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Structural requirements of salicylanilides for uncoupling activity in mitochondria: quantitative analysis of structure-uncoupling relationships

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The uncoupling activities of more than 20 salicylanilides were measured in rat liver mitochondria. The activities, expressed as the minimum concentrations required for full release of state-4 respiration, ranged over three orders of magnitude. The acid dissociation constant, pK_A, and the partition coefficient between octanol and water (P_{oct}) of some of the salicylanilides were determined. These two parameters were found to be well expressed in terms of the Hammett constant, σ, and the hydrophobic substituent coefficient, Π, respectively. The pK_A and log P_{oct} values of all the salicylanilides were predicted according to these relationships. Furthermore, the capacity factor, k', on high-performance liquid chromatography was determined on glyceryl-coated-controlled pore glass (gly-CPG). Values of log k' correlated well with those of log P_{oct}. The uncoupling activities of the salicylanilides were analyzed in terms of these three parameters. Both hydrophobic and electron-withdrawing properties were found to be essential for induction of potent uncoupling activity. The correlations using log k' were better than those using log P_{oct}.

Introduction

A wide variety of compounds, such as phenols, benzimidazoles and salicylanilides, are known to induce uncoupling of oxidative phosphorylation and photophosphorylation in mitochondria, chloroplasts and other energy-transducing membranes [1]. Their mode of action is thought to be due to their protonophoric activity, which renders proton-impermeable energy-transducing membranes permeable to protons, and thus dissipates the protonmotive force necessary for ATP synthesis [1]. In this mechanism, protons are carried across membranes by repeated interchange of the protonated and deprotonated forms of an acidic group of the uncoupler in the membrane. Thus, an acid dissociable group is a prerequisite for uncoupling activity [2]. The structural requirements of weakly acidic uncouplers for activity have been studied extensively, especially those of phenols [3–10]. However, little is known about the structural requirements of salicylanilides for uncoupling, although some of them such as S-13 are very potent uncouplers [11–13]. This is partly because structural characterization of salicylanilides is very dif-

Abbreviations: HPLC, high-performance liquid chromatography; DMSO, dimethylsulfoxide; gly-CPG, glyceryl-coated controlled pore-glass; S-13, 2′,5-dichloro-4′-nitro-3-*tert*-butylsalicylanilide.

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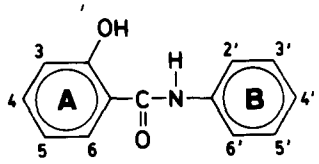


Scheme I. Chemical structure of salicylanides.

ficult due to the low solubility of these compounds in water.

In this study we determined the uncoupling activities of more than 20 derivatives of salicylanilides, in which various substituents such as halogens, NO_2, CH_3 and OCH_3 were introduced at the 3 and 5 positions of the salicylic acid moiety (A-ring), and at the 2′ and 4′ positions of the aniline moiety (B-ring) as shown in Scheme I. For examination of the structural characteristics necessary for uncoupling activity, we determined the acid dissociation constant pK_A in 20% DMSO, and the partition coefficient between *n*-octanol and water (P_{oct}) of some salicylanilides. We also determined the capacity factor k' of salicylanilides in HPLC, because this has recently been found to be a good index of the hydrophobic nature of compounds [14,15]. Using these experimentally determined physicochemical properties, and the electronic substituent constant σ (Hammett constant) and the hydrophobic substituent constant Π, the uncoupling activities of salicylanilides were analyzed statistically.

Materials and Methods

The salicylanilides used in this study were those reported previously, which were sufficiently pure for determination of physicochemical properties and uncoupling activities [16]. S-13 was the generous gift from Dr. P. Hamm, Monsanto Chemical Co., St. Louis. Other reagents were obtained from commercial sources, and were used without further purification.

Mitochondria were isolated from the liver of adult male Wistar rats [17]. The amount of protein was determined by the method of Lowry et al. with bovine serum albumin as a standard [18]. Uncoupling activities of salicylanilides were determined by measuring stimulation of state-4 respiration with succinate (plus rotenone) as substrate in medium consisting of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA and 10 mM potassium phosphate buffer (pH 7.2). Respiration was monitored polarographically with a Clark oxygen electrode (Yellow Springs, YSI 5331) at 25 °C in a total volume of 2.53 ml. A concentrated solution of salicylanilide in DMSO was added to state-4 mitochondria (0.7 mg/ml), and the minimum concentration of salicylanilide required for inducing full release of state-4 respiration, and the slope of the linear portion of the titration curve were taken as the uncoupling activity. Values for these uncoupling activities are the means of at least three runs. These values were quite reproducible.

The acid dissociation constant pK_A of salicylanilides was determined spectrophotometrically from the change in the absorption at λ_{max} on acid dissociation of their anionic form. Since the neutral forms of salicylanilides are sparingly soluble in water, we measured the absorbance change in 20% DMSO solution. Recordings were made in a Shimadzu dual-wavelength spectrophotometer, model UV 300.

The partition coefficient of salicylanilides between octanol and water (P_{oct}) was determined by the shaking-flask method. A volume of 2000 ml of phosphate solution (pH 2.2), was equilibrated with 5 ml of *n*-octanol containing salicylanilides, and then the salicylanilide transferred into the aqueous phase was extracted by vigorous shaking with 100 ml of CH_2Cl_2. This extraction was repeated 3–4 times, and the CH_2Cl_2 extracts were pooled and evaporated. The dried salicylanilide was dissolved in methanol, and determined by HPLC with a JASCO TRIROTOR-II equipped with an ultraviolet detector JASCO UVIDEC-100-II. Gly-CPG (Electro-Nucleonics, Fairfield) packed in a column (2.1 mm i.d. × 50 cm) was used as the stationary phase and a mixture of methanol and water (7 : 3 in volume), as the mobile phase.

The value of P_{oct} was determined from the initial concentration of salicylanilide in the octanol phase (C) and that in the aqueous phase after partition equilibrium (C_w) by Eqn. 1.

$$P_{oct} = \frac{CV_0 - C_wV_w}{C_wV_0} \quad (1)$$

where V_w and V_0 represent the volumes of the aqueous and octanol phases.

TABLE I

PHYSICOCHEMICAL PROPERTIES AND UNCOUPLING ACTIVITIES OF SALICYLANILIDES

No.	Substituents				$\Sigma\sigma_A$ [a]	$\Sigma\sigma_B$ [a]	$\Sigma\Pi_A$ [a]	$\Sigma\Pi_B$ [a]	pK_A [b]	pK_{calc} [c]	log k' [d]	log P_{SF} [e]	log P_{HPLC} [f]	log P_{calc} [g]	log($1/C_{unc}$) [h]
	3	5	2′	4′											
1	H	H	H	H	0.00	0.00	0.00	0.00	7.4	7.48	−0.03	3.50	3.44	3.49	4.51
2	H	H	CH_3	H	0.00	−0.17	0.00	0.56	7.5	7.67	–	–	–	4.19	4.56
3	H	H	NO_2	H	0.00	0.78	0.00	−0.28	–	6.91	−0.21	–	2.87	3.14	4.34
4	H	H	Cl	H	0.00	0.23	0.00	0.71	–	7.31	0.27	4.35	4.39	4.37	5.03
5	H	H	H	Cl	0.00	0.23	0.00	0.71	7.3	7.31	0.24	4.50	4.29	4.37	5.34
6	H	H	H	Br	0.00	0.23	0.00	0.86	–	7.31	0.31	–	4.51	4.56	5.79
7	H	H	CH_3	Cl	0.00	0.06	0.00	1.27	–	7.43	0.52	–	5.18	5.07	6.47
8	H	H	NO_2	Cl	0.00	1.01	0.00	0.43	–	6.74	0.19	4.09	4.13	4.02	5.18
9	H	H	Cl	Cl	0.00	0.46	0.00	1.42	–	7.14	0.62	–	5.49	5.25	6.57
10	H	NO_2	H	H	0.78	0.00	−0.28	0.00	–	3.03	0.04	–	3.66	3.33	5.67
11	H	Cl	H	H	0.23	0.00	0.71	0.00	–	6.17	0.14	4.05	3.98	3.90	5.79
12	H	F	CH_3	Cl	0.06	0.06	0.14	1.27	7.3	7.09	0.43	–	4.89	5.15	6.28
13	H	F	CH_3	Br	0.06	0.06	0.14	1.42	7.1	7.09	0.51	–	5.15	5.34	6.50
14	H	Cl	CH_3	H	0.23	−0.17	0.71	0.56	6.6	6.29	0.29	4.21	4.45	4.60	5.72
15	Cl	Cl	H	H	0.46	0.00	1.42	0.00	4.7 [i]	4.85	0.15	–	4.01	4.32	6.34
16	Cl	Cl	H	NO_2	0.46	0.78	1.42	−0.28	4.7 4.6 [i]	4.28	–	–	–	3.97	–
17	Cl	Cl	H	F	0.46	0.06	1.42	0.14	4.8 [i]	4.81	0.39	4.76	4.77	4.49	6.89
18	Cl	Cl	H	Cl	0.46	0.23	1.42	0.71	4.7 [i]	4.69	0.51	–	5.15	5.20	6.44
19	Cl	Cl	H	OCH_3	0.46	−0.27	1.42	−0.02	4.9 [i]	5.05	–	–	–	4.30	6.14
20	Cl	Cl	H	CH_3	0.46	−0.17	1.42	0.56	4.9 [i]	4.98	–	–	–	5.02	–
21	Cl	Cl	CH_3	NO_2	0.46	0.61	1.42	0.28	–	4.41	0.31	–	4.51	4.67	5.84
22	Cl	Cl	NO_2	Cl	0.46	1.01	1.42	0.43	–	4.11	0.49	–	5.08	4.85	6.95
23	Cl	Cl	Cl	NO_2	0.46	1.01	1.42	0.43	4.0 [i]	4.11	–	–	–	4.85	–
24	Cl	Cl	F	F	0.46	0.12	1.42	0.28	–	4.77	0.32	–	4.55	4.67	6.32
25	H	Br	H	Cl	0.23	0.23	0.86	0.71	–	6.00	0.47	–	5.02	4.87	6.76
26	Br	Br	NO_2	Cl	0.46	1.01	1.72	0.43	–	4.11	0.48	–	5.05	5.03	6.91
27	Br	Br	F	F	0.46	0.12	1.72	0.28	–	4.77	0.46	–	4.99	4.84	6.49
28	tBu	Cl	Cl	NO_2	0.03	1.01	2.69	0.43	6.4 [j] 5.8 [k]	6.57	–	–	–	5.60	7.50

[a] Values of benzenes cited from refs. 21 and 22.

[b] Values determined spectrophotometrically in 20% DMSO.

[c] Determined by Eqn. 4.

[d] Determined on gly-CPG with a mobile phase of 25% DMSO at pH 2.0.

[e] Determined by the shaking flask method between *n*-octanol and water (pH 2.2).

[f] Determined by Eqn. 7.

[g] Determined by Eqn. 10.

[h] Value in an M-scale.

[i] Values determined spectrophotometrically in 20% DMSO cited from Ref. 13.

[j] Value determined spectrophotometrically in 10% ethanol cited from Ref. 19.

[k] Value determined by conductance measurement in water cited from Ref. 20.

The capacity factor k' of salicylanilides in HPLC on gly-CPG was determined from the retention time of sample compounds (t_R), and that of potassium iodide as an unretained reference compound potassium iodide (t_0) according to Eqn. 2.

$$k' = \frac{t_R - t_0}{t_0} \quad (2)$$

In this case, phosphoric acid solution at pH 2 containing 25% DMSO was used as the mobile phase.

Quantitative structure-activity relationships were analyzed with a NEC personal computer PC-9801VX.

Results

Acid dissociation constant pK_A

The pK_A values of seven salicylanilides were determined spectrophotometrically in 20% DMSO solution, because their solubilities in water were very low. These values are summarized in Table I. As can be seen, the pK_A values of salicylanilides, substituted in the B-ring but not the A-ring, are all about 7.5. On the other hand, the introduction of electron-withdrawing substituents into the A-ring significantly decreases the pK_A value. These results indicate that the acid dissociation takes place at the phenolic OH group, not at NH of the aniline moiety.

Storey et al. [13] determined the pK_A values in 20% DMSO of various 3,5-dichlorosalicylanilides substituted at the 2′ and 4′ positions, and these values are also listed in Table I. Since their pK_A values range between 4.0 and 4.9, substituents in the B-ring had very small, but definite effects on the pK_A value, a fact which we also found. Our value of 4.7 for 3,5-dichloro-4′-nitrosalicylanilide (compound 16) is very similar to their value of 4.6.

The pK_A value of the very potent salicylanilide uncoupler S-13 (compound 28) was reported to be 6.4 in 10% ethanol [19], and 5.8 in water [20]. Hereafter we used the value of 6.4, because the pK_A values of other salicylanilides were determined in solutions containing the organic solvent DMSO. We analyzed pK_A in terms of σ(para) for all the salicylanilides for which the pK_A values are known. In the following analyses, we used 4.6 as the pK_A of compound 16, but the results were essentially the same when its pK_A was taken as 4.7. The results in Table I suggest that the electronic properties of substituents in the A- and B-rings have different effects. Thus, we first examined how the electron-withdrawing ability of substituents changes with their position in the two rings, as expressed by Eqn. 3.

$$pK_A = 7.285 - 5.650\sigma_3 - 5.109\sigma_5 - 2.110\sigma_{2'} - 0.383\sigma_{4'}$$
$$(\pm 0.155)(\pm 0.602)(\pm 0.890)(\pm 0.718)(\pm 0.254)$$
$$n = 14,\ r = 0.997,\ s = 0.111 \quad (3)$$

where the subscript on the σ value indicates the position of the substitution (cf. Scheme I), and n, r and s are the numbers of compounds used for the calculation, the correlation coefficient and the standard deviation, respectively. The values in parentheses are 95% confidence intervals. It is apparent from Eqn. 3 that on acid dissociation of salicylanilides, though the electron-withdrawing power of substituents in the B-ring had much less effect than that of those in the A-ring, its effect is definite.

Eqn. 4, where pK_A is expressed in terms of the sum of the σ's in the A− ($\Sigma\sigma_A$) and B-rings ($\Sigma\sigma_B$), is useful practically for prediction of the pK_A values of a wide variety of salicylanilides, although the correlation is slightly less than that of Eqn. 3.

$$pK_A = 7.478 - 5.704\Sigma\sigma_A - 0.733\Sigma\sigma_B \quad (4)$$
$$(\pm 0.170)(\pm 0.497)\quad(\pm 0.251)$$
$$n = 14,\ r = 0.992,\ s = 0.179$$

The pK_A value of 4.3 for 2′,3,4′,5,5′-pentachlorosalicylanilide calculated by this equation agrees well with the reported value of 4.4 [13]. It should be noted that in Eqn. 4 the electronic effect of substituents in the A-ring is about 8 times that of those in the B-ring. The values of pK_A determined by Eqn. 4 are listed in Table I as values of pK_{calc}, and agree well with those determined experimentally.

Partition coefficient

The values of P_{oct} of seven salicylanilides were determined by the standard shaking-flask method

and those are listed in Table I as log P_{SF} values. The value of log P_{SF} was analyzed in terms of $\Sigma\Pi$ values in the A- and B-rings, and the relation shown in Eqn. 5 was obtained.

$$\log P_{oct} = \underset{(\pm 0.490)}{3.607} + \underset{(\pm 0.486)}{0.591\Sigma\Pi_A} + \underset{(\pm 0.860)}{0.993\Sigma\Pi_B} \quad (5)$$

$$n = 7,\ r = 0.876,\ s = 0.235$$

The correlation is not statistically high. For obtaining a more significant correlation, the dependence of the hydrophobicity of substituents on their position of substitution should be examined. In fact, this treatment resulted in the more significant Eqn. 6, though the coefficient with Π_5 is below the 95% confidence level. However, too few compounds were examined to allow accurate statistical determination of the position-dependent contribution of Π.

$$\log P_{oct} = \underset{(\pm 0.453)}{3.605} + \underset{(\pm 0.917)}{1.016\Pi_3} + \underset{(\pm 0.726)}{0.384\Pi_5} + \underset{(\pm 0.766)}{0.902\Pi_{2'}} + \underset{(\pm 0.898)}{1.150\Pi_{4'}}$$

$$n = 7,\ r = 0.949,\ s = 0.217 \quad (6)$$

Capacity factor k' in HPLC

We next determined the capacity factor, k', of 22 derivatives of salicylanilide, since log k' has been reported to be correlated with log P_{oct}, though the correlation depends on the structure of the test compounds and experimental conditions [14,15,23,24]. In this study we used gly-CPG as a stationary phase and phosphoric acid solution (pH 2.0) containing 25% DMSO as a mobile phase.

As shown in Eqn. 7, the log k' values of seven compounds correlated well with their log P_{SF} values. Introduction of the electronic term into Eqn. 7 did not improve the correlation significantly (data not shown).

$$\log k' = \underset{(\pm 0.505)}{-1.116} + \underset{(\pm 0.120)}{0.316 \log P_{SF}} \quad (7)$$

$$n = 7,\ r = 0.945,\ s = 0.048$$

The values of log P_{oct} can be determined by Eqn. 7. Since these values of log P_{oct} are based on log k' in HPLC, they are referred to as log P_{HPLC}, and are listed in Table I. The values of log P_{HPLC} are very similar to those of log P_{SF}. Furthermore, the log k' values for 22 compounds were well represented by $\Sigma\Pi$ values of substituents in the A- and B-rings as shown in Eqn. 8, where the constant term can in fact be eliminated.

$$\log k' = \underset{(\pm 0.054)}{-0.014} + \underset{(\pm 0.041)}{0.185\Sigma\Pi_A} + \underset{(\pm 0.060)}{0.394\Sigma\Pi_b} \quad (8)$$

$$n = 22,\ r = 0.959,\ s = 0.061$$

Addition of the electronic parameter improved this correlation, as shown in Eqn. 9, but this treatment was statistically insignificant.

$$\log k' = \underset{(\pm 0.065)}{-0.054} + \underset{(\pm 0.052)}{0.151\Sigma\Pi_A} + \underset{(\pm 0.061)}{0.418\Sigma\Pi_B} + \underset{(\pm 0.163)}{0.162\Sigma\sigma_A} + \underset{(\pm 0.075)}{0.037\Sigma\sigma_B}$$

$$n = 22,\ r = 0.968,\ s = 0.057 \quad (9)$$

In both equations, the contribution of the hydrophobicity of substituents in the B-ring to log k' is more than twice that of these in the A-ring, suggesting the stronger interaction of the B-ring with the stationary phase of gly-CPG.

The value of log P_{HPLC} is expressed in terms of $\Sigma\Pi$ with high correlation in Eqn. 10.

$$\log P_{HPLC} = \underset{(\pm 0.173)}{3.489} + \underset{(\pm 0.131)}{0.585\Sigma\Pi_A} + \underset{(\pm 0.189)}{1.243\Sigma\Pi_B} \quad (10)$$

$$n = 22,\ r = 0.959,\ s = 0.194$$

This equation is very similar to Eqn. 5, and that derived from Eqns. 7 and 8. The relation in Eqn. 10 can be used to determine log P_{oct} values of salicylanilides when their log k' values are not known. The values of log P_{oct} determined by Eqn. 10 are referred to as log P_{calc} values and are summarized in Table I, where their good agreement with log P_{HPLC} can be seen.

Uncoupling activity

The uncoupling activities of 25 salicylanilides in rat liver mitochondria were determined by monitoring their stimulation of state-4 respiration with succinate as respiratory substrate. Almost all the salicylanilides stimulated state-4 respiration

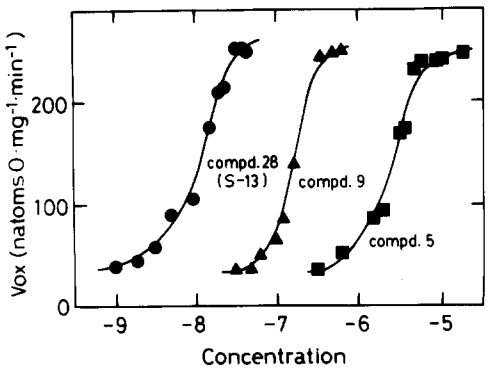


Fig. 1. Stimulation of state 4 respiration of mitochondria by salicylanilides. The respiration V_{ox} is a function of salicylanilide concentration (M) on a log scale. The numbers of compounds shown beside titration curves correspond to those of salicylanilides listed in Table I.

more than 5-fold, as observed with the typical weakly acidic uncoupler S-13 as shown in Fig. 1. The dependence of the respiratory release on the concentration of salicylanilides, plotted on an arithmetic scale, was first linear, and then the slope gradually decreased, and the plot reached a maximum. Thus, the uncoupling activities of all salicylanilides could be compared quantitatively from the minimum concentration required for full release of state-4 respiration (C_{unc}) and from the slope of the linear portion of the titration curve (S_{unc}), as observed with other weakly acidic uncouplers for activity have been studied extensively, especially those of phenols [3–10]. However, little is known about the structural requirements of salicylanilides for uncoupling, although some of them such as S-13 are very potent uncouplers [11–13]. This is partly because structural characterization of salicylanilides is very difficult due to the low solubility of these compounds in water. with a C_{unc} of 31.6 nM, and the weakest uncoupler is 2′-nitrosalicylanilide (compound 3) with a C_{unc} of 45.7 μM. The C_{unc} of 3,4′,5-trichloro-2′-nitrosalicylanilide (compound 22) is 112 nM. Thus, introduction of three Cl residues into compound 3 caused a 400-fold increase in the uncoupling activity. Interestingly, introduction of one Cl (compounds 4, 5 and 11) into salicylanilide (compound 1) increased the activity, and the increase depended on the position of the substitution.

Analysis of uncoupling activity in terms of physicochemical properties

The individual effects of Π, log k', log P_{HPLC}, log P_{calc}, σ and log K_{calc} ($= -pK_{calc}$) were first examined. Of these parameters, log k' showed the most significant correlation, given in Eqn. 11.

$$\log(1/C_{unc}) = 5.042 + 3.075 \log k' \quad (11)$$
$$(\pm 0.349)(\pm 0.936)$$
$$n = 22,\ r = 0.837,\ s = 0.423$$

For improving this relation, introduction of the electronic parameter log K_{calc} was necessary, as shown in Eqn. 12.

$$\log(1/C_{unc}) = 6.335 + 2.963 \log k' + 0.213 \log K_{calc} \quad (12)$$
$$(\pm 0.659)(\pm 0.678) \qquad (\pm 0.101)$$
$$n = 22,\ r = 0.923,\ s = 0.305$$

This correlation was better than those obtained using log P_{HPLC} and log P_{calc} instead of log k'. From the fact that addition of log K_{calc} term to Eqn. 11 resulted in higher correlation as shown in Eqn. 12, it can be concluded that the hydrophobicity of salicylanilides is a major factor for inducing uncoupling, and the electron-withdrawing property is auxiliary.

Of the salicylanilides listed in Table I, compounds 2, 19 and 28 (S-13) were not used in these regression analyses, because their log k' values are not known. For quantitative analysis of all the salicylanilides of which C_{unc} values are known, log P_{calc} was used instead of either log k' or log P_{HPLC}. The best correlation was obtained by analysis in terms of log P_{calc} and log K_{calc} as expressed in Eqn. 13.

$$\log(1/C_{unc}) = 2.886 + 1.044 \log P_{calc} + 0.272 \log K_{calc}$$
$$(\pm 0.372)(\pm 0.249) \qquad (\pm 0.114)$$
$$n = 25,\ r = 0.903,\ s = 0.370 \quad (13)$$

The addition of the term $(\log K_{calc})^2$ based on the idea that there should be an optimum pK_A for induction of uncoupling, did not improve the correlation of Eqn. 13. Though pK_A values of the salicylanilides used in this study were in a range between 7.7 and 3.0, the range seems to be not sufficient to determine whether there is an optimum pK_A for their uncoupling activity. Although

TABLE II

ANALYSIS OF UNCOUPLING ACTIVITY OF ALL THE SALICYLANILIDES LISTED IN TABLE I IN TERMS OF σ AND Π.

Equation	n	r	s	Eqn. No.
$\log(1/C_{unc}) = 4.878 + 0.874\Sigma\Pi_A + 0.979\Sigma\Pi_B$ ($\pm$0.359) ($\pm$0.239) ($\pm$0.409)	25	0.864	0.434	14
$\log(1/C_{unc}) = 4.910 + 0.891\Sigma\Pi$ [a] ($\pm$0.331) ($\pm$0.225)	25	0.862	0.427	15
$\log(1/C_{unc}) = 5.543 + 1.418\Sigma\sigma_A + 0.520\Sigma\sigma_B$ ($\pm$0.513) ($\pm$1.366) ($\pm$0.815)	25	0.459	0.764	16
$\log(1/C_{unc}) = 5.705 + 1.337\Sigma\sigma_A$ ($\pm$0.451) ($\pm$1.379)	25	0.385	0.777	17
$\log(1/C_{unc}) = 4.559 + 0.702\Sigma\Pi_A + 1.194\Sigma\Pi_B + 1.275\Sigma\sigma_A + 0.160\Sigma\sigma_B$ ($\pm$0.390) ($\pm$0.250) ($\pm$0.388) ($\pm$0.885) ($\pm$0.439)	25	0.908	0.378	18
$\log(1/C_{unc}) = 4.603 + 0.737\Sigma\Pi_A + 1.186\Sigma\Pi_B + 1.183\Sigma\sigma_A$ ($\pm$0.365) ($\pm$0.229) ($\pm$0.382) ($\pm$0.837)	25	0.905	0.374	19
$\log(1/C_{unc}) = 4.807 + 0.839\Sigma\Pi$ [a] $+ 0.685\Sigma\sigma_A + 0.033\Sigma\sigma_B$ ($\pm$0.347) ($\pm$0.238) ($\pm$0.770) ($\pm$0.463)	25	0.882	0.414	20
$\log(1/C_{unc}) = 4.812 + 0.844\Sigma\Pi$ [a] $+ 0.676\Sigma\sigma_A$ ($\pm$0.332) ($\pm$0.221) ($\pm$0.741)	25	0.882	0.405	21

[a] Sum of Π values of substituents in the A- and B-rings.

Eqns. 12 and 13 indicate the importance of both the hydrophobicity and electron-withdrawing ability of substituents, the position-dependent effects of substituents cannot be understood. For this purpose, the uncoupling activity was analyzed in terms of σ and Π, and results are summarized in Table II. As in Eqns. 12 and 13, the importance of both hydrophobic and electronic properties for uncoupling is noted in Eqns. 18 and 19. The uncoupling activity of 7.13 for S-13 calculated by Eqn. 19 agreed well with the observed value of 7.5. It is noteworthy that the contribution of Π in the B-ring is somewhat greater than its contribution in the A-ring, as observed in the cases of log P_{SF} (Eqn. 5) and log k' (Eqn. 8), and that the contribution of σ in the A-ring is much greater than its contribution in the B-ring, as observed in the case of pK_A (Eqn. 4).

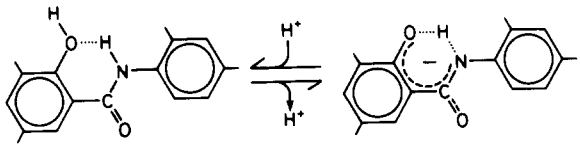
Discussion

As structural parameters, the acid dissociation constant pK_A and the partition coefficient log P_{oct} of salicylanilides were determined in this study. The values of pK_A for the phenolic OH were found to be well predicted in terms of $\Sigma\sigma_A$ and $\Sigma\sigma_B$ by Eqn. 4, where the contribution of the electron-withdrawing power of the substituents in the A-ring is about 8 times that of those in the B-ring. By Eqn. 4, values of pK_A of salicylanilides can be predicted accurately as shown in Table I.

The values of log P_{oct} were well expressed as a function of log k' on a gly-CPG column by Eqn. 7, indicating that gly-CPG is a very efficient stationary phase for determination of log P_{oct} [15]. Since log k' was well correlated with log P_{oct} and ($\Sigma\Pi_A + \Sigma\Pi_B$), as shown in Eqns. 7 and 8, log P_{oct} should be expressed in terms of ($\Sigma\Pi_A + \Sigma\Pi_B$). In fact, the values of log P_{oct} determined from log k' by use of Eqn. 7 were well correlated with those determined from ($\Sigma\Pi_A + \Sigma\Pi_B$) by Eqn. 10. The values of log P_{oct} of various salicylanilides could be predicted accurately by Eqn. 10 (see log P_{calc} in Table I).

The value of log P_{oct} of unsubstituted salicylanilide (compound 1) can be estimated by summation of the log of the partition coefficient of benzanilide [21] and Π_{OH} as shown in Eqn. 22.

$$\log P_{oct} = \log P(C_6H_5\text{-}CO\text{-}NH\text{-}C_6H_5) + \Pi_{OH}$$
$$= 2.62 - 0.67 = 1.95 \quad (22)$$



Scheme II. Formation of a hydrophobic six-membered, hydrogen bonded ring of salicylanilides, which stabilizes their anionic form.

This value is 1.55 log unit (about 35 times) smaller than the experimental value of 3.50 (cf. Table I). This could be due to the formation of a six-membered hydrophobic ring between an NH in the aniline moiety and a phenolic OH in the salicylic acid moiety by intramolecular hydrogen bonding, as depicted in the left-side structure of Scheme II, and also to the shielding from H_2O of lone pair electrons of the former nitrogen atom by two bulky benzene rings, as observed with *N*-phenylanthranilates [7]. The increased hydrophobicity should lead to higher uncoupling activity. The electron-withdrawing effect of the substituted groups in the B-ring, especially at the 2′ position, through the intramolecular hydrogen bond is expected to facilitate the acid dissociation of a phenolic OH group more than that through a covalent CO–NH bond when a hydrogen bonded ring is not formed (Eqn. 3).

Formation of the six-membered hydrogen bonded ring not only increases the hydrophobicity of salicylanilides about 40-fold, but also stabilizes the anionic form of salicylanilides, as suggested by Storey et al. [13]. For induction of protonophoric and uncoupling actions of weakly acidic uncouplers, the stability of uncoupler anion in a hydrophobic environment such as the mitochondrial membrane is thought to be decisive [1,20,25]. The dynamic electronic structure of the most potent uncoupler SF 6847 to reduce the polarity of its anionic form is especially noteworthy [1,4]. In the case of salicylanilides, the reduction of the polarity of the anionic forms is expected to be achieved by the hydrogen-bonded ring which is coplanar to the benzene ring of the salicylic acid moiety. The polar negative charge should be delocalized by this ring, having an aromatic nature as depicted in the right side structure in Scheme II. The stability of the hydrogen-bonded ring will be influenced by the steric effect of substituents at the 2′ and 6′ positions. Examination of the role of the hydrogen-bonded ring in uncoupling is under way.

The uncoupling activities of a series of salicylanilides reported so far were in a narrow range [11,13], and thus statistical analysis in terms of their physicochemical parameters seems unsuccessful [12]. The salicylanilides used in this study were structurally and biologically diverse, so allowing quantitative analysis of structure-uncoupling relationships. We found that both hydrophobicity and electron-withdrawing power are necessary for induction of uncoupling, as observed with phenols [3,5]. The electron-withdrawing ability could contribute mainly to the acid dissociation of the phenolic OH group, which is favorable for the shuttle-type protonophoric action of uncouplers [1]. The effect of hydrophobicity of substituents in the A-ring on the uncoupling was similar to, but definitely smaller than that of substituents in the B-ring (compare Eqns. 15, 20 and 21 with Eqns. 14, 18 and 19, respectively). The electron-withdrawing ability of substituents in the A-ring, but not in the B-ring, was found to be very important for high uncoupling.

In conclusion, the formation of a hydrogen-bonded ring endows the anionic forms of salicylanilides with high hydrophobicity and structural stability. Furthermore, the introduction of a highly hydrophobic substituent(s) into either the A- or B-ring, or both, and of a strong electron-withdrawing group into the A-ring should be favorable for strong uncoupling activity, as typically observed with compound 26. The very potent uncoupling action of S-13 was found to be mainly due to its high hydrophobicity. The present results should be useful for molecular design of very potent uncouplers.

Finally, the action of the most potent salicylanilide uncoupler S-13 should be considered. Terada [26] reported that the binding of S-13 to state 4 rat liver mitochondria was partition-like and that 96% of the added S-13 bound to mitochondria over a wide concentration range. S-13 showed maximum uncoupling at 31.6 nM (cf. Table I). As 0.7 mg mitochondria per ml was present in 2.53 ml of the incubation medium, on maximal uncoupling the amount of S-13 in mitochondria and its partition coefficient, P(mito), between mitochondria and the incuba-

tion medium (water), expressed as (mol of S-13 per kg of mitochondria)/(mol of S-13 per liter of water), were calculated to be 42.4 pmol/mg protein and $2.38 \cdot 10^4$ (log P(mito) = 4.38), respectively. Miyoshi et al. [27] reported that the partition of the neutral form of phenol uncouplers between phosphatidylcholine liposomes and water, P(liposome) = (mol of phenols per kg of phospholipid)/(mol of phenols per liter of water), was about the same magnitude as the partition coefficient between octanol and water, P(oct). However, in the case of S-13, P(mito) is about one order of magnitude smaller than P(oct) ($= 3.98 \cdot 10^5$). This could be due in part to the fact that P(mito) was the value at neutral pH at which S-13 exists as both neutral and anionic forms. However, the value of P(mito) of the neutral form should be about the same, because the values of P(liposome) of 2,4-dinitrophenol and trichlorophenol anions are reported to be only slightly smaller than those of the neutral forms [28].

It can be calculated that when all the S-13 in mitochondria is operative for uncoupling, about 0.2 mol S-13 per cytochrome aa_3 is enough for induction of full uncoupling, since 1 mg of mitochondrial protein contains 210 pmol cytochrome aa_3 [26]. Furthermore, according to the simple shuttle-type mechanism of uncoupling, one cycle of uncoupler molecule is necessary to carry one H^+ from the cytosolic phase to the matrix space [1,25]. Assuming that $H^+/O = 4$ with succinate as respiratory substrate, about 400 cycles per s of S-13 are required for induction of full uncoupling (V_{ox} = 250 natoms O/min per mg protein, cf. Fig. 1). This substoichiometric relation of the uncoupler molecule with the respiratory chain, and the high number of cycles required to dissipate the proton motive force, together with the fact that modification of an acidic moiety of an uncoupler molecule to a non-acid dissociable group resulted in complete loss of uncoupling activity [2], provide support for an uncoupling mechanism based on the protonophoric action of the uncoupler. For operation of uncoupling in mitochondria, the actual stoichiometry could be smaller and the number of cycles could be greater, as some uncoupler molecules may be trapped by binding to various membrane components other than phospholipids. A similar catalytic action of uncoupler was also observed with the most potent uncoupler SF 6847 [29].

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