Spectral Studies of Drug Interaction with Hepatic Microsomal Cytochrome

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SUMMARY

Two types of spectral changes are described as resulting from substrate interaction with a hepatic microsomal cytochrome; the magnitude of these spectral changes is dependent on protein concentration and substrate concentration as well as the substrate employed. In two of the three types of substrates examined, the concentration of substrate necessary to evoke half-maximal enzyme activity was similar to the concentration of the same substrate necessary for half-maximal spectral changes. The addition of NADPH, a corequirement for the microsomal mixed function oxidase, causes a modification of both types of spectral changes, without altering substrate affinity. Three possible hypotheses are advanced, based upon the experimental observations, to explain the two types of spectral changes observed.

INTRODUCTION

Studies in the early 1950's provided evidence that the liver endoplasmic reticulum, or its fragments the microsomes, contain an enzyme system capable of converting various drugs and aromatic substances to more polar compounds (1–3). These earlier findings provided great impetus to the study of drug metabolism. At present many different reactions are known to be catalyzed by liver microsomes, and to require NADPH[3] and molecular oxygen. Among these reactions are N, O, and S dealkylations, side-chain oxidations, and ring hydroxylations (4). Still unresolved is the problem of whether the various reactions are catalyzed by one nonspecific enzyme or by several reaction-type specific enzymes.

In 1958 Klingenberg (5) and Garfinkel (6) reported the presence of a carbon monoxide binding pigment in liver microsomes. This pigment was shown by Omura and Sato (7, 8) to be a cytochrome and was named cytochrome P-450 from the position of the absorption peak of the CO-complex of the reduced pigment. The photochemical activation spectra for a number of CO-inhibited microsomal mixed-function oxidases (9–11) have demonstrated the role of cytochrome P-450 as the oxygen activating enzyme for many microsomal oxidation reactions.

Recently (12) we have reported that various drugs, other substrates, and inhibitors of hepatic microsomal mixed-function oxidases react with microsomal cytochrome to give two characteristic types of spectral change. This observation has since been confirmed by Imai and Sato (13). It was concluded that the spectral changes observed are indicative of substrate inter-

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[3] The following abbreviations are employed: Tris, tris(hydroxymethyl) aminomethane; $NADP^+$ and NADPH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide phosphate; ETNC, ethylisocyanide; DPEA, Lilly 32391, 2,4-dichloro-6-phenyl phenoxyethylamine hydrochloride; SKF 525A, β-diethylaminoethyl diphenylpropyl acetate.

action—presumably representing the primary binding of substrate for enzymic hydroxylation. This report will describe in detail the experimental foundation for this hypothesis.

METHODS

Microsomes were prepared from 0.25 M sucrose homogenates of liver by differential centrifugation as previously described (12). Male albino rats (180–250 g) were used in this study, since differences have been observed in the extent of substrate interaction with liver microsomes of male and female rats (14). Difference spectra were recorded with a wavelength scanning recording spectrophotometer (15). The extent of spectral change, upon addition of varying amounts of different substrates, was determined as the difference in absorbance at two fixed wavelengths with an Aminco-Chance dual wavelength spectrophotometer (American Instrument Company, Silver Spring, Maryland). Microsomes used for spectral studies were suspended in 50 mM Tris-HCl buffer, pH 7.5. Enzyme activities were determined at 37° in a medium containing 50 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 0.33 mM $NADP^+$, 8 mM isocitrate, and 15 μg/ml isocitric dehydrogenase (Sigma type IV, 3 μmoles NADPH generated/min/mg at 25°). Aminopyrine, aniline, and hexobarbital were used as substrates for the determination of the different oxidative activities of the mixed-function oxidase. Demethylase activity was determined by measuring formaldehyde produced, using the pH 6.0 Nash reagent B (16). The demethylase reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid to 3 ml of a reaction medium containing 1 mg of microsomal protein per milliliter. After removal of the protein by centrifugation at about 18,000 *g* for 5 min, 1.5 ml of the supernatant fluid was mixed with 1.5 ml of Nash reagent B and incubated at 58° for 8 min in a water bath. The sample was then cooled to room temperature and the intensity of the yellow color was determined at 412 mμ. Aniline hydroxylase activity was determined by measuring *p*-aminophenol formation (method of Imai and Omura, personal communication). The hydroxylation reaction was terminated by the addition of 1.5 ml of

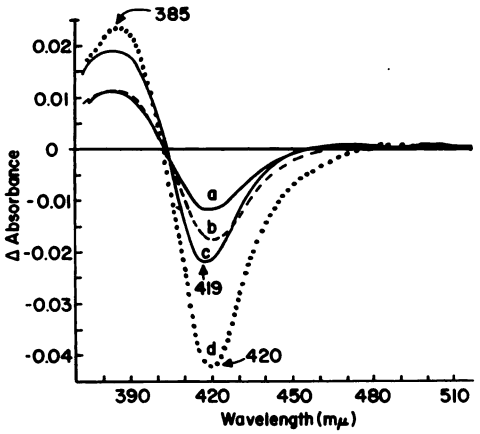

FIG. 1A

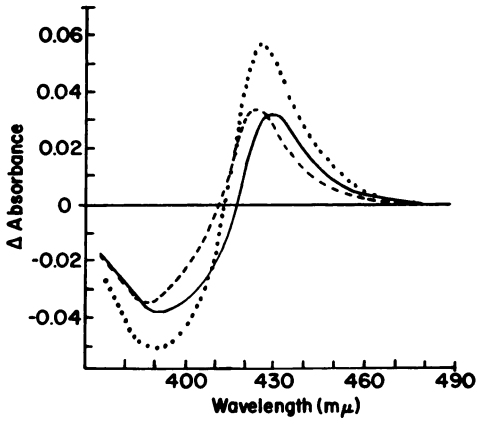

FIG. 1B

FIG. 1. *Spectral changes caused by addition of various substances to suspensions of rat liver microsomes*

Six milliliters of microsomal suspension (2 mg protein per milliliter) was diluted in 50 mM Tris buffer, pH 7.5, and divided into two cuvettes; a baseline of equal light absorbance was recorded. The baseline was subtracted from the change in light absorbance caused by the addition of the different chemicals to one cuvette, and the resultant difference spectra were plotted. Spectra were obtained at room temperature. Fig. 1A: Spectral changes caused by the addition of: (*a*) 5 mM aminopyrine; (*b*) 5 mM amobarbital; (*c*) 5 mM hexobarbital; (*d*) 0.083 mM SKF 525 A. Fig. 1B. Spectra shown in the solid line were obtained with 5 mM aniline; the dashed line, with 40 mM nicotinamide; and the dotted line, with 30 mM pyridine.

20% trichloroacetic acid to 3 ml of a reaction medium containing 1 mg of microsomal protein per milliliter, and the protein was removed by centrifugation at 18,000 *g* for 5 min. Of the supernatant fluid, 1 ml was added to 1 ml of 0.5 M NaOH containing 1% phenol; after mixing, 1 ml of M Na_2CO_3 was added. After 20 min incubation at room temperature, the intensity of the blue color was determined at 630 mμ. The metabolism of hexobarbital was measured by determining spectrally the concentration of unchanged hexobarbital at 242 mμ. The samples were extracted with petroleum ether, and the hexobarbital was reextracted into buffer solution, pH 10.8, as described elsewhere (17).

RESULTS

Types of Spectra

The addition of various substrates of the microsomal mixed-function oxidase system to aerobic liver microsomes causes two types of spectral change. One class of spectral change (termed type I) is characterized by the appearance of a trough at 420 mμ and an absorption peak at 385–390 mμ (Fig. 1A). This type of spectral change is similar to that reported by Narasimhulu *et al.* (18) for the interaction of 17-hydroxyprogesterone with adrenal cortex microsomes. In the present studies, those compounds so far tested causing this type of spectral change with hepatic microsomes

TABLE 1

Comparison of absorption maxima and minima and relative change in absorbance (ΔOD) of compounds causing type I and type II spectral changes with liver microsomes

Difference spectra were obtained at room temperature with freshly prepared microsomes from livers of adult male rats. Microsomes were suspended in 50 mM Tris-HCl, pH 7.5, to a concentration of 2 mg protein/ml. The change in optical density (ΔOD) values were determined from the difference spectra after addition of an excess of the listed substrates. In each instance the ΔOD values listed are expressed as the change in absorbance observed for a microsomal protein content of 1 mg/ml. The ΔOD of cytochrome P-450 was determined at 450 mμ relative to 500 mμ in samples treated with CO for 1 min, followed by the addition of a few milligrams of $Na_2S_2O_4$. Since the P-450 content of microsomal preparations with the same protein content may vary, the change in optical density is also related to the amount of P-450 present in the suspension. Spaces marked by dashes represent compounds for which difference spectra were obtained with liver microsomes of phenobarbital-treated rats.

Compound, type I	ΔOD/mg/ml t[a]-500 mμ	ΔOD/ΔOD P-450[b]	Absorption Minimum	Absorption Maximum
Aminopyrine	0.005	0.066	420	385
Phenobarbital	0.007	0.073	420	385
Amobarbital	0.009	0.096	420	385
Hexobarbital	0.010	0.128	419	385
SKF 525A	0.022	0.247	420	385
DDT[d]	0.005	0.099	422	388
Chlorpromazine	0.014[c]	0.181[c]	420	385
N,N-Dimethylaniline	0.003	0.052	421	390
Testosterone	—	—	418	388
Imipramine	—[c]	—[c]	420	388
β-Estradiol	—	—	425	390

Compound, type II	ΔOD/mg/ml p[a]-500 mμ	ΔOD/ΔOD P-450[b]	Absorption Minimum	Absorption Maximum
Nicotine	0.004	0.045	395	428
Aniline	0.011	0.175	392	430
Nicotinamide	0.017	0.191	390	425
DPEA[d]	0.022	0.241	395	431
Pyridine	0.028	0.120	390	426
p-Aminophenol	0.008	0.156	405	435

Compound, modified type II	ΔOD/mg/ml p[a]-500 mμ	ΔOD/ΔOD P-450[b]	Absorption Minimum	Absorption Maximum
Cortisol	—	—	365	409
Corticosterone	—	—	375	413
Acetanilide	0.004	0.068	388	420
Ethylisocyanide	—	—	410	433
Cyanide	0.021	0.341	410	445
Rotenone	—	—	390	420

[a] Absorbance of the absorption peak (p) or trough (t) relative to 500 mμ.

[b] P-450 obtained as described in legend.

[c] Accurate values could not be obtained because the addition of this compound caused the microsomes to agglutinate.

[d] DDT, dichlorodiphenylthrichloroethane; DPEA, 2,4-dichloro-6-phenyl phenoxyethylamine hydrochloride.

include barbiturates, aminopyrine, and chlorpromazine, as well as the inhibitor (19) of drug oxidations, β-diethylaminoethyl diphenylpropyl acetate (SKF 525A). Some steroids cause a similar type of spectral change; these include testosterone and β-estradiol. A detailed summary of the compounds examined is listed in Table 1.

The second class of spectral change (termed type II) is characterized by the appearance of an absorption peak at about 430 mμ and a trough at about 390 mμ (Fig. 1B). This type of change occurs upon the addition to aerobic liver microsomes of amines, like aniline and pyridine, and the inhibitor (20) of the microsomal oxidase, 2,4-dichloro-6-phenyl phenoxyethylamine hydrochloride (DPEA) (Lilly 32391). Several other compounds cause a slightly modified type II spectrum when added to aerobic liver microsomes. These include cortisol, corticosterone, and cyanide. The magnitude of the spectral changes, as well as the location of maxima and minima observed, are summarized for a number of compounds in Table 1.

Effect of Substrate Concentration

The magnitude of the spectral changes observed is dependent upon the concentration of substrate added to the microsomal suspension, as well as the concentration of microsomal protein employed. The spectra illustrated in Fig. 2 were obtained by recording the change in absorbance as a function of wavelength between microsomes with substrate and microsomes without added substrate, following repeated additions, for example, of hexobarbital (Fig. 2A) or aniline (Fig. 2B). A similar titration of these spectral changes can be obtained by determining the absorbance difference between two fixed wavelengths, a maximum or minimum and the isosbestic point. A plot of the change of absorbance at fixed wavelengths, as a function of aniline concentration at three different microsomal protein concentrations, was made. The hyperbolic shape of the resultant titration curve indicated that substrates may be interacting in a manner comparable to that observed in the determination of enzyme activity with

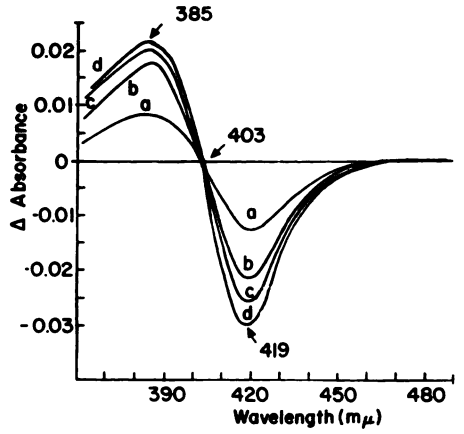

FIG. 2A

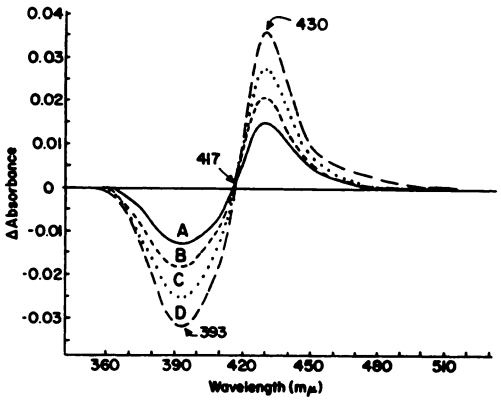

FIG. 2B

FIG. 2. *The effect of substrate concentration on the magnitude of the spectral change*

Fig. 2A. The effect caused by consecutive additions of hexobarbital: (*a*) 0.083 mM; (*b*) 0.33 mM; (*c*) 0.66 mM; (*d*) 4.7 mM. Fig. 2B. The effect of consecutive additions of aniline: (*a*) 0.27 mM; (*b*) 0.54 mM; (*c*) 1.2 mM; (*d*) 2.5 mM.

substrate concentration. Therefore the reciprocals of that data were plotted as illustrated in Fig. 3. Extrapolation of the reciprocal plot to the abscissa permits the determination of the concentration of substrate required for half-maximal spectral change. This constant will be termed here a "spectral dissociation constant." The spectral dissociation constants for a number of substances are included in Table 2.

It is interesting to note that nicotinamide also gives an absorbance change, and that the concentration necessary for saturation

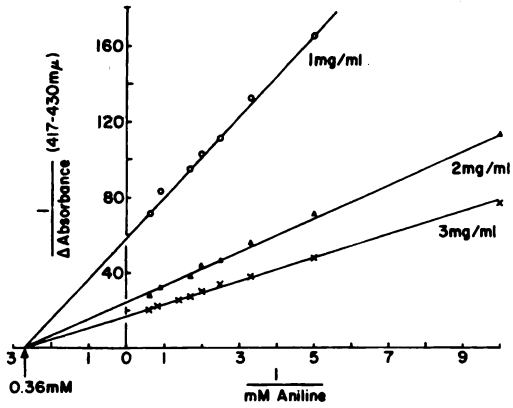

FIG. 3. *A reciprocal plot of changes in absorbance at 430 mμ relative to 417 mμ caused by consecutive additions of aniline to three different concentrations of microsomal protein*

Liver microsomes were suspended in 0.1 M Tris buffer, pH 7.5. Changes in absorbance were determined with an Aminco-Chance dual wavelength spectrophotometer at room temperature.

with this substance (about 15 mM) is below the concentration of this chemical usually added (20–50 mM) (2, 21) to the assay media to inhibit pyridine nucleotidase (22). Cyanide, which has been previously reported (4, 21) not to inhibit the mixed-function oxidase system of microsomes, also interacts with microsomal cytochrome, but the spectral dissociation constant for this compound is considerably higher than that of the usual substrates (see Table 2). In separate enzymic experiments it has been noted that concentrations of cyanide approaching saturation level (10 mM), as determined in spectral studies, do inhibit aminopyrine demethylation (unpublished experiments).

TABLE 2

Concentrations of substances required for half-maximal enzyme activities (K_m) and half-maximal spectral changes (K_s)

The spectral dissociation constants (K_s) were obtained by means of a double reciprocal plot of spectral changes associated with the addition of increasing concentrations of the respective substrates to suspensions of liver microsomes (1, 2, and 3 mg protein/ml). Changes in absorbance were determined at two fixed wavelengths by means of an Aminco-Chance dual wavelength spectrophotometer at room temperature. The Michaelis constants were obtained from reciprocal plots of enzyme activity at different substrate concentrations.

Substrate	K_m (mM)	K_s (mM)
Aminopyrine	0.36	0.33
Hexobarbital	0.10	0.08
SKF 525A	—	0.0005–0.001
Aniline	0.04	0.36
Nicotinamide	—	2.85
DPEA	—	0.005
KCN	—	2.1

Effect of NADPH

The addition of NADPH to a microsomal suspension pretreated with hexobarbital or aniline, for example, results in a modification of the spectral change and a shift in the location of the absorption maximum or minimum. The addition of excess NADPH to microsomes in the presence of hexobarbital causes a further decrease in absorbance and a shift of the trough minimum to 423 mμ. The addition of NADPH to microsomes in the presence of aniline causes a decrease in the absorption peak. Although the absorption peak is consistently observed to decrease in height, some experiments have shown an associated shift of the absorption maximum to 435 mμ (12).

Although the addition of NADPH causes spectral changes as reflected in the difference spectra, its addition is without effect on the affinity of substrates as determined by spectral titration experiments (Fig. 4), i.e., the spectral dissociation constants for aniline and hexobarbital as obtained from reciprocal plots are the same in the presence (solid triangles, Fig. 4) and absence (solid circles, Fig. 4) of NADPH. However the addition of an excess of the chemical reductant, sodium dithionite, obliterates the substrate-induced spectral changes.

Unlike the reaction of steroids with the cytochrome of adrenal microsomes (18), a disappearance of the substrate-induced spectral change is not observed with time in the presence of NADPH. This is possibly due to the relatively large concentrations of substrates required for observation of enzyme activity and substrate-induced spectral changes with liver microsomes, i.e.,

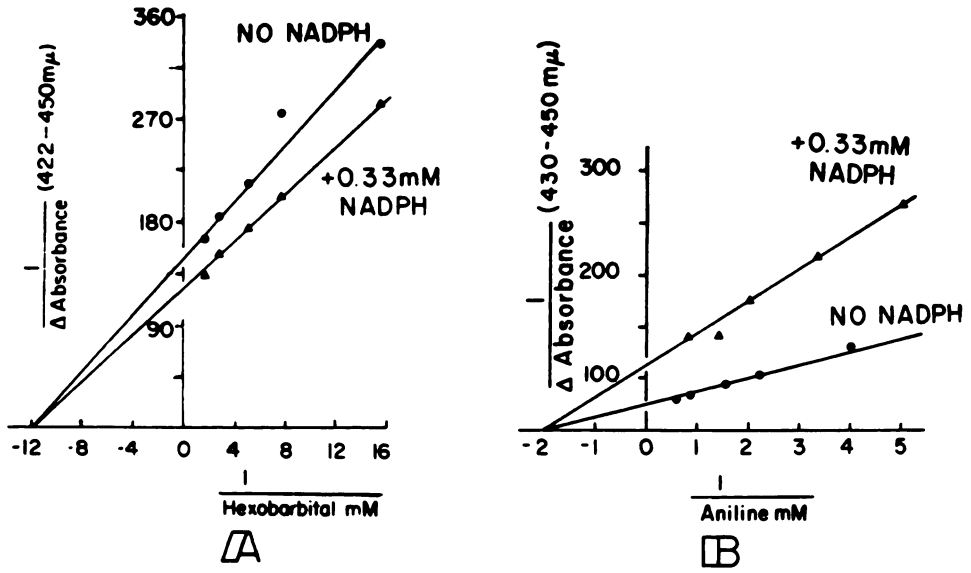

FIG. 4. *Reciprocal plots of the hexobarbital and aniline-dependent spectral changes in the presence and the absence of NADPH*

The microsomal protein concentration was 1.45 mg/ml in Fig. 4A, and 2 mg/ml in Fig. 4B. Microsomes were suspended in 50 mM Tris buffer, pH 7.5. The solid circles are in the absence of NADPH, and the solid triangles are in the presence of 0.33 mM NADPH.

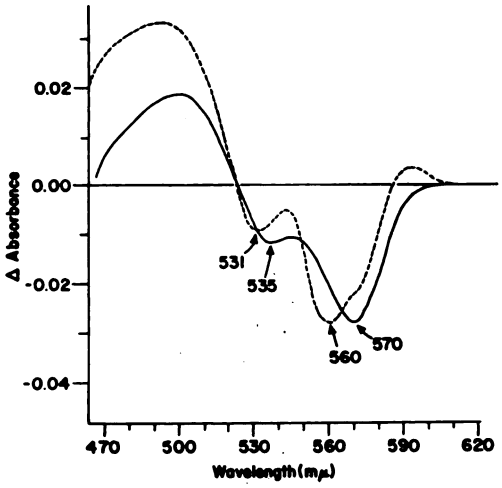

FIG. 5A

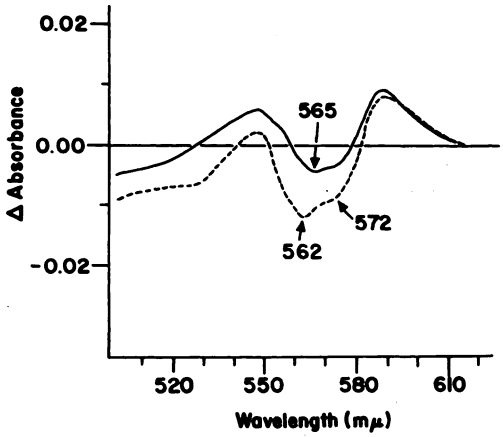

FIG. 5B

FIG. 5. *Spectral changes in the visible region of the spectrum accompanying changes in the Soret region and near UV*

In Fig. 5A the solid line indicates the effect of 4.2 mM hexobarbital addition to the experimental cuvette, and the dashed line indicates the subsequent addition of 0.17 mM NADPH to the microsomal suspension in the reference cuvette and to the microsomal suspension containing hexobarbital. Liver microsomes from phenobarbital-treated rats were suspended in 50 mM Tris buffer, pH 7.5, to a concentration of 2.5 mg/ml.

In Fig. 5B the solid line shows the effect of 3.7 mM aniline addition to the experimental cuvette, and the dashed line indicates the effect of a subsequent addition of 0.17 mM NADPH to the microsomal suspension in the reference cuvette and to the microsomal suspension containing aniline. Liver microsomes from phenobarbital-treated rats were suspended in 50 mM Tris buffer, pH 7.5, to a concentration of 2.1 mg/ml.

both the enzymically determined Michaelis constant and the spectrally determined dissociation constants for the mentioned drugs are on the order of 10^{-4} M. With adrenal cortex microsomes, the concentrations of steroids required for a spectral change are on the order of 10^{-6} M (18). Another difference between the adrenal cortex microsomal system and the liver microsomal system is the disappearance of the spectral change in the former in the absence of oxygen, when excess NADPH and excess substrate are present (18); the substrate-induced spectral changes observed with liver microsomes remain, even after the medium has become anaerobic in the presence of excess NADPH, or after deoxygenation with nitrogen.

Spectral Changes in the Visible Region

Concomitant with substrate-induced changes in the Soret and near-UV regions, spectral changes also occur in the visible region of the spectrum. Changes in the visible spectrum accompanying type I spectra, in the absence and presence of NADPH, are shown in Fig. 5A.

The spectral changes illustrated in Fig. 5B show the modification of the visible spectra which accompany the type II spectral changes in the presence and absence of NADPH. Higher concentrations of microsomal protein are required for observation of the spectral changes in the visible region, as they are not as intense as those in the Soret and near-UV regions. Liver microsomes from male phenobarbital-treated rats were used in these experiments in order to obtain greater changes in the difference spectra.

The addition of potassium ferricyanide to aerobic liver microsomes does not cause any spectral change per se, nor does it alter the spectral change normally seen with aniline or hexobarbital. Because potassium ferricyanide has an intense absorption band with a maximum at 420 mμ, the influence of this chemical oxidant was examined by measuring the visible spectral changes caused by substrate.

Reversibility of Substrate Interaction

In order to determine whether the interaction between substrate and the microsomal cytochrome is reversible or not, washing experiments were carried out. Saturating concentrations of hexobarbital (Fig. 6A) or aniline (Fig. 6B) were added

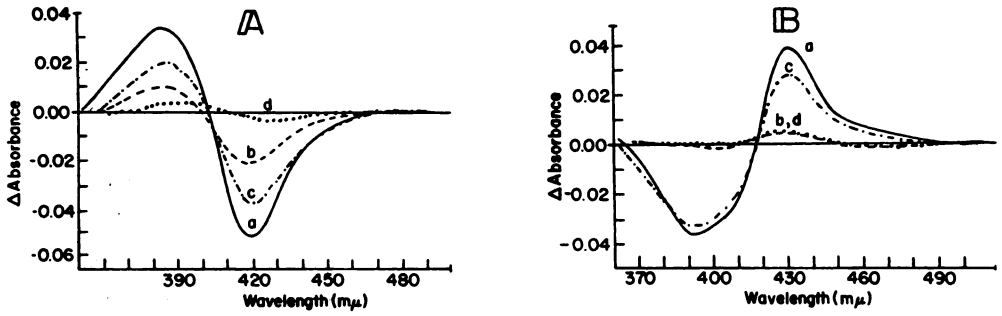

FIG. 6. *Reversibility of hexobarbital or aniline binding to rat liver microsomal cytochrome*

Microsomes were suspended to 2 mg/ml in 50 mM Tris buffer, pH 7.5. Aliquots (3 ml) were added to each of two cuvettes and a baseline of equal light absorbance was determined, as in Fig. 1. Curve *a* of Fig. 6A shows the effect of addition of 2 mM hexobarbital to one (experimental) cuvette, and curve *a* of Fig. 6B shows the effect of addition of 1.8 mM aniline to the experimental cuvette. An equal volume of buffer was added to the other (reference) cuvettes before the difference spectra were recorded. Curves *b* show the effect of sedimenting the microsomes in a Spinco at 50,000 rpm for 15 min and, after decanting all the supernatant fluid, resuspending the microsomal pellets to the previous volume with fresh buffer. Microsomes from the experimental and reference cuvettes were handled separately. Curves *c* show the effect of readdition of the previous concentration of substrates to the respective experimental cuvettes. Curves *d* show the effect of addition of an identical amount of substrates to the respective reference cuvettes.

to rat liver microsomes, and the difference spectra were recorded (curves *a*). The microsomal suspensions with substrate were then centrifuged at 50,000 rpm in a Spinco refrigerated ultracentrifuge for 15 min. The microsomal pellets were then resuspended in buffer to the original volume, and the difference spectra were recorded (curves *b*). Control samples of microsomes, used as reference for the difference spectral measurements, were subjected to the same washing procedure. These experiments show that the spectral changes caused by aniline (Fig. 6B, curve *b*) or hexobarbital (Fig. 6A, curve *b*) are largely obliterated when the suspending medium is replaced by the medium without substrate.

The addition of more of the respective substrates causes a restoration of the absorbance change (curves *c*), illustrating that substrate interaction is indeed reversible and does not result in the conversion of one stable form of the enzyme to another stable form. The inability to completely restore the substrate-induced spectral changes may be due in part to the destruction of some hemoprotein by the washing treatment employed, since the addition of an equal concentration of the respective substrates to the sample of control microsomes in the reference cuvettes (curves *d*), restores the baseline of equal absorption. Also, a small destruction of hemoprotein is evidenced by observing the difference spectrum between washed untreated microsomes and the same microsomes which have not been subjected to the washing procedure.

Studies by Ernster and Orrenius (23) with ^{14}C-labeled aniline and phenobarbital have indicated that these substances are bound to the microsomes. The fact that about three times as much labeled aniline was bound to liver microsomes of phenobarbital-pretreated rats, as compared with controls, and that the microsomal P-450 was elevated 3-fold in the microsomes of these induced animals, suggested that cytochrome P-450 might be involved in the binding of these substances. However, contrary to our findings, their data indicate that the binding of aniline to microsomes is not reversible, the aniline remaining bound even after 4–6 washings. It is interesting that the concentration of aniline used by these workers (0.3 mM) is only enough to half saturate the spectrally observed binding sites (see Table 2), whereas the amount of aniline remaining bound to their microsomes was equivalent to half the amount of CO-binding pigment present in their microsome preparation.

Effect of Substrates on the CO-Binding Spectrum

Experiments by Cooper *et al.* (10) and Orrenius (21) have related the microsomal carbon monoxide-binding cytochrome to the microsomal mixed-function oxidase of the liver. The carbon monoxide-binding pigment is also related to the drug-binding cytochrome, as illustrated in Fig. 7. The addition of increments of aniline to liver microsomes previously exposed to CO and NADPH causes a decrease in the magnitude of the 450 mμ absorption peak with the subsequent appearance of an absorption peak at 435 mμ, in a manner similar to that shown by Omura and Sato (7) with ethyl isocyanide. The addition of hexobarbital to

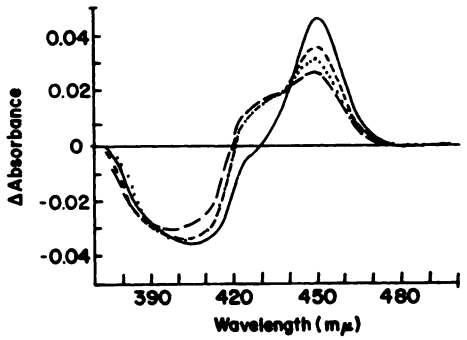

FIG. 7. *The effect of aniline on the 450 mμ CO-complex of cytochrome P-450*

The conditions were as described in Fig. 1. The microsomal suspension in the experimental cuvette was gassed with CO for 1 min, and then 0.17 mM NADPH was added to each cuvette (solid line). Aliquots of aniline were then added to the experimental cuvette, and the difference spectra were recorded after each addition. The dashed line represents the results obtained with 7 mM aniline; the dotted line, with 14 mM aniline; and the long dashes, with 21 mM aniline.

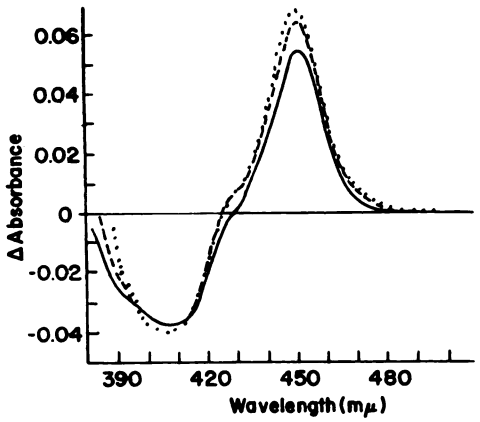

FIG. 8. *The effect of hexobarbital on the 450 mμ CO-complex of cytochrome P-450*

The conditions were as described in Fig. 7. After CO and NADPH additions (solid line), aliquots of hexobarbital were added and the difference spectra were recorded. The dashed line represents the results obtained with 0.33 mM hexobarbital, and the dotted line represents the results with 2.7 mM hexobarbital.

CO-pretreated microsomes, in the presence of NADPH, does not cause a disappearance of the CO-binding spectrum, but actually enhances it (Fig. 8).

Relation between Substrate Levels at Half-Maximal Enzyme Activity and Substrate-Induced Spectral Changes

The microsomal mixed-function oxidase and the pigment responsible for substrate-induced spectral changes can be related by a comparison of the substrate concentrations required to express half-maximal enzyme activity and half-maximal change in absorbance. The values obtained for the spectral dissociation constant (K_s) of several compounds are listed in Table 2, together with the enzymically obtained Michaelis constants of some of these substances. The two substrate dependence values obtained are similar, and are suggestive of a relationship between these two descriptive constants. This similarity suggests the hypothesis that the spectral changes observed are indicative of an enzyme substrate complex function. The constants obtained with aniline, however, differ by nearly one order of magnitude, the Michaelis constant being one-tenth that of the spectral affinity constant. Whether this indicates no relationship between K_m and K_s for type II compounds is not known at this time.

DISCUSSION

Two types of spectral change result from the interaction of various substances with a hepatic microsomal pigment. The intensity of these spectral changes is dependent on the concentration of both substrate and microsomal protein.

The interaction between the microsomal cytochrome and substrates of the two categories may be considered a freely reversible binding, since spectral evidence of substrate interaction can be largely removed by centrifuging the microsomes from the substrate containing medium and resuspending them in a medium free of substrate; a reintroduction of substrate causes a restoration of the spectral change.

The binding of substrates to the microsomal cytochrome presumably precedes enzymic oxidation; the appearance of a spectral change therefore may be considered as evidence for the formation of an enzyme-substrate complex. Certainly the similarity in two of the three substrates studied, between the spectral dissociation constant and the Michaelis constant obtained by determining the substrate concentration at half-maximal enzyme activity, is support for such a conclusion. The possibility does exist that the type II binding spectrum is merely the result of hemochrome formation with nitrogenous bases. However, the concentration of the bases required to observe the spectral change is about two orders of magnitude lower than that usually employed.

Direct proof as to the nature of the site at which the various substances interact requires a more homogeneous preparation of the microsomal mixed-function oxidase, a knowledge of the chemical bonds of the heme moiety of cytochrome P-450, and a knowledge of the coordination bonds of the fifth and sixth ligands of the heme iron. The experimental results suggest three

alternative hypotheses, which are currently under consideration:

1. The two types of spectral change are expressions of substrate interaction with two different heme-proteins or enzymes of the microsomal mixed-function oxidase. The great diversity of sizes and types of compounds oxidized by this enzyme system is certainly amenable to this hypothesis, but the fact that substrates causing one type of spectral change competitively inhibit metabolism of substrates, which cause the same and other types of spectral change (unpublished experiments), weakens this hypothesis.

2. Different substrates affect different ligands of the heme with the enzyme protein, the latter causing a spectral shift by modification of the heme-protein ligand interaction. An alternative of this hypothesis is that substances cause the different types of spectral changes by modifying the ligand on different sides of the heme of cytochrome P-450. Data obtained by electron paramagnetic resonance (EPR) spectroscopy (24) suggest that substrate interaction does, in fact, modify ligand interaction with the heme iron of cytochrome P-450. In other words, the spectral changes observed may be due to a modification of the molar extinction coefficient of the hemoprotein as more or less nucleophilic substances interact near the heme iron, and affect the heme-iron-protein interaction. Thus we would propose that substrates causing type II spectral change might affect the same site as does oxygen, since the addition of aniline displaces the CO-complex of P-450. This hypothesis suggests, but does not require, that type II substrates are activated, instead of oxygen, and that the activated substrate then interacts with oxygen to form the hydroxylated product.

3. The two types of spectral change are the result of alteration of the oxidation state of the cytochrome P-450. This hypothesis suggests that substrate addition results in the conversion of an "active oxygen" form of cytochrome P-450 to an oxidized form. This hypothesis implies an electron transfer during interaction with substrate. The experiments described, using the chemical oxidant potassium ferricyanide, as well as the washing experiments, appear to disprove this hypothesis, unless the oxidation-reduction potential of the "active oxygen" complex of cytochrome P-450 is significantly more positive than that of the potassium ferro- and ferricyanide couple.

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