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INTERACTION OF DRUGS AND STEROIDS WITH HUMAN PLACENTAL MICROSOMES*

PETER BERGHEIM, GÜNTHER H. RATHGEN and KARL J. NETTER†

Department of Pharmacology, Section on Toxicology, and Department of Gynaecology, University of Mainz, D 65 Mainz, Germany

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Abstract—Human term placental microsomes contain relatively small amounts (approx. 0·1 nmoles) of cytochrome P-450 per milligram of protein as determined by carbon monoxide complexing. The other components of the mixed function oxidase (NADPH-cytochrome *c*-reductase and NADPH-cytochrome P-450 reductase) are present in amounts comparable to those in rat liver microsomes. No oxidative metabolism could be detected when *p*-nitroanisole, acetanilide, amphetamine and aniline were incubated with placental microsomes. Type II compounds such as aniline, *p*-aminophenol, nicotinamide and metyrapone induce binding spectra. No spectrum, however, could be recorded with dithionite reduced material to which metyrapone was added. Type I compounds (aminopyrine, hexobarbital, naphthalene and pentobarbital) produce no difference spectra. Steroid hormones show either type I or II or mixed type spectra. The spectral types in placental microsomes are in most cases the opposite ones from those in rat liver microsomes. Metyrapone is bound to placental microsomes and can be either displaced or modified by steroids. It is concluded that each type of microsomes exhibits different complexing properties.

MOST DRUGS given during pregnancy penetrate the placental barrier and reach the foetus. Exceptions to this rule are few and include very highly polar substances such as D-tubocurarine or heparin, and have a lesser ability to cross the membranes "protecting" the foetus. In addition to its function as a transport organ the placenta is also able to catalyse practically all cellular metabolic reactions as is documented by the respectable list of enzymes found in placental tissues.[1] Indeed a distinctive similarity between placenta and liver structure was noticed by Bernard, as early as 1859.[2] In view of the histological similarity we investigated whether the placenta, particularly the human placenta, possesses a microsomal mixed function oxidase and is capable to metabolize drugs. The presence of the drug hydroxylating enzyme, cytochrome P-450, has been demonstrated spectrally in placental homogenates.[3] Accordingly drug oxidation should proceed in preparations of placenta as well as in those of liver, thus perhaps establishing a sort of chemical "protection" of the foetus against harmful substances.[4] However, evidence for drug metabolizing activity in placenta has been conflicting. Sometimes low activities have been postulated from substrate disappearance in rabbit placentas[5] or in two single human placentas,[6] sometimes no activity has been found.[7,8] In this situation it seemed interesting to investigate if drugs are bound to cytochrome P-450 by measuring their difference spectra.

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† To whom reprint requests should be addressed at the Department of Pharmacology, Section on Toxicology.

It is demonstrated in this paper that human placental microsomes show no difference spectra, measured by high resolution spectroscopy, with non-steroid type I substrates, and modified spectra with type II substrates. Certain steroids produce a mixed type of spectrum. Thus there is a distinct difference in the behaviour of placental microsomes compared to rat liver microsomes. No evidence was found for placental hydroxylation of type I and type II drugs. Therefore, in the case of human placental microsomes, there seems to be no correlation between binding and metabolism.

MATERIALS AND METHODS

Preparation of placental microsomes. Placentas of normal full term deliveries were used for this study. Placentas weighing between 300 and 500 g were immediately taken from the delivery room and perfused with about 10 l. of ice-cold 0·9% NaCl solution. For this purpose the solution was passed through a cooling bath of about 0°. The placenta was cannulated via the umbilical arteries. The pressure of the perfusing solution was about 500 mm Hg. Thus both the foetal blood and the maternal blood in the "intervillous spaces" were removed. The perfusion was performed until haemoglobin was no longer visible in the effluent and the tissue, normally for about 1 hr. The temperature of the tissue was maintained between 4° and 8°. The cotyledons were cleaned from connective tissue, cut into cubes and disintegrated in a high-speed blendor (Braun-Multimix). The resulting preparation was homogenized in an Ultra-Turrax rotating blade homogenizer for 20 sec. Due to the previous perfusion the material contained sufficient fluid and further addition of buffer was omitted. The homogenate was placed in the Sorvall Rotor SS 34 and centrifuged for 15 min at 2700 *g* and 30 min at 9000 *g* without changing the tubes. The microsomes were sedimented from the resulting supernatant in a Spinco L 65 B centrifuge at 105,000 *g* for 30 min and washed twice with a solution of 0·25 M sucrose, 0·054 M disodium EDTA and 0·02 M Tris–HCl, pH 7·4. These microsomes proved stable for about 6 weeks when frozen at −26°. Protein concentrations were determined by the method of Lowry *et al.*[9]

Assay of microsomal hydroxylase constituents. The spectral absorption at 450 nm of dithionite reduced microsomes was determined after gassing with carbon monoxide and the concentration of P-450 was derived using an extinction coefficient of 9100 $(\text{mole/l.})^{-1} \times \text{cm}^{-1}$.[10] NADPH-cytochrome *c* reductase was measured by the increase in absorption at 550 nm.[11] Horse heart cytochrome *c* was purchased from Boehringer-Mannheim. NADPH was regenerated in the cuvette by 1 mM glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase ("Boehringer", 0·4 U; G-6-PDH). Cytochrome P-450 reduction was measured using dual wavelength spectrometry at 450 and 490 nm. The phosphate buffer pH 7·85 contained 1 mM G-6-P and was gassed with oxygen free carbon monoxide to produce anaerobiosis. The NADPH generating system was the same as described above. Measurements were carried out at 37°.

Assay of drug metabolic activities. Although no drug metabolizing activities could be measured in human placental microsomes the methods of detection applied should be briefly documented.

*Acetanilide-*p*-hydroxylase.* Its activity was measured in microsomes or at 9000 *g* supernatant by assaying *p*-hydroxyacetanilide produced using Folin–Ciocalteu's

reagent.[12] The incubation mixture contained 0·67 to 1·3 mM substrate, 18 mM $MgCl_2$, 18 mM KCl, an NADPH regenerating system (G-6-P, G-6-PDH), 0·37 mM nicotinamide and either 1·6 mg microsomal protein/ml or 5 ml 9000 *g* supernatant in 0·07 M phosphate buffer, pH 7·4. After incubating for 1 hr at 37° in a shaking water bath, the mixture was extracted with ether and the blue colour resulting from the addition of the reagent was measured at 691 nm.

p-*Nitroanisole*-O-*demethylase*. This was measured directly in the cuvette at 405 nm;[13] the product *p*-nitrophenol absorbs at this wavelength in slightly alkaline solutions. The dual wavelength method, using 495 nm as a reference, was employed. The assay mixture contained 1 to 2·5 mg microsomal protein/ml, 0·5 mM substrate, 2·5 mM $MgCl_2$, 0·04 mM nicotinamid, and the above mentioned NADPH generating system in a total volume of 3 ml in 0·07 M phosphate buffer, pH 7·85.

Amphetamine hydroxylase. This was measured in a total volume of 6 ml; 0·07 M phosphate buffer, pH 7·4 containing either 9000 *g* supernatant corresponding to 5 g wet wt of cotyledon tissue or microsomes at a protein concentration of 1 mg/ml, 16·5 mM KCl, 12·5 mM $MgCl_2$, 0·04 mM nicotinamide and the NADPH regenerating system. The substrate concentration was 0·3 to 1·0 mM. After 1 hr of incubation at 37° the reaction was stopped by the addition of solid NaCl. The amphetamine not metabolized was extracted into benzene at pH 12 and reacted with methylorange.[14] The colour was measured at 547 nm.

Aniline-p-*hydroxylase*. This was estimated in 105,000 *g* supernatant and microsomes.[15] The product, *p*-aminophenol, reacts with phenol to form an indophenol dye. The assay mixture contained 0·1 M Tris–HCl buffer, pH 8, either 2 ml supernatant (= 3·3 g tissue) or 2·4 mg microsomal protein, 0·5 to 2 mM aniline, 3·3 mM $MgCl_2$, 7 mM nicotinamide and an NADPH regenerating system in a total volume of 3 ml. After 30 min at 37° the reaction was stopped with 1·5 ml of 20% trichloroacetic acid and the product reacted with phenol at alkaline pH. The absorption was measured at 630 nm.

Spectral binding constants (K_s). Spectral binding constants for drugs and steroids were determined with a Perkin–Elmer–Hitachi Model 356 spectrophotometer by reciprocal plotting of maximal spectral differences vs concentration. Steroids were added in microlitre quantities in dimethyl–formamide, the reference cuvette receiving the same amount of the solvent. Most of the difference spectra were recorded at a sensitivity of $A = 0{\cdot}03$ for the total recorder width.

RESULTS

Drug metabolizing activity. As has already been pointed out in the Materials and Methods section no drug metabolic activity could be shown in placental homogenates or microsomes. Thus even with a concentration of 3 mg microsomal protein/ml no demethylation of *p*-nitroanisole was demonstrated. Homogenates as well as microsomes also did not metabolize amphetamine and acetanilide. Also no hydroxylation of aniline could be demonstrated neither in microsomal preparations nor in the 105,000 *g* supernatant. Juchau *et al.*[16,17] described an aniline and benzpyrene hydroxylase, the first of which, however, depends on the presence of haemoglobin in the supernatant. Since, because of the perfusion, our supernatant contained only very little haemoglobin no activity could be found. When rat liver microsomes were used for comparison all substrates mentioned above were metabolized normally.

TABLE 1. MICROSOMAL PROTEIN AND CYTOCHROME P-450 IN HUMAN PLACENTA

Placenta (No.)	Microsomal protein (mg/g wet tissue)	Cytochrome P-450 (nmoles/mg protein)	Remarks
4	1·0	0·046	Not perfused
8	2·7	0·080	Not perfused
9	1·0	0·130	Perfused
10	0·5	0·054	Perfused, frozen, microsomes prepared after 21 days
11	1·3	0·20	Perfused
13	1·0	0·16	Perfused
14	1·6	0·12	Perfused
15	2·0	0·087	Perfused
16	0·9	0·067	Perfused
17	2·0	0·073	Perfused

Presence of mixed function oxidase constituents. Table 1 shows the recovery of microsomal protein and the concentration of cytochrome P-450 in human placental tissue per milligram of microsomal protein. In comparison to human liver studies[18] the amount of microsomal protein obtained is about ten- to thirty-times less, while the cytochrome P-450 content of the microsomes is only about half of that of human liver.[18–20] The table purposely does not show mean values to illustrate the degree of variation in human placentas. Thus on the whole the cytochrome P-450 content per gram of placenta is very markedly less than in liver. Figure 1 shows the carbon monoxide induced spectrum in reduced placental microsomes and illustrates the method of measuring the cytochrome P-450 concentration. Both cuvettes are reduced with sodium dithionite. If only the sample cuvette is reduced[21] the cytochrome b_5 peak at 425 nm will not be balanced and thus in our experience interferes with quantitative evaluation of the cytochrome P-450 peak. The method used is obviously only applicable in perfused haemoglobin free preparations where the difference between reduced haemoglobin and CO–haemoglobin is minimized.

The cytochrome *c* reductase was measured in placentas No. 8, 14 and 15 with the result that its activity at 0·05 mM cytochrome *c* was 26, 87 and 90 nmoles/mg protein/min, respectively. Compared to the values obtained with mouse, rat, rabbit and guinea pig liver microsomes[22] the activity in perfused human placenta microsomes is about half and thus does not correspond to the approximately 5-fold smaller cytochrome P-450 content. Figure 2 shows that 0·1 mM metyrapone inhibits the reduction of cytochrome *c* non-competitively and not very strongly. The inhibition was between 19 and 29 per cent, the Michaelis constant for cytochrome *c* was found to be between 2·2 and 5·2 $\times 10^{-5}$ M.

Preliminary experiments on the anaerobic reduction of cytochrome P-450 by NADPH in the presence of CO showed that this reaction proceeds extremely rapidly and kinetic measurements are difficult. But it may be concluded that cytochrome P-450 reductase is present in human placental preparations.

Thus the electron transport chain from NADPH to cytochrome P-450 is present also in human placental microsomes.

Drug binding. In order to determine the binding of drugs and steroids to placenta

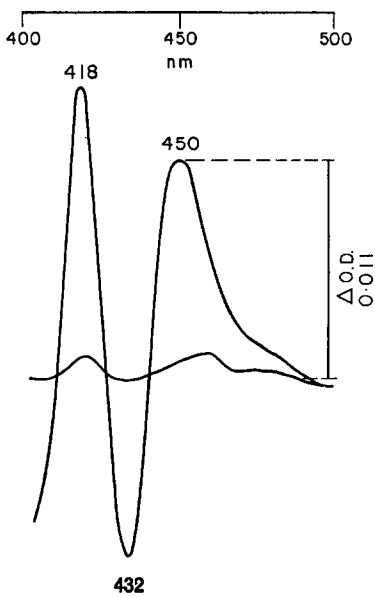

FIG. 1. Determination of cytochrome P-450 in human placenta microsomes. A suspension of microsomes (1 mg protein/ml) in 0·07 M phosphate buffer, pH 7·4, was reduced with sodium dithionite and divided equally between reference and sample cuvettes. (1) Baseline, (2) Sample cuvette bubbled with CO for 1 min. An absorbance difference of 0·011 corresponds to 0·12 nmole of cytochrome P-450/mg protein.

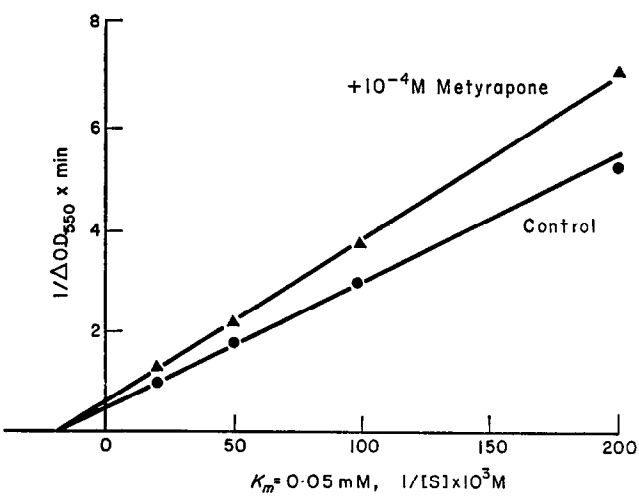

FIG. 2. Double reciprocal plot of NADPH-cytochrome *c* reductase activity vs cytochrome *c* concentration. S = cytochrome *c* concentration, 0·5 mg microsomal protein/ml; ● —— ●, without inhibitor; ▲ —— ▲, with 0·1 mM metyrapone.

microsomes difference spectra were recorded. Figure 3 shows the original tracings of the spectrum obtained after addition of various aniline concentrations to placental microsomes. It shows the spectral characteristics of a type II binding mechanism

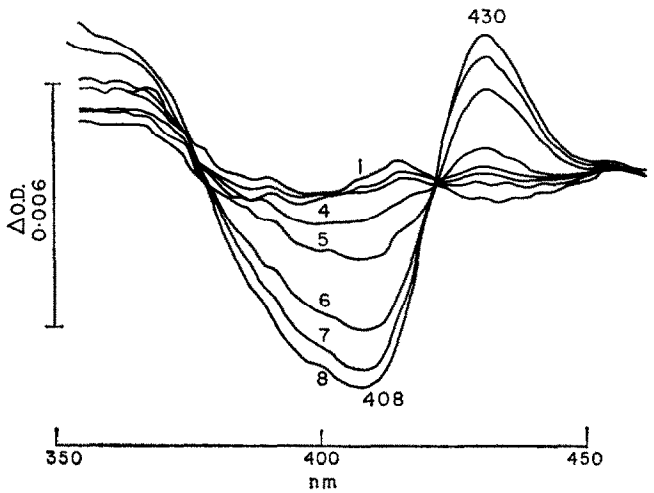

FIG. 3. Difference spectra of aniline in human placenta microsomes. One mg placenta microsomal protein per ml 0·07 M phosphate buffer, pH 7·4. Aniline solutions were made in the same buffer and added to the sample cuvette in 5 to 10 μl. quantities. The reference cuvette received an equal amount of buffer only. Final concentrations: (1), Baseline; (4), 5×10^{-5} M; (5), $1{\cdot}5 \times 10^{-4}$ M; (6), $5{\cdot}5 \times 10^{-4}$ M; (7), $1{\cdot}5 \times 10^{-3}$ M; (8), $5{\cdot}5 \times 10^{-3}$ M.

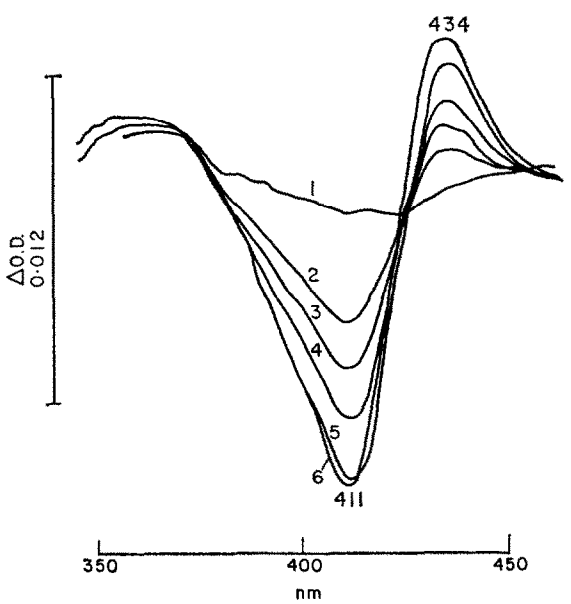

FIG. 4. Determination of K_s for *p*-aminophenol from difference spectra. Conditions as in Fig. 3. 1·6 mg protein/ml. (1), Baseline; (2), 10^{-5} M; (3), 2×10^{-5} M; (4), 4×10^{-5} M; (5), $1{\cdot}4 \times 10^{-4}$ M; (6), $3{\cdot}4 \times 10^{-4}$ M, *p*-aminophenol; K_s, $2{\cdot}1 \times 10^{-5}$ M.

which, however, exhibits maximum and minimum peaks of a slightly different wavelength to those obtained using human liver microsomes. In human liver Ackermann[19] found the maximum at 434 and the minimum at 398 nm. This difference in peak and trough wavelengths may point to a different form of cytochrome P-450 in human liver and placenta. Figure 4 shows the same experiment for *p*-aminophenol and demonstrates a slightly different and much more pronounced spectrum. Table 2 summarizes the results obtained with various compounds and shows that among these substances with a binding spectrum metyrapone and *p*-aminophenol have the lowest spectral binding constants suggesting a considerable binding affinity. It is notable that all these substrates belong to the type II class of compounds. Other members of this group, f.i. acetanilide, acenocoumarin, phenprocoumon, warfarin and dihydralazine, however, show no difference spectra. Type I drugs such as hexobarbital, pentobarbital, amidopyrine, and naphthalene also do not produce a binding spectrum in placental microsomes, in sharp contrast to their ability to produce a spectrum in rat liver microsomes. A further contrast between the two kinds of microsomes is the failure of dihydralazine and metyrapone to produce a spectrum in dithionite reduced placental microsomal preparations such as can be demonstrated in rat and mouse liver microsomes.

TABLE 2. PLACENTAL BINDING SPECTRA AND K_s OF SOME TYPE II COMPOUNDS

Compound	Trough (at nm)	Peak (at nm)	K_s (M)
Metyrapone	395	426	$1{\cdot}3 \times 10^{-5}$
Nicotinamide	408	426	$1{\cdot}1 \times 10^{-3}$
Aniline	408	430	$1{\cdot}0\text{–}3{\cdot}0 \times 10^{-4}$
p-Aminophenol	411	434	$2{\cdot}1 \times 10^{-5}$

Because of the physiological role of the placenta in the oxidative catabolism of steroid hormones we examined the binding of a number of steroids and found three separate types of difference spectra as depicted in Figs. 5–7. Figure 5 shows the redrawn spectrum of 17-α-OH-progesterone, which resembles a modified type I spectrum but shows relatively small spectral excursion for the concentrations employed. The same substance in non-stimulated rat liver microsomes shows a type II difference spectrum. Figure 6 shows an original tracing of a 17-α-OH-pregnenolone induced difference spectrum which must be classified as type II; again, in rat liver microsomes this substance elicits a type I spectrum. Finally a mixed type spectrum has been observed using androstenediol with two peaks and one trough resembling a combination of type I and II spectra (Fig. 7). Thus in this case and with *trans*-dehydroandrosterone and testosterone a striking difference in the spectra compared with rat liver microsomes can be seen; in rat liver microsomes addition of these steroids to the sample cuvette results in type I spectra. Table 3 illustrates the various spectral maxima and minima and the binding constants.

Interaction of metyrapone with steroid binding. In view of the inhibitory action of the type II compound metyrapone on various drug reactions[23] and also its peculiar enhancing effect in liver microsomes[24] the metyrapone effect on steroid binding in

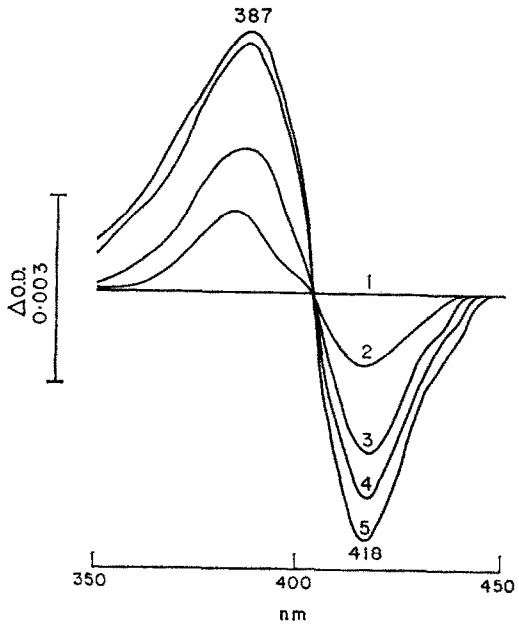

FIG. 5. Difference spectra of 17-α-hydroxyprogesterone. Protein concentration 0·75 mg/ml, pH 7·4. The steroid was dissolved in dimethylformamide and added in 5–10 μl. quantities. (1), Baseline; (2), $5{\cdot}5 \times 10^{-6}$ M; (3), $1{\cdot}05 \times 10^{-5}$ M; (4), $4{\cdot}05 \times 10^{-5}$ M; (5), $8{\cdot}05 \times 10^{-5}$ M; 17-α-OH progesterone.

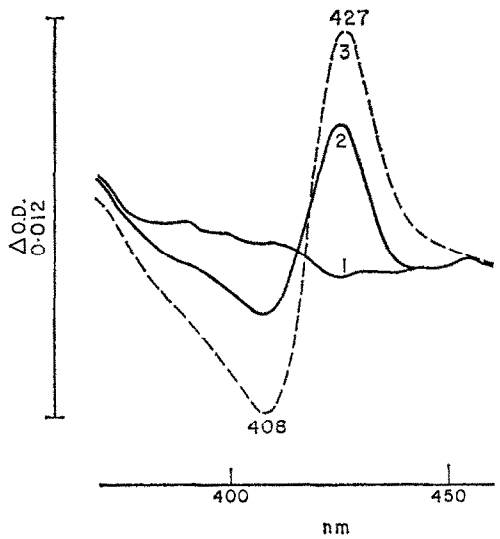

FIG. 6. Influence of metyrapone on the 17-α-OH pregnenolone induced difference spectrum. Protein concentration 1 mg/ml, pH 7·4. (1), Baseline; (2), Sample cuvette saturated with 5×10^{-5} M of the steroid in dimethylformamide; (3), 1×10^{-4} M metyrapone added to sample cuvette. Addition of steroid without dimethylformamide shows that the solvent has no influence on the induction of the difference spectrum.

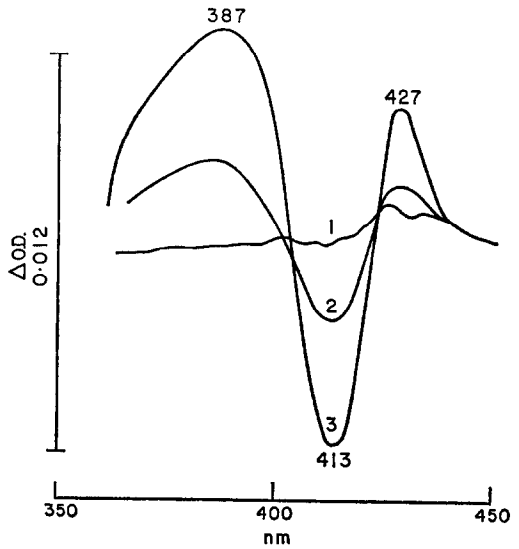

FIG. 7. Mixed-type difference spectrum induced by androstenediol. Protein concentration 1 mg/ml, pH 7·4. (1), Baseline; (2), 2×10^{-7} M; (3), 1×10^{-4} M androstenediol.

placenta seemed to be of interest. Figure 8 shows the alteration of the placental difference spectrum of metyrapone by 17-α-OH-pregnenolone. Metyrapone alone induces a regular type II spectrum; when the steroid is added to both cuvettes the trough is shifted to a longer wavelength. This indicates that the steroid determines the position of the minimum, as can be seen from comparison with Fig. 6, where 17-α-OH-pregnenolone causes a minimum at 408 nm. From both figures it can be concluded that, irrespective of the sequence of addition, both compounds are bound in a type II fashion, demonstrated by the increase in peak height at 426 nm (Fig. 6) and shift of the minimum (Fig. 8). This may suggest two slightly different type II binding sites in placental microsomes.

TABLE 3. STEROID DIFFERENCE SPECTRA

	Human placenta				Rat liver, non-stimulated			
	Min	Max	K_s	Type	Min	Max	K_s	Type
17-α-OH-Progesterone	418	387	$1{\cdot}3 \times 10^{-5}$	I	383	414	3×10^{-7}	II
Progesterone					383	414	3×10^{-7}	II
17-α-OH-Pregnenolone	408	427	$4{\cdot}6 \times 10^{-6}$	II	418	387	$1{\cdot}5 \times 10^{-6}$	I
Pregnenolone	408	427						
trans-Dehydroandrosterone	413	387 427	$2{\cdot}8 \times 10^{-7}$	Mixed	418	384	$1{\cdot}2 \times 10^{-6}$	I
Androstenediol	413	387 427	$1{\cdot}2 \times 10^{-7}$	Mixed	418	385	$1{\cdot}2 \times 10^{-6}$	I
Testosterone	413	387 427		Mixed				

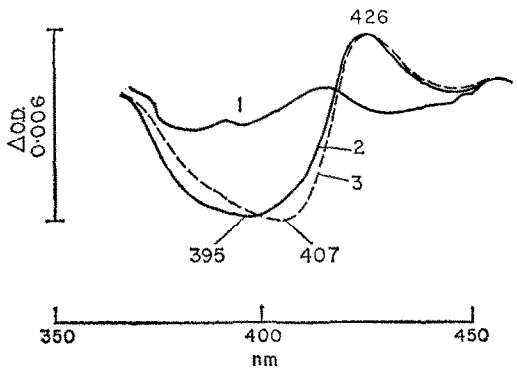

FIG. 8. Alteration of metyrapone difference spectrum by 17-α-OH pregnenolone. Protein concentration 1 mg/ml, pH 7·4. (1), Baseline; (2), $2{\cdot}2 \times 10^{-4}$ M metyrapone; (3), $2{\cdot}2 \times 10^{-4}$ metyrapone added to sample cuvette after addition of 5×10^{-5} M 17-α-OH pregnenolone to both cuvettes. Note the shift of the metyrapone induced minimum to longer wavelengths.

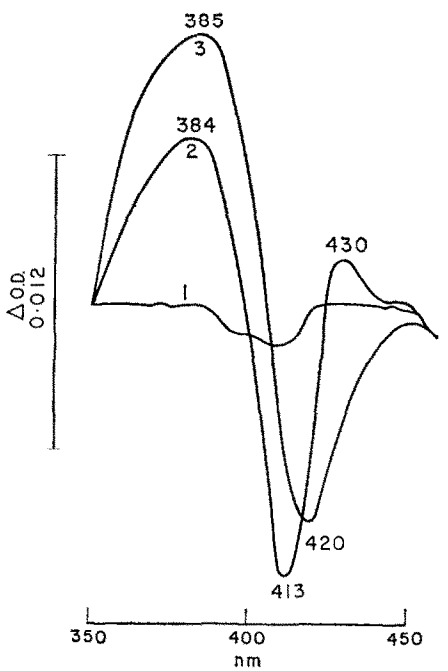

FIG. 9. Interaction of metyrapone with androstenediol induced difference spectrum. Protein concentration 1·5 mg/ml, pH 7·4. (1), Baseline; (2), 1×10^{-5} M androstenediol followed by 3×10^{-4} M metyrapone added to reference cuvette (3).

The situation seems to be different when mixed type steroids such as androstenediol and metyrapone are added and no change is apparent in the original steroid spectrum (Fig. 7), indicating a complete failure to bind metyrapone. Furthermore addition of metyrapone to the reference cuvette leads to a suppression of the type II and an accentuation of the type I component of the mixed spectrum. This compensation causes a slight dislocation of peak and trough wavelengths. Dimethylformamide, used as the solvent for the steroids, does not cause spectral changes in placental microsomes. Nevertheless the reference cuvettes always received the same amount of solvent as did the samples.

DISCUSSION

In this work some parameters of the microsomal mixed function oxidase were examined in an attempt to see if the human placenta can function as a "protective" organ for the foetus by oxidizing possibly harmful drugs.

The determination of the carbon monoxide binding cytochrome P-450 led to results similar to those of Meigs and Ryan[3] who found about half as much cytochrome P-450. This established that placental microsomes do contain the pigment. The cytochrome P-450 content, however, is about ten times less than rat liver and 2 to 3-times less than human liver.[18–21] Human placenta not only possesses a CO binding pigment but also the electron transport chain for its reduction. Measurements of the NADPH-cytochrome *c* reductase and the anaerobic NADPH-cytochrome P-450 reductase revealed activities directly comparable to those of rat liver preparations. Thus there exists a seemingly complete drug (and steroid) oxidizing system in placenta. Yet no drug metabolizing activity could so far be unambiguously demonstrated. In contrast to a few reports on placental drug oxidation[5,25] no metabolism could be found for the drugs examined in this paper. Similarly Yaffe *et al.*[26] found no metabolism of aminopyrine and 3,4-benzpyrene in human fetal liver preparations and corresponding results are described by Juchau.[27]

Since the oxidation of drugs requires their binding to the microsomal pigment, this phenomenon was studied spectrally. It was found that typical type I drugs[28] did not bind at all to placental microsomes. On the other hand a number of type II drugs (Table 2) show an aniline type difference spectrum and also possess spectral binding constants that are comparable to those in rat liver. However, they are not metabolized. Moreover the aniline spectrum in human placenta differs from that in human adult liver[19] by showing its minimum at 408 nm instead of 398 nm and the maximum at 430 nm instead of 434 nm. This can be taken as an indication for a genuinely different configuration of the cytochromes. Furthermore, dithionite reduced placental microsomes do not show a binding spectrum for metyrapone with a single peak at about 446 nm as observed by Hildebrandt *et al.*[29] in rat liver. Aminopyrine (type I) also does not bind, although it has been shown to produce a spectrum with human foetal liver microsomes which was classified as type II.[26] This may be taken as a second argument for a different configuration and specificity of foetal cytochrome P-450.

Further evidence for the special nature of the placental cytochrome P-450 can be derived from the binding experiments with steroids. As illustrated in Table 3 the steroids examined show very different spectral binding types in rat liver and human placenta microsomes. It appears as if there are structurally different binding sites for

the steroid ligands which may be the reason for the specificity of placenta microsomes towards steroid hormones. It remains unclear, whether the production of a mixed type spectrum is due to binding with two different sites or to a "special" complexing mechanism. Metyrapone does not seem to have any effect on mixed type spectra but does bind simultaneously with type II steroids, whereby the steroid determines the minimum. This may mean that placental cytochrome P-450 is different from adrenal cortex mitochondrial cytochrome P-450. In the latter material metyrapone displaces the deoxycorticosterone induced type I difference spectrum.[30] Our experiments suggest that the steroid binding is largely unaffected by metyrapone, and that the steroid compounds have a greater affinity for the binding sites. The lack of a metyrapone binding spectrum in reduced microsomes could be interpreted in the same way.

In conclusion it appears that the human full term placental microsomal mixed function oxidase is inactive towards drugs although spectral binding does occur. This points to distinct structural differences from the adult liver preparation, which may be connected with its higher specificity for steroid substrates.

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