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Physicochemical Properties and Uncoupling Activity of 3′-Substituted Analogues of N-Phenylanthranilic Acid

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SUMMARY

The relationship between the physicochemical properties and uncoupling activity of flufenamic acid [*N*-(3′-trifluoromethylphenyl)anthranilic acid] and its structural analogues, which vary in position 3′ of the phenyl ring, has been investigated. Some of these compounds are known to be potent anti-inflammatory agents. Their physicochemical properties were compared with their uncoupling activities, measured as their effects on State 4 respiration and on the ATP-P_i exchange reaction of rat liver mitochondria. The partition coefficients, determined in *n*-heptane–H_2O, *n*-hexane–H_2O, and chloroform–H_2O at pH 7, increased with introduction of hydrophobic groups such as —CF_3, —CH_3, and —C_6H_5 into *N*-phenylanthranilic acid, and decreased with introduction of hydrophilic groups such as —OH, —NH_2, and —SO_3H. Compounds with large partition coefficients had high uncoupling activity, and this correlation was highest in the chloroform–H_2O system. The formation of a dimer in the organic solvent phase was strongly suggested in chloroform, but not in *n*-hexane or *n*-heptane, with compounds possessing —H, —CF_3, —CH_3, or —NO_2 at position 3′. Higher uncoupling activity was correlated with larger pK_a values. Also, an important role of the hydrophobic nature of the acids with respect to uncoupling was confirmed for compounds that had similar ionization constants but different uncoupling activities.

INTRODUCTION

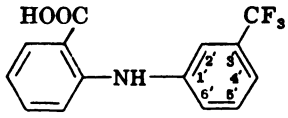
Nonsteroidal anti-inflammatory drugs are known to uncouple respiratory-chain phosphorylation in isolated mitochondria (1–4). However, little is known of the role of energy supply in mitochondrial metabolism by vascular endothelial cells and connective tissue during the inflammatory response. Moreover, the mechanism of action of known uncouplers, such as dinitrophenol and dicoumarol, in respiratory-chain phosphorylation is not completely understood. Several mechanisms have been proposed on the basis of chemical (5, 6) and chemiosmotic (7) hypotheses regarding the mechanism of respiratory-chain phosphorylation.

Of a series of *N*-phenylanthranilic acids reported to be analgesic and anti-inflammatory agents (8, 9), the most potent was flufenamic acid [*N*-(3′-trifluoromethylphenyl)anthranilic acid], which possesses a trifluoromethyl group at position 3′. Like other nonsteroidal anti-inflammatory agents, this compound is also known to be a potent uncoupler. Whitehouse (10) reported that an unsubstituted carboxylic group, an *o*-imino group, and aromaticity are required for the uncoupling activity of *N*-substituted anthranilates. In the present paper we have studied the relationship between physicochemical properties and uncoupling activity in rat liver mitochondria of a series of *N*-phenyl analogues of anthranilic acid in which the chemical structure of the phenyl ring was

changed by introduction of various groups at position 3'.

MATERIALS AND METHODS

Flufenamic acid and other derivatives were kindly supplied by Drs. Ikawa and Fujihira of Taisho Pharmaceutical Com-



I. Flufenamic acid

pany, Tokyo. The melting points of these acids are shown in Table 3 along with other physicochemical parameters. Rotenone was a gift from Dr. Nishizawa, Sumitomo Chemical Industry, Osaka. *n*-Heptane, *n*-hexane, and chloroform were purchased from Nakarai Chemicals, Ltd., Kyoto.

Rat liver mitochondria were isolated by the method of Hogeboom (11) as described by Meyers and Slater (12). Protein was determined by the biuret method (13). All reactions were carried out in mixtures containing 25 mM Tris-chloride buffer (pH 7.4) 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA, and 15 mM KCl in a final volume of 3 ml.

Oxygen consumption was measured with a Galvani electrode as described by Utsumi *et al.* (14). The ATP-$^{32}P_i$ exchange reaction was determined by the method of Hagihara and Lardy (15).

Partition coefficients were determined by shaking 10 ml of 10^{-4}–10^{-5} M solutions of each *N*-phenylanthranilic acid derivative (in 0.1 M phosphate buffer, pH 7.0, unless otherwise stated) with 10 ml of organic solvent for 48 hr in a water bath at 25 ± 0.1°. The acid concentrations in the aqueous phases were determined from optical density at an appropriate wavelength before and after equilibration, using a Hitachi double-beam spectrophotometer, model 124.

For determination of pK values, 2–3 mg of the compound to be tested were dissolved in deionized water or in dilute NaOH solution containing 5–10 (*v/v*) % acetone. The solution was titrated with 1 N HCl, using a pH-stat (Radiometer, type TTT-1). The influence of organic solvents on pK values was regarded as negligible, since this effect has been reported to be significant only when the solution contains at least 20% (*v/v*) organic solvent (16–18).

RESULTS

Uncoupling activity of N-phenylanthranilic acid derivatives. Flufenamic acid is a strong uncoupler of oxidative phosphorylation in rat liver mitochondria, since at a concentration of 50 μM it caused complete inhibition of the ATP-$^{32}P_i$ exchange reaction and induction of ATPase activity, and diminished respiratory control as effectively as dinitrophenol at the same concentration.

Figure 1 shows the effect of *N*-phenylanthranilates on the respiration of rat liver mitochondria in the absence of rotenone. The uncoupling activities of the acids were taken to be the same as the concentrations giving maximal effect on respiration, and are shown in Table 1. The order of potency of the compounds as respiratory stimulants was essentially the same as the order of their effects on the ATP-P_i exchange reaction (Fig. 2). Analogues possessing hydrophobic groups such as —CF_3 (flufenamic acid), phenyl, —Cl, naphthyl, and —CH_3 (mefenamic acid) displayed higher activity than compounds with hydrophilic groups such as —OH, —SO_3H, —NH_2, or —$N(CH_3)_2$.

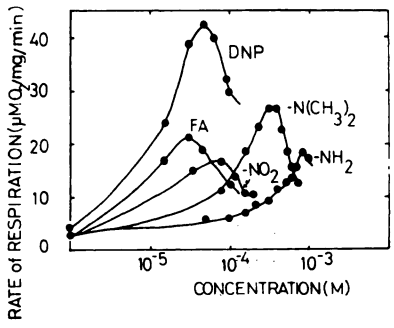


FIG. 1. *Effect of N-phenylanthranilates on respiration of mitochondria in the absence of rotenone*

Uncouplers were added to rat liver mitochondria (5 mg of protein per 3 ml) to promote succinate oxidation under State 4 conditions. Inorganic phosphate, 10 mM; succinate, 6 mM. DNP, 2,4-dinitrophenol; FA, flufenamic acid. —NO_2, —NH_2, and —$N(CH_3)_2$ represent *N*-phenylanthranilic acid derivatives in which groups are substituted at position 3' of the phenyl ring.

TABLE 1

Uncoupling activity of N-phenylanthranilic acid derivatives

Substituent	Uncoupling activity[a]	
	Concentration	Activity relative to flufenamic acid
	M	%
3′-H (*N*-phenylanthranilic acid)	2.4×10^{-4}	15
3′-CF_3 (flufenamic acid)	3.6×10^{-5}	100
3′-Cl	6.5×10^{-5}	55
3′-CH_3	1.3×10^{-4}	23
3′-NH_2	7.9×10^{-4}	5
3′-NO_2	1.2×10^{-4}	30
3′-SO_3H	1.1×10^{-3}	3
3′-$N(CH_3)_2$	3.8×10^{-4}	10
3′-OH	1.3×10^{-3}	0
3′-$COCH_3$	4.1×10^{-4}	9
3′-OCH_3	2.4×10^{-4}	15
N-Naphthyl	6.7×10^{-5}	54
3′-CH_6H_5	4.8×10^{-5}	75
2′,3′-$(CH_3)_2$ (mefenamic acid)	1.0×10^{-4}	36

[a] Concentration at maximal release of respiration, as described in the text.

Partition coefficients of N-phenylanthranilic acid derivatives. The apparent partition coefficients (Q) of *N*-phenylanthranilic acid derivatives were determined at 25° in *n*-hexane–H_2O (pH 7.5), *n*-heptane–H_2O (pH 7.0), and chloroform–H_2O (pH 7.0). In the *n*-heptane–H_2O system (Table 2), the apparent partition coefficients were approximately 0.04–0.40 for all the acid derivatives except for *N*-phenylanthranilic acid and the 3′-C_6H_5 homologue. These partition coefficients may be low because the ionized forms of these acids predominate in the aqueous phase at pH 7.0. This was confirmed by the observation that derivatives with larger pK_a values were more lipophilic. Introduction of a hydrophobic group such as —CH_3, —CF_3, or —C_6H_5 at position 3′ of *N*-phenylanthranilic acid increased the partition coefficient, while introduction of a hydrophilic group such as —OH, —SO_3H, or —NH_2 decreased it. Similar results were obtained in the *n*-hexane–H_2O system, in which the compounds tested were more hydrophilic. In the chloroform–H_2O system, almost all the compounds showed higher lipid solubility than in the other systems, perhaps because chloroform is more polar than the other two organic solvents (19).

The partition coefficient of the unionized form of the *N*-phenylanthranilic acid derivatives, i.e., the true partition coefficient Q_0, was calculated as follows. Assuming that the acid (AH) can dissociate to (A^-) in only the aqueous phase (dissociation constant, K) and that only the unionized form is present in the organic phase, where it associates as a dimer, $(AH)_2$ (association constant, K_d), we obtain Eq. 1.

$$Q = \frac{C_o}{C_w} = \frac{[AH]_o + 2[AH]_{2,o}}{[AH]_w + [A^-]_w} = Q_0 \frac{a}{K}\left\{\frac{1 + 2K_d Q_0 (a/K)[A^-]_w}{1 + (a/K)}\right\} \quad (1)$$

where square brackets mean concentration of the acid and the subscripts o and w indicate the organic and aqueous phases, respectively, in the region of low concentration (C) of the acid. When K is much greater than the hydrogen ion activities (a) of the solution, Eq. 1 can be expressed as follows.

$$Q = Q_0 \frac{a}{K} + 2K_d \left(Q_0 \frac{a}{K}\right)^2 [A^-]_w \quad (2)$$

Plotting $[A^-]_w$ against Q, therefore, it is possible to obtain the value of the true partition coefficient, Q_0, from the intercept of the Q-axis, and the value of the association constant (K_d) of the acids in the organic phase, from the slope of the straight line at a fixed value of a. Equation 2 can also be written as follows.

$$Q = Q_0 \frac{a}{K}\{1 + 2K_d Q_0 (C_w - [A^-]_w)\} \quad (3)$$

Therefore, in the region where almost all the acid dissociates to (A^-), Q is constant irrespective of the value of $[A^-]_w$ just as in the absence of association. It is necessary, however, to determine whether the association occurs in the concentration range employed.

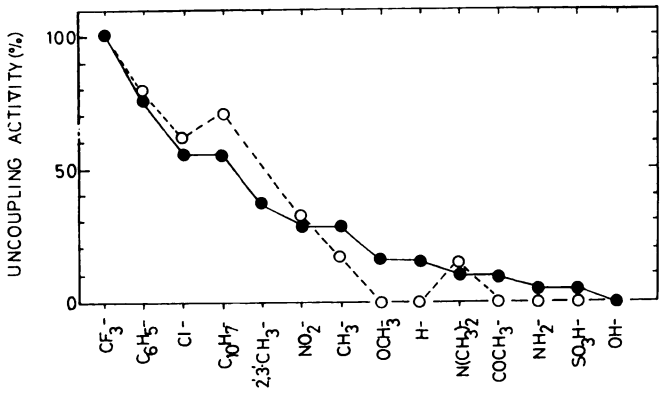


FIG. 2. *Comparative effects of N-phenylanthranilic acid derivatives on ATP-P_i exchange reaction and State 4 respiration*

The inhibitory effect on the ATP-P_i exchange reaction (○) was obtained from the percentage decrease of ^{32}P in the organic phosphate fraction in the presence of 50 μM concentration of each *N*-phenylanthranilic acid derivative. Percentage uncoupling activity, determined by the respiration method (●), was taken from the relative activity values of Table 1. The compound designated 2′, 3′—CH_3 = 2′, 3′—$(CH_3)_2$.

TABLE 2

Values of apparent partition coefficients of N-phenylanthranilic acid derivatives

Substituent	Apparent partition coefficient (log *Q*)		
	n-Heptane–H_2O (pH 7.0)	*n*-Hexane–H_2O (pH 7.5)	Chloroform–H_2O (pH 7.0)
3′-H (*N*-naphthylanthranilic acid)	−1.025	−2.000	0.651
3′-CF_3	−0.530	−0.959	1.019
3′-Cl	−0.721	−1.301	
3′-CH_3	−0.700	−1.222	1.017
3′-NH_2	−1.425	−2.301	−0.719
3′-NO_2	−1.393	−2.301	0.492
3′-SO_3H	−1.309	−2.000	−1.319
3′-$N(CH_3)_2$	−1.008	−1.398	0.740
3′-OH	−1.054	−2.000	−1.176
3′-$COCH_3$	−1.275	−2.000	0.553
3′-OCH_3		−1.699	
N-Naphthyl	−0.090	−0.538	
3′-C_6H_5	0.451	0.086	
2′,3′-$(CH_3)_2$		−0.745	1.043

The values of *Q* obtained with both the *n*-heptane–H_2O and *n*-hexane–H_2O systems were constant at various concentrations of the ionized forms of all the acid derivatives examined in the aqueous phase. On the other hand, in the chloroform–H_2O system, the value of *Q* increased linearly with the concentration of the ionized form of acid in the aqueous phase (Fig. 3). Equation 2 suggests that *N*-phenylanthranilic acid and some of its derivatives with —CF_3, —CH_3, or –NO_2 at position 3′ or 2′ may associate in chloroform, but not in *n*-heptane or *n*-hexane. The true partition coefficients and association coefficients of these acid deriva-

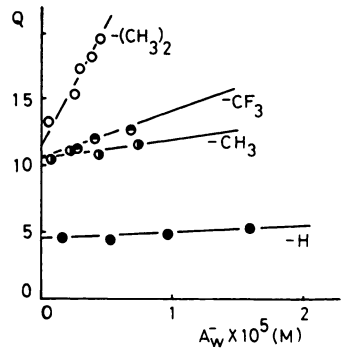


FIG. 3. *Relationship between apparent partition coefficient (Q) and concentration of ionized forms in the aqueous phase ($\bar{A_w}$)*

Q was obtained from the chloroform–H_2O system at 25° and pH 7.0. —H, *N*-phenylanthranilic acid; —CF_3, flufenamic acid; —$(CH_3)_2$, mefenamic acid; —CH_3, *N*-(3′-methylphenyl)-anthranilic acid.

TABLE 3

Physicochemical properties of N-phenylanthranilic acid derivatives

Substituent	Melting point	pK	True partition coefficient (log Q_0)		
			n-Heptane–H_2O	*n*-Hexane–H_2O	Chloroform –H_2O
3′-H (*N*-phenylanthranilic acid)	183°[a]	5.28	0.703	0.123	2.377
3′-CF_3	125°	5.84	0.659	0.613	2.179
3′-Cl	167°	5.77	0.534	0.339	
3′-CH_3	139°	5.82	0.507	0.370	2.200
3′-NH_2	165°[a]	4.72	0.857	0.380	1.562
3′-NO_2	218°	5.34	0.277	−0.239	2.152
3′-SO_3H		4.31	1.382	1.090	1.371
3′-$N(CH_3)_2$	155°	5.83	0.190	0.184	1.910
3′-OH	170–171.5°	4.65	1.298	1.450	1.076
3′-$COCH_3$	166°	5.05	0.680	0.352	2.504
3′-OCH_3		5.17		0.633	
N-Naphthyl	204–206°	6.40	0.607	0.504	
3′-C_6H_5	156–157°	7.00	0.752	0.632	
2′,3′-$(CH_3)_2$	230–231°	5.80		0.866	2.243

[a] Decomposition occurred.

TABLE 4

Association constants of N-phenylanthranilic acid derivatives in chloroform at 25° and pH 7.0

Substituent	Association constant (K_d)
	M
3′-H (*N*-phenylanthranilic acid)	1.02×10^3
3′-CF_3	1.77×10^3
3′-CH_3	0.75×10^3
3′-NO_2	0.35×10^3
2′,3′-$(CH_3)_2$	8.01×10^3

tives, evaluated from Eq. 2, are summarized in Tables 3 and 4, respectively. The *N*-phenylanthranilic acid derivatives were lipophilic in the un-ionized form, as shown clearly in the chloroform–H_2O system. It is also interesting that the association constants ranged from 3.5×10^2 to 8.0×10^3 M, indicating that a significant proportion of these acids was associated in chloroform (Table 4).

Correlations of uncoupling activity with physicochemical properties of N-phenylanthranilates. Comparison of the reciprocals of the concentrations of uncouplers ($1/C_u$) giving maximum uncoupling activity, with

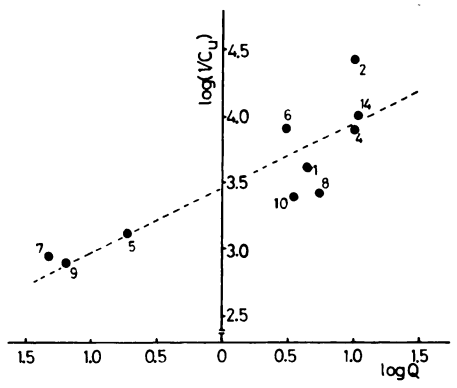


FIG. 4. *Relationship between uncoupling activity (log $1/C_u$) and apparent partition coefficient (log Q) of N-phenylanthranilic acid derivatives*

Log *Q* was obtained in the chloroform–H_2O system at 25° and pH 7.0. Numbers adjacent to the points represent *N*-phenylanthranilic acid derivatives: *1*, 3′-H (*N*-phenylanthranilic acid); *2*, 3′-CF_3; *4*, 3′-CH_3; *5*, 3′-NH_2; *6*, 3′-NO_2; *7*, 3′-SO_3H; *8*, 3′-$N(CH_3)_2$; *9*, 3′-OH; *10*, 3′-$COCH_3$; *14*, 2′,3′-$(CH_3)_2$. The values for log Q to the left of zero are negative.

their partition coefficients, showed that uncoupling activity increased with increases in the apparent partition coefficients (*Q*). The correlations differed with the particular organic solvent used. The correlation be-

tween log $(1/C_u)$ and log Q in the chloroform–H_2O system was especially close (Fig. 4). The magnitude of the uncoupling activity of *N*-phenylanthranilates is linearly related to their hydrophobic nature, as described by

$$\log\left(\frac{1}{C_u}\right) = 0.461 \log Q + 3.456 \quad (4)$$
$$(n = 10, r = 0.8551, s = 0.2792)$$

Eq. 4, which was calculated by the least-squares method; n is the number of compounds tested, r is the correlation coefficient, and s is the standard deviation.

The electronic properties of the carboxyl groups of the acids (ionization constants) (20) were then examined. Plots of the pK values of *N*-phenylanthranilic acid derivatives against their uncoupling activities revealed a linear relationship (Fig. 5); the correlations are expressed by Eq. 5.

$$\log\left(\frac{1}{C_u}\right) = 0.586pK + 0.494 \quad (5)$$
$$(n = 13, r = 0.8076, s = 0.2928)$$

Figure 5 shows that the uncoupling activities of the acid derivatives were greater when the pK values were high.

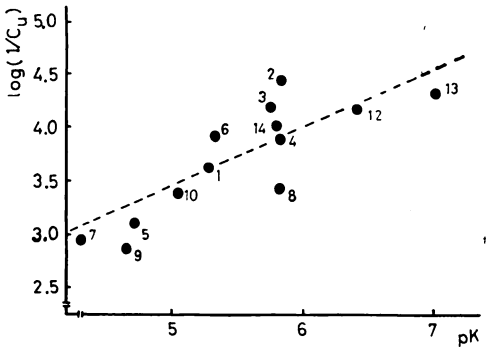


FIG. 5. *Relationship between uncoupling activity (log $1/C_u$) and pK value of N-phenylanthranilic acid derivatives*

Numbers adjacent to the points represent *N*-phenylanthranilic acid derivatives: *5*, 3′-Cl; *12*, *N*-naphthyl; *13*, 3′-C_6H_5. The others were the same as in Fig. 4.

DISCUSSION

Flufenamic acid is known to be a potent analgesic, antipyretic, and anti-inflammatory agent *in vivo* (8, 9). It also stabilizes lysosomal membranes (21), uncouples respiratory-chain phosphorylation (4), and inhibits hemolysis of erythrocytes (22, 23) and heat denaturation of serum albumin (24) *in vitro*. However, it is difficult to understand the mechanisms of all its actions either *in vivo* or *in vitro*; moreover, other nonsteroidal anti-inflammatory agents, such as salicylate and phenylbutazone, have similar effects (3). Therefore it seemed important to investigate the effects of flufenamic acid and its derivatives on isolated mitochondria as a model of interaction between drugs and organized biopolymers with a distinct biochemical function.

It is well known that since the mitochondrial membrane has an extremely high lipid content and is not attacked by proteolytic enzymes, such as Nagarse (25), it possesses a hydrophobic character. This is supported by reports that cations, such as K^+ and Na^+, do not move across the membrane appreciably in the absence of valinomycin (7), and that the uncoupling activity of alkyl-substituted nitrophenols rises with increases in the hydrophobic nature of these derivatives (26). The latter observation was confirmed by the present finding that derivatives with hydrophobic groups in position 3′ of *N*-phenylanthranilic acid possessed high uncoupling activity.

Some organic solvents have been used as models of biological membranes because of their hydrophobic nature, but there is no sound theoretical basis for their selection. 1-Octanol (20, 27), 2-butanol (28), xylene (26), and chloroform (29, 30) are frequently used for this purpose, since the physicochemical properties of certain compounds, such as lipid solubility, sometimes correlate well with their relative biological activities in these organic solvents. It is interesting that, in this study on the structure-activity relationships of *N*-phenylanthranilates, the best correlation was observed in chloroform–H_2O. Partition experiments demonstrated that only the unionized form of the acid penetrates into the oily phase, where it exists as a monomer or dimer.

Some *N*-phenylanthranilic acid derivatives seemed to be present as dimers in

chloroform, but not in *n*-heptane or *n*-hexane. Furthermore, the correlation between uncoupling activity and partition coefficient was closest in the chloroform–H_2O system, and acids that formed dimers in chloroform were highly lipophilic and exhibited potent uncoupling activities (Table 3). Therefore the dimeric form of the acids may be important for their uncoupling activities. It is not clear at present whether the dimer itself is necessary for uncoupling, or whether the basic properties required for dimer formation are also responsible for interaction with the coupling site on the mitochondrial membrane. The former explanation was suggested by Finkelstein (31), who proposed that a charged dimer transports hydrogen ions across the mitochondrial membrane, (as discussed below concerning Mitchell's hypothesis). The latter alternative is based on the concept of Weinbach and Garbus (32), who suggested that binding of an uncoupler molecule with specific sites of the energy-conservation system results in uncoupling of respiratory-chain phosphorylation.

On a theoretical basis it is reasonable that the apparent partition coefficient Q varies with the pH of the aqueous phase, since the concentration of an acidic compound in H_2O is the sum of the concentrations of its ionized and un-ionized forms. On the other hand, the true partition coefficient Q_0, which is a physicochemical constant of the acid, is constant irrespective of the pH value. As mentioned above, it is assumed that the ionized form of an acid does not penetrate into an oily phase. If so, it seems better to use Q_0 rather than Q as a physicochemical parameter, as pointed out by Fujita *et al.* (33). Figure 6 illustrates the relationship between the true partition coefficients of the *N*-phenylanthranilic acid derivatives in the chloroform–H_2O system and their uncoupling activities. The straight, dashed line indicates the correlation calculated by the least-squares method. This correlation is expressed by Eq. 6, which indicates that uncoupling activity rises with increases in hydrophobic character.

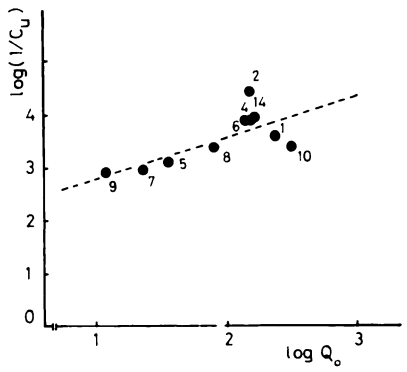


FIG. 6. *Relationship between uncoupling activity (log $1/C_u$) and true partition coefficient (log Q_0) of N-phenylanthranilic acid derivatives*

Values of log Q_0 were taken from the results of Table 3 (chloroform–H_2O system). Numbers adjacent to the points are the same as in Fig. 4.

$$\log\left(\frac{1}{C_u}\right) = 0.777 \log Q_0 + 2.043 \qquad (6)$$

$$(n = 10, r = 0.7226, s = 0.3689)$$

It is uncertain whether lipid solubility of the uncoupler is necessary for true uncoupling or for other rate-limiting processes, such as penetration across a membrane. According to Mitchell (7), weak acid uncouplers do not in themselves inhibit specific enzymatic reactions, but form lipid-soluble, proton donor-acceptor systems in the lipid phase of the mitochondrial membrane, which is normally impermeable to hydrogen ions. The presence of these compounds prevents formation of respiration-dependent pH gradients across the membrane by acting as H^+ carriers. This is supported by the results of experiments using an artificial bimolecular phospholipid membrane (34–36). An alternative mechanism, proposed by van Dam and Kraayenhof (5, 6), is that the ionized form of an acidic uncoupler enters the mitochondrion in exchange for endogenous anions, and that the uncoupler can leave the mitochondrion in an uncharged form. In this case, hydroxyl ions act as endogenous ions produced by energy-dependent hydrolysis of water. The lipid solubility of an uncoupler is accordingly related to the entry (Mitchell's hypothesis) or exit (van Dam and Kraayenhof's hypothesis) of the unionized form of an acidic uncoupling agent in the

hydrophobic region of the membrane. In addition, the hypothesis of van Dam and Kraayenhof suggests that the ionized form of the uncoupler molecule penetrates the mitochondrial membrane via an anion carrier common to the substrate anion. This is supported by the finding that 2,4-dinitrophenol (37–40) and flufenamic acid[1] competitively inhibit the penetration of succinate.

On the basis of the above hypotheses, the pK values of the acid uncoupler should influence the penetration of an uncoupler through a mitochondrial membrane, especially in the presence of anionic compounds such as substrates. Mitchell's hypothesis suggests that the uncoupler molecule penetrates the biomembrane in an un-ionized form, while according to van Dam and Kraayenhof the anionic form is translocated into the membrane via a substrate anion carrier. As shown in Fig. 5, the uncoupling activity of *N*-phenylanthranilates increased with decreases in dissociability at pH 7.4, which seems to be important both for entry (Mitchell) and for exit (van Dam and Kraayenhof) of the uncoupler molecule. The importance of relatively high pK values for the uncouplers was also emphasized by van Dam and Slater (41), whereas Parker (42) observed that uncoupling activity in a homologous series of phenols rose with decreases in pK values. Further study is necessary, using different series of acidic uncouplers under the same conditions, since it is unlikely that the actions of acidic uncouplers are based on different mechanisms.

A comparison of the role of two physicochemical parameters on the uncoupling activity is left for future investigation. However, the present report offers a good example in which five derivatives with similar ionization constants, of about 5.8 [i.e., flufenamic acid, mefenamic acid and the 3′-Cl-, 3′-CH_3-, and 3′-$N(CH_3)_2$-substituted acids], differed in uncoupling activity. Four of these compounds conferred hydrophobicity on uncoupling activity (the Q_0 value of the 3′-Cl homologue is unknown), as shown in Fig. 6, where uncoupling activity is seen to increase with the value of the partition coefficient.

[1] S. Muraoka, H. Terada, and S. Ikawa, unpublished observations.

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