Salicylate: A Structure-Activity Study of its Effects on Membrane Permeability

Abstract. *Salicylate and benzoate, and their analogs, reversibly increase the membrane potential and conductance of identified molluscan neurons by increasing potassium conductance and decreasing chloride conductance. The relative potencies of these compounds were closely correlated with their octanol-water partition coefficients and their* pK_a *values.*

The analgesic salicylate increases the membrane potential and conductance of identified invertebrate neurons by increasing potassium permeability and decreasing chloride permeability (*1*). We have suggested that similar ionic mechanisms might be involved in nonnarcotic analgesia. We have now related the physicochemical properties of salicylate and other aromatic monocarboxylic acids to their ability to alter the electrical properties of neuronal membranes. A high correlation was found between the ability of a compound to alter membrane potential and conductance and its lipid solubility.

Experiments were conducted on large identified neurons (*2*) in the buccal ganglion of the marine mollusk *Navanax inermis* (*3*). The ganglion was pinned to paraffin in a 5-ml Lucite chamber and bathed at room temperature (20° to 25°C) in a salt solution (*4*). The membrane potential and input resistance of the neurons were monitored with intracellular electrodes (*1*).

Since alterations in the membrane conductance to potassium and chloride are readily detected as membrane potential changes (*1*), we have used the latter as an easily measurable parameter to indicate the ability of a compound to alter membrane conductance. For convenience, this activity was evaluated relative to the membrane potential change produced by salicylate. The dose-response curves for salicylate and several analogs are shown in Fig. 1A. Approximately 10 m*M* salicylate was required to produce half the maximum increase in membrane potential. Therefore, the biologic activity of a compound was defined in terms of the concentration required to cause a change in membrane potential equivalent to that produced by 10 m*M* salicylate. For example, if 1 m*M* of a substance produced an increase in membrane potential equal to that produced by 10 m*M* salicylate, then the relative activity of that substance was ten times that of salicylate.

We have tested the ability of 37 aromatic acids (*5*) to alter neuronal membrane conductance and have quantitatively correlated their activity in this system with their pK_a values (describing the degree of dissociation of these compounds) and their octanol-water partition coefficients (indicating hydrophobicity) (Table 1 and Fig. 1B).

In Fig. 1B the relative biologic activity is plotted on a logarithmic scale as a function of the logarithm of the octanol-water partition coefficient *P*. The relative activity increases with increasing log *P* over a thousandfold range of activity and partition coefficient. A quantitative description of this relationship was obtained by applying the method of least squares to these data.

$$\log RA = 1.000 \log P - 2.440 \quad (1)$$

In this and subsequent equations, *RA* is the relative biologic activity. A statistical analysis of the data reveals the following: the number of data points used in the regression analysis, $n = 29$; the correlation coefficient, $r = .954$; the standard error of the estimate, $s = 0.250$; the *F* ratio derived from an analysis of variance of the data, with subscripts denoting degrees of freedom in numerator and denominator, $F_{1,27} = 274$, which is much greater than the *F* ratio at the .001 level of confidence (α), $F_{1,27\alpha} = 13.6$. A value of .954 for *r* indicates that Eq. 1 accounts for 91 percent ($r^2 = .910$) of the variability in the data. These results indicate that the ability of a compound to alter neuronal membrane permeability is highly correlated with its octanol-water partition coefficient.

An analysis in which the degree of dissociation of these organic acids is also considered improves the correlation between activity and physicochemical properties. The relative activity of these aromatic acids can be expressed as a function of both octanol-water partition coefficient and pK_a, as suggested by Hansch and his collaborators (*6*). Quadratic expressions relating these properties to activity were derived by the method of least squares for benzoates (Eq. 2) and salicylates (Eq. 3), where $\Delta pK_a = pK_a$ (aromatic acid) $- pK_a$(salicylate). [The statistical analysis

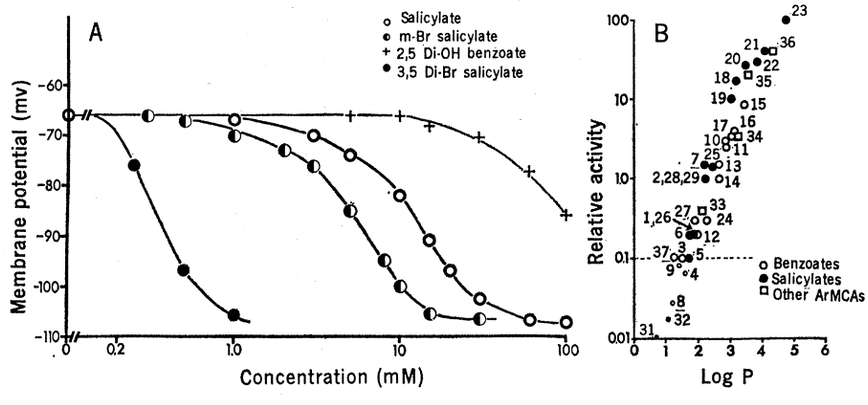

Fig. 1. (A) The dose-response curves for salicylate (open circles), 3,5 dibromosalicylate (filled circles), *m*-bromobenzoate (half-filled circles), and 2,5-dihydroxybenzoate (crosses) relate the increase in membrane potential to the concentration (logarithmic scale) of each substance added to the bathing medium. The concentration of each substance required to produce the same hyperpolarization as 10 m*M* salicylate indicated its relative activity. This was close to the concentration required to produce half the maximum response of 42 mv. Prior to the application of each drug the cell had a resting membrane potential of — 66 mv. Such dose-response curves are typical of all substances tested and listed in Table 1. (B) The logarithm of the relative biological activity of benzoate derivatives (open circles) and salicylate (*o*-hydroxybenzoate) derivatives (filled circles) is shown as a function of log *P*, where *P* is the octanol-water partition coefficient. Other active aromatic monocarboxylic acids (ArMCA's) (open squares) fall well into the general pattern described by benzoate and salicylate derivatives. The relative activity of compounds with less than one-tenth the potency of salicylate (dashed line indicating the limit of resolution of the system) were calculated from Eqs. 2 and 3. Their data points are represented by smaller symbols. Numbers adjacent to the data points refer to compounds listed in Table 1. The log *P* values are for the undissociated form of the molecules.

omits nitro-substituted derivatives from both groups (compounds 26 to 29).]

$$\log RA = -0.056(\log P)^2 + 1.166 \log P - 0.633\ \Delta pK_a - 1.935 \quad (2)$$

A statistical analysis yields: $n = 10$, $r = .992$, $s = 0.098$, $F_{3,6} = 122$, and $F_{3,6\alpha} = 23.7$ for $\alpha = .001$.

$$\log RA = -0.236(\log P)^2 + 2.422 \log P + 0.231\ \Delta pK_a - 4.133 \quad (3)$$

A statistical analysis yields: $n = 10$, $r = .992$, $s = 0.132$, $F_{3,6} = 128$, and $F_{3,6\alpha} = 23.7$ for $\alpha = .001$. Equations 2 and 3 account for 98.4 percent of the variability in the data ($r^2 = .984$). The fact that they adequately describe variations in activity suggests that both the solubility of an aromatic acid in the membrane (as reflected by its relative solubility in octanol and water) and the concentration of aromatic anions available (as reflected by its pK_a and its degree of dissociation at the *p*H used in these experiments) are the primary factors underlying ability to increase the membrane conductance to potassium and decrease conductance to chloride. The influence of steric factors is evidently minimal, which suggests that these drugs are not interacting with specific receptor sites on the membrane.

Table 1. The log *P*, pK_a, and relative activity of compounds tested. (*RA*) Relative ability of an aromatic acid to alter membrane permeability. Calculated values of log *RA* were obtained by using Eq. 2 (for benzoates) and Eq. 3 (salicylates). (Δ) Difference between the calculated and observed activity. (*P*) Octanol-water partition coefficient of undissociated acids, either obtained from the literature (*6*) or calculated (*) by summation of the substituent partition coefficients (*6*). (pK_a) Negative logarithm of the dissociation constant, either obtained from the *Handbook of Chemistry and Physics* (*16*) or, in the case of several salicylate derivatives (*), calculated by using the Hammett equation, $pK(\text{salicylate}) - pK(\text{salicylate derivative}) = (\sigma/\rho)(\text{salicylate})$, where σ is the Hammett sigma constant for substituents (*17*) and ρ (= 1.103) is the reaction constant for salicylates (*17*).

Number	Compound	log *P*	pK_a	RA_{obs}	log *RA*		
					Observed	Calculated	Δ
1	Benzoate	1.87	4.19	0.2	−0.70	−0.72	0.02
	Benzoate derivative						
2	*o*-OH	2.21	2.97	1.0	0.00	0.06	0.06
3	*m*-OH	1.50	4.06	0.1	−1.00	−1.00	0.00
4	*p*-OH	1.58	4.48	<0.1		−0.19	
5	2,4-di-OH	1.60*	4.70	0.1	−1.00	−0.52	0.48
6	2,5-di-OH	1.72*	2.97	0.2	−0.70	−0.65	0.05
7	2,6-di-OH	2.20	2.70	1.5	0.18	−0.00	0.18
8	3,4-di-OH	1.20*	4.48	<0.1		−1.57	
9	3,5-di-OH	1.40	4.04	<0.1		−1.09	
10	*m*-Br	2.84	3.86	3.0	0.48	0.36	0.12
11	*p*-Br	2.86	3.98	2.5	0.40	0.29	0.11
12	*o*-Cl	1.98	2.92	0.2	−0.70	−0.27	0.43
13	*m*-Cl	2.68	3.82	1.5	0.18	0.24	0.06
14	*p*-Cl	2.65	3.98	1.0	0.00	0.12	0.12
15	3,4-di-Cl	3.46*	3.66*	8.5	0.93	0.99	0.06
16	*m*-I	3.13	4.0	4.0	0.60	0.64	0.04
17	*p*-I	3.02	3.91	3.5	0.54	0.47	0.07
18	5-Br-2-OH	3.21*	2.62*	17.0	1.23	1.13	0.10
19	5-Cl-2-OH	3.02*	2.63*	10.0	1.00	0.95	0.05
20	5-I-2-OH	3.52*	2.65*	27.0	1.43	1.39	0.04
21	3,5-di-Br-2-OH	4.21*	2.26*	40.0	1.60	1.74	0.14
22	3,5-di-Cl-2-OH	3.88*	2.30*	30.0	1.48	1.57	0.09
23	3,5-di-I-2-OH	4.78*	2.33*	100.0	2.00	1.96	0.04
24	*p*-CH_3	2.27	4.36	0.3	−0.52	−0.47	0.05
25	5-OCH_3-2-OH	2.41*	2.86*	1.5	0.18	0.31	0.13
26	*m*-NO_2	1.83	3.47	0.2	−0.70	−0.30	0.40
27	*p*-NO_2	1.89	3.41	0.3	−0.52	−0.28	0.24
28	5-NO_2-2-OH	2.21*	2.32*	1.0	0.00	−0.08	0.08
29	3,5-di-NO_2-2-OH	2.21*	1.55*	1.0	0.00	−0.26	0.26
30	*o*-NH_2	1.21	6.97	<0.1		−3.14	
31	*p*-NH_2	0.68	4.92	<0.1		−2.42	
32	4-NH_2-2-OH	1.02*	3.57*	<0.1		−1.77	
33	Cinnamate	2.13	4.44	0.3	−0.04	−0.62	0.22
34	2-Naphthoate	3.25*	4.17	3.5	0.54	0.51	0.03
35	3-OH-2-Naphthoate	3.59*	2.80*	20.0	1.30	1.52	0.22
36	Mefenamate	4.34*	4.20	30.0	1.48	1.30	0.18
37	Acetylsalicylate	1.23	3.50	0.1	−1.00	−0.91	0.09

The increase in cation conductance and decrease in anion conductance resulting from adsorption of a negatively charged molecule to the membrane are consistent with current theories on mechanisms controlling membrane permeability to ions (*7*).

Equations 2 and 3 allow one to predict the relative activity of benzoate and salicylate derivatives from a knowledge of their pK_a's and octanol-water partition coefficients. On this basis one would predict that aspirin (acetylsalicylic acid), with a log *P* of 1.23 (*6*) and pK_a of 3.5, would be about 0.13 as active as salicylate (Eq. 2). Since aspirin was too insoluble to allow proper evaluation we used one of its stable salts, a mixture of calcium acetylsalicylate and urea (*8*). In this form we found aspirin to be 0.10 as active as salicylate (compound 37).

If one were interested in determining the activity of a substance whose partition coefficient had not been experimentally determined, its log *P* value could be estimated by algebraically summing the partition coefficients of the various substituents (*6*). This procedure was used to estimate the relative activity of three polycylic compounds, which are currently being studied as potentially useful analgesics: indomethecin (*9*), mefenamic acid (*10*), and naproxen (*11*). Mefenamate (*12*) was tested on these molluscan neurons and found to have about 30 times the potency of salicylate (Table 1). Using calculated log *P* values (*13*) and Eq. 2, we estimate that the relative activity of indomethecin and naproxen to mefenamate would be 8 : 0.8 : 1. This estimate compares favorably with the relative analgesic potency of these compounds, 8.6 : 1 : 1 (*11*), and thus lends support to the thesis (*1*) that the effects of these drugs on the molluscan neurons provide insight into the mechanisms underlying nonnarcotic analgesia. If the correlation between the relative activity of a substance in this model system and its relative analgesic activity is reliable over a wide range of log *P* values, it should be possible not only to predict the analgesic potency of as yet untested drugs, but also to design new nonnarcotic analgesics of any desired potency (*15*).

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References and Notes

1. J. L. Barker and H. Levitan, *Science* **172**, 1245 (1971).
2. H. Levitan, L. Tauc, J. P. Segundo, *J. Gen. Physiol.* **55**, 484 (1970).
3. Animals were obtained from the Pacific Bio-Marine Supply Co., Venice, California.
4. The salt solution consisted of: 500 m*M* NaCl, 1 m*M* KCl, 11 m*M* $CaCl_2$, 50 m*M* $MgCl_2$, and 10 m*M* tris(hydroxymethyl)aminomethane maleate buffered to *p*H 7.8 with NaOH.
5. The sodium salts of the acids were used because they were more soluble in the salt solution. When unavailable commercially, they were prepared by titrating the acid with NaOH in ethanol and then collecting the precipitate.
6. T. Fujita, J. Iwasa, C. Hansch, *J. Amer. Chem. Soc.* **86**, 5175 (1964); C. Hansch and S. M. Anderson, *J. Org. Chem.* **32**, 2583 (19(→ A. Leo, C. Hansch, D. Elkins, *Chem. Rev.* **71**, 525 (1971).
7. J. M. Diamond and E. M. Wright, *Annu. Rev. Physiol.* **31**, 581 (1969).
8. Calurin was provided by Dorsey Laboratories, Lincoln, Nebraska.
9. J. S. Fleming, M. E. Bierwagen, A. W. Pircio, M. H. Pindell, *Arch. Int. Pharmacodyn. Ther.* **178**, 423 (1969).
10. C. V. Winder, J. Wax, R. A. Scotti, E. M. Scherrer, E. M. Jones, F. W. Short, *J. Pharmacol. Exp. Ther.* **138**, 405 (1962).
11. A. P. Roszkowski, W. H. Rooks, A. J. Tomolonis, L. M. Miller, *ibid.* **179**, 114 (1971).
12. Mefenamic acid, sodium salt generously provided by Parke, Davis & Co., Detroit, Michigan.
13. We assume that these aromatic acids would behave more like benzoate than salicylate derivatives. The calculations of the log *P* values are as follows: log *P*(indomethacin) = π(indol) + π(benzaldehyde) + π(*p*Cl) + $2\pi(CH_2)$ + π(*m*-OCH_3) + π(COOH) = 5.82; log *P*(mefenamic acid) = π(anthranilic) + π(benzene) + $2\pi(CH_2)$ = 4.34; and log *P*(naproxen) = π(2-naphthalene) + $\pi(OCH_3)$ + $2\pi(CH_3)$ − π(branch) = 4.15. The pK_a's of indomethecin and naproxen in water are presumed equal to that of mefenamic acid [4.2 (*14*)]. The substituent constants (π) are from (*6*).
14. P. G. Stecher *et al.*, Eds., *The Merck Index* (Merck, Rahway, New Jersey, ed. 8, 1968), p. 648.
15. C. Hansch, in *Drug Design*, E. J. Ariens, Ed. (Academic Press, New York, 1971), vol. 1, p. 271.
16. R. C. Weast, Ed., *Handbook of Chemistry and Physics* (Chemical Rubber, Cleveland, ed. 52, 1971).
17. H. H. Jaffe, *Chem. Rev.* **53**, 191 (1953).

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Endosperm Protein Synthesis in Maize Mutants with Increased Lysine Content

Abstract. *The endosperm proteins of the maize mutants,* opaque-*2*, opaque-*7*, floury-*2*, brittle-*2, and the double mutant of* opaque-*2 and* brittle-*2, were separated into five soluble fractions by the Landry-Moureaux method. As compared to their isogenic normal counterparts, the mutant endosperms had higher concentrations of albumins, globulins, and glutelin-3, and lower concentrations of prolamines. The combination of the* opaque-*2 and* brittle-*2 genes enhanced this difference. Although the four mutant genes are located on three different chromosomes, they exert a similar effect on endosperm protein composition. Five other starch-modifying mutants with high lysine content resemble the* brittle-*2 mutant in endosperm protein composition, when the gene is present either singly or combined with* opaque-*2. Therefore, the pattern of protein synthesis in all maize mutants with high lysine concentrations may be either identical or very similar. Because no synergistic effect on lysine concentration is obtained when* floury-*2 is combined with* opaque-*2, different pathways leading to reduced zein synthesis may exist in the floury and starch-modifying mutants with high lysine concentrations.*

In 1964 Mertz *et al.* (*1*) reported that the *opaque*-2 (o_2) gene (chromosome 7) changed the protein composition and increased the lysine content of maize endosperm. Using a copper fractionation method (*2*), they found that the zein concentration of o_2 endosperm was lower, and the glutelin concentration was higher, than in normal endosperm. A second maize mutant with high lysine concentrations, *floury*-2 (fl_2) (chromosome 4), was identified and reported in 1965 (*3*). Recently, McWhirter (*4*) identified a third maize mutant with high lysine content, designated *opaque*-7 (o_7). This appeared as a spontaneous mutation in the inbred line W22; linkage studies (*4*) show it is located on chromosome 10.

In studies of starch-modifying mutant genes and their combinations with the o_2 gene, we found that the *sugary*-1 (su_1), *shrunken*-1 (sh_1), *shrunken*-2 (sh_2), *shrunken*-4 (sh_4), *brittle*-1 (bt_1), and *brittle*-2 (bt_2) genes increased the lysine content of the endosperm substantially above the isogenic normal control, and each gene had an enhanced effect on lysine when the gene was combined with o_2 (*5*). In addition, we found that the distribution of endosperm proteins in these six mutants resembled that found in the three floury maize types with high lysine content (o_2, o_7, and fl_2).

We present data on the bt_2 mutant and its combination with o_2 as a typical example of the starch-modifying mutants that exhibit more or less comparable degrees of endosperm defectiveness in the mature kernel. We have added, for comparison, the data on o_2 and fl_2 mutants in the same isogenic background, and have included the o_7 mutant, whose complete amino acid and protein patterns have not been reported previously. The bt_2 gene is located on chromosome 4 at a locus distinct from that of fl_2. The endosperm of the bt_2 mutant is translucent and shrunken (*6*).

Near isogenic sublines of o_2, fl_2, and bt_2 of inbred Oh43 were recovered after six backcrosses to the recurrent parent. The double mutant bt_2o_2 was isolated by a system of backcrossing and selfing, which permitted the classification for the segregation of the bt_2 gene in the background of the o_2 gene. The double mutant endosperm is opaque and shrunken, and the mature kernels are comparable in defectiveness to those of the bt_2 mutant (*7*).

The dry corn kernels were soaked in distilled water for 30 minutes, and were then separated with a scalpel into pericarp, embryo, and endosperm. The endosperms were dried in air overnight, were defatted with hexane, and were ground to a fine powder in a ball mill. The concentration of nitrogen was determined on the powder by the micro-Kjeldahl technique; 25-mg samples were hydrolyzed with 100 ml of 6*N* HCl under reflux for 24 hours, the acid was removed in a vacuum on a rotary evaporator, and the residue was taken up in *p*H 2.2 citrate buffer. This solution was applied to the columns of an automatic amino acid analyzer (Beckman-Spinco). Data were obtained on single hydrolyzates because duplicate hydrolyzates on many similar maize mutant samples did not differ by more than 5 percent in their lysine content. The concentration of tryptophan was determined in 100-mg samples of defatted powder by a colorimetric method (*8*).

Portions of the powdered defatted endosperms (1 g each) were subjected to fractionation sequence D of the procedure described by Landry and Moureaux (*9*). Fraction I contains proteins soluble in saline (albumins, globulins); fraction II, zein (prolamine); fraction III, glutelin-1; fraction IV, glutelin-2; and fraction V, glutelin-3.

Other data (*5*) show that the embryos