Structure-Activity Relationships of Three Groups of Uncouplers of Oxidative Phosphorylation: Salicylanilides, 2-Trifluoromethylbenzimidazoles, and Phenols

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The uncoupling activity in rat-liver mitochondria of a number of substituted salicylanilides, 2-trifluoromethylbenzimidazoles, and phenols has been successfully correlated by means of the Hansch free-energy relationships involving the lipophilic and electronic substituent constants. Steric effects seem to play a minor role. The new developed electronic substituent constant of Seth-Paul and Van Duyse has been compared with the commonly used Hammett σ constant. Evidence is presented indicating that the phenolic moiety adjacent to the amide function may be partly responsible for the high uncoupling activity of salicylanilide and its derivatives. Parabolic dependence of the activity is found on the ΔpK_a values. An equation is derived accounting for the concentration of phenols required for maximal stimulation (pC_{opt}) of the latent ATPase of rat-liver mitochondria as a function of the pH of the test medium. Application of the equation leads to the correct prediction of the pH dependence of pC_{opt} for six nitro-substituted phenols. The regression equations seem to be compatible with the Van Dam and Slater theory of uncouplers of oxidative phosphorylation.

Uncouplers and inhibitors of oxidative phosphorylation exhibit a wide range of biocidal activities. Salicylanilides have been shown to possess fungicidal, bacteriostatic, cestocidal, and molluscicidal properties.[1-4] 5-Chloro-2'-chloro-4'-nitrosalicylanilide† is known as a potent cestocidal drug.[5] Burton, *et al.*,[6] noted that 2-trifluoromethylbenzimidazoles have insecticidal and herbicidal activity. Davis, *et al.*,[7] reported on the fasciolicidal activity of 2-iodo-4-cyano-6-nitrophenol. Most members of these three groups of compounds are uncouplers of oxidative phosphorylation in mammalian mitochondria,[8-10] as well as in mitochondria of *Ascaris suum*[11] and *Hymenolepis diminuta*.[2] In order to gain a further insight into the structure-activity relationships of these three groups of compounds, the uncoupling activities of substituted salicylanilides[8] and those of substituted benzimidazoles[9] were investigated. Though the activity data of the substituted phenols of Hemker[12] have already been analyzed by Hansch, *et al.*,[13] and Fujita,[14] it is worthwhile to reconsider these results in terms of the newly developed electronic substituent constant of Seth-Paul and Van Duyse.[15] It will be shown in this paper that this set of electronic constants tends to provide statistically significant equations which in some cases are superior to those obtained by employing the usual Hammett constants.[16] In brief, the present study is an attempt to provide a number of structure-activity relationships describing the uncoupling activity of the three title compounds in rat-liver mitochondria.

Method

Hansch and coworkers[17-22] established that, in a homogenous series of compounds, where typical steric effects are absent, the biological response of a compound can be rationalized in terms of the hydrophobic substituent constant π and a suitable electronic substituent constant. In exploring the effect and the use of the various electronic constants, the following parameters σ, σ^-, σ^+, and E_R were found to be most useful.[23,24] The multiple regression equation relating the biological response to a linear combination of hydrophobic and electronic properties of a drug is

$$\log(1/C) = pC = a\pi^2 + b\pi + c\sigma + \ldots k \qquad (1)$$

where C stands for the molar concentration of a drug causing a standard response. The constants a, b, and c and the intercept k are computed by means of computerized regression techniques. In addition to the commonly used Hammett σ electronic substituent constant, which unfortunately lacks values for ortho substituents, the origin of the previously mentioned δ constant will be discussed briefly prior to its use in the Hansch type analysis. This constant has been developed not only to find new constants bridging the Hammett σ and Taft σ^* values (which are not defined in one and the same scale) but also for those groups which are lacking in the Hammett and Taft compilations. From ir measurements, many carbonyl frequencies of simple R'R''C=O compounds were measured in dilute carbon tetrachloride. Seth-Paul and Van Duyse[15] assigned numerical values X(R) to the substituents R' and R'' fitting the equation

$$\nu(C{=}O) = a + b\,[X(R') + X(R'')]$$

For over 450 carbonyl stretching frequencies, it was found that a = 1583 cm^{-1} and b = 38.2. Some typical X(R) values obeying this equation are given in Chart I. It will be noticed

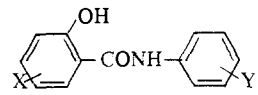
Chart I

R	X(R)	R	X(R)	R	X(R)
F	**3.750**	Br	**2.800**	H	**2.130**
Cl	**3.150**	OMe	2.770	Me	1.807
OH	3.100	OEt	2.570	*t*-Bu	1.658
OPh	3.090	CCl_3	2.440	PhNH	1.390
CF_3	2.925	I	**2.400**	Ph	1.070

that the bold-faced values resemble the Pauling electronegativities. Using the notation $\delta_\alpha{}^R = X(R) - X(\alpha)$, each X(R) value may be compared with that of the parent group α. Thus with α = Me the $\delta_\alpha{}^R$ values are positive for groups such as CF_3 and CCl_3 and negative for *t*-Bu. Likewise, with α = phenyl, the $\delta_\alpha{}^R$ value is positive for *p*-NO_2Ph and negative for *p*-NH_2Ph. The $\delta_{Ph}{}^R$ value of a polysubstituted aromatic ring equals $\delta_{Ph}{}^R = \Sigma_i^n \delta_i$. Only $\delta_{Ph}{}^R$ values will be used in this paper; the super- and subscripts R and Ph are therefore omitted. It has been shown that for substituted phenyl rings, $\delta = \beta\sigma/10$, where σ refers to the Hammett constant. The slope β equals 2 and 5 for para-substituted electron-attracting and electron-donating substituents, respectively, and 3 for meta-substituted groups. Thus the δ value is a measure of the electronic effect exerted by a substituent on an aromatic nucleus in a similar fashion as the Hammett constant σ. The main advantage of the δ set of constants lies in the

†Yomesan.

Table I. Biological Data and Physicochemical Constants of Substituted Salicylanilides



No.	X	Y	$\Sigma\pi^a$	$\Sigma\delta^b$	$\Sigma\sigma^c$	pI_{50} Obsd[d]	pI_{50} Calcd[e]	Δ obsd − calcd
1	H	H	0.00	0.000	0.000	4.88	5.07	−0.19
2	5-Cl-3-(4-Cl-C_6H_4)	4′-Cl	4.68	0.087	0.463	7.60	7.25	0.35
3	5-Cl	2′-Cl-4′-NO_2	2.12	0.466	1.724	7.69	7.61	0.08
4	5-Cl-3-C_6H_5	3′,4′-Cl_2	4.79	0.199	0.836	7.81	7.71	0.10
5	5-Cl-3-C_6H_5	2′,5′-Cl_2	4.55	0.318	0.836	7.84	8.04	−0.20
6	5-Cl-3-C_6H_5	2′,4′,5′-Cl_3	5.48	0.364	1.063	8.19	8.58	−0.39
7	5-Cl-3-C_6H_5	3′-Cl-4′-NO_2	4.36	0.408	1.879	8.21	8.30	−0.09
8	5-Cl-3-(4-Cl-C_6H_4)	2′-Cl-5′-NO_2	4.98	0.419	1.173	8.58	8.58	0.00
9	5-Cl-3-(4-Cl-C_6H_4)	2′-Cl-4′-CN	4.58	0.386	1.091	8.66	8.31	0.35
10	5-Cl-3-(4-Cl-C_6H_4)	2′-Cl-5′-CF_3	5.93	0.335	0.878	8.70	8.66	0.04
11	5-Cl-3-(4-Cl-C_6H_4)	2′,4′,5′-Cl_3	6.41	0.364	1.063	8.76	8.96	−0.20
12	5-Cl-3-*t*-Bu	2′-Cl-4′-NO_2	4.00	0.536	1.527	8.77	8.62	0.15

[a]From ref 20 (phenol system). [b]From ref 15. Position 5 is para in X-phenyl and meta in Y-phenyl. [c]From ref 16. [d]From ref 8. pI_{50} is not corrected for ionization effects. It may be expected that all compounds except 1 should have very similar pK_a values because of the minor variation of the X substituents. [e]Calculated using eq 3.

fact that this set is also defined for ortho substituents, hence eliminating the need of employing para σ values for the ortho substituents. It is therefore of interest to compare regression equations in terms of σ constants with those containing δ in order to assess the usefulness of this new set of constants in SAR studies. The use of the δ constant in a Hansch type of analysis has been reported.[25]

Results

Salicylanilides. The biological data and the substituent constant values are collected in Table I. The $\Sigma\pi$, $\Sigma\delta$, and $\Sigma\sigma$ values are the sum of the individual π, δ, and σ values for the substituents of both phenyl rings. Equations 2 and 3

	n	*S*	*r*	*F*	
$pI_{50} = 0.452\Sigma\pi\ (7.196) + 0.951\Sigma\sigma\ (4.541) + 5.027$	12	0.349	0.955	47.14	(2)
$pI_{50} = 0.391\Sigma\pi\ (8.476) + 3.810\Sigma\delta\ (6.957) + 5.066$	12	0.250	0.977	95.58	(3)

are derived from the data given in Table I. The figures in parentheses are the t-test values (Student's t test), *n* is the number of compounds submitted to the regression analysis, *S* the standard error of the estimate, *r* the correlation coefficient, and *F* is the overall statistical significance of the equation. It is obvious that eq 3 featuring $\Sigma\delta$ is superior to eq 2. Inspection of Table I reveals that all compounds, except the parent compound, possess ortho substituents relative to the OH group. It thus appears that the δ constant being defined for ortho substituents can be employed instead of the usual procedure of using para Hammett values for ortho substituents. In the hope of delineating the electronic requirements of the X and Y substituted phenyl rings, regression equations have been generated in terms of $\Sigma\pi$ and either $\Sigma\delta$(X), $\Sigma\delta$(Y), $\Sigma\sigma$(X), and $\Sigma\sigma$(Y). It was found that none of these could match the quality of eq 3 (see later discussion). Equation 3, covering a 7700-fold potency range, suggests that the activity of this class of compounds is enhanced by increasing the lipophilic and electron-withdrawing character of the substituents. A t test involving an additional $\Sigma\pi^2$ term in eq 3 shows this term to be significant only at the 90% confidence level ($t = -1.89$). The calculated pI_{50} values are listed in Table I.

2-Trifluoromethylbenzimidazoles. Preliminary correlations of the pK_a values with the electronic substituent constants δ and σ revealed that (i) the substituents should be considered relative to the NH group and (ii) the δ constant yields a better correlation. From the data presented in Table II, eq 4-6 were derived. Equation 6 accounts reasonably well for the variation of the pK_a values of the substituted 2-trifluoromethylbenzimidazoles over a range of

	n	*S*	*r*	*F*	
$pK_a = -6.682\Sigma\delta_A\ (-10.545) + 8.486$	22	0.580	0.921	111.20	(4)
$pK_a = -1.705\Sigma\sigma_B\ (-10.519) + 8.114$	22	0.581	0.920	110.66	(5)
$pK_a = -6.179\Sigma\delta_B\ (-15.934) + 8.182$	22	0.401	0.963	253.89	(6)

5.4 pK_a units. Since the benzimidazoles listed in Table II are weak acids, and therefore partially dissociated under the given test conditions, p*C* is corrected for ionization in the following manner

$$pC_{corr} = pC + \log([H^+] + K_a)/[H^+] \quad (7)$$

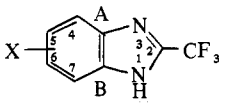
where K_a is the ionization constant and $[H^+]$ the hydrogen ion concentration of the test medium.

From the data of Table II, eq 8-10 are formulated. It is readily seen that eq 10 is to be preferred over eq 8 and 9 despite the excellent fit between pK_a and $\Sigma\delta$ in eq 6. Secondly, the equation featuring $\Sigma\delta$ is superior to the one containing $\Sigma\sigma$. Equation 10 indicates that the uncoupling of oxidative phosphorylation is enhanced by increasing the lipophilic character of the substituents. As far as the electronic behavior of the substituents is concerned, eq 10 indicates that low pK_a values exert a beneficial effect on the activity of these compounds. Differentiating eq 10 with respect to ΔpK_a leads to the optimal value $\Delta pK_a = 5.0$ (4.3-6.3).‡ It is felt that ΔpK_a should be considered as a measure of the electronic effect of the substituents[14] (see also Discussion).

Substituted Phenols. Hemker[12] determined the concentration of substituted phenols required for maximal stimulation of the latent ATPase in rat-liver mitochondria as a function

‡The 90% confidence levels were calculated by Mr. L. Gijpen. See also discussion in ref 26.

Table II. Biological Data and Physicochemical Constants of Substituted 2-Trifluoromethylbenzimidazoles



No.	X	ΔpK_a[a]	$\Sigma\delta_A$[b]	$\Sigma\delta_B$	$\Sigma\sigma_B$	$\Sigma\pi_B$	pC_{corr} Obsd[a]	pC_{corr} Calcd[c]	Δ obsd − calcd
1	H	0.0	0.000	0.000	0.000	0.00	3.54	3.56	−0.02
2	5-Me	−0.1	−0.020	−0.085	−0.170	0.49	3.67	3.60	0.07
3	5-Me-6-Cl	0.5	0.026	0.027	0.203	1.47	5.14	4.95	0.19
4	5-Cl	0.8	0.112	0.046	0.227	0.93	5.08	5.14	−0.06
5	5-CN	1.6	0.184	0.180	0.628	0.14	5.68	5.79	−0.11
6	4,6-Cl_2-5-Me	1.8	0.191	0.139	0.576	2.45	6.46	6.97	−0.51
7	5,6-Cl_2	1.4	0.158	0.158	0.600	1.91	6.40	6.31	0.09
8	4,5-Cl_2	1.8	0.277	0.158	0.600	1.91	6.82	6.74	0.07
9	4,5,6-Cl_3	2.6	0.323	0.270	0.973	2.89	7.76	7.88	−0.12
10	4-Br-5,6,7-Cl_3	3.5	0.520	0.440	1.218	3.77	8.78	8.81	−0.03
11	4,5,6,7-Br_4	3.0	0.530	0.530	1.246	4.36	8.31	8.78	−0.47
12	4,6,7-Cl_3	3.2	0.323	0.389	0.973	2.65	8.54	8.18	0.36
13	4,5,6,7-Cl_4	3.8	0.435	0.435	1.200	3.67	9.50	8.90	0.60
14	4-NO_2	2.0	0.400	0.213	0.710	0.47	5.73	6.34	−0.61
15	5-NO_2	2.1	0.213	0.255	1.270	0.49	6.97	6.44	0.53
16	4,6-$(NO_2)_2$	3.8	0.655	0.426	1.420	0.94	7.70	7.75	−0.05
17	5,6-$(NO_2)_2$	3.8	0.468	0.468	1.980	0.96	7.33	7.76	−0.43
18	6-Cl-4-NO_2	2.5	0.446	0.325	1.083	1.45	7.41	7.19	0.22
19	4-Cl-6-NO_2	3.3	0.420	0.325	1.083	1.45	8.03	7.73	0.30
20	6-Cl-5-NO_2	2.8	0.259	0.367	1.643	1.47	7.53	7.43	0.10
21	4,6-Cl_2-5,7-$(NO_2)_2$	5.4	0.637	0.879	3.286	2.78	8.59	8.72	−0.13
22	4,6,7-Cl_3-5-NO_2	4.7	0.536	0.644	2.243	3.15	8.88	8.89	−0.01

[a] From ref 9. $\Delta pK_a = 8.8 - pK_a$. [b] Subscript A refers to position A in the benzimidazole ring. Position 5 is para relative to point B and meta relative to A. [c] Calculated using eq 10.

Table III. Observed[a] and Calculated[b] Uncoupling Activity (pC_{opt}) of Substituted Phenols in the pH Range 5–8

Substituents				Physicochemical constants			pC_{opt} pH 5		pH 6		pH 7		pH 8	
2	3	4	6	Σπ	Σδ	Σσ	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd
H	H	NO_2	H	0.50	0.255	1.270	3.30	3.32	3.37	3.37	3.51	3.42	3.39	3.47
NO_2	H	H	NO_2	−0.21[c]	0.800	2.540	4.77	4.76	4.22	4.25	3.68	3.74	3.12	3.23
NO_2	Me	Me	NO_2	1.08	0.695	2.201	4.92	4.91	4.51	4.59	4.36	4.27	3.95	3.95
NO_2	H	*i*-Bu	NO_2	1.86	0.703	2.425	5.16	5.22	4.88	4.95	4.71	4.68	4.48	4.42
NO_2	H	*i*-Pe	NO_2	2.36	0.743	2.315	5.50	5.53	5.42	5.26	5.18	4.99	4.92	4.72
NO_2	H	*i*-Oct	NO_2	3.86	0.743	2.315	6.08	6.08	5.89	5.92	5.64	5.76	5.41	5.60

[a] Taken from ref 12. [b] Calculated using eq 20. [c] Determined from the log *P* values of phenol (log *P* = 1.46) and 2,6-dinitrophenol (log *P* = 1.25), ref 20, 21.

	n	*S*	*r*	*F*	
$pC_{corr} = 0.580\Sigma\pi_B\,(6.414) + 3.104\Sigma\sigma_B\,(8.625) - 0.680\Sigma\sigma_B{}^2(-6.090) + 3.839$	22	0.531	0.951	82.98	(8)
$pC_{corr} = 0.436\Sigma\pi_B\,(4.540) + 10.616\Sigma\delta_B\,(9.177) - 8.209\Sigma\delta_B{}^2(-5.988) + 4.161$	22	0.440	0.968	91.04	(9)
$pC_{corr} = 0.421\Sigma\pi_B\,(5.843) + 1.641\Delta pK_a\,(9.843) - 0.162\Delta pK_a{}^2(-5.383) + 3.560$	22	0.338	0.981	158.80	(10)
$pC_{opt}\,(pH\,5) = 0.366\Sigma\pi\,(31.033) + 3.172\Sigma\delta\,(37.177) + 2.305$	6	0.036	0.999	1668.80	(11)
$pC_{opt}\,(pH\,6) = 0.454\Sigma\pi\,(12.662) + 2.163\Sigma\delta\,(8.325) + 2.579$	6	0.111	0.995	160.92	(12)
$pC_{opt}\,(pH\,7) = 0.506\Sigma\pi\,(11.351) + 1.105\Sigma\delta\,(3.428) + 2.991$	6	0.138	0.992	90.04	(13)
$pC_{opt}\,(pH\,8) = 0.585\Sigma\pi\,(10.173) + 0.429\Sigma\delta\,(1.030) + 3.009$	6	0.178	0.988	60.89	(14)

of the pH of the medium. Hansch, *et al.*,[13] correlated these data (pH 5) in terms of the lipophilic character and the pK_a of the phenols. However, from the data collected in Table III, regression equations (11–14) can be formulated in terms of π and δ at four different pH values. Equations 11–14 indicate that the concentration necessary to produce maximal ATPase activity is lowered by enhancing the lipophilic and electron-withdrawing character of the substituents. The same state of affairs seems to prevail for the ATPase activity in *Ascaris suum* mitochondria.[11] It must be noted, however, that Stockdale and Selwyn[10] arrived at the opposite conclusion based upon a multiple regression equation derived from data of five substituted phenols. Their equation, featuring a negative coefficient for $\Sigma\sigma$, suggests that pC_{opt} increases with increasing pK_a. No explanation can be offered for these conflicting results.

Inspection of eq 11–14 reveals a linear relationship between the coefficients of $\Sigma\pi$ and $\Sigma\delta$ and pH (Figure 1). Due to the markedly reduced confidence level of the coefficient of $\Sigma\delta$ in eq 14, it is seen that the linear relationship between the coefficients of $\Sigma\delta$ and pH is no longer strictly obeyed at pH 8. The intercepts of eq 11–14 are approximately linearly related to the pH of the medium. On the basis of these results, the more general expression of eq 11–14 can be formulated as

$$pC_{opt} = f_1(pH)\Sigma\pi + f_2(pH)\Sigma\delta + f_3(pH) + k_1 \quad (15)$$

Analysis of the coefficients of $\Sigma\pi$ and $\Sigma\delta$ as a function of

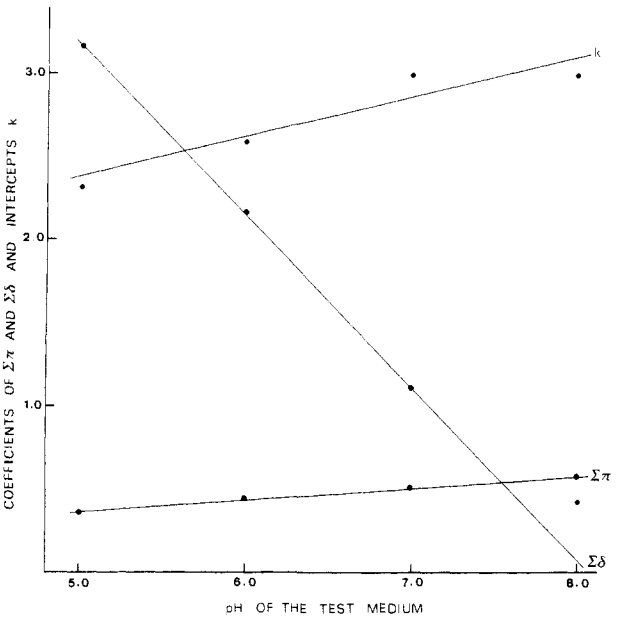


Figure 1. The coefficients of $\Sigma\pi$ and $\Sigma\delta$ and the intercepts k of eq 11-14 against pH.

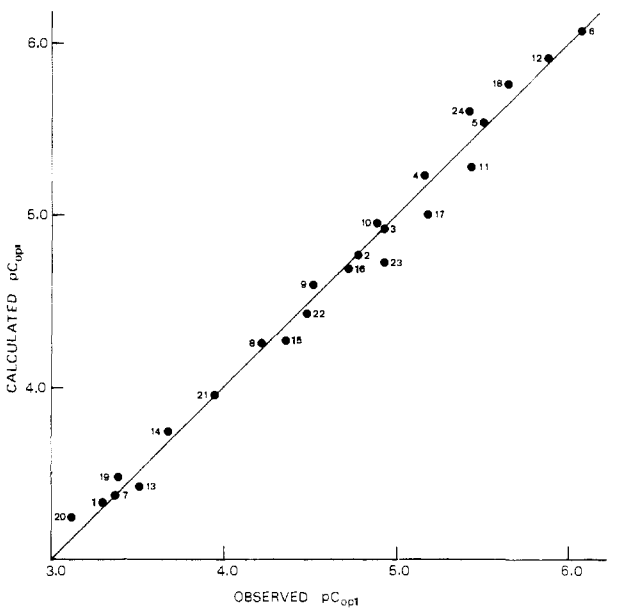


Figure 2. Calculated pC_{opt} against observed pC_{opt} of substituted phenols stimulating the latent ATPase in rat-liver mitochondria.

pH leads to the following equations

$$f_1 = a\text{pH} + c_0 \quad (16)$$

$$f_2 = b\text{pH} + c_1 \quad (17)$$

where $a = 0.073$, $b = -1.034$, $c_1 = 8.34$, and $c_0 = 0$; *i.e.*, f_1 passes through the origin.

For the present purpose it suffices to note that

$$f_3 = d\text{pH} + c_2 \quad (18)$$

Substitution of eq 16-18 into eq 15 allows one to express the pC_{opt} values of the six phenols at four different pH values into a single equation.

$$pC_{opt} = p(\Sigma\pi)(\text{pH}) + q(\Sigma\delta)(\text{pH}) + r(\Sigma\delta) + s(\text{pH}) + k_2 \quad (19)$$

The foregoing treatment is fully substantiated by taking a linear combination of the terms $(\Sigma\pi)$(pH), $(\Sigma\delta)$(pH), $(\Sigma\delta)$, and pH leading to the multiple regression equation.

Equation 20 covers a 910-fold activity range and "explains" 99% of the activity of the six phenols in the pH range 5-8. The excellent agreement between observed and calculated pC_{opt} values is depicted in Figure 2. It should be noted that eq 20 in terms of Hammett σ gives rise to a slightly worse equation (21). Rearrangement of eq 20 yields

	n	S	r	F	
$pC_{opt} = 0.073(\Sigma\pi)(\text{pH})\ (28.191) - 0.935(\Sigma\delta)(\text{pH})\ (-8.795) + 7.797(\Sigma\delta)\ (11.134) + 0.252(\text{pH})\ (3.488) + 1.081$	24	0.106	0.994	387.20	(20)
$pC_{opt} = 0.075(\Sigma\pi)(\text{pH})\ (22.538) - 0.398(\Sigma\sigma)(\text{pH})\ (-6.564) + 3.306(\Sigma\sigma)\ (8.280) + 0.501(\text{pH})\ (3.736) - 1.002$	24	0.139	0.989	224.04	(21)

$$pC_{opt} = (0.073\Sigma\pi - 0.935\Sigma\delta + 0.252)\text{pH} + 7.797\Sigma\delta + 1.081 \quad (22)$$

from which the pH dependence of pC_{opt} is easily obtained by taking the partial derivative of eq 22 with respect to pH

$$\partial pC_{opt}/\partial\text{pH} = 0.073\Sigma\pi - 0.935\Sigma\delta + 0.252 \quad (23)$$

Equation 23 indicates that the pH dependence of the activity is governed by the difference between the lipophilic and the electronic character of the substituents. Equation 23 rationalizes the fact that 4-nitrophenol with its relatively low $\Sigma\delta$ value has a positive $\partial pC_{opt}/\partial\text{pH}$ value compared with a negative value for the remainder of the series. The observed and calculated $\partial pC_{opt}/\partial\text{pH}$ values are listed in Table IV. The good agreement is taken as a justification of the approximations made in the derivation of eq 23. It is interesting to note that Fujita[14] also studied the data of Hemker[12] in terms of the lipophilic constant π and ΔpK_a. He considered two sets of equations, one for the neutral form of the phenols and the other for the ionized species. Inspection of the coefficients of the equations for the neutral form at pH 5, 6, 7, and 8 does reveal a fairly similar trend with respect to the change of the pH of the test medium. Analysis of the regression equations of ref 14 shows that the coefficients of ΔpK_a sharply decrease with increasing pH, whereas the coefficients of $\Sigma\pi$ have a gently increasing trend with pH.

The magnitude of the slope of the equation relating the $\Sigma\pi$ coefficients of eq 11-14 might suggest that the mitochondrial membranes stay fairly intact under the changing pH conditions of the test medium. The positive sign of the slope indicates that the compounds are increasingly desol-

Table IV. Observed[a] and Calculated[b] pH Dependence of the Uncoupling Activity of Substituted Phenols

	Substituent				$\partial pC_{opt}/\partial\text{pH}$	
No.	2	3	4	6	Obsd	Calcd
1	H	H	NO_2	H	0.041	0.050
2	NO_2	H	H	NO_2	-0.549	-0.511
3	NO_2	Me	Me	NO_2	-0.306	-0.319
4	NO_2	H	*i*-Bu	NO_2	-0.211	-0.269
5	NO_2	H	*i*-Pe	NO_2	-0.213	-0.271
6	NO_2	H	*i*-Oct	NO_2	-0.226	-0.161

[a] Determined from the experimental data from Table III. [b] Calculated using eq 23.

vated as a function of the pH. From the diminishing role of $\Sigma\delta$ (reflected in its coefficients) with increasing pH, it might be argued that the degree of ionization of these phenols under the given test conditions rapidly becomes irrelevant at higher pH values. On the other hand, it is seen that these compounds being almost completely ionized in the given pH range (except for 4-nitrophenol) are less active with increasing pH values of the test medium. However, 4-nitrophenol which is progressively ionized in the pH range 5-8, shows an increasing activity with increasing pH. In fact, Hemker[12] observed a linear relationship between pC_{opt} and the pH for a number of substituted phenols (see also Table IV). This linear relationship is, however, characterized by a reversal of the slope and a change of the magnitude of this slope once the concentration of the ionized species approximately equals that of the neutral species, that is, when $pH \approx pK_a$. From this result it could be inferred that the activity (pC_{opt}) is enhanced by increasing the [ionized]/[neutral] ratio until the optimal ratio 1:1 is reached. Any further increase of the pH of the test medium and possibly that of the biophase then causes an unfavorable ratio and a concomitant decrease of the activity. This hypothesis, however, is hard to reconcile with the fact that 2,6-dinitrophenol, for instance (pK_a = 3.7), is already almost completely ionized at pH 5 but still shows a pC_{opt} which is linearly related to the pH in the range 5-8. A more plausible explanation therefore would be the assumption of the existence of two opposing effects, *i.e.*, the favorable effect of the increase of the concentration of the ionized species, reflected by the positive sign of the coefficients of $\Sigma\delta$ in eq 11-14, and the opposing effect of the unfavorable alteration of the mitochondrial receptor due to the increase of the pH of the test medium. If so, it is easily seen that the degree of ionization of these phenols becomes irrelevant because of the reduced response capability of the receptor at an unfavorable pH. It is conceivable to think that profound conformational changes can take place at the receptor when the pH of the test medium sufficiently deviates from the optimal pH conditions.

It is to be noted that eq 22 is derived from biological data pertaining to only six nitro-substituted phenols, thereby casting some doubt on its general validity. It is therefore felt that eq 22 should be subjected to a more stringent test by its application to a larger number of data from phenols or other uncoupling compounds featuring wider structural variations. As to the physical meaning of the cross terms appearing in eq 22, no convincing explanation (apart from the fact that it works) can yet be offered.

Discussion

The regression eq 3, 10, and 11-14 for the three groups of uncouplers have in common that they account for the uncoupling activity solely in terms of the lipophilic and electronic character of the substituents. They all substantiate the fact that the activity is enhanced by increasing the lipophilic and the electron-withdrawing character of the substituents and that steric factors are of no or minor importance. It is interesting to note that a similar analysis[27] of the malate-induced inhibition of the P_i incorporation into organic phosphate in *Ascaris suum* mitochondria by 23 substituted phenols revealed a small but nevertheless significant contribution from the steric factor at the ortho position. This finding, however, might have been made possible by the careful selection of the compounds whose substituents covered a broad range of $\Sigma\sigma$, $\Sigma\pi$, and E_s values which is a prerequisite for entangling the roles of the various factors involved.

It is observed that the three groups of compounds have a chemical group from which a proton is dissociable which is taken as a general characteristic feature of an uncoupling compound.[28-30] For example, Skulachev, *et al.*,[31] reported that *N*-methyl-2-trifluoromethylbenzimidazole (*N*-methyl-TFB) is totally inactive as an uncoupler of oxidative phosphorylation in rat-liver mitochondria, whereas compound **13** (Table II) is seen to be very potent. Further evidence for the importance of the NH proton is furnished by the fact that *N*-methyl-4,5,6-trichloro-2-TFB was found to be inactive as an uncoupler in rat-heart mitochondria.[32] Salicylanilide differs structurally from phenol and benzimidazole by the presence of an OH and a CONH group. It could well be that the presence of both groups in one and the same molecule is partly responsible for the high uncoupling activity of this compound. This is supported by the fact that salicylanilide is 70 times more potent as an inhibitor of the malate induced P_i incorporation into organic phosphate in *Ascaris suum* mitochondria than phenol and 100 times more potent than benzimidazole.[11] With log *P* values ranging from log *P* = 1.46 (phenol[20] and benzimidazole[§]) to log *P* = 3.31 (salicylanilide[§,33]), it is rather hard to accept that merely due to its 70-fold larger lipophilic character, salicylanilide would possess an approximately 100-fold higher potency than phenol and benzimidazole. In fact, examination of the $\Sigma\pi$ coefficients of eq 3, 10, and 11-14 reveals that the activity increase due to the lipophilic character alone lies between 0.4 and 0.5 log activity units (at physiological pH); that is, a tenfold increase of lipophilicity results only in an approximately threefold activity increase. On the other hand, it is interesting to compare the pK_a values of phenol (9.99),[34] benzimidazole (5.55, 12.78),[35] and salicylanilide (7.11).[36] Assuming the local pH at the reaction site in the biophase to be equal to the bulk physiological pH, it follows that phenol and benzimidazole are virtually completely undissociated whereas salicylanilide is present as an almost equally populated mixture of dissociated and neutral species.

Hence, assuming the presence of an anion to be of major importance for high activity, it follows that only salicylanilide can meet this requirement to a certain extent. Although the lipophilic and electronic character of salicylanilide point toward a high activity, it is felt that structural features may also be involved. In other words, it is suggested that the presence of a phenolic moiety adjacent to an amide function represents an important feature of an uncoupling molecule.

It is recalled that $\Sigma\delta$ or $\Sigma\sigma$ in eq 2 and 3 are the sums of the electronic contributions of the substituents on *both* rings. It is felt that this sum is a reflection of the electronic behavior of the hydrogen-bonded amide-phenolic moiety in salicylanilide.

In addition, Whitehouse,[37] in a study of the uncoupling activity of nonsteroidal antiinflammatory drugs, reported that *N*-methyl and *N*-phenyl derivatives of salicylanilide were inactive as uncouplers at twice the concentration at which salicylanilide completely uncouples oxidative phosphorylation. Furthermore, 2-methoxybenzanilide, 2-benzamidophenol, and 4-hydroxybenzanilide are all virtually inactive as uncouplers.[37]

Questions may be raised regarding the biochemical inter-

[§] Experimentally determined value using the method described in ref 20.

pretation of the terms appearing in the regression equations. As has been pointed out by Cammarata, *et al.*,[38] a multiple regression equation can be used as a predictive tool or as a means of proposing a reaction mechanism model. Considering the current hypotheses on uncoupling activity, *i.e.*, the chemiosmotic hypothesis of Mitchell,[39] the view[30] that uncouplers act as acids or bases catalyzing the hydrolysis of a high-energy chemical intermediate and the Van Dam and Slater theory,[40,41] it appears that the equations can be reconciled with the last mentioned view. In fact, the equations are consistent with the requirement of an anionic uncoupling species U (low pK_a value). According to the theory U^- is actively transported through the membrane by an ion translocation system. Once inside, U^- recombines with H^+ to form UH which then passively diffuses outward through the membrane. This process is enhanced by the high log P values of UH.

The parabolic relationship of eq 10 appears to be in agreement with the hypothesis[41] that substrate ions and U^- competitively interact with the ion translocating system, in the sense that a more suitably charged U^- could be the better uncoupler.#

It has been shown by Weinbach and Garbus[42] that uncouplers interact with mitochondrial protein. In the light of this finding it is not unreasonable to suspect that the anomalous activity of salicylanilide is related to a near optimal interaction process between the ion translocating system and the salicylanilide ion.

In this paper it has been shown that by means of the extrathermodynamic approach structure-activity problems can be rationalized by putting them on a mathematical basis. To a large extent, the ultimate success of the treatment depends upon the availability of accurate biological data obtained from measurements on a series of structurally related compounds. In addition, these compounds should possess the widest possible variation in their lipophilic and steric substituents.

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#Preliminary simple Hückel calculations show a relationship between pC_{corr} and the excess π charge of compounds 1–13 of Table II except for compound **5**. No such correlation is found for the nitro-substituted compounds **14–22** which along with the CN-substituted compounds are all π electron deficient. In other words, all compounds possessing substituents with $\sigma_R^\circ > 0$ do not obey the relationship.