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Quantitative analyses of the uncoupling activity of substituted phenols with mitochondria from flight muscles of house flies

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Uncoupling activity with flight-muscle mitochondria from house flies was measured for a series of weakly acidic uncouplers (substituted phenols) and compared with the protonophoric potency across lecithin liposomal membranes. The activity was linearly related to the protonophoric potency when such factors as the stability of anionic species in the membrane phase and the difference in the pH conditions of the extramembranous aqueous phase were taken into account. Relationships of the flight-muscle activity with activities measured previously with rat-liver mitochondria and spinach chloroplasts were linear. Our findings were further evidence for the shuttle-type mechanism of the uncoupling action of weakly acidic uncouplers.

Introduction

Recent studies including ours [1–4] have provided evidence of the shuttle-type mechanism in which weakly acidic uncouplers work as protonophores across the inner mitochondrial membrane. Other studies [5,6], however, show the possibility that the uncoupler molecules specifically interact with functional proteinous components in the mitochondrial membranes. To identify the mode of action of acidic uncouplers, it is worthwhile to compare uncoupling activities between various energy-transducing biomembranes by use of a series of systematically selected chemicals. Not many investigations [7–9] have dealt with the uncoupling action of acidic uncouplers with insect mitochondria because of difficulties in the preparation of the mitochondria [10,11]. Ilivicky and Casida [8] and Holan and Smith [9] reported that the activity of weakly acidic uncouplers in insect and rat-liver mitochondria are not parallel. Some uncouplers are selectively active toward the insect mitochondria but some others are so to the rat-liver mitochondria. With a shuttle-type mechanism in which uncouplers do not interact with a specific membranous protein, however, it seems unlikely that there be marked selectivity in the uncoupling activity with mitochondria from various origins.

In this study, we prepared intact flight-muscle mitochondria from house flies, that are maintaining high respiration control, and measured the uncoupling activity using a number of variously substituted phenols. We examined quantitatively the relationships in the uncoupling activity be-

Abbreviations: SF6847, 3,5-di-*t*-butyl-4-hydroxybenzylidene malononitrile; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TCA cycle, tricarboxylic acid cycle; Chl, chlorophyll.

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tween flight-muscle and rat-liver mitochondria as well as between flight-muscle mitochondria and spinach chloroplasts. We found excellent linear relationships among uncoupling activities with biomembranes from three different species. Selectivity in the uncoupling activity was not observed for the compounds examined here. We also quantitatively analyzed variations in the uncoupling activity of these phenols with physicochemical substituent and molecular parameters using the regression technique. The protonophoric potency across such an artificial membrane system as lecithin liposomes was related with the uncoupling activity when the difference in the pH conditions of the extramembranous aqueous phase was taken in consideration. Moreover, the stability of anionic species in the mitochondrial membranes in terms of the dissociation constant of phenols as well as the shielding of the negative charge of phenolate anions by nearby substituents was found to govern the uncoupling potency markedly.

Materials and Methods

Materials. Uncouplers used in this study were the same samples as those used previously [1,2]. ATP and ADP were obtained from the Oriental Yeast Co. Oligomycin and rotenone were purchased from Sigma Chemical Co. House flies (*Musca domestica*) of both sexes were used between 4 and 8 days after emergence.

Isolation of flight-muscle mitochondria. Mitochondria were isolated from the flight-muscle of house flies by a procedure modified from that of Van den Berg and Slater [12]. After removal of the heads and abdomens, about 200 thoraces were disrupted by gentle squeezing without any strong grinding action with a loose glass homogenizer in 10 ml of an isolation medium containing 150 mM KCl, 1 mM EDTA, and 2 mM Tris-HCl (pH 7.4). The homogenate was filtered through four layers of gauze that had been washed with ice-cold isolation medium. The trapped residue was washed with 2 ml of the isolation medium. The filtrated homogenate was carefully homogenized with a Teflon pestle. The resulting homogenate was centrifuged at $500 \times g$ for 3 min in a refrigerated centrifuge and the supernatant again centrifuged at $5500 \times g$ for 8 min. The sedimented mitochondrial pellet was resuspended in a small volume of the isolation medium with a swab, and then centrifuged at $5500 \times g$ for 8 min. The mitochondrial pellet was suspended in 1 ml of the isolation medium to yield a suspension containing about 4 mg of protein per ml. All of these operations were carried out at 1–3°C. The amount of mitochondrial protein was measured by the method of Bradford [13] with bovine serum albumin as the standard. This procedure differs from that of Van den Berg and Slater in that the primary 'homogenization' with the glass homogenizer was not done to homogenize the tissues completely, and in that the glass pestle only loosely fit the tube of the homogenizer. The secondary homogenization was done after removal of pieces of hard, rigid cuticle.

Measurement of respiration rate of flight-muscle mitochondria. The standard reaction medium used in all experiments was 100 mM KCl, 20 mM KH_2PO_4, 20 mM pyruvate, 20 mM proline, 5 mM $MgCl_2$, 1 mM EDTA, 0.8 mM ATP, and 10 mM Tris-HCl (pH 7.4). Mitochondrial respiration was measured with a Clark-type oxygen electrode fixed with a thermostatted reaction cell (2.5 ml) at 25°C. The final protein concentration was 0.07 mg/ml. To this respiration system, each uncoupler as an ethanol stock solution was added one after the other (1–15 μl). The uncoupling activity of the test compounds was estimated as the concentration, C_{200}^{fly}, at which the respiration rate was twice that of state-4 respiration.

The stoichiometric relationships between uncoupler molecules and the phosphorylation assemblies under complete uncoupling conditions were examined for SF6847 and FCCP by the method of Terada and Van Dam [14] on the assumption that the amount of phosphorylation assemblies in flight-muscle mitochondria is 1.0 nmol per mg of mitochondrial protein [15].

The effect of the concentration of ATP in the reaction medium on the respiration rate stimulated by an uncoupler was examined with various concentrations of ATP. The difference in the respiration rate of flight-muscle and rat-liver mitochondria stimulated by uncoupler in the presence or absence of oligomycin was examined. The experimental details are provided in the legends to the figures.

Measurement of uncoupling activity with rat-liver mitochondria and spinach chloroplasts. The uncoupling potency with rat-liver mitochondria was expressed as log $1/C_{200}^{rat}$, where C_{200}^{rat} is the concentration needed to double the state-4 respiration rate. As the index for the uncoupling potency with spinach chloroplasts, log $1/C_{200}^{Chl}$ was used, where C_{200}^{Chl} is the concentration needed to increase the rate of electron transport to twice that of the control. The values of log $1/C_{200}^{rat}$ and log $1/C_{200}^{Chl}$ were from our earlier studies [2,16]. The amount of phosphorylation assemblies in the reaction medium was made to be almost equivalent among these three experimental systems, being about $7.0 \cdot 10^{-11}$ mol of phosphorylation assemblies per ml of medium for flight-muscle and rat-liver mitochondria and about $10.0 \cdot 10^{-11}$ mol for spinach chloroplasts [16].

Measurement of protonophoric potency and the acid dissociation constant. The protonophoric potency across the liposomal membrane, log P_p, in which P_p is the increment of the proton permeability in terms of cm/s per unit molar concentration of the uncouplers, and the dissociation constant, K_A, were taken from previous papers [1,2].

Results

Characterization of flight-muscle mitochondria

In each of the mitochondrial preparations, the respiration control ratio was constant, being 4.5 or more within 3 h after the preparation. ADP-stimulated respiration (state 3) was inhibited by the addition of oligomycin ($1.5 \cdot 10^{-7}$ M) to the level of state-4 respiration. The state-3 and state-4 respirations were completely inhibited by rotenone ($2.5 \cdot 10^{-6}$ M), because pyruvate was used as substrate in this study.

Uncoupler-stimulated respiration was not observed in the absence of ATP in the reaction medium for all of the test compounds with flight-muscle mitochondria. State-4 respiration, which is the respiration after the added ADP has been completely converted to ATP, was markedly stimulated by the addition of uncoupler. The relationship between the respiration rate stimulated by uncoupler and the concentration of ATP in the reaction medium was examined with $2 \cdot 10^{-8}$ M SF6847 as an example (Fig. 1). This concentration of SF6847 was chosen because it caused the maximum stimulation of the respiration rate in the rat-liver mitochondria [1]. With this concentration of SF6847, the minimum ATP concentration giving the maximum respiration rate was about 0.6 mM. Therefore, 0.8 mM ATP was added to the reaction medium to measure the uncoupling activity in the following experiments. The same concentration of SF6847 was confirmed to stimulate the respiration rate completely also with the flight-muscle mitochondria.

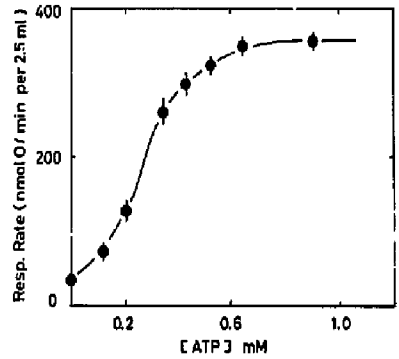


Fig. 1. Dependence of the respiration rate stimulated by SF6847 (20 nM) on the concentration of ATP in the reaction medium.

The effect of oligomycin on the uncoupler-stimulated respiration of flight-muscle mitochondria differed from that on the respiration of rat-liver mitochondria as shown in Figs. 2 and 3. With rat-liver mitochondria, the inhibition of the F_0 component of ATPase by oligomycin has no effect on the respiration rate stimulated by uncoupler [17]. This was also confirmed in this study (Fig. 2A and B). With flight-muscle mitochondria, however, the respiration rate was not stimulated by uncoupler with the addition of oligomycin, which inhibits the F_0 component of ATPase (Fig. 3A, B and C). Under these conditions for flight-muscle mitochondria, the addition of ADP was required to restore the stimulation of the respiration. The restored respiration rate was, however, slightly smaller than that in the absence of oligomycin. It should be also noted from Fig. 3D that uncoupler-stimulated respiration was observed even after the addition of oligomycin when ADP

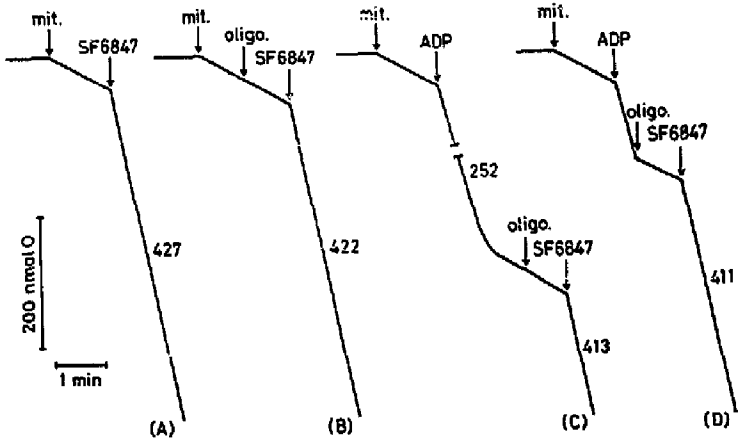


Fig. 2. Effect of oligomycin on the uncoupler-stimulated respiration of rat-liver mitochondria. The reaction medium contained 10 mM sodium succinate, 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA and 2.5 mM potassium phosphate (pH 7.4). The final mitochondrial protein concentration was 0.7 mg/ml. Arrows indicate the addition of 25 nM SF6847, 2.5 μM oligomycin, or 0.4 mM ADP. The numbers on the figure indicate the oxygen consumption rate as nmol O/min per 2.5 ml.

in the reaction medium was not completely converted to ATP.

Relationships of uncoupling activity among various energy-transducing membranes

The uncoupling activity of compounds measured with flight-muscle mitochondria, in terms of $\log 1/C_{200}^{fly}$, and the previous data for the rat-liver mitochondria, $\log 1/C_{200}^{rat}$, and spinach chloroplasts, $\log 1/C_{200}^{Chl}$, were listed in Table I together with molecular and substituent physicochemical parameters. In each system, SF6847 had the highest uncoupling activity.

The relationship among the uncoupling activi-

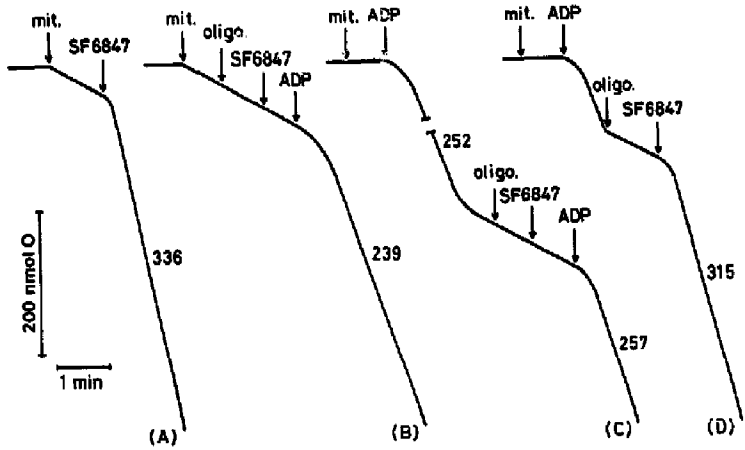
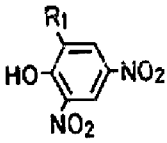
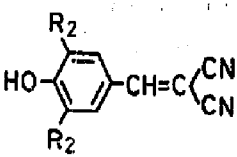
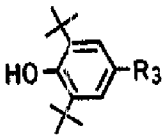


Fig. 3. Effect of oligomycin on the uncoupler-stimulated respiration of flight-muscle mitochondria. The reaction medium contained 100 mM KCl, 20 mM KH_2PO_4, 20 mM pyruvate, 20 mM proline, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) without (C, D) or with (A, B) 0.8 mM ATP. The final mitochondrial protein concentration was 0.07 mg/ml. Arrows indicate the addition of 25 nM SF6847, 0.15 μM oligomycin or 0.8 mM ADP. The numbers on the figure indicate the oxygen consumption rate as nmol O/min per 2.5 ml.

TABLE I

PHYSICOCHEMICAL CONSTANTS AND UNCOUPLING ACTIVITIES OF UNCOUPLERS

No.	Compound	$-\log P_P$ [a]	$-\log K_A$ [a]	$-\Sigma E_s^{ortho}$ [b]	$\log P_f$ [a]	$\log 1/C_{200}^{fly}$ obs.	$\log 1/C_{200}^{fly}$ calc. [c]	$\log 1/C_{200}^{rat}$ obs. [a]	$\log 1/C_{200}^{rat}$ calc. [d]	$\log 1/C_{200}^{Chl}$ obs. [e]	$\log 1/C_{200}^{Chl}$ calc. [f]
											
1	R_1 = H	6.22	4.09	1.65	−0.70	4.76	4.30	5.10	4.66	– [g]	–
2	Me	5.44	4.44	2.89	−0.44	5.52	5.57	5.60	5.71	– [g]	–
3	Et	4.94	4.43	2.96	−0.45	6.07	5.89	6.02	6.04	– [g]	–
4	*i*-Pr	4.53	4.47	3.36	−0.42	6.12	6.44	6.46	6.55	– [g]	–
5	*s*-Bu	4.18	4.51	4.02	−0.40	6.65	6.76	6.89	6.71	– [g]	–
6	*t*-Bu	4.16	4.80	4.43	−0.21	6.89	7.18	6.85	7.09	– [g]	–
											
7	R_2 = H	5.77	7.04	0	0.51	5.20	5.24	5.37	5.95	3.68	3.73
8	Me	5.02	6.91	2.48	0.51	6.11	6.54	6.39	6.82	4.79	5.11
9	Et	4.68	6.98	2.62	0.51	6.77	6.76	6.76	7.03	5.60	5.42
10	*i*-Pr	4.48	7.06	3.42	0.51	7.35	7.09	7.35	7.23	6.00	5.67
11	*s*-Bu	4.01	7.22	4.74	0.48	7.72	7.64	7.66	7.57	6.22	6.21
12	*t*-Bu	4.06	6.84	5.56	0.51	8.60	8.13	8.44	7.88	6.86	6.75
											
13	R_3 = CH=C(CN)COOEt	4.48	7.51	5.56	0.36	7.41	7.17	7.14	6.97	5.42	5.50
14	CH=C(CN)H	4.37	9.95	5.56	0	5.12	5.14	4.94	5.18	– [g]	–
15	CH=C(CN)CONH$_2$	5.47	7.73	5.56	0.22	5.85	6.11	5.92	5.91	4.64	4.18
16	CH=C(COMe)COOEt	4.77	8.67	5.56	0	5.51	5.56	5.42	5.49	– [g]	–
17	CH=CHNO$_2$	5.22	6.89	5.56	0.51	7.17	7.37	7.16	7.07	5.44	5.45
18	CH=C(CN)SO$_2$Me	4.62	6.77	5.56	0.50	7.70	7.79	7.73	7.51	5.79	6.24
19	CH=C(CN)COOMe	4.51	7.45	5.56	0.43	7.52	7.34	7.60	7.16	5.62	5.54
20	CHO	5.98	7.93	5.56	0.06	5.28	5.32	5.12	5.14	3.36	3.40
21	CN	4.85	8.89	5.56	0	5.40	5.39	5.42	5.34	3.24	3.46
22	FCCP	4.99	6.20	5.56	0.37	7.52 [h]	7.56	7.49 [h]	7.24	6.10 [h]	6.52
23	CCCP	4.30	5.95	5.56	0.30	7.33 [h]	7.96	7.30 [h]	7.67	5.58 [h]	7.55

[a] From Refs. 1 and 2.

[b] From Ref. 21. For the nitro group, the E_s^{ortho} 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. 18. This averaged E_s^{ortho} value was recently found to apply to the steric effect of an *o*-nitro group on the acid-catalytic hydrolysis of benzamides (Sotomatsu, T. and Fujita T., unpublished results).

[c] By Eqn. 8.

[d] By Eqn. 9.

[e] From Ref. 16.

[f] By Eqn. 10.

[g] Not studied because of the marked inhibition of the electron transport system.

[h] Not included in the correlation analyses.

ties with various energy-transducing membranes was examined by regression analysis, giving Eqns. 1 and 2.

$$\log 1/C_{200}^{fly} = \underset{(0.078)}{1.026} \log 1/C_{200}^{rat} - \underset{(0.509)}{0.198}$$

$$(n = 21,\ s = 0.171,\ r = 0.988) \quad (1)$$

$$\log 1/C_{200}^{fly} = \underset{(0.186)}{0.934} \log 1/C_{200}^{Chl} + \underset{(0.993)}{1.986}$$

$$(n = 13,\ s = 0.329,\ r = 0.958) \quad (2)$$

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 interval. The slopes of Eqns. 1 and 2, being close to 1, show that the variations in the uncoupling activity with flight-muscle mitochondria correspond to those with rat-liver mitochondria and spinach chloroplasts in an almost 1-to-1 way. The intercept of Eqn. 1 is close to 0, indicating that the activity of each compound with rat-liver mitochondria is almost equivalent to that with flight-muscle mitochondria. The intercept of Eqn. 2 shows, however, that the activity with flight-muscle mitochondria is about 100 times that with chloroplasts.

Stoichiometric study of uncoupler activity

The 'intrinsic' uncoupling activity was defined previously in terms of the number of uncoupler molecules needed for the complete uncoupling with a unit amount of phosphorylation assembly when all of the added uncoupler molecules in the test medium are bound to mitochondria. The value of the intrinsic uncoupling activity of SF6847 and FCCP with flight-muscle mitochondria is listed in Table II together with that in rat-liver mitochondria and spinach chloroplasts [16]. The intrinsic uncoupling activity measured with flight-muscle mitochondria was close to that measured with rat-liver mitochondria.

Quantitative analysis of uncoupling activity with physicochemical substituent and molecular parameters

In our earlier studies [1,2,16], the uncoupling activities with rat-liver mitochondria and chloroplasts were linearly related to the protonophoric potency across such a model membrane as liposomes, when differences between biomembranes and liposomes in the extramembranous pH conditions and the stability of the ionized form of the uncoupler molecules were taken into account, as shown by Eqns. 3 and 4.

$$\log 1/C_{200}^{rat} = \underset{(0.134)}{0.915} \log P_p + \underset{(0.065)}{0.440} \log K_A + \underset{(0.407)}{2.136} \log P_f + \underset{(0.719)}{13.500}$$

$$(n = 38,\ s = 0.554,\ r = 0.977) \quad (3)$$

$$\log 1/C_{200}^{Chl} = \underset{(0.429)}{1.263} \log P_p + \underset{(0.442)}{0.957} \log K_A + \underset{(3.391)}{18.270}$$

$$(n = 13,\ s = 0.394,\ r = 0.947) \quad (4)$$

In Eqns. 3 and 4, all of the terms are significant at the 99.5% level. log P_p, in which P_p is the increment of the proton permeability across liposomal membranes per unit molar concentration of

TABLE II

INTRINSIC AND APPARENT UNCOUPLING ACTIVITIES

	Intrinsic activity (molecules uncoupler per phosphorylation assembly)			Intrinsic activity Ratio			Apparent activity [C_{200} (M)]			Apparent activity Ratio		
	Fly [a]	Rat [b]	Chl [b]	Rat / Fly	Chl / Fly	Chl / Rat	Fly [a]	Rat [b]	Chl [b]	Rat / Fly	Chl / Fly	Chl / Rat
SF6847	0.12	0.09	2.7	0.75	23	30	$4.0 \cdot 10^{-9}$	$3.6 \cdot 10^{-9}$	$1.4 \cdot 10^{-7}$	0.90	35	38
FCCP	0.35	0.30	8.7	0.85	25	29	$3.0 \cdot 10^{-8}$	$3.2 \cdot 10^{-8}$	$8.0 \cdot 10^{-7}$	1.07	26	25

[a] Newly found.
[b] From Ref. 16.

uncouplers in bulk medium, was used as the index for the protonophoric potency across biomembrane systems. The proton conductivity of weakly acidic uncouplers across the lipid bilayer membrane is markedly dependent on the extramembranous pH condition [4]. When the uncoupling activity of uncouplers with mitochondria is correlated with the protonophoric potency across the liposomal membrane, it is important to consider the pH dependence of the proton conductivity of compounds because of differences in pH conditions between the intermembrane space of mitochondria (about 7.2) and the internal aqueous phase of liposomes (4.5–7.0) set in our previous studies. log P_f in Eqn. 3 is the parameter that takes into account this effect as mentioned in a previous paper [2]. P_f was formulated by Eqn. 5:

$$P_f = \frac{P_r(\text{mitochondria})}{P_r(\text{liposome})} \tag{5}$$

P_r in Eqn. 5 is defined by Eqn. 6:

$$P_r = \frac{[H^+] \cdot K_A}{(K_A + [H^+])(K_A + 200[H^+])} \tag{6}$$

where $[H^+]$ is the extramembranous proton concentration in either mitochondrial or liposomal measurements, and K_A is the dissociation constant of uncouplers. In Eqn. 4, the log P_f term was not significant with the chloroplast activity because of the small range of variations in the log P_f for compounds included in the study under experimental conditions.

Eqns. 3 and 4 suggest that the physico-chemical factors of phenols governing the variations in the uncoupling activity are almost identical for various energy-transducing biomembranes. Thus, we first analyzed the uncoupling activity with flight-muscle mitochondria using the same parameters as those used in Eqns. 3 and 4.

$$\log 1/C_{200}^{fly} = \underset{(0.366)}{0.945} \log P_p + \underset{(0.172)}{0.339} \log K_A + \underset{(0.702)}{1.956} \log P_f + \underset{(2.056)}{13.020}$$

$$(n = 21,\ s = 0.481,\ r = 0.909) \tag{7}$$

Although the correlation coefficient of Eqn. 7 seems good enough to explain the variations in flight-muscle activity, the standard deviation value is large. In elaborating the correlation, we observed that the deviation between the observed and calculated values tended to increase with increases in the bulk of the *ortho*-substituents. Anticipating that the deviation could be accounted for by some steric parameters relevant to *ortho*-substituents, we added the ΣE_s^{ortho} term to Eqn. 7, resulting in Eqn. 8.

$$\log 1/C_{200}^{fly} = \underset{(0.231)}{0.630} \log P_p + \underset{(0.115)}{0.525} \log K_A - \underset{(0.104)}{0.306} \Sigma E_s^{ortho} + \underset{(0.407)}{2.280} \log P_f + \underset{(1.271)}{11.411}$$

$$(n = 21,\ s = 0.267,\ r = 0.974) \tag{8}$$

ΣE_s^{ortho} is the sum of the Taft-Kutter-Hansch E_s^{ortho} parameters [18] of the two *ortho*-substituents relative to H (E_s^{ortho} (H) = 0). The quality of the correlation was highly improved in Eqn. 8.

Discussion

Gregg et al. [19] observed that 2,4-dinitrophenol stimulated the respiration of flight-muscle mitochondria of house flies only in the presence of ATP. Our results shown in Fig. 1 are consistent with their observations. Van den Bergh [7] found that the factor required for the stimulation of pyruvate oxidation by an uncoupler is not ATP itself but ADP derived from the added ATP by the uncoupler-stimulated ATPase. They also reported that ADP and inorganic phosphates are needed for the overall operation of the TCA cycle. Our findings shown in Fig. 3 that the uncoupler-stimulated respiration was not observed in the absence of ADP when ATPase was inhibited by oligomycin agreed with the findings of Van den Bergh. Under these conditions, the ADP essential for the operation of the TCA cycle is not available even if ATP exists in the reaction medium, because ATPase is inhibited.

Eqns. 1 and 2 show that the uncoupling activities measured with various energy-transducing membranes are linearly related to each other regardless of the difference in membrane composition. The difference in the intercept in Eqns. 1 and

2, however, reflects the difference in the 'apparent' activity of each uncoupler among biomembrane systems. Because the amount of phosphorylation assemblies in the unit volume of the medium was kept approximately equal for the three series of measurements, the difference in the 'apparent' activity should arise from the difference in the 'intrinsic' activity among various biomembranes. As shown in Table II the intrinsic activity indices of SF6847 and FCCP are indeed parallel to the apparent activity indices among the three biomembrane systems. The ratio of intrinsic as well as apparent activity between rat-liver and flight-muscle mitochondria is approx. 1, which is in accord with the intercept value in Eqn. 1. That between chloroplasts and mitochondria, being in the range between 25 and 40, is somewhat smaller but located within the 95% confidence interval of the intercept value of Eqn. 2.

Eqn. 7 seems to show that the factors governing the variations in the flight-muscle uncoupling activity of substituted phenols are the same as those for the rat-liver mitochondrial and spinach chloroplasts activities appearing in Eqns. 3 and 4. In Eqn. 8, however, the steric effect of ortho-substituents in terms of ΣE_s^{ortho} was shown to be an important physico-chemical factor governing the uncoupling activity with flight-muscle mitochondria.

The E_s parameter was defined originally for the steric effect of aliphatic substituents by Taft [20]. The more negative the value, the bulkier the substituents. It was extended later by Kutter and Hansch [18] and shown to apply to the steric effect of aromatic substituents at various positions. The steric effect of two *ortho*-substituents may differ from each other. Therefore, we attempted to analyze them by classifying substituents according to their bulkiness in terms of E_s^{ortho}. The collinearity in the E_s^{ortho} value between bulkier and less bulky substituents was, however, quite high for the compounds used here. Thus, E_s^{ortho} values were combined to give Eqn. 8.

We showed that the steric effect of *ortho*-substituents on the log K_A value of substituted phenols in the aqueous phase can indeed be analyzed with the Taft-Kutter-Hansch E_s^{ortho} parameter [21]. For this dissociation equilibrium, the sign of the E_s^{ortho} term was positive, indicating that the bulkiness of the *ortho*-substituent is unfavorable, perhaps inhibiting possible hydration, leading to destabilization of the anionic form in the aqueous phase. The negative sign of the ΣE_s^{ortho} term in Eqn. 8 means that the steric bulk of *ortho*-substituents is favorable to the uncoupling activity. The membrane phase is much less polar than the aqueous phase and the solvation of the anionic form of phenols should be much less significant in the membrane phase than in the aqueous phase. The steric inhibition of solvation by *ortho*-substituents could be less important, but steric shielding of the negative charge from the non-polar medium to stabilize the anions should become more important in the membrane phase than in the aqueous phase. The log K_A value is used as the parameter representing the stability of the anionic form of phenols in the membrane phase in Eqns. 3, 4, 7 and 8. It was, however, measured in the aqueous phase. The ΣE_s^{ortho} term in Eqn. 8 represents a correcting factor for the stability of anionic phenols in the membrane relative to the aqueous phase.

We anticipated that the addition of the ΣE_s^{ortho} term into Eqns. 3 and 4 for rat-liver and chloroplast activity would be also significant.

$$\log 1/C_{200}^{Rat} = \underset{(0.170)}{0.676}\ \log P_p + \underset{(0.055)}{0.453}\ \log K_A - \underset{(0.065)}{0.123}\ \Sigma E_s^{ortho} + \underset{(0.343)}{2.110}\ \log P_f + \underset{(1.016)}{11.958}$$

$$(n = 38,\ s = 0.299,\ r = 0.985) \qquad (9)$$

$$\log 1/C_{200}^{Chl} = \underset{(0.344)}{1.070}\ \log P_p + \underset{(0.365)}{1.191}\ \log K_A - \underset{(0.122)}{0.172}\ \Sigma E_s^{ortho} + \underset{(2.490)}{18.287}$$

$$(n = 13,\ s = 0.285,\ r = 0.976) \qquad (10)$$

In Eqns. 9 and 10, to which the ΣE_s^{ortho} term is added, the quality of correlation, especially in terms of the standard deviation, was indeed much improved. In each case, the sign of the term is negative, supporting the discussion made for the term in Eqn. 8.

To examine the general applicability of Eqns. 8–10, we attempted to extend the study of correlation beyond the phenolic uncouplers toward phen-

ylhydrazone uncouplers such as FCCP and CCCP. The steric parameter was, however, difficult to estimate on the same standards as those for *ortho*-substituted phenols. The steric effect was taken to be that on the negative charge center of the anions, so we estimated the degree of shielding effect of nearby substituents or substructures by calculating the surface area of either phenolic oxygen or α-nitrogen of phenylhydrazones accessible to solvent. The area was calculated by use of MOLAREA, provided by Hermann [22] with the standard van der Waals radii of the elements [23]. For negative nitrogen and oxygen atoms, the radius of neutral atoms was used. For the calculation, we selected conformations that have minimum steric repulsion energy, the geometry of which was derived with use of ANCHOR, a program system for molecular modeling (Kureha Chemical Industry Co. Ltd. and Fujitsu Ltd., Tokyo), using standard bond lengths and bond angles. The solvent-accessible surface areas were compared for water and methane as the 'solvent'. The methane molecule was used as an approximate model of a lipid. The radius of 1.5 Å for water [22] and 2.2 Å for methane [24] was selected. Although the individual values in Table III were not very accurate owing to a number of assumptions involved in the calculations, they show a definite correspondence with the bulkiness of di-*ortho*-substituents. The bulkier the substituent, the lower the solvent-accessible surface for 2,6-substituted phenolate anions in both 'solvent' phases. The surface area for the deprotonated nitrogen atom of FCCP (CCCP) is very close to that of the oxygen atom of 2,6-di-*t*-butylphenol, the model of SF6847. Thus, we assumed that the steric parameter for FCCP (CCCP) can be approximated by that for SF6847 in terms of ΣE_s^{ortho}. Then, the log $1/C_{200}$ values were estimated by use of Eqns. 8 and 9, with experimentally measured log P_p and log K_A and calculated log P_f values, for these phenylhydrazone uncouplers taken from a previous paper [2]. The estimated values agreed very well with the experimental indices as shown in Table I for flight-muscle and rat-liver mitochondria. For the chloroplasts activity, the estimated values with Eqn. 10 lacking the log P_f term for phenylhydrazone uncouplers deviated considerably from those experimentally measured. As mentioned earlier in this paper, the variations in the log P_f term for 13 phenolic uncouplers for which the activity was measurable were too low to disclose the significance of this term. In other words, extramembranous pH conditions are not much different between chloroplasts and liposomal membranes in our experimental systems. That the chloroplast activity for phenylhydrazone uncouplers was not predicted well by Eqn. 10 suggests that factors governing the uncoupling activity may not be completely separated in Eqn. 10 for only 13 compounds of similar log K_A values. Nevertheless, the good quality of the correlation of Eqns. 8–10, along with the activity of phenylhydrazone uncouplers with mitochondria conforming well with that of phenolic uncouplers rationalized by Eqns. 8 and 9, indicates the strong possibility of explaining the variations of the activity of weakly acidic uncouplers in general.

TABLE III

SOLVENT-ACCESSIBLE SURFACE AREAS OF THE OXYGEN ATOM OF 2,6-DI-SUBSTITUTED PHENOLS AND THE α-NITROGEN ATOM OF FCCP

Compound	Surface areas (Å^2)	
	CH_4	H_2O
2,6-H_2	73.8	52.8
2,6-$(Me)_2$	43.2	34.4
2,6-$(Et)_2$	41.8	33.4
2,6-$(i\text{-}Pr)_2$	37.7	31.7
2,6-$(s\text{-}Bu)_2$	37.7	31.7
2,6-$(t\text{-}Bu)_2$	7.1	7.1
FCCP (CCCP)	9.2	10.5

Previously, Draber et al. [25] suggested the importance of the hydrophobic shielding effect of substituents on the acidic dissociation group in a structure-activity study of phenylhydrazone uncouplers. Our results quantitatively showed the significance of the shielding of the dissociation group by nearby substituents. Storey et al. [26] reported that the uncoupling activity of salicylanilides is much affected by the steric bulk of substituents on the salicylate-benzene ring, implying that the uncoupler molecules would bind with a specific binding site fulfilling particular steric requirements. This study shows, however, that the steric demand of substituents for the uncoupling

activity did not necessarily indicate the participation of a particular binding site.

Our results suggesting an identical mode of action for acidic uncouplers among energy-transducing membranes from different sources were further evidence for the shuttle-type mechanism of the uncoupler action.

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References

1 Miyoshi, H., Nishioka, T. and Fujita, T. (1987) Biochim. Biophys. Acta 891, 194–204.
2 Miyoshi, H., Nishioka, T. and Fujita, T. (1987) Biochim. Biophys. Acta 891, 293–299.
3 Terada, H., Kumazawa, N., Juichi, M. and Yoshikawa, K. (1984) Biochim. Biophys. Acta 767, 192–199.
4 Benz, R. and McLaughlin, S. (1983) Biophys. J. 41, 381–398.
5 Herweijer, M.A., Berden, J.A. and Slater, E.C. (1986) Biochim. Biophys. Acta 849, 276–287.
6 Decker, S.J. and Lang, D.R. (1977) J. Biol. Chem. 252, 5936–5938.
7 Van den Bergh, S.G. (1964) Biochem. J. 93, 128–136.
8 Ilivicky, J. and Casida, J.E. (1969) Biochem. Pharmacol. 18, 1389–1401.
9 Holan, G. and Smith, D.R.J. (1986) Experimentia 42, 558–560.
10 Wojtczak, L. and Wojtczak, A.B. (1960) Biochim. Biophys. Acta 39, 277–286.
11 Ilivicky, J., Chefurka, W. and Casida, J.E. (1967) J. Econ. Ent. 60, 1404–1407.
12 Van den Bergh, S.G. and Slater, E.C. (1962) Biochem. J. 82, 362–371.
13 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
14 Terada, H. and Van Dam, K. (1975) Biochim. Biophys. Acta 387, 507–518.
15 Sacktor, B. (1974) in The Physiology of Insecta (Rockstein, M., ed.), Vol. 4, pp. 271–353, Academic Press, New York.
16 Miyoshi, H. and Fujita, T. (1987) Biochim. Biophys. Acta 894, 339–345.
17 Lardy, H. (1981) in Inhibitors of Mitochondrial Function (Erecinska, M. and Wilson, D.F., eds.), pp. 187–198, Pergamon Press, Oxford.
18 Kutter, E. and Hansch, C. (1969) J. Med. Chem. 12, 647–652.
19 Gregg, C.T., Johnson, J.R., Heisler, C.R. and Remmert, L.F. (1964) Biochim. Biophys. Acta 82, 343–349.
20 Taft, R.W. (1965) in Steric Effects in Organic Chemistry (Newman, M.S., ed.), pp. 556–675, John Wiley, New York.
21 Fujita, T. and Nishioka, T. (1976) Prog. Phys. Org. Chem. 12, 49–90.
22 Hermann, R.B. (1972) J. Phys. Chem. 76, 2754–2759.
23 Bondi, A. (1964) J. Phys. Chem. 68, 441–451.
24 Charton, M. (1969) J. Am. Chem. Soc. 91, 615–618.
25 Draber, W., Büchel, K.H. and Schäfer, G. (1972) Z. Naturforsch. 27, 159–171.
26 Storey, B.T., Wilson, D.F., Bracey, A., Rosen, S.L. and Stephenson, S. (1975) FEBS Lett. 49, 338–341.