The Use of Substituent Constants and Regression Analysis in the Study of Enzymatic Reaction Mechanisms

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Electronic, steric, and "hydrophobic bonding" substituent constants have been used with regression analysis to separate electronic and adsorption effects on the rates of enzymatic reactions. It is shown by the use of π that one can establish the stereospecific nature of hydrophobic bonding. The steric substituent constant E_s has been applied for the first time to reactions occurring on an enzyme. Regression analysis and substituent constants, especially π and σ, provide a new approach for the difficult task of mapping the sites of action on enzymes.

Recent work[1-3] in this laboratory has resulted in the development of eq. 1 for the correlation of chemical constitution with biological activity. C_x is the molar

$$\log 1/C_x = -k\pi^2 + k'\pi + \rho\sigma + k'' \quad (1)$$

concentration of a derivative, S_x, in a family of related compounds causing an equivalent biological response, *e.g.*, $L.D._{50}$. The free energy related substituent constant,[4,5] π, is defined as

$$\pi = \log P_X - \log P_H \quad (2)$$

where P_H is the partition coefficient of the parent molecule between 1-octanol and water and P_X that of the derivative. The Hammett constants, $\rho\sigma$, have their usual meaning.[6] It has been assumed for a first approximation that one chemical reaction or physical process is rate limiting as far as the observed relative biological response is concerned. Equation 1 can be regarded as a Hammett equation in which the π term has been introduced to account for the very complex processes by which an organic molecule makes its way *via* a random walk through living tissue to its ultimate site of action which may be more or less hidden within a cellular organelle.

The results obtained with eq. 1 and 2 have prompted us to consider simpler systems involving reactions with isolated enzymes, which can be expressed in terms of enzyme kinetics as follows

$$E + S_x \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES_x \underset{k_4}{\overset{k_3}{\rightleftarrows}} EPr_x \underset{k_5}{\overset{k_5}{\rightleftarrows}} Pr_x + E \quad (3)$$

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E is a fixed amount of enzyme under constant conditions. S_x is a substrate which can be varied by changing the substituent X, and Pr_x is the corresponding product. ES_x is the substrate adsorbed to the enzyme and EPr_x is the modified substrate still held by the enzyme before the desorption step. For the early stages of the reaction Pr_x will be small and, neglecting the recombination of Pr_x and E, we may write an expression for the over-all velocity of formation of Pr_x as

$$V_x = k_3[ES_x] \quad (4)$$

k_3 has its usual significance; it is the rate constant for the conversion of ES_x to $Pr_x + E$. The equilibrium expression for the formation of ES_x is

$$[ES_x] = \frac{[E][S_x]k_1}{k_2} \quad (5)$$

Substituting eq. 5 into eq. 4 and taking logarithms yields

$$\log V_x = \log k_3 + \log k_1/k_2 + \log [E] + \log [S_x] \quad (6)$$

For a given set of experimental conditions, [E] is a constant and, if relatively small amounts of S_x are consumed, $[S_x]$ may be considered constant.

For the first approximation we can assume that the chemical or physical change as governed by k_3 is susceptible to the Hammett treatment and that log k_3 may be replaced by $\rho\sigma$ + constant or by other substituent constants.[7] Making a similar extrathermodynamic assumption that partitioning between the aqueous phase and the hydrophobic sites of the enzyme as governed by log (k_1/k_2) is linearly related to log P_x or π, eq. 6 can be converted to the form

$$\log V_x = k\pi + \rho\sigma + c \quad (7)$$

In eq. 7, k and c are constants and the linear combination of the two parameters, π and σ, is subject to the usual limitations[7] of extrathermodynamic relationships. In the examples in this paper where moderate structural changes were made in a related series of derivatives and only the over-all reaction rates were measured, eq. 7 appears to give quite a good account of substituent effects on the over-all reaction velocities.

A more sophisticated approach to the study of enzyme specificity is to make an independent evaluation of substituent effects on k_1/k_2 and k_3. In so doing, the assumption is usually made that k_3 is small in comparison to k_2 so that the reciprocal of the Michaelis constant can be used as the equilibrium constant governing adsorption

$$k_1/k_2 = 1/K_m \qquad K_m = (k_2 + k_3)/k_1 \quad (8)$$

For the general case, one must consider the steric, electronic, and partitioning effects of a substituent, and

(1) C. Hansch, R. M. Muir, T. Fujita, P. P. Maloney, C. F. Geiger, and M. J. Streich, *J. Am. Chem. Soc.*, **85**, 2817 (1963).
(2) C. Hansch and T. Fujita, *ibid.*, **86**, 1616 (1964).
(3) C. Hansch and A. R. Steward, *J. Med. Chem.*, 7, 691 (1964).
(4) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964).
(5) J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, **8**, 150 (1965).
(6) H. H. Jaffé, *Chem. Rev.*, **53**, 191 (1953).
(7) J. E. Leffler and E. Grunwald, "Rates and Equilibria of Organic Reactions," John Wiley and Sons, Inc., New York, N. Y., 1963.

a linear combination of suitable parameters is shown in eq. 9.

$$\log k_1/k_2 = k_a\pi + k_b\sigma + k_cE_s + k_d \quad (9)$$

The parametric functions π, σ, and E_s are simply representing the above-mentioned substituent effects. For example, instead of π, one could consider using log P, parachor or R_m constants obtained from chromatography. We have recently shown[5] the relationship of the latter two to π or log P. The electronic constant σ can be replaced by quantum mechanical calculations or spectral measurements. Any steric constant such as E_s (obtained from relative rates of ester hydrolysis) probably has limited use, and it will greatly simplify matters when this variable can be held constant. We have shown[4] that π itself has a small dependence on σ so that, unless one uses log P from the particular molecules under study, the electronic term in eq. 9 will often be needed to compensate for this effect.

Since k_3 covers at least two processes, the reaction occurring on the enzyme and desorption, hydrophobic bonding will also be important in this part of the overall reaction and a suitable parameter should be included for this effect. This can be approximated by the linear combination of two processes depicted in eq. 3.

$$\log k_3^* = k_f\sigma + k_gE_s + k_h \quad (10)$$

$$\log k_5 = -k_i\pi - k_j\sigma + k_lE_s + k_m \quad (11)$$

$$\log k_3 = \log k_3^* + \log k_5 = k_n\pi + k_p\sigma + k_qE_s + k_r \quad (12)$$

Negative signs have been written with the first two terms in eq. 11 to indicate that desorption will impose opposite demands for these effects when compared with adsorption (eq. 9). Assuming steric effects to be constant, substitution of eq. 9 and 12 into 6 again results in eq. 7, where it is now apparent that the constants associated with the parameters π and σ result from the combination of several processes dependent on substituent effects. As long as the dependencies are linear in nature, eq. 7 can be used to make useful studies of substituent effects on enzymatic reactions.

The type of bonding of substrate to enzyme characterized by π or log P is probably best defined as hydrophobic.[8,9]

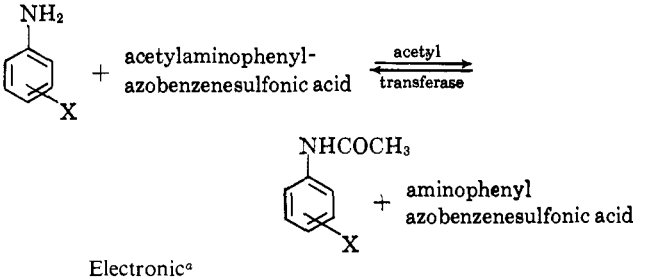
We have used the solvents 1-octanol and water at ~25° for standard conditions.[4] In establishing π-constants for various substituents, we have worked with low solute concentrations (~10^{-3} M) so that it can be assumed that the solute in each phase is approximately at unit activity. π is, in effect, the logarithm of the partition coefficient for a given function, *e.g.*, Cl or NO_2, and we have shown[4] that it is approximately constant (when strong interactions are absent) from one aromatic system to another.

Several examples of the combination of free energy related substituent constants in substituent effects on enzyme specificity are considered in the following sections of this paper.

Application I. The interesting results of Jacobson[10] in which pigeon liver acetyl transferase was used in the acetylation of different aromatic amines provides a test of eq. 7. A least-squares fit of the data in Table I to eq. 7 yields eq. 14 and, omitting the π term, eq. 13. Although Jacobson did not make a correlation of the type shown by eq. 13, he was aware that the rate of reaction was influenced by the electron density on the amino nitrogen. For the above equations, s represents the standard deviation, r the multiple correlation coefficient, and n the number of points used to derive the constants. For σ we have used σ^- values, and for π we have used values obtained from aniline and its derivatives rather than benzene to minimize interaction effects. The importance of the extra parameter governing the formation of the enzyme–substrate complex and eventual desorption of product is seen by comparison of eq. 13 and 14. As is apparent from r^2, eq. 13 accounts for only 64% of the variance in the data while eq. 14 accounts for better than 99%. The fact that hydrophobic bonding of the substituent accounts for almost 40% of the variance in rate due to substituent effects explains the failure of both Jacobson and Perault and Pullman[11] to obtain satisfactory interpretation of substituent effects since they considered only an electronic parameter. The excellent correlation obtained with eq. 14 also reveals the absence of steric effects, at least for those groups studied. By a study of larger and larger substituents one can find the point where eq. 14 fails and in this way map the free space around the enzymatic reaction site.

	n	s	r^2	r	
$\log A_x = -0.465\sigma - 0.022$	6	0.218	0.638	0.799	(13)
$\log A_x = 0.252\pi - 0.335\sigma - 0.155$	6	0.025	0.997	0.998	(14)

Table I. Enzymatic Acylation of Aromatic Amines[a]



X	Electronic[a] charge on N of NH_2	σ^-	π^b	Calcd.[c] log A_x	Obsd. log A_x	Δ log A_x
4-Br	1.849	0.23	1.13	0.0533	0.049	0.004
4-Cl	1.849	0.23	0.93	0.0028	0.037	0.034
4-CH_3	1.853	−0.17	0.48	0.0234	0	0.023
H	1.851	0	0	−0.1548	−0.155	0.000
4-NO_2	1.827	1.27	0.50	−0.4542	−0.468	0.014
4-SO_2NH_2	1.841	0.91	−1.16[d]	−0.7525	−0.745	0.008

[a] From ref. 10. [b] Obtained from aniline and its derivatives, ref. 4. [c] Calculated using eq. 14. [d] This value was estimated using the linear relationship connecting small changes in π with σ.[4]

While Jacobson studied ten amines, comparative constants are available for only six; thus, only six

(8) W. Kauzmann, *Advan. Protein Chem.*, **14**, 37 (1959).
(9) G. Némenthy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).
(10) K. B. Jacobson, *J. Biol. Chem.*, **236**, 343 (1961).
(11) A. Perault and B. Pullman, *Biochim. Biophys. Acta*, **66**, 86 (1963).

points were used in deriving the constants for eq. 13 and 14. Nevertheless, the π term in eq. 14 is highly significant statistically. An F test shows it to be significant at much better than the 0.995 level of significance.

Perault and Pullman[11] have also considered the results of Jacobson and have shown that the electron density on the amino nitrogen estimated by quantum mechanical calculations and the rate of acylation more or less parallel each other. Using their values as shown in Table I, we have derived eq. 15 and 16. In

	n	s	r^2	r	
$\log A_x = 22.912\epsilon - 42.487$	6	0.265	0.469	0.685	(15)
$\log A_x = 0.291\pi + 18.163\epsilon - 33.815$	6	0.042	0.990	0.995	(16)

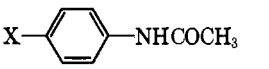
eq. 15 and 16, ϵ represents the calculated[11] electron density. Comparison of eq. 13 and 14 with 15 and 16 provides another illustration of the parallel results which can be obtained using experimentally determined relative electron densities and quantum mechanically calculated values. The coefficients of π in eq. 14 and 16 are quite close, indicating the same role for hydrophobic bonding in the two different electronically based models. The minus sign associated with σ in eq. 13 and 14 indicates that functions releasing electrons to the amino group increase the reaction rate. In eq. 15 and 16 the electron density on the amino group is proportional to ϵ. This supports the conclusions of Pullman and Pullman[12] that quantum mechanically calculated electron densities provide a particularly useful approach to the understanding of electronic interactions of biologically important molecules which are usually so complex in character that parameters such as Hammett constants are not easy to obtain.

Jacobson also considered a variety of acetyl donors in the transacetylation reaction. Relative rates were calculated for four of these using aminophenylazobenzenesulfonic acid as an acceptor. A least-squares fit of the data in Table II to eq. 7 yields eq. 18 and, omitting the π term, eq. 17. An F test indicates the π

	n	s	r^2	r	
$\log A_x = 0.625\sigma - 0.773$	4	0.289	0.745	0.863	(17)
$\log A_x = 0.768\pi + 0.691\sigma - 1.075$	4	0.069	0.993	0.996	(18)

term to be significant at slightly less than the 0.90 level of significance. σ^- values were used in eq. 17 and 18 since the correlation using σ is not as good ($r^2 = 0.954$). The importance of hydrophobic bonding as revealed by the π term is quite useful in understanding the over-all reaction mechanism. The final picture of the active site must include an area for hydrophobic bonding. Some care must be exercised in interpreting each of the terms in the equation for the *over-all* relative reaction velocity, *e.g.*, eq. 18. Since the π term in eq. 18 has a positive sign, an obvious conclusion would be that the enzyme contains a hydrophobic site and that groups binding to this site in the adsorption step promote the over-all reaction; k_a in eq. 9 would be larger than k_i in eq. 11. In fact, it is conceivable that the opposite is true and k_i in eq. 11 is positive and larger than a negative k_a in eq. 9. This would mean that there is a net over-all rate gain due to repulsion between a hydrophobic group in S_x and a hydrophilic site on the enzyme. This ambiguity can be resolved by studying substituent effects on k_1/k_2 and k_3 independently.

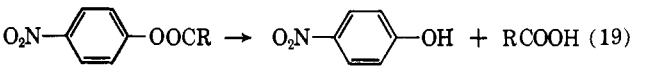
Table II. Acetanilides as Sources of the Acyl Function in Transacetylation



X	σ^-	π^a	Calcd.[b] log A_x	Obsd. log A_x	Δ log A_x
4-NO_2	1.27	0.24	−0.0139	0	0.014
4-Cl	0.23	0.70	−0.3791	−0.420	0.041
4-CH_3	−0.17	0.52	−0.7936	−0.745	0.049
4-H	0	0	−1.0754	−1.097	0.022

[a] π-values were obtained from the phenoxyacetic acid system.[4] [b] These values were obtained using eq. 18.

Application II. The enzymatic hydrolysis of esters provides a different and interesting case for studying the effect of substituents on reaction rates. Huggins and Lapides[13] studied the esterase activity of human serum using various *p*-nitrophenyl esters as indicated in eq. 19.



From the data in Table III, the following equations have been derived.

	n	s	r^2	r	
$\log A_x = 2.996E_s + 2.598$	6	0.634	0.896	0.947	(20)
$\log A_x = 0.955\pi + 3.572E_s + 1.573$	6	0.508	0.950	0.975	(21)
$\log A_x = -7.614\sigma^* + 0.389\pi + 3.808E_s + 1.552$	6	0.381	0.981	0.991	(22)

The best model is that described by eq. 22; however, using eq. 20, the single function E_s accounts for 90% ($r^2 = 0.896$) of the variance in the data. E_s is a free energy related substituent constant for steric effects developed by Taft.[14] Equations 21 and 22 reveal small roles for π and σ^*, the latter being a polar substituent constant for aliphatic functions comparable[14] to σ. F tests indicate that both the π and σ^* terms are significant between the 0.75 and the 0.90 level of significance.

Comparison of eq. 20 and 21 brings out the great importance of steric effects compared to hydrophobic bonding.

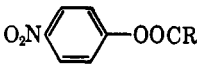
It is of particular interest that E_s values derived for simple organic molecules in solution should give such a

(12) B. Pullman and A. Pullman, "Quantum Biochemistry," Interscience Publishers, Inc., New York, N. Y., 1963.

(13) C. Huggins and J. Lapides, *J. Biol. Chem.*, 170, 467 (1947).

(14) M. S. Newman, "Steric Effects in Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1956 p. 556.

Table III. Enzymatic Hydrolysis of 4-Nitrophenyl Esters by Human Serum at pH 7



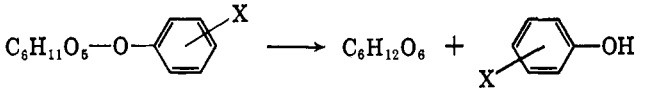
R	σ^{*a}	π^b	E_s^c	Calcd.[d] log A_x	Obsd.[e] log A_x	Δ log A_x
Me	0.00	0.50	0.00	1.746	1.93	0.184
Et	−0.10	1.00	−0.07	2.436	2.00	0.436
i-Pr	−0.19	1.37	−0.47	1.742	1.98	0.238
t-Bu	−0.17	1.78	−1.54	−2.326	−2.40	0.074
n-Pr	−0.12	1.50	−0.36	1.678	1.73	0.052
Bu	−0.13[f]	2.00	−0.39	1.835	1.87	0.036

[a] Obtained from ref. 14. [b] π-values from phenol used.[4] [c] Obtained from ref. 14. [d] Calculated using eq. 22. [e] Although Huggins and Lapides[13] reported a value for the *sec*-butyl group, we have not included these because no value for E_s is available. [f] Estimated value.

reasonable correlation for an enzymatic reaction presumably occurring on the surface of a protein. E_s may become more useful in the study of enzymatic reactions than one would, *a priori*, have any right to expect.

Application III. An especially interesting case for substituent constant analysis comes from the elegant study by Nath and Rydon[15] of the enzymatic hydrolysis of substituted phenyl β-D-glucosides by emulsin. In their study they reported values for the constants k_1/k_2 and k_3'. The constant k_3' is proportional to k_3 and arises because of the uncertainty in the concentration of enzyme used. *meta*, *para* and *ortho* substituents have been treated separately. Regression analysis using the data from Table IV leads to eq. 23–26 for the effects of substituents on k_1/k_2.

Table IV. Enzymatic Hydrolysis of Phenyl Glucosides[a]



X	log (k_1/k_2 × 10)	log (k_3 × 10^8)	π^b
H	0.565	1.352	0
3-CH_3	0.724	1.754	0.56
3-OCH_3	0.586	2.121	0.12
3-Cl	1.011	2.358	1.04
3-CN	1.260	2.839	−0.24
3-NO_2	1.347	2.663	0.54
4-CH_3	0.886	1.233	0.48
4-*i*-Pr	1.377	0.960	1.40
4-*t*-Bu	1.196	0.559	1.78
4-OCH_3	0.728	1.769	−0.12
4-Cl	1.077	1.644	0.93
4-CN	1.623	2.456	0.14
4-NO_2	1.699	2.586	0.50
2-CH_3	0.638	2.956	0.68
2-*i*-Pr	1.538	1.749	1.50
2-*t*-Bu	1.121	0.825	1.88
2-OCH_3	0.764	2.985	−0.33
2-Cl	1.346	2.920	0.69
2-NO_2	1.208	3.546	0.33

[a] Rate and equilibrium constants were taken from ref. 15. σ-constants used are those reported by Jaffé, ref. 6. [b] π-values were obtained from phenol and its derivatives, ref. 4.

(15) R. L. Nath and H. N. Rydon, *Biochem. J.*, 57, 1 (1954).

para Substituents

	n	s	r^2	r	
$\log \frac{k_1}{k_2} = 0.519\sigma + 2.033$	8	0.291	0.567	0.753	(23)
$\log \frac{k_1}{k_2} = 0.330\pi + 0.615\sigma + 1.802$	8	0.189	0.848	0.921	(24)

meta Substituents

	n	s	r^2	r	
$\log \frac{k_1}{k_2} = 0.954\sigma + 1.628$	6	0.120	0.901	0.949	(25)
$\log \frac{k_1}{k_2} = 0.121\pi + 0.960\sigma + 1.585$	6	0.118	0.928	0.963	(26)

In deriving the above equations we have used σ^- values as did Nath and Rydon, rather than σ, because slightly better correlations resulted. π constants were those obtained from phenols.[4]

The most striking result coming from a comparison of eq. 23–26 is that hydrophobic bonding appears to be very important for *para* substituents, but impossible for functions in the *meta* position. Although the correlation with eq. 26 seems to be better than with eq. 25, an F test shows that the π term in eq. 26 is not significant even at the 0.75 level of significance. This information is of the utmost importance in the complete mapping of the electronic and structural features of the active site on the enzyme where the hydrolytic reaction occurs. With the information that π makes no contribution for *meta* groups, one equation can be derived for the effect of both *para* and *meta* substituents on k_1/k_2:

meta and *para* Substituents

	n	s	r^2	r	
$\log \frac{k_1}{k_2} = 0.358\pi + 0.664\sigma + 1.763$	13	0.163	0.842	0.917	(27)

In the least-squares derivation of the constants for eq. 27, zero was used for π for all *meta* substituents. While the correlation with eq. 27 is not as good as that obtained with eq. 25 for the *meta* substituents, it is as good as that obtained with eq. 24. One reason the *meta* substituents might give a better correlation is that Nath and Rydon did not include the bulky isopropyl and *t*-butyl groups in this series.

It is interesting to consider the significance of the positive coefficient associated with σ in eq. 23–26. This indicates that electron withdrawal by the substituent from the phenolic oxygen and sugar moiety increases the binding of the substrate by the enzyme. One's first impulse might be to assume that binding occurs to an electron-rich site on the enzyme. This deduction is not the only one possible. We have found[4] that electron-withdrawing substituents, when associated with functions having lone-pair electrons, reduce the hydrogen bonding of the lone pair and cause an increase above simple additivity in the preference of the molecule for the hydrophobic phase.

The substituent effects on k_3' are summarized in the following equations:

para Substituents

	n	s	r^2	r	
$\log k_3' = 1.011\sigma - 6.646$	8	0.388	0.736	0.858	(28)
$\log k_3' = -0.466\pi + 0.875\sigma - 6.320$	8	0.221	0.929	0.964	(29)

meta Substituents

$\log k_3' = 1.522\sigma - 6.278$	6	0.242	0.851	0.922	(30)
$\log k_3' = 0.078\pi + 1.526\sigma - 6.305$	6	0.275	0.855	0.925	(31)

Again, regression analysis reveals that *meta* substituent effects do not involve hydrophobic bonding (compare eq. 30 and 31). As one might expect, a negative sign is associated with the π term in eq. 29, indicating that hydrophobic bonding slows down the desorption step in eq. 3. It occurred to us that the negative sign associated with π in eq. 29 might fortuitously be due to steric effects of the large *t*-butyl and isopropyl groups which, to a certain extent, parallel the hydrophobic bonding tendency. To test this possibility, a least-squares fit to eq. 7 was made, omitting these two functions. Equation 32 is the result. The coefficients of

	n	r	
$\log k_3' = -0.315\pi + 0.839\sigma - 6.347$	6	0.932	(32)

π and σ in eq. 32 are quite close to those in eq. 29 and offer convincing evidence that increasing hydrophobic bonding decreases the rate of desorption resulting in lower values for k_3' and that this effect is important for functions other than large alkyl groups. This confirms the fact that steric effects from the *meta* and *para* positions must be very small.

Just as in the case of k_1/k_2, one equation can be derived for the effects of both *meta* and *para* substituents on k_3'. In obtaining the constants for this equation we have used $\pi = 0$ for *meta* substituents:

	n	s	r^2	r	
$\log k_3' = -0.605\pi + 0.938\sigma - 6.148$	13	0.244	0.901	0.949	(33)

Combining eq. 27 and 33, we obtain eq. 34 for the relative over-all rates of reaction.

	n	s	r^2	r	
$\log k_3' \frac{k_1}{k_2} = -0.247\pi + 1.601\sigma - 4.385$	13	0.318	0.897	0.947	(34)

The π term in eq. 34 is not very important since it is significant at the 0.75 level but not at the 0.90 level of significance. The advantages gained by hydrophobic bonding in the adsorption step are almost exactly cancelled by the disadvantages in the desorption step. Equation 34 can be, for practical purposes, simplified to:

	n	s	r^2	r	
$\log k_3' \frac{k_1}{k_2} = 1.685\sigma - 4.504$	13	0.339	0.871	0.933	(35)

Nath and Rydon also examined the hydrolysis of a number of *ortho* derivatives. While steric interactions preclude high correlation between rate of hydrolysis and the two parameters σ and π, useful conclusions may be drawn from the results of the regression analysis summarized in eq. 36–39. Comparison of eq.

ortho Substituents

	n	s	r^2	r	
$\log \frac{k_1}{k_2} = 0.496\sigma + 0.981$	7	0.378	0.149	0.386	(36)
$\log \frac{k_1}{k_2} = 0.330\pi + 0.666\sigma + 0.742$	7	0.285	0.614	0.783	(37)
$\log k_3' = 2.035\sigma + 2.150$	7	0.906	0.339	0.582	(38)
$\log k_3' = -0.604\pi + 1.722\sigma + 2.588$	7	0.836	0.550	0.741	(39)

37 with 36 and eq. 39 with 38 shows that hydrophobic bonding by *ortho* substituents is important. As with the *para* substituents, it is of interest to note the opposite signs associated with the π term in eq. 37 and 39.

From a consideration of the above three cases, certain inferences may be made about the adsorption and desorption steps in an enzymatic reaction when only the over-all relative rates of a series of derivatives are known. If, through regression analysis using π, evidence for hydrophobic bonding can be established, as for example in application I, then there must be a difference in bonding involved in the adsorption step of the reactants and the desorption step of the products. If π is not significant, then the free energy change in one step is canceled by that in the other. An alternative deduction would be that the main part of the molecule bonds hydrophobically and fills the site so that this type of bonding is not possible for the substituents. While a large amount of work has gone into the evaluation of k_1/k_2 in enzyme kinetic studies, very little attention has been given to the desorption step (k_5/k_6). k_6 will not be important in the early stage of a reaction, whereas k_5 will, since as long as a site is occupied by products, reactant molecules cannot be adsorbed. Our analysis of Nath and Rydon's work indicates how information on k_5 can be obtained. Most important, we have shown how, by means of substituent constants and regression analysis for substituents in various positions of a parent compound, one can delineate the stereospecific nature of hydrophobic bonding. The use of π with σ provides the first means of separating with some assurance steric effects from electronic and lipophilic binding effects.

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