SPECTROPHOTOMETRIC STUDIES OF INTERACTIONS BETWEEN VARIOUS SUBSTRATES IN THEIR BINDING TO MICROSOMAL CYTOCHROME P-450*

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SUMMARY

The question of whether liver microsomes contain a single cytochrome P-450 with broad substrate specificity or a multitude of different cytochromes P-450 with high substrate specificity has been examined by determining the patterns of spectral changes in the presence of various combinations of substrates. The results can be explained in terms of the existence of two spectrally distinguishable forms of cytochrome P-450, which can associate with various substrates to form complexes which are spectrally similar to, but not necessarily identical to, one or the other of these forms.

Cytochrome P-450 is the terminal oxygenase of many mixed-function oxidase reactions catalyzed by liver microsomes, as well as those of adrenal organelles and of certain bacterial systems (1-4). Spectral changes have been observed upon addition of various substrates and modifiers of mixed-function oxidases to liver and adrenal microsomes; two general classes of substrates have been delineated which cause the production of two distinct types of difference spectra (5-7). Studies of the absolute spectra of the CO-binding hemoprotein induced by treatment of animals with phenobarbital or with 3-methylcholanthrene have indicated that cytochrome P-450 exists in two spectrally distinct forms (8). The characteristic difference spectra produced by the addition of substrates to microsomes has been explained by the disappearance of one form of P-450 concomitant with formation of the other (9).

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The presence of more than one molecular species of cytochrome P-450, however, has been suggested from experiments demonstrating the different patterns of product formed during microsomal oxidation of substrates, the wide diversity of substrates oxidatively metabolized, the variation in relative rates of metabolism observed with various animal species, and the preferential influence of inducing agents for the metabolism of some substrates but not others (10-12).

In contrast, it has been noted for many years that substrates of microsomal mixed-function oxidases often inhibit the oxidation of other substrates. The extensive studies of Rubin et al. (13) and of Schenkman (unpublished) have shown that many substrates are competitive inhibitors of each other's enzymic oxidation. In addition, many modifiers of mixed-function oxidase activity have been studied, some of which cause enhancement of certain activities (14-16). In order to investigate further these phenomena at the level of the binding of substrates to cytochrome P-450, we have studied with liver microsomes the effects of substrates upon the difference spectra produced by other substrates.

For these experiments an aliquot of liver microsomes from phenobarbital-treated rats was suspended in 50 mM Tris - 15 mM KCl buffer, pH 7.4, containing one compound or substrate, called here the modifier. In control experiments the modifier was omitted. The diluted sample was then divided equally between two cuvettes and the spectral baseline was recorded with an Aminco-Chance double beam wavelength scanning spectrophotometer. Varying aliquots of another compound, here called the substrate, were then added to the sample cuvette and the resultant difference spectrum recorded after each such addition. The sum of the absorbance changes at the wavelengths of the peak and trough of the difference spectrum was determined in each case. These data were then treated as in the classical enzyme-substrate-inhibitor models, using the absorbance change (ΔA) in place of initial velocity.

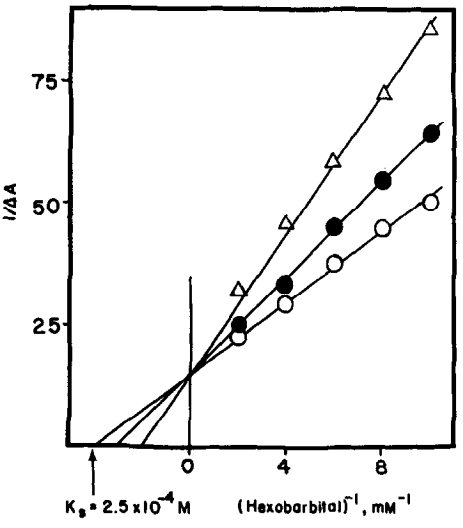


Fig. 1. Effect of aniline upon the difference spectra produced by hexobarbital. Rat liver microsomes were diluted with Tris-KCl, pH 7.4, containing aniline, to a final concentration of 1 mg protein/ml, and distributed between cuvettes. The sample cuvette was then titrated with aliquots of 50 mM hexobarbital. Aniline concentrations: open circles, 0.0; filled circles, 0.45 mM; triangles, 2.25 mM.

When a substance of type II‡ was used as the modifier, and a compound of type I as the substrate in such an experiment, results such as that shown in Fig. 1 were obtained. In this instance aniline acted as a competitive inhibitor of the spectral change produced by addition of hexobarbital. The K_s for hexobarbital (0.25 mM) was increased in its apparent value in the presence of aniline. The dissociation constant for aniline which may be calculated in a manner analogous to that for a K_i in an enzyme-substrate-competitive inhibitor reaction was 1.8 mM.

When both the substrate and modifier were of type II, the inhibition was never found to be competitive. In some cases, as when the substrate was metyrapone [2-methyl-1,2-bis(3-pyridyl)-propan-1-one] and the modifier aniline, the inhibition of the spectral change was analogous to the classic example of noncompetitive inhibition in an enzyme-substrate-inhibitor reaction (Fig. 2).

‡Type I and type II compounds are those which give one or the other of the two distinct types of difference spectra (5,6).

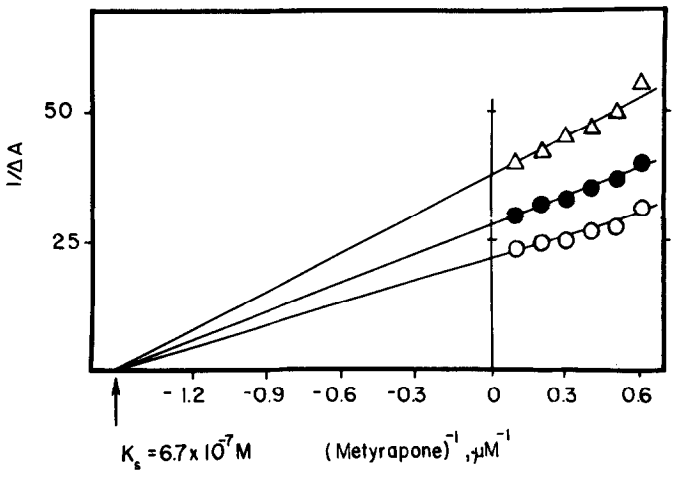


Fig. 2. Effect of aniline upon the difference spectra produced by metyrapone. Conditions were similar to those of Fig. 1, except that protein concentration was 0.5 mg/ml, and titration was conducted using aliquots of 1 mM metyrapone. Aniline concentrations: open circles, 0.0: filled circles, 0.45 mM; triangles, 1.13 mM.

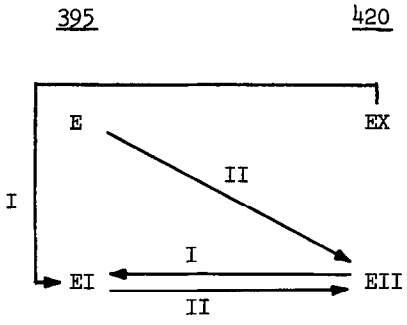
The K_s of metyrapone, 0.67 μM, was not changed in the presence of aniline, while the maximal absorbance change (ΔA_{max}) was decreased progressively with increasing concentrations of aniline. The "K_i" for aniline calculated from these data was 1.4 mM, which was quite similar to that found for the competitive inhibition of hexobarbital binding, and to the K_s of aniline found by spectrophotometric titration of liver microsomes (6). In instances where the substrate and modifier were more closely structurally related, for example aniline and acetanilide, the inhibition was of the mixed type, with both the apparent K_s and the ΔA_{max} changes.

A somewhat similar result was obtained when both substrate and modifier were of type I. Although when both substrate and modifier were barbiturates (for example hexobarbital and amobarbital), the inhibition was competitive, when the modifier was quite unrelated (isooctane), the inhibition was analogous to noncompetitive enzyme inhibition.

When the modifier was a type I substance and the substrate was a compound of type II, however, a very different result was obtained. The dif-

ference spectrum caused by addition of aniline to liver microsomes was increased in amplitude when hexobarbital was present as the modifier.

Some of these results may be explained by the following schema for the interaction of compounds of the two types (I and II) with two forms of the microsomal P-450 hemoprotein:



In this diagram, E represents the form of the oxidized hemoprotein whose absolute spectrum shows a peak at about 395 nm, and EX that form, which may represent a complex of E with an endogenous substrate or membrane constituent, that has a peak at about 420 nm (8). The former (E) can react with type II substrates to produce a family of complexes which have spectral properties similar to, but not necessarily identical to, that of EX. This would result in an increase in absorbance at about 425 nm and a decrease in absorbance around 395 nm, resulting in a type II difference spectrum. In a similar manner, reaction of EX with type I substances results in a complex spectrally similar to E, giving rise to a typical type I difference spectrum. The two enzyme-substrate complexes are pictured as interconvertible. After maximal conversion of EX to EI, all the hemoprotein would be in a form in which it could readily react with a type II substrate. In this way the enhancement of the type II difference spectrum obtained after modification with a type I compound could be explained. The fact that a similar enhancement did not occur when the modifier was of type II and the substrate of type I suggests that other, unreactive complexes might also be formed. Such complexes would also explain inhibition of spectral changes by compounds

of the same type in manners other than competitive. The results presented here would therefore be compatible with the presence of a single cytochrome P-450 in liver microsomes capable of interacting in two different ways with the two classes of compounds, both endogenous and exogenous.

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