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Quantitative relationship between protonophoric and uncoupling activities of analogs of SF6847 (2,6-di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol)

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Uncoupling activity with rat liver mitochondria and protonophoric activity across the lecithin liposomal membranes were measured for a series of non-classical uncouplers related to the most potent uncoupler known until now, SF6847 (2,6-di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol). The correlation between uncoupling and protonophoric activities for a number of uncouplers, both non-classical and classical (simply substituted phenols), was examined quantitatively. Correlation was excellent 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. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which are structurally different, were correlated in a way that resembled the correlation of phenolic compounds, so we think that the mode of action of weakly acidic uncouplers was the same regardless of the structural type. Our findings were evidence for the shuttle-type mechanism of uncoupling action.

Introduction

We have measured uncoupling activity with the oxidative phosphorylation of rat liver mitochondria and protonophoric activity across lecithin liposomal membrane for a series of phenols substituted by such simple substituents as alkyl, halogen, nitro, cyano, and trifluoromethyl [1]. We have examined quantitatively the possible relationships between the two activities and found that the activities correspond to each other in a 1-to-1 way when the difference in the stability of the ionized form of the uncoupler molecules in the membrane phase between mitochondria and lecithin liposomes is taken into account in terms of the dissociation constants of the uncouplers. We think that the results were evidence for a shuttle-type mechanism of uncouplers in which they work as a protonophore across the inner mitochondrial membrane to dissipate the proton gradient.

We have also found that the uncoupling potency of the very potent uncoupler SF6847 (X_2=X_3=CN) is much stronger than expected from the quantitative relationship with the protonophoric activity. This might be due to the mechanism of this SF6847 being other than the shuttle-type mechanism suggested for classical uncouplers, or perhaps we have overlooked some other physico-chemical factors in the structure of SF6847. The extramembranous pH values are different between mitochondria under uncoupling conditions and lecithin liposomes under protonophoric conditions. Benz and McLaughlin [2] have found that the proton conduc-

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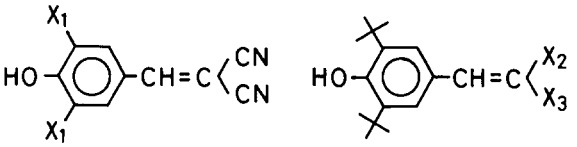
tivity of a certain protonophoric compound across the membrane is governed by the pH conditions of the extramembranous aqueous phase. Thus, we measured uncoupling and protonophoric activities of non-classical uncouplers structurally analogous to SF6847 such as 2,6-di-alkyl-4-(2′,2′-dicyanovinyl)phenols (I) and 2,6-di-*t*-butyl-4-(2′,2′-disubstituted vinyl)phenols (II).



(I) (II)

Using a parameter defining the difference in the effect of the extramembranous pH along with the dissociation constant, we analyzed the quantitative relationship between the two activities again. Here we report that the uncoupling activity for the whole series of phenols including classical and non-classical uncouplers indeed corresponds to the protonophoric activity and that it is evident for the shuttle-type mechanism for weakly acidic uncouplers.

Materials and Methods

Materials. The non-classical uncouplers used are listed in Table I. 2,6-Di-alkyl-4-(2′,2′-dicyanovinyl)phenols [1–5] prepared by the procedure of Horiuchi et al. [3] were kindly supplied by Professor H. Terada (Tokushima University, Japan) [4]. SF6847 [6] was the same as that used previously [1]. 2,6-Di-*t*-butyl-4-substituted phenols [7–19] were generous gifts from Dr. K. Watanabe of the Kanegafuchi Chemical Co. [5]. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) of reagent grade were purchased from the Sigma Chemical Co. Lecithin prepared from fresh egg yolk was purified by the method of Singleton et al. [6].

Methods. The acid dissociation constant (K_A) was measured spectrophotometrically at 25 °C from the values of absorbance at a specified wavelength measured at different pH as reported previously; the uncoupling activity was measured using rat-liver mitochondria [1]. The potency was expressed by C_{200}, which is the concentration at which the rate of respiration was twice that of state 4. The proton permeability across the liposomal membrane was evaluated from first-order kinetics of the proton efflux monitored by the pH shift in the internal aqueous phase using a fluorescent pH indicator [1]. As the index for the protonophoric activity of uncouplers, the increment of the proton permeability, Pp, per unit molar concentration of each compound in the external aqueous phase was used.

Results

Dissociation constant

The log K_A values of compounds 1–6 where the *para* substituent is fixed as dicyanovinyl and the two *ortho* substituents are varied were close to those found by Terada et al. [4]. With increasing bulkiness and branching, the K_A value first increased and then decreased up to the 2,6-di-*sec*-butyl derivatives. The irregular increase in the log K_A value of SF6847 [6] is explained as being due to the tumbling motion of the dicyanovinyl group, which is much less than that in other di-*ortho* compounds [4], which leads to a stronger electron-withdrawing effect of the *para* substituent.

The log K_A value for compounds 7–16 and 18 and 19 were newly measured. In general, those compounds with two strongly electron-withdrawing substituents at the terminal position had high log K_A values. The range of variation in the log K_A value, however, was larger than expected although the substituents were quite far from the site of dissociation. Cohen and Jones [7] observed a similar substituent effect for the log K_A value of 2,6-di-*t*-butyl phenols substituted at the *para* position with simpler substituents.

Uncoupling activity

The uncoupling activity of the series of compounds studied here in terms of log $1/C_{200}$ at pH 7.4 is listed in Table I. The range of variation in log $1/C_{200}$ was between 4 and 8.5 compared with the range between 2 and 7 for classical uncouplers (Table II). SF6847 [6] had the highest and compounds 5 and 13 had the second highest activity. FCCP and CCCP also had high uncoupling activity.

Protonophoric activity

The protonophoric activity in terms of log *Pp* is also shown in Table I. The most potent protonophores were SF6847 [6] and compound 5. However, the correspondence between log *Pp* and log $1/C_{200}$ was not complete.

Discussion

The relationship between uncoupling and protonophoric activities for the non-classical uncouplers was examined by regression analysis, which gave Eqn. 1.

$$\log 1/C_{200} = 1.174 \log Pp + 0.748 \log K_A + 17.887 \quad (1)$$
$$(0.431) \qquad (0.148) \qquad (2.465)$$

$$(n = 19,\ s = 0.447,\ r = 0.948)$$

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. Eqn. 1 shows that the variations in the uncoupling activity are decided by the protonophoric activity and dissociation constant in a way similar to variations for classical uncouplers [1], as shown in Eqn. 2.

$$\log 1/C_{200} = 0.813 \log Pp + 0.293 \log K_A + 11.422 \quad (2)$$
$$(0.155) \qquad (0.066) \qquad (0.712)$$

$$(n = 19,\ s = 0.246,\ r = 0.987)$$

The quality of the correlation in Eqn. 1 is much poorer than than in Eqn. 2. The much larger standard deviation, s, in Eqn. 1 means that there are more deviants from the regression line in non-classical uncouplers. Moreover, the regression coefficient of each term in Eqn. 1 is significantly larger than that of their counterparts in Eqn. 2. For non-classical uncouplers, the uncoupling activity seems to depend on the log *Pp* and log K_A values more than for classical uncouplers. This feature is illustrated in Fig. 1, which shows that the classical and non-classical uncoupler groups constitute two separate clusters. The correlation for the whole set of compounds was, of course, much poorer (Eqn. 3), the s value being much larger than that in Eqn. 1.

$$\log 1/C_{200} = 1.119 \log Pp + 0.331 \log K_A + 13.859 \quad (3)$$
$$(0.266) \qquad (0.127) \qquad (1.478)$$

$$(n = 38,\ s = 0.728,\ r = 0.898)$$

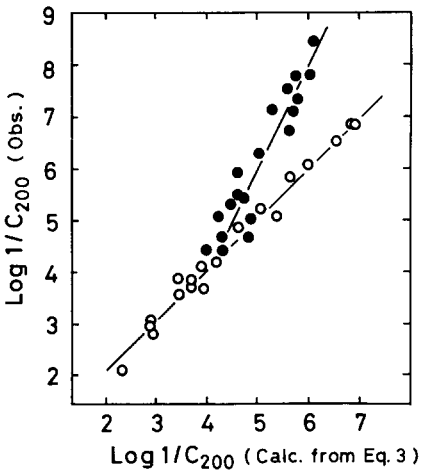


Fig. 1. Plot of the observed uncoupling activity (log $1/C_{200}$) vs. the calculated value from Eqn. 3 for classical (○) and non-classical uncouplers (●).

The different behaviors of the two groups of phenols might be due to the fact that their uncoupling mechanism is different. It is more likely that there are physico-chemical factors involved in addition to the protonophoric activity and dissociation constant considered in Eqns. 1–3, since these two characteristics are at least important in deciding the uncoupling potency.

McLaughlin et al. [2,8] showed that the proton flux induced by such weakly acidic uncouplers as FCCP and CCCP across artificial lipid bilayer membranes and mitochondrial membranes is dependent on the extramembranous pH value. Using bilayer membranes and charge-pulse and voltage-clamp techniques, they examined the effect of FCCP and CCCP on the current attributed to proton transport across the membrane. By analyzing the kinetics of the current variations under various experimental conditions, they proposed a model that rationalizes the protonophoric mechanism. The model predicts well the variation in the uncoupling activity of FCCP measured at various extramembranous pH values. According to this model, the proton flux produced by a certain uncoupler is proportional to the *Pr* value defined by Eqn. 4:

$$Pr = \frac{[H^+]}{\left(1 + \frac{[H^+]}{K_A}\right)\left(1 + 2\frac{[H^+]}{K'_A}\frac{k_{HA}}{k_{A^-}}\right)} \quad (4)$$

In this equation, $[H^+]$ is the proton concentration of the bulk aqueous phase, and K_A and K'_A are the dissociation constants of the acidic uncoupler in the bulk aqueous phase and at the membrane surface, respectively; k_{AH} and k_{A^-} are the first-order rate constant for movement across the membrane of the non-ionized and ionized form, respectively. We think that their assumption that $K_A = K'_A$ for FCCP and CCCP is also applicable to phenolic uncouplers. They estimated that $k_{AH} = (10\,000/700) \cdot k_{A^-}$ for FCCP and $k_{AH} = (12\,000/175) \cdot k_{A^-}$ for CCCP, so we first assumed that $k_{AH}/k_{A^-} = 100$ for the phenols. Then, Eqn. 4 was converted to Eqn. 5.

$$Pr = \frac{[H^+]K_A}{(K_A + [H^+])(K_A + 200[H^+])} \tag{5}$$

The plot of Pr against $[H^+]$ has a maximum at $[H^+] = K_A/\sqrt{200}$, which corresponds to an optimal $pH = pK_A + 1.15$. Since the value of Pr varies depending on the pH of the aqueous phase, and since the pH conditions were different when uncoupling and protonophoric activities were measured, a new parameter based upon Eq. 5 that takes into account the pH dependence of the proton flux should be found for analysis of the relationship between two activities.

In Fig. 2, the Pr value is plotted as a function of pH. At both sides of the optimal pH, the value decreased sharply to 1/10 the maximum value at about $pH_{opt} \pm 2$. McLaughlin et al. [2] found good

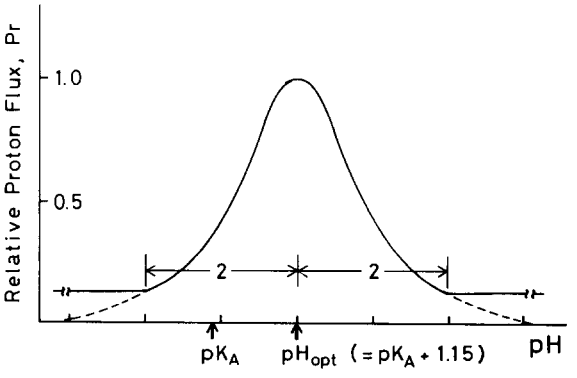


Fig. 2. Dependence of the proton flux on pH. The Pr value reaches an optimum at $pH = pK_A + 1.15$. The value beyond the range of $pH = pH_{opt} \pm 2$ according to Eqn. 5 is shown by the broken line and the value according to our assumption, $P'r$, by the solid line (see text).

correspondence in the pH dependence for uncoupling activity and proton flux estimated by Eqn. 5 in the range of $pH = pH_{opt} \pm 2$ for FCCP. We have also examined previously the pH dependence of phenolic uncouplers between 6.4 and 8.4 [1]. The uncoupling activity tends to have a broad maximum in the region where the pH value is higher than the pK_A of the compound comforming to Eq. 5 where $pH_{opt} = pK_A + 1.15$. For compounds of which the pK_A is far beyond the pH range of 6.4–8.4, uncoupling activity is almost unchanged. Therefore, we assumed that the Pr value beyond the range of $pH_{opt} \pm 2$ is unchanged as shown in Fig. 2. The value calculated from the modified function was designated as $P'r$. We defined the new parameter for the difference in the pH conditions in Eqn. 6.

$$P_f = \frac{P'r(\text{mitochondria, at pH 7.2})}{P'r(\text{liposome, at pH 4.5–7.0})} \tag{6}$$

For the uncoupling activity with mitochondria, the pH is about 7.2 in the intermembrane space and 7.4 in the external aqueous phase [9]. These pH values stay almost constant during measurement. For the protonophoric activity with lecithin liposome, the pH of the internal aqueous phase varies from 4.5 to 7.0, whereas that of the external aqueous phase is almost unchanged between 7.0 and 7.2 [1]. Therefore, the value of $P'r$(mitochondria) was taken as that at pH 7.2. For $P'r$(liposome), $P'r$ value was calculated at 250 points with intervals of 0.01 in the pH, and the 250 values were averaged between pH 4.5 and 7.0 for each compound. In Fig. 3, the situation is shown with SF6847 given as the example. The log value of P_f is shown in Table I for non-classical and in Table II for classical uncouplers.

Using log P_f as the new parameter, we analyzed again the relationship between protonophoric activity with lecithin liposome and uncoupling activity with mitochondria for the whole set of classical and non-classical uncouplers, which lead to Eqn. 7.

$$\log 1/C_{200} = \underset{(0.135)}{0.915} \log Pp + \underset{(0.065)}{0.440} \log K_A + \underset{(0.409)}{2.136} \log P_f + \underset{(0.722)}{13.500} \tag{7}$$

$(n = 38,\ s = 0.354,\ r = 0.977)$

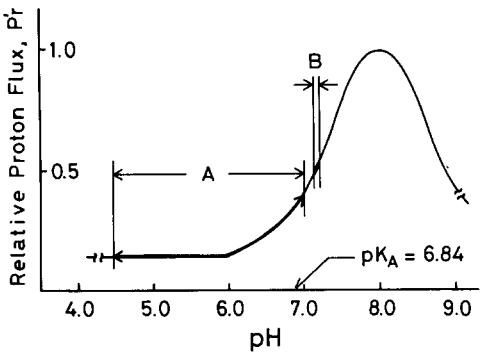


Fig. 3. Dependence of $P'r$ value of SF6847 on pH. Region A is the pH range of the internal aqueous phase of liposomes and B that of the intermembrane space of the mitochondria. The $P'r$ value for the liposomal proton flux was evaluated by averaging over the values in region A.

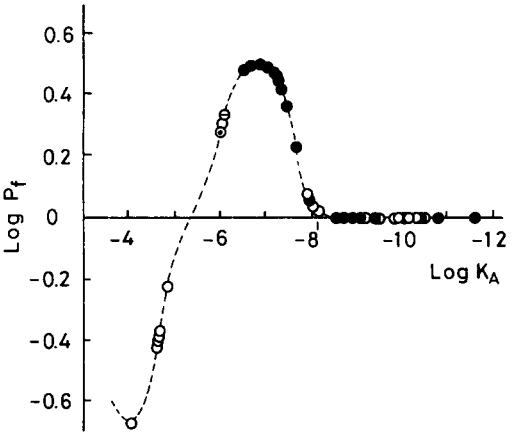


Fig. 4. Relationship between log K_A and log P_f for classical (○), non-classical uncouplers (●), CCCP (⊙), and FCCP (⊖).

The log P_f value did not vary much when we used the $P'r$ value averaged over 2500 data points with intervals of 0.001 in the pH (data not shown). When the P_f value was calculated with k_{AH}/k_{A^-} taken as 10 instead of 100, which was called P_f', the counterpart of Eqn. 7 was expressed as Eqn 8.

$$\log 1/C_{200} = 1.107 \log Pp + 0.441 \log K_A + 1.225 \log P_f' \quad (8)$$
$$(0.132) \qquad (0.067) \qquad (0.240)$$
$$+14.524$$
$$(0.744)$$
$$(n = 38,\ s = 0.361,\ r = 0.976)$$

The quality of the correlation was almost the same as in Eqn. 7. The regression coefficient of log P_f' was about half that in Eqn. 7, which meant that log P_f' is almost proportional to log P_f. Even if the value of k_{AH}/k_{A^-} was regarded as being either 1 or 1000, the quality of the correlation was only slightly poorer than that found in Eqns. 7 and 8 (data not shown). The correlation was quite stable regardless of the estimation of the k_{AH}/k_{A^-} value.

In Fig. 4, log P_f is plotted against the log K_A of phenols. For phenols the log K_A of which was more negative than -8, the value was zero. Even for the set of classical uncouplers, the log P_f value was not constant. So we reexamined Eqn. 2 for classical uncouplers using log P_f as an additional parameter, which gave Eqn. 9.

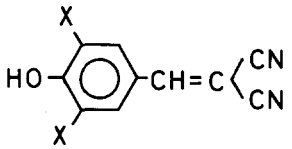
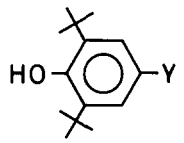
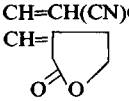
$$\log 1/C_{200} = 0.829 \log Pp + 0.346 \log K_A + 0.668 \log P_f \quad (9)$$
$$(0.135) \qquad (0.079) \qquad (0.659)$$
$$+12.041$$
$$(0.713)$$
$$(n = 19,\ s = 0.210,\ r = 0.991)$$

The terms are significant at a level higher than 99.5%, except for the log P_f term, which is justified at slightly higher than the 95% level. This is probably attributable to the fact that the collinearity between log K_A and log P_f is fairly large, with $r = 0.749$, so that the two factors are not completely separate. Nevertheless, the slope of the log K_A term was closer to that in Eqn. 7 than that in Eqn. 2. The addition of the log P_f term to Eqn. 1 was not justified over the 95% level, since the collinearity of log K_A with log P_f is high as far as non-classical uncouplers are concerned. This type of collinearity was reduced to an insignificant level ($r = 0.239$) for the whole set of compounds in Eqn. 7.

FCCP and CCCP are included in Fig. 5. Although these compounds are not phenolic, they behave like phenols. Along with this observation, the high quality of the correlation of Fig. 7 shows that the mode of action of weakly acidic uncouplers is the same regardless of structural differences. As reported elsewhere [1], the log K_A

TABLE I

PHYSICO-CHEMICAL CONSTANTS AND UNCOUPLING AND PROTONOPHORIC ACTIVITIES OF NON-CLASSICAL UNCOUPLERS

No.	Compound	$-\log K_A$ [a]	$\log P_f$ [b]	$-\log Pp$	$\log 1/C_{200}$	
					observed	calculated [c]
						
1	X = H	7.04	0.51	5.77	5.37	6.21
2	Me	6.91	0.51	5.02	6.39	6.95
3	Et	6.98	0.51	4.68	6.76	7.24
4	*i*-Pr	7.06	0.51	4.48	7.35	7.38
5	*s*-Bu	7.22	0.48	4.01	7.66	7.68
6	*t*-Bu (SF6847)	6.84	0.51	4.06	8.44	7.87
						
7	Y = CH=C(COOEt)$_2$	9.29	0	4.53	4.70	5.27
8	CH=C(CN)COOEt	7.51	0.36	4.48	7.14	6.87
9	CH=CHCN	9.95	0	4.37	4.94	5.13
10	CH=C(CN)CONH$_2$	7.73	0.22	5.47	5.92	5.56
11	CH=C(COMe)COOEt	8.67	0.00	4.77	5.42	5.32
12	CH=CHNO$_2$	6.89	0.51	5.22	7.16	6.78
13	CH=C(CN)SO$_2$Me	6.77	0.50	4.62	7.73	7.36
14	CH=CHCOOEt	10.82	0	4.69	4.37	4.45
15	CH=CH(CN)COOMe	7.45	0.43	4.51	7.60	7.05
16		9.89	0	5.03	4.62	4.55
17	H	11.70 [d]	0	4.75	4.24	4.01
18	CN	8.89	0	4.85	5.42	5.08
19	CHO	7.93	0.06	5.98	5.12	4.67
20	FCCP	6.20	0.37	4.99	7.49 [e]	7.05
21	CCCP	5.95	0.30	4.30	7.30 [e]	7.59

[a] Unless otherwise noted, newly measured.
[b] From Eqn. 6.
[c] By Eqn. 7.
[d] From Ref. 7.
[e] Not included in the analysis of Eqn. 7.

term in Eqn. 7 reflects a difference in the stability of the ionized form of uncouplers in mitochondrial and liposomal membranes under our experimental conditions. Considering the factors arising from this difference and the effect of the difference in the extramembranous pH conditions, the results reported here show that the uncoupling activity is closely related to the protonophoric activity across the liposomal membrane, in support of the theory of the shuttle-type of mechanism for weakly acidic uncouplers.

Recently, the shuttle-type mechanism where no

TABLE II

PHYSICO-CHEMICAL CONSTANTS AND UNCOUPLING ACTIVITY OF SUBSTITUTED PHENOLS AS CLASSICAL UNCOUPLERS

Substituent	$-\log K_A$ [a]	$\log P_f$ [b]	$\log 1/C_{200}$ observed [c]	$\log 1/C_{200}$ calculated [d]
H	9.98	0	2.15	2.30
4-Me	10.14	0	2.75	2.88
4-Et	10.21	0	2.96	2.92
4-*n*-Pr	10.21	0	3.43	3.29
4-*t*-Bu	10.23	0	3.70	3.65
4-*t*-Pent	10.23	0	4.08	3.81
4-Cl	9.38	0	3.56	3.84
2,4-Cl_2	7.89	0.09	4.85	5.17
2,4,6-Cl_3	5.99	0.31	5.20	5.69
3-CF_3	8.95	0	4.10	4.36
4-CN	7.95	0.04	3.80	3.60
4-COMe	8.05	0.02	2.95	2.97
3-NO_2	8.40	0	3.77	3.73
2,4-$(NO_2)_2$	4.09	−0.70	5.10	4.53
2-Me-4,6-$(NO_2)_2$	4.44	−0.44	5.60	5.63
2-Et-4,6-$(NO_2)_2$	4.43	−0.45	6.02	6.07
2-*i*-Pr-4,6-$(NO_2)_2$	4.47	−0.42	6.46	6.68
2-*t*-Bu-4,6-$(NO_2)_2$	4.80	−0.21	6.85	7.14
2-*s*-Bu-4,6-$(NO_2)_2$	4.51	−0.40	6.89	6.78

[a] From Ref. 1.
[b] From Eq. 6.
[c] From Ref. 1.
[d] By Eq. 7.

specific binding site for uncouplers is considered has been criticized in reports [10–13]. The studies were based upon the development of mutant strains of bacteria resistant to uncouplers. Such resistance was said to arise from some modification of the specific proteinous binding sites or receptors. Other investigations showed, however, that resistance developed because of a decrease in the fluidity of the inner mitochondrial membrane [14] and changes in the energization system of phosphorylation [15,16]. Thus these observations do not rule out the possibility of the shuttle-type mechanism.

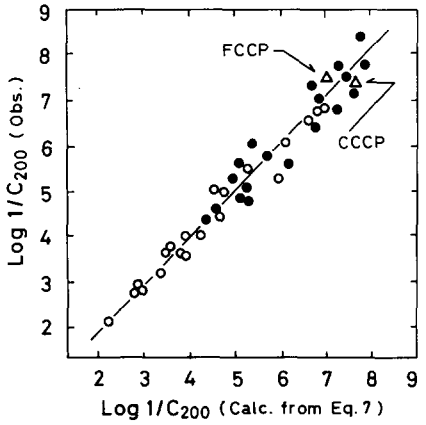


Fig. 5. Plot of the observed uncoupling activity ($\log 1/C_{200}$) vs. the calculated value from Eq. 7 for classical (○) and non-classical uncouplers (●). CCCP and FCCP were not phenolic but behaved like non-classical uncouplers.

Acknowledgments

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