MOLECULAR PHARMACOLOGY, 9, 383–397

Involvement of a Hydrophobic Site in the Inhibition of the Microsomal p-Hydroxylation of Aniline by Alcohols

G. M. COHEN[1] AND G. J. MANNERING

Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

(Received November 8, 1972)

SUMMARY

COHEN, G. M., AND MANNERING, G. J.: Involvement of a hydrophobic site in the inhibition of the microsomal *p*-hydroxylation of aniline by alcohols. *Mol. Pharmacol.* **9**, 383–397 (1973).

Ethanol in millimolar concentrations inhibited the *p*-hydroxylation of aniline, a type II compound, but had no effect on the *N*-demethylation of ethylmorphine, a type I compound. The inhibitory effect of the ethanol on aniline *p*-hydroxylation was shown to occur at some stage beyond the reduction of cytochrome P-450. Aniline *p*-hydroxylation was inhibited competitively by all the primary alcohols from ethanol through heptanol, and the inhibitory potency of the alcohols increased with increasing carbon chain length. The increment in the free energy of binding, −0.48 kcal/mole/CH_2 group, provided evidence for an alcohol hydrophobic binding site in either the microsomal membrane or the protein. Studies with the fluorescent probe 1-anilino-8-naphthalenesulfonate suggested that the hydrophobic catalytic site resides in the protein. The first requirement for inhibition of aniline *p*-hydroxylation appeared to be hydrogen bond formation between the hydroxyl group of the alcohol and the enzyme. Steric requirements were also shown to be important. A good correlation between inhibitory potency and logarithm of the partition coefficient was observed for the straight-chain alcohols, but this no longer held for branched-chain compounds. An analysis of the inhibitory data by the method of Hansch showed this system to be one of the most sensitive ascribed to the effects of alcohols.

INTRODUCTION

During a study of the inhibitory effects of Δ^9-tetrahydrocannabinol on the metabolims of type I and II compounds[2] by hepatic microsomes, it was observed that millimolar concentrations of ethanol, added as the solvent for Δ^9-tetrahydrocannabinol, markedly inhibited the hydroxylation of aniline, a type II compound, but had no effect on the *N*-demethylation of ethylmorphine, a type I compound. Ethanol in molar concentrations is known to inhibit the microsomal

This work was supported by United States Public Health Service Grant GM 15477. Some of this work was presented in abstract form [*Fifth Int. Congr. Pharmacol.* (*San Francisco*) p. 44 (1972)].

[1] Present address, Department of Clinical Pharmacology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS, England.

[2] Type I compounds produce a difference spectrum with oxidized microsomes with a maximum at about 390 nm and a minimum at about 420 nm. Type II compounds produce a difference spectrum with oxidized microsomes with a minimum at about 390 nm and a maximum in the region of 425–435 nm.

metabolism of both type I and II compounds, probably by acting nonspecifically through membrane perturbation or protein denaturation. The differential inhibitory effect of ethanol in low concentrations on the type I and II substrates used in this preliminary study suggested a more specific action of ethanol than has been observed when high concentrations of ethanol were employed. Additional preliminary studies with aliphatic alcohols of increasing carbon chain length suggested that the alcohols might interact with a hydrophobic metabolic site. This possibility was explored using concepts developed by Hansch and co-workers (1–3).

As a working hypothesis to explain the reaction of drugs with biological systems, Hansch *et al.* proposed that drugs reach their site of action by a "random walk" process (1). During this process they may act reversibly or irreversibly with proteins, lipids, water, and other cellular constituents, and eventually they react with the site of action at a critical region of the enzyme or membrane. Hansch has postulated that substituents on drugs may promote hydrophobic binding in three ways: by facilitating the random walk of the drug to the site of action, by directing the orientation of the drug on the enzyme or protein, and by causing allosteric effects in the protein or enzyme, which may or may not affect the binding process.

Hansch has also shown that the biological actions of many compounds can be correlated with two equations:

$$\text{RBR} = \log \frac{1}{C} = a \log P + b \qquad (1)$$

$$\text{RBR} = \log \frac{1}{C} = -d (\log P)^2 + e \log P + f \qquad (2)$$

where RBR is the relative biological response, a, b, d, e, and f are constants for a given system, and P is the octanol/water partition coefficient. Equation 1, showing a linear relationship between log $(1/C)$ and log P, is a special case of the more general equation (Eq. 2) seen either when the range of log P studied is both small and on the "linear" portion ot the parabolic relationship or when a one-step partition is involved. The latter case arises when drug action occurs on the outside of a cell membrane and in certain reactions of drugs with enzymes. Equation 2 is the more general of the two equations and shows the parabolic dependence of log (1/C) on log P. This means that there is an optimal P value, designated P_o. Below P_o an increase in P causes an increase in biological activity as more of the drug reaches the active site, but when P_o is exceeded, increases in P cause a decrease in biological activity. This parabolic relationship is predicted by the random walk process.

With these principles in mind, a study was made of the relationship between log P and the inhibitory potency of members of a series of aliphatic and substituted alcohols on aniline *p*-hydroxylation by hepatic microsomes.

MATERIALS AND METHODS

Male Holtzman rats (200–350 g) were employed. Hepatic microsomes, prepared as described previously (4), were used on the day of their preparation.

The type II binding spectrum of aniline was determined by the method of Remmer *et al.* (5). Cytochrome *c* reductase activity was measured at 37° essentially as described by Gigon *et al.* (6). The reaction was started by the rapid addition of NADPH (final concentration, 0.83 mM), and the rate of reduction was determined from the initial linear portion of the reaction. An extinction coefficient of 18.5 mM^{-1} cm^{-1} was used for the oxidized minus reduced cytochrome *c*. NADPH oxidase activity was determined at 37° by the method of Gillette and coworkers as modified by Gigon *et al.* (6). The rate of anaerobic reduction of cytochrome P-450 by NADPH was determined in the presence of CO by recording the increase in optical density between 450 and 490 nm as described by Gigon *et al.* (6). The reaction was initiated by adding 10 µl of 0.25 M NADPH to the mixture (3 ml). All spectral measurements were made with an Aminco DW-2 spectrophotometer. Protein was determined by the method of Lowry *et al.* (7).

Rates of *N*-demethylation of ethylmorphine, aminopyrine, methadone, and *p*-chloro-*N*-methylaniline were determined by

measuring formaldehyde formed, as described by Anders and Mannering (4). The reaction mixture was the same as that described previously (4), except that nicotinamide was omitted. Aniline *p*-hydroxylation was measured as described by Imai *et al.* (8), with minor modifications as follows. The incubation mixture contained 5 mM Mg^{++}, 0.33 mM NADP, 3.3 mM sodium isocitrate, 17 mM $PO_4^{=}$ (pH 7.4), 1 unit of isocitrate dehydrogenase (Sigma type IV), and substrate or inhibitor in a volume of 2.5 ml. The less soluble alcohols were added directly to the microsomal suspension to aid solubility, although similar I_{50} values were obtained whether the alcohol was mixed with the enzyme preparation or added to the incubation mixture before addition of the enzyme. The mixture was incubated at 37° for 3 min before the reaction was initiated by adding 0.5 ml of microsomal preparation containing 6 mg of protein per milliliter. The mixture was incubated for 20 min and centrifuged at 1800 × *g* for 20 min after the reaction had been stopped with 1.5 ml of 20% trichloracetic acid solution. Two milliliters of the supernatant fluid were removed, 2 ml of 1 M Na_2CO_3 solution and 2 ml of 0.5 M NaOH–1% phenol solution were added with mixing, and the absorbance was read 35 min later at 630 nm in a Beckman B spectrophotometer. Recovery of *p*-aminophenol from tissue blanks was approximately 90%. In both the presence and absence of inhibitor, the reaction rates were linear throughout the incubation period. Rates of acetanilide *p*-hydroxylation and acetophenetedin *O*-dealkylation were determined by measuring *p*-aminophenol formation by the method of Shimazu (9). Fluorescence measurements using the hydrophobic fluorescent probe 1-anilino-8-naphthalenesulfonate were made with an Aminco-Bowman spectrofluorometer as described by DiAugustine *et al.* (10). Partition coefficients were obtained from the data of Hansch *et al.* (2, 11). Values for Taft's steric parameter E_s (12), Hancock's corrected steric substituent (13), and the polar substituent constant σ^* (12) were obtained from the literature.

Aniline hydrochloride was obtained from Eastman Organic Chemicals. $NADP^+$, isocitrate, and isocitrate dehydrogenase were obtained from Sigma Chemical Company.

The Michaelis constant (K_m), the inhibitor dissociation constant (K_i), and the maximum velocity (V_{max}) were determined by the method of Wilkinson (14), using an Olivetti computer. Inhibition was interpreted as being competitive when V_{max} values obtained in the presence or absence of inhibitor were not different ($p > 0.05$). The concentration of inhibitor required to reduce enzyme activity to 50% of control (I_{50}) was determined from a plot of enzyme activity against the logarithm of the concentration of drug, with the aid of a linear regression program. The correlations of inhibitory potency and log partition coefficient were determined with the aid of a multiple regression program (supplied by Dr. P. S. Portoghese).

RESULTS

Effects of alcohols on metabolism of type I and type II compounds. The I_{50} concentration of ethanol for the *N*-demethylation of ethylmorphine, a typical type I compound, was approximately 50 times that seen for the *p*-hydroxylation of aniline, a typical type II compound (Table 1). Similarly, ethanol in low millimolar concentrations did not inhibit the *N*-demethylation of the other type I compounds, aminopyrine and methadone, or the *O*-dealkylation of acetophenetedin, also a type I compound. A high concentration of ethanol (100 mM) reduced the *N*-demethylation of aminopyrine (1 mM) by

TABLE 1

Inhibitory effects of ethanol on ethylmorphine N-demethylation and aniline p-hydroxylation

The ethylmorphine concentration was 1 mM (approximately 4 times the K_m); the aniline concentration was 0.2 mM (approximately 4 times the K_m). Values are the means ± standard errors of the numbers of experiments indicated in parentheses.

Substrate	I_{50}
	mM ± *SE*
Ethylmorphine (4)	660 ± 70
Aniline (6)	12.5 ± 0.49

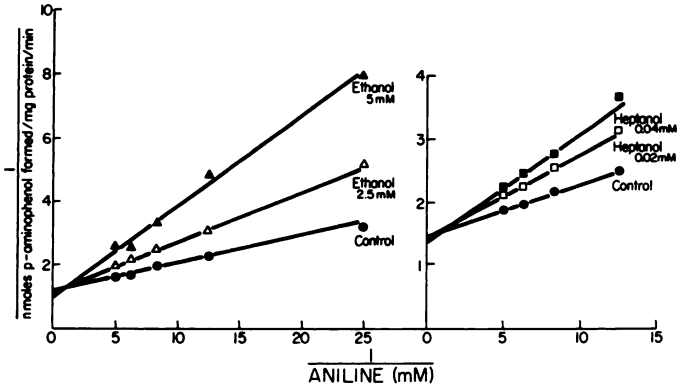

FIG. 1. *Kinetics of inhibition of microsomal aniline p-hydroxylation by ethanol and heptanol*
Microsomes (1 mg of protein per milliliter) were incubated with aniline in the presence or absence of ethanol or heptanol. K_i of ethanol = 2.10 mM; K_i of heptanol = 0.032 mM.

only 20% and was without effect on the *O*-de-ethylation of acetophenetedin. Low concentrations of ethanol inhibited the *p*-hydroxylation of acetanilide, a type II compound, but did not affect the *N*-demethylation of *p*-chloro-*N*-methylaniline, also a type II compound. The I_{50} value of ethanol was 22 mM for inhibition of the *p*-hydroxylation of acetanilide (1 mM), whereas ethanol (100 mM) reduced the *N*-demethylation of *p*-chloro-*N*-methylaniline by only 20%.

It seemed possible that type I compounds might behave like type II compounds with respect to the inhibitory effects of ethanol on their rates of metabolism if the type I site was removed from microsomes by treating them with phospholipase C (15) or by inactivating the site by occupying it irreversibly with 2-diethylaminoethyl 2,2-diphenylvalerate HCl (16). This proved not to be the case; ethanol was still a very poor inhibitor of the *N*-demethylation of ethylmorphine by microsomes that had been treated with either phospholipase C or SKF 525-A.[3]

Kinetics of inhibition of aniline p-hydroxylation by alcohols. As can be seen in Fig. 1, the inhibition of aniline *p*-hydroxylation by ethanol and heptanol was competitive. Similarly, all the other primary alcohols studied were competitive inhibitors of this reaction.

[3] The abbreviations used are: SKF 525-A, 2-diethylaminoethyl 2,2-diphenylvalerate HCl; ANS, 1-anilino-8-naphthalenesulfonate.

This suggests a relatively specific action of the alcohols rather than a nonspecific action due to membrane perturbation or protein denaturation, for which noncompetitive inhibition kinetics would have been predicted.

Effects of ethanol on components of the microsomal drug-metabolizing system. NADPH–cytochrome *c* reductase, NADPH–cytochrome P-450 reductase, NADPH oxidase, and the binding of drugs to cytochrome P-450 have been implicated in the microsomal mixed-function oxidase system responsible for drug metabolism (17, 18). It was therefore of interest to determine which, if any, of these components of the system would be inhibited by ethanol. Table 2 shows that none was inhibited by ethanol. The sequence of events leading to product formation is thought to proceed as follows: an electron from NADPH is donated via NADPH–cytochrome *c* reductase (or NADPH–cytochrome P-450 reductase) to the oxidized cytochrome P-450–substrate complex; the reduced complex then combines with molecular oxygen and accepts a second electron from NADPH to form an oxygenated, reduced cytochrome P-450–substrate complex which dissociates to give the product and oxidized cytochrome P-450. The failure of ethanol in a concentration that inhibited the metabolism of aniline by 50% to affect either the reductases or aniline binding shows that both the inhibitory ac-

TABLE 2

Effects of aniline and ethanol on components of hepatic microsomal drug-metabolizing system

All values represent the means ± standard errors of at least three determinations.

Compound added	NADPH–cytochrome *c* reductase	NADPH oxidase	NADPH–cytochrome P-450 reductase	Binding ($\Delta A_{390-430}$)
	nmoles/min/mg protein			
None	144 ± 9.9	8.41 ± 0.61	12.3 ± 1.5	
Ethanol (12 mM)	135 ± 3.8	8.54 ± 0.86	11.8 ± 2.0	
Aniline (0.2 mM)	135 ± 7.5	8.04 ± 0.84	12.2 ± 1.6	0.0077 ± 0.0003
Ethanol (12 mM) + aniline (0.2 mM)	133 ± 7.1	8.20 ± 0.68	12.0 ± 1.8	0.0071 ± 0.0003

tion of ethanol and the rate-limiting step for aniline *p*-hydroxylation occur at some stage beyond the formation and reduction of the cytochrome P-450–substrate complex.

Evidence for hydrophobic nature of the inhibitory site. The inhibitory potency of the alcohols increased with increasing carbon chain length (Table 3). For hydrophobic binding without steric restriction, a linear increase in binding energy with chain length has been predicted by theory (19) and found in practice for inhibitors of alcohol dehydrogenase (20). As seen in Fig. 2 from a plot of the logarithm of the reciprocal of K_i against increasing carbon chain length, $\Delta\Delta F$, the change in free energy of binding per methylene group, can be calculated from the following formula:

$$\Delta\Delta F = 2.303\, RT\Delta pK_i$$

where R is the gas constant, T is the temperature in degrees Kelvin, and ΔpK_i is the change in the logarithm of the reciprocal of K_i obtained from the graph. Two plots are shown, using corrected and uncorrected (observed) K_i values. The corrected K_i values were calculated by the following equation:

$$K_i\,(\text{corrected}) = \frac{K_i\,(\text{observed}) \times P}{P + 1} \quad (3)$$

The theoretical reason for use of these corrected values will be discussed later. The free energy of binding per methylene group was calculated using both corrected and uncorrected K_i values and found to be 0.36 and 0.48 kcal/mole, respectively. These values are within the range of 0.36–0.95 kcal/mole calculated by Webb (21) for hydrophobic interactions, the actual values depending on the degree of fit. This suggests a hydrophobic binding site for the alcohols.

TABLE 3

Inhibition of aniline p-hydroxylation by primary alcohols (K_i values)

Corrected values were calculated according to the equation $K_{i\,corr} = (K_{i\,obs} \times P)/(P + 1)$, where P is the octanol/water partition coefficient of the alcohol. Numbers in parentheses are the number of experiments.

Alcohol	K_i	$K_{i\,corr}$
	mM ± *SE*	*mM*
Ethanol (6)	2.10 ± 0.45	0.68
Propanol (4)	0.35 ± 0.02	0.24
Butanol [4]	0.20 ± 0.01	0.18
Pentanol (4)	0.083 ± 0.016	0.080
Hexanol (4)	0.057 ± 0.003	0.056
Heptanol (4)	0.032 ± 0.003	0.032

Structural requirements of hydrophobic binding site. In order to obtain further information on structural requirements of the hydrophobic binding site, the studies were extended to include primary alcohols with branched side chains, and secondary and tertiary alcohols. The preceding studies utilized K_i values. To minimize effort, I_{50} rather than K_i values were determined. It can be shown by Michaelis-Menten kinetics with competitive inhibitors that the ratio of K_i values is equal to the ratio of I_{50} values, provided that the latter are determined at a constant substrate concentration. The validity of this proposal was tested by repeating the work with primary alcohols, using I_{50}

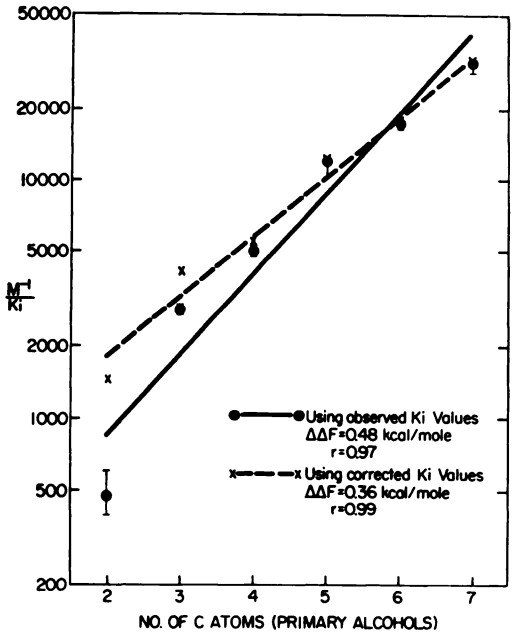

FIG. 2. *Relationship of logarithm of reciprocal corrected and uncorrected inhibitor dissociation constants to carbon chain lengths of primary alcohols*

Corrected and uncorrected K_i values were obtained from Table 3.

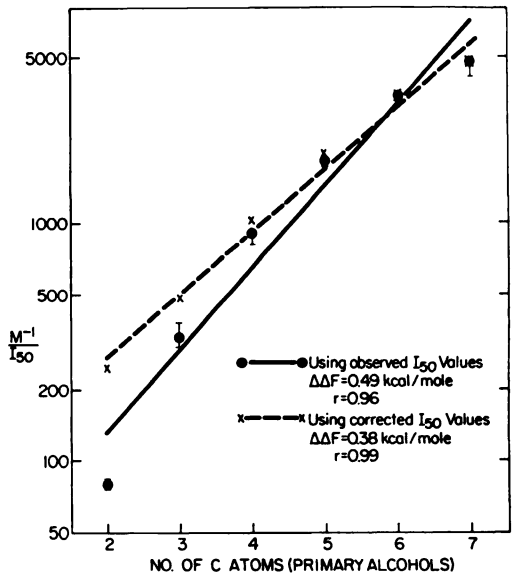

FIG. 3. *Relationship of logarithm of reciprocal corrected and uncorrected I_{50} values to carbon chain lengths of primary alcohols*

Corrected and uncorrected I_{50} values were obtained from Table 4.

rather than K_i determinations. A plot of log $(1/I_{50})$ against increasing carbon chain length (Fig. 3) enabled the free energy of interaction per methylene group to be calculated in a manner analogous to that described previously. Values of 0.38 and 0.49 kcal/mole were obtained using corrected and uncorrected I_{50} values, respectively. These results were in very good agreement with the values obtained using K_i values.

The over-all free energy change may be calculated from K_i values, but this usually includes energy terms other than those defining the direct interaction of enzyme and inhibitor. These other terms, for example, those for the displacement of water or for the ionic atmosphere, are difficult to determine accurately. The use of relative binding energies with related inhibitors enables one to attribute energy changes to structural changes of the inhibitor, which in turn provide further details of the hydrophobic binding site. As shown by Webb (21), the difference in binding energies is given by the equation

$$\Delta F_1 - \Delta F_2 = 1.422 \log \frac{I_1}{I_2}$$

where ΔF_1 and ΔF_2 are the free energies of binding of the respective inhibitors and I_1 and I_2 are the concentrations of inhibitors required for 50% inhibition at 37°. All binding energies were calculated relative to ethanol. These values, as well as the I_{50} and log P values for the primary alcohols from methanol through heptanol, are shown in Table 4.

The inhibitory potencies of the secondary alcohols are given in Table 5. In all cases except propanol the primary alcohol was a more potent inhibitor than the corresponding secondary alcohol. The free energy of binding per methylene group for secondary alcohols was calculated to be 0.28 kcal/mole from a plot of log $(1/I_{50})$ against increasing carbon chain length (Fig. 4). This was less than that obtained for primary alcohols and less than predicted by theory for hydrophobic interactions.

Steric factors might hinder the binding of the inhibitor to the hydrophobic site. As may be seen in Table 6, 3-pentanol was a more potent inhibitor than 2-methyl-3-pen-

TABLE 4

Inhibition of aniline p-hydroxylation by primary alcohols (I_{50} values)

The aniline concentration was 0.2 mM. Numbers in parentheses are the number of experiments.

Alcohol	I_{50}	Relative binding energy[a]	$I_{50\ corr}$[b]	Log *P*
	mM ± *SE*	*kcal/mole*	*mM*	
Methanol (5)	1240 ± 150	+2.84	223	−0.66
Ethanol (6)	12.50 ± 0.49		4.05	−0.32
Propanol (6)	3.00 ± 0.38	−0.88	2.06	+0.34
Butanol (6)	1.11 ± 0.14	−1.50	0.98	+0.88
Pentanol (3)	0.54 ± 0.03	−1.94	0.52	+1.40
Hexanol (4)	0.29 ± 0.01	−2.32	0.287	+2.03
Heptanol (4)	0.21 ± 0.03	−2.52	0.209	+2.53

[a] Relative to ethanol and calculated by the equation $\Delta F_1 - \Delta F_2 = 1.422 \log (I_1/I_2)$ (17).

[b] Calculated according to the equation $I_{50\ corr} = (I_{50\ obs} \times P)/(P + 1)$, where *P* is the octanol/water partition coefficient of the alcohol.

TABLE 5

Inhibition of aniline p-hydroxylation by secondary alcohols

The aniline concentration was 0.2 mM. Numbers in parentheses are the number of experiments.

Alcohol	I_{50}	Log *P*
	mM ± *SE*	
2-Propanol (6)	2.96 ± 0.26	0.14
2-Butanol (4)	2.23 ± 0.17	0.61
2-Pentanol (3)	1.18 ± 0.14	1.16
2-Hexanol (3)	0.71 ± 0.13	1.83
2-Heptanol (3)	0.56 ± 0.06	2.33

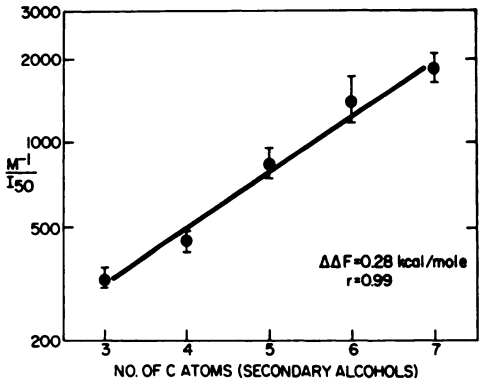

FIG. 4. *Relationship of logarithm of reciprocal I_{50} values to carbon chain lengths of secondary alcohols*

The I_{50} values were obtained from Table 5.

tanol, which in turn was more potent than 2,4-dimethyl-3-pentanol. Similarly, steric factors could be of importance in the inhibitory effects of a series of 5-carbon alcohols (Table 7). The most highly sterically hindered alcohol in the series, *tert*-pentyl alcohol, was also the weakest inhibitor of aniline *p*-hydroxylation.

Relationship between inhibitory potency and lipophilicity of alcohols. The relative rates of metabolism of many compounds have been correlated with their lipophilic character. A correlation was also observed by Kato *et al.* (22) between the ability of hydrazine derivates to inhibit microsomal drug metabolism and their relative lipid solubility. It therefore seemed possible that the inhibitory potency and lipophilicity of the alcohols might be related. The logarithm of the partition coefficient (log *P*) obtained from Hansch's data was used as a measure of the lipophilicity of the alcohol. A plot of log $(1/K_i)$ or log $(1/K_{i\ corr})$ against log *P* revealed excellent correlation of the two parameters (Fig. 5). The inhibitory potency of the alcohol for the primary aliphatic alcohols was linearly related to log *P* by the following equations:

$$\log \frac{1}{K_i} = 0.60 \log P + 3.08, \quad r = 0.97 \tag{4}$$

$$\log \frac{1}{K_{i\ corr}} = 0.45 \log P + 3.38, \quad r = 0.99 \tag{5}$$

TABLE 6

Inhibition of aniline p-hydroxylation by sterically hindered alcohols

Values are expressed as the means ± standard errors of three determinations. The aniline concentration was 0.2 mM.

Alcohol	I_{50}	Log P
	mM ± *SE*	
3-Pentanol	2.32 ± 0.63	1.16
2-Methyl-3-pentanol	7.78 ± 1.56	1.44
2,4-Dimethyl-3-pentanol	23.9 ± 6.0	1.74

TABLE 7

Inhibition of aniline p-hydroxylation by 5-carbon alcohols

The aniline concentration was 0.2 mM. Numbers in parentheses are the number of experiments.

Alcohol	I_{50}	Relative binding energy[a]	Log P
	mM ± *SE*	*kcal/mole*	
Pentanol (3)	0.54 ± 0.03	−1.94	1.40
2-Pentanol (3)	1.18 ± 0.14	−1.46	1.16
2-Methyl-1-butanol (3)	1.40 ± 0.03	−1.35	1.16
3-Methyl-1-butanol (4)	1.56 ± 0.11	−1.29	1.16
3-Pentanol (3)	2.32 ± 0.63	−1.04	1.16
Neopentanol (3)	4.68 ± 0.98	−0.61	1.36
tert-Pentyl alcohol (3)	367 ± 24.8	+2.08	0.89

[a] Relative to ethanol and calculated as described in Table 4.

Similarly, using I_{50} determinations, an extremely good correlation was seen between inhibitory potency and log partition coefficient for both primary and secondary alcohols (Figs. 6 and 7, respectively). The equations from these plots were:

Primary alcohols ($C_2 \rightarrow C_7$):

$$\log \frac{1}{I_{50}} = 0.62 \log P + 2.27, \quad r = 0.98 \tag{6}$$

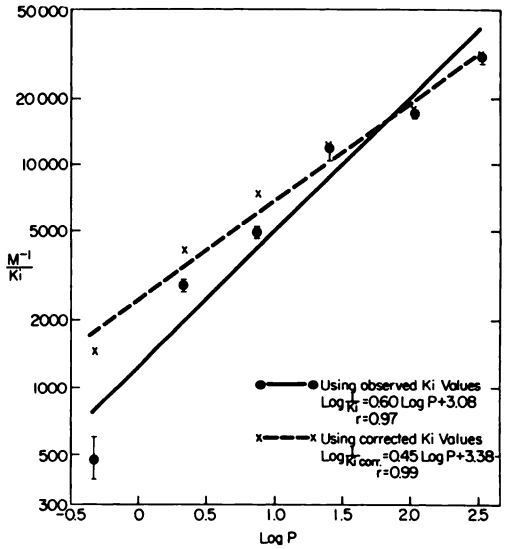

FIG. 5. *Relationship of logarithm of reciprocal corrected and uncorrected K_i values to log P for primary alcohols*

Corrected and uncorrected K_i values were obtained from Table 3. P is the octanol/water partition coefficient obtained from the data of Hansch and colleagues (2, 11).

Primary alcohols ($C_2 \rightarrow C_7$):

$$\log \frac{1}{I_{50\,\mathrm{corr}}} = 0.47 \log P + 2.56, \quad r = 0.99 \tag{7}$$

Secondary alcohols:

$$\log \frac{1}{I_{50}} = 0.35 \log P + 2.48, \quad r = 0.99 \tag{8}$$

The use of corrected I_{50} values did not improve the correlation obtained for the secondary alcohols, possibly because of the high inhibitory potency of 2-propanol. A combination of the data in Tables 4 and 5 for these primary and secondary alcohols, excluding methanol, which appeared to be acting by a different mechanism, gave the following equation:

$$\log \frac{1}{I_{50}} = 0.52 \log P + 2.33, \quad r = 0.95 \tag{9}$$

The excellent correlations observed for primary and secondary alcohols between

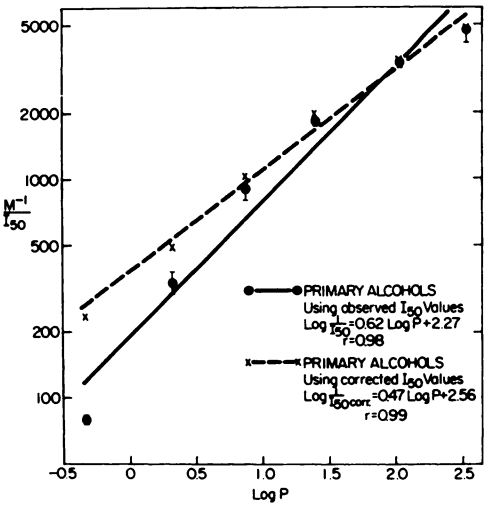

FIG. 6. *Relationship of logarithm of reciprocal corrected and uncorrected I_{50} values to log P for primary alcohols*

Corrected and uncorrected I_{50} values were obtained from Table 4. *P* is the octanol/water partition coefficient obtained from the data of Hansch and colleagues (2, 11).

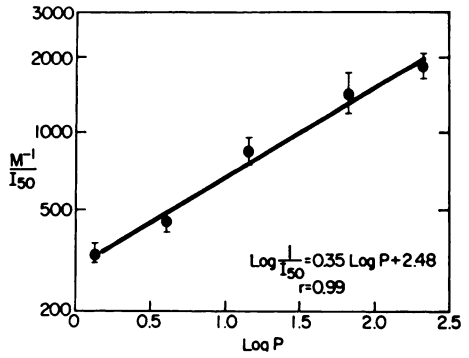

FIG. 7. *Relationship of logarithm of reciprocal I_{50} values to log P for secondary alcohols*

The I_{50} values were obtained from Table 5. *P* is the octanol/water partition coefficient obtained from the data of Hansch and colleagues (2, 11).

inhibitory potency and lipophilicity did not appear to hold when the studies were extended to include other branched-chain alcohols. As may be seen from Table 6, an increased partition coefficient of an alcohol did not increase its inhibitory potency. This was also seen with the series of 5-carbon alcohols, in which no correlation of inhibitory potency and log *P* was observed (Table 7). The most striking example of this disparity was the very weak inhibitory potency of *tert*-pentyl alcohol. *tert*-Butyl alcohol was unique among the 25 alcohols examined because it stimulated rather than inhibited aniline *p*-hydroxylation. In a concentration of 12 mM it caused a 35% stimulation of the rate of *p*-hydroxylation of aniline (0.2 mM).

It was thought that Taft's steric parameter E_s (12), Hancock's corrected steric substituent E_s^c (13), or the polar substituent constant σ^* (12), together with log *P*, might improve some of the weaker correlations obtained with the branched-chain alcohols. E_s constants were originally defined by Taft, using the hydrolysis of aliphatic esters as the model reaction, and thus are probably of limited use in this study. For the 17 alcohols (Tables 4 and 5, 3-pentanol I_{50} = 2.32 mM, neopentanol I_{50} = 4.68 mM, 3-hexanol I_{50} = 2.95 mM, 2-methyl-1-propanol I_{50} = 2.44 mM, and benzyl alcohol I_{50} = 0.48 mM) for which these substituent constants were available, the data were analyzed by use of the Hansch multiple regression program.[4] The following results were obtained:

$$\log \frac{1}{I_{50}} = 0.82(\pm 0.33) \cdot \log P + 1.83(\pm 0.44), \quad (10)$$
$$s = 0.53,\ r = 0.80,\ n = 17$$

$$\log \frac{1}{I_{50}} = 1.50(\pm 0.62) \cdot \log P - 0.36(\pm 0.29) \cdot (\log P)^2 + 1.75(\pm 0.38), \quad (11)$$
$$s = 0.44,\ r = 0.98,\ n = 17$$

$$\log \frac{1}{I_{50}} = 2.09(\pm 0.50) \cdot \log P - 0.58(\pm 0.22) \cdot (\log P)^2 + 0.63(\pm 0.31)E_s + 2.03(\pm 0.29), \quad (12)$$
$$s = 0.29,\ r = 0.95,\ n = 17$$

[4] We thank Dr. Corwin Hansch, one of the reviewers of the manuscript, for his suggestions of the use of E_s^c and σ^* in the analysis of our data and his derivation of Eqs. 10–12.

where s is the standard deviation from the regression, n is the number of experimental points used in deriving the expression, and the figures in parentheses are the 95% confidence intervals. The most significant single-variable equation was that of log P (Eq. 10), whereas the most significant two-variable equation was Eq. 11, indicating that $(\log P)^2$ was the next most important variable. Equation 12 was the most significant three-variable equation, and the inclusion of E_s greatly improved the correlation. Addition of σ^* resulted in a lowering of the variance but was not significant at $\alpha = 0.1$. Similarly, the addition of E_s^c did not result in significant improvement of the data.

Inhibitory effect of butylamine on ethylmorphine and aniline metabolism. Jefcoate *et al.* (23), while studying the type II spectral binding properties of *N*-alkylamines, noted that concentration-dependent spectral changes obeyed two binding constants, K_1 and K_2. A linear correlation was observed between the logarithm of the spectral binding constants and increasing alkylamine chain length. The free energies of binding per methylene group were 0.75 (K_1) and 0.65 (K_2) kcal/mole. This led to their postulation of a hydrophobic spectral binding site in the heme region of cytochrome P-450. A limitation of binding values was reached with alkylamines with more than 8 carbon atoms, and branching of the alkyl chain severely weakened the binding. Their failure to observe spectral binding with *tert*-pentylamine and their observation of the adverse effect of branching on binding, together with our observation of a much lesser inhibitory potency of the branched-chain alcohols, suggest the equivalence of their hydrophobic spectral binding site and our alcohol hydrophobic binding site. Further support for this idea comes from our observation that butylamine resembles the alcohols in its selective inhibition of aniline *p*-hydroxylation. The inhibition was competitive, with a K_i of 0.52 mM, which compares favorably with the K_i of 0.20 mM (Table 3) for the inhibition of aniline *p*-hydroxylation by butanol. Butylamine also resembled the alcohols in its lack of potency as an inhibitor of ethylmorphine metabolism; a 40 mM concentration of butylamine inhibited the *N*-demethylation of ethylmorphine (1 mM) by only 30%.

The lack of binding of alkylamines of chain length greater than 8 carbon atoms, as well as some tendency of the inhibitory potency of the alcohols to fall off at 7 carbon atoms, suggests that the size of the hydrophobic site may be limited to 7 or 8 carbon atoms. The additional studies that would be necessary to support this view are not practical because of the insolubility of the higher alcohols in the aqueous medium.

Use of a fluorescent probe to explore location of hydrophobic site in microsomes. The hydrophobic binding site could reside either in the microsomal membrane or in the microsomal protein. ANS has been used as a fluorescent probe for hydrophobic binding sites of many systems, including erythrocyte membranes, mitochondria, and sarcoplasmic reticulum. Recently DiAugustine *et al.* (10) showed that ANS binds to hepatic microsomes and that it binds to phospholipid rather than to protein or heme. An enhancement of the ANS fluorescence was observed in the presence of the type I compound benzphetamine, and a decrease in the presence of the type II compound warfarin. We were able to confirm these observations. Eling and DiAugustine (24) suggested that ANS fluorescence in microsomes was due to its binding to membrane phospholipids. This conclusion was supported by their observation that digestion of microsomes with phospholipase C or D caused a decrease of ANS fluorescence, which was related to the phospholipid content of the microsomes. ANS fluorescence was also increased by the presence of phospholipid micelles, and the modification of the ANS-microsome and ANS-phospholipid fluorescence by drugs was similar. We observed that aniline (0.2 mM) and ethanol (12 mM), either alone or in combination, did not alter the ANS-enhanced fluorescence of microsomes. This suggests that the hydrophobic binding site revealed by our kinetic studies resides in the protein rather than the phospholipid portion of the microsomal membrane.

DISCUSSION

The marked inhibitory effects of primary alcohols on the metabolism of aniline, a

type II compound, and the relative inability of the same alcohols to inhibit the metabolism of ethylmorphine and other type I compounds are in agreement with the observation of Rubin *et al.* (25) that ethanol selectively inhibited the metabolism of type II compounds. This differential inhibition might be explained in several ways. (a) Alcohols might cause conformational changes which alter the binding of type II compounds, but not that of type I compounds. (b) The type I and type II compounds may cause different conformational changes in either the protein or the lipid of the membrane, after which ethanol can inhibit the metabolism of some type II compounds, but not that of type I compounds. (c) Different enzymes or different sites on the same enzyme may be responsible for the metabolism of Type I and II compounds; only that enzyme or site associated with the metabolism of type II compounds may be susceptible to alcohol inhibition.

With the present evidence it is difficult to decide among these alternatives, but (c) seems more likely than the others. Ethanol and other primary alcohols in molar concentrations give a modified type II difference spectrum with oxidized microsomes. The similarity of the modified type II spectrum to an inverted type I spectrum has led to the speculation that it represents the inverse spectral expression of an unidentified endogenous type I compound associated with microsomes in the reference cuvette, which is made to appear by displacement of the same compound by alcohol in the sample cuvette (26). However, much higher concentrations of alcohols are required to produce these inverse type I spectra than are required for inhibition of aniline *p*-hydroxylation; for example, an I_{50} concentration of ethanol does not affect aniline spectral binding (Table 2), nor does this concentration of ethanol produce changes in the difference spectrum of oxidized microsomes. This tends to eliminate (a), but does not exclude the possibility that ethanol may cause a conformational change that is not expressed spectrally or that the spectral binding site of aniline is not related to the metabolic site. The lack of agreement between the spectral dissociation constant (K_s) and the K_m for aniline (27) provides some evidence for the latter possibility.

That the type I conformational change was not required to prevent the inhibition by ethanol was shown by the failure of ethanol to inhibit the *N*-demethylation of ethylmorphine from microsomes treated with either phospholipase C (15) or SKF 525-A (16). Ethanol (12 mM) and aniline (0.2 mM), either alone or in combination, did not markedly affect ethylmorphine *N*-demethylation. Thus the type II conformational change produced by aniline did not increase the inhibitory effect of ethanol on ethylmorphine *N*-demethylation. These observations tend to rule out (b), leaving (c) the most likely possibility.

Since generally it is those drugs with high lipid solubility which react with the drug-metabolizing system, the active area of cytochrome P-450 has been assumed to be in contact with or embedded in a highly hydrophobic part of cytochrome P-450 protein or in lipids of the microsomal membrane. Since this generalization applies to type I compounds, and since the hydrophobic site, as revealed by our inhibition studies, does not appear to be related to the metabolism of type I compounds, at least two hydrophobic regions must exist in microsomes. This is in agreement with recent findings of Ullrich and Weber (28), who obtained biphasic Lineweaver-Burk plots for the *O*-dealkylation of 7-ethoxycoumarin and observed that of the two apparent dealkylation reactions, the reaction with the higher affinity was much more sensitive to inhibition by 1-hexanol.

Ethyl isocyanide combines with reduced cytochrome P-450 to form Soret peaks at 430 nm and 455 nm (29). The relative heights of these peaks depend on pH and ionic strength. Low concentrations of some alcohols affected the A_{455}:A_{430} ratio of the ethyl isocyanide difference spectrum of cytochrome P-450 without conversion of P-450 to P-420, and the ability of the alcohols to alter the spectrum increased with increasing chain length and hydrophobicity.[5] It was suggested that this was due to an increase

[5] Unpublished results of Y. Imai and H. S. Mason, cited by Imai and Siekevitz (30).

in the hydrophobicity of the environment of cytochrome P-450 by the addition of hydrophobic side chains of the alcohols. The 455 nm peak was also proposed to be specifically related to a hydrophobic interaction of heme with the environment within the membrane. We found that the I_{50} concentrations of ethanol (12 mM) and aniline (0.2 mM), either alone or in combination, did not affect either the 455 or 430 nm peak heights produced by ethyl isocyanide binding to dithionite-reduced microsomes. This suggests that the hydrophobic binding site in our studies is not associated with the hydrophobic interaction of the heme with its membrane environment, as was suggested by the ethyl isocyanide spectrum. However, the possibility cannot be excluded either that the measurement of aniline *p*-hydroxylation is a more sensitive index of the interaction of heme with its environment or that the affinity of ethyl isocyanide for this site is much greater than that of ethanol. The latter possibility seems unlikely, because higher alcohols did affect the A_{455}:A_{430} ratio (30).

The observation that a concentration of ethanol (12 mM) which caused 50% inhibition of aniline *p*-hydroxylation (0.2 mM) had no effect on NADPH oxidase, NADPH–cytochrome *c* reductase, NADPH–cytochrome P-450 reductase, or aniline spectral binding suggests that the rate-limiting step of aniline *p*-hydroxylation is at some stage beyond the reduction of cytochrome P-450, possibly at the addition of the second electron. Aniline and other type II compounds have been shown by Gigon *et al.* (6) to slow the rate of reduction of cytochrome P-450. However, the concentration of aniline (2 mM) used in their experiments was much greater than those used in our metabolism studies, namely, 0.04–0.2 mM, at which concentrations no effect on NADPH–cytochrome P-450 reductase activity was observed (Table 2).

The competitive nature of the alcohol inhibition (Fig. 1) and the low concentrations required (Table 3) suggest a specific action of the alcohols rather than a nonspecific action due to protein denaturation or membrane perturbation. The specificity of the reaction was further shown by equations derived from plots of log $(1/K_i)$ and log $(1/I_{50})$ against log P. Hansch and Dunn (2) showed that many systems fit the general Eq. 1, where C is the molar concentration of drug or, in our case, the molar I_{50} concentration. The slope a (Eq. 1) is a measure of the sensitivity of the system to perturbation by hydrophobic effects of the drugs. Hansch and Dunn (2) grouped the slopes in three classes. (a) Equations with slopes greater than 0.85 describe those systems most sensitive to hydrophobic effects. In most all these systems the biological response appears to be mediated through membranes. Examples are the partitioning of alcohols between red cell ghosts and water and the lysis of protoplasts. (b) Equations with slopes in the range of 0.40–0.85 describe systems of intermediate hydrophobic sensitivity. A pertinent example is the conversion of cytochrome P-450 to cytocheome P-420 by aniline or phenols (Table 8). (c) Equations with slopes less than 0.4 describe systems which are relatively insensitive to hydrophobic effects. One of the few examples of these systems is the denaturation of cytochrome *c* by alcohols.

The inhibition of aniline *p*-hydroxylation by primary alcohols falls into the category of intermediate hydrophobic sensitivity as determined by Eqs. 4–6; this agrees with our conclusions regarding the hydrophobic nature of the alcohol-binding site from the calculation of the change of free energy of inter-

TABLE 8

Drug-microsome interactions: conversion of cytochrome P-450 to P-420 and inhibition of aniline p-hydroxylation by primary alcohols

	Equation
Phenols[a]	$\log \frac{1}{C} = 0.57 \log P + 0.36$
Aniline[a]	$\log \frac{1}{C} = 0.67 \log P + 0.34$
Inhibition of aniline[b]	$\log \frac{1}{K_i} = 0.60 \log P + 3.08$

[a] Data taken from Ichikawa and Yamano as calculated by Hansch and Dunn (2) for the conversion of cytochrome P-450 to P-420.

[b] Equation obtained from Fig. 5.

action. The slope of 0.35 obtained for the secondary alcohols suggests a system of low hydrophobic sensitivity. This may mean that the secondary alcohols do not fit the hydrophobic site as well as the primary alcohols. However, the slope is lowered considerably by the inclusion in the series of 2-propanol, which exhibits an exceptionally high inhibitory potency, and of 2-heptanol, with which there appears to be some falling off of activity. The high inhibitory potency of 2-propanol also explains why the use of corrected I_{50} values for the secondary alcohols does not improve the correlation.

The value of the intercept b (Eq. 1) is a function of the sensitivity of the biochemical system and the intrinsic activity of a given set of congeners. It also will depend in part on the level of standard response used in the experiment; for example, if an I_{75} had been used rather than an I_{50}, a lower intercept would have been obtained. The intercepts permit comparisons between different sets of congeners acting on different systems. A comparison of intercepts means a comparison under isolipophilic conditions because, when $\log P = 0$, $P = 1$. Table 8 shows a comparison of some of our data with those of Ichikawa and Yamano for the conversion of P-450 to P-420 as calculated by Hansch and Dunn (2). Their intercepts of 0.34 and 0.36 characterize the conversion of P-450 to P-420 as a nonspecific process involving the perturbation of macromolecules by organic compounds. The very high intercept of about 3 found for the inhibition of aniline *p*-hydroxylation suggests a system of very high sensitivity. The increased sensitivity also implies increased specificity. In general, equations correlating simple neutral compounds do not yield intercepts above 2. This system appears to be one of the most sensitive ascribed to alcohol inhibition. The only example given by Hansch and Dunn (2) of a biological system more sensitive to alcohols is the I_{25} for sheep liver esterase, which has an intercept of 3.69. High intercepts are also obtained when alcohols are given in the vapor phase, as was shown in studies of the toxicity of alcohol vapors to tomato plants and red spiders.

Extremely good correlation of $\log (1/K_i)$ against both increasing carbon chain length and $\log P$ was observed, despite the fact that the values for ethanol fit the plots poorly (Figs. 2 and 5). This deviation of ethanol from the pattern established with the higher alcohols suggested that the increase in inhibitory effect seen with increasing chain length might simply reflect the partition of the alcohols between membrane lipid and the aqueous medium as determined by the P values of the alcohols, in which case one would not require a hydrophobic site on the enzyme to explain the inhibitory effect; all the alcohols would exhibit an identical inhibitory effect at a given molar concentration of alcohol in the lipid of the membrane. Accordingly, ethanol, with the lowest P value, would be expected to have the $\log (1/K_i)$ value most deviant from the linear decline in $\log (1/K_i)$ values observed with increasing chain length. Although the energy relationships and inhibition kinetics argue for a more specific hydrophobic site than one which simply reflects the partition between the medium and the membrane lipid, it seemed that a more accurate assessment of the hydrophobic site might be obtained if the suspended microsome were visualized as a three- rather than a two-compartment system, with one equilibrium of the drug established between the medium and the membrane lipid and another between the membrane lipid and the enzyme. This would seem a reasonable model because cytochrome P-450, the terminal oxidase of the enzyme system, is thought to be thoroughly embedded in the lipid membrane. This has been deduced from the dependence of membrane-bound cytochrome P-450 on the membrane for certain of its spectral and biochemical properties and from the difficulties that have been experienced in attempts to solubilize the cytochrome in a form that retains all of its native characteristics (18). The corrected K_i and I_{50} values calculated according to Eq. 3 represent the concentration of the alcohol in the lipid phase of the microsomes, assuming a single partition between the aqueous medium and the membrane lipid. The largest differences between corrected and observed K_i and I_{50} values occur with the lowest members of the series, particularly with ethanol (Tables 3 and 4). A plot of $\log (1/K_{i\ corr})$

or log ($1/I_{50\ corr}$) against increasing carbon chain length and log P now gives straight lines with slightly decreased slopes which pass through all the points, including that representing ethanol (Figs. 2, 3, 5, and 6). The slopes obtained using corrected K_i values (Figs. 2 and 5) show that the increasing carbon chain length of the alcohol is responsible for the inhibition and not simply that the increase in carbon chain length causes an increased concentration of the alcohol in the lipid phase of the membrane. In other words, the inhibitory effect results from an interaction of the alcohol with the enzyme rather than from the accumulation of equimolar amounts of the alcohols in the lipid phase of the membrane.

The very low activity of methanol as an inhibitor of aniline *p*-hydroxylation is striking. The binding energy of methanol relative to ethanol is 2.8 kcal/mole (Table 4). This is typical of the value expected for hydrogen bonding (21) and suggests that the first requirement for inhibition of aniline *p*-hydroxylation is hydrogen bond formation between the hydroxyl group of the alcohol and some site on the enzyme, conceivably the site where the amino group of aniline might bind. The *tert*-pentyl alcohol is also a very poor inhibitor of aniline *p*-hydroxylation (Table 7), probably because hydrogen bond formation is sterically hindered by the methyl groups. The wide differences between methanol and *tert*-pentyl alcohol in their lipid solubilities and in their potentials for hydrogen bonding suggest that methanol is a poor inhibitor even though it is capable of hydrogen bonding, because it is lacking in lipophilicity, whereas *tert*-pentyl alcohol fails as an inhibitor even though it is highly lipophilic, because it is lacking in hydrogen-bonding capability. Once the hydrogen bond has formed, hydrophobic interactions assume importance. This is clearly shown by the increasing inhibitory potency of the primary alcohols with increasing carbon chain length. The $\Delta\Delta F$ per methylene group of 0.48 kcal/mole is typical of a hydrophobic interaction between the alcohol and some nonpolar site on the enzyme.

Except for 2-propanol, the secondary alcohols are all less potent than corresponding primary alcohols of the same carbon chain length (compare Tables 4 and 5). Thus it appears that the methyl group attached to the carbon bearing the hydroxyl group does not bind to the enzyme, or that this methyl group interacts with the protein, causing a conformational change such that the rest of the alcohol can no longer interact as effectively with the enzyme. The results with pentanol, 2-pentanol, and 3-pentanol support the first of these hypotheses. 2-Pentanol had an activity similar to that of butanol; 3-pentanol was less active than 2-pentanol, but more active than propanol (Tables 4 and 7). Branching of the alkyl side chain severely hinders the inhibitory potency of the alcohol, although log P is increased (Tables 6 and 7). This could be due to interference with hydrogen bond formation between the hydroxyl group of the alcohol and the enzyme or to prevention of the hydrophobic interaction. The former appears to be the case with *tert*-pentyl alcohol, but with the other alcohols it is difficult to choose among these alternatives. The importance of the hydrophobic nature of the site was further demonstrated by the very weak inhibitory action of 1,3-propanediol (I_{50} = 73.4 mM) and 1,4-butanediol (I_{50} = 25.8 mM) on aniline *p*-hydroxylation.

For the 17 alcohols for which the substituent constants E_s, E_s^c, σ^*, and log P were available, Eq. 10, using log P, gave the most significant single-variable equation, stressing the importance of lipophilicity in the alcohol inhibition of aniline *p*-hydroxylation. The fact that the most significant two-variable equation was that containing $(\log P)^2$ indicated that parabolic dependence on log P as predicted by the random walk process was very important ($\log P_o$ = 2.1). A parabolic dependence of inhibitory potency on P with a P_o of 2.1 would offer an alternative explanation for the lower activity of both ethanol and heptanol ($\log P$ = 2.53). However, for the 23 alcohols for which both P values and inhibition data were available, addition of a $(\log P)^2$ term did not significantly improve the correlation over log P alone.

Equation 12, containing an E_s term, was the most significant three-variable equation; E_s greatly improved the correlation. The significance of the E_s term supports our

earlier suggestion of steric interference by the alkyl chain of the alcohol either with hydrogen bond formation between the hydroxyl group of the alcohol and the enzyme or with prevention of the hydrophobic binding.

ACKNOWLEDGMENTS

We should like to thank Virginia R. Kickertz for technical assistance, and Dr. Patrick E. Hanna for his helpful views on structure-activity relationships.

REFERENCES

1. C. Hansch, A. R. Steward, J. Iwasa, and E. W. Deutsch, *Mol. Pharmacol.* **1**, 205–213 (1965).
2. C. Hansch and W. J. Dunn, *J. Pharm. Sci.* **61**, 1–19 (1972).
3. C. Hansch, *in* "Drug Design" (E. J. Ariëns, ed.), Ch. 2. Academic Press, New York, 1971.
4. M. W. Anders and G. J. Mannering, *Mol. Pharmacol.* **2**, 319–327 (1966).
5. H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper, and O. Rosenthal, *Mol. Pharmacol.* **2**, 187–190 (1966).
6. P. L. Gigon, T. E. Gram, and J. R. Gillette, *Mol. Pharmacol.* **5**, 109–122 (1969).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265–275 (1951).
8. Y. Imai, A. Ito, and R. Sato, *J. Biochem.* (*Tokyo*) **60**, 417–428 (1966).
9. T. Shimazu, *Biochim. Biophys. Acta* **105**, 377–380 (1965).
10. R. P. DiAugustine, T. E. Eling, and J. R. Fouts, *Chem.-Biol. Interactions* **2**, 17–27 (1970).
11. A. Leo, C. Hansch, and D. Elkins, *Chem. Rev.* **71**, 525–616 (1971).
12. J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions." Wiley, New York, 1963.
13. C. K. Hancock, E. A. Meyers, and B. J. Yager, *J. Amer. Chem. Soc.* **83**, 4211–4213 (1961).
14. G. N. Wilkinson, *Biochem. J.* **80**, 324–332 (1961).
15. M. D. Chaplin and G. J. Mannering, *Mol. Pharmacol.* **6**, 631–640 (1970).
16. G. J. Mannering, in "Concepts in Biochemical Pharmacology," "Handbook of Experimental Pharmacology" (B. B. Brodie and J. Gillette, eds.), Vol. XXVIII/2, p. 465. Springer, New York, 1971.
17. J. R. Gillette, *Advan. Pharmacol.* **4**, 219–261 (1966).
18. G. J. Mannering, *in* "Fundamentals of Drug Metabolism and Disposition" (B. N. La Du, H. G. Mandel, and E. L. Way, eds.), Ch. 12. Williams & Wilkins, Baltimore, 1971.
19. G. Némethy and H. A. Scheraga, *J. Phys. Chem.* **66**, 1773–1789 (1962).
20. B. M. Anderson, M. J. Reynolds, and C. D. Anderson, *Biochim. Biophys. Acta* **99**, 46–55 (1965).
21. J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. I, Ch. 6. Academic Press, New York, 1963.
22. R. Kato, A. Takanaka, and H. Shoji, *Jap. J. Pharmacol.* **19**, 315–322 (1969).
23. C. R. E. Jefcoate, J. L. Gaylor, and R. L. Calabrese, *Biochemistry* **8**, 3455–3463 (1969).
24. T. E. Eling and R. P. DiAugustine, *Biochem. J.* **123**, 539–549 (1971).
25. E. Rubin, H. Gang, P. S. Misra, and C. S. Lieber, *Amer. J. Med.* **49**, 801–806 (1970).
26. H. Diehl, J. Schädelin, and V. Ullrich, *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1359–1371 (1970).
27. J. B. Schenkman, H. Remmer, and R. W. Estabrook, *Mol. Pharmacol.* **3**, 113–123 (1967).
28. V. Ullrich and P. Weber, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1171–1177 (1972).
29. Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.* **23**, 5–11 (1966).
30. Y. Imai and P. Siekevitz, *Arch. Biochem. Biophys.* **144**, 143–159 (1971).