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The Analysis of Physiological Activity of Substituted Phenols with Substituent Constants[1]

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A recently developed method for the correlation of biological activity and chemical structure using the Hammett σ constant and a substituent constant π for "lipohydrophilic" character (or hydrophobic bonding ability) has been applied to analyze the physiological activity of the substituted phenols which exist partly as neutral molecules and partly in the ionized form at physiological pH. Taking the effect of dissociation into account, the physiological activity such as toxicity to plants and bacteria and uncoupling activity with oxidative phosphorylation are clearly shown to be associated linearly with the electronic and "lipohydrophilic" character of the substituents.

Recently, a method for correlating the physiological activity of a series of variously substituted compounds using substituent constants has been developed by Hansch and his co-workers.[2-6] They found that using an electronic parameter such as the Hammett σ constant with a substituent constant π, which is a free-energy-related parameter evaluating the lipohydrophilic character or hydrophobic bonding power of a substituent, the physiological activity of a series of substituted compounds can be well accommodated by eq 1. In some cases, eq 2, a special form of eq 1, was

$$\log \frac{1}{C} = a\pi - b\pi^2 + \rho\sigma + c \quad (1)$$

$$\log \frac{1}{C} = a\pi + \rho\sigma + c \quad (2)$$

found to be capable of rationalizing the physiological actions including highly specific enzymatic reactions.[3,5] In these equations, C is the equieffective molar concentration of compounds (the concentration causing a standard response such as LD_{50}, ED_{50}, isotoxic concentration, isonarcotic concentration, minimum inhibitory concentration etc.), and a, b, and c are constants. π is defined as: $\log P_X - \log P_H$. P_X and P_H are the partition coefficients determined in a 1-octanol–water system of the substituted and unsubstituted compound, respectively.[7,8]

The purpose of this paper is to apply this approach to the physiological actions of the substituted phenols under conditions of physiological pH, where the molecule is partly in the dissociated form. Since the dissociation constant of the phenols is quite susceptible to substituent variation, the ratio of the neutral phenol molecule to the dissociated ion varies with varying substitution of the phenol.

First, we consider the cases where the phenols exert their activity on the cell membranes, *i.e.*, essentially outside the cell. The microbial cell membranes have recently been shown to be a network to which many enzymes are fixed.[9] If we assume that the physiological action is triggered by a complex formation in which both the ionic and neutral forms of phenols take their parts simultaneously and the magnitude of the biological response is the sum of the effects of the ionic and neutral forms, the rate of biological response, BR, would be expressed by eq 3, where α is the degree

$$dBR/dt = C(1 - \alpha)ARk + C\alpha A'R'k' \quad (3)$$

of ionization, A is a factor governing the process of adsorption so that the product $C(1 - \alpha)A$ represents the concentration of the neutral form at the site of action, R is a factor proportional to the number of receptor sites, and k is a rate constant of a critical reaction to form the complex for the neutral molecule. A', R', and k' are those for the ionized form having the same significance as A, R, and k for the neutral molecule. As a first approximation, the neutral or ionized phenols are expected to interact with the complementary groups in the receptor, in the neutral or ionized form, respectively. Since the degree of ionization of the complementary groups is constant at a certain pH, the number of the neutral receptor sites (R) and that of the ionized sites (R') is assumed to be constant under a standard experimental condition.

When a critical reaction does not occur but a critical equilibrium—not a phase equilibrium—takes place at

(1) Studies on Structure–Activity Relationship. I.

(2) C. Hansch, R. M. Muir, T. Fujita, P. P. Maloney, F. Geiger, and M. Streich, *J. Am. Chem. Soc.*, **85**, 2817 (1963).

(3) C. Hansch and T. Fujita, *ibid.*, **86**, 1616 (1964).

(4) C. Hansch and A. R. Steward, *J. Med. Chem.*, **7**, 691 (1964).

(5) C. Hansch, E. W. Deutsch, and R. N. Smith, *J. Am. Chem. Soc.*, **87**, 2738 (1965).

(6) C. Hansch, K. Kiehs, and G. L. Lawrence, *ibid.*, **87**, 5770 (1965).

(7) T. Fujita, J. Iwasa, and C. Hansch, *ibid.*, **86**, 5175 (1964).

(8) J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, **8**, 150 (1965).

(9) M. R. Pollock, "The Bacteria," Vol. IV, I. C. Gunsalus and R. Y. Stanier, Ed., Academic Press Inc., New York, N. Y., 1962, p 149.

the site of action, the biological response is not a function of time after an initial stage and is expressed by eq 3', where k_e and k_e' are the equilibrium constants of the complex formation. If a certain physiological

$$BR = C(1 - \alpha)_1 R k_e + C\alpha_1 R' k_e' \qquad (3')$$

action of a series of phenols is considered to be due to a physical action of the molecule and if the magnitude of the biological response can be only attributed to a phase equilibrium concentration at the site of action as in the cases where the Ferguson's principle is applicable,[10] we can assume $k_e = k_e' =$ constant, so that the situation could be regarded as a special case of eq 3'.

In most cases, the biological results are determined in terms of concentrations required for a constant equivalent response (see above) obtained in a definite time interval. For these conditions we can replace dBR/dt in eq 3 and BR in eq 3' with constants. Taking the unsubstituted phenol as a standard, *i.e.*, at a condition of $(dBR/dt)_t = (dBR_0/dt)_t$ or $BR = BR_0$, we obtain eq 4 for a certain physiological action of a series of phenols, where k and k' are either equilibrium or rate constants of the complex formation. Equation 4

$$C(1 - \alpha)_1 R k + C\alpha_1 R' k' = C_0(1 - \alpha_0)_1 R k_0 + C_0\alpha_{0_1} R' k_0' \qquad (4)$$

contains too many independent variables to be useful for the analysis of structure–activity relationship. However, if eq 4 can be separated into eq 5 and 6, and these equations are satisfied simultaneously, eq 4 is fulfilled so that the structure–activity relationship could be analyzed by using them.

$$C(1 - \alpha)_1 k = C_0(1 - \alpha_0)_1 k_0 \qquad (5)$$

$$C\alpha_1 k' = C_0\alpha_{0_1} k_0' \qquad (6)$$

Equation 5 is obeyed when only the neutral molecule is considered to be the active form, *i.e.*, when BR is proportional to concentration of the complex formed from the neutral molecule. Likewise, eq 6 holds if the dissociated phenoxide ion is regarded to be responsible for the physiological action. Hence, it seems as if eq 5 and 6 can not be satisfied at the same time. However, as will be shown later in this paper, eq 5 and 6 (actually equations derived from eq 5 and 6, eq 18 and 19) are interrelated *a priori* by simple relationships (eq 21 and 22). Therefore, if either eq 5 or 6 is obeyed, they both should be fulfilled simultaneously.

Secondly, when we consider cases where the site of action is located intracellularly, the distribution of the neutral molecule and the ionized form in the extracellular and intracellular phases should be represented as follows, where α and α' are the degrees of ionization

	extracellular phase		intracellular phase
concn of the neutral form	$C(1 - \alpha)$	$\rightleftharpoons$	$C(1 - \alpha)T$
	⇓⇑		⇓⇑
concn of the ionized form	$C\alpha$	$\rightleftharpoons$	$C(1 - \alpha)T\dfrac{\alpha'}{1 - \alpha'}$

outside and inside the cell, respectively, and T is a factor governing the process of transfer so that the product $C(1 - \alpha)T$ represents the concentration of the neutral form in the intracellular biophase. Generally, dissociable organic compounds are considered to penetrate cells through membranes much more easily as the undissociated neutral molecule than as the dissociated ion.[11,12] However, even if the ionized form penetrates through membranes to some extent, the expressions for the concentration of the neutral and ionized forms in the intracellular phase do not change, since the ratio of the neutral and ionized forms for a certain compound should be determined only by the pH value of the intracellular phase.

The biological response in this case would be expressed by eq 7. Taking the biological response of

$$(dBR/dt)_t \text{ or } BR = C(1 - \alpha)TRk + C(1 - \alpha)T\frac{\alpha'}{1 - \alpha'}R'k' \qquad (7)$$

the unsubstituted phenol as a standard, we obtain eq 8.

$$C(1 - \alpha)TRk + C(1 - \alpha)TR'k'\frac{\alpha'}{1 - \alpha'} = C_0(1 - \alpha_0)T_0Rk_0 + C_0(1 - \alpha_0)T_0R'\frac{\alpha_0'}{1 - \alpha_0'}k_0' \qquad (8)$$

Proceeding in a manner similar to that used for the case outside the cell, we obtain the simultaneous equations (9) and (10). Substitutions of $1 - \alpha = [H^+]/([H^+] + K_A)$ and $\alpha'/(1 - \alpha') = K_A/[H^+]'$, where $[H^+]$ and $[H^+]'$ are the hydrogen ion concentrations of extracellular and intracellular phases, respectively, into eq 10, yield eq 11. Dividing both sides of eq 11 by $[H^+]/[H^+]'$, we obtain eq 12. Because the product,

$$C(1 - \alpha)Tk = C_0(1 - \alpha_0)T_0k_0 \qquad (9)$$

$$C(1 - \alpha)T\frac{\alpha'}{1 - \alpha'}k' = C_0(1 - \alpha_0)T_0\frac{\alpha_0'}{1 - \alpha_0'}k_0' \qquad (10)$$

$$C\frac{[H^+]}{[H^+] + K_A}\frac{K_A}{[H^+]'}Tk' = C_0\frac{[H^+]}{[H^+] + K_{A_0}}\frac{K_{A_0}}{[H^+]'}T_0k_0' \qquad (11)$$

$$C\frac{K_A}{[H^+] + K_A}Tk' = C_0\frac{K_{A_0}}{[H^+] + K_{A_0}}T_0k_0' \qquad (12)$$

$CK_A/([H^+] + K_A) = C\alpha$, which is the concentration of ionized form in the extracellular phase, we can replace eq 12 by eq 13. Thus the simultaneous equations (9) and (13) have the same form as eq 5 and 6.

$$C\alpha Tk' = C_0\alpha_0T_0k_0' \qquad (13)$$

Taking logarithms of the simultaneous equations, we obtain eq 14 and 15 for the cases where the sites of action are located outside the cell, and eq 16 and 17 for the intracellular actions. Our basic assumption is that free-energy-related parameters of adsorption

$$\log\frac{1}{C(1 - \alpha)} = \log\frac{1}{C_0(1 - \alpha_0)} + \log\frac{A}{A_0} + \log\frac{k}{k_0} \qquad (14)$$

$$\log\frac{1}{C\alpha} = \log\frac{1}{C_0\alpha_0} + \log\frac{A'}{A_0'} + \log\frac{k'}{k_0'} \qquad (15)$$

$$\log\frac{1}{C(1 - \alpha)} = \log\frac{1}{C_0(1 - \alpha_0)} + \log\frac{T}{T_0} + \log\frac{k}{k_0} \qquad (16)$$

$$\log\frac{1}{C\alpha} = \log\frac{1}{C_0\alpha_0} + \log\frac{T}{T_0} + \log\frac{k'}{k_0'} \qquad (17)$$

such as $\log(A/A_0)$ and $\log(A'/A_0')$ and a similar parameter for transfer process like $\log(T/T_0)$ are governed by "lipohydrophilic character" (or hydrophobic bonding ability) of the substituent and can be

(10) J. Ferguson, *Proc. Roy. Soc.* (London), **B127**, 387 (1939).

(11) M. E. Krahl and G. H. A. Clowes, *J. Cellular Comp. Physiol.*, **11**, 21 (1938).

(12) B. B. Brodie and C. A. M. Hogben, *J. Pharm. Pharmacol.*, **9**, 345 (1957).

formulated by $f(\pi)$.[3] In general, $f(\pi)$ is expressed as a second-order formula of π like $a\pi - b\pi^2$. However, in the cases discussed in this paper, the π^2 term can be deleted as discussed earlier.[3] For a given biological system, the formula for log (A/A_0) is considered to be equal to that for log (A'/A_0'), since the lipohydrophilic character, π, for a certain substituent and the susceptibility of adsorption factor to substituent variation remain approximately constant whatever the parent molecular species may be, neutral or ionic.[7] Then, log (A/A_0), log (A'/A_0'), and log (T/T_0) in these equations can be expressed as $a\pi$. Log (k/k_0), a relative electronic factor for a given substituent on the neutral phenol molecule, is regarded to be different from log (k'/k_0'), the factor for the same substituent on the dissociated phenoxide ion, so that they can be expressed, according to the Hammett relationship, as $\rho\sigma$ and $\rho'\sigma$, respectively, since the electrical charge may play a role here. Substitutions of $1/(1 - \alpha) = ([H^+] + K_A)/[H^+]$, $1/\alpha = ([H^+] + K_A)/K_A$, log (A/A_0), log (A'/A_0'), and log $(T/T_0) = a\pi$, log $(k/k_0) = \rho\sigma$, and log $(k'/k_0') = \rho'\sigma$ into eq 14–17 yield the simultaneous equations (18) and (19), where $c = \log(1/C_0) +$

$$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{[H^+]} = a\pi + \rho\sigma + c \qquad (18)$$

$$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{K_A} = a\pi + \rho'\sigma + c' \qquad (19)$$

log $[(K_{A_0} + [H^+])/[H^+]] \fallingdotseq \log(1/C_0)$ and $c' = \log(1/C_0) + \log[(K_{A_0} + [H^+])/K_{A_0}] \fallingdotseq \log(1/C_0) + \log([H^+]/K_{A_0})$, since K_{A_0} (K_A of phenol) is about 10^{-10} and $[H^+]$ is usually near 10^{-7}. Hence, wherever the sites of action may be located inside or outside the cell for a particular physiological action of the phenols, the structure–activity relationship can be analyzed by the simultaneous equations (18) and (19).

One would think, if a physiological action of the phenols is solely due to the neutral molecule, the structure–activity correlation could be described by eq 18 and, if the active form is the phenoxide ion, the physiological activity would be accommodated by eq 19. However, eq 18 can be modified to eq 18′.

$$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{K_A} + \log \frac{K_A}{[H^+]} = a\pi + \rho\sigma + \log \frac{1}{C_0} \qquad (18')$$

Substituting eq 19 into eq 18′, we obtain eq 20. Therefore, if we use ΔpK_A instead of the Hammett σ constant for a measure of the electronic effect of the substituent, eq 20 can be converted to eq 20′. Hence, eq 18 and 19

$$(\rho - \rho')\sigma = \log \frac{K_A}{K_{A_0}} \qquad (20)$$

$$\log \frac{K_A}{K_{A_0}} \equiv \Delta pK_A = (\rho - \rho')\Delta pK_A \qquad (20')$$

are interrelated *a priori* by eq 21 and 22. Therefore,

$$\rho - \rho' = 1 \qquad (21)$$

$$c' = c + \log \frac{[H^+]}{K_{A_0}} = c + pK_{A_0} - pH \qquad (22)$$

if a physiological action of a series of the phenols at a certain fixed pH is well correlated by eq 18, it does not necessarily mean that the neutral form is responsible for the action, since eq 19 for the dissociated ion can be expected to accommodate the same physiological action. Thus, even if a structure–activity relationship can be analyzed by either eq 18 or 19, we cannot draw a definite conclusion as to the active form. The active form may be either the neutral or the ionized form or both. In order to determine in which form the phenols exert the physiological activity, we have to have the activity data obtained at several different exophase pH values as well as information on the dissociation characteristics of the receptor site(s).[13]

For the apparent relative potency, log $(1/C)$, we obtain biphasic plots: for the compounds of which $K_A \ll [H^+]$, *i.e.*, log $[(K_A + [H^+])/[H^+]]$ in eq 18 approaches zero

$$\log \frac{1}{C} = a\pi + \rho\Delta pK_A + c \qquad (23)$$

and for those of which $K_A \gg [H^+]$, *i.e.*, log $[(K_A + [H^+])/K_A]$ in eq 19 approaches zero

$$\log \frac{1}{C} = a\pi + \rho'\Delta pK_A + c' \qquad (24)$$

The most favorable dissociation constant for the relative potency is calculated by eq 25 and 26 which are derived from eq 18 or 19.

$$\frac{\partial \log \frac{1}{C}}{\partial \log K_A} = \rho - \frac{K_A}{K_A + [H^+]} = 0 \qquad (25)$$

$$K_A = -\frac{\rho}{\rho'}[H^+] \qquad (26)$$

In the following section, the physiological action of phenols will be analyzed on the basis of these mathematical expressions, in particular, with eq 18 and 19 using ΔpK_A instead of the Hammett σ by means of regression analyses. In deriving the equations we have assumed as a first approximation that the adsorption and transfer processes of the substituted phenols relative to those of the unsubstituted are solely determined by "lipohydrophilic" or hydrophobic bonding character of the substituent. However, even if the adsorption and transfer processes would be governed by an electronic effect of the substituent to some extent, eq 18 and 19 would be still obeyed. In this situation, a pure "lipohydrophilic" part of the adsorption and transfer processes could be expressed by the π term, and the electronic factors for adsorption, transfer processes, and for the complex formation could be collected together and represented by the ΔpK_A term in eq 18 and 19. It should be noticed, therefore, that even if the ΔpK_A term is analyzed to make a significant contribution to a certain physiological action of the substituted phenols, we are unable to assign the electronic factor only to the complex formation at the site of action.

Example 1. Toxicity of Phenols to Plants.—Taking the effect of dissociation into account, Blackman and his co-workers tried to analyze the relationship between the activity of phenols causing chlorosis in *Lemna minor* and their structure.[14] Their data for the comparative potency of the phenols are expressed in terms of the concentration of the neutral molecule (C_{cor}) which is present in the apparent equieffective solution.

(13) For a further discussion, see E. J. Ariëns, A. M. Simonis, and J. M. van Rossam, "Molecular Pharmacology," Vol. 1, E. J. Ariëns, Ed., Academic Press Inc., New York, N. Y., 1964, p 363.

(14) G. E. Blackman, M. H. Parke, and G. Garton, *Arch. Biochem. Biophys.*, **54**, 45, 55 (1955).

TABLE I
ACTIVITY OF PHENOLS CAUSING CHLOROSIS IN *Lemna minor*

Substituent	pK_A[a]	ΔpK_A	π	Log $(1/C_{cor})$ Calcd[b]	Log $(1/C_{cor})$ Found	Log $(1/C_{cor})$ + log $([H^+]/K_A)$ Calcd[d]	Log $(1/C_{cor})$ + log $([H^+]/K_A)$ Found
H	9.9	0	0	1.76	1.8	6.56	6.6
4-Cl	9.4	0.5	0.9	2.61	2.7	6.91	7.0
2,4-Cl_2	7.8	2.1	1.6	3.45	3.4	6.15	6.1
2,4,6-Cl_3	6.1	3.8	2.3	4.30	4.5	5.30	5.5
2,4,5-Cl_3	7.0	2.9	2.7	4.52	5.1	6.42	7.0
2,3,4,6-Cl_4	5.3	4.6	3.4	5.37	5.6	5.57	5.8
Cl_5	4.8	5.1	4.4	6.31	6.2	6.01	5.9
2-Me-4-Cl	9.6	0.3	1.4	3.01	3.2	7.51	7.7
2-Me-6-Cl	8.7	1.2	1.2	2.97	2.4	6.57	6.0
3-Me-4-Cl	9.4	0.5	1.5	3.13	3.2	7.43	7.5
4-Me-2,6-Cl_2	7.6	2.3	1.9	3.74	3.4	6.24	5.9
3-Me-2,4,6-Cl_3	6.6	3.3	2.9	4.75	4.7	6.25	6.2
3-Me-2,4,5,6-Cl_4	5.9	4.0	4.0	5.80	5.4	6.60	6.2
2-Me	10.0	−0.1	0.5	2.18	2.2	7.08	7.1
2,6-Me_2	10.6	−0.7	1.0	2.52	2.4	8.02	7.9
2,4-Me_2	10.4	−0.5	1.0	2.55	2.6	7.85	7.9
2,5-Me_2	10.3	−0.4	1.1	2.65	2.6	7.85	7.8
3,5-Me_2	10.1	−0.2	1.1	2.68	2.7	7.68	7.7
2,4,6-Me_3	10.9	−1.0	1.5	2.91	2.8	8.71	8.6
2,3,5-Me_3	10.6	−0.7	1.6	3.04	3.0	8.54	8.5
3-Me-5-Et	10.1	−0.2	1.5	3.03	3.1	8.03	8.1
3,5-Me_2-4-Cl	9.6	0.3	2.0	3.53	3.6	8.03	8.1
2,5-Me_2-4-Cl	9.7	0.2	2.0	3.52	3.6	8.12	8.2
2,6-Me_2-4-Cl	9.9	0	1.9	3.40	3.4	8.20	8.2
3-Me-5-Et-4-Cl	9.6	0.3	2.4	3.88	4.0	8.38	8.5

[a] Taken from Table IV and Figure 6 in ref 14. [b] Calculated by eq 27c. [c] $[H^+] = 10^{-5.1}$. [d] Calculated by eq 28c.

While Blackman, *et al.*, studied 32 substituted phenols, 25 compounds are included in Table I, since as they indicated, the data for the others were obtained under somewhat different experimental conditions. They tried to correlate the activity with pK_A and solubility of the phenols and found that, in mono-, di-, and some of the trisubstituted phenols, log C_{cor} − log solubility is linearly correlated with their pK_A values. However, with the other phenols, in particular, those substituted at *ortho* positions, this kind of correlation is rather poor. They postulated that combined steric and electronic effects of the *ortho* substituent on the hydrogen bond formation between hydroxyl group and biosurface might be operative in these *ortho*-substituted compounds.

Using the method of least squares with the 25 derivatives, the following equations were derived in terms of the equieffective concentration of the neutral form obtained at pH 5.1, where n is the number of points used in the regression, s is the standard deviation, and r is the correlation coefficient. Equation 27c is an

	n	s	r	
$\log \frac{1}{C_{cor}} = 1.077\pi + 1.531$	25	0.275	0.972	(27a)
$\log \frac{1}{C_{cor}} = 0.546\Delta pK_A + 2.902$	25	0.572	0.873	(27b)
$\log \frac{1}{C_{cor}} = 0.146\Delta pK_A + 0.865\pi + 1.758$	25	0.230	0.981	(27c)

application of eq 18 and eq 27a and 27b are those of eq 18 with one term each deleted. The π values for polysubstituted phenols which have not been determined experimentally are those obtained by summing up π values of the individual substituents.[7] Comparison of eq 27a–c would indicate that the role of lipohydrophilic (or hydrophobic bonding) character of the substituent is very important, whereas that of the electronic effect is only of subsidiary significance. An F test indicates, however, that both ΔpK_A and π terms in eq 27c are justified at better than 0.995 confidence level when compared with eq 27a and 27b, respectively (for ΔpK_A term: $F_{1,22} = 10.79$, for π term: $F_{1,22} = 119.87$; $F_{1,22,0.005} = 9.72$). The calculated values for log $(1/C_{cor})$ in Table I were obtained with eq 27c.

The same action of the substituted phenols is also examined in terms of the concentration of the ionic phenolate at pH 5.1 using the value, log $(1/C_{cor})$ + log $([H^-]/K_A)$, for the 25 compounds. Equations 28a–c were obtained by the method of least squares. The difference between coefficients of ΔpK_A term of eq 27c and 28c is equal to 1 and that between constant

	n	s	r	
$\log \frac{1}{C_{cor}} + \log \frac{[H^+]}{K_A} = -0.374\pi + 7.885$	25	0.947	0.385	(28a)
$\log \frac{1}{C_{cor}} + \log \frac{[H^+]}{K_A} = -0.454\Delta pK_A + 7.702$	25	0.572	0.830	(28b)
$\log \frac{1}{C_{cor}} + \log \frac{[H^+]}{K_A} = -0.854\Delta pK_A + 0.865\pi + 6.558$	25	0.230	0.976	(28c)

terms is equal to pK_{A_0} − pH = 9.9 − 5.1 = 4.8, as theoretically expected by eq 21 and 22. It is apparent, therefore, that no definite conclusion can be drawn about the active form. It is noteworthy that eq 27c and 28c give as good a correlation for the polysubstituted as for the mono- and disubstituted phenols. Phenols with and without *ortho* substituents are equally well accomodated by eq 27c or 28c, *i.e.*, steric effects or any other proximity effects of the *ortho* substituent(s)

TABLE II

BACTERIOSTATIC ACTIVITY OF NITROPHENOLS

Substituent	ΔpK_A[a]	π	Log (1/C) + log [(K_A + [H⁺])/[H⁺]][b,c] pH 5.5 Calcd	pH 5.5 Found	pH 6.5 Calcd	pH 6.5 Found	pH 7.5 Calcd	pH 7.5 Found	pH 8.5 Calcd	pH 8.5 Found
3-NO_2	1.6	0.54	2.49	2.6	2.43	2.6	2.39	2.6	2.62	2.7
4-NO_2	2.8	0.50	3.27	3.2	3.33	3.1	3.40	3.1	3.76	3.8
2-NH_2-4-NO_2	2.9	−1.13[d]	2.78	2.7	2.72	2.6	2.86	2.7	3.27	3.1
2,5-$(NO_2)_2$	4.8	0.29[e]	4.52	4.5	4.76	4.9	5.04	5.4	5.61	5.8
2-NH_2-4,6-$(NO_2)_2$	5.5	−1.59[d]	4.34	4.4	4.50	4.6	4.92	5.1	5.60	5.8
2,4-$(NO_2)_2$	5.9	0.04[e]	5.16	5.1	5.49	5.5	5.89	5.8	6.58	6.3
2,6-$(NO_2)_2$	6.3	−0.21[e]	5.33	5.4	5.69	5.7	6.14	5.9	6.87	6.7
2,4,6-$(NO_2)_3$	9.1	−0.12[e]	7.21	7.2	7.86	7.8	8.58	8.6	9.60	9.7

[a] Obtained from ref 17 taking pK_{A_0} = 9.9. [b] C is the lowest molar concentration preventing visible growth of *E. coli* for 4 days. [c] Calculated values are obtained by using eq 29c and 30–32. [d] Calculated values. [e] Determined by Hansch, *et al.* (private communication).

on the hydroxyl group seem to be unimportant. The correlation coefficients 0.981 of eq 27c and 0.976 of eq 28c would indicate unusual precision for the biological test.

The plus sign of the coefficient of the π term in these equations suggests that the higher the π value, the higher the potency of the phenols as weed killers. However, the dissociation of the phenols does not necessarily correlate with the apparent potency. With eq 26, a maximum contribution from the ΔpK_A term to the apparent potency, log $(1/C)$, would be made when $K_A = (0.146/0.854)[H^+]$, *i.e.*, $pK_A = 5.9$.

Example 2. Toxicity of Phenols to Bacteria.—The bactericidal activity of phenols has been analyzed by Hansch and Fujita with the two substituent constants.[3] The results showed little importance for the electronic effect in determining the toxicity of the phenols to both gram-positive and gram-negative bacteria. They used for the analysis the molar phenol coefficients calculated from the extensive work of Klarmann, *et al.*[15] However, the selection of phenols does not cover those phenols for which the electronic effect of substituents varies greatly. The phenols used in the analysis were mostly 3- and 4-alkoxy- and alkylphenols for which ΔpK_A values are very small and do not vary significantly. Since the phenol coefficients were measured by means of the Reddish test in which the pH of the test medium is supposed to be approximately 6.8,[16] those phenols chosen for the analysis should exist exclusively as neutral molecules in the test medium.

Here, we have chosen the work of Cowles and Klotz[17] as another example where the dissociation constant of the phenols varies considerably in order to examine the role of the electronic effect of the substituent. In Table II, the bacteriostatic activity of eight nitrophenols against gram-negative *Escherichia coli* determined at several pH values of the culture medium was recalculated from their data in terms of the equieffective concentration of the neutral molecule. Cowles and Klotz[17] suggested from the pH dependence of the apparent bacteriostatic potency [log $(1/C)$] that the phenol is active essentially in its nonionized form.

The following equations result from the data obtained at pH 5.5. Comparison of the coefficients of correlation for eq 29a–c suggests that, in this case, the electronic effect of the substituent is highly important in determining the bacteriostatic activity of the phenols. Although an F test reveals that the π term in eq 29c is justified at better than 0.995 confidence level ($F_{1,5} = 66.5$, $F_{1,5,0.005} = 22.785$), the role of the π term in this case is much less important than is expected from the earlier analysis by Hansch, *et al.*[3] Probably, the toxicity of phenols against bacteria is governed by both the electronic and lipohydrophilic character of the substituents. Insofar as the mode of toxic action remains unchanged by the structural change, an analysis should include a wide variety of phenols in terms of both electronic and lipohydrophilic character before definite conclusions about the structure–activity relationship can be drawn.

$$\log \frac{1}{C} + \log \frac{K_A + [H^-]}{[H^+]} = -0.039\pi + 4.379 \quad n = 8,\ s = 1.678,\ r = 0.019 \quad (29a)$$

$$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{[H^+]} = 0.639\Delta pK_A + 1.283 \quad n = 8,\ s = 0.287,\ r = 0.985 \quad (29b)$$

$$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{[H^-]} = 0.339\pi + 0.659\Delta pK_A + 1.257 \quad n = 8,\ s = 0.083,\ r = 0.999 \quad (29c)$$

Equations 30–32 were obtained from the data at pH 6.5, 7.5, and 8.5. The fact that the coefficients of these equations do not differ markedly from each other, whereas those of equations derived in terms of the equieffective concentration of the ionized molecule vary considerably with the pH change, might support Cowles and Klotz's view[17] that the active form is essentially nonionic and suggests that the pK_A value (s) of the receptor site (s) would be located at least outside the pH range examined. It has been indicated by Judis[18] that the phenolic disinfectants seem to act at the cell membrane and damage its structure or inhibit respiratory enzymes located at the cell surface so that the site of toxic action of the phenols might be under more or less direct influence of the pH changes of the exophase. A gradual increase of the contribution from the electronic term with increase of the medium pH would suggest an increasing susceptibility of the neutral phenol molecule to interact with electron-donating center(s) of the receptor. The receptor site(s) would be made more nucleophilic in the more basic solution.

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(16) G. F. Reddish, *Am. J. Public Health*, **17**, 320 (1927).

(17) P. B. Cowles and I. M. Klotz, *J. Bacteriol.*, **56**, 277 (1948).

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TABLE III
UNCOUPLING ACTIVITY OF PHENOLS

Substituent	ΔpK_A[a]	π[a]	Log (1/C)[b]	Log (1/C) = log [K_A + (H^+)]/(H^+) Calcd[d]	Found	Log (1/C) = log [K_A + (H^+)]/K_A Calcd[e]	Found
Cl_3	5.1	4.4	1.7	4.15	4.3	1.54	1.7
2,3,4,6-Cl_4	4.6	3.4	1.3	3.51	3.4	1.40	1.3
2,3,5-Cl_3	2.9	2.7	1.3	1.81	1.8	1.40	1.4
2,4-Cl_2	2.1	1.6	0.7	0.88	0.8	1.27	1.2
3-Cl	0.8	1.0*	−0.4	−0.43	−0.4	1.26	1.3
4-Cl	0.5	0.9*	−0.4	−0.72	−0.4	1.26	1.6
2,4-Br_2	2.3	2.0	0.7	1.14	1.0	1.32	1.1
2,4-$(NO_2)_2$	5.9	0.0*	1.0	4.14	4.4	0.74	1.0
2,6-$(NO_2)_2$	6.2	−0.2*	0.6	4.38	4.3	0.68	0.6
4-NO_2	2.9	0.5*	0.6	1.44	1.1	1.03	0.7

[a] Taken from Table II in ref 23. [b] C is the millimolar concentration of the phenol required for the complete uncoupling. [c] $[H^+] = 10^{-7.4}$. [d] Calculated by eq 34c. [e] Calculated by eq 35c. [f] Calculated values except for those with asterisks which were obtained experimentally.

	n	s	r	
$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{[H^+]}$ (at pH 6.5) =				
$0.421\pi + 0.760\Delta pK_A + 0.992$	8	0.112	0.999	(30)
$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{[H^+]}$ (at pH 7.5) =				
$0.385\pi + 0.859\Delta pK_A + 0.802$	8	0.279	0.993	(31)
$\log \frac{1}{C} + \log \frac{K_A + [H^+]}{[H^+]}$ (at pH 8.5) =				
$0.363\pi + 0.962\Delta pK_A + 0.885$	8	1.213	0.997	(32)

In Table II, π values are primary ones except for the amino substituted phenols, for which π values are summed to get a figure, assuming the value for the 2-amino group to be equal to that for the 4-amino group.[5]

Example 3. Uncoupling Activity of Phenols.—Much work has been done on the uncoupling effects of various substituted phenols on oxidative phosphorylation and it has been generally accepted that the uncoupling activity of the phenols is associated with their lipophilic character as well as their degree of dissociation.[19-23] Weinbach and Garbus studied a series of phenols with isolated rat liver mitochondria and determined the relative uncoupling activity by the apparent concentration of phenols required for complete uncoupling, *i.e.*, a minimum concentration at which the P:O ratio becomes zero.[23] From the data in Table III, we have derived eq 33-35.

Although eq 33 shows a good correlation for the apparent concentration, this would be only of practical value perhaps to know a general trend. Equations 34a-c are obtained for the neutral form. From an F test which reveals that the π term in eq 34c is significant at better than 0.975 confidence level ($F_{1,7} = 10.4$, $F_{1,7,0.025} = 8.073$), it can be concluded that both ΔpK_A and π terms are of importance for the equieffective concentration of the neutral form. When we take the equieffective concentration of the phenoxide ion, eq 35a-c were obtained. Although the correlations are not so good as those for eq 34a-c because of the small variance of the value for log $(1/C)$ + log $[([H^+] + K_A)/K_A]$ throughout the series, eq 35c

	n	s	r	
$\log \frac{1}{C} = 0.228\Delta pK_A +$				
$0.273\pi - 0.493$	10	0.315	0.915	(33)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]} =$				
$0.993\Delta pK_A - 1.076$	10	0.345	0.986	(34a)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]} =$				
$0.220\pi + 1.672$	10	2.004	0.172	(34b)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]} =$				
$0.928\Delta pK_A - 0.172\pi - 1.340$	10	0.226	0.995	(34c)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{K_A} =$				
$-0.064\Delta pK_A + 1.404$	10	0.349	0.368	(35a)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{K_A} =$				
$0.166\pi + 0.919$	10	0.267	0.705	(35b)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{K_A} =$				
$-0.069\Delta pK_A + 0.170\pi + 1.144$	10	0.237	0.808	(35c)

relates to eq 34c by eq 21 and 22 as it theoretically should.

Weinbach and Garbus studied the adsorption of phenols to mitochondrial protein and concluded that the phenols reacted with the protein moiety of the intact mitochondria to exert the uncoupling activity.[23] In fact, Hansch and his co-workers have shown recently that the substituent constant π is linearly correlated to the logarithm of the binding constant for a series of phenols and serum albumin.[6] Our result would suggest that the uncoupling activity is linearly associated with hydrophobic bonding ability of the substituent onto a proteinous surface.

As to which is the active agent for the inhibition of oxidative phosphorylation, the neutral or ionized form, there has been some controversy. While DeDeken concluded that the active agent was the neutral form, from the pH dependence of the uncoupling activity of 2,4-dinitrophenol expressed in terms of I_{50} concentration against phosphorylation in yeast,[19] Parker pointed out that it is those phenols which are highly dissociated at a physiological pH which are the most potent uncouplers of oxidative phosphorylation in rat liver mitochondria and considered the ionized form to be the

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TABLE IV
UNCOUPLING ACTIVITY OF PHENOLS

Substituent	ΔpK_A[a]	π	$-\text{Log } (1/C) + \log [(K_A + [H^-])/[H^+]]$[b] pH 5 Calcd	pH 5 Found	pH 6 Calcd	pH 6 Found	pH 7 Calcd	pH 7 Found	pH 8 Calcd	pH 8 Found
2,6-$(NO_2)_2$	6.2	−0.2	6.10	6.1	6.52	6.5	7.05	7.0	7.43	7.4
3,4-$(CH_3)_2$-2,6-$(NO_2)_2$	5.7	0.8	5.82	5.8	6.35	6.3	7.19	7.2	7.84	7.8
4-*i*-Bu-2,6-$(NO_2)_2$	5.6	1.6	6.00	6.0	6.62	6.6	7.50	7.4	8.25	8.2
4-*i*-Am-2,6-$(NO_2)_2$	5.8	2.1	6.45	6.5	7.13	7.3	7.87	8.1	8.58	8.8
4-*i*-Oct-2,6-$(NO_2)_2$	5.8	3.6	7.02	7.0	7.88	7.8	8.59	8.5	9.40	9.3

[a] Taken from Table I in ref 21. [b] Calculated values are obtained using eq 36c and 37–39.

active agent.[20] This might be attributable to work done in two different systems. Hansch, *et al.*, also have analyzed the uncoupling activity of phenols with pK_A and π using Weinbach's data.[6] They suggested that a phenoxide anion might react with an electron-deficient species. However, from the data obtained at a certain fixed pH of the medium, we are unable to conclude whether the uncoupling effect of the phenols is due to either the neutral or the ionized form or both. From Weinbach's data, we are only able to say that if the uncoupling effect is due to the neutral form, the higher the electron-withdrawing and hydrophobic bonding power of the substituent, the higher is the uncoupling efficiency; if the effect is by the ionic form, the lower the electron-withdrawing and the higher the hydrophobic bonding ability of the substituent, the more effective is the uncoupling of the phenoxide ion.

Although both terms of ΔpK_A and π in eq 34c are significant as judged by the F test, the comparison of the correlation coefficients of eq 34c and eq 34a would suggest that the contribution of the lipophilic character is not so important in determining the uncoupling activity of the phenols as that of the electronic effect of the substituent. We have analyzed the work by Hemker[21] as another example to get more insight into the role of lipophilic character. He measured the uncoupling activity of the alkyl derivatives of 2,6-dinitrophenol determining the concentration which induces the highest ATPase activity of rat liver mitochondria. From the data obtained at pH 5 in Table IV, eq 36a–c are derived for the neutral molecule. Although an F test indicates that the ΔpK_A term in eq 36c is significant at better than 0.99 confidence level ($F_{1,2} = 158$, $F_{1,2,0.01} = 98.50$), it is apparent by comparison of the correlation coefficients for eq 36a and 36c that the role of the ΔpK_A term is much less significant than that of the π term in this example. Although the relative significance of the level of the ΔpK_A and π term varies according to the selection of compounds, it is most probable that, for a wide variety of phenols, the two roles for the substituent are equally significant for the uncoupling activity.

	n	s	r	
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]} =$				
$0.278\pi + 5.841$	5	0.306	0.831	(36a)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]} =$				
$0.154\Delta pK_A + 5.385$	5	0.549	0.074	(36b)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]} =$				
$0.382\pi + 1.325\Delta pK_A - 2.034$	5	0.642	0.998	(36c)

Hemker also studied the pH dependence of the concentration for the optimum ATPase activity. From the data at pH 6, 7, and 8, eq 37, 38, and 39 are obtained for the neutral molecule, respectively. The good correlation coefficients in these equations as well as eq 34c would indicate the absence or constancy of steric effects for the uncoupling action of the phenols. The coefficients for eq 36c and 37–39, however, varies considerably according to pH change of the test medium. Conversion of these equations using eq 21 and 22 to those for the ionized form does not improve the situation. This fact would suggest that both the neutral and ionized forms are responsible for the activity, and/or a mitochondrial receptor is pH sensitive.

	n	s	r	
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]}$ (at pH 6) =				
$0.499\pi + 1.337\Delta pK_A - 1.671$	5	0.137	0.988	(37)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]}$ (at pH 7) =				
$0.478\pi + 0.678\Delta pK_A + 2.937$	5	0.189	0.978	(38)
$\log \frac{1}{C} + \log \frac{[H^+] + K_A}{[H^+]}$ (at pH 8) =				
$0.548\pi + 0.286\Delta pK_A + 5.770$	5	0.179	0.986	(39)

As shown in the above analyses, the two roles of substituents of phenols are nicely interpreted through the use of π with ΔpK_A. The relativistic approach by means of substituent constants has been used to make the analysis feasible; *i.e.*, wherever the site of action may be located and whatever the true active form may be, the structure–activity correlation can be analyzed by essentially the same procedure. It should be emphasized that the electronic effect of the substituents, ΔpK_A, is used in the analyses as a counterpart of the Hammett σ constants. The effect of the dissociation should not be confused with ΔpK_A. The analysis using the two substituent constants with correction for the effect of dissociation should help in analyzing the physiological activity of certain classes of ionizable drugs.

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