**Acknowledgments.** We thank Mr. Robert B. Bicknell and his staff for the large-scale preparations; Dr. Peter Lim and his staff for the spectral data; and Dr. David W. Henry for helpful discussions.

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# Structure–Activity Relationships of Fenamic Acids†

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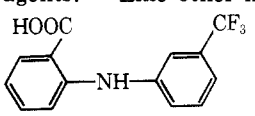
Physicochemical properties such as the dissociation constant in $H_2O$ and partition coefficient with the 1-octanol–$H_2O$ system have been determined for a set of 3′-substituted fenamic acids including flufenamic and mefenamic acids. It has been found that the log $K_A$ value is linearly related with the Hammett $\sigma$ constants and the $\Pi$ value of 3′ substituents is practically identical with those derived from substituted phenols. With the use of substituent parameters, $\sigma$ and $\Pi$, the BSA binding affinity and uncoupling activity of the acids have been analyzed. An important role of hydrophobicity is found for each of the activities. The uncoupling activity of fenamic acids is indicated to vary according to common features with other sets of uncouplers such as phenols, benzimidazoles, and methylenebisphenols, hydrophobically as well as electronically, suggesting that they interact quite similarly with the energy conservation mechanism of mitochondria.

Flufenamic acid [*N*-(3′-trifluoromethylphenyl)anthranilic acid] and mefenamic acid [*N*-(2′,3′-dimethylphenyl)anthranilic acid] are known to be potent analgesic and antiinflammatory agents.[1,2] Like other nonsteroidal anti-




flufenamic acid

inflammatory agents, these compounds have uncoupling activity of respiratory chain phosphorylation in isolated mitochondria.[3-7] Recently, we measured the uncoupling activity of a series of 3′-substituted fenamic acids (*N*-phenylanthranilic acids) with the use of rat liver mitochondria. We also determined partition coefficients with *n*-hexane–$H_2O$, *n*-heptane–$H_2O$, and chloroform–$H_2O$ systems, and the dissociation constant in acetone–$H_2O$, and attempted to elucidate their uncoupling activity in terms of these physicochemical properties. An important role of hydrophobic character in the activity of the molecules was found.[8]

In order to obtain more precise information on the biological activity of this class of compounds, the present study was undertaken to analyze the effect of 3′ substituents on uncoupling activity as well as binding affinity with serum albumin in terms of the electronic and hydrophobic substituent parameters, $\sigma$ and $\Pi$, which were evaluated from the dissociation constant in $H_2O$ and the partition coefficient with the 1-octanol–$H_2O$ system, respectively.

**Physicochemical Parameters.** The 14 fenamic acids used in this study are listed in Table I. Since the potentiometric titration cannot be applied due to their limited solubility, we determined the dissociation constant, $K_A$, by the "pH-dependent solubility method."[9] p$K_A$ values are listed in Table I. They are considerably different from corresponding values obtained by potentiometric titration in 5–10% aqueous acetone.[8] The 5–10% acetone may not be enough to solubilize the acids during titration. The present values are linearly related to the Hammett $\sigma$ constant of the 3′ substituent as shown in eq 1. In eq 1, $n$ is the number of acids studied, $s$ is the standard deviation, $r$ is the correlation coefficient, and the values in parentheses are 95% confidence intervals. The p$K_A$ values for some derivatives which were not determined experimentally are calculated from eq 1 by means of $\sigma$ constants.

$$\log K_A = 0.738\sigma\ (\pm 0.388) - 4.144\ (\pm 0.097) \qquad (1)$$

| $n$ | $s$ | $r$ |
|---|---|---|
| 6 | 0.076 | 0.935 |

The apparent partition coefficient, $P'$, of fenamic acids was measured with the 1-octanol–phosphate buffer system. The partition coefficient, $P$, for the neutral form was then calculated by eq 2. The $P$ value was also determined directly for some acids by equilibrating the 1-octanol solution with $10^{-2}$ $N$ HCl. The log $P'$ and log $P$ values are listed in Table I. The coincidence of log $P$ values from

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**Table I.** Physicochemical Properties of Fenamic Acids

| Substituents | $pK_A$[a] | $\sigma$[b] | Log $P'$[c] | Log $P$[d] I | Log $P$[d] II | $\Pi$[d] I | $\Pi$[d] II | $\Pi_{phenol}$[e] |
|---|---|---|---|---|---|---|---|---|
| H | 4.15 | 0.00 | 1.54 | 4.39 | 4.36 | 0.00 | 0.00 | 0.00 |
| 3′-$CF_3$ | 3.85 | 0.43 | 1.74* | 5.89 | 5.62 | 1.50 | 1.26 | 1.49 |
| 2′,3′-$(CH_3)_2$ | 4.33* | −0.24 | 2.37 | 5.04 | 5.37 | 0.65 | 1.01 | 1.04 |
| 3′-Cl | 3.86* | 0.37 | 2.43 | 5.57 | | 1.18 | | 1.04 |
| 3′-$CH_3$ | 4.15 | −0.07 | 1.95 | 4.80 | 4.88 | 0.41 | 0.52 | 0.56 |
| 3′-$NH_2$ | 4.35 | −0.16 | 0.34 | 2.99 | | −1.40 | | −1.29 |
| 3′-$NO_2$ | 3.59* | 0.71 | 1.30 | 4.71 | 4.57 | 0.32 | 0.21 | 0.54 |
| 3′-OH | 4.05* | 0.12 | 0.81 | 3.76 | 3.49 | −0.63 | −0.87 | −0.66 |
| 3′-$OCH_3$ | 3.95 | 0.12 | 1.41 | 4.56 | | 0.17 | | 0.12 |
| 3′-$COCH_3$ | 3.90 | 0.38 | 1.21 | 4.31 | | −0.08 | | −0.07 |
| 3′-$N(CH_3)_2$ | 4.31* | −0.21 | | | | | | 0.10 |
| 3′-$COC_6H_5$ | 3.87* | 0.35 | | | | | | 1.56[f] |
| 3′-$C_6H_5$ | 4.10* | 0.06 | | | | | | 1.89[g] |
| 2′,3′-$(CH)_4$ | 4.11* | 0.04 | | | | | | 1.24[g] |

[a]The values with an asterisk are estimated from eq 1 with the $\sigma$ value. Mean deviation of the values is ±0.06. [b]Taken from D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23,** 420 (1958), except the value of 3′-$COC_6H_5$, which is taken from W. N. White, R. N. Schlitt, and D. Gwyn, *ibid.*, **26,** 3613 (1961). [c]The $P'$ values are determined with pH 7.0 buffer except for the one with an asterisk with pH 8.0 buffer. Mean deviation of the values is ±0.08. [d]The values of series I are estimated from eq 2; those of series II are directly determined. Mean deviation of the values in series II is ±0.10. [e]Taken from ref 10. [f]Estimated as follows: $\Pi$(*m*-$COCH_3$ in phenol) − $\Pi(-CH_3)$ + $\Pi(-C_6H_5)$ = −0.07 − 0.50 + 2.13 = 1.56. [g]Values from substituted phenoxyacetic acids.

**Table II.** Biological Activity of Fenamic Acids

| Substituents | BSA binding, log $K$ Obsd[a] | BSA binding, log $K$ Calcd[b] | Uncoupling, log $1/C$; mitochondria from Liver Obsd[c] | Liver Calcd[d] | Heart Obsd[c] | Heart Calcd[e] |
|---|---|---|---|---|---|---|
| H | 5.65 | 5.59 | 3.62 | 3.53 | 4.18 | 4.32 |
| 3′-$CF_3$ | 5.83 | 5.79 | 4.44 | 4.29 | 5.30 | 5.06 |
| 2′,3′-$(CH_3)_2$ | 5.71 | 5.73 | 4.00 | 4.06 | 5.00 | 4.84 |
| 3′-Cl | | | 4.19 | 4.06 | 4.82 | 4.84 |
| 3′-$CH_3$ | 5.68 | 5.67 | 3.89 | 3.82 | 4.33 | 4.60 |
| 3′-$NH_2$ | 5.39 | 5.41 | 3.10 | 2.88 | 3.70 | 3.68 |
| 3′-$NO_2$ | 5.60 | 5.66 | 3.92 | 3.81 | 5.00 | 4.59 |
| 3′-OH | 5.56 | 5.50 | 2.89 | 3.20 | | |
| 3′-$OCH_3$ | | | 3.62 | 3.59 | 4.22 | 4.38 |
| 3′-$COCH_3$ | 5.52 | 5.58 | 3.39 | 3.50 | | |
| 3′-$N(CH_3)_2$ | | | 3.42 | 3.58 | | |
| 3′-$COC_6H_5$ | | | | | 5.10 | 5.10 |
| 3′-$C_6H_5$ | | | 4.32 | 4.49 | 4.89 | 5.26 |
| 2′,3′-$(CH)_4$ | | | 4.17 | 4.16 | 5.08 | 4.94 |

[a]Mean deviation of $K$ values is ±1.5. [b]By eq 4. [c]Mean deviation of $C$ values is ±0.2. [d]By eq 5. [e]By eq 6.

two sets of experiments is excellent considering the difficulty involved in determining very low concentration of acids in the aqueous phase. The result also indicates the reliability of the presently determined $K_A$ values.

$$\log P = \log P' + \log ([H^+] + K_A)/[H^+] \simeq \log P' - pK_A + pH \quad (2)$$

Values of the hydrophobic substituent constant, $\Pi$, were calculated from two sets of log $P$ values. They are very close to the values from those of substituted phenols, $\Pi_{phenol}$.[10]

As $\Pi$ is an additive-constitutive parameter, an estimation of log $P$ of fenamic acid (FAA) could be based on the log $P$ of anthranilic acid (AA)[10,11] where a similar intramolecular hydrogen bond formation is expected as shown in eq 3. The experimentally obtained log $P$(FAA), 4.36, is about one log unit larger than the estimated value, 3.34. The difference could be due to a shielding of lone-pair electrons on the nitrogen from hydrogen bond formation with $H_2O$ molecules by two bulky benzene rings.

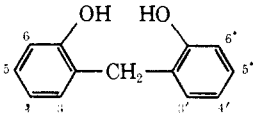
$$\log P(\text{FAA}) = \log P(\text{AA}) + \log P(\text{benzene}) = 1.21 + 2.13 = 3.34 \quad (3)$$

In the following sections, we analyze biological activities with substituent constants by multiple regression analysis. The addition of a $\Pi^2$ term does not improve the correlation in any of the analyses. Unless otherwise noted, the $\Pi_{phenol}$ is used for $\Pi$ values. Since the $pK_A$ value of this set of compounds is around 3.5–4.5, they exist exclusively as the anionic form under physiological conditions. In this situation, the effect of dissociation need not be considered as far as the activity in terms of the anionic form is concerned.

## Results

The binding behavior of this class of compounds with bovine serum albumin (BSA) obeys the Langmuir's isotherm. The equilibrium constant of the isotherm, $K$, at pH 7.0 of eight compounds in Table II was analyzed using $\sigma$ and $\Pi$ parameters singly and together. The best correlation is eq 4; $\sigma$ was not correlated with log $K$, and the addition of $\sigma$ term does not improve the correlation with $\Pi$. The variation in the binding affinity is determined solely by the hydrophobicity of 3′ substituents. Linear relationships between BSA binding constants and hydrophobic parameters such as eq 4 have been observed for other sets of congeners.[11-13] In most cases, the slope associated with $\Pi$ or log $P$ is 0.6 ± 0.1. In the present case, the slope is considerably smaller (0.14). The binding affinity of this class of compounds seems to be determined mostly by the anthranilic acid moiety and the 3′ substituents may not

**Table III.** Physicochemical Properties and Uncoupling Activity of Methylenebisphenols



| Substituent | p$K_A$[a] | $\Delta \log K_A$ | Log $P_{hexane}$[b] | Log $P_{octanol}$[c] | Log $1/C$ + log $(K_A + [H^+]^d)/[H^+]$ Obsd[e] | Calcd |
|---|---|---|---|---|---|---|
| H | 10.00 | 0.00 | −0.40 | 1.95 | 4.80 | 4.53 |
| 4,4′-$Cl_2$ | 9.20 | 0.80 | 0.70 | 3.51 | 5.11 | 5.29 |
| 3,3′,4,4′-$Cl_4$ | 8.10 | 1.90 | 0.72 | 4.15 | 5.74 | 6.07 |
| 4,4′,6,6′-$Cl_4$ | 6.95 | 3.05 | 1.54 | 5.63 | 6.82 | 7.02 |
| 3,3′,5,5′-$Cl_4$ | 7.60 | 2.40 | 0.82 | 4.54 | 6.62 | 6.44 |
| 3,3′,4,6,6′-$Cl_5$ | 6.05 | 3.95 | 2.86 | 7.45 | 7.97 | 7.88 |
| 3,3′,4,4′,6,6′-$Cl_6$ | 5.65 | 4.35 | 2.34 | 7.16 | 8.27 | 8.07 |
| 1,3,4,4′,6,6′-$Cl_6$[f] | 8.55 | 1.45 | 1.88 | 5.11 | 5.99 | 5.96 |
| $Cl_5$-$C_6H_5OH$ | 4.80[g] | 5.20 | | 5.12[h] | 8.12 | 8.23 |
| 2,4-$(NO_2)_2$-$C_6H_5OH$ | 4.11[g] | 5.89 | | 1.51[h] | 8.07 | 8.03 |

[a]The p$K_A$ values are kindly supplied by Dr. H. S. Nakaue, Oregon State University. [b]Recalculated from $P'$ values with pH 6.5 buffer in ref 30. [c]Estimated by an equation derived by Leo and coworkers;[11] log $P_{octanol}$ = log $P_{cyclohexane}$ + 1.20 log $K_{HB}$ + 2.35, replacing log $P_{cyclohexane}$ and log $K_{HB}$ (hydrogen bonding parameter) defined by T. Higuchi, J. H. Richards, S. S. Davis, A. Kamada, J. P. Hou, M. Nakano, M. I. Nakano, and I. H. Pitman, *J. Pharm. Sci.*, **58**, 661 (1969), with log $P_{hexane}$ and $\Delta \log K_A/2.11$, respectively. For meta- and para-substituted phenols, log $K_{HB}$ and $\Delta \log K_A$ are expressed with $\sigma^-$ values as log $K_{HB}$ = $0.941\sigma^-$ (±0.195) + 0.101 (±0.122), $n$ = 14, $s$ = 0.171, $r$ = 0.950, and $\Delta \log K_A = 2.11\sigma^-$ [H. H. Jaffè, *Chem. Rev.*, **53**, 191 (1953)], respectively. The values of log $K_A$ of unsubstituted phenol and methylenebisphenol are almost identical and the relation log $K_{HB} \doteqdot \Delta \log K_A/2.11$ derived from the above two equations is assumed to hold even for ortho-substituted derivatives of phenol and methylenebisphenol. [d][$H^+$] = $10^{-7.4}$. [e]From ref 30. [f]2,3,3′,5,5′,6-Hexachloro-2′,4-dihydroxydiphenylmethane. [g]From ref 31. [h]From ref 11.

be bound so tightly as to be embedded completely into the hydrophobic region of BSA.

$$\log K = 5.590\ (\pm 0.048) + 0.137\Pi\ (\pm 0.055) \quad (4)$$

$n$ = 8, $s$ = 0.054, $r$ = 0.927

The uncoupling activities in Table II were analyzed to yield eq 5 and 6, respectively. For liver mitochondria

$$\log 1/C = 3.533\ (\pm 0.110) + 0.507\Pi\ (\pm 0.115) \quad (5)$$

$n$ = 13, $s$ = 0.160, $r$ = 0.947

For heart mitochondria

$$\log 1/C = 4.323\ (\pm 0.219) + 0.497\Pi\ (\pm 0.192) \quad (6)$$

$n$ = 11, $s$ = 0.242, $r$ = 0.890

$\sigma$ was not significantly correlated with log $1/C$. Correlation observed in the two parameter equations is not improved over that of eq 5 and 6, respectively. The slope of the $\Pi$ term is not significantly different in eq 5 and 6; this indicates that the mitochondria from two different tissues are susceptible to the hydrophobic nature of uncouplers to almost the same extent. The difference in the intercepts between two equations shows that the heart mitochondria are approximately six times more sensitive to any one of the uncouplers than the liver mitochondria. This might be ascribed to a difference in the chemical constitution between mitochondrial membranes from two sources.

## Discussion

Nonsteroidal antiinflammatory drugs have been known to exhibit various biochemical and physiological effects. Among examples at the subcellular level are the uncoupling with the mitochondrial respiratory chain phosphorylation[3-7] and the stabilization of lysosomal membranes in polymorphonuclear leucocytes.[14] They also stabilize the erythrocyte membrane against hemolysis[15,16] and the serum albumin structure against heat denaturation[17] and tryptic digestion.[18] The stabilizing effects on protein and membrane structure have been considered to be due to a potent interaction of drugs with proteins where an electrostatic attraction between anionic drugs and positively charged groups in proteins as well as the hydrophobic bonding between hydrophobic counterparts of partners may play a role.[19] In addition, it was recently reported that some nonsteroidal drugs including flufenamic acid and mefenamic acid inhibit the biosynthesis of prostaglandin.[20-23]

The present result expressed as eq 4 shows that, within the set of fenamate anions, the most fundamental factor enhancing the BSA binding is the hydrophobicity of substituents. However, if the drugs are bound as the neutral form which exists in the dissociation equilibrium, the binding constant, $K_n$, should be expressed as eq 7.[24] Substitution of eq 1 and 4 into eq 7 yields eq 8. Here, the hydrophobicity as well as the electron-withdrawing ability of 3′ substituents contributes significantly to the binding affinity.

$$\log K_n = \log K + \log (K_A + [H^+])/[H^+] \simeq \log K + \log K_A - \log [H^+] \quad (7)$$

$$= 8.446 + 0.738\sigma + 0.137\Pi \quad (8)$$

It might be unrealistic to consider the binding affinity of the neutral form of such a low concentration under the present experimental conditions. Recently, Chignell has suggested from the extrinsic Cotton effect generated by the binding with human serum albumin that the substituted aromatic moiety of fenamic acids is inserted into a hydrophobic crevice of the protein, while the carboxylate anion interacts with a cationic site of the protein surface.[25] However, from their data, as well as the present binding constants determined at a single pH, we should refrain from concluding which is responsible for the binding, the neutral or the anionic form or both.

There have been some studies which indicate a close relationship between binding affinity with BSA and uncoupling activity of antiinflammatory drugs including 2,4-dinitrophenol and its analogs. Whitehouse reported the case for this series of compounds.[6] Weinbach and Garbus

**Table IV.** Solubility of Fenamic Acids (in mol/l.)

| Substituents | Solubility | Substituents | Solubility |
|---|---|---|---|
| H | $1.1 \times 10^{-5}$ | 2′,3′-$(CH_3)_2$ | $<10^{-6}$ |
| 3′-$CF_3$ | $4.6 \times 10^{-6}$ | 3′-OH | $2.9 \times 10^{-4}$ |
| 3′-Cl | $5.0 \times 10^{-6}$ | 3′-$OCH_3$ | $1.57 \times 10^{-5}$ |
| 3′-$CH_3$ | $8.8 \times 10^{-6}$ | 3′-$COCH_3$ | $1.38 \times 10^{-5}$ |
| 3′-$NH_2$ | $7.4\text{–}9.0 \times 10^{-5}$ | 3′-$COC_6H_5$ | $<10^{-6}$ |
| 3′-$NO_2$ | $3.7 \times 10^{-7}$ | 2′,3′-$(CH)_4$ | $<10^{-6}$ |

proposed that the binding of uncoupling phenols with mitochondrial protein is essential for their activity.[26,27] Hansch and coworkers demonstrated that the binding affinities of phenols with BSA and mitochondrial protein are linearly related to the hydrophobic parameter, Π, of aromatic substituents.[28]

The present result would suggest that apparent relationships between protein binding and the uncoupling effect of antiinflammatory drugs, if any, are also mediated by the hydrophobicity of drugs. The greater the hydrophobic nature of the drugs, the greater the affinity with protein and uncoupling activity become although the susceptibility is different in each case.

Similar analyses have been performed for other series of uncouplers by Hansch.[29] For the uncoupling activity of a number of substituted phenols in terms of reciprocal of the concentration required for the complete uncoupling with rat liver mitochondria, eq 9 was derived. The activity of substituted trifluoromethylbenzimidazoles determined by the concentration required for 50% uncoupling with rat liver mitochondria was similarly analyzed to give eq 10. For a set of compounds including methylenebisphenols, pentachlorophenol, and 2,4-dinitrophenol, the 50% uncoupling activity was determined by Nakaue and associates with the use of rat liver mitochondria.[30] We have analyzed their data shown in Table III to obtain eq 11. For phenols (at pH 7.4)

$$\log 1/C + \log (K_A + [H^+])/[H^+] = 0.286 \log P + 0.906\ \Delta \log K_A + 1.170 \quad (9)$$

$n$ = 43, $s$ = 0.559, $r$ = 0.960

For trifluoromethylbenzimidazoles (at pH 7.4)

$$\log 1/C + \log (K_A + [H^+])/[H^+] = 0.462 \log P + 0.809\ \Delta \log K_A + 2.903 \quad (10)$$

$n$ = 22, $s$ = 0.425, $r$ = 0.963

For methylenebisphenols (at pH 7.4)

$$\log 1/C + \log (K_A + [H^+])/[H^+] = 0.173\ (\pm 0.093) \log P + 0.606\ (\pm 0.094)\ \Delta \log K_A + 4.194\ (\pm 0.476) \quad (11)$$

$n$ = 10, $s$ = 0.222, $r$ = 0.989

In these equations, $\Delta \log K_A$ is defined as $\log K_A - \log K_{A,H}$. $K_A$ and $K_{A,H}$ are the dissociation constants of the substituted and unsubstituted compounds, respectively. Since the $pK_A$ value of compounds in these series varies considerably at both sides of the experimental pH value, the effect of dissociation should be separated from the other effects.[31] Thus, these equations are expressed in terms of uncoupling activity of the neutral form. Equations 5 and 6 are those which correlate the activity of fenamate anions. Since the comparison should be made at the same standard, eq 5 and 6 are modified to eq 12 and 13 with $\log P$ and $\Delta \log K_A$ in terms of the neutral form. For fenamic acids with rat liver mitochondria

$$\log 1/C + \log (K_A + [H^+])/[H^+] = 0.507 \log P + \Delta \log K_A + 4.573 \quad (12)$$

For fenamic acids with rat heart mitochondria

$$\log 1/C + \log (K_A + [H^+])/[H^+] = 0.497 \log P + \Delta \log K_A + 5.393 \quad (13)$$

It is immediately apparent that the slopes in these equations associated with $\log P$ and $\Delta \log K_A$ terms are close to each other. Thus, regardless of which form is responsible for the uncoupling activity, phenols, trifluoromethylbenzimidazoles, methylenebisphenols, and fenamic acids can be considered as belonging to the same type of uncouplers and their mechanisms of action are quite similar. The intercept varies according to the compound series. This is partly due to a difference in the definition of $\log 1/C$, *i.e.*, the $\log 1/C$ value for 50% uncoupling is larger than that for the complete uncoupling. Besides this, the intercept should be related to the intrinsic activity of each pharmacophoric structure. If the values of intercept for various sets of uncouplers could be elucidated in physicochemical terms, they should provide valuable information as to structural requirements for lead compounds from which to start designing of novel potent uncouplers. Thus, the analyses of this type should be made with the use of data obtained under standard experimental conditions for more and more sets of uncouplers to accumulate the intercept values. Since the mechanism of antiinflammatory activity of fenamic acids at the ultimate site of action has not been definitely elucidated, it remains to be shown whether the activity has a common basis not only physiologically but also physicochemically with the uncoupling effect.

## Experimental Section

3′-Substituted fenamic acids used in this study were kindly supplied by Drs. Shunichi Ikawa and Eiichi Fujihira of Taisho Pharmaceutical Co., Tokyo.

**Solubility and Dissociation Constant.** Solubility of the compounds in $H_2O$ was measured at 25 ± 0.1° at various pH points between 2 and 6. pH values were adjusted with sodium acetate and acetic acid and the ionic strength was maintained at 0.11 by adding NaCl. Buffer solution (50–100 ml of each) was saturated by continuous shaking with an excess of solid acid for at least 3 hr. It was then quickly filtered and the final pH values were checked with a Hitachi-Horiba pH meter/F-5. The concentration of the saturated solution was determined spectrophotometrically. In lower pH regions, $S$ is nearly constant. This value was taken as the solubility of the neutral molecular form of the acid, $S_0$. Values of $S_0$ are listed in Table IV. We determined the dissociation constant, $K_A$, with the use of eq 14 by means of a plot of $\log (S/S_0 - 1)$ against the final pH.

$$pK_A = pH - \log (S/S_0 - 1) \quad (14)$$

**Partition Coefficient.** The apparent partition coefficient, $P'$, of fenamic acids was determined with the 1-octanol–phosphate buffer system, the pH of aqueous phase being 7.0 or 8.0. Each acid was dissolved in 10 ml of a 0.1 mol/l. phosphate buffer and equilibrated with 1–10 ml of 1-octanol for at least 3 days at 25°. $P'$ was determined by eq 15, where $C$ and $V$ are the equilibrium concentration and volume of aqueous (subscript w) and organic (subscript o) phases, respectively. $C_i$ is the initial concentration in the aqueous phase. The $P'$ value was determined with various values of $C_i$, $10^{-5}$–$10^{-4}$ mol/l. In every case, $P'$ was independent of the

$$P' = (C_i - C_w)V_w/C_wV_o \quad (15)$$

conditions. The true partition coefficient, $P$, was measured by equilibrating a 1-octanol solution of the acids, 1–2 ml, the initial concentration, $C_0$, of which is $10^{-3}$–$10^{-2}$ mol/l., with 50 ml of $10^{-2}$ $N$ HCl. Under this condition, acid molecules exist exclusively as the neutral form. After equilibrium was attained by shaking at least for 1 hr, concentration in the aqueous phase was measured and $P$ was determined by eq 16, since the concentration change in the organic phase during the partition process can be neglected for such highly lipophilic compounds. Under experimental conditions, the concentration in the aqueous phase was always lower than $10^{-6}$ mol/l. The concentration dependence of $P$ was not observed in any case.

$$P = C_o/C_w \tag{16}$$

In above described experiments, the concentration in the aqueous phase was determined spectrophotometrically. In the case where it was greater than $10^{-6}$ mol/l, 2.5 $N$ NaOH (3 ml) was added to the sample solution (3 ml) and the absorbance of this solution was measured at $\lambda_{max}$ (280–300 nm), since the absorbance was stable under alkaline conditions. For concentrations lower than $10^{-6}$ mol/l., we measured the difference in absorbance, $\Delta A$, between $\lambda_{max}$ and $\lambda_{trough}$ (300–320 nm), with the use of a two-wavelength spectrophotometer ($\Delta A$, 0.03 full scale).[32]

**BSA Binding.** Binding affinity with BSA was determined by measuring the ability to displace 2-(4′-hydroxyphenylazo)benzoic acid (HABA) competitively under conditions of pH 7.0 and 25°. BSA was fraction V of the Armour Co., Chicago. It was used without further purification. Its molecular weight was taken as 70,000. HABA was purchased from Wako Pure Chemical Industries, Osaka, and purified by three recrystallizations from methanol. Various amounts of each acid were added to 10 ml of a mixed solution of HABA ($10^{-4}$ mol/l). and BSA ($5 \times 10^{-5}$ mol/l.) so as to give final acid concentrations of $0.5–5 \times 10^{-4}$ mol/l/. and the metachromatized absorbance of HABA was measured at 475 nm. The absorbance was decreased with the increase in the acid concentration. From the variation in the HABA absorbance, we determined the binding constant, $K$, with BSA according to the method of Moriguchi.[33] The present $K$ value for flufenamic acid, $6.5 \times 10^5$ l. mol$^{-1}$, seems to conform to a value, $1.3 \times 10^6$ l. mol$^{-1}$, obtained by Chignell with human serum albumin at pH 7.4.[25]

**Uncoupling Activity.** The effect on the state 4 respiration of isolated rat heart mitochondria was measured in the absence of rotenone at pH 7.4. The mitochondria were isolated with the Nagarse method described by Tyler and Gonze.[34] The reaction mixture for mitochondrial respiration contained 10 mmol/l. of succinate, 50 mmol/l. of Tris–HCl buffer (pH 7.4), 180 mmol/l. of sucrose, 0.25 mmol/l. of EDTA, 10 mmol/l. of KCl, and 1.8 mg of mitochondria in a total volume of 3 ml. The rate of oxygen consumption was measured with a Galvani electrode by the method of Utsumi and associates[35] at 25° as described earlier.[8] The reciprocal of acid concentration required for the maximum respiration rate was taken as the uncoupling activity. The uncoupling data with rat liver mitochondria were taken from our earlier publication.[8]

**Acknowledgments.** The authors are indebted to Misses Masae Ikawa, Reiko Miyake, and Yuko Hamada for skillful technical assistance. They also thank Dr. H. S. Nakaue for providing the numerical values of p$K_A$ of methylenebisphenols.

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