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Influence of Hydrophobic Character on the Relative Rate of Oxidation of Drugs by Rat Liver Microsomes

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The relative Michaelis constant (K_m) of a series of 14 drugs for the mixed-function oxidases of rat liver microsomes increases with an increase in the octanol–water partition coefficient of the drug. For ionized drugs a correction for ionization improved the results. In a series of dimethylanilines there was no correlation of K_m with σ or σ^+.

The relative rate of oxidative metabolism of drugs has long been considered to be related to the relative lipophilic character of the molecule. Although the correlation was not analyzed statistically, Gaudette and Brodie presented evidence that the relative rates of demethylation of a series of a variety of drugs was related to their $CHCl_3$–pH 7.4 buffer partition coefficients.[1] Mazel and Henderson have reported similar studies with the opposite conclusion: in a series of 14 compounds there was no relationship between the extent of N-demethylation and $CHCl_3$–pH 7.4 buffer partition coefficient.[2] In neither of these studies was a distinction made between structure–activity relationships in substrate binding to the enzymes and in the maximal velocity.

In another study, Hansch and coworkers[3] showed that the relative rates of demethylation of a series of aliphatic tertiary amines originally studied by McMahon was indeed related to the partition coefficient and that the data could be expressed by eq 1.[3] In this

$$\log BR = 0.470 \log P - 0.268 (pK_a - 9.5) - 1.305$$
$$n = 18 \; r = 0.890 \; s = 0.222 \qquad (1)$$

equation n is the number of points used in the regression analysis, r is the multiple correlation coefficient, and s is the standard deviation. The biological response, BR, is the relative rate of *in vitro* demethylation by rat microsomes, P is the octanol–H_2O partition coefficient, and K_a is the ionization constant. Both the partition coefficient and the pK_a were statistically significant predictors of the relative rate of demethylation.

(1) L. E. Gaudette and B. B. Brodie, *Biochem. Pharmacol.*, **2**, 89 (1959).
(2) P. Mazel and J. Henderson, *ibid.*, **14**, 92 (1965).
(3) C. Hansch, A. R. Steward, and J. Iwasa, *J. Med. Chem.*, **8**, 868 (1965).

It was thus of interest to extend such studies to include a wider variety of molecules and to expand the reactions studied to several of the oxidations catalyzed by the NADPH-dependent, mixed-function oxidases of liver microsomes.

Experimental Section

Enzyme Activity.—Adult male rats were pretreated with 100 mg/kg of phenobarbital ip daily for 5 days. After 18-hr starvation the liver microsomes were isolated[4] and stored frozen at −20° until use. The rate of oxidation of the drugs by liver microsomes (0.5 mg of protein) was studied in pH 7.4 phosphate buffer at 37° by following the rate of disappearance of 10^{-3} M NADPH spectrophotometrically.[5] This system was chosen because it is not necessary to add other commonly used cofactors such as nicotinamide or $MgCl_2$ and because the system is relatively free from extraneous protein. The advantages of such a system are evident from the reports that nicotinamide[6] and protein[7] can interfere with the reaction. Only those substrates for which the rate of NADPH consumption was double that of the endogenous rate were considered. The K_m's and V_{max}'s were calcd from the data with a FORTRAN program which incorporates the statistical treatment of Wilkinson.[8]

Partition Coefficients.—The partition coefficients in Table I marked by an asterisk are experimental values. The others are calcd values. The derivs of $PhNMe_2$ were calcd by adding π_x from the aniline[9] series to log P for $PhNMe_2$. Log P for N,N-dimethyl-β-naphthylamine was calcd by adding $\pi_{N(CH_3)_2}$ of 0.18 from the benzene system to log P of naphthalene (3.37). Log P for codeine was calcd from log P (0.76) for morphine. To this was added: π = log $P_{anisole}$ − log P_{phenol} = 2.11 − 1.46 =

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(5) Y. C. Martin, *Biochem. Pharmacol.*, **16**, 2041 (1967).
(6) J. B. Schenkman and R. W. Estabrook, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **26**, 729 (1967).
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TABLE I

MICHAELIS CONSTANT AND V_{max} FOR VARIOUS SUBSTRATES OF RAT MICROSOMAL NADPH OXIDASE

No.	Compd	Log P	pK_a	Log fraction not ionized	Log K_m, M Obsd	Log K_m, M Calcd[d]	V_{max} nmoles/min per mg of protein	Log V_{max}
1	N,N-Dimethyl-β-naphthylamine	3.55			5.63	5.38	172	2.236
2	m-Chloro-N,N-dimethylaniline	3.29			4.94	5.19	109	2.037
3	m-Methyl-N,N-dimethylaniline	2.81			4.73	4.84	220	2.342
4	p-Methyl-N,N-dimethylaniline	2.80			4.70	4.84	177	2.230
5	Pentobarbital	2.07*	7.68[a]	0.19	4.48	4.15	185	2.267
6	Hexobarbital	1.49*	7.90[a]	0.13	4.22	3.76	311	2.493
7	N,N-Dimethylaniline	2.31*			4.19	4.47	195	2.290
8	Codeine	1.41	7.95[b]	0.66	3.36	3.31	376	2.575
9	p-Amino-N,N-dimethylaniline	1.08			3.87	3.56	151	2.179
10	m-Amino-N,N-dimethylaniline	1.08			3.85	3.56	136	2.134
11	Ephedrine	0.93*	9.21[c]	1.81	1.97	2.11	569	2.755
12	Barbital	0.65*	7.46[a]	0.43	2.77	3.07	161	2.207
13	Physostigmine	1.05*	6.12[b]	0	2.94	3.53	251	2.400
14	Caffeine	−0.07*			2.86	2.71	298	2.474

[a] M. E. Krahl, *J. Phys. Chem.*, **44**, 449 (1940). [b] Merck Index, 8th ed, Paul G. Stecher, Ed., Merck and Co., Rahway, N. J., 1968. [c] L. G. Sillen and A. E. Martell, *Chem. Soc. Spec. Publ.*, 17 (1964). [d] From eq 4, Table II.

0.65. Log P for $PhCH_2NMe_2$ was calcd from log P (1.57) for $PhCH_2NHMe$. To this was added: π = log P_{Me_3N} − log P_{Me_2NH} = 0.57.

Results

The results of the enzymatic studies as well as the partition coefficients of the molecules are in Table I. It can be seen that the most striking variation within this series is the difference in K_m between substrates. The variation in V_{max} is not suitable for regression analysis since experimental error could be a substantial fraction of the variation between substrates. However, the exception to this is ephedrine, for which the V_{max} is very much higher than that of the other substrates. Since ephedrine is a benzyl alcohol this observation supports the recent suggestion[10] that benzyl compounds often exhibit unusually high activity in reactions with radical enzymes, which the microsomal enzymes could be.[3,11]

The K_m values determined by this method agree with those previously published for codeine.[12,13] Gaudette and Brodie also observed that whereas caffeine is demethylated, theophylline is not.[1]

Equation 2 (Table II) expresses the relationship between the pK_m and log P. It can be seen that log P is indeed a statistically significant predictor of pK_m. However, the compounds investigated could be subdivided into two categories: the first, those which are not ionized at the pH of assay, and the second, those which are partially ionized at this pH. For the nonionized drugs eq 4 was calcd. Thus a substantially better correlation was seen with this subgroup than with the total list of compounds. For the drugs which are partially ionized at pH 7.4 the logarithm of the fraction not ionized was calcd.[14] If one postulates that only the uncharged species of drug is a substrate for the microsomal enzymes then this correction would be applied to the pK_m. It would be increased so that the pK_m now represented the concn of unionized substrate at half-maximal velocity. Equation 6 was calcd on this basis; it represents a slight improvement over the uncorrected case, eq 5. Finally, one may then include all drugs in equations with corrections; eq 3 results.

TABLE II

EQUATIONS WHICH RELATE pK_m TO PHYSICAL PROPERTIES

	n	r	F	s	Eq
All Compounds					
pK_m = 2.460(±0.58) + 0.821(±0.29) · log P	14	0.874	39.1	0.508	2
Corrected pK_m = 2.900(±0.38) + 0.693 · (±0.19) log P	14	0.920	66.1	0.330	3
Nonionized Drugs					
pK_m = 2.996(±0.40) + 0.641(±0.17) · log P	8	0.967	87.9	0.230	4
Drugs Partially Ionized at pH 7.4					
pK_m = 1.259(±1.8) + 1.604(±0.14) · log P	6	0.855	10.9	0.546	5
Corrected pK_m = 2.24(±1.29) + 1.226 (±0.96) log P	6	0.871	12.6	0.387	6
Dimethylanilines					
pK_m = 3.295(±0.40) + 0.487(±0.17) · log P	6	0.971	65.61	0.126	7
pK_m = 4.494(±0.46) + 0.995(±1.41)σ	6	0.700	3.85	0.377	8
pK_m = 3.193(±0.84) + 0.525(±0.33) · log P − 0.143(±0.93)σ	6	0.973	53.35	0.141	9

The quality of these correlations is especially impressive when one considers that the reactions catalyzed by the microsomes in this study include N-demethylation of substituted dimethylaminoanilines, O- and N-demethylation of complex heterocyclic compounds, and hydroxylation of aliphatic side chains. The high correlation of pK_m with log P reinforces the earlier conclusions that steric factors are of little importance in the oxidation of drugs by liver microsomes.[3]

The dimethylanilines were included in the study in order to investigate the contribution of electronic effects to the K_m or V_{max} of the reactions. Those studies were specifically chosen to include a range of σ values and low correlation between log P and σ. Equations 7–9 in Table II summarize the results of these studies. Equation 9 was not a significantly

(10) C. Hansch and R. Kerley, *J. Med. Chem.*, **13**, 957 (1970).
(11) C. Hansch, *ibid.*, **11**, 920 (1968).
(12) J. B. Schenkman, J. A. Ball, and R. W. Estabrook, *Biochem. Pharmacol.*, **16**, 1071 (1967).
(13) A. Rubin, T. R. Tephly, and G. N. Mannering, *ibid.*, **13**, 1007 (1964).
(14) A. Albert, "Selective Toxicity," Methuen & Co. Ltd., London, 1965, pp 346–347.

better predictor of pK_m than eq 7. The use of σ^+ did not improve the correlation. Thus it was not possible to demonstrate any dependence of the reaction on electronic effects.

The demonstration of a decrease in K_m with an increase in log P is especially interesting in view of the recent studies by Lu, *et al.*,[15] on the subfractionation of the microsomal NADPH oxidase system. These workers have separated the microsomes into 3 components which are necessary for oxidation: (1) a NADPH-dependent reductase, (2) cytochrome P450 (which binds the substrates), and (3) a heat-stable, lipid fraction. The lipid fraction is not necessary for substrate *binding* but is necessary for *oxidation* of the substrate.

It seems likely that the increase in pK_m with increasing log P can be translated to *in vivo* rate and/or extent of metabolism. For example, it is known that pentobarbital is extensively and rather quickly metabolized whereas barbital is largely excerted unchanged.[16]

These structure–activity relationships observed with the nonspecific microsomal oxidases are different from those previously reported for three other common pathways of drug metabolism; oxidative deamination by monoamine oxidase and the formation of glucuronide and glycine conjugates.[17] For these reactions there was evidence of an optimum log P of approximately 2. Table I includes compounds with log P values ranging from −0.07 to 3.64; no evidence of an optimum log P is seen. In the cases of oxidative deamination and glucuronide formation there was also evidence of a steric requirement for maximal oxidation. As noted above no such effect was seen with the microsomal oxidases.

The relationships discussed above may help explain why it is not always possible to predict *in vivo* pharmacological activity of a series of drugs from *in vitro* studies. For example, *in vitro* there may be no dependence of activity on log P. Thus two drugs might appear equally active *in vitro* but differ dramatically *in vivo* when the difference in metabolic rates must also be considered. The medicinal chemist should remember that it may be possible to alter the rate of metabolism of a "lead" compound by synthesizing a derivative with a different partition coefficient.

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(17) C. Hansch, E. Lien, and F. Helmer, *Arch. Biochem. Biophys.*, **128**, 319 (1968).

Analogs of Bradykinin Containing β-2-Thienyl-L-alanine[1]

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Three analogs of bradykinin containing β-2-thienyl-L-alanine in place of Phe were synthesized by the solid phase method: [5-thienylalanine]-, [8-thienylalanine]-, and [5,8-bis(thienylalanine)]bradykinin. These analogs were more active than bradykinin in the rat uterus and rat blood pressure assays.

The problem of finding a generally applicable rule for the synthesis of antimetabolites of peptide hormones is as yet unsolved. Many structural analogs of these hormones have been synthesized, but the relatively few examples of antihormonal activity found do not seem to exemplify any general rule for structural modification of the parent compounds for the purpose of producing inhibitors. Among the analogs of bradykinin described,[2] there are only a few peptides with any antibradykinin activity. The biological properties of these peptides leave much to be desired for a good antibradykinin.

One logical approach to the synthesis of peptide antimetabolites would be the incorporation into peptides of amino acids which in other systems have been found to be antimetabolites of the amino acids they replace. This approach was followed by Nicolaides, *et al.*,[3] who synthesized [8-*p*-fluoro-L-phenylalanine]-bradykinin, but they found it to have only bradykinin-like activity; indeed, its activity was higher than that of bradykinin itself. A likely candidate for replacement of phenylalanine would be β-(2-thienyl)alanine, which has long been recognized as having antiphenylalanine activity in both microbial and mammalian systems.[4,5] β-(2-Thienyl)alanine has been used in the synthesis of several di- and oligopeptides.[6–9] Dunn and coworkers have shown that under their testing conditions most thienylalanine-containing peptides are much more potent inhibitors of bacterial growth than is

(1) This work was aided by U. S. Public Health Service Grant HE12325 and Population Council Grant M70.64C (JMS) and a grant from The Robert A. Welch Foundation of Houston, Texas (FWD). All optically active amino acid residues are of the L configuration. Abbreviations used are: Boc = *tert*-butyloxycarbonyl; Thi = β-(2-thienyl)alanine; TFA = trifluoroacetic acid.

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