Biochimica et Biophysica Acta 891 (1987) 194–204
Elsevier

BBA 42517

Quantitative relationship between protonophoric and uncoupling activities of substituted phenols

Hideto Miyoshi, Takaaki Nishioka and Toshio Fujita

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto (Japan)

(Received 23 October 1986)

Key words: Acidic uncoupler; Mitochondrial membrane; Liposomal membrane; Protonophoric potency; Quantitative structure-activity relationship

The protonophoric activity through liposomal membranes was measured and compared with the uncoupling activity with the oxidative phosphorylation of rat-liver mitochondria for 19 substituted phenols. Quantitative analyses of the protonophoric activity of the phenols in terms of physicochemical molecular parameters showed that the activity was mostly decided by two factors: the partition coefficient between the liposome and aqueous buffer phases and the acid dissociation constant. Correlation was excellent between protonophoric and uncoupling activities when the difference in the effect of acidity of phenols between liposomal and mitochondrial membranes was taken into account. The results were further evidence for the shuttle-type of mechanism of weakly acidic uncouplers based on the Mitchell chemiosmotic hypothesis.

Introduction

Possible relationships between the ability of weakly acidic uncouplers to release respiration control in mitochondria and their effect on proton conductivity of artificial lipid membrane have been explored [1–4]. Most of these studies are examining the effect of uncouplers on proton conductivity in terms of the potency to increase electric conductance in the black lipid membrane. For instance, Skulachev [1], using a number of weak acids, found a fairly good correlation between these two activities and proposed a shuttle-type mechanism in which the molecule of uncouplers functions as a protonophore and discharges the membrane potential in the inner mitochondrial membrane. Ting and coworkers [2], however, using a similar set of compounds presented a critical view to this mechanism. Although this apparent paradox was partly resolved by McLaughlin and Dilger [5] favoring conclusions of Skulachev, no general description of the relationship between uncoupling activity and proton conductivity has been made yet.

Here, we measured the potency to increase proton permeability across a lecithin liposomal membrane and the uncoupling activity for the 19 substituted phenols listed in Table I. The phenols were chosen so that physico-chemical properties such as hydrophobicity and electronic properties vary as independently as possible. Using quantitative parameters for these physico-chemical characters and regression analysis, we analyzed factors influencing these activities. The potency to increase proton permeability and the uncoupling activity of phenols were governed by hydrophobic and electronic factors of the molecule. The uncoupling activity was closely related to the potency to

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

Correspondence: H. Miyoshi, Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto, Japan.

increase proton permeability when the difference in the effect of acidity between liposomal and mitochondrial membranes was taken into account.

Materials and Methods

Materials

Lecithin was prepared from fresh egg yolk and purified by the method of Singleton et al. [6]. 2-Alkyl-4,6-dinitrophenols were synthesized by nitration of 2-alkylphenols [7]. 2-*i*-Propyl-4,6-dinitrophenol (m.p. 56°C) and 2-*t*-butyl-4,6-dinitrophenol (m.p. 131°C) have not been described in the literature. Their structures were confirmed by elementary analysis and spectra. Phenols, including those purchased from Nakarai Chemicals, Ltd., were purified by either distillation or silica-gel column chromatography before use. SF6847 (2,6-di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol) of the purest grade was purchased from the Wakenyaku Co. Cholesterol was of reagent grade and was purified by recrystallization from ethanol. Reagent grade pyranine (sodium 8-hydroxy-1,3,6-pyrenetrisulfonate) was purchased from the Eastman Kodak Co. and used without further purification.

Measurement of partition coefficient. Bilayered liposome was prepared by a procedure similar to that reported previously [8]. The dry lecithin, 100 mg, was suspended in 20 ml of an aspartate buffer solution (0.04 M sodium aspartate/0.25 M Na_2SO_4/0.2 mM EDTA), adjusted to pH 7.2 by addition of NaOH, and sonicated in an ice-cooled bath under Ar gas. Bilayered liposome was separated from the multilayered liposome by gel filtration on Sepharose 4B at 4°C. The partition coefficient, $P(L/W)$, of each substituted phenol between the liposomal membrane and the external aqueous phase was measured by an equilibrium dialysis method at pH 7.2 [8]. Using an equilibrium dialysis cell (10 × 1 ml chambers, Sanko Plastic Co.), the liposome suspension (1 ml) was equilibrated with the phenol solution (1 ml) through a cellophane dialysis membrane at 25°C for 12 h. The solution of each phenol, the concentration of which was 10^{-5}–10^{-4} M, was prepared with the same pH 7.2 aspartate buffer as for the liposome suspension. At this pH, some phenols exist as mixture of nonionized and ionized forms in equilibrium. The $P(L/W)$ value was, however, not corrected for the ionization effect but the apparent one. The ionic strength in the aqueous phase was much higher than that used previously, since we wanted the $P(L/W)$ value measured under conditions equivalent with those for the measurement of proton permeability as shown below. The log $P(L/W)$ was used as an index of the hydrophobicity of the compounds. The partitioning of the phenols into liposome did not follow the regular pH-partition rule observed for that into the usual organic neutral solvents as shown elsewhere [8].

Measurement of potency to increase proton permeability. For the measurement of proton permeability across liposomal membranes, liposomes were prepared in aspartate buffer (0.04 M sodium aspartate/0.25 M Na_2SO_4/0.2 mM EDTA, with pH adjusted to 4.5 by H_2SO_4) containing 1 mM pyranine as the pH indicator [9]. Pyranine outside the liposome was removed by gel filtration on Sepharose 4B at 4°C. The pH indicator, once trapped inside, does not readily diffuse through the liposomal bilayer. The amount of lecithin in the preparation was estimated as that of phosphorus by a modification of the Bartlett method [10]. The resultant suspension of liposomes contained about 1 mg of lecithin/ml. The surface area of liposomal membrane was calculated from the diameter of liposomes observed by electron microscopy (JME-100) [8]. The mean value of the membrane area was $(2.0 \pm 1.0) \cdot 10^3$ $cm^2/\mu mol$ lecithin. Aspartate buffer, the pH of which was adjusted to 7.8, and containing various amounts of each phenol, was placed in a fluorescence cell and stirred at 25°C. The liposome suspension (0.2 ml) in the pH 4.5 buffer was added to the cell. After the addition, the pH of the external phase rapidly changes to 7.2. The time-dependent change of the pH inside the liposomes was followed by fluorescence emission of pyranine with a Shimadzu RF-503A difference spectrophotometer. When an aqueous solution of pyranine was excited at 400 and 450 nm, the ratio of the emission intensities at 510 nm increased with the pH of the medium (Fig. 1). Since the solution inside the liposomes was buffered, the net proton efflux was not reflected by the pH change. Thus, the aspartate buffer was titrated with HCl from pH 9 to 4 in the presence of pyranine. From this titration curve, we derived

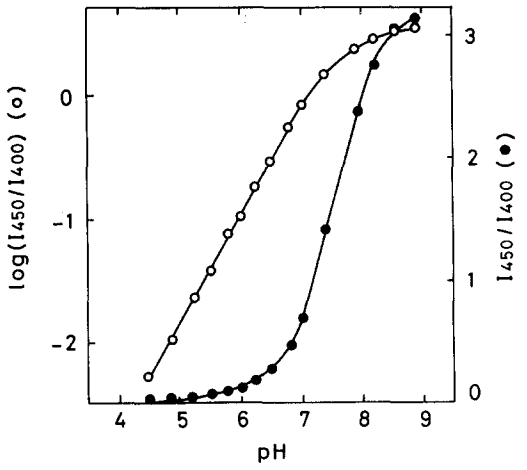

Fig. 1. Ratio of the emission intensities at 510 nm excited at 450 and 400 nm in a $5.0 \cdot 10^{-7}$ M aqueous solution of pyranine as a function of pH at 25°C.

a relationship between the pH change and the change in the amount of protons (Fig. 2). The internal pH of the liposomes, monitored for the first 0.5–5 h with a fluorescence spectrometer, increased from pH 4.5 to 7.0 with time. The time-dependent pH shift was converted to the net proton efflux from the buffered interior with the relationship given in Fig. 2. Under our experimental conditions, the charge which may be arisen by

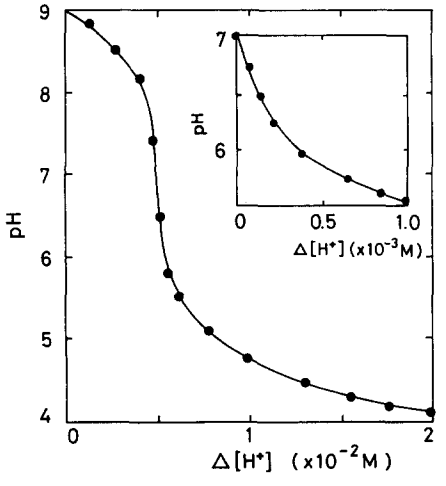

Fig. 2. Variation in pH of the aspartate buffer induced by successive additions of protons. The inset is an expansion of part of the curve.

the proton efflux across the membrane is compensated by the influx of Na^+ as demonstrated by Deamer and Nichols [11]. The salt concentrations on the two sides of the membranes were equal and much higher than the order of the initial proton concentration gradients, so no significant potential difference across the membrane should have been established [12]. The potency of phenols to increase the proton permeability was estimated by their successive additions to the outer phase. Their potency was also measured with lecithin-cholesterol liposomes (lecithin/cholesterol, 10 : 1, m/m) prepared from the lipid mixture dissolved in CH_3Cl.

Measurement of uncoupling activity. Rat-liver mitochondria were isolated by the method of Hogeboom [13] as described by Myers and Slater [14]. The amount of mitochondria was measured as the amount of protein by the Biuret method [15]. The mitochondrial preparation 20–30 μl was added to 3.25 ml of the 2.5 mM phosphate buffer (pH 7.4) containing 10 mM sodium succinate/200 mM sucrose/2 mM $MgCl_2$/1 mM EDTA to give a final protein concentration of 0.7 mg/ml. The rate of mitochondrial respiration at the State 4 was observed at 25°C by the oxygen consumption being followed with a Garbani-type oxygen electrode fixed in a thermostatted reaction cell (3.25 ml, Central Kagaku Co.). To this respiration system, each phenol as an ethanol stock solution was added one after the other (2–30 μl). The uncoupling activity of the phenols was estimated as the concentration (C_{200}) at which the rate of respiration increased to 200% of that of the control. For nine phenols selected so that the pK_A value ranged from 4 to 10.5, the C_{200} value was also measured at pH 6.4 and 8.4.

Measurement of dissociation constant. The dissociation constant was measured spectrophotometrically for 2-alkyl-4,6-dinitrophenols and SF6847. All operations were at 25°C. A methanol solution of each phenol (0.02 ml) was added to 2.5 ml of acidic, alkaline, or buffer solutions in a 3 ml quartz cell with rapid stirring. After the spectral measurement, the pH of the solution in the cell was found with a Radiometer Model PHM26 pH meter; no large change from the original solution was observed. From the values of absorbance, A, measured at different pH, the log K_A value was

TABLE I

PHYSICOCHEMICAL CONSTANTS AND PROTONOPHORIC AND UNCOUPLING ACTIVITIES OF SUBSTITUTED PHENOLS

Substituent	log P (L/W) [a]	log K_A [b]	log Pp		log Pp^{ch}		log $1/C_{200}$		
			obsd. [c]	calcd. [d]	obsd. [c]	calcd. [e]	obsd. [f]	calcd. [g]	calcd. [h]
H	1.97 (0.03)	−9.98	−7.44	−7.69	−7.92	−7.73	2.15	2.20	2.45
4-Me	2.42 (0.02)	−10.14	−6.73	−7.14	−6.88	−7.01	2.75	2.63	2.98
4-Et	2.88 (0.02)	−10.21 [i]	−6.54	−6.55	−6.65	−6.93	2.96	3.11	3.03
4-*n*-Pr	3.26 (0.02)	−10.21 [j]	−6.25	−6.04	−6.39	−6.53	3.43	3.54	3.35
4-*t*-Bu	3.45 (0.04)	−10.23 [i]	−5.85	−5.80	−6.52	−6.12	3.70	3.74	3.67
4-*t*-Pent	3.81 (0.05)	−10.23 [k]	−5.67	−5.32	−5.83	−4.15	4.08	4.15	3.82
4-Cl	2.86 (0.01)	−9.38	−5.94	−6.34	−6.40	−6.21	3.56	3.52	3.85
2,4-Cl_2	3.11 (0.01)	−7.89 [i]	−5.52	−5.58	−5.88	−5.79	4.85	4.59	4.63
2,4,6-Cl_3	3.31 (0.03)	−5.99 [i]	−4.96	−4.77	−5.43	−5.22	5.20	5.84	5.64
3-CF_3	3.25 (0.02)	−8.95 [l]	−5.69	−5.70	−6.07	−5.96	4.10	4.19	4.18
4-CN	2.17 (0.03)	−7.95	−7.09	−6.85	−7.28	−7.38	3.80	3.50	3.33
4-COMe	1.80 (0.05)	−8.05	−7.63	−7.37	−7.93	−7.93	2.95	3.03	2.86
3-NO_2	2.41 (0.02)	−8.40	−6.64	−6.66	−6.84	−6.92	3.77	3.53	3.57
2,4-$(NO_2)_2$	2.12 (0.03)	−4.09 [i]	−6.20	−5.81	−6.50	−6.48	5.10	5.47	5.19
2-Me-4,6-$(NO_2)_2$	2.49 (0.04)	−4.44 [m]	−5.44	−5.42	−5.85	−5.71	5.60	5.70	5.70
2-Et-4,6-$(NO_2)_2$	2.84 (0.04)	−4.43 [m]	−4.94	−4.95	−5.14	−5.20	6.02	6.10	6.11
2-*i*-Pr-4,6-$(NO_2)_2$	3.03 (0.05)	−4.47 [m]	−4.33	−4.71	−4.74	−4.58	6.46	6.30	6.59
2-*t*-Bu-4,6-$(NO_2)_2$	3.26 (0.05)	−4.80 [m]	−4.16	−4.50	−4.22	−4.41	6.85	6.38	6.64
2-*s*-Bu-4,6-$(NO_2)_2$	3.42 (0.03)	−4.51 [m]	−4.18	−4.20	−4.24	−4.49	6.89	6.72	6.66
SF6847	− [n]	−6.84 [m]	−4.06	− [o]	−4.22	−4.31	8.44	− [o]	6.12 [p]

[a] For the ionization mixture at pH 7.2, see text. The figures in parentheses are the standard deviation.

[b] Unless otherwise noted, from Albert, A. and Serjeant, E.P., Ionization Constants of Acids and Bases, Methuen, London (1962), p. 130.

[c] Pp and Pp^{ch} values are listed in Table II.

[d] By Eqn. 6.

[e] By Eqn. 5.

[f] The C_{200} value is the concentration at which the rate of respiration is doubled over that of State 4; see text.

[g] By Eqn. 8.

[h] By Eqn. 9.

[i] From Tables of Rate and Equilibrium Constants of Heterolytic Organic Reactions (1975) (Palm, V.A., ed.), Tartu State University, Vol. 1.

[j] Taken as the same as that of 4-ethylphenol.

[k] Taken as the same as that of 4-*t*-butylphenol.

[l] Estimated according to a correlation equation shown in Fujita, T. (1976) Prog. Phys. Org. Chem. 12, 58.

[m] Newly found.

[n] Not studied because the spectrum was unstable during equilibrium dialysis.

[o] Not calculable because of the lack of log P(L/W) value.

[p] [illegible]

evaluated by Eqn. 1:

$$\log K_A = -\mathrm{pH} + \log \frac{\varepsilon_{RH} C - A}{A - \varepsilon_{R^-} C} \quad (1)$$

where C is the total concentration of the phenol and ε_{RH} and ε_{R^-} are the molar absorptivity of nonionized and ionized forms, respectively.

Results

Partition coefficient and dissociation constant

The log P(L/W) and log K_A values are listed in Table I. The standard deviation of log P(L/W) was 0.02–0.05 and that of log K_A was 0.02 for newly measured values.

Potency to increase proton permeability

The net proton efflux from liposomes obeyed first-order kinetics according to Eqn. 2:

$$\log([\mathrm{H}^+]_t^{in} - [\mathrm{H}^+]_{t+\Delta t}^{in}) = \frac{-kt}{2.303} \quad (2)$$

where $[\mathrm{H}^+]^{in}$ is the proton concentration of the internal phase and k is the first-order rate constant. An example is shown in Fig. 3 for the control examination (phenol-free). The apparent permeability coefficient of protons, $P'm(\mathrm{H}^+)$, was defined as in Eqn. 3:

$$P'm(\mathrm{H}^+) = k\frac{V_i}{A} \quad (3)$$

where V_i and A are the internal volume and the surface area of liposomes, respectively. The $P'm(\mathrm{H}^+)$ value was measured with various concentrations of each phenol in the outer aqueous phase. The value of $P'm(\mathrm{H}^+)$ increased almost linearly with the concentration of the phenol as shown in Fig. 4 for 2,4-dinitrophenol. The relationship was formulated by Eqn. 4.

$$P'm(\mathrm{H}^+) = a[\mathrm{phenol}] + c \quad (4)$$

The a value is the increment of proton permeability per unit molar concentration of phenols in the external phase. It is an index of their potency to increase proton permeability. The a value was thus designated as Pp and estimated by the least-squares method. In Table II the Pp and c values for 19 substituted phenols and SF6847, the most potent uncoupler known to date, are listed along with statistical data for the correlation. The correlation was excellent; the correlation coefficient was higher than 0.95 for all 19 phenols. With the lecithin-cholesterol liposome, the potency to increase the proton permeability, Pp^{ch}, was also measured (Table II). The log of Pp and Pp^{ch} values are listed in Table I.

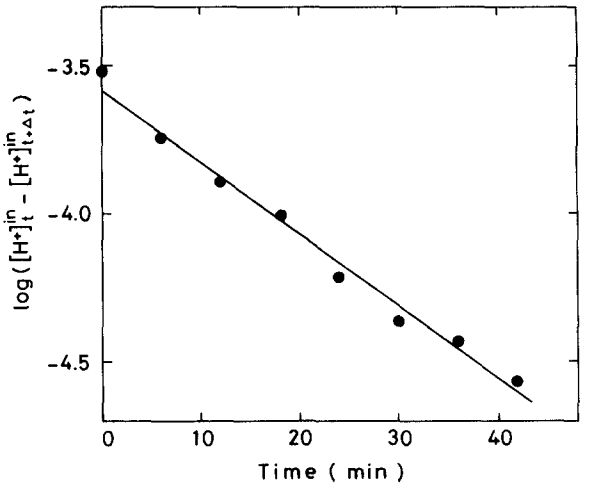

Fig. 3. Release kinetics of protons. Data were plotted with Eqn. 2, used for proton release under phenol-free conditions.

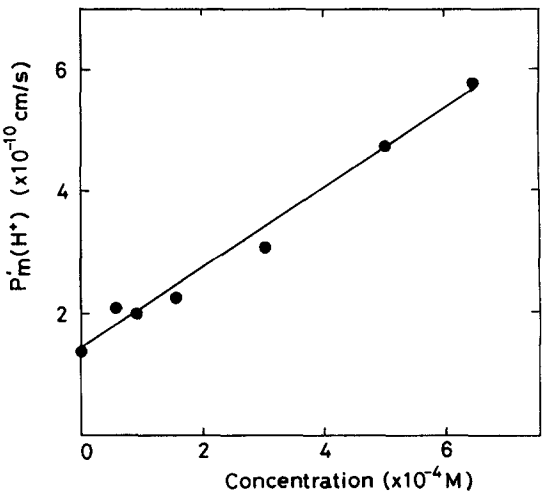

Fig. 4. Plot of the apparent proton permeability coefficient against the concentration of 2,4-dinitrophenol. Data were analyzed by the least-squares method with Eqn. 4.

Analysis of potency to increase proton permeability

The potency to increase the proton permeability in terms of log *Pp* in liposomes with and without cholesterol corresponded well, as seen in Eqn. 5. The Pp^{ch} value was almost universally lower than the *Pp* value by a factor of $10^{0.19} = 1.5$.

$$\log Pp^{ch} = \underset{(0.097)}{1.014}\ \log Pp - \underset{(0.578)}{0.189} \quad (n = 19,\ s = 0.202,\ r = 0.983) \tag{5}$$

In this and the following equations, *n*, *s*, and *r* are the number of compounds included in the correlation, the standard deviation, and the correlation coefficient, respectively. The figures in parentheses are the 95% confidence interval.

The potency to increase the proton permeability varied about $10^{3.5}$-fold according to the structure. The structural effect was examined by multiple regression analysis with physico-chemical molecular parameters. The best correlation was Eqn. 6, where hydrophobic and electronic parameters are significant.

$$\log Pp = \underset{(0.236)}{1.328}\ \log P(\mathrm{L/W}) + \underset{(0.055)}{0.286}\ \log K_A - \underset{(0.783)}{7.455} \quad (n = 19,\ s = 0.268,\ r = 0.969) \tag{6}$$

Addition of any steric parameter such as molar volume [8] did not improve the correlation. Eqn. 6 shows that the potency to increase proton permeability increased with the partitioning into the liposomal membrane phase and with the acidity of phenols. P(L/W) was defined as an overall partition coefficient from aqueous to liposomal membranes based on the total concentration including both neutral and ionized species present at pH 7.2. With the log P(O/W) value for the nonionized phenol in the 1-octanol/water system [8] instead of log P(L/W), the correlation was poorer (data not shown).

Analysis of uncoupling activity

The uncoupling activity in terms of $\log(1/C_{200})$ at pH 7.4 is listed in Table I along with the value for SF6847. At pH 7.4, phenols substituted with strongly electron-withdrawing groups were present as the ionized species, but those substituted with alkyl groups were present as the neutral form. To examine whether either or both of the neutral and ionized forms of phenols contributed to the uncoupling activity, the C_{200} value of eight substituted phenols and SF6847 was measured at pH conditions (pH 6.4, 7.4 and 8.4) (Table III). From these values, uncoupling activities attributable to the neutral and ionized species were estimated separately by Eqn. 7:

$$\log \frac{1}{C_{200}} = (1 - \alpha)A + \alpha B \tag{7}$$

where α is the degree of dissociation of phenols at a given pH and A and B are the uncoupling activities of the neutral and ionized forms, respectively. The A and B values obtained for the three pH values did not differ much and their means gave the following results.

2,4-Cl_2 ($A = 4.78\ (\pm 0.01)$, $B = 5.07\ (\pm 0.02)$)

4-Cl ($A = 3.57\ (\pm 0.06)$, $B = 3.88\ (\pm 0.07)$)

3-NO_2 ($A = 3.66\ (\pm 0.05)$, $B = 4.71\ (\pm 0.09)$)

SF6847 ($A = 8.40\ (\pm 0.03)$, $B = 8.50\ (\pm 0.05)$)

For the five other compounds in Table I that exist as almost one species at the three different pH values, the A and B values were not estimated accurately. Although the uncoupling activity of the neutral form was slightly lower than that of the ionized form, the two species had almost the same order of uncoupling activity. Since the uncoupling activity was not attributable to one of the two species, but probably to both, the C_{200} values observed for the 'equilibrated mixture' at pH 7.4 were used in the following analysis. The uncoupling activity at pH 7.4 was correlated to log P(L/W) at pH 7.2 and log K_A values by Eqn. 8.

$$\log \frac{1}{C_{200}} = \underset{(0.239)}{1.291}\ \log P(\mathrm{L/W}) + \underset{(0.054)}{0.526}\ \log K_A + \underset{(0.745)}{5.226} \quad (n = 19,\ s = 0.262,\ r = 0.985) \tag{8}$$

Eqn. 8 shows that the critical factors in the mitochondrial uncoupling activity were the partitioning of phenols into the lipid phase of the mitochondrial membrane and the acidity.

We previously analyzed quantitatively the uncoupling activity of alkyl derivatives of 2,4-di-

TABLE II

Pp AND Pp^{ch} VALUES ESTIMATED FROM Eqn. 4

Substituent	Lecithin liposome						Lecithin-cholesterol liposome					
	Pp [a] (10^{-6} cm/s per M)	*c* [a] (10^{-10} cm/s)	range (M) [b]	*n* [c]	*s* [d]	*r* [e]	Pp^{ch} [a] (10^{-6} cm/s per M)	*c* [a] (10^{-10} cm/s)	range (M) [b]	*n* [c]	*s* [d]	*r* [e]
H	0.0365 (0.0113)	1.20 (0.262)	$(1.20–3.59)\cdot10^{-3}$	6	0.118	0.976	0.0119 (0.00185)	1.06 (0.759)	$(2.59–6.47)\cdot10^{-3}$	6	0.333	0.994
4-Me	0.187 (0.0460)	2.02 (0.484)	$(0.26–2.58)\cdot10^{-4}$	7	0.377	0.978	0.127 (0.0622)	3.74 (1.78)	$(1.51–4.52)\cdot10^{-4}$	6	0.825	0.955
4-Et	0.226 (0.081)	0.988 (0.261)	$(1.34–5.34)\cdot10^{-4}$	6	0.129	0.968	0.308 (0.0810)	3.85 (0.998)	$(0.58–2.90)\cdot10^{-4}$	7	0.775	0.974
4-*n*-Pr	0.575 (0.175)	1.13 (0.300)	$(0.74–2.97)\cdot10^{-4}$	6	0.150	0.977	0.413 (0.0810)	3.53 (0.998)	$(0.21–2.66)\cdot10^{-4}$	6	0.657	0.989
4-*t*-Bu	1.43 (0.370)	1.86 (0.419)	$(0.22–2.20)\cdot10^{-5}$	6	0.244	0.983	0.297 (0.0694)	1.69 (1.11)	$(0.68–2.73)\cdot10^{-4}$	6	0.550	0.984
4-*t*-Pent	2.16 (1.350)	1.32 (0.477)	$(1.25–6.25)\cdot10^{-5}$	5	0.210	0.957	1.47 (0.424)	2.22 [h] (2.80)	$(0.09–1.13)\cdot10^{-4}$	6	1.550	0.979
4-Cl	1.16 (0.222)	0.528 [f] (0.433)	$(0.47–9.40)\cdot10^{-4}$	7	0.694	0.986	0.318 (0.143)	4.79 (1.78)	$(0.54–2.14)\cdot10^{-4}$	6	0.879	0.968
2,4-Cl_2	3.05 (0.0090)	1.57 (0.364)	$(1.17–7.82)\cdot10^{-5}$	6	0.205	0.978	1.31 (0.206)	2.00 (0.858)	$(1.43–7.10)\cdot10^{-5}$	6	0.439	0.977
2,4,6-Cl_3	10.8 (3.02)	1.76 (0.601)	$(0.76–3.80)\cdot10^{-5}$	6	0.322	0.980	3.68 (0.730)	1.21 [f] (0.926)	$(0.43–2.26)\cdot10^{-5}$	6	0.482	0.989
3-CF_3	2.08 (0.816)	0.613 [h] (0.280)	$(0.25–1.52)\cdot10^{-4}$	6	0.369	0.962	0.850 (0.195)	0.786 [g] (0.225)	$(0.35–2.15)\cdot10^{-4}$	6	1.310	0.987
4-CN	0.0821 (0.0340)	1.47 [h] (1.21)	$(1.10–6.25)\cdot10^{-4}$	6	0.623	0.958	0.0527 (0.0146)	0.727 (0.761)	$(0.63–9.88)\cdot10^{-4}$	6	0.456	0.981

4-COMe	0.0236 (0.0076)	1.06 (0.412)	$(0.52–2.03)\cdot10^{-3}$	6	0.451	0.974	0.0117 (0.00506)	0.685 [h] (1.33)	$(0.83–4.12)\cdot10^{-3}$	6	0.645	0.955
3-NO_2	0.231 (0.099)	1.03 [h] (0.605)	$(3.61–9.38)\cdot10^{-4}$	5	0.223	0.974	0.144 (0.0404)	0.883 [g] (0.552)	$(2.00–8.67)\cdot10^{-4}$	6	1.035	0.980
2,4-(NO_20_2	0.638 (0.120)	1.45 (0.305)	$(0.47–6.32)\cdot10^{-5}$	7	0.262	0.986	0.313 (0.122)	0.932 (0.557)	$(0.99–5.23)\cdot10^{-5}$	6	0.611	0.983
2-Me-4,6-$(NO_2)_2$	3.68 (1.20)	0.576 (0.303)	$(0.49–4.92)\cdot10^{-5}$	6	0.177	0.974	1.40 [f] (0.733)	0.302 (0.967)	$(0.56–2.34)\cdot10^{-5}$	5	0.410	0.962
2-Et-4,6-$(NO_2)_2$	11.6 (1.53)	0.660 [f] (0.313)	$(1.01–9.09)\cdot10^{-6}$	6	0.150	0.998	7.17 (3.58)	0.441 (0.136)	$(0.82–4.36)\cdot10^{-6}$	6	0.431	0.963
2-*i*-Pr-4,6-$(NO_2)_2$	47.4 (7.35)	0.542 [f] (0.303)	$(1.02–5.09)\cdot10^{-6}$	6	0.253	0.996	18.01 [f] (9.05)	0.528 (0.129)	$(1.97–8.45)\cdot10^{-6}$	6	0.432	0.960
2-*t*-Bu-4,6-(NO_20_2	68.5 (12.55)	0.887 (0.662)	$(0.53–6.63)\cdot10^{-6}$	6	0.266	0.985	59.63 [f] (34.8)	0.457 (0.113)	$(0.51–5.55)\cdot10^{-6}$	6	0.571	0.963
2-*s*-Bu-4,6-$(NO_2)_2$	58.5 (11.0)	1.43 (0.565)	$(0.13–3.09)\cdot10^{-6}$	7	0.376	0.987	65.59 (8.95)	0.723 [g] (0.521)	$(0.82–4.14)\cdot10^{-6}$	6	1.01	0.995
SF6847	89.0 (49.8)	0.767 [h] (0.650)	$(0.35–4.18)\cdot10^{-6}$	7	0.357	0.973	59.74 (14.4)	1.42 [h] (1.34)	$(0.46–2.29)\cdot10^{-6}$	6	0.912	0.985

[a] Slope and intercept in Eqn. 4. Unless otherwise noted, the correlation and the values were justified above the 99.5% level; figures in parentheses are the 95% confidence interval.
[b] Concentration range.
[c] Number of points.
[d] Standard deviation of the correlation.
[e] Correlation coefficient.
[f] Justified at the 99% level.
[g] Justified at the 97.5% level.
[h] Justified at the 95% level.

TABLE III

UNCOUPLING ACTIVITY OF SUBSTITUTED PHENOLS AT DIFFERENT pH

Substituent	pK_A [a]	$\log 1/C_{200}$ [b]		
		pH 6.4	pH 7.4	pH 8.4
4-Cl	9.38	3.58 (0.1)	3.56 (1.0)	3.64 (9.5)
2,4-Cl_2	7.89	4.78 (3.1)	4.85 (24.4)	5.00 (76.4)
2,4,6-Cl_3	5.99	5.10 (71.8)	5.20 (96.2)	4.77 (99.6)
3-NO_2	8.40	3.65 (1.0)	3.77 (9.1)	4.05 (50.0)
2,4-$(NO_2)_2$	4.09	5.45 (99.5)	5.10 (99.9)	5.00 (99.9)
2-*s*-Bu-4,6-$(NO_2)_2$	4.51	7.06 (99.0)	6.89 (99.9)	6.46 (99.9)
4-*t*-Bu	10.23	3.67 (0.01)	3.70 (0.1)	3.64 (1.5)
4-*n*-Pr	10.21	3.43 (0.01)	3.43 (0.2)	3.39 (1.5)
SF6847	6.84	8.42 (28.9)	8.44 (80.3)	8.44 (97.6)

[a] From Table I.

[b] The figures in parentheses are the degree of ionization (%).

nitrophenol measured at four different pH values [16]. The analysis showed that the activity is attributable to that of the neutral form but not to that of the ionized mixture. The discrepancy in these results probably came from the fact that structural variations in the compounds reported previously were so limited that the range of log K_A was narrow and that the index used for the activity did not necessarily reflect the ultimate uncoupling effect.

Eqn. 8 is similar to Eqn. 6 for potency to increase proton permeability. This suggests a relationship between the uncoupling activity in mitochondria and the potency to increase proton permeability across liposomal membranes. In fact, the two activities are correlated by Eqn. 9.

$$\log\frac{1}{C_{200}} = \underset{(0.155)}{0.813}\ \log Pp + \underset{(0.066)}{0.293}\ \log K_A + \underset{(0.712)}{11.422} \qquad (9)$$
$$(n = 19,\ s = 0.246,\ r = 0.987)$$

In Eqn. 9, the log K_A term is significant at the 99.5% level. The effect of acidity on the uncoupling activity is more important than that on the potency to increase proton permeability. The powerful uncoupler SF6847 behaved as an outlier from the correlation and was not included in Eqn. 9. This compound had an uncoupling activity much higher than expected. The observed uncoupling activity was 8.44 in logarithmic units, but the calculated activity from Eqn. 9 was 6.12.

Discussion

Intrinsic proton permeability of lecithin membrane

Nozaki and Tanford [12] investigated the apparent permeability of protons across lecithin-bilayered liposomes by measuring the increase in proton concentration in the outer aqueous phase. They examined the effects of the flux of hydroxide anions and the protonated counter-anions on the apparent permeability of protons. Using an approximation for the intrinsic value of the hydroxide permeability coefficient and neglecting the proton flux by aspartate used as the buffer component, they estimated the intrinsic proton permeability coefficient, $Pm(H^+)$, as being $5 \cdot 10^{-12}$ cm/s. They reported that the apparent proton permeability $P'm(H^+)$ is expressible by Eqn. 10:

$$P'm(H^+) = Pm(H^+) + Pm(HX)\,K_{HX}[X^-] \qquad (10)$$

where $Pm(HX)$ is the intrinsic permeability coefficient of protonated counter-anions, K_{HX} is its association constant, and $[X^-]$ is the concentration of counter-anions. We calculated the $P'm(H^+)$ value from the changes in the proton concentration in the internal aqueous phase with various Na_2SO_4 concentrations on the two sides of the membranes. The $P'm(H^+)$ value was linearly related to the concentration of Na_2SO_4. From the intercept, the $Pm(H^+)$ value of our system was $2.0 \cdot 10^{-11}$ cm/s. Our value is close to

that obtained by Nozaki and Tanford. In our procedure, we did not account for the contribution of hydroxide flux. As far as the increment in the apparent permeability coefficient by phenol is concerned, the contribution of hydroxides and protonated counter-anions is probably constant.

Effect of phenols on proton permeability

The apparent permeability of protons through liposomal membranes was increased linearly with the concentration of each phenol in the external buffer solution. The proton permeability might be increased when the structure of the liposomal membrane is perturbed. We have evaluated the degree of perturbation of the liposomal membrane by phenols in terms of the change in permeability to glucose [8]. The quantitative analysis showed that the liposomal membrane was perturbed by the partitioning of phenols into the membrane and by their steric bulk. The concentration in the outer aqueous phase necessary to double the glucose permeability was 0.71 mM for 4-*tert*-butylphenol and 0.43 mM for 2-*sec*-butyl-4,6-dinitrophenol. For the potency to increase proton permeability, the steric bulk of phenols was not significant, as was shown by Eqn. 6. The concentration in the outer aqueous phase required to double the proton permeability was 0.13 mM for 4-*tert*-butylphenol and 0.0024 mM for 2-*sec*-butyl-4,6-dinitrophenol. These outerphase concentraitons corresponded to the membrane concentration of 1.06 and 0.25 mol/kg lipid for 4-*tert*-butylphenol and 0.62 and 0.0053 mol/kg lipid for 2-*sec*-butyl-4,6-dinitrophenol. The membrane concentrations that induce proton leakage were 1/4 to 1/120 those necessary for glucose leakage. The change in the polarity of the membrane surface calculated previously showed that the structure of the membrane surface is perturbed significantly when appreciable amounts of glucose are released [8]. Within the concentration range of phenols for proton release, however, the membrane surface structure was probably not perturbed. These findings indicate that, under experimental conditions for proton permeability, perturbation of the liposomal membrane could be disregarded. That the effect of steric bulk was not observed in the potency to increase proton permeability in Eqn. 6 is also evidence for this statement. We concluded that the enhancement of proton permeability of phenols is due to their function as protonophores.

Eqn. 6 shows that the protonophoric activity of phenols depends on two physico-chemical factors; partitioning into the liposomal membrane from the buffer phase, log P(L/W), and acidity, log K_A. The best explanation of the meaning of Eqn. 6 is that the protonophoric behavior of phenols is due to a shuttle-type mechanism. In this mechanism, the partitioned phenolate anion first catches a proton existing in the internal aqueous phase at the inner surface, moves across the membrane as a neutral species, releases the proton into the external aqueous phase, and then returns to the inner surface of the membrane. The movement of a neutral species across the lipid membrane should be much easier than that of the ionized form. Thus, the rate-limiting step of the shuttle mechanism is probably the movement of the ionized phenols. The more stable the phenolate anion, i.e., the more strongly delocalized the negative charge on the molecule, the easier would be the movement. Eqn. 6, which indicates that the higher log K_A values are more favorable to protonophoric activity, is consistent with this mechanism. The log P(L/W) term in Eqn. 6 shows that the higher the partition coefficient, the lower is the outer-phase concentration of phenols to give an equivalent protonophoric activity.

The protonophoric activity in lecithin-cholesterol liposomes was lower than that in lecithin liposomes by a factor of about 1.5 for each phenol (Eqn. 5). It is generally thought that cholesterol molecules cause a decrease in the membrane fluidity above the phase-transition temperature of the membrane [17]. The lower protonophoric activity in the lecithin-cholesterol liposome may be caused by a decrease in membrane fluidity which retards the movement of compounds, supporting the suggestion of the shuttle-type mechanism.

Relationship between protonophoric and uncoupling activities of phenols

That Eqn. 8 is similar to Eqn. 6 is also evidence for the shuttle-type mechanism for phenols under uncoupling conditions. Eqn. 9 shows that the protonophoric and uncoupling activities of phenols correspond one-to-one when the electronic effect of the molecule was separated in terms of log K_A.

The log K_A term in Eqn. 9 would reflect a difference in the physical properties such as the surface-charge density and the dielectric constant between liposome and inner mitochondrial membranes.

McLaughlin and coworkers [18] found that an acidic and potent uncoupler, CCCP (carbonylcyanide *m*-chlorophenylhydrazone), produced a conductance one order of magnitude higher on a bilayered membrane formed from phosphatidylethanolamine, a zwitterionic lipid, than on such a membrane formed from a mixture containing 20% cardiolipin, a negatively charged lipid. They thought that the mitochondrial membrane which contains cardiolipin would have a surface negative-charge density higher than the zwitter-ionic artificial membrane. The surface concentration of the negatively ionized protonophore would be lower in the mitochondrial membrane than in liposomes. Thus, the conductance in the mitochondrial membrane could be lower than that in liposomes. They also suggested another factor; the protonophoric activity of CCCP could be higher in the mitochondrial membrane which have a dielectric constant higher than the lecithin bilayer, since the ionized species would be more stable in the higher dielectric medium.

The above two factors operate in opposite directions to the protonophoric activity. That the sign of the log K_A term is positive in Eqn. 9 indicates that the effects of ionizability of phenols and stability of phenolate anions are more important in the protonophoric activity under uncoupling conditions in mitochondria than in lecithin liposome. The effects of the dielectric constant of the membrane that stabilize the anionic species may outweigh those of the membrane surface charge that depress the ionization of phenols in the overall process. The above argument supports the idea of a shuttle-type mechanism for acidic uncouplers consistent with Mitchell's chemiosmotic hypothesis.

The behavior of SF6847 outlying from the relationship represented by Eqn. 9 for 'simply' substituted phenolic uncouplers might be due to an uncoupling mechanism different from that of the shuttle type. In fact, there have recently been some questions about this mechanism [19–22]. To establish the shuttle-type mechanism, explanation of the outlying behavior of the extremely potent SF6847 is important.

Acknowledgements

The calculations were done with a FACOM M382 computer at the Data Processing Center of this university. We thank Professor Hiroshi Terada, Faculty of Pharmaceutical Sciences, Tokushima University for his invaluable discussion.

References

1 Skulachev, V.P. (1971) Curr. Top. Bioenerg. 4, 127–190
2 Ting, H.P., Wilson, D.G. and Chance, B. (1970) Arch. Biochem. Biophys. 141, 141–146
3 Liberman, E.A., Topaly, U.P., Tsofina, L.M., Jasaitis, A.A. and Skulachev, V.P. (1969) Nature 222, 1076–1078
4 Bakker, E.P., Van den Heuvel, E.J., Wiechmann, A.H.C. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 78–87
5 McLaughlin, S.G.A. and Dilger, J.P. (1980) Physiol. Rev. 60, 825–863
6 Singleton, S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53–56
7 Oettmeier, W. and Masson, K. (1980) Pestic. Biochem. Physiol. 141, 86–97
8 Miyoshi, H., Nishioka, T. and Fujita, T. (1986) Bull. Chem. Soc. Jap. 59, 1099–1107
9 Kano, K. and Fendler, J.H. (1978) Biochim. Biophys. Acta 509, 289–299
10 Shibuya, I. (1967) Agr. Biol. Chem. 31, 111–114
11 Deamer, D.W. and Nichols, J.W. (1983) Proc. Natl. Acad. Sci. USA 80, 165–168
12 Nozaki, Y. and Tanford, C. (1981) Proc. Natl. Acad. Sci. USA 78, 4324–4328
13 Hogeboom, G.H. (1955) Methods enzymol. 1, 16–19
14 Myers, D.K. and Slater, E.C. (1957) Biochem. J. 67, 558–572
15 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766
16 Fujita, T. (1966) J. Med. Chem. 9, 797–803
17 Dufourc, E.j., Parish, E.J., Chitrakon, S. and Smith, I.C.D. (1984) Biochemistry 23, 6062–6071
18 Kasianowicz, J., Benz, R. and McLaughlin, S. (1984) J. Membrane Biol. 82, 179–190
19 Hatefi, Y. (1975) J. Supramol. Struct. 3, 201–213
20 Hanstein, W.G. (1976) Biochim. Biophys. Acta 456, 129–148
21 Decker, S.J. and Lang, D.R. (1977) J. Biol. Chem. 252, 5936–5938
22 Guffanti, A.A., Blumenfeld, H. and Krulwich, T.A. (1981) J. Biol. Chem. 256, 8416–8421