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Quantitative analysis of uncoupling activity of substituted phenols with a physicochemical substituent and molecular parameters

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The uncoupling potency of a series of substituted phenols with rat-liver mitochondria was analyzed quantitatively with physicochemical substituent and molecular parameters such as log *P*, *P* being the partition coefficient in a phosphatidylcholine liposome/water system, log K_A, K_A being the acid dissociation constant, and the Taft-Kutter-Hansch steric constant, E_s, for *ortho*-substituents. The potency evaluated from the concentration in the medium required for a defined response was analyzed, showing that the incorporation of compounds in terms of log *P*, a certain balance between neutral and ionized forms expressible by a parabolic function of log K_A and the steric shielding effect of the *ortho*-substituents on the negatively charged center of ionized form are highly significant factors governing the variations in potency. The potency was also quantitatively separated into the intrinsic potency as the protonophore inside the inner mitochondrial membrane and the incorporation factor in terms of log *P*. Some phenols found as outliers from the correlations and some others distorting the quality of the correlations were shown to have inhibitory effects on the respiratory chain by specific and non-specific modes of action, respectively, besides uncoupling activity.

Introduction

The shuttle-type mechanism of acidic uncouplers with bioenergy-transducing membranes such as mitochondria and chloroplasts has been widely accepted [1–3]. The mechanism assumes that uncoupler molecules incorporated in the membrane phase shuttle protons and collapse the electrochemical proton gradient across the membrane. Attempts [4–9] have been made to analyze the physicochemical factors governing the uncoupling potency of a number of series of uncouplers in terms of physicochemical parameters of the molecule.

We have shown [3,10] that the uncoupling potency with rat-liver mitochondria of a wide variety of substituted phenols including SF6847, the most potent uncoupler so far known, is quantitatively analyzable with the protonophoric potency across phosphatidylcholine liposomal membranes when such factors as the statibility of anionic species in the membrane phase related to their dissociation constant and the difference in the pH conditions of the extramembranous aqueous phase between mitochondrial and liposomal experiments are taken into consideration. Although this finding has been believed to strongly support the shuttle-type mechanism of the uncoupling action of weakly acidic uncouplers, the protonophoric potency itself of phenols across the phosphatidylcholine membrane is related not only to their hydrophobicity, defined as the partition coefficient into liposomal phase, but also to their dissociation constant [8]. Thus, it is not unreasonable that a number of earlier quantitative structure–activity analyses [4–9] have used the dissociation constant or the Hammett σ as its counterpart along with hydrophobicity as explanatory independent parameters.

In the earlier analyses, the potency of the uncoupling action has been expressed in terms of the reciprocal of the uncoupler concentration in the incubation media required for such certain defined responses as C_{200} [8], the molar concentration at which the respiration rate is increased to 200% that of state 4 respiration, and I_{50} [4], the molar concentration at which the ATP synthesis is inhibited to half that at state 3. If the overall potency of uncouplers defined as above could be separable quantitatively into fundamental factors such as the ease of incorporation into the membrane phase and the 'intrinsic' potency as the protonophore inside the mem-

Abbreviations: SF6847, 2,6-di-*t*-butyl-4-(2,2-dicyanovinyl)phenol; Dinoseb, 2-*s*-butyl-4,6-dinitrophenol.

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brane phase, this should further substantiate the shuttle-type mechanism of the weakly acidic uncouplers.

In this study, we measured the overall 'apparent' and 'intrinsic' uncoupling potencies for a set of substituted phenols mostly included in our previous analyses [8] with a few additional ones with the rat-liver mitochondria. We examined the quantitative relationships of these potencies with physicochemical parameters and found that the previously used protonophoric indices could be replaced by more fundamental parameters, leading to an important observation that an optimum pK_A value does exist for the potencies with a few outliers. We also analyzed the quantitative relationship between 'apparent' and 'intrinsic' potencies and have found that the 'apparent' potency is indeed correlated with the 'intrinsic' potency and the incorporation factor in terms of partition coefficient with a phosphatidylcholine liposome/aqueous buffer. We have attempted to rationalize the behaviors of the outliers and found that the observed uncoupling potency is, in effect, perturbed by inhibition of the electron transport chain, either specifically or nonspecifically.

Materials and Methods

Materials

Uncouplers used in this study, the same samples as those used in our previous studies [8,10], are listed in Table I. Rotenone was purchased from Sigma.

Methods

Mitochondria were isolated from the liver of adult male Wistar rats as described by Myers and Slater [11]. The amount of mitochondrial protein was measured by the method of Bradford [12] with bovine serum albumin as the standard. Mitochondrial respiration with 10 mM succinate (plus 1 μg of rotenone/ml) as the respiration substrate was monitored with a Clark-type oxygen electrode at 25°C, the final mitochondrial protein concentration in the medium being 0.7 mg/ml. The incubation medium consisted of 200 mM sucrose/2 mM $MgCl_2$/1 mM EDTA/2.5 mM potassium phosphate buffer (pH 7.4), and the total volume was 2.5 ml. In each of the mitochondrial preparations, the respiration control ratio was constant, being 4.5 or more.

For each phenol, the molar concentration in the incubation medium needed to double the state 4 respiration rate (C_{200}) was measured (Fig. 1). The values of C_{200} were taken from our previous studies [8,10] except for those of 4-methyl- (2), 4-*t*-butyl- (5) and 4-*t*-pentylphenol (7), which were newly measured. The log ($1/C_{200}$) was used as the index of the apparent uncoupling potency and is shown in Table I.

We also measured the minimum molar concentration of uncouplers inducing the maximum respiration release (C_{max}, Fig. 1) using various concentrations of mitochondria. In the correlation analysis for the apparent uncoupling potency inducing the maximum respiration release, we used the log ($1/C_{max}$) value obtained with 0.7 mg mitochondrial protein/ml, equivalent to that used to measure the C_{200} value. The plot of the moles of uncouplers per unit amount of mitochondrial protein at C_{max} concentration gave a straight line against the reciprocal of the protein concentration (Fig. 2). From the intercept, we decided the intrinsic uncoupling concentration of uncouplers (C_{int}), i.e., the 'concentration' (mol/mg protein) needed for inducing the maximum respiration release under conditions where all of the added uncoupler molecules are bound to mitochondria [13]. The index of the intrinsic uncoupling potency was defined as log ($1/C_{int}$) and is listed in Table I.

The maximum rate of respiration was evaluated with 0.7 mg mitochondrial protein/ml in terms of nmol O_2 consumption/mg protein per min. Each respiration rate

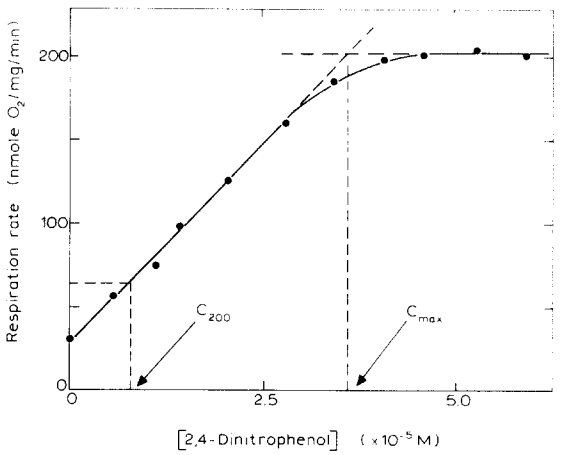

Fig. 1. Estimation of apparent uncoupling activity, C_{200} and C_{max}, respectively. The final mitochondrial concentration was 0.7 mg protein/ml.

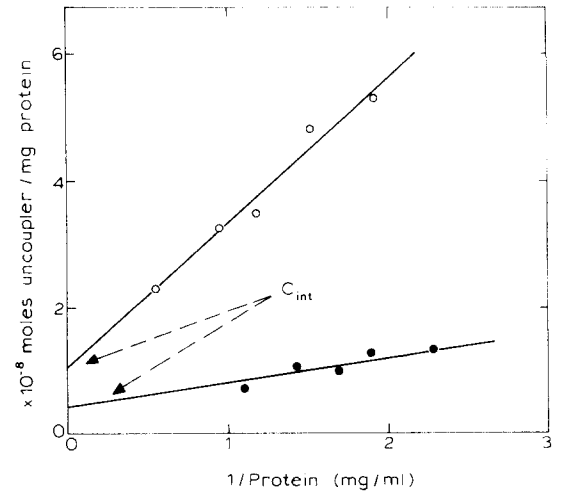

Fig. 2. Relationship between the moles of uncoupler per unit amount of mitochondria at C_{max} concentration and the reciprocal of the mitochondrial concentration. ○, 2,4,-dinitrophenol; ● 2-ethyl-4,6-dinitrophenol.

TABLE I

Physicochemical constants and uncoupling activities of phenols

	Substituent	log P(L/W) [a]	$-\log K_A$ [a]	$-\Sigma E_s^0$ [b]	log P_f [a]	$-\log P_p$		log $1/C_{200}$			log $1/C_{max}$			log $1/C_{int}$	
						Obs. [a]	Calc. [c]	Obs. [a]	Calc. [d]	Calc. [e]	Obs.	Calc. [f]	Calc. [g]	Obs.	Calc. [h]
1	H	1.97	9.98	0	0	7.44	7.49	2.15	2.10	2.32	– [i]	–	–	– [i]	–
2	4-Me	2.42	10.14	0	0	6.73	6.99	2.50 [j]	2.46	2.82	2.00	2.09	2.44	6.30	6.06
3	4-Et	2.88	10.21	0	0	6.65	6.46	2.96	2.91	2.86	– [i]	–	–	– [i]	–
4	4-*n*-Pr	3.26	10.21	0	0	6.25	6.01	3.43	3.34	3.18	– [i]	–	–	– [i]	–
5	4-*t*-Bu	3.45	10.23	0	0	5.85	5.80	3.60 [j]	3.53	3.65	3.15	3.00	3.12	6.57	6.39
6	2-*t*-Bu	3.51	11.16	2.78	0	– [i]	–	3.55 [k]	3.40	3.50	3.38	3.18	3.29	6.70	6.75
7	4-*t*-Pent	3.81	10.23	0	0	5.67	5.37	3.74 [j]	3.94	3.65	– [i]	–	–	– [i]	–
8	4-Cl	2.86	9.38	0	0	5.94	6.34	3.56	3.63	3.79	2.82	3.07	2.88	6.55	6.71
9	2,4-Cl	3.11	7.89	0.97	0.09	5.52	5.60	4.85	5.19	4.90	3.92	4.47	3.94	7.40	7.84
10	2,4,6-Cl_3	3.31	5.99	1.94	0.31	4.96	4.86	5.20	6.33 [l]	6.47 [l]	4.61	5.51 [l]	5.16	8.40	8.63 [l]
11	3-CF_3	3.25	8.95	0	0	5.69	5.80	4.10	4.40	4.18	3.50	3.72	3.34	6.83	7.08
12	4-CN	2.17	7.95	0	0.04	7.09	6.91	3.80	3.82	3.53	2.98	3.21	3.15	7.00	7.09
13	4-COMe	1.80	8.05	0	0.02	7.63	7.36	2.95	3.35	2.99	– [i]	–	–	– [i]	–
14	3-NO_2	2.41	8.40	0	0	6.64	6.70	3.77	3.83	3.65	3.24	3.23	3.11	6.89	7.01
15	2,4-$(NO_2)_2$	2.12	4.09	1.77	−0.70	6.20	5.96	5.10	4.85	4.90	4.58	4.23	4.40	8.11	7.91
16	2-Me-4,6-$(NO_2)_2$	2.49	4.44	3.01	−0.44	5.44	5.34	5.60	5.70	5.72	4.96	5.07	4.86	8.40	8.60
17	2-Et-4,6-$(NO_2)_2$	2.84	4.43	3.08	−0.45	4.94	4.91	6.02	6.11	6.12	5.31	5.43	5.16	8.55	8.76
18	2-*i*-Pr-4,6-$(NO_2)_2$	3.03	4.47	3.48	−0.42	4.33	4.62	6.46	6.45	6.64	5.76	5.75	5.89	9.13	8.99
19	2-*s*-Bu-4,6-$(NO_2)_2$	3.42	4.51	4.14	−0.40	4.18	4.04	6.89	7.08	6.72	6.15	6.36	6.46	9.51	9.40
20	2-*t*-Bu-4,6-$(NO_2)_2$	3.26	4.80	4.55	−0.21	4.16	4.19	6.85	7.06	6.92	6.08	6.38	6.12	9.26	9.54
21	4-(2,2-dicyanovinyl)	2.68 [m]	7.04	0	0.51	5.77	6.15	5.37	4.80	5.64	4.50	4.03	4.21	7.77	7.53
22	2,6-$(Me)_2$-4-(2,2-dicyanovinyl)	3.05 [m]	6.91	2.48	0.51	5.02	5.21	6.39	5.98	6.30	5.75	5.28	5.81	9.05	8.64
23	2,6-$(Et)_2$-4-(2,2-dicyanovinyl)	3.49 [m]	6.98	2.62	0.51	4.68	4.67	6.76	6.49	6.55	6.15	5.73	6.04	9.12	8.84

[a] Unless otherwise noted, from Refs. 8 and 10.

[b] From Ref. 20. For the nitro group, the E_s^o value was taken as the mean of those for the minimum perpendicular (−1.01) and the maximum coplanar (−2.52) dimensions, as defined in Ref. 15. This averaged E_s^o value was recently found to apply to the steric effect of an *o*-nitro group on the acid-catalytic hydrolysis of benzamides [21].

[c] By Eqn 8.

[d] By Eqn 2.

[e] By Eqn 7.

[f] By Eqn 3.

[g] By Eqn 5.

[h] By Eqn 4.

[i] Not studied.

[j] Reinvestigated in this study.

[k] Newly found.

[l] Not included in the correlation analyses.

[m] Newly measured according to the method in Ref. 14.

TABLE II

Maximum respiration rate (V_R) and pK_A of phenols

Substituent	pK_A [a]	V_R	Substituent	pK_A [a]	V_R
H	9.98	29	2-Et-4,6-$(NO_2)_2$	4.43	98
4-Me	10.14	37	2-*i*-Pr-4,6-$(NO_2)_2$	4.47	89
4-Et	10.21	39	2-*s*-Bu-4,6-$(NO_2)_2$	4.51	65
4-*n*-Pr	10.21	34	2-*t*-Bu-4,6-$(NO_2)_2$	4.80	101
4-*t*-Bu	10.23	34	4-(2,2-dicyanovinyl)-		
2-*t*-Bu	11.16	33	2,6-H_2	7.04	103
4-*t*-Pent	10.23	42	2,6-$(Me)_2$	6.91	70
4-Cl	9.38	64	2,6-$(Et)_2$	6.98	83
2,4-Cl_2	7.89	90	2,6-$(i\text{-}Pr)_2$	7.06 [b]	103
2,4,6-Cl_3	5.99	41	2,6-$(s\text{-}Bu)_2$	7.22 [b]	102
3-CF_3	8.95	53	2,6-di-*t*-$(Bu)_2$-		
4-CN	7.95	83	4-CH = C(CN)(COOEt)	7.51 [b]	92
4-COMe	8.05	80	4-CH = $C(COOEt)_2$	9.29 [b]	65
3-NO_2	8.40	81	4-CH = C(COMe)(COOEt)	8.67 [b]	71
2,4-$(NO_2)_2$	4.09	99	4-CH = $CH(NO_2)$	6.89 [b]	90
2-Me-4,6-(NO_2)	4.44	102	4-CH = C(CN)(SO_2Me)	6.77 [b]	103
			4-CH = CH(CN)	9.95 [b]	60
			4-CH = C(CN)(COOMe)	7.45 [b]	96

[a] Unless otherwise noted, from Table I.
[b] From Ref. 10.

was normalized to adjust possible differences in individual experimental conditions using the rate value with 30 nM SF6847 as the standard and expressed by the relativistic value, V_R, as shown in Eqn. 1.

$$V_R = \left\{ \frac{\text{rate induced by each phenol} - \text{rate (state 4)}}{\text{rate induced by SF6847} - \text{rate (state 4)}} \right\} \times 100 \quad (1)$$

The log $(1/C_{max})$ and V_R values are listed in Tables I and II, respectively.

To estimate the inhibitory effects of some phenols on the respiration chain, the decreases in the respiration rate stimulated by 30 nM SF6847 showing the maximum respiration rate were measured with various concentrations of uncouplers.

The partition coefficient in phosphatidylcholine liposome/aqueous buffer system, P(L/W), and the acid dissociation constant, K_A, of phenols were taken from our previous studies [8,10].

Results

Analysis of the apparent uncoupling potency

The structural effects on variations in the uncoupling potency in terms of log $(1/C_{200})$ were examined by regression analysis with physicochemical substituent and molecular parameters shown in Table I. For 22 phenols, Eqn. 2 was derived as that of the best quality.

$$\log(1/C_{200}) = \underset{(0.281)}{1.126}\log P(\text{L/W}) - \underset{(0.536)}{1.050}\log K_A - \underset{(0.033)}{0.100}(\log K_A)^2 - \underset{(0.156)}{0.290}\Sigma E_s^0 - \underset{(2.071)}{0.681} \quad (2)$$

$(n = 22,\ s = 0.265,\ r = 0.988)$

In this and following equations, n is the number of compounds included in the correlation, s is the standard deviation, and r is the correlation coefficient. The figures in parentheses are the 95% confidence interval. The P(L/W) value was defined as an overall partition coefficient from the aqueous phase to liposomal membranes based on the total concentration including both neutral and ionized species present at pH 7.2 [14]. ΣE_s^0 is the sum of the Taft-Kutter-Hansch E_s^0 parameter [15] of the two *ortho*-substituents relative to H (E_s^o (H) = 0), being defined so that the more negative the value, the greater is the steric bulk. Eqn. 2 indicates that the uncoupling poptency increases with the hydrophobicity of compounds in terms of log P(L/W) and the steric bulk of *ortho*-substituents. The potency varies parabolically with increases in the log K_A values, the optimum log K_A being at about −5.3. 2,4,6-Trichlorophenol (10) was not included in Eqn. 2, since the preliminary examination showed its significantly outlying behavior. This compound showed a great inhibitory effect on respiration chains as will be discussed later and the deletion of this compound from the set was thought to be rationalized.

For the quantitative analysis of the uncoupling potency in terms of log $(1/C_{max})$, Eqn. 3 was formulated.

$$\log(1/C_{max}) = \underset{(0.625)}{0.956}\log P(\text{L/W}) - \underset{(0.850)}{0.890}\log K_A - \underset{(0.052)}{0.084}(\log K_A)^2 - \underset{(0.266)}{0.346}\Sigma E_s^0 - \underset{(2.944)}{0.656} \quad (3)$$

$(n = 17,\ s = 0.348,\ r = 0.975)$

In Eqn. 3, the 2,4,6-trichloro derivative also was not included. Each term in Eqn. 3 was very similar to the

corresponding term in Eqn. 2. The quality of the correlation in terms of the standard deviation (s) as well as the 95% confidence interval of each term was, however, poorer than that of Eqn. 2. The C_{max} concentration was measured at a concentration higher than the C_{200}, where the maximum respiration rate was attained. As shown in Table II, the maximum rate, V_R, varied depending upon the structure. The lower V_R value is possibly brought about by inhibition of the respiration chain as will be discussed later. Since the log $(1/C_{max})$ value is perturbed by the inhibitory factor in the total effect of uncouplers much greater than the log $(1/C_{200})$ value, the poorer quality of Eqn. 3 is understandable.

Analysis of the intrinsic uncoupling potency

The intrinsic uncoupling potency was analyzed with the substituent and structural parameters to give Eqn. 4.

$$\log(1/C_{int}) = \underset{(0.674)}{-1.119}\log K_A \underset{(0.042)}{-0.088}(\log K_A)^2 \underset{(0.212)}{-0.497}\Sigma E_s^0 \underset{(2.353)}{+3.978} \quad (4)$$

$$(n = 17,\ s = 0.296,\ r = 0.971)$$

The log P(L/W) term was not justified over the 90% level. Except for this, Eqn. 4 is very similar to Eqn. 3 for 17 phenols included in this set. Eqn. 3 and 4 strongly suggest that the hydrophobic term in Eqn. 3 is taking care of the degrees of the incorporation into the mitochondrial membrane of phenols and the effects of other physicochemical factors are essentially common between apparent and intrinsic potencies.

Relationship between apparent and intrinsic potencies

The relationship between apparent and intrinsic uncoupling potencies was examined with the log $(1/C_{max})$ value obtained at the 0.7 mg protein/ml as the dependent variable for the apparent potency as mentioned in the Materials and Methods section to yield Eqn. 5.

$$\log(1/C_{max}) = \underset{(0.114)}{1.133}\log(1/C_{int}) + \underset{(0.273)}{0.309}\log P(L/W) \underset{(1.043)}{-5.433} \quad (5)$$

$$(n = 18,\ s = 0.235,\ r = 0.986)$$

In Eqn. 5, the log P(L/W) term is highly significant. The regression coefficient of log $(1/C_{int})$, being close to 1, shows that the variations in the apparent potency correspond to those in the intrinsic potency in an almost 1-to-1 way. In Eqn. 5, 2, 4, 6-trichlorophenol was well accommodated. The inhibitory factors observed for this compound are almost equivalent between log $(1/C_{max})$ and log $(1/C_{int})$ values. Being conformed with results from Eqns. 3 and 4, Eqn. 5 should indicate that the apparent potency was indeed separable into two components, the intrinsic potency in the inner mitochondrial membrane and the degree of the partitioning of compounds to the membrane phase in terms of log

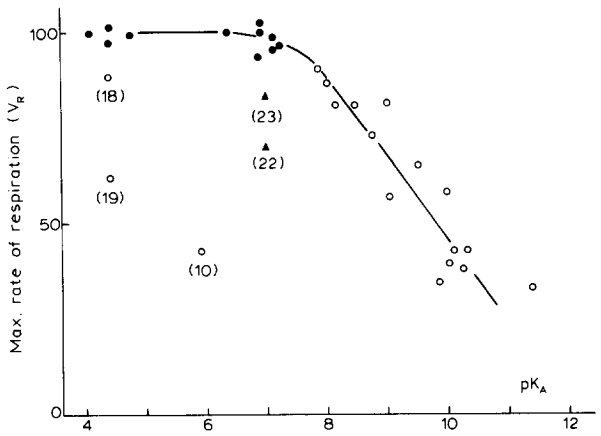

Fig. 3. Relationship beween the maximum rate of respiration (V_R) induced by phenols and the pK_A. V_R and pK_A values are listed in Table II. Phenols with irreversible (○) and reversible (▲) inhibitory effects on the respiration chain, respectively. ●, Those with no inhibitory effect on respiration chain. Figures in parentheses show the compound number listed in Table I. The maximum rate of respiration of each phenol is the mean for three replicates and is normalized according to Eqn. 1.

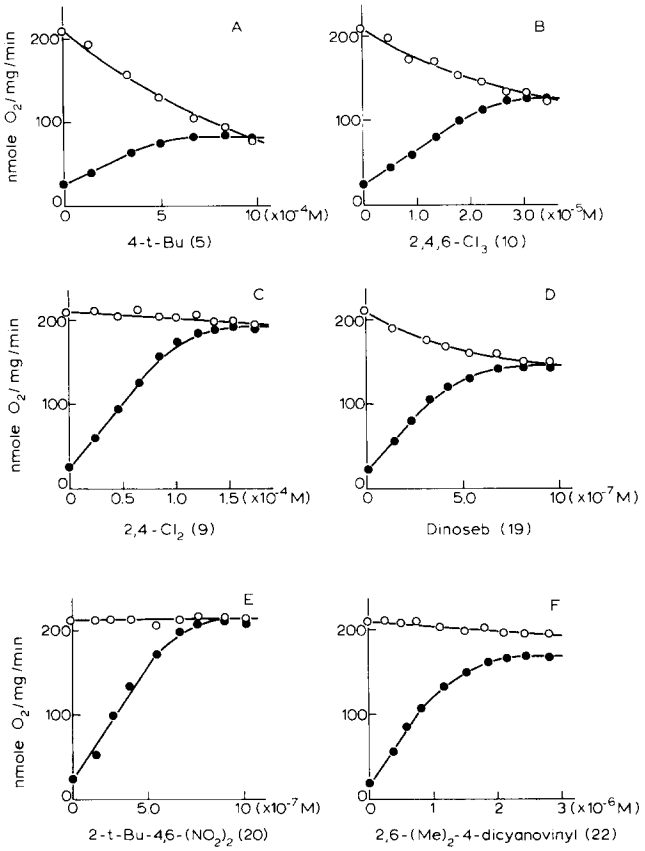

Fig. 4. Effects of 4-*t*-butylphenol (A), 2,4,6-trichlorophenol (B), 2,4-dichlorophenol (C), Dinoseb (D), 2-*t*-butyl-4,6-dinitrophenol (E), and 2,6-dimethyl-4-(2,2-dicyanovinyl) phenol (F) on the maximum rate of respiration induced by 30 nM SF6847. The reaction medium was described in the Materials and Methods section. The final mitochondrial protein concentration was 0.7 mg/ml. ●, The dose–response relationship of individual phenol without addition of SF6847. ○, Respiration rate of 30 nM SF6847 in the presence of various concentrations of each phenol.

P(L/W) value. The correlation using the log $(1/C_{200})$ as the dependent variable was apparently poorer than Eqn. 5 (data not shown).

Maximum rate of respiration

In Fig. 3, the relative maximum rate of respiration, V_R, is plotted against the pK_A value of phenols. For phenols the pK_A of which was higher than 8, a clear trend is obserbed so that the V_R value is decreased with increasing pK_A values. The lower maximum rates induced by these phenols ($pK_A > 8$) were not stimulated by the addition of 30 nM SF6847. As the concentration was increased toward C_{max}, these phenols even inhibited the maximum respiration rate induced by 30 nM SF6847, as shown in Fig. 4A.

The maximum rate of 2,4,6,-trichlorophenol (10) and Dinoseb (19) was considerably lower than their analogs with similar chemical structures. Like those, the pK_A value of which is higher than 8, the maximum rate was no longer accelerated by the addition of 30 nM SF6847. The inhibitory effect observed for 2,4,6,-trichlorophenol and Dinoseb was very specific to their structure. The situation is compared with that for their closely related analogs, 2,4-dichlorophenol (9) and 2-*t*-butyl-4,6-dinitrophenol (20), in Fig. 4B, C, D, and E.

The maximum rate of respiration induced by 2,6-dimethyl-4-(2,2-dicyanovinyl)phenol (22) and 2,6-diethyl-4-(2,2-dicyanovinyl) phenol (23), the pK_A of which is about 7, was appreciably lower than that of SF6847. The lower V_R value of these compounds was, however, stimulated by the addition of 30 nM SF6847 to the level of about 90, as shown in Fig. 4F for Compound 22. The higher concentration of these compounds close to C_{max} did not significantly inhibit the maximum rate of SF6847, the situation being apparently different from that shown in Fig. 4A, B and D.

Other phenols with V_R values close to 100 did not reduce the maximum rate of SF6847 (data not shown).

Discussion

As mentioned earlier in this paper, we have shown previously that the variations in the uncoupling potency (log $1/C_{200}$) of substituted phenols covering a wider range of substituents ($n = 38$) are nicely analyzable quantitatively with the protonophoric potency across phosphatidylcholine liposomal membrane. Unfortunately, we included 2,4,6,-trichlorophenol mistakenly in this analysis using an erroneous parameter (log $P_f = 0.31$ corrected). Eqn. 6 shows the corrected correlation where this compound is not included, since it shows a significantly lower activity as an outlier, conforming with those observed in Eqns. 2 and 3.

$$\log (1/C_{200}) = \underset{(0.163)}{0.669} \log P_p + \underset{(0.053)}{0.466} \log K_A - \underset{(0.062)}{0.131} \Sigma E_s^0 + \underset{(0.331)}{2.103} \log P_f + \underset{(0.991)}{11.994} \quad (6)$$

$(n = 37,\ s = 0.283,\ r = 0.988)$

In Eqn. 6, the log P_p is the index for protonophoric potency, P_p being the increment of proton permeability of the phosphatidylcholine membrane induced by the unit molar concentration of uncouplers in the medium. Log P_f is a parameter that takes into account the effects of the difference in extramembranous pH conditions between mitochondrial (pH $\approx$ 7.2) and liposomal (pH 4.5–7.0) experiments. It was estimated from a theoretical equation proposed by Benz and McLaughlin [16] for the effects of pH of the medium on the kinetics of protonophore shuttling in the lipid membranes. We repeated the same procedure for this set of phenols to give Eqn. 7.

$$\log (1/C_{200}) = \underset{(0.119)}{0.812} \log P_p + \underset{(0.060)}{0.430} \log K_A + \underset{(0.346)}{1.371} \log P_f + \underset{(0.535)}{12.651} \quad (7)$$

$(n = 21,\ s = 0.193,\ r = 0.993)$

Among 22 phenols included in Eqn. 2, the log P_p value of 2-*t*-butyl phenol (6) was not measured, so that $n = 21$. In Eqn. 7, the ΣE_s^o term was not significant. This is probably due to a higher collinearity ($r = 0.86$) between ΣE_s^0 and log K_A values than that for compounds included in Eqn. 6 ($r = 0.17$). The lower slope of the log P_f term in Eqn. 7 is perhaps due to the fact that compounds with relatively high log P_f values are not included in Eqn. 7.

In this study, Eqn. 2 was formulated for the log $(1/C_{200})$ value with physicochemical parameters more fundamental than protonophoric parameters requiring consideration of differences in experimental conditions as well as approximations for the kinetics of protonophore in membranes. In effect, Eqn. 2 and Eqn. 7 are linked with each other mathematically by such relationships as Eqns. 8 and 9 for the same set of compounds included in Eqn. 7. Although the log K_A and ΣE_s^0 terms are significant, in spite of the colinearity between the two parameters in this set of 21 compounds as mentioned above, both terms are not very important in Eqn. 8.

$$\log P_p = \underset{(0.260)}{1.178} \log P(L/W) + \underset{(0.116)}{0.172} \log K_A - \underset{(0.171)}{0.194} \Sigma E_s^0 - \underset{(0.861)}{8.093} \quad (8)$$

$(n = 21,\ s = 0.228,\ r = 0.978)$

$$\log P_f = \underset{(0.322)}{-0.899} \log K_A - \underset{(0.022)}{0.058} (\log K_A)^2 - \underset{(1.076)}{3.281} \quad (9)$$

$(n = 21,\ s = 0.176,\ r = 0.850)$

In cases when the pH-partition theory [17] applies, i.e., if only the neutral form of weak acids is responsible for the biological activity, being incorporated in the lipophilic phase and interacting or interfering with the action 'sites', parabolic or biphasic dependencies of the overall activity index measured at a constant pH on the log K_A are sometimes observed [18]. As previously shown [14], the substituted phenols partition into the liposomal membrane phase without according to the pH-partition theory: both the ionized and neutral forms are incorporated almost equally irrespective of the pH-variations in a range of $pK_A \pm 2$–3. In the shuttle-type mechanism, the ionized and neutral forms of compounds are equally at work. Although the P(L/W) was measured at pH 7.2, the value should be regarded as the ratio of compounds in total between the two phases. Since phosphatidylcholine liposomes mimic the inner mitochondrial membrane very well as shown in Eqn. 6, the biphasic relationship with log K_A value should be taken as indicating that there should be an optimum pK_A for a series of phenols so that the concentrations of two forms are well balanced inside the mitochondrial membrane.

The higher log K_A value is favorable for anions to be stabilized in the membrane phase, since the negative charge is more delocalized. According to Benz and McLaughlin [16], the mobility of anions is critical, being much lower than that of neutral molecules. This should be, however, up to an optimum value beyond which the concentration of the neutral form in the membrane is decreased too much, unbalancing the ratio of the two forms, both of which are necessary to work efficiently as protonophores.

In previous analyses by other investigators for sets of different types of uncoupling compound, clear biphasic dependencies on log K_A (or σ) have been observed, at least when variations in the log K_A value of compounds used in the sets are wide enough on both sides of the pH value of the medium. Draber et al. [5] have measured the uncoupling potency of substituted α-acyl-α-cyanocarbonylphenylhydrazones with rat-liver mitochondria at pH 7.4. They have observed that the apparent potency in terms of pI_{50} (M) yielding the half-maximal stimulation of O_2 uptake varies parabolically with log K_A, the optimum value being about 5.5. This value is very close to those found from Eqn. 2, 3 and 4 for the present set of substituted phenols, 5.3–6.4.

The negative sign of ΣE_s^0 term in Eqns. 2, 3, 4, 6 and 8 means that the steric bulk of *ortho*-substituents is favorable to the uncoupling and protonophoric potencies. The membrane phase is much less polar than the aqueous environment and the stabilization of the negative charge in anions by solvation should be much less significant in the membrane phase than in the aqueous phase. The steric shielding of the negative charge from the non-polar environment in the membrane phase could stabilize the ionized form leading to the increase in the protonophoric potency [19]. In Eqn. 6, the ΣE_s^0 term was not as important as those in Eqns. 2, 3, 4, and 8. This is probably due to the fact that the ΣE_s^0 term in Eqn. 6 represents the difference in steric shielding effects between uncoupling and protonophoric potencies. Draber et al. [5] have also pointed out the importance of bulky substituents at the *ortho* positions for the potency of their phenylhydrazone uncouplers. Although they have expressed the effect of bulkiness using the hydrophobic parameter, π, of *ortho* substituents instead of E_s^0, there is a certain high colinearity between E_s and π in their set of compounds.

According to the shuttle-type mechanism, a low maximum rate of respiration observed with a fixed amount of mitochondria could be due to a decrease in the turnover numbers in terms of the cycling movement of uncoupler molecules in the inner mitochondrial membrane per unit time. Even poorer uncouplers with lower turnover numbers could, however, increase the respiration rate to a maximum level similar to that of potent uncouplers if the concentration in mitochondrial membranes could be increased without any side-effects other than the protonophoric action. The higher the pK_A value, i.e., the lower the amount of the ionized form, the higher concentration in mitochondrial membrane could be required to bring about an equivalent stimulation of respiration. Under such conditions, the uncoupling potency could be reduced with increasing the pK_A, but without lowering the maximum rate of respiration.

As a matter of the fact, however, the maximum respiration rate in terms of V_R was decreased almost linearly with increasing pK_A in the region higher than 8, as shown in Fig. 3. Therefore, it is readily assumed that the higher concentration of phenols should damage the function of respiration chains. The extent of the damage could be higher as the pK_A value increases, although they still act as protonophores simultaneously.

In contrast to the above phenols ($pK_A > 8$), 2,4,6-trichlorophenol (10) and Dinoseb (19), which are expected to have high turnover numbers from their lower pK_A value, had appreciably lower V_R values (Fig. 3). We examined the maximum rate of their analogs under the same experimental conditions and observed that 2,4-dichlorophenol (9) and 2-*t*-butyl-4,6-dinitrophenol (20) showed a high maximum respiration rate almost equivalent to that of SF6847. Contrarily, 2,6-dichlorophenol, another close relative of 2,4,6-trichlorophenol, the pK_A of which is 6.79 [14], had a very low maximum respiration rate ($V_R = 50$) almost equal to that of 2,4,6-trichlorophenol. 2-*i*-Propyl-4,6-dinitrophenol (18), an analog very similar to Dinoseb, had a maximum rate slighlty lower than that of other 2-alkyl-4,6-dinitro derivatives (Fig. 3). Therefore, the lower V_R of 2,4,6-trichlorophenol and Dinoseb should be governed by structurally highly specific inhibitory effects on the

components of the respiration chains. Dinoseb was included in Eqns. 2 and 3, but 2,4,6-trichlorophenol was not, as mentioned earlier. At least up to the C_{200} concentration, Dinoseb was thought not to have significant inhibitory effects. In Eqn. 3, the deviation of the observed potency value of Dinoseb from the predicted was not very high compared with others in Eqn. 2. This is probably due to the fact that Eqn. 3 was formulated with compounds showing the inhibitory effect, so that an otherwise apparent deviation was hidden in the correlation.

The inhibitory effects observed for phenols ($pK_A > 8$), 2,4,6-trichlorophenol (10) and Dinoseb (19) would apparently be due to some irreversible effects as indicated by concomitant application experiments with SF6847 (Fig. 4A, B and D). That shown by the less acidic phenols was considered as belonging to a nonspecific high-concentration dependent type and that for each of 2,4,6-trichlorophenol and Dinoseb was due to respective specific modes of action highly dependent on each of their structural patterns. Compounds 22 and 23 gave a V_R of about 70–80. The V_R of these compounds was raised by SF6847 up to about 90. The inhibitory effect of these phenols was 'reversible'.

Some phenols included in these analyses have both the uncoupling activity and the various extents and types of inhibitory effect on the respiration assembly at the same time. If the inhibitory effects could be separated from the uncoupling potency, the quality of the correlations of Eqns. 2, 3 and 4 would be more improved, providing us with more accurate information about the modes of action of this series of weakly acidic uncouplers.

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