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

Ze-jian Guo [1], Hideto Miyoshi [1], Terumasa Komyoji [2], Takahiro Haga [2] and Toshio Fujita [1]

[1] *Department of Agricultural Chemistry, Kyoto University, Kyoto and* [2] *Central Research Institute, Ishihara Sangyo Kaisha, Ltd., Kusatsu (Japan)*

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Variations in the uncoupling potency of a series of substituted diphenyl- and phenylpyridylamines with rat-liver mitochondria were analyzed quantitatively by regression analysis by use of two physicochemical parameters, log $P_{(M/W)}$ and log K_A^m. $P_{(M/W)}$ is the partition coefficient of compounds for incorporation into mitochondria from the aqueous phase and K_A^m is the acid dissociation constant in nonionic micellar system. The results of the analysis were similar to those observed previously for phenolic uncouplers, showing that the incorporation of compounds into the mitochondrial phase and a certain balance between neutral and ionized forms in the membranous phase were significant factors in governing the uncoupling potency. The findings were in accord with the hypothesis that the acidic uncouplers act primarily by working as protonophores in the inner mitochondrial membrane. In contrast to results obtained with phenols, however, the variations in the steric effect of the *ortho* substituents in shielding the negatively charged center of the ionized form did not significantly affect variations in the uncoupling potency of the diarylamines studied here.

Introduction

Variations in the uncoupling activity of a series of substituted phenols are governed by such physicochemical factors as the ease of incorporation of the compounds from an aqueous medium into the inner mitochondrial membrane, the stability of the ionized form in the nonpolar interior of the membrane, and the proper balance between the amounts of the neutral and ionized forms [1]. These findings support the hypothesis that phenolic uncouplers act as protonophores in the mitochondrial membrane. However, a number of recent studies have suggested that the uncoupling activity of other types of uncoupler arises not only from transmembrane proton transport but also by interaction with the proton pumps of respiration assemblies [2–5]. Thus, the uncoupling mechanism of acidic uncouplers may differ depending upon the structural class of the uncoupler. It is of interest to compare physicochemical factors governing the uncoupling potency of different classes of acidic uncouplers when examining differences, if any, in the modes of uncoupling action.

3-Chloro-*N*-(3-chloro-2,6-dinitro-4-trifluoromethylphenyl)-5-trifluoromethyl-2-pyridylamine (fluazinam) has powerful uncoupling potency with rat-liver mitochondria, greater than that of SF6847, the most potent uncoupler known at the time of writing [6]. Fluazinam gradually loses its uncoupling activity by metabolic decomposition owing to its conjugation with the glutathione in mitochondria. The structural profiles of fluazinam and its analogs, which are NH acids, are quite different from those of phenolic uncouplers. Here, we measured the uncoupling potency for a series of substituted diphenyl- and phenylpyridylamines with rat-liver mitochondria and examined the quantitative relationship of their potency with physicochemical molecular and substituent parameters. The physicochemical factors governing the uncoupling potency of these weakly acidic aromatic amines were similar to

Correspondence: H. Miyoshi, Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan.

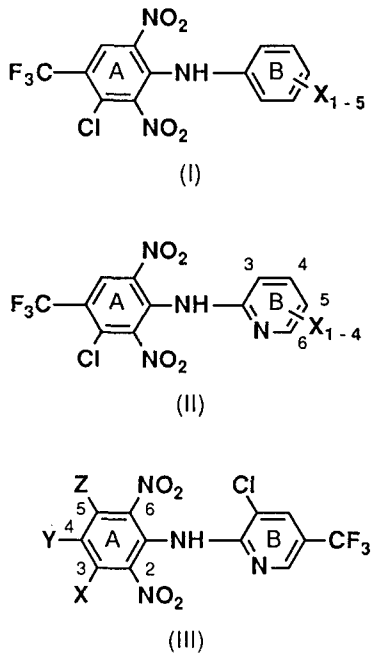

Fig. 1.

those observed for substituted phenols, showing that the uncoupling action is due to the protonophoric action in the mitochondrial membrane, regardless of the structural type of the uncoupler.

Materials and Methods

Compounds

Diarylamine uncouplers of series I, II and III, shown in Fig. 1, were used: they were the same as those used previously [7]. 2,4-Dinitrochlorobenzene (DNCB) and rotenone were purchased from Sigma. *n*-Dodecyloctaoxyethylene glycol monoether ($C_{12}E_8$) was from Nikko Chemical Co. Other reagents were of the purest grade commercially available.

Mitochondrial respiration measurements

Mitochondria were isolated as described by Myers and Slater [8] from the liver of adult male Wistar rats in a medium containing 250 mM sucrose and 2 mM Tris-HCl (pH 7.4). The amount of mitochondrial protein was measured by the method of Bradford [9], with bovine serum albumin as the standard. Mitochondrial respiration with 10 mM succinate as the respiration substrate was measured 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 μM rotenone, and 2.5 mM potassium phosphate buffer (pH 7.4) in a total volume of 2.5 ml. The uncoupling activity of each compound was estimated from the concentration, C_{200} (M), at which the respiration rate was twice that of state 4 respiration. The uncoupling activity of some test compounds with substituents that could be eliminated by metabolic transformation disappeared with time [6], so the initial respiration rate immediately after the addition of the compound to the mitochondrial suspension was used to estimate the C_{200} value. The log of $1/C_{200}$ was used as the index of the uncoupling activity. The $\log(1/C_{200})$ was measured at least twice, as a rule, and averaged. The standard deviation was within ± 0.08.

The maximum rate of respiration was evaluated with 0.7 mg of mitochondrial protein per milliliter in terms of nanomoles of O consumed per milligram of protein per minute. The respiration rate in each experiment was normalized to adjust possible differences among individual experimental conditions by use of the rate value with 30 nM SF6847 as the standard, and this rate was expressed by the relative value, V_R, as shown in Eqn. 1:

$$V_R = \frac{\text{rate induced by each amine} - \text{rate (state 4)}}{\text{rate induced by SF6847} - \text{rate (state 4)}} \quad (1)$$

Partition coefficient with mitochondria / aqueous system

The partition coefficient of each compound between the mitochondria and the aqueous medium, $P_{(M/W)}$, was measured by the method of Bakker et al. [10]. The concentration in the medium was estimated not directly but by the potency of the uncoupling action of the medium. Mitochondria (1.0 mg/ml) were incubated with each compound for 3 min in 1 ml of a buffer solution (pH 7.4) with a composition was identical to that used for the respiration measurement (the respiration medium) except for the addition of 30 μM DNCB in the plastic Eppendorf centrifuge cup. This concentration of DNCB prevents the metabolic transformation of the metabolizable compounds in this series of diarylamines [6]. For each compound, five different concentrations were used to treat the mitochondria. The concentrations covered were in ranges of about 5-fold selected from the range of from $5 \cdot 10^{-7}$ to $1 \cdot 10^{-5}$ M, depending on the uncoupling potency of the compound in question. Therefore, the higher the potency, the lower was the range of concentrations. After mitochondria had been spun down ($15\,000 \times g$ for 3 min) with a TOMY MC-15A centrifuge, a portion (0.1–0.8 ml) of supernatant (A) was diluted by fresh respiration medium (B) including fresh mitochondria (0.7 mg/ml) to give a final volume of 2.5 ml. The O_2 uptake by the fresh mitochondria was measured in respiration medium B mixed with the portion of the supernatant A. The concentration range of each compound in the total volume was always within the linear part of the titration curve for the respiration rate. The uncoupler concentration of the diluted respiration medium (C) could be read out by extrapolation of the

respiration rate to the standard titration curve as a function of the concentration of the uncoupler used. The uncoupler concentration of supernatant A was estimated from that of the medium C by correction with the dilution factor. Test compounds were adsorbed to the centrifuge cup to various extents, so we examined the amount of compounds adsorbed using a mitochondria-free system as a control under the same experimental conditions. The partition coefficient of the uncoupler between mitochondria and the aqueous medium was calculated according to Eqn. 2,

$$P_{(M/W)} = \frac{\Phi_{bound}/V_m}{\Phi_{free}/V} \quad (2)$$

where Φ_{free} is the amount of uncoupler left in supernatant A and Φ_{bound} is the difference between the total amount and the amount free. For the estimation of Φ_{bound} and Φ_{free}, the amount adsorbed to the centrifuge cup was corrected for. V_m is the volume of the mitochondria, estimated as 1 μl/mg protein [11], and V is the volume of the incubation medium (1.0 ml). The log $P_{(M/W)}$ value was used as a hydrophobicity index representing the ease of incorporation into the mitochondrial phase. The log $P_{(M/W)}$ measurement was repeated at least twice with various amounts of mitochondrial and different diarylamine concentrations, and the results were averaged. The standard deviation of the log $P_{(M/W)}$ was ± 0.03–0.06.

Acid dissociation constant in the micellar system

The acid dissociation constant in the micellar interfacial environment, K_A^m, was measured at 25 ± 0.5 °C by the method of Drummond et al. [12]. The aqueous micellar solution was prepared by use of a nonionic surfactant $C_{12}E_8$ (82 mM) with distilled water. A small portion (< 200 μl) of an ethanol solution of each test compound was added to 50 ml of the micellar solution to give the final concentration of $(1–2) \cdot 10^{-5}$ M. The UV-Vis. absorption spectrum was recorded under various pH conditions with a Shimadzu UV-3000 spectrophotometer. The bulk pH was adjusted with small portions of conc. NaOH and HCl (0.01–10 M) to decrease the dilution effects. A Radiometer (Model PHM 26) pH meter was used to monitor the pH. The apparent log K_A^m value in the micellar solution was estimated from the UV-Vis. absorption spectra as a function of the aqueous pH with the use of Eqn. 3,

$$\log K_A^m = -pH + \log\{[A_{(R^-)} - A]/[A - A_{(RH)}]\} \quad (3)$$

where A is the absorbance at λ_{max} at a certain pH, and $A_{(RH)}$ and $A_{(R^-)}$ are the absorbances measured under levels at which each compound exists almost

TABLE I

Uncoupling activity and physicochemical constants of series I compounds

No.	Compound	$\log(1/C_{200})$		$-\log K_A^m$	$\log P_{(M/W)}$	V_R
		obsd.	calcd.[a]			
1	3-CF_3	7.52	7.38	8.71	3.58	1.03
2	4-Cl-3-CF_3	8.08	7.92	8.10	3.55	1.02
3	3,5-$(CF_3)_2$	8.60	8.55	7.05	3.58	0.99
4	4-Br*	6.70	6.75	9.20	3.46	0.92
5	4-Cl*	6.74	6.66	9.32	3.57	0.93
6	4-CF_3	7.90	7.77	8.38	3.69	0.98
7	4-OCF_3*	6.95	6.67	9.23	3.40	0.92
8	2-Br*	6.85	7.11	8.86	3.40	0.94
9	2-Cl-5-CF_3	8.25	8.42	7.39	3.61	1.00
10	2,4-Cl_2-5-CF_3	8.61	8.59	6.62	3.46	1.03
11	2-Cl-4-CF_3	8.64	8.61	7.00	3.65	1.02
12	2,4-F_2	7.00	7.26	8.75	3.45	0.98
13	2-CF_3	7.67	7.93	8.13	3.60	0.97
14	4-Cl-2-CF_3	8.60	8.62	7.07	3.71	1.01
15	2,4,6-Br_3-3-CF_3	8.02	8.06	4.89	3.06	0.99
16	2,6-Br_2-4-CF_3	8.28	8.14	4.90	3.18	1.02
17	2-Br-4-Cl-6-CF_3	8.32	8.28	4.96	3.35	0.97
18	2-Br-6-Cl-4-CF_3	8.10	8.22	4.92	3.28	1.04
19	2,4,6-Cl_3	8.66	8.67	6.36	3.53	1.03
20	2,4-Cl_2-6-CF_3	8.20	8.24	5.01	3.24	1.02
21	2,6-Cl_2-4-NO_2	7.32	7.35	3.32	4.02	1.01
22	2,4,6-Cl_3-3-CH_3	8.44	8.35	6.97	3.22	1.04
23	2,4,6-Cl_3-3,5-$(CF_3)_2$	7.20	7.17	3.56	3.29	1.03

[a] Calculated from Eqn. 4.

entirely as the neutral and ionized form, respectively. The log K_A^m value was calculated as the average of the values calculated by Eqn. 3 at 5–7 different pH values. This log K_A^m estimation was repeated at least twice and the result were averaged. The standard deviation of log K_A^m was ± 0.04.

Results

Uncoupling activity

The uncoupling activity of compounds in terms of $\log(1/C_{200})$ is listed in Tables I and II. Some of the compounds were very potent uncouplers. For instance, compounds **3**, **9–11**, **14**, **16–20**, and **22** in series I, **24**, **29–30**, and **37** in series II, and **46** and **49–50** in series III had such activity at concentrations on the order of 10^{-9} M. Of these, compounds **3**, **10**, **11**, **14**, **19**, **30**, **37**, **46** and **50** were more potent than SF6847 with the $\log(1/C_{200})$ value of 8.44 [13].

In Tables I and II, the diarylamines, the uncoupling activities of which did not apparently disappear, are marked with asterisks. With series I and II, the disappearance of the uncoupling activity was not observed for compounds possessing $-\log K_A^m$ values larger than about 9. This is probably due to the fact that the respiration measurements of these compounds were done with high concentrations of compounds because of their lower uncoupling potencies owing to the larger $-\log K_A^m$ values. The uncoupling activity of series III compounds which lack the suitable leaving group as well as strongly electron-withdrawing substituents did not disappear.

TABLE II

Uncoupling activity and physicochemical constants of series II – III compounds

No.	Compound	$\log(1/C_{200})$			$-\log K_A^m$	$\log P_{(M/W)}$	V_R
		obsd.	calcd.[a]	calcd.[b]			
Series II							
24	6-Cl-5-CF_3	8.42	8.26	8.30	7.24	3.92	1.03
25	5-Br*	7.10	7.21	7.35	8.75	3.80	0.92
26	5-Cl*	7.03	7.05	7.20	8.87	3.71	0.94
27	5-NO_2	8.07	8.29	8.28	6.20	3.60	1.03
28	5-CF_3	7.87	7.59	7.69	8.28	3.76	1.02
29	3-Br-5-Cl	8.24	8.21	8.23	7.05	3.69	1.03
30	3-Br-5-CF_3	8.49	8.35	8.35	6.11	3.72	1.01
31	3-Br-6-Cl-5-CF_3	8.02	8.10	8.11	4.90	3.83	0.99
32	3-Cl-5-Br	8.13	8.18	8.19	7.02	3.62	1.04
33	3,5-Cl_2-4-Me	8.10	7.90	7.94	7.56	3.50	1.03
34	3,5-Cl_2-4,6-Me_2	7.70	7.59	7.66	7.99	3.39	0.97
35	3,5,6-Cl_3-4-CF_3	7.62	7.48	7.49	4.47	3.13	1.07
36	3,5-Cl_2-6-Me	7.93	7.79	7.83	7.59	3.33	1.05
37	3-Cl-5-CF_3	8.56	8.44	8.44	6.18	3.88	1.04
38	3,6-Cl_2-5-CF_3	8.10	8.10	8.11	4.83	3.89	1.03
39	6-Cl-3-NO_2	8.25	8.50	8.51	5.77	4.06	0.97
40	3-CH_3*	5.89	6.10	6.26	9.25	2.69	0.87
41	3-NO_2	7.68	7.86	7.86	7.23	3.15	0.96
42	3-NO_2-5-CF_3	8.06	8.10	8.12	4.83	3.90	0.98
43	3-CF_3	8.19	8.35	8.37	6.85	3.86	0.97
44	5-Br-6-Cl-3-CF_3	7.68	7.52	7.56	4.11	3.66	1.05
45	6-Cl-3-CF_3	8.15	8.32	8.33	5.16	4.03	1.03
Series III							
46	3-Br-4-CF_3	8.60		8.31	6.38	3.66	0.96
47	3-Cl-4-CH_3*	5.57		5.37	10.14	2.93	0.88
48	3-Cl-4-SO_2Me	7.90		8.01	5.36	3.36	1.02
49	3-Cl-4-SO_2NEt_2	8.40		8.38	6.17	3.78	1.00
50	3-I-4-CF_3	8.60		8.37	6.72	3.81	0.96
51	3-OBu(i)-4-CF_3*	7.62		7.57	8.06	3.32	0.99
52	3-OEt-4-CF_3*	7.94		7.90	7.94	3.76	0.97
53	3-OPr(n)-4-CF_3*	7.82		7.56	7.97	3.21	0.94
54	3,5-Cl_2-4-CH_3*	6.40		6.14	9.53	2.99	0.91
55	4-COOEt*	6.44		6.79	8.84	2.95	0.96

[a] Calculated from Eqn. 5.
[b] Calculated from Eqn. 6.

Quantitative structure–activity analyses

First, the effects of structure on the uncoupling potency of series I compounds were examined by regression analysis with the physicochemical parameters shown in Table I. For the 23 diphenylamines, Eqn. 4 was derived as the equation of the best quality.

$$\log(1/C_{200}) = \underset{(0.019)}{-0.203}(\log K_A^m)^2 - \underset{(0.253)}{2.502} \log K_A^m + \underset{(0.288)}{0.622} \log P_{(M/W)} - \underset{(1.332)}{1.120} \quad (4)$$

$n = 23$, $s = 0.131$, $r = 0.984$ and $F_{3,19} = 191.7$

In this and the 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 intervals. F is the ratio of regression and residual variances. Eqn. 4 indicates that the uncoupling potency increases with the hydrophobicity of compounds in terms of log $P_{(M/W)}$. The potency varies parabolically with increases in the log K_A^m values, the optimum log K_A^m being about -6.2.

We next proceeded to series II (Table II), including fluazinam (compound **37**). This series of compounds has features in common with series I compounds, except for the aza function in the B ring, so the uncoupling potency was analyzed with the parameter sets used for series I compounds to give Eqn. 5:

$$\log(1/C_{200}) = \underset{(0.038)}{-0.183}(\log K_A^m)^2 - \underset{(0.505)}{2.273} \log K_A^m + \underset{(0.270)}{0.537} \log P_{(M/W)} - \underset{(1.644)}{0.698} \quad (5)$$

$n = 22$, $s = 0.170$, $r = 0.964$ and $F_{3,18} = 78.0$

Each of the terms in Eqn. 5 was very close in value to the corresponding one in Eqn. 4 except for the intercept. The optimum log K_A^m value was -6.2 for series II. By combination of the results from series II and III, Eqn. 6 was formulated.

$$\log(1/C_{200}) = \underset{(0.030)}{-0.167}(\log K_A^m)^2 - \underset{(0.411)}{2.081} \log K_A^m + \underset{(0.265)}{0.573} \log P_{(M/W)} - \underset{(1.421)}{0.280} \quad (6)$$

$n = 32$, $s = 0.195$, $r = 0.970$ and $F_{3,28} = 148.0$

The quality of the correlation and the regression coefficient of each term are almost identical in Eqns. 5 and 6. The optimum log K_A^m value was also -6.2 for the combination of series II and III.

When the three sets of compounds, series I–III, were combined, Eqn. 7 was derived as the equation of the best quality.

$$\log(1/C_{200}) = \underset{(0.018)}{-0.182}(\log K_A^m)^2 - \underset{(0.242)}{2.259} \log K_A^m + \underset{(0.180)}{0.444} \log P_{(M/W)} + \underset{(0.102)}{0.270} I_b - \underset{(0.903)}{0.280} \quad (7)$$

$n = 55$, $s = 0.183$, $r = 0.970$ and $F_{4,50} = 199.4$

In Eqn. 7, the effect of the structural change in the B ring moiety was analyzed by use of an indicator variable, I_b, which equals unity for series I, but equals zero for the pyridylamine series II and III. The optimum log K_A^m value was -6.2, corresponding to the values estimated for each series from Eqns. 4–6.

These results suggest that the physicochemical factors deciding the uncoupling potency are the same for series I, II and III compounds except for the effect of the structural modification in the B ring. Each of the terms in Eqns. 4–7 is significant at a level higher than the 99.9% level if examined by the t-test. Additions of other structural parameter terms representing steric dimensions of the molecule and substituents in each equation were insignificant.

The use of the log K_A value measured in 50% ethanol [7] instead of log K_A^m afforded correlations similar to those of Eqns. 4–7. The addition of any steric parameter terms for the substituents and substructural dimensions had no significant effect. In that case, the quality of the correlation was slightly but significantly poorer; for instance, $s = 0.228$, $r = 0.953$ and $F_{4,50} = 123.2$ for the counterpart of Eqn. 7.

Inhibitory effect on the respiratory chain

For diarylamines with a $-\log K_A^m$ lower than about 9, the V_R values were close to 1. This indicated that these amines did not significantly inhibit the respiration chain within the concentration range (0.02–3 μM) studied here [1]. However, a slight inhibition was observed for compounds **4**, **5**, **7**, **8**, **25**, **40**, **47** and **54**, all with $-\log K_A^m$ higher than about 9. Their V_R value was slightly but significantly lower than 1 (0.87–0.92).

Discussion

In structure–activity studies of phenolic uncouplers, we have previously derived Eqn. 8 as the equation with the best correlation [1].

$$\log(1/C_{200}) = \underset{(0.033)}{-0.100}(\log K_A)^2 - \underset{(0.536)}{1.050} \log K_A + \underset{(0.281)}{1.126} \log P_{(L/W)} - \underset{(0.156)}{0.291} \Sigma E_s^o - \underset{(2.071)}{0.681} \quad (8)$$

$n = 22$, $s = 0.265$, $r = 0.988$ and $F_{4,17} = 167.7$

$P_{(L/W)}$ is the overall partition coefficient from the aqueous phase to the liposomal membrane based on

the net concentration including the neutral and ionized species present at pH 7.2. K_A is the acid dissociation constant measured in water. ΣE_s^o is the sum of the Tast-Kutter-Hansch E_s parameter [14] of the two *ortho* substituents relative to H ($E_s(H) = 0$), defined so that the more negative the value, the greater is the steric bulk. The coefficient of the hydrophobic log $P_{(L/W)}$ term is close to 1. Although liposome is used as a model of the mitochondrial membrane, Eqn. 8 indicates that the variations in the uncoupling activity are related to the extent of incorporation of phenols in the membranous phase with almost a one-to-one relationship when other factors are separated. In spite of the use of intact mitochondria for estimation of the ease of incorporation, the coefficient of the log $P_{(M/W)}$ terms in Eqns. 4–7 for diarylamines was smaller than 1 (0.5–0.6). This is probably because compounds are partitioned not only into the lipid phase but also into the proteinaceous components of the mitochondrial membrane, because of the large amount of proteins in mitochondria (50–70% by weight) [11,15]. It is difficult to separate the partition effects on lipid and proteinaceous components. Not all molecules incorporated into the mitochondrial membrane necessarily work as protonophores in the lipid phase. The net amount of compounds partitioned into the lipid phase should be lower than that represented by log $P_{(M/W)}$, leading to the lower coefficients in Eqns. 4–7. In fact, Bakker et al. [10] and Weinbach and Garbus [11] experimentally showed that various kinds of uncoupler molecule are fairly tightly bound to the proteinaceous components of mitochondrial membrane.

The $P_{(M/W)}$ value was defined as an overall partition coefficient from the aqueous phase to the mitochondrial membrane based on the net concentration including neutral and ionized species at pH 7.4. Both the ionized and neutral forms should participate in the protonophoric mechanism of uncoupling actions in the mitochondrial membrane, so the use of the overall partition coefficient should be explained. The use of this parameter here is similar to that of $P_{(L/W)}$ for the phenolic uncouplers in Eqn. 8. The hydrophobicity parameter, log $P_{(octanol/H_2O)}$, more generally used for the neutral form for phenols gives poorer results [16]. For phenolic uncouplers, the log $P_{(L/W)}$ value does not follow the pH-partition principle [17], but it also does not vary much with variations in the pH of the aqueous phase [18]. Thus the log $P_{(M/W)}$ value is regarded to be almost independent from the pH. The log $P_{(M/W)}$ value of the compounds used here seems to vary without depending significantly on the hydrophobicity of substructures and substituents. Steric dimensions and charge delocalizations in the molecule may be more important.

In Eqn. 8, the steric bulk of the *ortho* substituents of phenolic uncouplers in terms of ΣE_s^o is favorable to their uncoupling potency. The *ortho* substituent(s) stabilize ionized species by shielding the negative charge from the nonpolar interior of the mitochondrial membrane [1]. In Eqns. 4–7 for series I–III compounds, all steric parameter terms including those for *ortho* substituents on the B ring are insignificant. The difference in the steric effects between phenols and diarylamines may be explained as follows. First, the negative charge of the ionized form of diarylamines is more widely delocalized into two aromatic rings that have a number of electron-withdrawing substituents [7] than the negative charge of phenols. The negative charge may be delocalized from the amino-nitrogen atom, so that the shielding effects of the *ortho* substituents are not a significant factor. Second, as the negatively charged center of all test compounds is ultimately surrounded by the bulky di-nitro groups on the A ring and by the B ring itself, the shielding effect could be saturated so that *ortho* substituents do not have much effect. Third, the A and B rings in these compounds are not coplanar owing to the steric repulsion between these two heavily substituted rings. A molecular orbital study has shown that the interplane angle between the A and B rings of the neutral forms of compounds **13** and **20** is 65° and 85°, respectively [7]. That of fluazinam (compound **37**) is 63°. Under conformational conditions in which the A and B rings are almost perpendicular to each other, the larger *ortho* substituent on the B ring projects outside the molecular 'bend' [7] and acts as a wedge together with the B ring against the parallel arrangement of the lipid bilayer of the mitochondrial membrane. This kind of steric hindrance could inhibit compounds from moving across the ordered interior of the mitochondrial membrane. Such steric hindrance and the shielding effect of *ortho* substituents may operate in opposite ways to the protonophoric action, and offsetting each other.

The optimal log K_A^m value, −6.2, was the same for compound series I–III, as shown in Eqns. 4–7. This finding is understandable because their chemical structures are similar. For substituted phenols, the optimum log K_m value in Eqn. 8 in terms of that measured in water is about −5.3 [1] at pH 7.4. To compare the optimum log K_A value between diarylamines and phenols using the same standards, we also measured the log K_A^m values of the set of phenols used in Eqn. 8 under the same conditions as we used for diarylamines. Using log K_A^m value instead of log K_A, we reanalyzed the data used to derive Eqn. 8, obtaining Eqn. 9.

$$\log(1/C_{200}) = \underset{(0.017)}{-0.070}\,(\log K_A^m)^2 \underset{(0.272)}{-0.720}\log K_A^m + \underset{(0.250)}{1.493}\log P_{(L/W)} \underset{(0.132)}{-0.170}\,\Sigma E_s^o \underset{(1.238)}{-0.318} \quad (9)$$

$n = 22$, $s = 0.216$, $r = 0.992$ and $F_{4,17} = 254.1$

The quality of the correlation and the regression coefficient of each term are similar to those in Eqn. 8. From Eqn. 9, the optimal log K_A^m value of substituted phenols was estimated to be -5.1, which was not significantly different from that estimated from Eqn. 8. The optimum log K_A^m value was slightly more positive than that for substituted diarylamines.

If the protonophoric potency (uncoupling activity) profiles could be identified by the acidity of uncouplers after any contributions from other molecular parameters such as hydrophobicity and steric dimensions are eliminated, we could expected an almost equivalent dependency of the protonophoric potency on the acid dissociation constant regardless of the structural type of the uncoupler. In fact, the slopes (negative) of log K_A^m and $(\log K_A^m)^2$ terms of Eqns. 4–7 were significantly larger than those of Eqn. 9, although the optimum log K_A^m values of diarylamines and phenols were slightly different. Based on the model of protonophoric action of acidic uncouplers across the phospholipid bilayer membrane proposed by Benz and McLaughlin [19] and by Kasianowicz et al. [20], the rate constant for the movement of the anionic form of uncouplers across the membrane and the acid–base dissociation equilibrium at the membrane surface are important factors in transmembrane proton transport. These two kinds of effects are reflected in the overall dependence of the uncoupling activity on the acid dissociation constant of uncouplers. The acid–base dissociation equilibrium at the membrane surface is directly related to that in aqueous or micellar systems. In fact, the mode of variations in the log K_A^m value of diarylamines is very similar to that in the log K_A value in 50% ethanol solution, the simple correlation coefficient between the two parameters for 55 diarylamines being 0.98 [7,21]. On the other hand, the ease of movement of the anionic form is affected by their stability and shape in the nonpolar and highly ordered interior of the mitochondrial membrane. The stability of the ionized form is related to the acid dissociation constant, but the shape of the molecule and the steric shielding effect of nearby substructures on the charged center are also important. Thus, the effect on the movement of the anionic form is composed of factors that may vary depending on the structural type of the uncoupler.

Another possible factor in the stability of the anionic form of uncouplers in the nonpolar membrane interior is the formation of intramolecular hydrogen bonds. Storey et al. [22] and Terada et al. [23] have suggested that the anionic form of salicylanilides can be stabilized by the formation of a six-membered hydrogen-bonded ring between NH in the aniline moiety and the phenolic O^- in the salicylic acid moiety. This also suggests that the contribution of the electronic effect in terms of the acid dissociation constant to the stability of the anionic form differs among different types of uncoupler. Therefore, even if the uncoupling activity profile could be described with the 'molecular' parameter log K_A^m, the relationship between the uncoupling activity and log K_A^m would not necessarily be uniform, depending on the structural type of the uncoupler.

The significance of the I_b term in Eqn. 7 indicates that diphenylamines are almost uniformly about two times ($10^{0.27} \approx 1.9$) more active than phenylpyridylamines, other things being equal. For the ionized form of phenylpyridylamines, we have previously suggested that the negative charge on the bridge amino-nitrogen is effectively delocalized on the aza nitrogen of the pyridine ring [7]. The negative charge on the aza nitrogen seems to be exposed to the membrane phase more than that on the bridge nitrogen, which is effectively shielded by the two ortho substituents on the A and B rings. Therefore, the anionic form of diphenylamines lacking an aza nitrogen would be more stable in the nonpolar interior of the mitochondrial membrane than that of phenylpyridylamines. The higher activity of diphenylamines may be due to this increased stability of ionized species in mitochondrial membranes.

All protonophoric uncouplers may induce the same degree of maximum respiration by increasing the concentration in the mitochondrial membrane phase without any inhibitory effect on the respiration chain [1]. The higher the $-\log K_A^m$ value, the lower the amount of the ionized form, the movement of which significantly affects the protonophoric potency. A higher concentration of compounds would be required to increase the concentration of the ionized form in the membrane for the maximum stimulation of the respiration. High concentrations of diarylamines could, however, damage the function of the respiration chain leading to the inhibitory effect. Compounds **4**, **5**, **7**, **8**, **25**, **40**, **47** and **54**, all of which have a $-\log K_A^m$ that is higher than about 9, inhibited the respiration chain slightly but significantly. The inhibition highly specific to the substitution patterns observed in phenolic uncouplers such as 2,4-dinitro- and 2,6-dihalophenols [1] was not revealed in diarylamine uncouplers studied here.

Our findings suggest that acidic uncouplers act by working as a protonophore in the inner mitochondrial membrane. Rottenberg [5] has suggested that acidic uncouplers could work as intramembrane proton carriers by removing protons from proton pumps and transporting them to the membrane surface, besides working as transmembrane proton carriers. He has suggested, further, that the physicochemical factors required for intra- and transmembrane protonophores are similar. Herweijer et al. [24] suggest that protonophoric uncouplers could perform the intramembrane proton-shuttling between ATPase and redox enzyme complexes. If the intramembrane proton-shuttling oc-

curs, these two mechanisms (intra- and transmembrane proton shuttling) would be indistinguishable from each other by structure–activity studies that deal with overall activity profiles being governed by similar physicochemical factors as suggested by Rottenberg [5]. To ascertain whether transmembrane proton-shutting is the sole mechanism of acidic uncouplers, a different approach is required.

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