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Studies on the Nature of the Type I and Type II Spectral Changes in Liver Microsomes*

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ABSTRACT: The spectral changes which occur when substrates (type I) and basic amines (type II) interact with the terminal oxidase (cytochrome P-450) of the hepatic microsomal mixed-function oxidase have been reexamined. The type I spectral change which has been called the spectral manifestation of the enzyme–substrate complex, was also found to be present in the type II spectral change, and to be the cause of the asymmetrical shape of the trough in difference spectrum. When correction is made for the type I component in the type II spectral change, the resultant spectral change becomes both symmetrical and greatly enlarged, as well as shifted slightly toward the blue end of the spectrum. In 3,4-benzpyrene-treated rats, the lack of type I spectral change when type I substrates are added is not due to absence of the type I binding site; rather, it is caused by an inability of type I substrate to interact. That the type I binding site is present in liver microsomes of 3,4-benzpyrene-treated rats was shown by the presence of types I and II composite spectral change.

Hepatic microsomal cytochrome P-450 has been implicated as the oxygen-activating terminal oxidase of a number of drug oxidase (Cooper *et al.*, 1965), steroid oxidase (Conney *et al.*, 1968), and polycyclic hydrocarbon oxidase (Silverman and Talalay, 1967) reactions. Because of the tremendous variation in size, shape, and functional groups (organic chemical classification) of the substrate molecules, it is difficult to consider the mixed-function oxidase as a single enzyme, although the terminal oxidase moiety, cytochrome P-450, has, until recently, been thought of as a single hemoprotein and different substrates have been shown to competitively inhibit the metabolism of one another (Rubin *et al.*, 1964).

Studies by a number of investigators (Imai and Sato, 1966; Sladek and Mannering, 1966) have suggested, on the basis of the double Soret-banded ethyl isocyanide complex of cytochrome P-450, that two forms of the hemoprotein exist in liver microsomes. Sladek and Mannering (1969) have suggested that rather than two forms, two different species exist, based upon an apparent selective induction of one form with polycyclic hydrocarbons (Alvares *et al.*, 1967; Hildebrandt *et al.*, 1968), and the selective elevation of substrate-metabolizing activity (Sladek and Mannering, 1969; Conney, 1967). Xenobiotics interacting with the hepatic microsomal mixed-function oxidase can be categorized into two groups with respect to their affect on the spectral properties of microsomal suspensions. One group, composed only of known substrates of the mixed-function oxidase, cause what has been termed the type I spectral change (Schenkman *et al.*, 1967a) when added to liver microsomal suspensions. It is characterized by the appearance in difference spectrum of an absorption peak at 388 nm and the disappearance of an absorption band at 420–22 nm. The second group of compounds is composed of basic amines, of which only aniline is a known substrate. These form the type II spectral change (Schenkman *et al.*, 1967a), which is characterized by the appearance of an absorption peak in the Soret region, and the disappearance of absorption at 390 nm; the position of the absorption peak is a characteristic of the compound added, and has been suggested as being due to the formation of a ferrihemochrome with cytochrome P-450 (Schenkman *et al.*, 1967a). However, the type II spectral changes differ from most basic amine ferrihemochromes by having an asymmetrical trough in difference spectrum.

The type I spectral change has been implicated as the spectral manifestation of a complex between the substrate and the mixed-function oxidase (Schenkman *et al.*, 1967a), and shown to parallel enzyme activity (Schenkman *et al.*, 1967b). Indeed, after pretreatment of rats with the inducer phenobarbital, which elevates drug metabolism, the type I spectral change is increased in parallel. A disturbing finding was the observation that the polycyclic hydrocarbon inducer 3,4-benzpyrene, which elevates the level of cytochrome P-450 in liver microsomes did not increase the magnitude of the type

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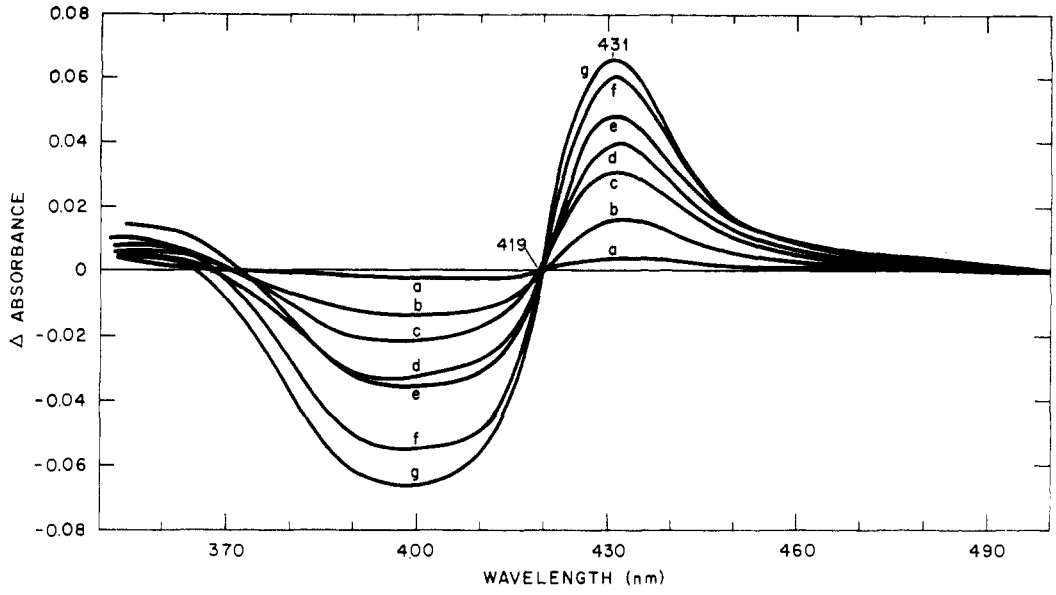

FIGURE 1: Spectral titration of the aniline-induced type II spectral change. Liver microsomes from adult male rats were suspended to 1 mg of protein/ml in 0.1 M Tris-HCl (pH 7.6) and distributed between two cuvets. Aniline was added to one cuvet and buffer was added in equal volumes to the reference cuvet. Total volume of additions was 23 μl to 3 ml of suspension: (a) 0.037 mM, (b) 0.224 mM, (c) 0.69 mM, (d) 1.62 mM, (e) 3.7 mM, (f) 11.2 mM, and (g) 18.5 mM aniline.

I spectral change, but actually decreased it (Remmer *et al.*, 1969). After 3,4-benzpyrene pretreatment of rats, it was noticed that although the type I spectral change was depressed, the aniline-induced type II spectral change was increased in the pretreated animals.

The purpose of this communication is to provide further evidence that the two P-450 hemoproteins are merely interconvertible forms of the same hemoprotein. The data show the reason for the asymmetry in the type II spectral change, indicate that only one type I binding site is available in liver microsomes, and explains recent findings (Schenkman, 1969; Leibman *et al.*, 1969) that type I substrates enhance type II spectral changes.

Materials and Methods

Liver microsomes were prepared from 0.25 M sucrose–1 mM EDTA homogenates as described previously (Schenkman *et al.*, 1967a). Difference spectra were recorded with an Aminco-Chance dual-wavelength recording spectrophotometer, as were dual-wavelength titrations. All chemicals and biochemicals were of the highest purity available commercially, and with the exception of aniline, were not purified further.

When difference spectra were recorded, the same microsomal suspension was divided between two cuvets. Compounds were added in aqueous medium to the sample cuvet and equal volumes of buffer were added to the reference cuvet, unless otherwise stated in figure legends.

Spectral titrations at fixed wavelengths were subjected to double-reciprocal plots, derived from the kinetic equation as

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_3} E + P$$

where E is enzyme, C is enzyme-substrate complex, and S is substrate. Titrations were performed at room temperature in the absence of NADPH, hence the reaction does not proceed to product P. The reversibility of the first step has been demonstrated in washing experiments (Schenkman *et al.*, 1967a).

The dissociation constant, K_s, termed "spectral dissociation constant" (Schenkman *et al.*, 1967a), is

$$K_s = \frac{([E_t] - [C])[S]}{[C]}$$

where E_t is the total amount of enzyme. By manipulation

$$K_s = \frac{[E_t][S]}{[C]} - [S]$$

and

$$[C] = \frac{[E_t][S]}{K_s + [S]}$$

The reciprocal of this equation

$$\frac{1}{[C]} = \frac{K_s}{[E_t]}\left(\frac{1}{[S]}\right) + \frac{1}{[E_t]}$$

is the equation of a straight line; when the reciprocal of the type I or type II spectral change $1/[C]$ is plotted against the reciprocal of substrate level used $1/[S]$, the Y intercept is equal to $1/[E_t]$ or the reciprocal of $[C]_{max}$, and the X intercept is equivalent to $-(1/K_s)$.

Results

Figure 1 shows the increase in the type II spectral change with increasing aniline concentration from 0.037 to 23 mM.

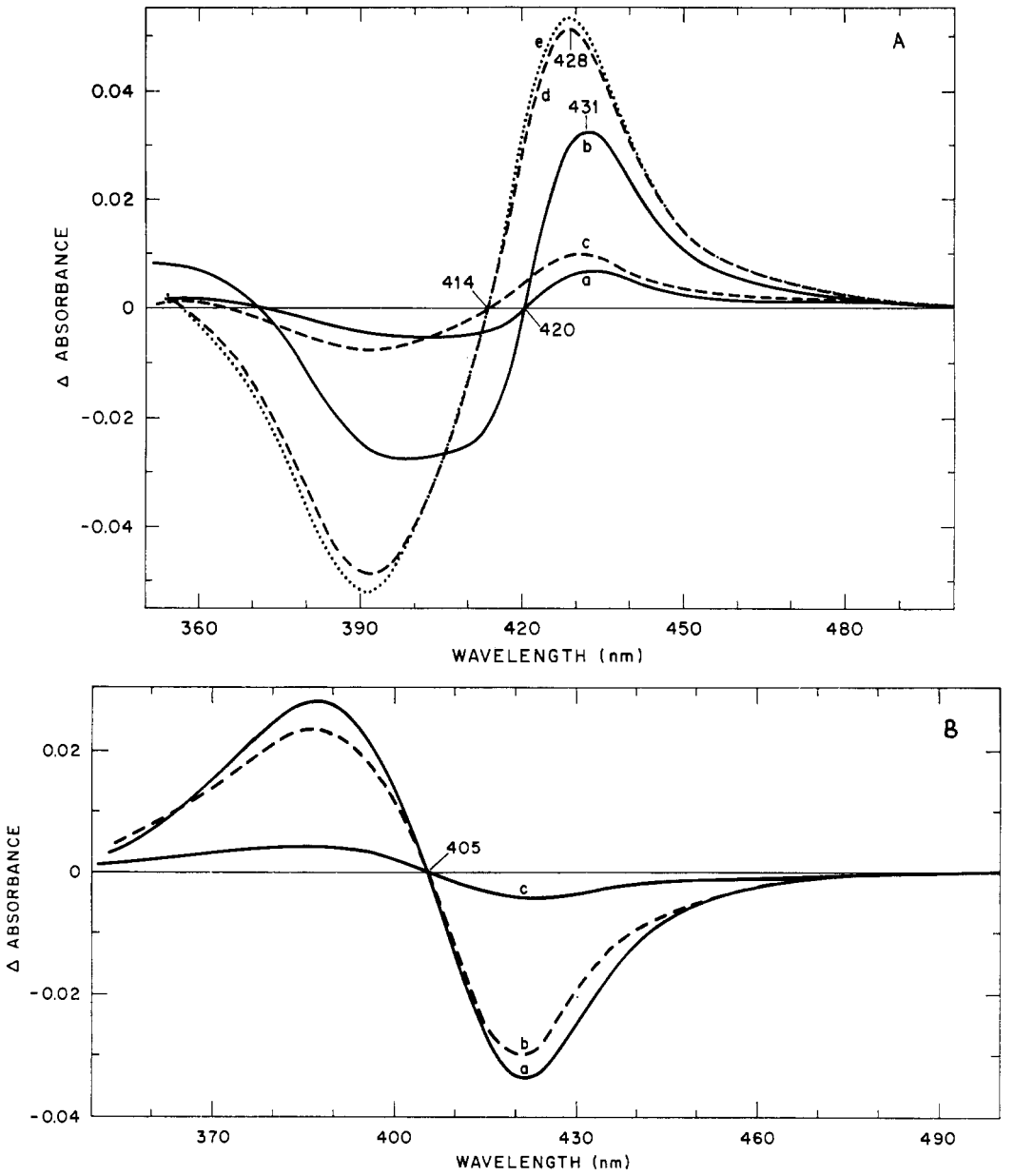

FIGURE 2: Separation of the type I and type II spectral overlap caused by aniline addition to hepatic microsomes. (A) Liver microsomes of adult male rats were suspended in 0.1 M phosphate buffer (pH 7.6) to a concentration of 1.75 mg of protein/ml (1.0 μM cytochrome b_5; 1.8 μM cytochrome P-450) and were divided between two cuvets. Spectra were run after the following additions: (a) 1.9 mM aniline, (b) 20.5 mM aniline, (c) 1.9 mM aniline + 2.5 mM hexbarbital in one cuvet and 2.5 mM hexobarbital in the reference cuvet, (d) 20.5 mM aniline +2.5 mM hexobarbital in one cuvet and 2.5 mM hexobarbital in the reference cuvet, (e) 20.5 mM aniline in one cuvet and 2.5 mM hexobarbital in the reference cuvet. (B) (a) 2.5 mM hexobarbital, same microsomal suspension as in part A; (b) curve d in part A subtracted from curve b to obtain the difference between the two spectra; (c) curve c in part A subtracted from curve a to obtain the difference between the two spectra.

Notice the very broad trough which gives an asymmetrical appearance to the spectrum, even at lower aniline concentrations. The isosbestic point is at 419 nm, the absorption maximum is at 431 nm, and the trough is spread out between 390 and 410 nm. Similar broad troughs were noted in the type II spectral changes caused by pyridine and nicotinamide.

The broad troughs could almost be construed as a combination of the type I and type II spectral changes; such an effect could be expected in the case of aniline, at least, since the compound is a substrate of the microsomal mixed-function oxidase, and competitively inhibits aminopyrine demethylase (J. B. Schenkman and R. W. Estabrook, 1966, unpublished data).

The effect of superimposition of type I spectral changes on type II spectral change was determined by adding hexobarbital to a microsomal suspension containing aniline. At low concentrations of aniline (1.87 mM), the addition decreases the size of the absorbance peak about 60% and further deepens the trough at 410 nm. Addition of the same amount of hexobarbital to the reference cuvet restores the type II spectral change to a somewhat greater extent, removing the asymmetry of the trough, and shifting the peak from 432

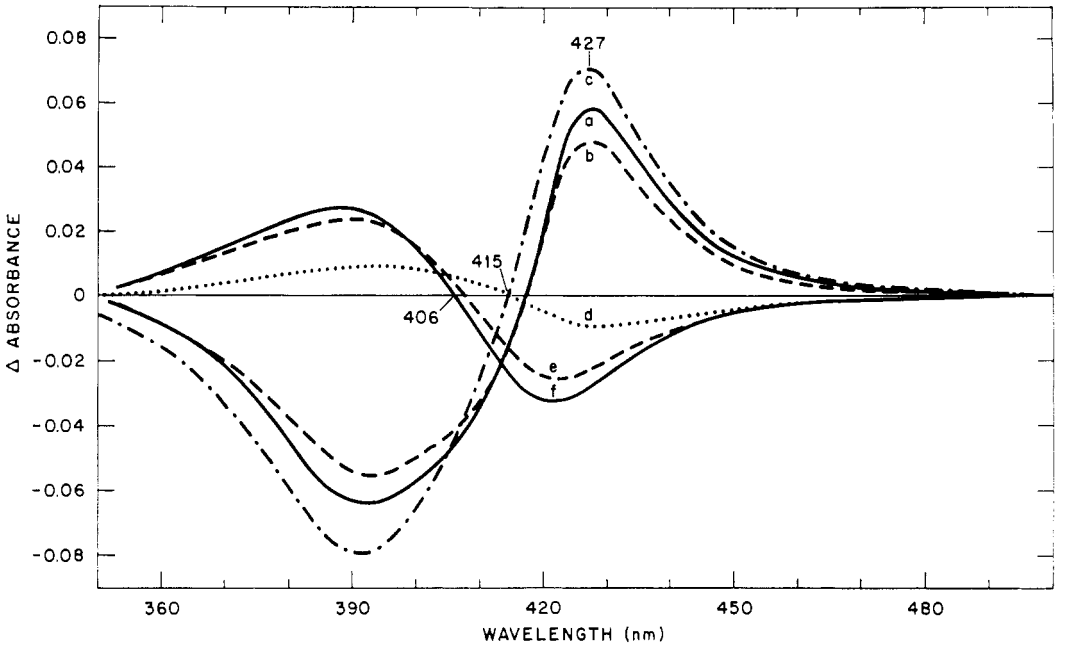

FIGURE 3: The effect of hexobarbital on the pyridine-induced type II spectral change. Hepatic microsomes were obtained from adult male rats and were suspended to 2.4 mg of protein/ml (1.5 μM cytochrome b_5; 2.5 μM cytochrome P-450) in phosphate buffer, pH 7.6. (a) 73 mM pyridine; (b) 73 mM pyridine +3.3 mM hexobarbital in one cuvet; (c) 73 mM pyridine +3.3 mM hexobarbital in one cuvet and 3.3 mM hexobarbital in the reference cuvet; (d) curve a subtracted from curve b; (e) curve c subtracted from curve a; (f) same microsomal suspension with 3.3 mM hexobarbital.

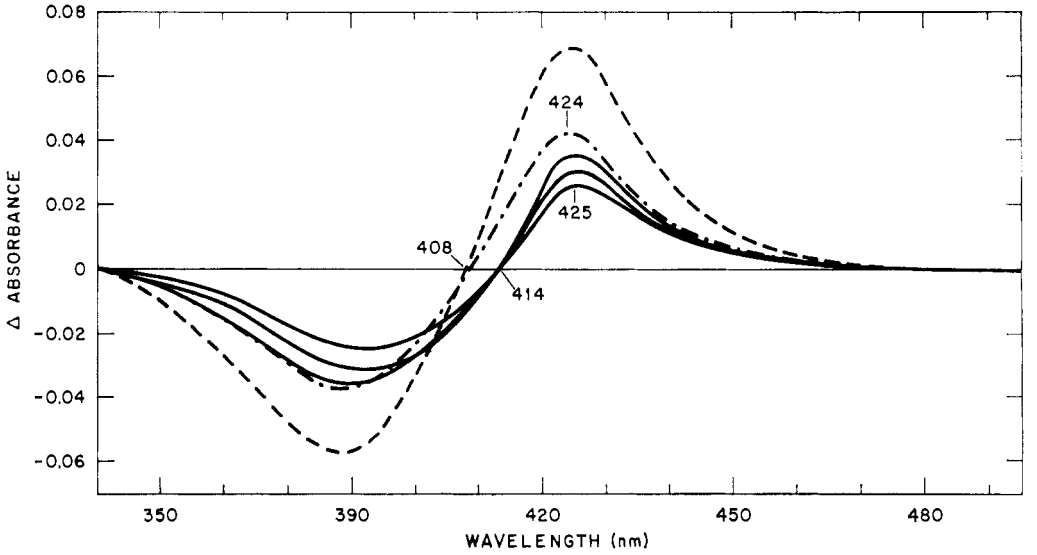

FIGURE 4: The effect of hexobarbital on the nicotinamide-induced type II spectral change. Hepatic microsomes were obtained from adult male rats and were suspended to 2.4 mg of protein/ml (1.5 μM cytochrome b_5 and 2.4 μM cytochrome P-450) in 0.1 M phosphate buffer (pH 7.6). (a) 20 mM nicotinamide, lowest solid curve; (b) 40 mM nicotinamide, middle solid curve; (c) 60 mM nicotinamide, highest solid curve; (d) 60 mM nicotinamide in one cuvet and 3.3 mM hexobarbital in the reference cuvet, dashed curve; (e) 60 mM nicotinamide +3.3 mM hexobarbital in one cuvet, and 3.3 mM hexobarbital in the reference cuvet, dash-dotted curve.

to 429 nm. In the presence of higher concentrations of aniline hexobarbital addition to the same cuvet has less of an effect, but when added to both cuvets it has the same effect on the spectral symmetry and the magnitude of the spectral change.

Figure 2a,b indicates that the asymmetry of the aniline binding trough is due to a combination of type I and type II spectral changes. Two different levels of aniline, 1.85 and 20.5 mM, caused the usual asymmetrical type II spectral change with a 420-nm isosbestic point (curves a and b of Figure 2A). Addition of hexobarbital to each cuvet in both instances elevated the spectral change markedly, and changed the isosbestic point to 414 nm (curves c and d). When a high level of aniline was used, a spectral change similar to that obtained by adding hexobarbital to both cuvets was seen

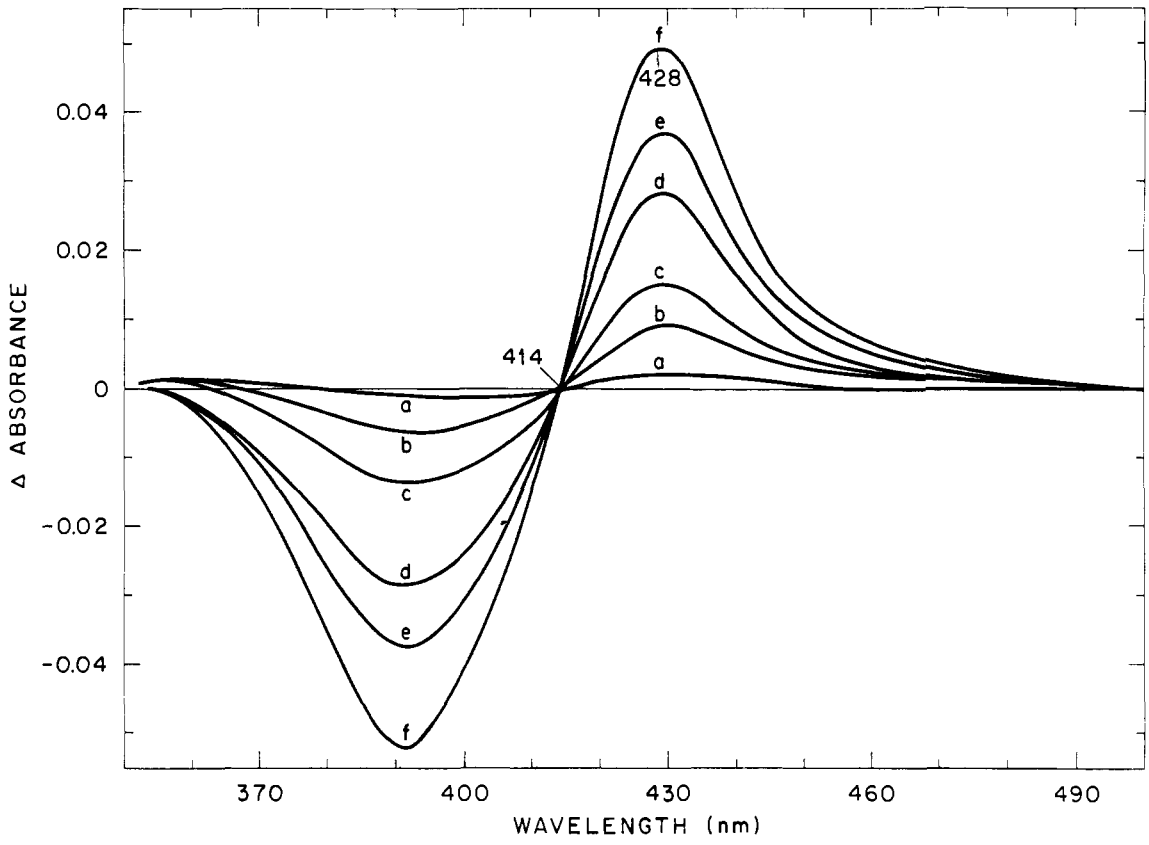

FIGURE 5: Spectral titration of the aniline-induced type II spectral change after removal of type I binding sites. Liver microsomes from adult male rats were suspended in 0.1 M Tris-HCl (pH 7.6) to 2 mg of protein/ml, and 3.3 mM hexobarbital was added before dividing the suspension between two cuvets. (a) 0.037 mM, (b) 0.22 mM, (c) 0.6 mM, (d) 2.4 mM, (e) 6.2 mM, and (f) 24.9 mM aniline. Total volume of additions was less than 25 μ to 3 ml.

when hexobarbital was added only to the reference cuvet (curve e). Figure 2B shows how the hexobarbital addition alters the type II spectral change. The solid curve (a) is the type I spectral change obtained by addition of hexobarbital in the absence of aniline. When aniline is added to liver microsomes, it too causes the type I spectral change. Low levels of aniline cause a small type I spectral change (Figure 2B, curve c), and high levels cause a large one (curve b). This is seen when the type I spectral change is canceled out by hexobarbital, the spectral change of aniline is obtained, and the latter is substracted from the spectral change obtained by addition of aniline alone.

The nonsubstrate amines, too, cause a composite type I and type II spectral change. However, since the peak of their type II spectral changes are at lower wavelengths, closer to the wavelengths of the type I spectral change, asymmetry is less obvious. Figure 3 shows the spectral change caused by addition of pyridine to liver microsomes. The absorption maximum is at 427 nm (curve a). Addition of hexobarbital to one cuvet has an effect similar to that seen when aniline is used (curve b); there is a decrease in the absorption peak and trough. Hexobarbital addition to both cuvets (curve c) results in an increase in the magnitude of the type II spectral change, makes the difference spectrum more symmetrical, and shifts the isosbestic point from 417 to 415 nm. Pyridine, even at 73 mM, does not remove all of the type I binding, as seen by the small change observed when hexobarbital is added only to the same cuvet as the pyridine (curve d), and when its amount of type I spectral change (curve e) is compared with that of hexobarbital alone (curve f). Nicotinamide, when added to liver microsomes also causes a type II spectral change of asymmetrical shape (Figure 4, solid curves), with an absorption maximum at 425 nm, isosbestic point at 414 nm, and broad trough. Addition of hexobarbital to both cuvets causes a slight elevation of the magnitude of the spectral change (— · — · — · —). It is obvious, when the spectra here are compared with that of Figures 2 and 3, that even at a concentration of 60 mM, nicotinamide has not complexed to the same extent with the hemoprotein as the other type II compounds, since hexobarbital addition to the reference cuvet alone indicates the extent of possible interaction (dashed curve); in the presence of hexobarbital in both cuvets, complete complexing would cause formation of a peak of the same height as that obtained on addition of hexobarbital to the reference cuvet alone (see Figure 2A, curve e).

Figure 5 shows the spectral titration of aniline binding in the absence of complicating type I spectra. This latter was accomplished by including 3.3 mM hexobarbital in the microsomal suspension prior to dividing the suspension between two cuvets. The absorption maximum is at 428 nm, the trough is at 392 nm, and the isosbestic point is 414 nm. The K_s obtained by taking reciprocal plots of the data in this figure was about 2 mM aniline.

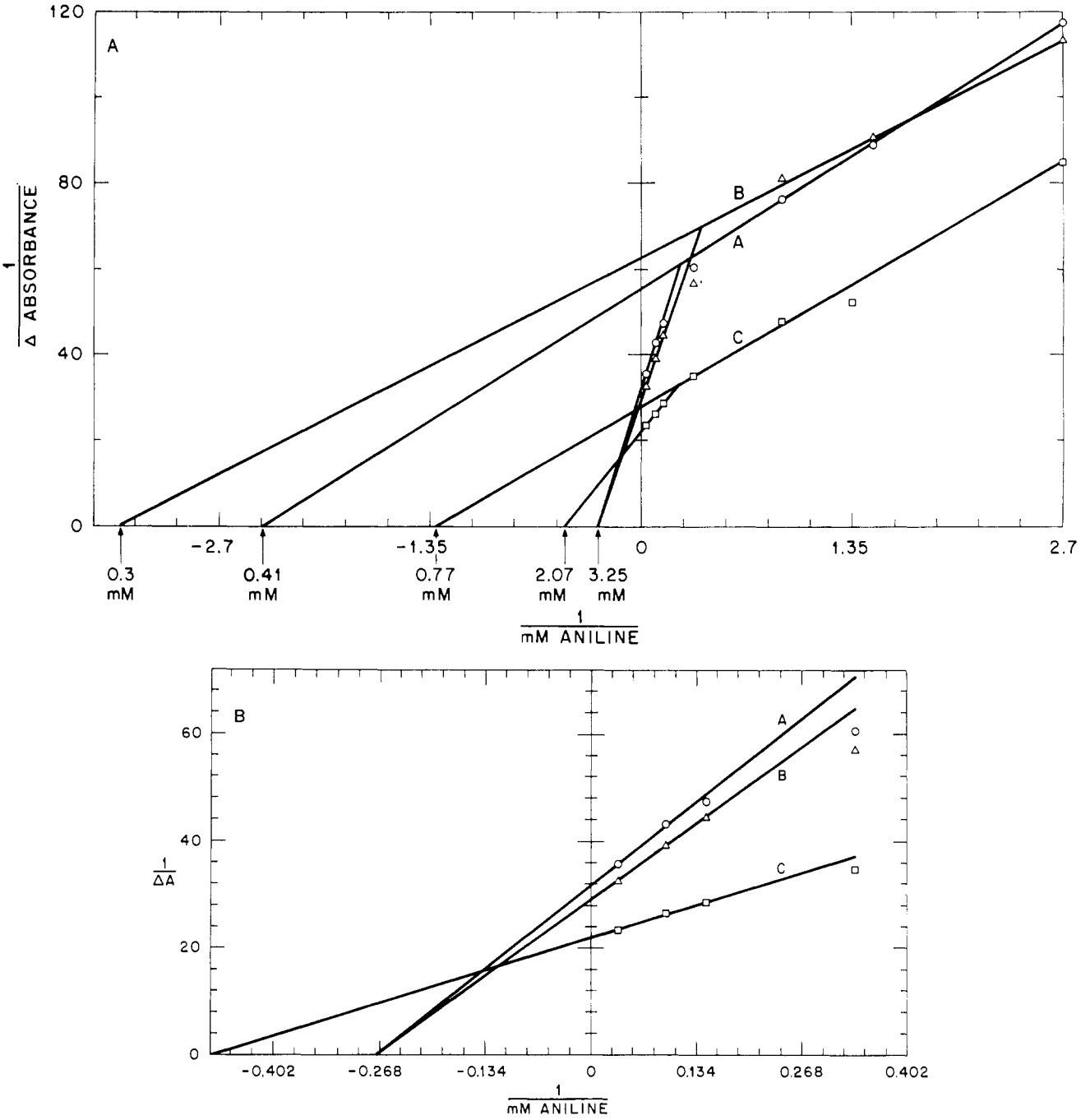

FIGURE 6: Double-reciprocal plot of the aniline-induced type II spectral change in the presence and absence of type I binding sites. Microsomes from adult male rats were suspended to 1.5 mg of protein/ml in 0.1 M phosphate buffer (pH 7.6) (1.86 μM cytochrome P-450). The dual monochromators of the Aminco-Chance spectrophotometer were set at 429 and 460 nm for lines A and C, and 429 and 418 mm for line B. Microsomal suspension C contained 3.3 mM hexobarbital. The maximum volume added to the 3-ml samples was 25 μl. (A) Full titration picture. (B) Expansion of the last 40% of the titration.

In order to determine the effect of the complicating type I spectrum on the titration of the type II spectral change, microsomal suspensions were prepared in the presence and absence of hexobarbital. Figure 6 shows the double-reciprocal plot of the spectral change at different substrate levels. As shown in Figure 6A, the double-reciprocal plot appears to be slightly convex upward, suggesting a facilitation of interaction by higher aniline levels. Figure 6B shows an expansion of the region of the plot at higher aniline concentrations. Lines A and B are titrations of the same sample of microsomes in which different reference wavelengths were used. In line A the wavelength 460 nm was chosen since it is well above the absorption maximum. In line B, 418 nm was used since this represents an isosbestic point for the aniline-induced spectral change (see Figure 1). The two titrations gave similar lines, differing only slightly in X axis

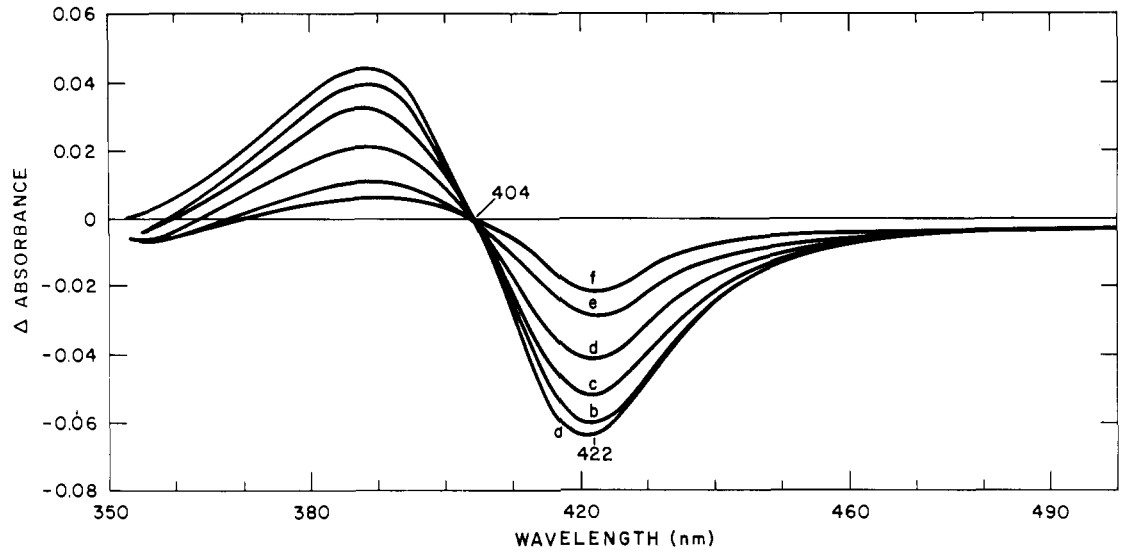

FIGURE 7: The effect of aniline addition on the hexobarbital-induced type I spectral change. Liver microsomes from adult male rats were suspended in 0.1 M Tris-HCl (pH 7.6) to 1 mg of protein/ml. (a) 1.7 mM hexobarbital; (b) 1.7 mM hexobarbital + 0.19 mM aniline in one cuvet, and 0.19 mM aniline in the reference cuvet; (c) as in part b, but with 0.56 mM aniline; (d) as in part b, but with 1.5 mM aniline; (e) as in part b, but with 5.2 mM aniline; (f) as in part b, but with 12.7 mM aniline. At 23 mM aniline the base line was restored.

intercept (A = 0.42 mM; B = 0.31 mM) at the lower aniline levels (Figure 6A). It is possible, however, that the spectral overlap does affect such titrations, because in the absence of the type I binding site (line C), the X intercept was 0.78 mM aniline for the initial part. In all three lines, the plots display convex shapes. The K_s values obtained from extrapolation of the points at higher aniline concentration indicated that no difference was obtained when the isosbestic point or a higher wavelength was chosen as the reference wavelength (Figure 6B, lines A and B); the value 3.25 mM aniline was obtained for K_s. Spectral overlap did, however, cause altered K_s values. When these lines were compared with the titration in the presence of hexobarbital (line C) the K_s value obtained was 2.07 mM aniline. The Y intercept values, $[C]_{max}$, obtained by extrapolating from the lower aniline levels was considerably lower than that obtained by extrapolating points at higher aniline levels; the latter intercept corresponded more closely with maximum absorbance values obtained in difference spectra (like Figures 1 and 5). It is possible that the effect is the result of spectral overlap, since the $[C]_{max}$ value obtained from line C is only 17% lower (true K_s is 2.7 times higher), while those of lines A and B are 44% (K_s is 7.9 times higher) and 56% (K_s is 11 times higher) lower, respectively.

Type I Binding. It is clear from the above that the type II substances also affect a type I spectral change. In order to learn their effect on the type I substrate interaction, increasing amounts of aniline were added to two cuvets, one of which contained 1.67 mM hexobarbital (Figure 7). As the level of aniline was increased, the magnitude of the type I spectral change decreased due to the type I component of the aniline spectrum in the reference cuvet. Similar results were obtained when aminopyrine was used, but the initial magnitude of the type I spectral change was lower. At 23 mM aniline the type I spectral change disappeared completely.

All of the type I compounds appear to cause the same type I spectral change. Maximal spectral change was obtained on addition of about 2.5 mM aminopyrine, ethylmorphine, or hexobarbital to liver microsomes. Addition of up to 5 mM levels of the respective compounds caused no greater spectral change.

All of the compounds caused the same minimum, maximum, and have the same isosbestic point. Since it was previously shown that the type I spectral change is caused by an indirect effect on the heme moiety (Schenkman and Sato, 1968), it seemed probable that one type I compound could affect the type I spectrum caused by another type I compound. Figure 8 shows the maximal spectral changes produced by hexobarbital (curve a) and by aminopyrine (curve b). When aminopyrine is already present in both cuvets, the magnitude of spectral change caused by hexobarbital (curves c, d, and e) is much lower. The magnitude of the hexobarbital-induced spectral change alone is 0.065A in this experiment. That of aminopyrine is 0.029A. Addition of 5 mM hexobarbital after aminopyrine was present in both cuvets only produced a 0.032-Å change (curve e), indicating the spectral change produced by the different type I substrates is the result of interaction with the same binding site. This is further indicated by the finding that increasing amounts of aminopyrine in the suspension cause increasingly greater type I spectral change, to a point. A further addition of hexobarbital causes a further increase in the magnitude of the spectral change. The latter increase is to about the same magnitude obtained by hexobarbital alone. Extrapolation of titration data on three different type I substrates to infinite substrate concentration (Figure 9) indicates that the different compounds cannot interact with the type I site to the same extent. The double slopes obtained in this experiment differ from that obtained with aniline (see Figure 6) in that they are not reproducible from experiment to experiment; the plots range from a single slope to double slopes shown in Figure 9, with lower X intercept values being inconstant. K_s values extrapolated from the higher values generally always agreed with those in legend to Figure 9.

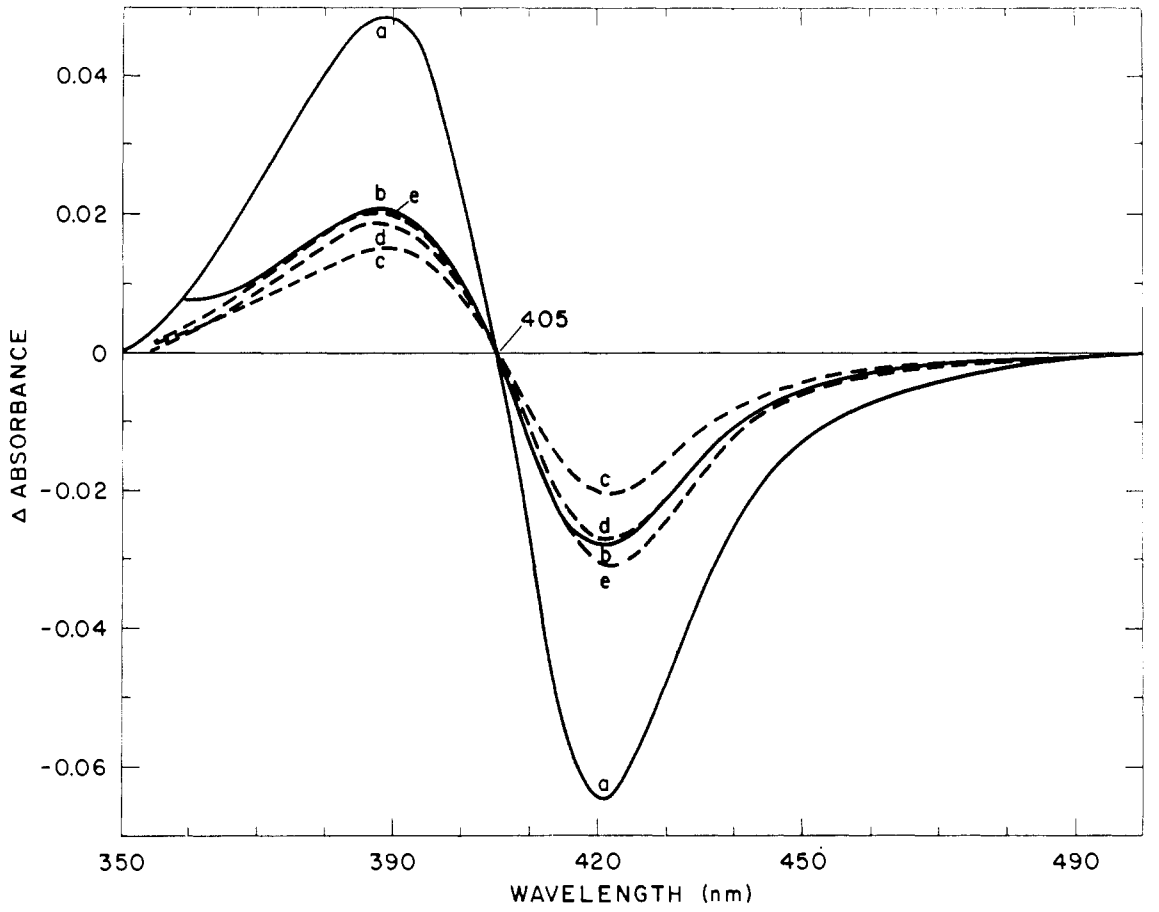

FIGURE 8: Interaction between different substrate-induced type I spectral changes. Liver microsomes of adult male rats were suspended in 0.1 M Tris-HCl (pH 7.6) to 1 mg of protein/ml. (a) Solid curve, 2.6 mM hexobarbital; (b) solid curve, 3.6 mM aminopyrine; (c) 1.67 mM hexobarbital +5 mM aminopyrine in one cuvet, and 5 mM aminopyrine in the reference cuvet; (d) same as part c, but with 3.4 mM hexobarbital; (e) same as part c, but with 5 mM hexobarbital.

Effect of Pretreatment with Phenobarbital or 3,4-Benzpyrene. Previously it was reported that the CO difference spectrum of reduced cytochrome P-450 of animals pretreated with polycyclic hydrocarbons differs from normal or phenobarbital pretreated animals (Alvares *et al.*, 1967).

In agreement with Alvares *et al.* (1967), the absorption maximum in polycyclic hydrocarbon-pretreated rats is shifted a few nm's toward the lower wavelengths. In addition, the shoulder which generally appears at 430 nm is somewhat more pronounced in microsomes of polycyclic hydrocarbon-pretreated rats.

The addition of aniline to liver microsomes of phenobarbital-pretreated or benzpyrene-pretreated rats (Figure 10) causes a spectral change very similar to that obtained with untreated rats (Figure 1); in all cases there is a marked asymmetry in the trough (curves a and d). Although the microsomal suspensions were adjusted to equal cytochrome P-450 levels, the magnitude of the spectral change was slightly higher in the microsomes from 3,4-benzpyrene-treated rats (curve a *vs.* curve d).

The addition of hexobarbital to the cuvet containing aniline, and to both cuvets had the same effect on the spectral change with phenobarbital rat microsomes as with microsomes from untreated rats (curves e and f). However, hexobarbital only produced minor changes in the aniline-induced type II spectral change in microsomes of the benzpyrene-treated rats (curves b and c). The addition of the type I substrate hexobarbital, in agreement with the report of Remmer *et al.* (1969), caused a markedly enhanced type I spectral change in microsomes of the phenobarbital-treated rats, and a markedly reduced type I spectral change in the liver microsomes of the benzpyrene-treated rats.

Discussion

The data shown above clearly indicates that the type II spectral change, or ferrihemochrome formed by interaction between cytochrome P-450 and basic amines, also contains a type I component, although of these amines, only aniline is a known substrate. The type I spectral change has been shown to be a characteristic of protoheme itself (Schenkman and Sato, 1968) and to be caused by an increase in the electronegativity of one ligand of the heme. The type I spectral change is a loss of an absorption band at 420 nm due to the loss of some prior ligand to the heme. Thus, replacing this ligand by any amine will also cause a decrease in absorption at 420 nm, superimposed upon the amine ferrihemochrome spectral change. However, if this ligand is already displaced, as by prior addition of a type I substrate to the liver microsomes, there will be no overlapping type I spectral component,

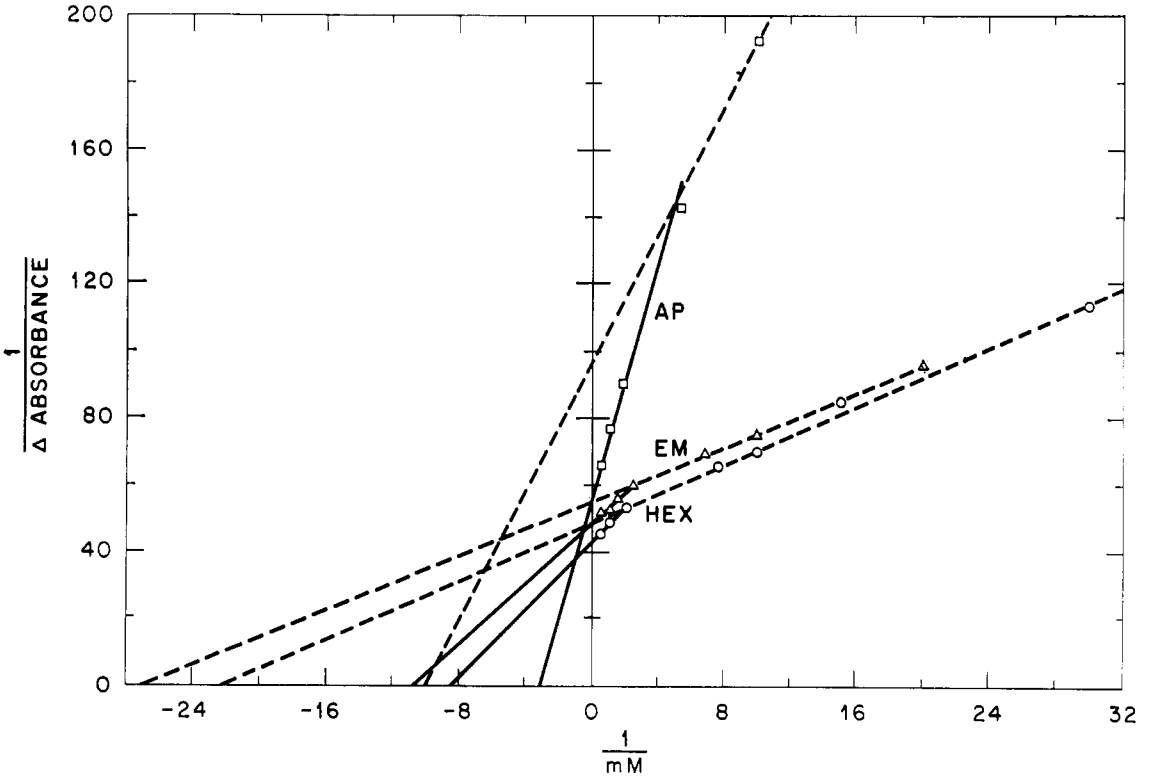

FIGURE 9: Double-reciprocal plot of type I substrate-induced spectral changes caused by three different type I substrates. Liver microsomes of adult male rats were suspended in 0.1 M phosphate buffer (pH 7.6) to 1.75 mg of protein/ml (1 μM cytochrome b_5 and 1.8 μM cytochrome P-450). The dual monochromators of the Aminco-Chance spectrophotometer were set at 421 and 455 nm. The maximal final volume added to the 3-ml sample of microsomal suspension was 25 μl. $[C]_{max}$ hexobarbital = $0.023A$; $[C]_{max}$ ethylmorphine = $0.020A$; $[C]_{max}$ aminopyrine = $0.018A$. K_s values were 0.33 mM aminopyrine, 0.12 mM ethylmorphine, and 0.09 mM hexobarbital.

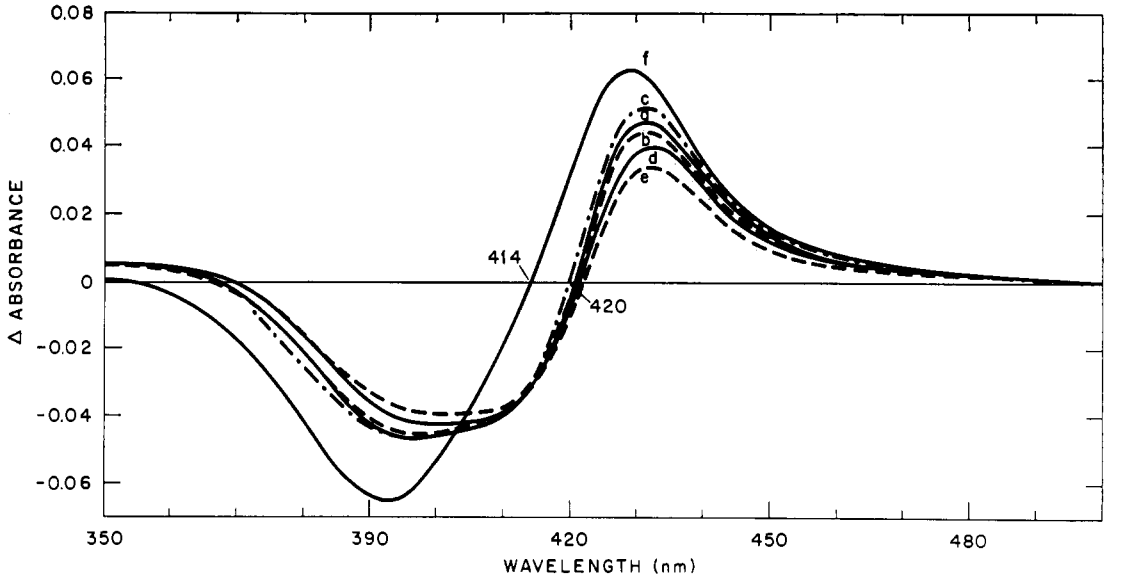

FIGURE 10: Comparison of aniline-induced type II spectral change and the effect of hexobarbital on this change in liver microsomes of phenobarbital- and 3,4-benzpyrene-treated rats. Treatments were: 80 mg of phenobarbital/kg daily for 3 days, or 20 mg of 3,4-benzpyrene/kg[1] daily for 3 days; animals were killed 24 hr after the last interperitoneal injection. Benzpyrene rat microsomal suspension contained 1.43 mg of protein/ml (2.1 μM cytochrome P-450 and 1.2 μM cytochrome b_5) in 0.1 M phosphate buffer (pH 7.6). Phenobarbital rat microsomal suspension contained 0.7 mg of protein/ml (2.2 μM cytochrome P-450 and 0.61 μM cytochrome b_5) in phosphate buffer (pH 7.6). Curves a, b, and c are with microsomes of benzpyrene-treated rats. Curves d, e, and f are with microsomes of phenobarbital-treated rats. (a) 25.7 mM aniline; (b) 25.7 mM aniline + 1.67 mM hexobarbital in one cuvet; (c) 25.7 mM aniline + 1.67 mM hexobarbital in one cuvet, and 1.67 mM hexobarbital in the reference cuvet; (d) 25.7 mM aniline; (e) 25.7 mM aniline + 1.67 mM hexobarbital in one cuvet; and (f) 25.7 mM aniline +1.67 mM hexobarbital in one cuvet, and 1.67 mM hexobarbital in the reference cuvet.

as shown in Figure 5, and a symmetrical type II spectral change will be obtained.

Remmer *et al.* (1969) and Shoeman *et al.* (1969) have recently reported that in microsomes of benzpyrene-pretreated rats, the type I spectral change is either reduced or absent. Similar results were observed by Schenkman *et al.* (1969), who suggested that something is already bound to the type I site. The addition of type I substrates of the enzyme system decreased the 419-nm absorption maximum of cytochrome P-450 of normal animal liver microsomes in absolute spectrum, forming a shoulder at 394 nm; the absolute spectrum of cytochrome P-450 of 3,4-benzpyrene-pretreated rats was very similar to this latter spectrum, suggesting that its inability to interact with type I substrates was due to its prior binding with benzpyrene, another type I substrate. The absolute spectrum of cytochrome P-450 of 3-methylcholanthrene-pretreated rabbits had a full peak at 394 nm, with a shoulder at 419 nm, suggesting a more complete binding with the inducer substrate. That different substrates interact with the enzyme at the same active site, but to different maximal extents, was shown in Figures 8 and 9.

Further indication that the reduced or absent type I spectral change was due to bound inducer was shown by Schenkman *et al.* (1969), who reported that 24–48 hr after pretreatment of animals, polycyclic hydrocarbon-like absorption was found in liver microsomes in amounts up to 4% of the cytochrome P-450 content. Unfortunately, this method would not have revealed unextracted polycyclic hydrocarbons, which may bind tightly to the microsomes (Silverman and Talalay, 1967) or open-ringed metabolites. However, 4 days after injecting a single dose of [^{14}C]-3-methylcholanthrene into rabbits, Jefcoate and Gaylor (1969) found 1.22 mμmoles of polycyclic hydrocarbon (or its metabolites) per mg of P-450 particle protein, containing 1.6 mμmoles of P-450 (76% of P-450). Of this, 0.1 mμmole (6% of P-450) could not be extracted with organic solvent. This method must be regarded as the more accurate, since it measures all of the xenobiotic in the liver microsomes, inducer, and metabolites. The consequence of so much substrate being present in the liver microsomes would be a lack of response when a type I substrate was added in the usual levels, due to the sixth ligand of the heme already being displaced. These microsomes would be expected to cause a symmetrical type II spectral change (as in Figure 5, this paper), and this is what was shown (Jefcoate *et al.*, 1969, Figure 1).

Jefcoate *et al.* (1969) have interpreted their observation that the binding of *n*-octylamine to cytochrome P-450 of methylcholanthrene-pretreated rabbits gives a symmetrical spectral change (like that in Figure 5, this paper), while the spectral change in control and phenobarbital-pretreated rabbits has a broad trough (like that of Figure 1), as indicating the presence of two types of cytochrome P-450, type a, and type b. It is apparent from the data presented in this paper that what these authors term type a and type b binding is merely the overlapping type I and type II difference spectra, which can be separated as above, and the pure type II difference spectrum, respectively, and not a combination of amine with two different types of cytochrome P-450.

The aniline binding spectrum in Figure 10 is the same for liver microsomes of control, phenobarbital-, and 3,4-benzpyrene-pretreated rats, indicating that although in the latter two cases the amount of hemoprotein was markedly elevated, the newly formed hemoprotein did not differ from that previously present in the microsomes. The microsomes of the 3,4-benzpyrene-treated rats do not interact with type I substrates with a resultant type I spectral change. However, the type I site is *not* occupied, and the sixth ligand is intact, since aniline addition causes a composite or asymmetrical spectral change, instead of a symmetrical one. It is possible that pretreatment of rats with benzpyrene induces synthesis of a different binding protein, one which does not recognize the usual type I substrates. It is equally possible that some product of benzpyrene metabolism is bound to an allosteric site, preventing interaction with type I substrates.

Since aniline is a substrate of the microsomal mixed-function oxidase, it would be expected to form a type I spectral change. Since the K_m for aniline is 0.04 mM (Schenkman *et al.*, 1967c), while the K_s for aniline is about 2 mM (Figure 6), it was thought possible to show a type I spectral change with aniline at sub K_m levels, without complication of the spectral change with ferrihemochrome formation. However, as shown in Figure 1, no type I spectral change was observed, only a composite (type I + type II) difference spectrum. This suggests that aniline does not bind to the type I site at all, but only to the heme of cytochrome P-450, an action which would be expected to inhibit the aniline hydroxylase reaction, since it would interfere with oxygen activation by the hemoprotein. Aniline hydroxylase activity in rat liver microsomes is between $^1/_{15}$ and $^1/_{20}$ that of aminopyrine demethylase activity (Schenkman *et al.*, 1967c), and may be an expression of such interference. However, the lowered rate may also reflect a different phenomenon, substrate activation; the reactive species may be activated aniline, which then interacts with oxygen. This possibility is currently under investigation.

The binding of aniline to the hemoprotein, in the presence or absence of type I binding site, does not observe a true first-order plot; *i.e.*, the plot of spectral change *vs.* aniline is not in the form of a rectangular hyperbola, but continues to rise slightly where it should appear to plateau. The result of this occurrence is that the double-reciprocal plot of the spectral change *vs.* aniline level shows two slopes. The slope of the lower aniline levels intersects the ordinate at a point approximately 50–60% of the maximal spectral change (see Figure 6). The fact that essentially the same picture was seen in the absence of type I binding site (although the ordinate intercept was only 17% below true $[C]_{max}$) suggests that the result, if not caused by spectral overlap, is certainly enhanced by it, and may reflect differences in difficulty of replacing the sixth ligand of the heme with aniline. Since the equation for the spectral change is valid for the binding of only one molecule per enzyme (or heme), the two slopes obtained could be an expression of the formation of a species containing two anilines per heme. However, since liganding with more than one basic amine per heme should alter the difference spectrum (Keilin, 1949), and since the same spectral change was obtained at high and low aniline levels (Figures 1 and 5), this possibility may be discarded. Another possibility to consider is that two hemoproteins, both capable of binding aniline (Shoeman *et al.*, 1969) are present in liver microsomes. But since different hemoproteins would be expected to have different ferrihemochrome spectra, this possibility must also be discarded. A last possibility, and one which may be difficult to prove, is that the binding of aniline to the heme

is facilitated by the presence of higher amounts of aniline due to a solvent effect. This possibility is currently under investigation.

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