The Interaction of Hepatic Cytochrome P-450 with Organic Solvents

THE EFFECT OF ORGANIC SOLVENTS ON APPARENT SPECTRAL BINDING CONSTANTS FOR HYDROCARBON SUBSTRATES

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Studies have been undertaken to explain the observed variation of the apparent association constant for water-insoluble substrates, which were diluted in common organic solvents, as a direct function of the solvent/solute ratio. By the use of suitable equations, the solvents methanol, ethanol, propanol, and acetone are shown to interact with hydrocarbon substrates in a competitive manner in PB-treated male rats, with the solvent producing a type I spectral component. Such solvents are shown to elicit, in addition to the type I component, a modified type II component. In untreated rats, ethanol does not produce a type I component, and also does not affect the apparent association constant for the hydrocarbon substrates when used as a solvent for those substrates. All perturbations of the enzyme which cause a change in the apparent association constant of the substrate also cause a quantitatively similar change in the apparent association of the solvent for the enzyme. A sex difference, with respect to competitive solvent binding, is also observed. Cytochrome P-450 from untreated male rats is apparently unable to bind small polar solvent substrates at the hydrocarbon binding site, whereas untreated female rats possess such an ability. In PB-treated rats, solvent binding is found to be sex-dependent. With respect to PB induction in female rats, the binding affinity for ethanol in the PB-treated animals is significantly larger than that observed in untreated females.

The addition of many substances to oxidized cytochrome P-450 causes changes in the absorption characteristics of the enzyme observed by difference spectroscopy (1, 2). There are three major types of ligand-induced difference spectra, type I, type II, and modified type II (or reverse type I) responses.

The type I change is caused primarily by substrates of the mixed function oxidase system. The spectral change is characterized by a peak at 385 nm and a trough at 420 nm (1, 2). The addition of increasing quantities of a type I compound produces a proportionate increase in the magnitude of the spectrum. As demonstrated by Schenkm an et al. (2, 3), a plot of the reciprocal of the substrate concentration versus the reciprocal of the change in absorbance results in a linear relationship from which an association constant may be determined. Type I compounds are generally thought to produce their spectra by binding to the active site of cytochrome P-450.

The addition of compounds such as aniline, aliphatic amines, and imidazole result in a type II spectrum when added to cytochrome P-450. Type II compounds produce difference spectra with peaks ranging from 425 to 435 nm and troughs from 390 to 410 nm (2, 3). The spectra produced by type II compounds are not symmetrical; type II spectra exhibit a broad trough. The lack of symmetry has been attributed to the presence of a type I component in the type II response (3).

The modified type II spectral change is produced by alcohols, ketones, and certain drugs (e.g., phenacetin) and is characterized by a peak at 420 nm and a trough at 385 nm, the apparent mirror image of the type I change. Schenkm an et al. (4) investigated the significance of the modified type II spectrum and concluded that these changes were the result of the interaction of MII compounds at both the type I site and another site, apart from the type II site. Yoshiida and Kumaoka (5) also studied the significance of the MII spectrum and concluded that the spectrum is the result of a type I interaction and a second interaction at the type II site. An additional hypothesis has recently been proposed (6) with respect to the action of MII compounds. It is based on the effect of the MII compounds (alcohols and ketones) on the dielectric constant of the aqueous phase of the microsomal preparation. As a MII compound is added to the microsomal preparation, a decrease in dielectric constant of the aqueous phase should be observed which would effectively "leach" endogenous substrate from the type I site of cytochrome P-450 into the aqueous phase (6).

When working with water-insoluble substrates such as hydrocarbons, steroids, and hydrophobic drugs, minute quantities of such compounds must be added in order to obtain an accurate value for an association constant. This problem is generally circumvented by dilution of such substrates in organic solvents. Since most organic solvents have been shown to elicit a MII spectral change, an accurate determination of the association constant for the type I substrate requires knowledge of the mode of interaction of the substrate with the enzyme. It appears that the MII spectral change is the least understood of the above types of spectra. Previous studies from this laboratory (7, 8) have demonstrated that solvents such as methanol, ethanol, and acetone can have a profound effect upon the apparent association constant for the type I substrate ethylbenzene. This present paper is concerned with the determination of a reliable value for the association constant for water-insoluble type I substrates, by correcting for the effect of the solvent employed. In addition, quantitative evidence for the mechanism of interaction of MII compounds is presented, using a series of equations developed here. The

* This work was supported by the West Virginia Medical Corporation and Department of Energy/Morgantown Energy Research Center Contract EY-77-C-21-8087. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: MII, modified type II spectrum; PB, phenobarbital; RI, reverse type I.
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approaches proposed here are applicable to the study of hydrophobic ligands in other systems as well, including kinetic studies.

EXPERIMENTAL PROCEDURES

Male and female Wistar rats were obtained from Hilltop Farms, Scottsdale, PA. For certain studies, Sprague-Dawley rats were used and were also obtained from Hilltop Farms. All rats ranged from 250 to 350 g and were killed by decapitation. Livers were quickly removed and homogenized in 0.15 M KCl. Washed microsomal suspensions were prepared by the method of Omura and Sato (9). The final microsomal pellet was suspended in 0.15 M KCl, 0.05 M Tris buffer (pH 7.4, at 25 °C). Cytochrome P-450 levels were measured according to the method of Estabrook et al. (10).

Substrate-induced Spectral Determinations

Three ml of a microsomal suspension were added to each of two cuvettes. These preparations were then placed in the reference and sample compartments of a Cary 17D dual beam scanning spectrophotometer at 25 °C. A base-line of equal light absorbance was recorded. When water-soluble substrates were used, stock solutions were dissolved and diluted in water. Microcrystalline increments of substrate solutions were added to the sample cuvette, with equivalent aliquots of water being added to the reference cuvette. After each addition, the resulting spectra were recorded between 450 and 370 nm subsequent to a fresh aliquot of the contents of the cuvettes. The total volume of the cuvettes was changed by less than 2% (v/v). The magnitude of the spectral change was measured between the peak and trough of the difference spectrum. For type I compounds, the peak and trough generally were found at 386 nm and 421 nm, respectively. The reciprocal of the magnitude of the spectral change was plotted against the reciprocal of the total concentration of substrate after each addition (a spectral analog of the Lineweaver-Burk plot for a rate process). Linear plots were obtained as described by Schenkman et al. (2). The apparent spectral association constant \( K_{app} \) was calculated from the slope of the line.

Procedure I

For determinations of spectral binding constants for water-insoluble substrates, a method analogous to that for water-soluble substrates was utilized. In this case, the microsomal preparation was added to the reference and sample cuvettes as described above. Stock solutions of a water-insoluble substrate were prepared by dilution in an organic solvent (e.g., methanol, ethanol, propanol, or acetone). After a base-line was obtained, microliter increments of substrate solutions were added to the sample cuvette, with equivalent volumes of the organic solvent being added to the reference cuvette. Upon addition of each increment, the spectral change was determined.

Next, a volume of 9 ml of a fresh microsomal suspension was added to a glass-stoppered centrifuge tube. While blending on a Vortex mixer, 75 µl of ethylbenzene (pure liquid) were slowly added to the suspension. After addition, the tube was closed and the preparation was blended vigorously on a Vortex mixer. Then, 3 ml of this solution were added to both the reference and sample cuvettes. A base-line was determined, and microliter aliquots of the organic solvent used for the addition of the hydrophobic substrate were added to the reference cuvette while equivalent volumes of water were added to the sample. The absorbances after each addition were recorded and added to the absorbances obtained with the previous preparation as described in Fig. 6. The reasoning behind these manipulations will be explained under "Results."
I spectrum elicited by ethylbenzene is shown in Fig. 1.

The apparent binding constant \( K_{s}^{app} \) for a number of hydrophobic substances was determined in untreated and phenobarbital-pretreated male rats, using working solutions of different concentrations in ethanol. Each of these compounds, when added to a liver microsome preparation, elicited a type I change, which has been demonstrated to be indicative of substrate binding to cytochrome P-450 (3, 13). By plotting the reciprocal of the magnitude of the spectral change against the reciprocal of the substrate concentration, a linear relationship analogous to the Lineweaver-Burk plot for enzyme kinetics may be obtained and an association constant may be determined.

The results in Table I demonstrate that in phenobarbital-pretreated male rats \( K_{s}^{app} \), the apparent binding constant increased as the concentration of the stock solution decreased for both an aliphatic and an aromatic hydrocarbon. In untreated rats, a change in the concentration of the hydrocarbon stock solutions did not affect \( K_{s}^{app} \).

There are at least three possible explanations for this effect. First, the differences in apparent binding constants may be due to the presence of multiple forms of cytochrome P-450. Second, the organic solvent may, in some manner, affect the apparent association constant. Finally, the presence of multiple forms of cytochrome P-450 and the interaction of solvent due to the presence of multiple forms of cytochrome P-450, then addition of increasing volumes of the 20.4 mM stock solution to the microsomes to a final concentration of 0.66 mM, should cause a downward break in the double reciprocal plot demonstrating biphasic behavior as suggested by Klotz and Hunston (14, 15). This should occur due to saturation of the high affinity binding site, permitting the subsequent titration of the binding site possessing the smaller affinity. Since a final concentration of 0.66 mM ethylbenzene should be well into the titration of the second binding site (neither the 81.7 mM nor the 20.4 mM solutions alone demonstrated biphasic nature), biphasic results should then clearly be observed. However, if variation of \( K_{s}^{app} \) is due to a contribution of the solvent rather than the presence of multiple forms of cytochrome P-450, changes in the ratio of solvent to substrate should cause a change in the apparent association constant.

To demonstrate that the change in \( K_{s}^{app} \) observed for some substrates in PB-treated rats is due to changes in the concentration of the working solutions rather than the presence of multiple enzyme forms both capable of binding the same substrate with different affinities, increments of a dilute substrate solution were added to the microsomal preparation, then part way through the titration, a concentrated substrate solution (different substrate/solvent ratio) was used to complete the titration. The results are shown in Fig. 2. The double reciprocal plot for ethylbenzene/ethanol. Ethylbenzene was diluted to various concentrations in ethanol as described in the legend to Table I. Closed circles represent spectral titration using a 3.27 mM ethylbenzene stock solution. In the titration depicted by the open squares, the first four additions \((1/EB)\) ranges from 30 to 100 mM \(^{-1}\) were made using 3.27 mM ethylbenzene, with the final five additions \((5 to 20 \text{ mM})\) using 16.34 mM ethylbenzene. Phenobarbital-treated male rat liver microsomes containing 3 mg of microsomal protein/ml were used in these studies. Similar results were obtained with pregnenolone and hexane diluted in ethanol in PB-treated rats.
The general equation (written in the reciprocal form for convenience) describing the dependence of the spectral change upon the concentrations of both \( A \) and \( B \) is as follows:

\[
\frac{1}{\Delta A_{\text{total}}} = 1 + \frac{\bar{K}_E[B] + \bar{K}_A[A] + \bar{K}_{AB}[B][A]}{(\Psi_B\bar{K}_E[B] + \Psi_A\bar{K}_A[A] + \Psi_{AB}\bar{K}_{AB}[B][A])[E]_{o}} \tag{1}
\]

Where \([E]_o\) is the total enzyme concentration, \( A_{\text{total}} \) is the sum of the absorbances of the \( EA, EB, \) and \( EAB \) complexes at equilibrium and \( \Psi_A, \Psi_B, \) and \( \Psi_{AB} \) are the molar absorbances of \( EA, EB, \) and \( EAB, \) respectively. The simplest possible case would be seen in the absence of \( A \).

Under conditions where substrates \( A \) and \( B \) are added to the assay mixture in a fixed ratio, a mathematical linkage of the two substrates can be introduced to equation 1. We may therefore write \([A]/[B] = N \) or \([B]/[A] = [A] \). If the solution of substrate \( B \) in solvent \( A \) (which we are treating here as a second substrate) is reasonably dilute, then \( N \) is simply the reciprocal of the mole fraction of \( B \) dissolved in solvent \( A \). Substituting \([B]/[A] = [A] \) in equation 1 produces, upon some rearrangement:

\[
\frac{1}{\Delta A_{\text{total}}} = 1 + \frac{\bar{K}_E[B] + \bar{K}_A[A] + \bar{K}_{AB}[B][A]}{(\Psi_B\bar{K}_E[B] + \Psi_A\bar{K}_A[A] + \Psi_{AB}\bar{K}_{AB}[B][A])[E]_{o}} \tag{2}
\]

It will be easily seen that the above equation does not predict a linear double reciprocal plot when one plots \( 1/\Delta A_{\text{total}} \) versus \( 1/[B] \). This equation represents the general noncompetitive case where \( EB, EA, \) and \( EAB \) complexes all absorb. It can be shown that other noncompetitive mechanisms also yield nonlinear double reciprocal plots under these conditions (8).

**Competitive Interactions**—If the \( EB \) and \( EA \) complexes are mutually exclusive, thus prohibiting the formation of \( EAB, \) equation 3 can be reduced to a competitive interaction. In this case, \( K_A \) and \( K_B \) are both equal to zero, resulting in the following equation:

\[
\frac{1}{\Delta A_{\text{total}}} = \frac{1}{(\Psi_B\bar{K}_E[B] + \Psi_{AB}\bar{K}_{AB}[B][A])[E]_{o}} + \frac{\bar{K}_A + \bar{K}_N}{(\Psi_A\bar{K}_A[A] + \Psi_{AB}\bar{K}_{AB}[B][A])[E]_{o}} \tag{3}
\]

For this case, plotting \( 1/\Delta A_{\text{total}} \) versus \( 1/[B] \) will yield a linear plot. If \( 1/\Delta A_{\text{total}} \) is set equal to zero, from the \( x \) intercept it follows that \( K_A^{\text{app}} = (N\bar{K}_A + \bar{K}_B) \). Equation 3 is illuminating, in that it indicates that if one dissolves a substrate \( B \) in a solvent \( A \) (which competes for the enzyme binding site), a linear double reciprocal plot will be obtained. Of course \( K_A^{\text{app}} \), the association constant, and \( \Delta A_{\text{total}} \), will have different significances than would be the case if substrate \( B \) were added in water or a noninteracting organic solvent. By preparing solutions of different concentrations (different \( N \) values) for our experiments, the binding constants for both substrates can be determined. This can be accomplished by determining \( K_A^{\text{app}} \) at different values of \( N \) and plotting \( K_A^{\text{app}} \) against \( N \). This procedure will result in a linear plot with the \( y \) intercept equaling \( \bar{K}_B \) and a slope of \( \bar{K}_A \). A plot of the reciprocal of the slope versus \( N \) will also result in a linear relationship as follows:

\[
\frac{1}{\text{slope}} = \Psi_A[E]_{o} + \Psi_{AB}[E]_{o}N \tag{3a}
\]

with a slope of \( \bar{K}_A\Psi_{A}[E]_{o} \) and intercept of \( \bar{K}_B\Psi_{B}[E]_{o} \). Replotting the \( y \) intercept against \( N \) does not yield linearity.
If $\Psi_A = \Psi_B$, the following linear relationship is obtained:

$$
\frac{1}{\Delta A_{\text{obs}}} = \frac{1}{\Psi_A K_A + \Psi_B K_B} + \frac{1}{\Psi_A [E]_B}
$$

By setting $1/\Delta A_{\text{Total}}$ equal to zero, $\Delta A_{\text{obs}}$ can be determined from the $x$ intercept. The significance of this replot is the same as that from equation 3. A replot of $1/\text{slope}$ versus $N$ also yields a linear plot.

$$
\frac{1}{\text{slope}} = \frac{1}{\Psi_A K_A [E]_0} + \frac{1}{\Psi_A [E]_B} (4a)
$$

with $y$ intercept of $\Psi_A K_A [E]_0$ and slope of $\Psi_A K_A [E]_0$. A replot of $1/\Delta A_{\text{max}}$ (equation 4) versus $N$ now results in a relationship that is independent of $N$.

A special case of equation 3 is when the $EA$ complex can form but does not absorb; therefore, $\Psi_A$ equals zero. Equation 3 then simplifies to:

$$
\frac{1}{\Delta A} = \frac{1}{\Psi_B K_A [E]_0} + \frac{K_B}{\Psi_B [E]_B K_A}
$$

which also results in a linear relationship. When $1/\Delta A$ is set equal to zero, the $x$ intercept has exactly the same significance as in equation 3. Replotting the $y$ intercepts from double reciprocal plots having different $[A]/[B]$ ratios versus $N$ results in a linear plot as shown below:

$$
\frac{1}{\Delta A_{\text{max}}} = \frac{K_A N}{K_A [E]_B} + \frac{1}{\Psi_A [E]_B} (5a)
$$

with a slope of $\Delta A/K_A [E]_B$ and intercept of $1/\Psi_A [E]_B$. A replot of the slope from equation 5 versus $N$ will result in no dependence.

According to the above equations, nonlinear double reciprocal plots should be obtained with noncompetitive interactions. Since we do indeed obtain linear double reciprocal plots for ethylbenzene/ethanol binding in PB-treated male rats, the data suggest a competitive mechanism.

**Barbiturate Competition**—In order to confirm the validity of equation 3 (general competitive case), two barbiturates (methohexital and hexobarbital) were selected. These compounds were chosen because of differences in the association constants for each compound. The extinction coefficients of both substrates can be readily obtained in the absence of any solvent other than water for comparison with extrapolated values. Fig. 3a shows the double reciprocal plots for mixtures of hexobarbital and methohexital from PB-treated male rats. Microliter quantities protein/ml were used in this experiment. (a) Double reciprocal plots for the apparent association of methohexital in the presence of hexobarbital were plotted as a function of methohexital concentration. Each stock solution with a particular methohexital/hexobarbital ratio was used for generation of the data points. Concentrations of the substrate stock solutions were (a) 0.78 mm, 15.82 mm; (b) 0.84 mm, 15.47 mm; (c) 1.57 mm, 14.06 mm; (d) 3.14 mm, 10.55 mm; for methohexital and hexobarbital, respectively. (b) Replot of the $x$ intercept ($K_A^{\text{obs}}$) versus $N$ (hexobarbital/methohexital). (c) Replot of $1/\text{slope}$ from (a) versus $N$. (d) Replot of $1/\Delta A_{\text{max}}$ versus $N$. Solid line represents the theoretical curve predicted from equation 3 using the equilibrium constants determined from $b$ and $c$ above.
of stock solutions of varying methohexital/hexobarbital ratios were added to the microsomal suspension and the reciprocal of the spectral change was plotted against the reciprocal of the methohexital concentration as described under "Experimental Procedures." The effect of varying quantities of hexobarbital on the apparent association constant for methohexital is clearly demonstrated from these results.

As predicted by equation 3 and shown in Fig. 3b, plotting $K_{app}$ for methohexital against $N$ ([hexobarbital]/[methohexital] ratio) results in a linear relationship with intercept of $K_{app}$ for methohexital and slope of $K_{app}$ for hexobarbital. The extrapolated values for the association constants for methohexital and hexobarbital (41,000 M$^{-1}$) compare quite well with values obtained by conventional direct determination (42,000 M$^{-1}$ and 9,000 M$^{-1}$ for methohexital and hexobarbital, respectively).

Replotting 1/slope against $N$ also yields a linear relationship as predicted by equation 3 and shown in Fig. 3c. The significance of the y intercept is $K_{app}^0(A)[E]_0$, the slope is equal to $K_{app}^0(A)[E]_0$ and $K_{app}^0(B)$, respectively. Since the association constants for both substrates were determined from Fig. 3b, utilization of these values permitted the determination of $[E]_0$ and $[A]_0$ in the whole system.

Values from the extrapolation ($[E]_0 = 0.18, [A]_0 = 0.17$) agree well with the directly determined values ($[E]_0 = 0.14, [A]_0 = 0.07$). A plot of $1/\Delta A_{max}$ versus $N$ was consistent with the non-linearity predicted by equation 3. Using the values derived from Fig. 3, b and c, the theoretical curve for $1/\Delta A_{max}$ versus $N$ can be plotted as shown in Fig. 3d. Experiments from untreated male rats using the same substrates gave qualitatively similar results (data not shown).

**Effect of Ethanol on $K_{app}$ for Ethylbenzene**—The concentration of the stock solution of ethylbenzene diluted in ethanol, which is added to the microsomal suspension, has a profound effect on the apparent association constant obtained in PB-treated male rats as shown in Table I and Fig. 2.

From the characteristics of the double reciprocal substrate dependence plots and the secondary plots of $K_{app}$, 1/slope, and $1/\Delta A_{max}$ versus $N$, the mechanism of interaction between solvent and substrate with the enzyme may be determined, in addition to enabling determination of association constants and extinction coefficients for both substrate and solvent complexes. In a manner analogous to the barbiturate studies, as the value of $N$ ([ethanol]/[ethylbenzene]) increased, the $K_{app}$ for ethylbenzene also increased. These results are shown in Fig. 4a. Replotting $K_{app}$ versus $N$ resulted in a linear plot from which $K_{app}$ for ethylbenzene can be obtained from the y intercept and $K_{app}$ for ethanol from the slope (Fig. 4b). The association constants for ethylbenzene and ethanol were 5,000 M$^{-1}$ and 12 M$^{-1}$, respectively.

No solvent dependence was observed in the untreated male, indicating no demonstrable binding of solvent to the ethyl-
benzene type I spectral site. The association constant for ethylbenzene in the untreated rat was 2,800 M\(^{-1}\).

Replotting the reciprocal of the slopes of the lines from Fig. 4a against \(N\) produced a linear relationship from which \(\Psi(E)\), and \(\Psi(E)\) may be determined (Fig. 4c). Values of 0.21/mg of protein and 0.07/mg of protein were obtained for \(\Psi(E)\) and \(\Psi(E)\), respectively. It should be noted that a positive sign for \(\Psi(E)\) indicates a type I response. A negative sign for \(\Psi(E)\) indicates either a type II or modified type II response. (Discerning the difference between a type II and a modified type II response would require careful examination of the data at wavelengths over the entire range of spectral interaction.)

In the untreated male, the slope of the line in Fig. 4c is essentially zero, which is consistent with the absence of an interaction of ethanol with the type I site. The extinction coefficient for ethylbenzene in the untreated rat was 0.06/mg of protein. The slope of the line for the PB-treated male is positive, suggesting that \(\Psi\) represents a type I response similar to \(\Psi\). Alcohols have been shown to possess a modified type II (also called a reverse type I) spectrum when added to liver microsomes (4). If this component were the competing species, then \(\Psi\) would be negative, rather than the positive response actually obtained.

These spectral studies were done by adding ethylbenzene diluted in ethanol to the sample cuvette, and ethanol to the reference cuvette, a common procedure (6, 16-20). This method causes inconsistencies in the application of the above-mentioned equations. These equations were derived in a form which assumes that both substrates were to be added to the sample cuvette and the solvent added to the reference, only if the solvent is a noninteracting one such as water. If substrate and solvent bind to separate noninteracting sites, then adding solvent to the reference cuvette would be proper procedure and no solvent dependence would be observed. Such is the case with the untreated male rat. A method for correcting the procedure for phenobarbital-treated males follows.

The points from the \(1/\Lambda_{\text{max}} \) versus \(N\) plot in the phenobarbital-treated rat are consistent with the curve predicted by equation 3 (Fig. 4d). In the untreated male, \(1/\Lambda_{\text{max}}\) was independent of \(N\) as would be expected if ethanol did not interact with ethylbenzene at the type I site (not shown). Similar results were obtained using either Sprague-Dawley or Wistar rats (data not shown for Sprague-Dawley). Thus, this characteristic is not peculiar to Wistar rats.

**Dual Interaction of Ethanol in Phenobarbital-treated Rats**—In the phenobarbital-treated male rat there appears to exist a more complex interaction between ethylbenzene, ethanol, and the enzyme than is observed in the untreated male. Ethanol exhibits a modified type II interaction in the phenobarbital-treated male, which can be observed by simply adding the alcohol compound to the microsomal preparation and recording the spectral change. This spectral change is also observed in the untreated rat and must be eliminated to obtain linear double reciprocal plots when these compounds are used as solvents for type I substrates. This end is commonly attained by adding ethanol to the reference cuvette. By analogy, the absorbance from the apparently noninteracting MII change in phenobarbital-treated rats must also be eliminated. The data from Fig. 4 suggest that in PB-treated rats, ethanol possesses a type I spectral change that appears to compete with ethylbenzene. Since this component is accounted for by equation 4, it should not be eliminated as is the MII component by the addition of solvent to the reference cuvette. Therefore, for the system with which we are concerned, there appear to be two sites. One site will bind solvent and exhibit a MII spectral change (\(E\)), the other site will bind the hydrocarbon and exhibit a type I spectral change (\(E\)). In the untreated male, ethanol does not bind to \(E\); however, ethanol apparently does bind to \(E\) in the PB male. If this is indeed the case, ethanol spectra from the different animals should have different characteristics. In the untreated male, the addition of saturating amounts of the type I substrate ethylbenzene to both cuvettes should not affect the MII response to ethanol. In the PB-treated male, however, the MII change observed should be the sum of two spectral components, a type I and a type II. Therefore, the addition of saturating amounts of the type I substrate to both cuvettes should increase the magnitude of the MII spectral change by effectively blocking any solvent interaction with the type I site. This is indeed the case, as clearly demonstrated in Fig. 5, b and d. This effect was observed over a wide range of ethanol concentrations. Fig. 5, a and c, demonstrates the lack of effect in the untreated rat.

The interaction of ethanol with the type I site in the PB-treated rat requires that the experimental procedure be redesigned to permit proper determination of the equilibrium constants. Fig. 6 describes the protocol and the species involved.

Fig. 7 compares the results obtained with and without the above correction in PB males. A double reciprocal plot comparing corrected with uncorrected data is shown in Fig. 7a. The data clearly show that either method for determining \(K_{II}^{w} \) results in approximately the same value for \(K_{II}^{w}\) and \(K_{II}^{w}\) (Fig. 7b). Introducing the correction causes a small decrease in both apparent association constants. Likewise, Fig. 7c shows a small increase in both \(\Psi(E)\) values. Using the constants obtained from Fig. 7, b and c, a theoretical curve can be calculated using equation 3. The points from the experimental determinations agree well with those predicted by the theoretical curve (Fig. 7d).

Careful observation of Fig. 7a will reveal that the double reciprocal plots deviate from linearity at very high substrate concentrations. A situation that appears to be analogous to substrate inhibition for kinetic data occurs, in that the plots appear to be slightly concave upward. Introduction of the correction causes the disappearance of this deviation exemplified by a general increase in the correlation coefficients for the double reciprocal plots.

An additional complication would be encountered if ethylbenzene binds to the MII site. Although this treatment does not account for such a possibility, it is generally thought that hydrocarbons are pure type I compounds; therefore, not exhibiting the type II response (5, 21).

When comparing corrected versus uncorrected data, three characteristics may be observed: 1) an increase in linearity in the double reciprocal plots, in addition to a displacement of the corrected curve to smaller \(1/\Lambda_{\text{A}}\) values, 2) a decrease should be noted in \(K_{II}^{w}\) and \(K_{II}^{w}\) of the \(K_{II}^{w}\) versus \(N\) replots, and 3) the replot should be linear.

Equation 6 describes the substrate dependence of two competing substrates that are both able to bring about an absorbance change when only one of the substrates (substrate A) is added to the reference cuvette; this describes the uncorrected data (Fig. 4).

\[
\frac{1}{\Delta \Lambda_{\text{total}}} = \frac{\Delta \Lambda_{A}}{K_{A}[N]} + \frac{\Delta \Lambda_{B}}{K_{B}[N]}\psi_{A}[E] + \frac{\Delta \Lambda_{B}}{K_{B}[N]}\psi_{B}[E] + \psi_{A}[E] + \psi_{B}[E] + \psi_{A}[E] + \psi_{B}[E] \\
(1 + K_{A}[N]) + (1 + K_{B}[N])
\]

Equation 6 describes the substrate dependence of two competing substrates that are both able to bring about an absorbance change when only one of the substrates (substrate A) is added to the reference cuvette; this describes the uncorrected data (Fig. 4).

According to this equation, nonlinear double reciprocal plots are predicted. Therefore, by assuming arbitrary values for \(K_{A}\), \(K_{B}\), \(N\), \(\psi_{A}[E]\), \(\psi_{B}[E]\), and \(B\), the characteristics predicted...
FIG. 5. Demonstration of type I component in modified type II spectrum. Microsomal suspensions from both (a) untreated and (b) PB-treated male rats containing 2.8 and 3.2 mg of microsomal protein/ml, respectively, were used in this study. A total volume of 3 ml of liver microsomes was added to both reference and sample cuvettes. Solid lines represent microsomes to which no exogenous substrates were added prior to placement in spectrophotometer. The curves shown by the dashed lines represent microsomes to which 68 mM ethylbenzene was added prior to placement in spectrophotometer. A base-line was then obtained. In each case, the spectral change was produced by addition of 40 μl of ethanol to the sample cuvette; 40 μl of H2O was added to the reference cuvette to correct for volume changes. (c) double reciprocal plot for ethanol in the absence (●) and presence (○) of 68 mM ethylbenzene in untreated male rats; (d) double reciprocal plot for ethanol in the absence (●) and presence (○) of 68 mM ethylbenzene in PB-treated male rats.

FIG. 6. Modified procedure for removal of noninteracting MI1 component in phenobarbital-treated rats.

Desirable results of such an experiment are presented in Table II. The association constant for ethylbenzene was determined in the absence of solvent by taking advantage of the very limited solubility of the hydrocarbon in an aqueous buffer. An aqueous KCl/Tris buffer (0.15 M, 50 mM, pH 7.4) will dissolve ethylbenzene to a concentration of approximately 2 mM. The concentration of ethylbenzene presented to the microsomes was varied by mixing various ratios of buffer containing the hydrocarbon with buffer lacking the hydrocarbon and adding these solutions to microsomal preparations that were concentrated to 14 mg of microsomal protein/ml as described under “Experimental Procedures.” The results clearly demonstrate good agreement between values obtained by the extrapolation method and those obtained in the absence of solvent for both $K_a^{extr}$ and $\Psi_{q,E}$. Large amounts of microsomal material were required in determining $K_a^{extr}$ for ethylbenzene in the absence of solvent (many times that necessary for an extrapolation). Furthermore, direct addition of hydrocarbon is a tedious and time-consuming process which is impractical for the determination of large numbers of binding constants.
method for the removal of endogenous substrates from microsomes by extracting with defatted bovine serum albumin (11). By utilizing this method, the effect of extraction of endogenous substrates on the solvent extrapolation was determined. The results in Fig. 9a clearly show that the binding of ethanol to the type I site in PB-treated males was not due to the presence of different levels of endogenous substrates in the microsomes of the PB versus untreated rats. In the untreated male the extraction process caused essentially no change in the apparent association constant for ethylbenzene. Independence from solvent effects remained. Endogenous substrate extraction of microsomes from PB-treated male rats did not cause the disappearance of the solvent dependence. In fact, the association constant for ethanol as determined from the slope of the $K_{	ext{app}}$ versus $N$ plot actually increased very slightly, as did that for ethylbenzene.

The Effect of Microsome Concentration on Ethylbenzene/Ethanol Extrapolation—As predicted by Parry et al. (22) and demonstrated by Ebel et al. (6), the apparent association constants for type I substrates are affected by microsomal concentration. This effect is caused by a decrease in the

$$K_{	ext{app}} = \frac{R_B}{1 + K_A[A]}$$

which quantitatively indicates that the presence of an endogenous substrate causes a decrease in the apparent binding constant for an exogenous substrate.

Fig. 7. Effect of ethanol on the apparent association of ethylbenzene for the type I site in PB-treated male rats. Ethylbenzene was diluted to different stock concentrations in ethanol. Each solution was then used for the determination of $K_{Bpp}$ by both procedures previously described in Figs. 4 and 6. No shifts in peaks or troughs were observed throughout the titration or as the concentration of the stock solutions were varied by either procedure. Microsomes containing 3.2 mg of microsomal protein/ml were prepared from PB-treated male rats. (a) Double reciprocal plots comparing uncorrected (procedure I) and corrected (procedure II) data. Solid circles represent uncorrected (procedure I) data, and open circles represent corrected data. (b) Replot of $K_{Bpp}$ versus $N$ (ethanol)/[ethylbenzene]). In this and each of the following panels of this figure, solid and open circles represent uncorrected and corrected data, respectively. (c) Replot of 1/slope versus $N$. (d) Replot of 1/Δ$A_{max}$ versus $N$. In each case, the lines represent theoretical curves predicted from equation 3 using constants determined from $b$ and $c$ above.
The association constants and $\Delta$A depend on the solvent used. It is clear that...in the preceding section; however, since a microsomal concentration was 2.7 mg of microsomal protein/ml.

**TABLE II**

Comparison of solvent extrapolation with direct determination of $K_{app}$ for ethylbenzene in the absence of solvent in PB-treated rat liver microsomes.

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The solvent dependence of ethylbenzene/ethanol was determined at a variety of microsome concentrations. The reciprocal of the solvent dependence of ethylbenzene as described in Table III using the relationship \( \Delta G = -RT \ln K \). The \( \Delta G \) of binding of the type II component of the alcohol-induced MII spectrum (A) was determined by blocking the type I site with 68 mM ethylbenzene. Determinations were made on PB-treated male rats as described in the legend to Table III. The alcohols represented in this figure are (progressing from 1 to 4 carbon atoms): 1) methanol, 2) ethanol, 3) 1-propanol and acetone (open symbols), and 4) 1-butanol.

Table IV. Two hydrocarbons and solvent dependence with various substrates

| Solvent  | Ethylbenzene | Ethylbenzene | Ethylbenzene | Ethylbenzene |
|----------|--------------|--------------|--------------|--------------|
|          | \( K_s \)    | \( \Psi(E) \) | \( K_s \)    | \( \Psi(E) \) |
| Methanol | 2800         | 0.14         | 0.029        | 8500         | 0.13         | 5.3          | 0.027        |
| Ethanol  | 2300         | 0.15         | 0.002        | 4000         | 0.16         | 15           | 0.050        |
| Acetone  | 1600         | 0.13         | -0.2         | 6000         | 0.16         | 23           | 0.060        |
| Propanol | 2500         | 0.15         | 2.8          | 8500         | 0.14         | 39           | 0.046        |

Also illustrated in Fig. 11 is the size dependence of the type II component of the ethanol spectral change. In these experiments, alcohol spectra were determined in the presence of saturating ethylbenzene to block type I binding of the alcohol. A linear plot of \( AG \) apparent versus number of carbon atoms added was obtained; the slope was \(-0.3 \) kcal/mol/carbon atom added. A linear plot of this type was not observed unless type I binding was blocked as described in the legend to Fig. 5.

The association constant and \( \Psi(E) \) for ethylbenzene in the untreated male rat were independent of the solvent employed. The solvents used did not appear to interact significantly with the type I site, resulting in negligible values for \( K_s \) and \( \Psi(E) \) for the solvent.

Solvent Dependence for Different Hydrocarbon Substrates—If the solvent dependence observed with ethanol and other organic solvents is the result of binding of solvent to cytochrome P-450 and competition with substrate, then a similar solvent dependence should be observed with different substrates. This should occur since the significance of the slope of the \( K_s \) versus \( N \) replot is simply \( K_s \), which should be independent of the competing substrate if both substrate and solvent are large enough to exclude simultaneous binding to the same site. Results of such an experiment are shown in Table IV. Two hydrocarbons of similar structure were used, since it would be reasonable to assume that both substrates would bind to the same species of cytochrome P-450. The results demonstrate that these hydrocarbons possess greatly different affinities for cytochrome P-450; however, in each case the apparent binding constant for the solvent ethanol is the same.

Effect of Ethanol on the \( K_s \) for Ethylbenzene in Female Rats: Demonstration of Sex Differences—The results of extrapolations of ethylbenzene diluted in ethanol for both untreated and phenobarbital-treated females are shown in Table V. The results are consistent with those predicted by equation 3. Table V shows the interaction of ethanol in the PB-treated female rat having a \( K_s \) for ethylbenzene and ethanol of 2,400 to 9,000 \( M^{-1} \) and 9 \( M^{-1} \), respectively. (The reason for the high degree of variability in the \( K_s \) for ethylbenzene is unknown at this time, but may be due to the interaction of cyclic hormonal changes within the individual female animal with the drug effect.) When compared with untreated females, a 2-fold increase in cytochrome P-450 levels was observed in PB-treated females. The \( K_s \) for ethanol in the PB-treated female is significantly different from that found in the PB-treated male (Table V). Although a difference in the \( K_s \) between induced males and females could not be demonstrated for ethylbenzene binding, a sex difference in \( K_s \) for
ethanol binding does exist. Results from the untreated females when compared to untreated males (Fig. 4) also show a sex difference with respect to apparent ethanol binding to the type I site (Table V); ethanol does bind to the type I site in the untreated female; the association constant for ethanol being 5 \text{ M}^{-1}. Since ethanol dependence was not observed in the untreated male, this indicates a sex difference for ethanol binding to the type I site, despite the similarity in the association constant for ethylbenzene (Table V).

**DISCUSSION**

Data obtained from PB-treated male rats where ethylbenzene is diluted in ethanol are consistent with the equations describing the solvent (ethanol) as a competitive substrate. According to this treatment, the type I site is capable of binding either ethanol or hydrocarbon with both complexes (EA and EB) eliciting a spectral change. In addition, this competing solvent possesses a type I component which is superimposed on the MI1 spectrum in PB-treated male rats. Evidence for this interpretation is 2-fold. 1) The slope of the l/slope versus N replot has a positive sign (Figs. 4c and 7c), indicating that the competing species (solvent) causes an absorbance that is in the same direction as that of the type I substrate. 2) Ethanol and other solvents are able, when added alone, to elicit a spectral change (4, 17), which has been called either a modified type II or reverse type I spectrum. If the MI1 spectrum represents the sum of a type I and a type II component, then blocking type I binding of the solvent by addition of saturating quantities of a type I substrate (ethylbenzene) to both cuvettes will cause an increase in the magnitude of the MI1 change. This point has also been demonstrated by other investigators (4, 5). This effect was only observed in the present work when a solvent dependence consistent with that presented in Fig. 7 was also present; the untreated male rat produced neither of these results.

The concept of dual interaction, where the MI1 spectral change is the result of two overlapping spectral components, has been proposed by both Schenkan’s laboratory (3, 4) and Yoshida and Kumaoka (5). Schenkan et al. used phenacetin as the MI1 compound and demonstrated that the magnitude of the phenacetin spectrum increased in untreated rat liver microsomes when a type I compound (aminopyrine or hexobarbital) was present in both the reference and sample cuvettes. Yoshida and Kumaoka (5) presented similar evidence with the MI1 compound butanol by demonstrating an increase in the magnitude of the butanol spectral change when the type I compound pentane was added to both sample and reference cuvettes containing liver microsomes from PB-treated male rats. The treatment presented here gives strong quantitative support to this proposed mechanism.

The results presented in Table I and Fig. 2 demonstrate the large discrepancies that may occur as a result of the use of organic solvents to dilute those hydrophobic substrates. Although the magnitude of the effect may vary depending on the solvent used, it is by no means negligible. Therefore, in the study of sparingly water-soluble substances, the substrate must either be added without an organic solvent or the affinity of the solvent for the site in question must be determined so that \( K_{o/w} \) in the absence of solvent can be calculated by extrapolation. Use of the former method (as described under “Experimental Procedures”) is tedious and utilizes much larger quantities of microsomes when compared with the solvent extrapolation. In addition, the microsomes which are added to the reference and sample cuvettes must be diluted separately; therefore, small pipetting errors will affect the base-line which cannot be directly determined. This will increase the amount of scatter observed with the experimental points. Despite these shortcomings, it may be advantageous to use this method under certain circumstances.

Hydrocarbons, steroids, and water-insoluble drugs are commonly added to the cytochrome P-450 system using organic solvents as the vehicle. Results obtained from many of these studies may be seriously in error due to effects of the solvent. Therefore, if solvents are used as the vehicle for a substrate, the very least an extrapolation similar to that shown in Fig. 4 must be performed to determine the magnitude of any effect caused by the presence of solvent. As shown in Fig. 2, if solvent interacts with the enzyme, then simply changing the ratio of solvent to substrate should cause a break in the linearity of the double reciprocal plot at the point where the substrate concentration of the stock solution is changed. This is a simple and rapid method for determination of the presence of a “solvent effect” of the type studied here. Such a procedure (Fig. 2) should be utilized when nonaqueous solvents must be used in order to determine whether or not a solvent has an effect on the formation of the enzyme-substrate complex.

As noted in Fig. 8a, deviation from linearity at very high substrate concentrations was observed using procedure I (uncorrected method) where solvent was simply added to the reference cuvette. When the uncorrected method was utilized, this deviation was characterized by a concave upward curvature in the double reciprocal plots. Guegrich (16) also noted...
such an effect with 7-ethoxycoumarin (acetone was used as the solvent) binding to purified cytochrome P-450 from PB-treated rats, but was unable to explain the cause of such a deviation. (The effect was attributed to nonspecific binding at high substrate concentrations.) Our results clearly demonstrate an increase in linearity of the double reciprocal plots by redesigning the experiments as described in Fig. 6. The presence of the concave upward curvature with 7-ethoxycoumarin in acetone (Fig. 5, Ref. 16) on a purified cytochrome P-450 preparation suggests that phenobarbital-induced rat P-450 B5 has the ability to bind small molecules such as acetone and presumably alcohols, and may be the same binding site with which we are dealing in our microsomal preparations.

Despite the ability of the correction procedure (Fig. 6) to increase the linearity of the double reciprocal plots, it must be stressed that the correction is only an approximation. The accuracy of the values obtained by this methodology is based on the ability of the blocking (type I) substrate to exclude solvent binding at the type I site. An increase in the accuracy of such a correction may be accomplished by using a substrate with a very large affinity for that type I site.

In this study, the suggestion has been made that in the PB-treated rat, the ethanol-induced MI1 spectrum is the sum of both a type I and a MI1 component, where in the untreated rat, the ethanol-induced spectrum is simply the result of the MI1 component. The possibility still exists that ethanol can bind to a type I site of an isozyme in the untreated rat that was not detected by these methods; however, type I binding is not a necessary characteristic for the categorization of MI1 substrates. The presence of a type I component in MI1 substrates would be expected to depend not only on the substrate employed, but also on the isozymic profile of the tissue preparation.

Throughout this study, anything that was done to perturb the apparent type I binding of the hydrocarbon substrate also affected the apparent binding of the solvent in a similar manner. When the PB-pretreated microsomal preparation was treated with defatted bovine serum albumin in order to extract endogenous substrates, a small increase was observed in $K_{pp}$ for ethylbenzene with a corresponding small increase in $K_{pp}$ for ethanol. Results of this type would be expected if the bovine serum albumin treatment removed a small quantity of an endogenous substrate (probably including phenobarbital) that was competing for the type I site. The presence of this "inhibitor" should cause a decrease in the apparent binding constant as previously predicted by equation 7. If the increase in $K_{pp}$ for the hydrocarbon was simply due to the removal of a competitive inhibitor, an increase in $K_{pp}$ for ethanol should also be observed, as demonstrated in Fig. 9.

Parry et al. (22) have quantitatively treated the effect of membrane concentration on the association of substrates for sites on membrane associated enzymes. Ebel et al. (6) demonstrated this effect by measuring the apparent binding of type I substrates to liver microsomal cytochrome P-450 at various microsomal concentrations. According to this treatment, as the microsomal concentration increases, the reciprocal of the association constant ($1/K_{pp}$) also increases in a linear fashion. Therefore, if the solvent dependence observed in the PB-treated rat is due to competition for ethanol for the type I (ethylbenzene) binding site, then both ethylbenzene and ethanol should vary in a similar manner. According to Fig. 10, both ethylbenzene and ethanol follow the same kind of law with respect to the effect of microsome concentration. Again the solvent ethanol is acting as though it were simply an alternative substrate.

Further evidence that the observed solvent dependence is the result of competition between ethanol and ethylbenzene for the type I site is demonstrated by the invariance of $K_{pp}$ for ethanol when the affinities of different hydrocarbon substrates were measured (Table IV). In these studies, two hydrocarbons were chosen as substrates since structural similarity would suggest that these substrates would bind to the same enzyme site. If these hydrocarbons do bind to the same fraction of cytochrome P-450, and ethanol competes with one of the hydrocarbons, then ethanol should also compete with the other hydrocarbon and exhibit the same solvent dependence, since the solvent dependence simply reflects $K_{pp}$ for ethanol. Deviations from these predicted results may occur. One possible case is where multiple binding sites are present. As shown by Klotz and Hunston (14, 15), if two sites are present that bind the same compound (in this case, ethanol), the apparent association constant for the first site titrated (high affinity site) equals $-K_{pp}$, while the apparent association constant for the low affinity site equals $-2K_{pp}/(K_{pp} + K_{dd})$. If the latter site is being studied, deviations would be expected. Another possible case is where the substrate and solvent are well enough that both compounds may simultaneously bind in the single binding site. In this case, the characteristics of the double reciprocal plots might be expected to fit either a noninteracting (simple one substrate case), or a noncompetitive situation.

The dependence of $K_{pp}$ (association constant for enzyme-competitive inhibitor complex) upon molecular size of the inhibiting molecule has been demonstrated for a-chymotrypsin (23, 24), yeast alcohol dehydrogenase (26), and C-1 esterase (27). The inhibitors used in each were an isochromat series of aromatic hydrocarbons similar to that used here. This dependence was then compared with that obtained for the distribution of the same compounds between an organic solvent and water. Similar reports have been published with the cytochrome P-450 system where the size dependence of binding of a series of hydrocarbons (19), barbiturates (29), and alkylamines (28) on molecular size were determined.

If the solvent dependence is a result of the solvent simply acting as an alternative substrate, then the use of different solvents should not affect the extrapolated value of $K_{pp}$ for ethylbenzene; however, $K_{pp}$ for the alcohols might be expected to exhibit a linear size dependence of $DG^0$ of binding if hydrophobicity of the alcohol is important in binding. The results shown in Table III and Fig. 11 are consistent with those predicted.

It is interesting to note that the variation of $DG^0$ with size has a slope of approximately $-0.6$ kcal/mol/carbon atom increase in chain length for the alcohol series. This result is similar to the slope obtained for partitioning of the same compounds between octanol and water (approximately $-0.7$ kcal/mol/carbon atom). In this respect, the organic alcohols exhibit a linear size dependence and again behave as though they are simply alternative substrates. Although a small increase in the slope of the size dependence plot might be observed by extrapolating to zero microsome concentration, these results offer a very good estimate of the extrapolated values since the microsome concentration is relatively low in these studies (8). The dependence of $DG^0$ of binding for the alcohol series on the number of carbon atoms added compares well with the dependence of $DG^0$ of binding for a hydrocarbon series which binds to the same site in the PB-treated rat (8). Since both sets of compounds (alcohols and hydrocarbons) share a basic structural feature (alkane moiety), similar size dependences might be expected. These results indicate that hydrophobicity is a major factor in binding of these compounds to the type I site. However, these results do not indicate that the hydroxyl group is unimportant with respect to alcohol binding, only that the increase in $K_{pp}$ observed by
adding a methylene group is unaffected by the presence of the hydroxyl group. Therefore, the hydroxyl group apparently makes approximately the same contribution to the binding of the various alcohols to cytochrome P-450.

It is interesting to note that a linear size dependence was also observed for the binding of the type II component of the MII spectrum, again suggesting substrate binding. The slope of this size dependence was \(-0.3\) kcal/mol/carbon atom. Although quantitative data are not yet available, Yoshida and Kumaoka (5) demonstrated possible competition between type II and MII compounds at the type II site using rat liver microsomes. Similar results were demonstrated by Van den Berg et al. (30) using mouse liver microsomes. Studies by Schenkman et al. (4) clearly demonstrated that the MII (RI) compound phenacetin would not affect the carbon monoxide spectrum when cytochrome P-450 was in the reduced form, while type II substrates did produce such an interaction. From these data, Schenkman et al. (4) suggested that the MII spectral change is the result of substrate binding to a unique site different from the type II (heme) site. Studies from our laboratory have demonstrated that methanol and ethanol, which are considered to be MII compounds, are able to decrease the magnitude of the carbon monoxide spectrum of reduced cytochrome P-450 (data not shown). The variability of the interaction between reduced CO spectra and different MII substrates (e.g., phenacetin and methanol) suggest a qualitative difference between MII binding of different substrates, and that some MII compounds (e.g., alcohols) may actually interact at the heme as do type II compounds. Since type II compounds affect CO spectra in a manner similar to the MII compounds ethanol and methanol, a similarity in mode of interaction between MII and type II compounds might be expected.

Jefcoate et al. (28) determined the size dependence for a series of alkylamines (type II compounds), from which a slope of approximately \(-0.7\) kcal/mol/carbon atom was obtained. If the type II component of the alcohol spectrum and that of the amine spectrum represent binding to the same site, quite different size dependences might be expected if the contribution of the amine functions and hydroxyl functions to the entropy of binding were different. Comparison of the size dependence of amine binding (\(-0.7\) kcal/mol/carbon atom), as determined by Jefcoate, with that obtained for MII alcohol binding (\(-0.3\) kcal/mol/carbon atom), as determined in this study, supports the above possibility. It must be stressed, however, that this suggestion is based on presumptive evidence. Strong support for this argument may be obtained by a quantitative demonstration of competition between the type II component of an amine and that of an alcohol. Use of the equations previously presented should be of particular value in this determination. These experiments are in progress.

A number of investigators have suggested that the MII spectral change is the result of the removal of endogenous substrate from the type I site (4, 6, 11). Ebel et al. (6) have refined this hypothesis by suggesting that the modified type II spectral change is due to a perturbation in the dielectric constant of the aqueous phase which would increase the relative solubility of substrates in that phase and thus essentially “leach” endogenous substrates from the type I site. Although the evidence presented here cannot totally discount this possibility, preliminary experiments have demonstrated only a small decrease in the chemical potential of the substrate in the aqueous phase by the addition of ethanol to a final concentration of 225 mM (approximately 50 \(\mu\)l in 3 ml of aqueous buffer) (41). Studies by Vore et al. (31) conclusively demonstrated that butanol extraction of liver microsomes from PB-treated rats did not decrease the magnitude of the ethanol- or phenacetin-induced MII spectra. In fact, the magnitude of the MII spectral change was actually increased. Butanol, added to a lyophilized microsomal pellet, was shown to remove a significant amount of endogenous substrates. If the MII spectrum is caused by “leaching” of endogenous substrates from the type I site, the magnitude of the MII spectrum in butanol-extracted microsomes should be decreased. From their results, Vore et al. (31) concluded that the MII spectrum is not due simply to displacement of endogenous substrates. Butanol treatment exposes the microsomes to a medium with a dielectric constant of approximately 17.1 (32). The dielectric of a solution to which ethanol has been added in concentrations commonly encountered in spectral studies varies from 80 (water) to approximately 78 (32). Therefore, it seems unlikely that “endogenous substrates” that could not be removed by butanol could then be “leached” from the enzyme by the small change in dielectric caused by ethanol addition to the aqueous suspension and in turn bring about a MII spectral change.

The possibility may arise that endogenous substrate could not be removed from the enzyme due to steric factors caused by protein conformation. If such a case occurs, however, the endogenous substrate should be considered to be acting in a sense as a prosthetic group (i.e., a functional part of the enzyme) and should not be thought of as an endogenous substrate. This laboratory has recently reported that a reverse type I spectral change can be observed by addition of a hydrophobic substance to a prototiferric enzyme solution (33). Further studies have demonstrated that the heme monomer is the form responsible for this spectral change. It has been proposed that, by analogy, a hydrophobic substance (possibly phospholipid) may be in the vicinity of the heme in the enzyme and a change in the position of this moiety (conformational change) with respect to the heme could elicit the type I spectral change (33). Although the presence of a prosthetic group (endogenous substrate) may mediate each of the different types of spectral changes, it is not necessary to assume that a prosthetic group is present, at least for explanation of the results discussed here.

Since various organic solvents have been shown to possess different binding constants and extinction coefficients, the preferred solvent would be that which exhibits the smallest effect on the binding of the substrate. As a general rule, the best solvent to use in such determinations would be methanol; however, use of this solvent does not preclude determination of \(K_{\text{app}}\) for methanol. The extrapolation is necessary for the determination of a corrected association constant for the substrate in question. As demonstrated above, this extrapolation method is particularly important when working with PB-treated male rats and treated or untreated female rats.

It is conceivable that a previously determined solvent dependence might be assumed in order to correct for the effect of solvent in the study of various substrate interactions without actually ascertaining the solvent effect each time, thus saving some effort; however, caution must be exercised if such a procedure is to be used, since a number of factors may affect this dependence. For example, \(K_{\text{app}}\) for ethanol has been shown to be affected by changes in microsome concentration. Therefore, if association constants are to be determined at various microsome concentrations, then the solvent dependence should be determined at each of the microsome concentrations. If the microsome concentration is not varied, then a predetermined solvent dependence might then be assumed, since studies from this laboratory have produced quite consistent results at a particular microsome concentration, even when comparing different preparations from the same species.

Another variable to be considered involves the multiplicity

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of cytochrome P-450. Substrates which bind to the same site should exhibit similar solvent dependences; however, a particular solvent dependence should not necessarily be assumed for structurally different compounds. The reasons for this are 2-fold: 1) two different substrates might not bind to the same form of cytochrome P-450; if this occurs, different solvent dependences may be expected. 2) One of the substrates may have a different significance, as explained previously (14, 15); association constant for the second (low affinity) site would exhibit similar solvent dependences; however, a partic-

2-fold: 1) two different substrates might not bind to the same site in untreated female rats, but not in untreated male rats.

The binding of ethanol was found to be significantly different when comparing untreated with PB-treated female rats, although microsomes from female rats under both treatments were able to bind the solvent. It is interesting to note that a large variation was found in the association constant for ethylbenzene in the PB-treated female rat, but only a small variation was observed in the association constant for ethanol. The reason for this anomaly is at this time unexplained, but may be related to cyclic hormonal changes in the female rat. Studies are currently in progress to clarify this situation and to elucidate the mechanism involved in the sex-dependent hormonal regulation of solvent binding.

Mezey et al. (34) demonstrated that ethanol was oxidized by a liver microsomal component containing high concentrations of cytochrome P-450 and cytochrome c reductase, which were found to be required for maximal ethanol-oxidizing ability. Additional studies demonstrated inhibition of ethanol-oxidizing capacity by the addition of inhibitors of drug metabolism such as SKF-525A, carbon monoxide, and cytochrome c (34–36). Microsomal ethanol-oxidizing activity was also shown to be induced by ethanol pretreatment (37, 38). Despite this evidence, the involvement of cytochrome P-450 in micro-

since substrates for the cytochrome P-450 system have been shown to elicit a type I spectrum, such a spectral change may be expected with ethanol if it was metabolized by cyto-

chrome P-450. This study clearly demonstrates that ethanol can produce a type I component to the MI spectrum in PB-treated male rats, supporting the possibility of mixed function oxidation of ethanol; however, the type I component of the ethanol spectrum was not detected in untreated male rats (Figs. 4 and 5). It is interesting to note that in all studies where cytochrome P-450-dependent oxidation of ethanol was discounted (35, 39, 40), microsomes from untreated male rats were used, but in the study where cytochrome P-450-dependent ethanol oxidation was most clearly demonstrated (34), PB-treated male rats were used. These results support the possibility of differential involvement of cytochrome P-450 in ethanol oxidation when comparing PB-induced and untreated male rats.

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