385	423	$2{\cdot}9 \times 10^{-7}$	20	22
Testosterone	396	425	$1{\cdot}4 \times 10^{-6}$	7	4·3	385	420	$1{\cdot}3 \times 10^{-7}$	10	11
19-Nortestosterone	384	418	$3{\cdot}2 \times 10^{-6}$	9	5·7	385	420	$3{\cdot}5 \times 10^{-7}$	8	8
β-Estradiol	390	416	$5{\cdot}9 \times 10^{-7}$	16	10·2	388	418	$4{\cdot}0 \times 10^{-7}$	10	11
β-Estradiol-3-hemisuccinate	390	417	$4{\cdot}8 \times 10^{-7}$	5	3·4	NDT	NDT	NDT	NDT	NDT
β-Estradiol-17-hemisuccinate	390	417	$2{\cdot}4 \times 10^{-7}$	5	4·9	NDT	NDT	NDT	NDT	NDT
Nicotinamide	435	416	$1{\cdot}2 \times 10^{-4}$	3	1·8	428	390	$2{\cdot}9 \times 10^{-4}$	8	8
Aniline	435	416	$4{\cdot}0 \times 10^{-4}$	4	2·5	430	391	$3{\cdot}8 \times 10^{-4}$	9	9
Aminoglutethimide	436	413	$6{\cdot}1 \times 10^{-7}$	19	12·4	430	398	$3{\cdot}1 \times 10^{-7}$	4	4

* Direct comparisons were made by utilizing the same sample of partially purified placental cytochrome P-450 for all determinations. Protein concentration in the cuvettes was 1·6 mg/ml. NDT indicates that no determination was made.

1. The results obtained in these studies are in rather good agreement with less extensive earlier studies [1–5]. Examination of the data indicated that when maximal spectral changes were expressed in terms of the CO complex, two groups of values could be discerned. One group of steroids exhibited maximal values approximately double those of a second group; i.e., androstenedione, 19-hydroxyandrostenedione, 19-oxoandrostenedione, 1,4,6-androstatriene-3,17-dione and β-estradiol all produced maximal values in the range of 0·010 to 0·012 absorbance units/nmole of cytochrome P-450. Other steroids produced maximal values in the range of 0·005 to 0·006 absorbance units/nmole of cytochrome P-450. The apparent affinities (as measured by K_s values) of most of the steroids studied were approximately two orders of magnitude greater for placental cytochrome P-450 than for the hepatic cytochrome. Exceptions to this, however, included testosterone, 19-nortestosterone and β-estradiol. Androstenedione also appeared to bind either to two separate type-I binding sites on the same cytochromal molecule or to two separate cytochromes. Linear transforms of the binding data revealed a high affinity, low capacity primary binding site and a secondary site with lower affinity but higher capacity (Fig. 2). This phenomenon was observable in all preparations in which lower concentrations were studied except for two preparations which had been stored for extended periods. Scatchard and Lineweaver Burk plots both indicated the presence of two separate binding sites. K_s values varied considerably in different preparations; some approached 10^{-7} M for the secondary site in partially purified preparations and others were as low as 10^{-10} M for the primary site usually observed in fresh microsomes prepared by ultracentrifugation (see Methods). Essentially identical binding constants for androstenedione were observed when microsomes were analyzed in the presence of high concentrations ($>10^{-4}$ M) of NADPH.

Ascorbate, reduced glutathione or succinate did not appear capable of reducing placental microsomal cytochrome P-450 at concentrations exceeding 10^{-3} M. NADH could partially reduce the cytochrome in certain preparations, but exhibited a very high degree of variability for this capacity. In general, NADPH was much more effective than NADH as a reducing agent, but likewise exhibited considerable variability which appeared related to the stability of the reductase. Additions of sodium hydrosulfite to NADH- or NADPH-reduced cytochrome P-450 always produced an additional large increase in the concentration of reduced cytochrome P-450, even though as much as 30 min was allowed for reduction by the nucleotides. NADH produced no further reduction of the cytochrome in the presence of excess NADPH (3×10^{-4} M), but NADPH normally produced a

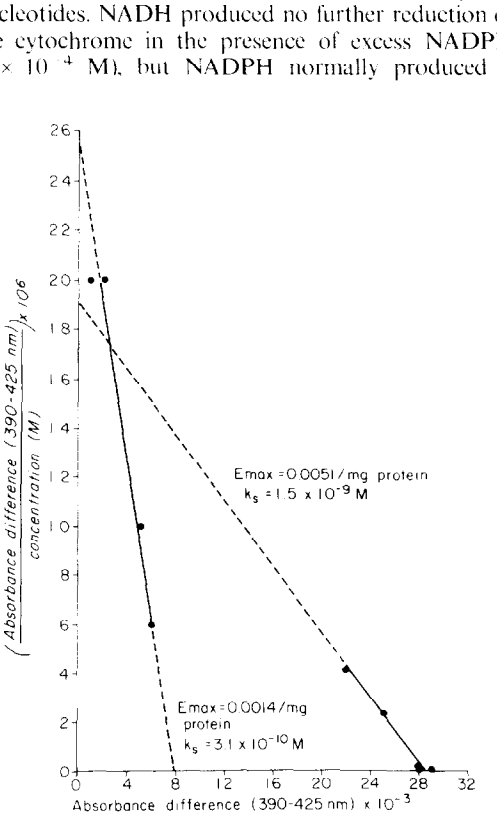

Fig. 2. Scatchard plot of the binding of androstenedione to cytochrome P-450 in freshly prepared human placental microsomes. Microsomes were resuspended in potassium phosphate buffer (0·1 M, pH 7·4); protein concentration was 3·6 mg/ml.

Table 2. Effects of potential inhibitors and activators on aromatase activity *in vitro*

Compound tested	Final concn added	% of Control	Type of spectral change*	Apparent K_s
Aminoglutethimide	5×10^{-4} M	47	II	$6{\cdot}1 \times 10^{-7}$ M
Diethylstilbestrol	10^{-5} M	37	ND	
Potassium cyanide	10^{-3} M	38	NDT	
Aniline	10^{-3} M	72	II	$4{\cdot}0 \times 10^{-4}$ M
Nicotinamide	5×10^{-3} M	98	II	$1{\cdot}2 \times 10^{-3}$ M
Metyrapone	10^{-3} M	97	II	$1{\cdot}3 \times 10^{-5}$ M†
Carbon monoxide	95% CO, 5% O_2	110‡	450 nm maximum (reduced form)	
Benzo[a]pyrene	10^{-4} M	96	ND	
Hexobarbital	10^{-3} M	101	ND	
Aminopyrine	10^{-3} M	98	ND	
Clofibrate	10^{-3} M	99	NDT	
N-acetylaminofluorene	10^{-3} M	94	ND	
Sodium cholate	10^{-4} M	4	I	$3{\cdot}1 \times 10^{-5}$ M
Sodium lauryl sulfate	5×10^{-3} M	41	NDT	
Dithiothreitol	10^{-3} M	84	ND	
Acetone	10^{-2} M	104	ND	
Theophylline	10^{-3} M	100	NDT	
NADH	5×10^{-4} M	123	427 nm maximum, 409 nm minimum	
Hydroxylamine	5×10^{-2} M	140	NDT	

* I refers to a spectral change with an absorption maximum near 390 nm and a minimum near 420 nm; II refers to a spectral change with an absorption maximum near 430 nm and a minimum near 400 nm; ND indicates that no spectral change was detectable at the concentration indicated; NDT indicates that no determination was made.

† Data taken from Bergheim *et al.* [1].

‡ Control for this experiment was 95% N_2 and 5% O_2.

small increase in the absorbance maximum at 450 nm when added to microsomal preparations containing high concentrations (3×10^{-4} M) of NADH.

Compounds exhibiting type-I spectral changes in both placental and hepatic microsomal preparations exhibited much more intense changes in the placenta if these changes were expressed in terms of the cytochrome P-450 concentrations (Table 1). When expressed in terms of protein concentrations the changes observed in hepatic microsomes were slightly more pronounced.

The only nonsteroidal compounds that exhibited significant binding were those which produced type-II (or similar) difference spectra. Not all of the compounds exhibiting type-II difference spectra were effective inhibitors of placental aromatase activity (Table 2). Metyrapone and nicotinamide exhibited no detectable inhibitory effects at concentrations of 10^{-3} M or greater. Aminoglutethimide, aniline and potassium cyanide, on the other hand, were relatively effective inhibitors. CO, which binds to ferrous heme of cytochrome P-450, displayed no inhibitory effects on the conversion of androstenedione to estrogens. No inhibition could be detected in the presence of limiting O_2 and high concentrations of NADPH (Table 3) in agreement with previous observations by Meigs and Ryan [14]. Under identical reaction conditions and with the same microsomal preparations, conversion of benzo[a]pyrene to 3-hydroxybenzo[a]pyrene was readily inhibited by CO (Fig. 3). The capacity of CO to inhibit benzo[a]pyrene hydroxylation in placental microsomes was roughly equivalent to its capacity to inhibit the reaction in rat hepatic microsomes.

The presence of relatively low concentrations ($<10^{-7}$ M) of androstenedione and closely related steroids in placental microsomal preparations could completely prevent the appearance of an absorbance maximum at 450 nm when the cytochrome was reduced by NADPH (Table 4). Interestingly, substrates for placental mixed-function oxidation reactions that are inhibited by CO did not exhibit this effect. Compounds which exhibited no significant type-I binding to the placental cytochrome likewise did not produce the effect. Addition of androstenedione to both the sample and reference cuvettes also eliminated the absorbance maximum at 450 nm subsequent to reduction with excess NADPH and equi-

Table 3. Effect of CO on rates of conversion of androstenedione to metabolites at low concentrations of O_2*

Gas phase	Rates of product formation (pmoles mg protein min)			
	Testosterone	Estrone	β-Estradiol	Total estrogens†
100% O_2	574	288	19	307
100% N_2	522	14	5	20
5% O_2, 95% N_2	646	128	4	133
5% O_2, 95% CO	616	122	23	146
5% O_2, 19% CO, 76% N_2	626	120	19	140
20% O_2, 80% N_2	685	172	26	199

* This experiment was repeated three times with similar results except that absolute values varied more than 10-fold and rates of β-estradiol formation were proportionately higher in the other experiments.

† Total of estrone plus β-estradiol.

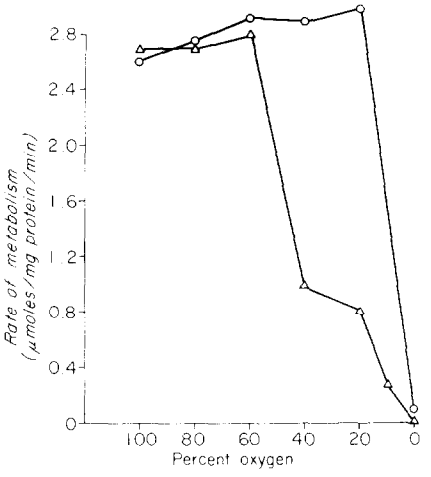

Fig. 3. Inhibition of the 3-hydroxylation of benzo[a]pyrene with carbon monoxide. ○ ○ . Rate of the reaction with nitrogen; △ △ , rate of the reaction with carbon monoxide as the balance of the gas phase.

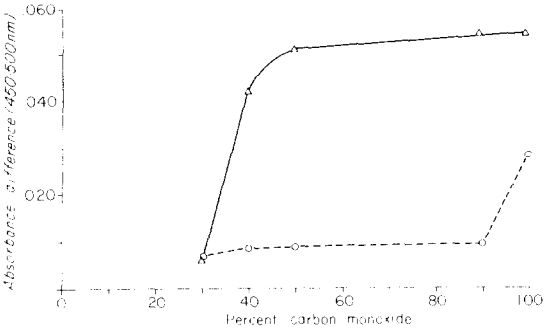

Fig. 4. Absorbance differences (ΔA 450–500) measured in human placental microsomes as a function of CO concentration (△ △): sample and reference cuvettes contained microsomes (5·2 mg protein/ml) and carbon monoxide at the concentrations indicated. The sample cuvette contained sodium dithionite. ○ ○. Same as above except NADPH (5×10^{-4} M, final concentration) was utilized as the reducing agent. Balance of the gas phase was oxygen in both cases.

libration with CO. Thus the effect was observed regardless of the sequence of addition of components to the cuvettes.

In order to gain preliminary insights into the relative affinity of the placental microsomal cytochrome for CO vs O_2, varying ratios of these gases were bubbled through sample and reference cuvettes for 60 sec and analyzed by split beam spectrophotometry. Immediately prior to recording of the difference spectra, the reducing agent was added to the sample cuvette and repetitive scans were run for a period of approximately 10 min. After no further change in the spectrum could be detected, the absorbance difference between 450 and 500 nm was measured and recorded at varying O_2:CO ratios. The results are presented in Fig. 4. With sodium hydrosulfite, maximal absorbance differences could be observed with as high as 50% concentrations of O_2. With NADPH, however, a marked decrease in the absorbance difference was observed with only 10% concentrations of O_2. Since O_2 is rapidly removed in the presence of dithionite but not by reduced pyridine nucleotides, this effect probably explains the differences observed with the two reducing agents. The results suggested that the affinity of the cytochrome for CO was relatively low compared with its affinity for O_2. More detailed studies will be required for confirmation of this suggestion, since the results may also indicate a more rapid reoxidation of the cytochrome in the presence of O_2.

Table 4. Effects of steroids on the 450–500 nm absorption maximum of the placental cytochrome P-450–CO complex

Compound tested*	Final (M) concn	Absorption (450–500 nm)
None		0·018
Androstenedione	5×10^{-5}	0·000
19-Hydroxyandrostenedione	5×10^{-5}	0·005
19-Oxoandrostenedione	5×10^{-5}	0·009
Testosterone	10^{-4}	0·003
19-Nortestosterone	10^{-4}	0·019
19-Norandrostenedione	10^{-4}	0·020
Pregnenolone	10^{-4}	0·018
Benzo[a]pyrene	10^{-4}	0·018

* Compounds were dissolved in absolute ethanol; a maximum volume of 10 μl was added to sample and reference cuvettes. Both cuvettes contained 2 ml of the microsomal suspensions (ultracentrifuged samples, see Methods), CO and steroids; NADPH (2×10^{-4} M, final concentration) was present only in the sample cuvette. Protein concentration was 4·3 mg protein/ml. Spectra were run immediately after addition of steroids to sample and reference cuvettes. Fifteen min was allowed for reduction after addition of NADPH.

DISCUSSION

The results of the present investigation appear to indicate the presence of at least two cytochromes P-450 in human placental microsomes apart from the cross-contaminating mitochondrial cytochrome P-450 [4]. One of these cytochromes binds androstenedione-similar steroids quite specifically and seems to represent a predominant form in the endoplasmic reticulum of placental cells. It would seem highly probable that this cytochrome would function as the terminal oxidase in the mixed-function oxidative conversion of androstenedione to estrone, a major function of the placental syncitiotrophoblast. The demonstration of discrete populations of binding sites for androstenedione, however, recalls the question of whether different cytochromes may be involved in the three hydroxylation reactions required in the aromatization sequence. Although androstenedione, 19-hydroxyandrostenedione and 19-oxoandrostenedione did not appear to produce additive effects on the type-I difference spectrum with respect to the secondary binding site, the question of additivity on the primary site was difficult to assess. However, neither of the latter two steroids appeared to bind to discrete populations of binding sites in any of the preparations investigated (in which androstenedione did exhibit biphasic binding) and their K_s values also tended to indicate that they probably bind to the secondary site. Testosterone also appeared to bind only to one site probably the secondary site. These observations would tend to corroborate those of Bellino and Osawa [17], who contended that androstenedione

and testosterone undergo aromatization catalyzed at separate enzyme sites, and of Menini and Engel [18], who suggested that androstenedione is the primary substrate for the aromatization reaction. Two populations of binding sites could indicate the presence of two sites on the same molecule or of two separate cytochromes. The latter possibility would seem the more probable but the former has not been ruled out.

Observations on the placental hydroxylation of benzo[a]pyrene provide an additional suggestion of multiple cytochromes P-450 in placental microsomes. Inhibition by CO of this mixed-function oxidation implies, but does not prove, the involvement of cytochrome P-450 in the reaction. Lack of observable binding of benzo[a]pyrene to placental microsomal cytochrome P-450 (as determined with tandem cuvettes according to methods described by Jakobsson *et al.* [19]) as well as lack of any correlations between aryl hydrocarbon hydroxylase activity and concentrations of cytochrome P-450 or positions of spectral maxima [4] indicates that if a cytochrome P-450 (or P-448) is involved, it may constitute only a very small fraction of the total present in placental microsomes.

A somewhat elusive β-estradiol-binding cytochrome P-450 may represent still another form of this placental microsomal pigment. Its absence from some preparations that exhibited strong androstenedione-binding spectra speaks for a separate cytochrome (which also exhibited greater lability than the androstenedione-binding form). β-Estradiol undergoes variable rates of hydroxylation at positions 2 [20, 21] and 6 [22, 23], and it seems feasible that a cytochrome P-450 which binds β-estradiol could function in those reactions. Binding of the estrogen to placental cytochrome P-450 could suggest a possible negative feedback on the aromatization reaction. The studies of Schwarzel *et al.* [24], however, tend to rule out any negative feedback role for estrogens.

Elimination of the NADPH-dependent absorption maximum at 450 nm by prior or subsequent additions of low concentrations of androstenedione, 19-hydroxyandrostenedione or 19-oxoandrostenedione appears to provide a reasonable explanation for the lack of inhibition of aromatase by CO. This hypothesis seems particularly attractive since substrates for CO-inhibited placental mixed-function oxidations did not exhibit this effect. Such observations may remove the last serious objections to earlier suggestions regarding the functionality of placental microsomal cytochrome P-450 in estrogen biosynthesis from endogenous androgens [5, 7]. Details of these phenomena will be published elsewhere. It is interesting to note, however, that Bergheim *et al.* [1] reported that steroids could modify the difference spectrum produced by metyrapone, possibly displacing it from binding. A possible explanation for these kinds of observed spectral changes is based on considerations of interactions of carbon monoxide with polymers of cytochrome *c* [25]. It is suggested that the binding of androstenedione or similar structurally related steroids to placental microsomal cytochrome P-450 may produce a conformational change in the apoprotein which results in a change in the reactivity at the sixth liganding position. This change could then prevent CO from binding to the heme as well as displace previously bound CO. Careful studies will be required to verify this hypothesis. If the observed spectral changes truly represent a change in the availability of the sixth coordination site for CO, an important question which remains concerns the capacity of O_2 to bind to the same site. Preliminary studies presented in this report (Fig. 4) tend to indicate that placental microsomal cytochrome P-450 possesses a relatively low affinity for CO in the presence of small amounts of O_2 following reduction with NADPH. Quantitative effects of androstenedione on the relative affinities of the cytochrome for O_2 vs CO remain to be investigated. It is known that relative affinities of CO vs O_2 can vary markedly among heme proteins and that such affinities can be altered considerably by the presence of other substances, i.e. organic phosphates [26]. It should be noted, however, that low concentrations of androstenedione could completely eliminate the absorbance maximum at 450 nm, even in the presence of deoxygenated CO. Also, it is of significance that the absorption maximum was much less readily decreased in the presence of dithionite. In some dithionite-reduced preparations, even high concentrations ($> 10^{-4}$ M) produced little effect on the absorption at 450 nm. After reduction with NADPH, however, the absorption maximum consistently was abolished completely with low concentrations of androstenedione ($< 10^{-7}$ M). Although Thompson and Siiteri [5] have suggested that lack of inhibition of aromatase may be explained in terms of a low electron flux, it now seems more reasonable to believe that lack of inhibition may be due to displacement of CO from the cytochrome by androstenedione, which normally is present in reaction vessels in relatively high concentrations ($> 10^{-5}$ M). (We were unable to observe inhibition at androstenedione concentrations of 10^{-6} M and at lower concentrations the substrate is depleted rapidly.) Displacement could occur as a result of an allosteric transition as suggested above or could be due to a very rapid reoxidation of the ferrous cytochrome to the ferric form. Additional research will be required to resolve this question.

It should be reemphasized that considerable variability in certain measured parameters was observed between placentas. This was particularly noticeable in measurements of K_s values, quantities of NADPH-reducible CO cytochrome P-450 complex, and binding with β-estradiol. In addition, we also observed variability in the types of spectra produced with 3,6,17-androstenetrione. In most preparations [4] we observed classical type-I binding spectra with variable intensity and high apparent affinity. In two others, however, we observed difference spectra with absorption minima near 483 nm and maxima near 413 nm. In both of the latter cases the preparations had been solubilized and partially purified, but no such phenomenon was observed in two other similar preparations. We observed no definitive correlation between the intensities of the absorbance maxima of the CO vs androstenedione complexes, in contrast to data reported by Thompson and Siiteri [5]. Androstenedione could produce very high values even following 70–80 per cent conversion of the cytochrome P-450 to P-420.

Further significant progress in the resolution of the above problems and hypotheses probably will require

the preparation of highly purified cytochromes. The maximum purification obtainable via ammonium sulfate fractionation was 4-fold in our experiments. Attempts to purify further by means of affinity chromatography have met thus far with only partial success. A 25-fold purification was achieved but with only an extremely low (< 2 per cent) yield. Further work in this area is currently in progress.

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