Drug Interaction with Hepatic Microsomal Cytochrome

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SUMMARY

The addition to liver microsomes of various substrates, such as hexobarbital, aminopyrine, or aniline, causes two types of spectral changes. Spectrophotometric studies suggest that these changes are related to substrate interaction with a microsomal hemoprotein. On the basis of these observations, two hypotheses are suggested to explain the interaction of substrates with the liver microsomal mixed function oxidase system.

Studies on the mechanism of enzymic hydroxylations associated with TPNH oxidation have established (1, 2) the role of cytochrome P-450 as the oxygen activating enzyme for many mixed function oxidations, e.g., drug and carcinogen hydroxylations by liver microsomes and steroid hydroxylations by microsomes and mitochondria of adrenal cortex. The isolation (3–6) from adrenal cortex of a flavoprotein and a nonheme iron protein, required for electron transfer from TPNH to cytochrome P-450, furthers our understanding of the pathway of TPNH utilization during mixed function oxidations. Recently Narasimhulu *et al.* (7) have demonstrated spectral changes during the C-21 hydroxylation of 17-OH progesterone, using adrenal cortex microsomes. Since cytochrome P-450 is a component of the adrenal cortex steroid hydroxylases as well as many drug metabolizing enzymes of liver microsomes (2), it was of interest to determine whether spectral changes, indicative of substrate interaction, also could be observed when various drugs were added to liver microsomes.

Livers of male rats (Holtzman, 220 g weight) were homogenized with ten volumes of 0.25 M sucrose containing 1 mM sodium ethylenediamine tetraacetate (Versene), pH 7.4. Cell debris, nuclei, and mitochondria were removed by 10-min centrifugations at 480 *g*, 5000 *g*, and 11,200 *g* with the SS-34 rotor of a Servall refrigerated centrifuge. All *g* values have been calculated for the center of the centrifuge tube. The supernatant fraction was centrifuged for 1 hr at 78,000 *g* in a Spinco Model L centrifuge, and the microsomal

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pellet was suspended in 0.15 M KCl and recentrifuged to ensure removal of adventitious hemoglobin. The washed microsomal pellet was suspended in 0.15 M KCl containing 0.05 M Tris buffer, pH 7.4, to give a protein concentration of about 20 mg/ml as measured by the biuret method. In induction experiments, weanling Holtzman male rats (50–60 g) were injected intraperitoneally with daily doses of 80 mg of phenobarbital per kilogram body weight or 20 mg of benzpyrene per kilogram body weight for 4 days. The rats were killed by decapitation 40 hr after the last injection, and liver microsomes were prepared as described above.

Difference spectra were recorded with the wavelength scanning recording spectrophotometer (8). The effects of various substrate concentrations on the difference in absorbance at two fixed wavelengths was measured with the Aminco-Chance dual wavelength spectrophotometer.

When various substrates of drug metabolizing enzymes were added to liver microsomes, either in the presence or absence of TPNH, the spectral changes illustrated in Figs. 1 and 2 were obtained. One group of compounds, such as hexobarbital, phenobarbital, aminopyrine, and amobarbital, caused the appearance of a trough at about 420 mμ (Fig. 1) and the simultaneous appearance of an absorption band at about 385 mμ. These spectral changes are similar to those observed by Narasimhulu *et al.* (7) on addition of 17-OH progesterone to adrenal cortex microsomes. When the substrates mentioned above were added to liver microsomes in

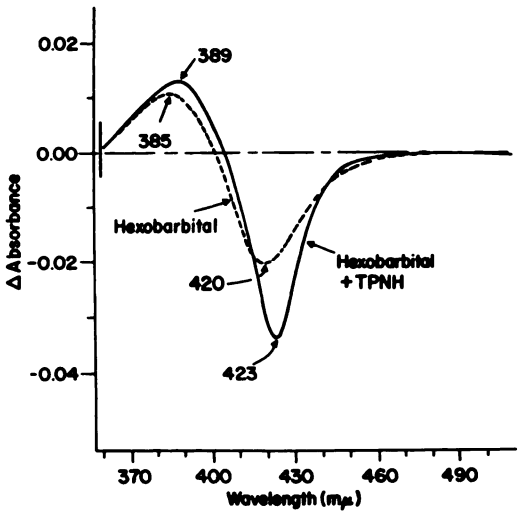

FIG. 1. *The spectral shift obtained on addition of hexobarbital to rat liver microsomes*

A 0.5 ml sample of rat liver microsomes was diluted with 5.5 ml of 50 mM tris buffer, pH 7.5, to give a protein concentration of 1.8 mg/ml. The diluted sample was divided into two cuvettes and the baseline of equal light absorbance recorded. Hexobarbital was added to the sample cuvette to give a final concentration of 3.8 mM, and the change in light absorbance recorded (dashed line). TPNH (0.025 ml of a 20 mM solution) was added to both the sample and reference cuvettes, and the difference spectrum (solid line) was recorded.

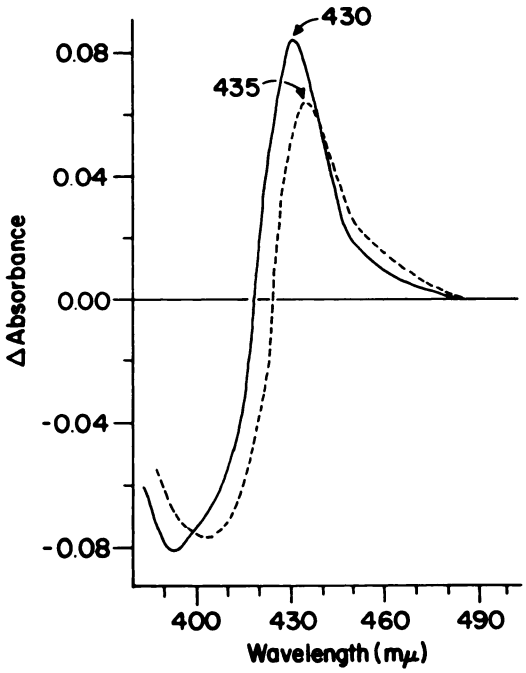

FIG. 2. *The spectral change observed on addition of aniline to rat liver microsomes*

Microsomes prepared from rats pretreated with phenobarbital were diluted as described in Fig. 1 to a final concentration of 2.1 mg of protein per milliliter. After establishment of a baseline, aniline (final concentration, 3.7 mM) was added to the sample cuvette, and the difference spectrum was recorded. TPNH was then added to both the sample and reference cuvettes, and the difference spectrum (dashed line) was recorded.

the presence of excess TPNH, the trough was intensified and shifted slightly to about 423 mμ. Of interest is the appearance of a similar trough when β-diethylaminoethyldiphenylpropylacetate (SKF 525-A), an inhibitor of the drug metabolizing enzymes (9), was added to liver microsomes.

A second type of spectral change (Fig. 2) was obtained on addition of a substrate, such as aniline or 2,4-dichloro-6-phenylphenoxyethylamine hydrochloride (DPEA) (Lilly 32391), another inhibitor (10) of drug oxidation. In this case an absorption band appeared at about 430 mμ with the concomitant disappearance of an absorption band at about 390 mμ. In the presence of TPNH the absorption peak (430 mμ) obtained with aniline was frequently shifted to 435 mμ.

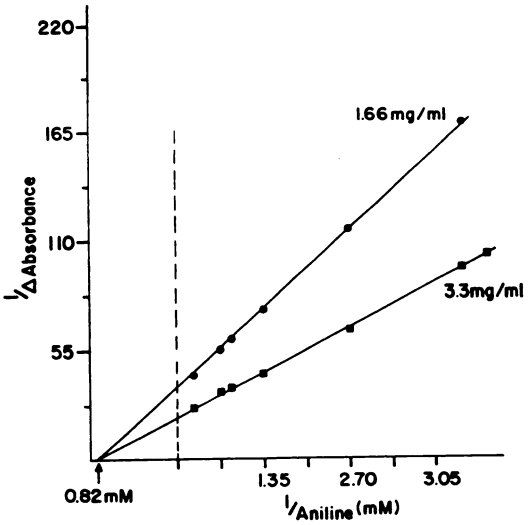

FIG. 3. *Reciprocal plots illustrating the influence of aniline on the change in absorbance observed at 430 minus 455 mμ*

Microsomes were diluted to 3 ml in 50 mM Tris buffer, pH 7.4, and changes in absorbancy were determined on addition of aliquots of aniline.

The magnitude of both types of spectral change was dependent on the concentration of substrate added. In Fig. 3 the relationship between absorbance change and aniline concentration at two concentrations of microsomal protein is shown in the form of a Lineweaver-Burk plot. Interestingly, the apparent dissociation constant, K_s, of 8×10^{-4} M obtained from this plot, is similar to the apparent K_m of 7×10^{-4} M determined by independent direct measurement of the *p*-aminophenol produced during *in vitro* metabolism of aniline. An apparent dissociation constant of 4×10^{-4} M was obtained for aminopyrine by spectrophotometric assay, while a K_m of 8×10^{-4} M was obtained when the product of dealkylation (HCHO) was measured (11).

Correlative studies on the effect of various "inducing agents" on the magnitude and pattern of spectral changes were carried out with the various substrates. Pretreatment of rats with phenobarbital, which causes an increase of the microsomal mixed function oxidase (12–15) and an increase in cytochrome P-450, enhances the spectral changes observed after adding hexobarbital or aniline to microsomal suspensions. In contrast, pretreatment of rats with benzpyrene also increases the amount of cytochrome P-450 and the magnitude of the spectral change observed with aniline, but diminishes the spectral change caused by hexobarbital. These diverse effects observed with various inducing agents may permit a direct assessment of the differences occurring on modification of the liver microsomal oxidation system during induction, and are currently under investigation.

The locus of substrate interactions during hydroxylation reactions still remains to be elucidated. The present results illustrate that substrates interact with a microsomal pigment, presumably cytochrome P-450, and cause two types of spectral shifts. Currently two hypotheses are under consideration:

(a) Either substrates substitute for a ligand of the heme of cytochrome P-450, resulting in a modification of the molar extinction coefficient of the hemoprotein, or substrates cause two types of conformational changes of the cytochrome P-450, modifying the heme-protein ligand interaction. The two types of spectral shifts may result from substrate interaction with a ligand on different sides of the heme of cytochrome P-450.

(b) Substrate addition results in the

conversion of an "activated" oxygen form of cytochrome P-450 (16) to its oxidized state. The latter hypothesis suggests a mechanism similar to the interaction of donor with complex II of peroxidase (17), but also implies an electron change (oxidation of the hemoprotein) on addition of substrate.

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