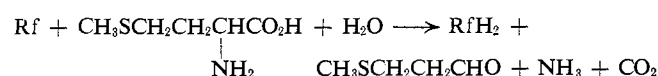
Table IV. Anaerobically Irradiated[a] Methionine–Riboflavin Solutions[b]

Expt.	Riboflavin photoreduced,[c] %	Molar ratio methional/ reduced riboflavin
1	88.3	1.02
2	89.3	1.06
3	87.7	1.01
4	90.0	1.04

[a] Irradiation duration 3 min.; conditions described in section on Anaerobic Irradiation. [b] Solutions: 0.3 mM in riboflavin, 6.0 mM in methionine, 0.1 M in KCl, 0.1 M in phosphate, pH 7.8. [c] Solutions were analyzed polarographically at pH 7.8. There was no polarographic evidence of any photodegradation of riboflavin. All of the riboflavin was recovered on oxidation with air.

nine by riboflavin in air-free solutions appears to be



$$\mathrm{Rf} + \mathrm{CH_3SCH_2CH_2CH(NH_2)CO_2H} + \mathrm{H_2O} \longrightarrow \mathrm{RfH_2} + \mathrm{CH_3SCH_2CH_2CHO} + \mathrm{NH_3} + \mathrm{CO_2}$$

As in the case of the EDTA–riboflavin reaction the stoichiometry suggests that water is the source of the oxygen for the aldehyde formation.

It is not known why Patton[15] and we appear to obtain methional and Nickerson and Strauss[4] methionine sulfoxide. Since the polarographic technique does not detect methionine sulfoxide in the methionine–riboflavin solutions, there is no positive evidence that it was not produced. However, under our experimental conditions where about 1 mole of methional is obtained per mole of riboflavin reduced, it seems that very little, if any, methionine sulfoxide is formed.

It is interesting to note that the behavior of methionine is unusual in that it is easily photooxidized by riboflavin, whereas primary amines in general are not.[5] The unusual behavior is probably due to the presence of the sulfur atom, but its role in this reaction is not understood.

Acknowledgments. This work was supported by research funds of the Department of Chemical Engineering, University of Waterloo, and by a grant from the Advisory Committee on Scientific Research, University of Toronto.

The Role of Substituents in the Hydrophobic Bonding of Phenols by Serum and Mitochondrial Proteins

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The adsorption of 19 phenols by bovine serum albumin was studied. It was found that binding depends on the lipophilic character of the substituent and that a linear free-energy relationship exists between the logarithm of the binding constants and substituent π. It was also shown that the hydrophobic binding constant π can be used to study the mechanism of the uncoupling of phosphorylation in mitochondria.

Introduction

The binding of organic compounds by proteins and, in particular, serum albumin has received extensive attention from theoretical as well as practical points of view.[1–3] In our own work[4] on the analysis of the activity of a series of penicillin derivatives we came to the conclusion that the adsorption of the penicillins to serum albumin could be rationalized using substituent constants. In particular, it was shown that using a substituent constant, π, to estimate the hydrophobic bonding of functions such as Cl, NO_2, etc., the activity of the penicillins could be explained in terms of their binding capacity for serum albumin. We have defined[5,6] π as $\pi = \log P_X/P_H$, where P_H is the partition coefficient of a parent compound between octanol and water and P_X is that of the derivative X. Thus, π is the logarithm of the partition coefficient of a function and, as such, represents the free energy of transfer of the substituent from an aqueous phase to a lipophilic phase.

More recently we have found[7] that using π and the Hammett constant σ it is possible to rationalize the highly specific binding of a congeneric series of substrates to enzymes. In fact, π appears to be an extremely useful parameter for many biochemical and pharmacological problems.[8]

The purpose of the work in this report was to investigate a simpler system than the above mentioned in which more careful control of the variables would be possible. For this purpose we chose to study the adsorption of phenols by bovine serum albumin (BSA). Phenols were chosen because of their well-known tendency to bind to protein, because of their ease of spectrophotometric determination, and because an extensive series of π constants are available.[5] The adsorption of 19 monosubstituted phenols by BSA was measured using the equilibrium dialysis technique of Klotz.[9]

(1) J. T. Edsall, *Advan. Protein Chem.*, **3**, 463 (1947).
(2) W. Scholtan, *Arzneimittel-Forsch.*, **14**, 1234 (1964).
(3) L. Pauling and H. A. Itano, "Molecular Structure and Biological Specificity," American Institute of Biological Sciences, Washington, D. C., 1957, p. 91.
(4) C. Hansch and A. R. Steward, *J. Med. Chem.*, **7**, 691 (1964).
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(7) C. Hansch, E. W. Deutsch, and R. N. Smith, *J. Am. Chem. Soc.*, **87**, 2738 (1965).
(8) C. Hansch, A. R. Steward, and J. Iwasa, *Mol. Pharmacol.*, **1**, 87 (1965).
(9) D. Glick, *Methods Biochem. Analy.*, **3**, 265 (1956).

Experimental Section

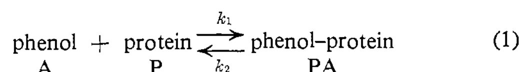
Electrophoretically pure, crystalline BSA (grade A) was obtained from Pentex Co., Kankakee, Ill. The phenols were commercial products purified by recrystallization or distillation. Their ultraviolet spectra served as purity controls.

In each experiment 10 ml. of protein stock solution (450 mg./250 ml., 2.5×10^{-5} M) in a dialysis bag was placed in 10 ml. of phenol solution in specially made, ground-glass-stoppered tubes. These were placed horizontally in a rack and gently shaken in a constant temperature room (3–5°). Experience showed that equilibrium was reached in 20 hr. The amount of phenol outside the bag was then determined by means of a Cary Model 14 spectrophotometer. Separate determinations on the amount of phenol adsorbed by the bag were made. For each phenol, runs were made at three to six concentrations to find r (r = moles of bound phenol/moles of albumin). These points lay in the range r = 0.2 to 5.0. In this range a plot of r against C gave a straight line for each of the phenols. From this plot the concentration of phenol necessary to produce a one-to-one complex of phenol and protein was determined. We have assumed a value of 69,000 for the molecular weight of the protein. It was necessary to correct for the fact that the commercial protein contained about 3.4% water.

The binding of the phenols in both water (doubly distilled water was used throughout) and in phosphate buffer was determined. The buffer was 0.035 M in KH_2PO_4 and 0.035 M in $NaHPO_4$. Its pH was 6.9.

Results and Discussion

In Figure 1 we have plotted log $1/C$ against π. C is the molar concentration of phenol necessary to produce a one-to-one complex of phenol and protein, π is a measure of the lipophilic character of the substituent, and $1/C$ is related to the binding constant as follows.



$$\underset{A}{\text{phenol}} + \underset{P}{\text{protein}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{PA}{\text{phenol–protein}} \quad (1)$$

If we take k to represent the association or binding constant and proceed according to Edsall and Wyman,[10] it can be shown that

$$\frac{\bar{\nu}}{n - \bar{\nu}} = k(\text{A}) \quad \text{or} \quad \frac{1}{(\text{A})} = k\frac{n - \bar{\nu}}{\bar{\nu}} \quad (2)$$

In the above equations $\bar{\nu}$ is the average number of molecules of phenol bound per molecule of protein and n is the total number of binding sites. In our work, $\bar{\nu}$ is 1 and n is a constant. Thus, $1/C$ is directly related to the binding constant.

The plot of log $1/C$ against π in Figure 1 is a good example of a Hammett-type linear free-energy relationship. The quality of the fit is illustrated in eq. 3 which was derived by the method of least squares from the data in Table I.

$$\log 1/C = 0.681\pi + 3.483 \qquad \begin{matrix} n & r & s \\ 19 & 0.962 & 0.133 \end{matrix} \quad (3)$$

In the above equation n is the number of points used in the regression, r is the correlation coefficient, and s is the standard deviation.

(10) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press Inc., New York, N. Y., p. 610.

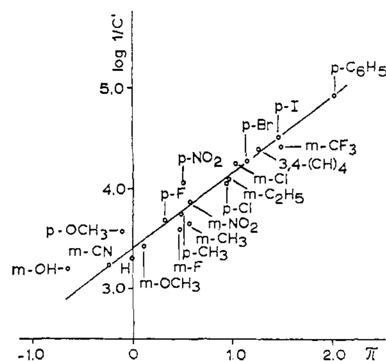


Figure 1.

A number of important conclusions can be drawn from eq. 3 or Figure 1. Most important is the fact that the adsorption of the phenols onto the protein very closely parallels the transfer of the phenols from a water phase to octanol. This indicates that there is not a highly specific kind of orientation of the phenol onto the protein. Actually, the OH group itself may not play a specific role. This is indicated by the reasonable fit obtained with resorcinol, a compound containing two phenolic OH groups. The uncertainty of this point is rather high since it is very poorly bound by the protein and rather strongly bound by the bag. Also, the wide variety of functional groups, NO_2, Br, CN, OCH_3, etc., do not appear to play special roles in binding other than those contained in the π values derived from the octanol–water model. This is also true of the large phenyl function as well as the –CH=CHCH=CH– function in β-naphthol. The fact that binding by these two large functions is well predicted by eq. 3 indicates that steric factors are not important, at least for groups of moderate size and the situation where $\bar{\nu}$ = 1. Also, the consistent results with these aromatic functions indicate no highly specific role for the π electrons.

Table I. Concentrations of Phenol Necessary to Produce One-to-One Phenol–Protein Complex

Substituent	Without buffer, log $1/C$	With buffer, log $1/C$	π
H	3.32	3.32	0
3-F	3.60	3.86	0.47
3-Cl	4.26	4.30	1.04
3-CH_3	3.65		0.56
3-Et	4.10	4.22	0.94
3-CF_3	4.43	4.52	1.49[a]
3-CN	3.25	3.26	−0.24
3-OH	3.23	3.15	−0.66
3-OCH_3	3.43	3.54	0.12
3-NO_2	3.89		0.54
4-F	3.70	3.46	0.31
4-Cl	4.07	4.00	0.93
4-Br	4.29	4.22	1.13
4-I	4.52	4.40	1.45
4-CH_3	3.76	3.70	0.48
4-C_6H_5	4.92		2.00[a]
4-OCH_3	3.59	3.40	−0.12
4-NO_2	4.07		0.50
3,4-$(CH)_4$	4.38		1.24[a]

[a] These values were estimated from phenoxyacetic values according to our previously described method.[5]

For the formation of the one-to-one complex, the results in the presence and absence of buffer are the same. From the data in Table I for the adsorption of phenols in the presence of buffer, we have derived eq. 4.

$$\log 1/C = 0.688\pi + 3.450 \qquad n = 14 \quad r = 0.974 \quad s = 0.109 \qquad (4)$$

The constants in eq. 4 are, within experimental error, the same as those in eq. 3.

Recently Weinbach and Garbus[11] have studied the binding of a series of phenols by mitochondrial protein. From the data in Table II we have derived eq. 5 and 6 by the method of least squares. In eq. 5, B represents

$$\log B = 0.196\pi + 1.112 \qquad n = 7 \quad r = 0.894 \quad s = 0.164 \qquad (5)$$

the per cent of phenol applied which is bound by protein under specified conditions. The correlation coefficient indicates a moderately good linear relationship between log B and the lipophilic character (π) of the substituents. Since in this series of phenols the degree of dissociation is much greater than that of the monosubstituted phenols studied with BSA, eq. 6 was derived to investigate the dependence of binding

$$\log 1/C = 0.069\mathrm{p}K_a + 0.228\pi + 0.583 \qquad n = 7 \quad r = 0.984 \quad s = 0.074 \qquad (6)$$

on the anionic character of the phenols. The linear combination of free-energy-related terms in eq. 6 is subject to the usual assumptions involved in the formulation of extrathermodynamic relationships.[12] An F test indicates that the additional term in eq. 6 is quite significant ($F_{1,4} = 21$; $F_{1,4}$ (when $\alpha = 0.01$) is 21.198). The comparison of correlation coefficients for eq. 5 and 6 also shows the dependence of bonding on the degree of dissociation. The positive coefficient associated with the pK_a term indicates that the less dissociated the phenol, the more firmly it is bound.

Table II. Binding of Phen ols to Mitochondrial Protein

Phenol	Log %[a] bound	pK_a[a]	$\Sigma\pi$[b]
Penta-Cl	1.85	4.8	4.39
2,3,4,6-Tetra-Cl	1.76	5.3	3.35
2,4,5-Tri-Cl	1.68	7.0	2.66
2,4-Di-Cl	1.58	7.8	1.62
3-Cl	1.45	9.1	1.04
4-Cl	1.36	9.4	0.93
2,4-Di-NO_2	0.85	4.0	0.04

[a] From ref. 11. [b] Calculated from data in ref. 5 except for 2,4-dinitrophenol which was subsequently determined by T. Fujita (unpublished data).

As in the case of binding of phenols by the BSA, eq. 5 and 6 indicate a nonspecific type of binding of the phenol by the mitochondrial protein. This is probably best described as hydrophobic binding.[13] It is interesting to note that eq. 6 gives just as good correlation for the polysubstituted as for the monosubstituted phenols. Phenols with and without *ortho* substituents are also equally well accommodated by eq. 6.

The use of π as a measure of the hydrophobic binding power of a substituent in conjunction with other substituent constants and regression analysis can provide new insights into biochemical reactions in which proteins are involved.[4] This can be shown by a further analysis of the work on the action of phenols in uncoupling phosphorylation which was the primary object of the study of the interaction of phenols with mitochondrial protein undertaken by Weinbach and Garbus.[11] It was their conclusion as well as that of earlier workers[14,15] that the uncoupling ability of various phenols was associated with lipophilic character of the phenol as well as the degree of dissociation. Weinbach and Garbus concluded that the phenols reacted with a protein to produce the uncoupling effect. This conclusion was reached in part by a study of the concentration of phenol necessary to cause complete uncoupling of phosphorylation in isolated rat liver mitochondria. To test their conclusion that two kinds of reversible bonds of phenol to protein are involved in uncoupling, we have derived eq. 7 from the data in Table III. In eq. 7, C is the

$$\log 1/C = -0.224\mathrm{p}K_a + 0.278\pi + 4.718 \qquad n = 10 \quad r = 0.909 \quad s = 0.325 \qquad (7)$$

molar concentration of phenol necessary to cause complete uncoupling.

A much more extensive investigation of the uncoupling action of phenols was made by DeDeken.[14] He investigated the inhibiting action of a series of phenols on phosphorylation in yeast. From the data in Table III we have derived eq. 8 and 9. The correlation

$$\log 1/C = -0.422\mathrm{p}K_a + 6.965 \qquad n = 14 \quad r = 0.826 \quad s = 0.621 \qquad (8)$$

$$\log 1/C = -0.491\mathrm{p}K_a + 0.620\pi + 6.792 \qquad 14 \quad 0.936 \quad 0.406 \qquad (9)$$

obtained with eq. 9 is quite good, especially when the possible analytical errors are taken into consideration. DeDeken also came to the conclusion that the uncoupling action of the phenols depended upon their pK_a and their degree of lipophilic character. This view is quantitatively supported by eq. 9. The negative coefficient associated with the pK_a term indicates that the lower pK_a value (the greater the tendency to dissociate), the higher the uncoupling activity. The positive coefficient with π indicates that increasing lipophilicity of substituents leads to larger log $1/C$ values and, therefore, higher activity.

Weinbach and Garbus postulated that phenols exert their uncoupling action by reaction with a protein. To investigate this possibility, they isolated mitochondrial protein and studied the adsorption of phenols by this material. Our analysis of this study is expressed by eq. 6 where it is seen that adsorption of the phenols is increased by increasing values of pK_a. The positive coefficient in the pK_a term in eq. 6 is just the opposite of the negative term in eq. 9. Although eq. 6

(11) E. C. Weinbach and J. Garbus, *J. Biol. Chem.*, **240**, 1811 (1965).
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(13) W. Kauzmann, *Advan. Protein Chem.*, **14**, 37 (1959).
(14) R. H. DeDeken, *Biochim. Biophys. Acta*, **17**, 494 (1955).
(15) H. C. Henker, *ibid.*, **63**, 46 (1962).

Table III. Concentrations of Phenols Inhibiting Phosphorylation

Phenol	Log $1/C^a$	$pK_a{}^a$	$\Sigma\pi^b$	Phenol	Log $1/C^f$	$pK_a{}^f$	$\Sigma\pi^g$
2,4-Di-NO_2-6-Et	5.92	4.55	1.04[d]	4-NO_2	3.3	7.0	0.50
2,4-Di-NO_2-6-*i*-Pr	5.60	4.60	1.34[d]	2,6-Di-NO_2	4.77	3.7	0.04
2,4-Di-NO_2-6-CH_3	5.40	4.40	0.54[d]	3,4-Di-CH_3-2,6-di-NO_2	4.92	4.2	1.08
2,4,5-Tri-Cl	4.96	7.00	2.66	4-*t*-Bu-2,6-di-NO_2	5.16	4.3	1.86
2,4-Di-NO_2	4.80	4.00	0.04	4-*t*-Am-2,6-di-NO_2	5.55	4.1	2.36
2,6-Di-NO_2	4.55	3.70	0.04[e]	4-*t*-Octyl-2,6-di-NO_2	6.08	4.1	3.86
2,4,6-Tri-Cl	4.54	6.42	2.31				
2,4-Di-Cl	3.70	7.75	1.62	Penta-Cl	4.7[h]	4.8[h]	4.39
4-NO_2	3.37	7.00	0.50	2,3,4,6-Tetra-Cl	4.3	5.3	3.35
2-I	3.22	9.40[c]	1.19	2,4,5-Tri-Cl	4.3	7.0	2.66
4-Br	3.14	9.40[c]	1.13	2,4-Di-Cl	3.7	7.8	1.62
2-NO_2	3.14	7.12	0.33	3-Cl	2.6	9.1	1.04
4-Cl	3.00	9.40	0.93	4-Cl	2.6	9.4	0.93
3-NO_2	3.00	8.00	0.54	2,4-Di-Br	3.7	7.6	2.02
				2,4-Di-NO_2	4.0	4.0	0.04
				2,6-Di-NO_2	3.6	3.7	0.04
				4-NO_2	3.6	7.0	0.50

[a] These values were used in the derivation of eq. 8 and 9 and are from the work of DeDeken.[14] [b] From ref. 5. [c] pK_a for these compounds was assumed to be the same as for 4-chlorophenol. [d] π values for these di-*ortho*-substituted phenols are not known. These values were estimated by adding the appropriate value for the alkyl group to 2,4-dinitrophenol. π values have been shown[5,6] to be an additive and constitutive property. [e] The π value for 2,6-dinitrophenol is not known for the octanol–water system. We have taken it to be the same as for 2,4-dinitrophenol. Partition coefficients determined for 2,6- and 2,4-dinitrophenol in the xylene–water system[15] show that they are close to each other. [f] These values were used in the derivation of eq. 10 and 11 and are taken from the work of Henker.[15] [g] π for the isoamyl and isooctyl functions was calculated by adding 0.50 for each additional CH_2 to the value of 1.82 obtained for the *sec*-butyl function in the phenoxyacetic acid systems.[5] π for alkyl groups is known to be constant from system to system.[5] [h] These values were used in the derivation of eq. 7 and are from ref. 11.

and 9 represent work done in two different systems (yeast and liver mitochondria), the conclusion can be drawn that protein isolated by Weinbach and Garbus is not the one involved in the phosphorylation reaction or, if it is, it must have changed in the process of isolation. Further support for this view comes from the work of Henker.[15]

Henker studied the uncoupling activity of six phenols on rat liver mitochondria. His conclusions about the physical and electronic requirements for activity of the phenols were similar to those of the other workers. In fact, he has formulated a mathematical model based on partition coefficients and pK_a, in many respects similar to ours, to rationalize the activity of six phenols. However, his more absolute equation is, in practice, more difficult to work with than our relativistic approach. From the data in Table III we have derived eq. 10 and 11 relating structure and activity for the action of the six phenols on rat mitochondria. Although Henker studied the activity of the phenols at several pH values, we have used the values at pH 5 since these are closest to the work of DeDeken (pH 4.5). Comparison of eq. 9 and 11 brings out a quite

$$\log 1/C = -0.644pK_a + 7.905 \quad n = 6,\ r = 0.827,\ s = 0.592 \quad (10)$$

$$\log 1/C = -0.501pK_a + 0.400\pi + 6.607 \quad n = 6,\ r = 0.999,\ s = 0.039 \quad (11)$$

striking similarity of action of two quite different sets of phenols acting in two different biological systems. The constants in these equations are quite close in value and have the same signs. The strong dependence of uncoupling action on low pK_a indicates that a phenolic anion reacts with an electron-deficient species. The dependence of activity on π indicates that adsorption from the aqueous phase to the sites of action is promoted by lipophilic groups. Two roles for the substituent may be in operation here. Lipophilic functions may simply aid in the diffusion through the surface of the mitochondria to the internal sites of action. They may also be instrumental in binding the phenol in a particular way to a particular enzyme as we have shown for systems involving purified enzymes.[7] The constants in eq. 7 probably differ from eq. 9 and 11 because of pH differences in the studies.

It is of interest to note that the *ortho*- and di-*ortho*-substituted phenols are almost as well accommodated by eq. 9 and 11 as the other phenols. The poorest fit to eq. 9 is obtained with 2,4-dinitro-6-ethylphenol; however, the corresponding *o*-isopropyl and *o*-methyl derivatives fit reasonably well. This would indicate that the $-O^-$ group of the phenol cannot be reacting with a very bulky protein or enzyme in causing the uncoupling reaction. In fact, such a lack of pronounced steric effect suggests that the phenol might play its critical role by accepting a proton at some key enzymatic site.

The role of substituents in hydrophobic binding of phenols to proteins as shown in our above study with BSA does not appear to be unique.[7] The practical insights that can be gained through the use of π in protein studies has been demonstrated with phosphorylating uncoupling. Other work in progress indicates that there are many situations where the use of π as a measure of hydrophobic bonding can be of great help in understanding biochemical processes.

Acknowledgment. This work was supported under Research Grant GM-07492 from the National Institutes of Health.