J. Membrane Biol. 25, 361–380 (1975)

Mitochondrial Uncoupling Agents

Effects on Membrane Permeability of Molluscan Neurons

Jeffery L. Barker and Herbert Levitan

Behavioral Biology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014 and Department of Zoology, University of Maryland, College Park, Maryland 20742

Received 16 June 1975; revised 5 August 1975

Summary. Agents which uncouple oxidative phosphorylation in mitochondria were applied to identified neurons in an isolated ganglion of the marine mollusc *Navanax inermis.* Aromatic monocarboxylic acids, acetanilides, benzamides, benzaldehydes and phenols all caused a rapid, reversible, dose-dependent increase in the membrane potential and conductance of the neurons tested. These events were due primarily to an increase in the membrane's conductance to potassium, relative to chloride. All active compounds also produced a reversible, dose-dependent decrease in the permeability of alkali-cations relative to potassium. The relative activity of congeners in each group of substances was directly correlated with the octanol-water partition coefficients of the various compounds, indicating that hydrophobicity was important in determining drug effect and suggesting that steric requirements were minimal. The results suggest that the observed changes in membrane electrical properties and cation selectivity are due to an increase in the membrane's anionic field strength caused by the hydrophobic interaction of anionic and nonionic agents with the neuronal membrane.

Compounds which uncouple oxidation from phosphorylation in mitochondria also affect the permeability and thus the physiology of excitable cells. For example some of these agents have been shown to increase the K^+ permeability of muscle (Webb & Hollander, 1956; Manchester, Randle & Smith, 1958; Hicklin, 1959; Haas, Kern & Einwächter, 1970; Bryant & Morales-Aguilera, 1971; McDonald & Macleod, 1972), nerve (Godfraind, Kawamura, Krnjević & Pumain, 1971; Meech, 1974*b*), and receptor cells (Gorman & McReynolds, 1974). Since uncoupling agents depress the ability of mitochondria to store intracellular Ca^{++} (Vasington & Murphy, 1962; Lehninger, Carafoli & Rossi, 1967; Caswell, 1969), and since an increase in intracellular Ca^{++} activity produces an increase in the K^+ permeability of some neuronal membranes (Meech, 1972, 1974*b*), it has been suggested that the effects of uncoupling agents on nerve membranes are mediated through their actions on mitochondria

(Godfraind *et al.*, 1971; Meech, 1974*b*). That is, uncoupling agents are thought to exert an *indirect* influence on membrane permeability by penetrating the plasma membrane and affecting the amount of Ca^{++} bound by mitochondria. An alternative explanation for the effects of uncoupling agents on excitable cells is that they act *directly* to alter the membrane permeability.

We have used identified neurons in an isolated molluscan ganglion to investigate the biophysical and physico-chemical mechanisms underlying the action of uncoupling agents on excitable cells. Our results suggest that uncoupling agents alter the electrical and cation-selective properties of these cells by adsorbing to, and increasing the anionic field strength of their membranes. Some aspects of this research have been reported in short communications (Barker & Levitan, 1971; Levitan & Barker, 1972*a, b*; Barker & Levitan, 1974).

Materials and Methods

Preparation and Recording

Specimens of the mollusc *Navanax inermis* were obtained from Pacific Bio-Marine Supply Co. (Venice, California). The buccal ganglion was dissected from the animal and pinned to paraffin in a 5 ml plastic dish which was perfused with an artificial salt solution at room temperature (20–22 °C). The capsule enveloping the ganglion was incised and large identified neurons were impaled with double-barreled micropipettes filled with 3 M KCl (recording barrel resistance: 2–10 MΩ; coupling resistance: $>10^4$ ohms). The neurons were identified according to the system of Levitan, Tauc & Segundo (1970). One barrel was used for recording membrane potential while the other was used to pass current. Membrane potentials were conducted through unity gain cathode followers with negative capacitance compensation, displayed on an oscilloscope and recorded on a pen recorder.

Solutions and Chemicals

Stock solutions were prepared with compositions listed in Table 1. Chemicals were purchased from K & K Laboratories (Plainview, N.Y.), Kodak (Rochester, N.Y.), Aldrich Chemical Co. (Cedar Knolls, N.J.), and Pfaltz and Bauer Chemical Co. (Flushing, N.Y.). When available, the sodium salt was obtained. If not available, the undissociated aromatic carboxylic acid or phenol was dissolved in pure ethanol and titrated with excess base (NaOH). The precipitate was then collected, washed with cold ethanol and dried. Chemicals were dissolved in the physiological solution immediately prior to perfusion.

Analysis of Ionic Conductances and Relative Cation Permeability of Membrane

The ionic basis for the effects of these organic compounds on membrane potential and input resistance was evaluated as follows: The contribution of potassium to the membrane

Table 1. Composition of solutions (mM)[a]

	A (0 K^+)	B (100 K^+)	C (low Cl^-)	D (low Na^+)	E (low cation)	F (high cation)
NaCl	500	400	–	10	–	–
KCl	–	100	–	10	–	–
$CaCl_2$	10	10	10	10	–	–
$MgCl_2$	50	50	–	300	–	–
Tris-Cl	10	10	10	10	–	–
Na Isethionate	–	–	500	–	–	–
K_2SO_4	–	–	20	–	–	–
Mannitol	–	–	–	400	0	130
$MgSO_4$	–	–	50	–	100	100
Tris-SO_4	–	–	–	–	620	10
X_2SO_4[b]	–	–	–	–	–	250

[a] The pH of all solutions was adjusted to 7.8 by titrating the Tris base with either HCl or H_2SO_4.
[b] X=K^+, Rb^+, Cs^+, Na^+ or Li^+.

potential was evaluated by monitoring changes in the membrane potential as a function of $[K^+]_o$, varied by mixing appropriate amounts of solutions A and B. The contribution of chloride to the membrane potential was assessed by observing variations in the membrane potential as the $[Cl^-]_o$ was abruptly reduced by perfusing with solution C. The contribution of sodium to the membrane potential was evaluated by observing the membrane potential changes caused by reducing the $[Na^+]_o$ from 500 to 10 mM (solution D).

The relative permeability of these neuronal membranes to alkali cations was investigated according to the scheme of Hagiwara, Toyama and Hayashi (1971). The assumption was made that the membrane potential is adequately described by a diffusion equation of the form:

$$V=(RT/F)\ln\left[\frac{[K^+]_o+(P_{X_1}/P_K)[X_1^+]_o+(P_{X_2}/P_K)[X_2^+]_o+\cdots}{[K^+]_i+(P_{X_1}/P_K)[X_1^+]_i+(P_{X_2}/P_K)[X_2^+]_i+\cdots}\right] \quad (1)$$

where V is the membrane potential, R is the gas constant, T is the absolute temperature, F is Faraday's constant, $[K^+]$ and $[X^+]$ are the concentrations of potassium and other alkali cations, P_{X_1}/P_K, P_{X_2}/P_K are the permeabilities of alkali cations relative to potassium and the subscripts o and i denote concentrations outside and inside the cell, respectively. It follows from this assumption that if all but one permeant cationic species were replaced by an impermeant one (such as $Tris^+$), then the membrane potential would be determined by the concentration gradients and permeabilities of the permeant species. Assuming the internal cation concentrations to be constant during manipulations of the exterior milieu, Eq. (1) reduces to:

$$V=(RT/F)\ln\left[\frac{(P_{X_1}/P_K)[X_1^+]_o}{k}\right] \quad (2)$$

where k is equal to the denominator of the term in the logarithm in Eq. (1). Thus, at a given membrane potential, V, the right-hand side of Eq. (2) can be equated for a series of cations, and referred to that for potassium. This gives

$$(RT/F)\ln\left[\frac{(P_{X_1}/P_K)[X_1^+]_o}{k}\right]=(RT/F)\ln\left[\frac{[K^+]_o}{k}\right] \quad (3)$$

which simplifies to:

$$P_{X_1}/P_K = [K^+]_o/[X^+]_o. \tag{4}$$

The contribution of anions to the membrane potential can be neglected if permeant anionic species are replaced with impermeant ones (i.e., SO_4^{2-}). Alternatively, the contribution of anionic species may be treated as constant. Under the present conditions, the counterion (SO_4^{2-}) makes some contribution to the membrane potential, since the slope of the curve of membrane potential as a function of $[K^+]_o$ is much less than the predicted 58 mV/10-fold change in $[K^+]_o$, in a solution containing K^+ as the only external cation. Furthermore, the contribution of the anion species to the membrane potential apparently is not constant, but increases as the $[K^+]_o$ increases (Strickholm & Wallin, 1967). This may cause the curves of membrane potential as a function of $[X^+]_o$ to become nonlinear at the higher concentrations of X^+. Relative cation permeability has therefore been evaluated over a linear portion of these curves at 20 mM $[K^+]_o$, near the physiological $[K^+]_o$. The external alkali-cation concentration $[X^+]_o$ was varied by mixing appropriate amounts of solutions E and F.

Relative Activity and Physico-Chemical Correlations

Relative activity of various uncoupling agents was assessed by comparing drug concentrations necessary to produce half the maximal change in membrane potential, as determined from dose-response curves. This data was obtained from cells bathed in saline solutions with $[K^+]_o = 1$ mM, since the membrane potential change in response to drug application was maximized under these conditions, usually approaching 40 mV.

Various classes of chemicals were evaluated as a group. Thus, dose-response curves were obtained for a series of benzoates, salicylates, acetanilides, benzaldehydes, benzamides and phenols in separate preparations. Relative activities were calculated from the responses observed in at least three preparations. The concentrations of chemical necessary to produce dose-response curves of relatively inactive substances ranged up to 10^{-1} M. (Osmotic control for the latter concentration consisted of adding 10^{-1} M NaCl to 1 mM $[K^+]_o$ seawater. This did not affect either the membrane potential or input resistance.)

The electronic properties of the substances were expressed in terms of the dissociation constant, pK_a. These values were obtained either from the Handbook of Chemistry and Physics (Weast, 1971) or calculated by using the Hammett equation (Jaffé, 1953):

$$\sigma/\varrho = pK_a \text{ (parent compound)} - pK_a \text{ (derivative)} \tag{5}$$

where σ is the Hammett sigma constant for substituents and ϱ is the reaction constant for the class of compounds. The hydrophobic properties of the substances were expressed in terms of their octanol-water partition coefficient P and were either obtained from the literature (Hansch, Kiehs & Lawrence, 1965; Fujita, 1966; Hansch & Anderson, 1967; Hansch, 1971; Leo, Hansch & Elkins, 1971) or calculated according to rules formulated by Hansch and his colleagues (Leo *et al.*, 1971; Hansch, 1971). Hansch's π constant can be defined as

$$\pi_I = \log P_I - \log P_H \tag{6}$$

where P_I is the octanol-water partition coefficient of the derivative, P_H is the octanol-water partition coefficient of the parent compound, and π_I is the logarithm of the partition coefficient of the substituent I.

The relative activities of the substances tested were correlated with their octanol-water partition coefficients and dissociation constants by the method of least squares. In the statistical notations used in this paper n is the number of data points used in the analysis, r is the correlation coefficient and s is the standard error of the estimate. The statistical

significance of the equations was examined by F tests, with $F_{k,\,n-k-1}$ compared with that at a specified level of confidence (α), where k is the number of independent variables and n the number of data points.

Results

The effects of 37 aromatic monocarboxylic acids, eight phenols, six acetanilides, five benzaldehydes, and three benzamides were tested on the membrane properties of *Navanax* neurons. All substances tested caused an increase in membrane potential and decrease in input resistance of all *Navanax* neurons examined. Representative examples from three classes of compound are illustrated in Fig. 1.

In each case the drugs caused a rapid change in membrane potential which was complete within 60–90 sec and was maintained as long as the drug was present. The magnitude of the increase in membrane potential was dependent upon the drug concentration applied and was accompanied by an increase in membrane conductance, as reflected by the decrease in voltage response to a constant current stimulus. Similar changes in membrane conductance were not observed following hyperpolarization of the membrane through current injection. The drug's effects on membrane potential and conductance were readily reversed by perfusing with normal solutions (*see also* Barker & Levitan, 1971, 1974, 1975). Dose-

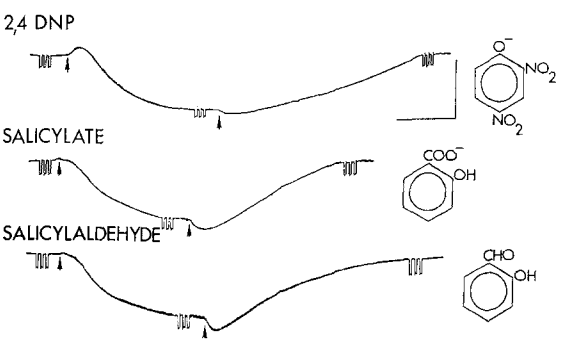

Fig. 1. Effects of metabolic uncouplers on membrane potential and conductance of molluscan neurons. Membrane potential traces recorded in 3 *G-R* cells bathed in physiological saline with three times the normal $[Mg^{++}]_o$ to prevent synaptic activity. Application of 5 mM 2,4-dinitrophenol (2,4-DNP), 10 mM salicylate, or 4 mM salicylaldehyde to the cell (initial arrow of each trace) results in an increase in membrane potential of 12–13 mV and conductance (as evidenced by the decrease in the voltage response to 4 nA constant current hyperpolarizing pulses—downward deflections from the baseline). Washing with drug-free solution (second arrow) restores the membrane properties to their control values. Calibration: 10 mV, 2 min

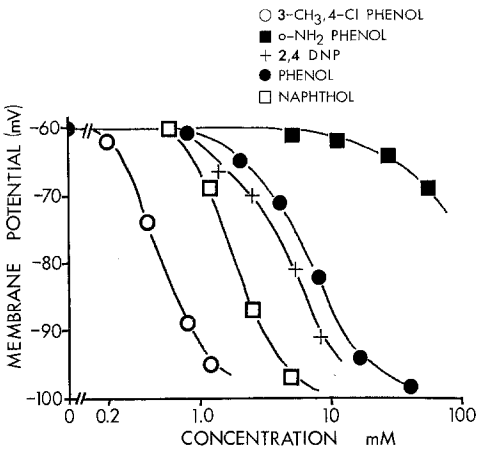

Fig. 2. Dose-response curves of the effects of phenols on membrane potential. The membrane potential as a function of the concentration of the phenol analogue. (Naphthol is 1-naphthol.) The data are averages from three cells. In each case increasing concentrations of the weaker, then stronger phenol analogues were applied to a cell bathed in physiological saline with 1 mM $[K^+]_o$, and the membrane potential response observed. The cells were washed extensively between the different drug assays

response curves were constructed for all substances examined. The change in membrane potential produced by increasing concentrations of several phenol analogs is illustrated in Fig. 2 (*see also* Levitan & Barker, 1972*a*).

Ionic Mechanisms

The ionic mechanisms underlying the increase in membrane potential and conductance were examined by varying the external concentration of one of the predominant ions at a time. All substances tested increased the slope of the curve of membrane potential as a function of $[K^+]_o$ in a dose-dependent, reversible manner (Fig. 3; *see also* Barker & Levitan, 1971, 1974, 1975), indicating an increase in the dependence of membrane potential on the potassium ion concentration gradient. Over the range of $[K^+]_o$ from 8–100 mM the curve approached a slope of 58 mV per 10-fold change in $[K^+]_o$ in the presence of saturating concentrations of each class of compound.

To determine the effect of the drugs on the membrane's conductance to chloride, the cells were first bathed in a solution containing 40 mM potassium. This caused the membrane potential to shift to a level where the addition of a drug produces little or no change in membrane potential

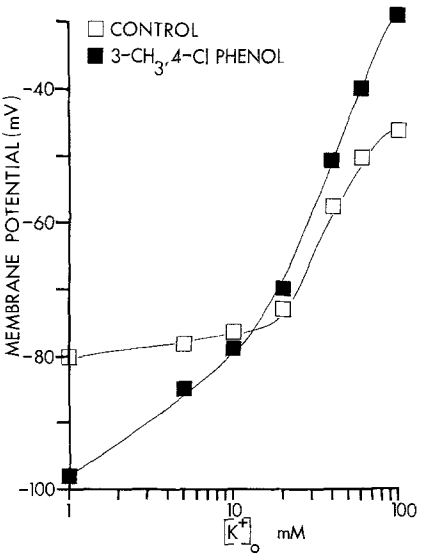

Fig. 3. Effects of 3-CH_3, 4-Cl phenol on the contribution of K^+ to the membrane potential. Membrane potential of cell G-L is plotted as a function of $[K^+]_o$ under control conditions and in the presence of 1 mM 3-CH_3, 4-Cl phenol. An increase in the slope of the curve of membrane potential as a function of $[K^+]_o$ is evident in 1 mM 3-CH_3, 4-Cl phenol

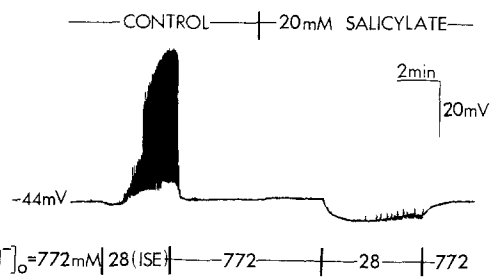

Fig. 4. Salicylate attenuates the depolarizing response to abrupt reduction in $[Cl^-]_o$. Membrane potential recorded in the cell *G-R* bathed in 100 mM excess of Mg, in the form of MgCl, to decrease synaptic activity, and four times normal $[K^+]_o$. Replacing most of the $[Cl^-]_o$ with less permeant anion isethionate results in a rapid depolarization and initiation of spike activity. Return to the full complement of Cl^- (772 mM) restores the membrane potential. Addition of 20 mM salicylate completely abolishes the depolarizing response. *See text* for details

even though the chloride and/or potassium conductance was greatly increased (as reflected in the decrease in voltage-response to constant current pulses). In this way we could examine the drug's effect on membrane potential changes induced by decreasing $[Cl^-]_o$ from the same initial membrane potential level. Consider the experiment illustrated in Fig. 4. In drug-free medium, reducing the $[Cl^-]_o$ to 28 mM (by replacement with

isethionate) caused a 10–15 mV depolarization of the membrane with the initiation of action potentials (Fig. 4). Upon returning to the initial $[Cl^-]_o$, the membrane potential returned to its previous value. The addition of 20 mM salicylate produced little change, if any, rather than a hyperpolarization of the membrane potential. This probably was due to the fact that the potassium equilibrium potential was close to the resting membrane potential in 40 mM $[K^+]_o$ (*see* Fig. 3). Reducing the $[Cl^-]_o$ in the presence of salicylate abolished the depolarizing response, reflecting the insensitivity of the membrane potential to changes in $[Cl^-]_o$. The hyperpolarization observed under these conditions reflected, we believe, the large isethionate gradient established and a slight membrane permeability to this anion which may be enhanced when salicylate is present (Barker & Levitan, *in preparation*) [A drug-induced increase in transmitter release (leading to the fast, upward-going events during the ion change) and contribution of transmitter-mediated membrane property changes cannot at present be excluded.] All classes of drugs tested reduced the amplitude of the depolarization observed upon decreasing $[Cl^-]_o$.

None of the substances tested had any observable effect on the membrane hyperpolarization produced upon reduction of $[Na^+]_o$. The foregoing results suggest that the ionic mechanisms underlying the increase in membrane potential and decrease in input resistance are primarily an increase in potassium conductance relative to chloride conductance.

Effects on Alkali-cation Permeability

The permeability of the membrane to alkali-cations relative to K^+ was examined by determining the concentration of each cation required to produce a membrane potential equal to that produced by 20 mM $[K^+]_o$ (*see* Materials and Methods). In drug-free conditions (Fig. 5, left), 20 mM $[K^+]_o$ produced a membrane potential of -50 mV. Since this membrane potential was also produced by 12 mM $[Rb^+]_o$, 40 mM $[Cs^+]_o$ and 300 mM $[Na^+]_o$ (or $[Li^+]_o$), the relative permeabilities were calculated to be Rb (1.67) > K (1.0) > Cs (0.40) > Na = Li (0.05).

Application of representatives of each type of weak acid metabolic inhibitor altered the relative cation permeability of these cells (*see also* Levitan & Barker, 1972*b*; Barker & Levitan, 1974, 1975). In each case the alkali-cation permeability relative to K^+ was reduced. For example, in the presence of 1 mM 5-bromosalicylate, a greater concentration of each of the alkali-cations was required to reproduce the same potential

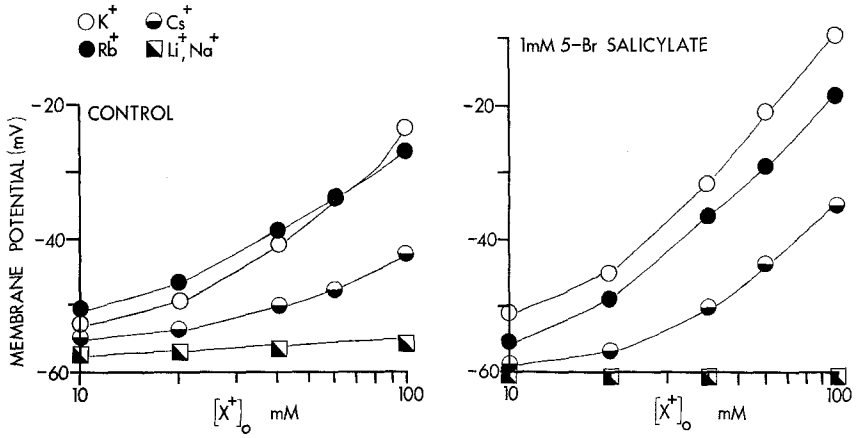

Fig. 5. Effect of 5-Br-Salicylate on cation selectivity. The data are from cell *G-L* and are representative of all the results observed. Membrane potential is plotted as a function of external cation concentration under control conditions and in the presence of 1 mM 5-Br-Salicylate. The curves of membrane potential as a function of Na^+, Li^+, Cs^+ and Rb^+ are all shifted to the right (relative to K^+) in salicylate. *See text* for details

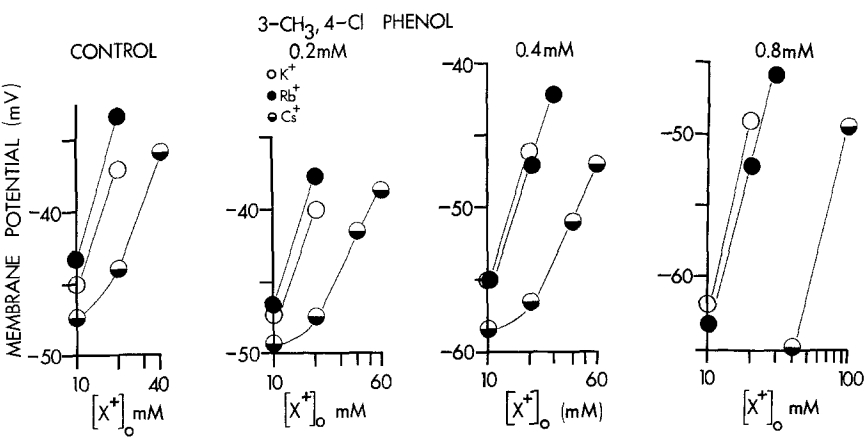

Fig. 6. Effect of 3-CH_3, 4-Cl phenol on cation selectivity. The data are from cell *G-L*. Membrane potential is plotted as a function of either $[K^+]_o$, $[Rb^+]_o$, or $[Cs^+]_o$ under control conditions and in the presence of increasing concentrations of 3-CH_3, 4-Cl phenol. The curves of potential as a function of $[Rb^+]_o$ and $[Cs^+]_o$ shift to the right (relative to the curve of potential *vs.* $[K^+]_o$ as the concentration of phenol increases

in 20 mM $[K^+]_o$ (−45 mV) than was observed under control conditions: 28 mM $[Rb^+]_o$ and 60 mM $[Cs^+]$. The relative cation permeability was calculated to be K (1.0) > Rb (0.71) > Cs (0.33) > Na = Li (< 0.01).

The permeability of the membrane to K^+, Rb^+ and Cs^+ was examined at various concentrations of drug. In Fig. 6 the membrane potential is

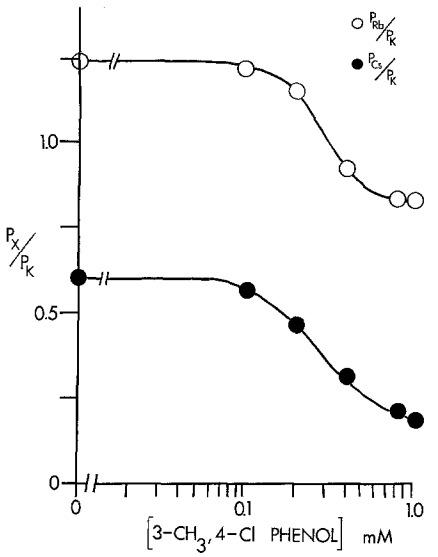

Fig. 7. Relative alkali-cation permeability is a function of the concentration of 3-CH_3, 4-Cl phenol. Data taken from experiments performed on same cell as illustrated in Fig. 6. The P_{Cs}/P_K and P_{Rb}/P_K ratios are plotted as a function of the concentration of 3-CH_3,4-Cl phenol. The points were obtained by examining the concentrations of Cs^+ and Rb^+ giving the same membrane potential as 20 mM $[K^+]_o$. *See text* for details

plotted as a function of alkali-cation concentration $[X^+]_o$ for several concentrations of a phenol analog. From these data we have calculated the permeability of Cs^+ and Rb^+ relative to K^+ and plotted the results in Fig. 7. As can be seen, increasing the concentration of the drug produces a parallel decrease in the ratio of P_{Rb}/P_K and P_{Cs}/P_K. The concentration producing half the maximal change in relative cation permeability was approximately the same as that producing half the maximal change in membrane potential.

Physico-Chemical Correlations

The relative activity of the drugs was obtained by comparing concentrations of the test substance producing half the maximal increase in membrane potential. The relative activity was then correlated with the physico-chemical properties of the compounds, including octanol-water partition coefficient P as a measure of hydrophobicity or lipid solubility, dissociation constant, K_a (or $pK_a = -\log K_a$) and Hammett constant σ as a measure of the electronic character of the drug.

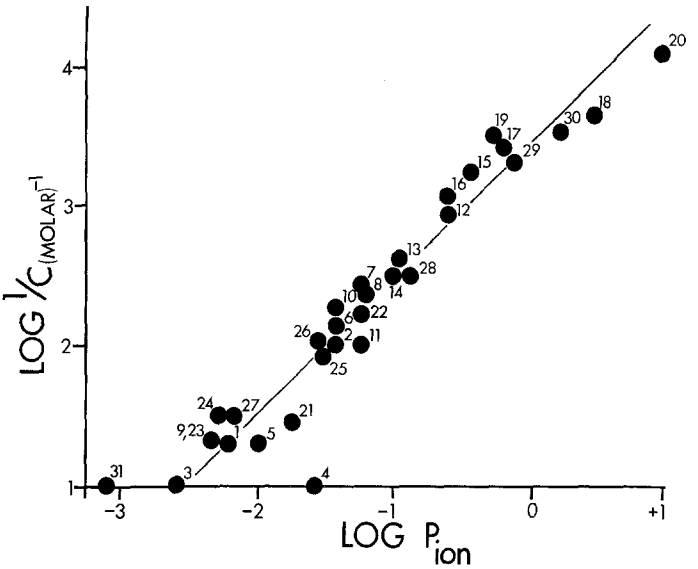

Fig. 8. Relative activity of aromatic monocarboxylic acids is plotted as a function of the octanol-water partition coefficient of the dissociated acid, $\log P_{ion}$. C is the drug concentration producing half the maximum response. The least-squares regression line is superimposed on the data. Numbers refer to compounds in Table 2

The activity of aromatic monocarboxylic acid derivatives of benzoates and salicylates was highly correlated with the octanol-water partition coefficient of the dissociated acid P_{ion} over four orders of magnitude of change in activity and partition coefficient (Fig. 8). The data are tabulated in Table 2 and the linear regression line for these data is presented as Eq. (7) in Table 5. Correlating drug activity with both partition coefficients and pK_a produces a statistically significant increase in the regression line only at a level of $P=0.1$ (Eq. 8). The partition coefficient for the dissociated form rather than the undissociated form was used here because all the active compounds were at least 99.9% ionized ($pK_a < 4.8$) and many were 99.999% ionized ($pK_a < 2.8$), at the pH of the experimental conditions (7.8).

The relative activity of the neutral compounds tested (acetanilides, benzamides and benzaldehydes) was also highly correlated with their partition coefficients (Fig. 9). The data are tabulated in Table 3A, B and C with linear regression analysis yielding Eqs. (9), (11) and (12) in Table 5. A least-squares analysis reveals that addition of the Hammett sigma constant, a term representing the electronic character of the molecule, does not add significantly to the correlation ($P > 0.1$) for acetanilide derivatives (Eq. (10), Table 5). There was insufficient data to perform meaningful

Table 2. Physico-chemical properties and relative activities of aromatic monocarboxylic acids

Compound	$\log P_{ion}$[a]	pK_a[b]	$\log 1/C$[e]	
			obs	calc[d]
1. Benzoate	−2.23	4.19	1.300	1.386
2. BZ, 2-OH	−1.43	2.97	2.000	2.078
3. BZ, 3-OH	−2.61	4.06	1.000	1.057
4. BZ, 2,4-diOH	−1.63	4.70	1.000	1.905
5. BZ, 2,5-diOH	−1.95	2.97	1.300	1.628
6. BZ, 2,6-diOH	−1.49	2.70	2.180	2.026
7. BZ, 3-Br	−1.23	3.86	2.400	2.251
8. BZ, 4-Br	−1.24	3.98	2.400	2.243
9. BZ, 2-Cl	−2.38	2.92	1.300	1.256
10. BZ, 3-Cl	−1.42	3.82	2.180	2.087
11. BZ, 4-Cl	−1.24	3.98	2.000	2.243
12. BZ, 3,4-diCl	−0.53	3.66[c]	2.930	2.857
13. BZ, 3-I	−0.95	4.00	2.600	2.494
14. BZ, 4-I	−1.09	3.91	2.540	2.372
15. BZ, 2-OH-5-Br	−0.44	2.62[c]	3.230	2.935
16. BZ, 2-OH-5-Cl	−0.60	2.63[c]	3.000	2.796
17. BZ, 2-OH-5-I	−0.19	2.65[c]	3.430	3.151
18. BZ, 2-OH-3,5-diBr	0.55	2.26[c]	3.600	3.792
19. BZ, 2-OH-3,5-diCl	−0.23	2.30[c]	3.480	3.117
20. BZ, 2-OH-3,5-diI	1.13	2.33[c]	4.000	4.294
21. BZ, 4-CH_3	−1.73	4.36	1.480	1.819
22. BZ, 2-OH-5-OCH_3	−1.29	2.86[c]	2.180	2.199
23. BZ, 3-NO_2	−2.28	3.47	1.300	1.343
24. BZ, 4-NO_2	−2.21	3.41	1.480	1.403
25. BZ, 2-OH-5-NO_2	−1.48	2.32[c]	2.000	2.034
26. BZ, 2-OH,3,5-diNO_2	−1.53	1.55[c]	2.000	1.992
27. Cinnamate	−2.23	4.44	1.480	1.386
28. 2-Naphthoate	−0.88	4.17	2.540	2.554
29. 3-OH-2-Naphthoate	−0.08	2.80[c]	3.300	3.246
30. Mefenamate	0.28	4.20	3.480	3.558
31. Acetylsalicylate	−3.13	3.50	1.000	0.607
32. *BZ, 4-OH	−2.62	4.48		1.048
33. *BZ, 3,4-diOH	−3.00	4.48		0.720
34. *BZ, 3,5-diOH	−2.80	4.04		0.893
35. *BZ, 2-NH_2	−2.99	6.97		0.728
36. *BZ, 4-NH_2	−3.52	4.92		0.270
37. *BZ, 2-OH,4-NH_2	−3.18	3.57[c]		0.564

[a] From Hansch (1975), calculated from the partition coefficient of the undissociated acid by subtracting 4.1 from $\log P$ for benzoic acid derivatives and subtracting 3.69 from $\log P$ of salicylic acid derivatives.

[b] Weast (Handbook of Chemistry and Physics, 1971).

[c] Levitan and Barker (1972*a*).

[d] Calculated from Eq. (7).

[e] Molar concentration producing half the maximum increase in membrane potential (~20 mV).

* Compounds not included in regression analysis because they were inactive.

obs = experimentally observed. calc = calculated. BZ = Benzoate.

Table 3. The activity and physico-chemical properties of nonionic compounds

A. Acetanilide derivatives

Compound	log P[a]	σ[b]	log $1/C$[e]	
			obs	calc[d]
1. Acetanilide	1.16	0.00	2.301	2.196
2. 4-OH Acetanilide	0.55	−0.37	1.478	1.664
3. 4-Cl Acetanilide	1.86	0.23	2.892	2.807
4. 4-Br Acetanilide	2.18	0.23	2.954	3.086
5. 4-I Acetanilide	2.42[c]	0.18	3.210	3.278
6. 4-NO_2 Acetanilide	1.40	0.78	2.602	2.406

[a] Hansch and Helmar (1968).
[b] Hansch, Leo, Unger, Kim, Nikaitani and Lien (1973).
[c] Calculated using π4-I from phenoxyacetate system of Fujita, Iwasa and Hansch (1964).
[d] Calculated from Eq. (9).
[e] Molar concentration producing half the maximum increase in membrane potential.

B. Benzamide derivatives

Compound	log P[a]	σ[a]	obs	calc[b]
1. Benzamide	0.64	0.00	1.506	1.517
2. 2-OH Benzamide	1.28		2.176	2.216
3. 4-CH_3 Benzamide	1.18	−0.17	2.114	2.063

[a] Hansch, Kim and Sarma (1973).
[b] Calculated from Eq. (11).

C. Benzaldehyde derivatives

Compound	log P[a]	σ[b]	obs	calc[d]
1. Benzaldehyde	1.48	0.00	2.778	2.806
2. 2-OH Benzaldehyde	1.70		2.903	2.975
3. 3-OH Benzaldehyde	1.25	0.12	2.602	2.594
4. 4-OH Benzaldehyde	0.85	−0.37	2.301	2.255
5. 4-Br Benzaldehyde	2.50[c]	0.23	3.700	3.654

[a] Leo, Hansch and Elkins (1971).
[b] Hansch, Leo, Ungar, Kim, Nikaitani and Lien (1973).
[c] Calculated using $\pi_{4\text{-Br}}$ from phenoxyacetate system of Fujita, Iwasa and Hansch (1964).
[d] Calculated from Eq. (12).

multivariate regression analyses on the data for benzamides and benzaldehydes.

The activity of phenol derivatives was also found to be highly correlated with the octanol-water partition of the undissociated compounds (Fig. 10). The data are tabulated in Table 4 and the linear regression line for these

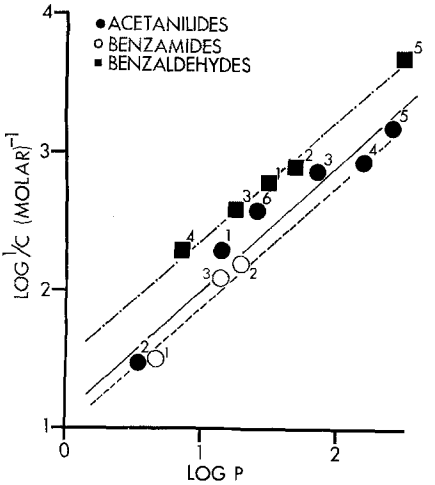

Fig. 9. Relative activity of acetanilides, benzamides, and benzaldehydes is plotted as a function of the undissociated octanol-water partition coefficient $\log P$. C is the drug concentration producing half the maximum response. Numbers refer to Table 3

data is presented as Eq. (13) in Table 5. Correlating drug activity with both partition coefficient and pK_a (Eq. (14), Table 5) does not significantly increase the correlation ($P > 0.1$). It is perhaps noteworthy that using Eq. (7) one can predict the activity of the two phenols which are essentially completely ionized at physiological pH. Utilizing the fact that the $\log P$

Table 5. Relationships between the activity of several types

Chemical class	Equation
Aromatic mono-carboxylic acids	$\log 1/C = 0.87\ (\pm 0.10)\ \log P_{ion} + 3.32\ (\pm 0.15)$ $\log 1/C = 0.83\ (\pm 0.10)\ \log P_{ion} - 0.10\ (\pm 0.12)\, pK_a + 3.62\ (\pm 0.40)$
Acetanilides	$\log 1/C = 0.87\ (\pm 0.30)\ \log P + 1.18\ (\pm 0.52)$ $\log 1/C = 0.79\ (\pm 0.28)\ \log P + 0.36\ (\pm 0.51)\ \sigma + 1.26\ (\pm 0.44)$
Benzamides	$\log 1/C = 1.97\ (\pm 0.85)\ \log P + 0.82\ (\pm 0.91)$
Benzaldehydes	$\log 1/C = 0.85\ (\pm 0.15)\ \log P + 1.53\ (\pm 0.25)$
Phenols	$\log 1/C = 0.69\ (\pm 0.26)\ \log P + 1.10\ (\pm 0.61)$ $\log 1/C = 0.69\ (\pm 0.25)\ \log P - 0.043\ (\pm 0.08)\ pK_a + 1.40\ (\pm 0.81)$

Numbers in brackets represent 95% confidence intervals.
C = Molar concentration producing half-maximal increase in membrane potential.
P = Octanol-water partition coefficient of undissociated compound.
P_{ion} = Octanol partition coefficient of dissociated compound.
n = number of compounds tested.

Table 4. Activity and physico-chemical properties of phenol derivatives

Compound	log P[a]	pK_a	log $1/C$[f]	
			obs	calc[e]
1. Phenol	1.46	9.9	2.32	2.11
2. 2,4,-diNO_2 Ph	1.52	4.1[c]	2.40	2.15
3. 3-NO_2 Ph	2.00	8.4	2.40	2.48
4. 2,4,6-triNO_2 Ph	2.03	0.3	2.78	2.50
5. 2-NH_2 Ph	0.50	9.4	1.08	1.44
6. 3-CH_3, 4-Cl Ph	3.10	9.4[d]	3.40	3.25
7. 2,4,6-triCl Ph	3.69	6.4[c]	3.60	3.65
8. 1-Naphthol	3.00	9.3	2.78	3.18

[a] Leo, Hansch and Elkins (1971).
[b] Weast (*Handbook of Chemistry and Physics*, 1971).
[c] Hansch, Kiehs and Lawrence (1965).
[d] Machleidt, Roth and Seeman (1972).
[e] Calculated from Eq. (13).
[f] Molar concentration producing half the maximum increase in membrane potential.
Ph = phenol.

of the dissociated form of a phenol derivative is about 3.2 less than the undissociated form (C. Hansch, *personal communication*), the relative activity, $\log 1/C$, of 2,4-dinitrophenol ($\log P_{ion} = 1.68$, $pK_a = 4.1$) and of 2,4,6-trinitrophenol ($\log P = 1.10$, $pK_a = 0.3$) are calculated from Eq. (7) to be

of compounds and their physico-chemical characteristics

n	r	s	F_{ratio}		Eq. No.
31	0.958	0.255	$F_{1,29} = 353$	$F_{\alpha=0.001} = 13$	(7)
31	0.962	0.247	$F_{2,28} = 190$	$F_{\alpha=0.001} = 8.9$	(8)
6	0.970	0.168	$F_{1,4} = 67.8$	$F_{\alpha=0.005} = 31.3$	(9)
6	0.989	0.120	$F_{2,3} = 67.2$	$F_{\alpha=0.005} = 49.8$	(10)
3	0.998	0.032	$F_{1,1} = 274$	$F_{\alpha=0.05} = 161$	(11)
5	0.995	0.059	$F_{1,3} = 315$	$F_{\alpha=0.001} = 167$	(12)
8	0.940	0.290	$F_{1,6} = 49.6$	$F_{\alpha=0.001} = 35.3$	(13)
8	0.956	0.268	$F_{2,5} = 29.0$	$F_{\alpha=0.005} = 18.3$	(14)

r = correlation coefficient.
s = standard error of the estimate.
F_{ratio} = statistical significance test from an analysis of variance.
α = level of confidence.

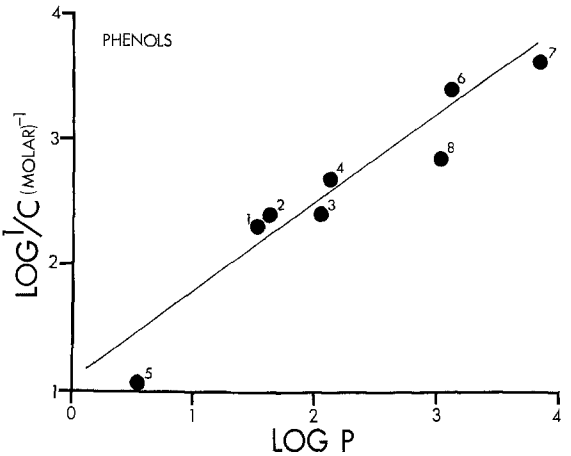

Fig. 10. Relative activity of phenols is plotted as a function of the undissociated octanol-water partition coefficient, log P. C is the drug concentration producing half the maximum response. Numbers refer to compounds in Table 4

1.8 and 2.37, respectively. Their activity was found experimentally to be 2.4 and 2.78, respectively. This would tend to support the implication of Eq. (7) that the activity of any anionic organic acid can be substantially predicted from a knowledge of the partition coefficient of the dissociated acid. On the other hand, the activity of the phenol derivatives which were substantially undissociated at physiological pH were more closely predicted by equations relating the activity of the neutral weak acids to hydrophobicity.

Discussion

In examining the effects of a variety of uncoupling agents on the membrane permeability of molluscan neurons, we have found that all substances reversibly increased the potassium conductance relative to the chloride conductance in a dose-dependent, reversible manner. Each class of compound examined also decreased the permeability of Rb^+, Cs^+, Na^+ and Li^+ relative to K^+ in a dose-dependent, reversible manner. These latter observations may be interpreted according to Eisenman's theory of ion permselectivity (Eisenman, 1961, 1963, 1965). Eisenman found that cation discrimination by glass was dependent on the field strength of anionic sites in the glass and proposed that similar mechanisms may be involved in the permselectivity of ions through biological membranes (Eisenman, 1963, 1965). Others have used this theory to account

for the ion selective nature of a wide variety of biological membranes (Diamond & Wright, 1969). According to Eisenman's theory the changes in relative cation permeability we have observed would be expected following an increase in the anionic field strength of membrane sites. Since the activity of a substance in changing relative cation permeability was comparable to its ability to increase membrane potential, it is possible that the increase in potassium conductance relative to chloride conductance responsible for the increase in membrane potential is due to an increase in the anionic field strength of the membrane.

The most important physico-chemical characteristic for a compound's activity was its partition coefficient (Eqs. (7), (9), (11), (12), (13) of Table 5). The significance of the correlation between activity and hydrophobicity for all the compounds tested suggests that steric factors play a minimal role in the interaction between the molecules and the membrane. Since the slopes of the linear functions relating activity with partition coefficient are quite similar, it is evident that activity varies in a fairly constant manner with $\log P$. A significant difference, however, lies in the fact that the intercept of the equations describing the activity of the dissociated carboxylic acids is about two log units higher than that for the neutral or undissociated compounds. Thus, one can conclude that given two compounds with approximately the same partition coefficient (e.g., 3,5-di-I-salicylate, $\log P_{ion} = 1.13$ and 4-CH_3-benzamide, $\log P = 1.18$), the dissociated compound (the salicylate) will be approximately 100 times more potent than the neutral compound (the benzamide). In similar fashion the ability of dissociated molecules to perturb the red cell's membrane is reported to be 100–1,000 times greater than for neutral molecules (Hansch & Glave, 1971).

The fact that changes in relative cation permeability were well-correlated with the hydrophobic nature of the anionic and nonionic organic molecules suggests that the increase in anionic field strength is due to the hydrophobic adsorption of the compounds to the membrane. The observed changes in cation selectivity would not have resulted if the primary effect of the agents had been to increase the negative surface potential of the membrane, as suggested by McLaughlin for his work on the interaction of salicylate and 2,4-DNP with artificial membranes (McLaughlin, 1972, 1973).

Because of the relative speed of onset and reversibility of the effects of the uncoupling agents on membrane properties as well as the apparent shift in ion selectivity of the membrane, we believe that these molecules act directly on the cell membrane rather than indirectly through an action

on the mitochondria. In order that mitochondrial uncoupling play a role in eliciting the charges in membrane permeability produced by these compounds, the anionic and nonionic uncoupling agents would have to permeate the neuronal membrane with equal facility, and rapidly depress the energy-dependent storage of calcium by the mitochondria. The increase in potassium permeability produced in cortical neurons (Godfraind *et al.*, 1971) and invertebrate photoreceptors (Gorman & McReynolds, 1974) is also more rapid than the increase in potassium conductance produced by direct intracellular injection of calcium ions (Meech, 1972; 1974*b*), the former occurring within seconds rather than minutes. Moreover, recent attempts to prevent any increase in the free internal calcium by intracellular injection of EGTA did not alter the increase in potassium conductance produced by external application of salicylate (H. Levitan, *unpublished observation*). The concentration of EGTA was, however, sufficient to produce the prolongation of action potentials in a train (Meech, 1974*a*). Presumably, the direct membrane actions of these agents accounts entirely for their ability to increase the K^+ permeability of human erythrocytes which respire anaerobically (Omachi, 1964).

Although we believe it is unlikely that uncoupling of oxidative phosphorylation is a prerequisite to the observed increase in K^+ conductance in the neuronal system used in the present study, it is possible that the mechanism proposed to account for the actions here may be applicable to the uncoupling activity of these agents. An increase in the anionic field strength of the mitochondria membrane should significantly increase its permeability to hydrogen ions relative to other cations, following the isotherms of permselectivity to cations developed by Eisenman (1965), and such an increase in H^+ permeability would uncouple oxidative phosphorylation according to Mitchell's chemiosmotic hypothesis (Mitchell, 1966; Mitchell & Moyle, 1967, 1969).

This work was supported in part by Grant GB-43141 from the National Science Foundation and in part through the facilities of the Computer Science Center at the University of Maryland.

References

Barker, J.L., Levitan, H. 1971. Salicylate: Effects on membrane permeability of molluscan neurons. *Science* **172**:1245

Barker, J.L., Levitan, H. 1974. Phenols: Effects on invertebrate membrane permeability related to uncoupling activity in mitochondria. *Brain Res.* **67**:555

Barker, J.L., Levitan, H. 1975. Acetanilides: Effects on invertebrate neurons correlated with analgesic activity in vertebrates. *J. Pharmacol. Exp. Ther.* **193**:892

Bryant, S.H., Morales-Aguilera, O. 1971. Chloride conductance in normal and mytonic muscle fibres and the action of monocarboxylic aromatic acids. *J. Physiol. (Lond.)* **219**:367

Caswell, A.H. 1969. Proton permeability and the regulation of potassium permeability in mitochondria by uncoupling agents. *J. Membrane Biol.* **1**:53

Diamond, J.M., Wright, E.M. 1969. Biological membranes: The physical basis of ion and nonelectrolyte selectivity. *Annu. Rev. Physiol.* **31**:581

Eisenman, G. 1961. On the elementary atomic origin of equilibrium ion specificity. *In*: Symposium on Membrane Transport and Metabolism. A. Kleinzeller and K.A. Kotyk, editors. p. 163. Academic Press, New York

Eisenman, G. 1963. The influence of Na, K, Li, Rb, and Cs on cellular potentials and related phenomena. *Bol. Inst. Estud. Med. Bio. (Univ. Nac. Auton. Mex.)* **21**:155

Eisenman, G. Some elementary factors involved in specific ion permeation. *Proc. 23rd Int. Cong. Physiol. Sci. (Tokyo)*, p. 489

Fujita, T. 1966. The analysis of physiological activity of substituted phenols with substituent constants. *J. Med. Chem.* **9**:797

Fujita, T., Iwasa, J., Hansch, C. 1964. A new substituent, constant, π, derived from partition coefficients. *J. Amer. Chem. Soc.* **86**:5175

Godfraind, J.M., Kawamura, H., Krnjević, K., Pumain, R. 1971. Actions of dinitrophenol and some other metabolic inhibitors on cortical neurones. *J. Physiol. (London)* **215**:199

Gorman, A.L.F., McReynolds, J.S. 1974. Control of membrane K^+ permeability in a hyperpolarizing photoreceptor: Similar effects of light and metabolic inhibitors. *Science* **185**:620

Haas, H.G., Kern, R., Einwächter, H.M. 1970. Electrical activity and metabolism in cardiac tissue: An experimental and theoretical study. *J. Membrane Biol.* **3**:180

Hagiwara, S., Toyama, K., Hayashi, H. 1971. Mechanism of anion and cation permeations in the resting membrane of a barnacle muscle fiber. *J. Gen. Physiol.* **57**:408

Hansch, C. 1971. Quantitative structure-activity relationships in drug design. *In*: Drug Design. E.J. Ariens, editor. Vol. 1, p. 271. Academic Press, New York

Hansch, C. 1975. Biosterism. Intra-Science Chem. Reports (*in press*)

Hansch, C., Anderson, S. 1967. The effect of intramolecular hydrophobic bonding on partition coefficients. *J. Org. Chem.* **32**:2583

Hansch, C., Glave, W.R. 1971. Structure-activity relationships in membrane-perturbing agents. *Mol. Pharmacol.* **7**:337

Hansch, C., Helmer, F. 1968. Extrathermodynamic approach to the study of the adsorption of organic compounds by macromolecules. *J. Polymer Sci.: Part A-1* **6**:3295

Hansch, G., Kiehs, K., Lawrence, G.L. 1965. The role of substituents in the hydrophobic bonding of phenols by serum and mitochondrial proteins. *J. Amer. Chem. Soc.* **87**:5570

Hansch, C., Kim, K.H., Sarma, R.H. 1973. Structure-activity relationship in benzamides inhibiting alcohol dehydrogenase. *J. Amer. Chem. Soc.* **95**:6447

Hansch, C., Leo, A., Unger, S.H., Kim, K.H., Nikaitani, D., Lien, E.J. 1973. "Aromatic" substituent constants for structure-activity correlations. *J. Med. Chem.* **16**:1207

Hicklin, J.A. 1959. Salicylate and potassium fluxes of rat diaphragm. *Nature* **184**:2029

Jaffé, H.H. 1953. A re-examination of the Hammett equation. *Chem. Rev.* **53**:191

Lehninger, A.L., Carafoli, E., Rossi, C.S. 1967. Energy-linked ion movements in mitochondrial systems. *Adv. Enzymol.* **29**:259

Leo, A., Hansch, C., Elkins, D. 1971. Partition coefficients and their uses. *Chem. Rev.* **71**:525

Levitan, H., Barker, J.L. 1972*a*. Salicylate: A structure-activity study of its effects on membrane permeability. *Science* **176**:1423

Levitan, H., Barker, J.L. 1972*b*. Membrane permeability: Cation selectivity reversibly altered by salicylate. *Science* **178**:63

Levitan, H., Tauc, L., Segundo, J.P. 1970. Electrical transmission among neurons in the buccal ganglion of a mollusc, *Navanax inermis*. *J. Gen. Physiol.* **55**:484

Machleidt, H., Roth, S., Seeman, P. 1972. The hydrophobic expansion of erythrocyte membranes by the phenol anesthetics. *Biochim. Biophys. Acta* **255**:178

Manchester, K.L., Randle, P.J., Smith, G.H. 1958. Some effects of sodium salicylate on muscle metabolism. *Brit. Med. J.* **1**:1028

McDonald, T.F., Macleod, D.P. 1972. The effect of 2,4-dinitrophenol on electrical and mechanical activity, metabolism and ion movements in guinea-pig ventricular muscle. *Brit. J. Pharmacol.* **44**:711

McLaughlin, S.G.A. 1972. The mechanism of action of DNP on phospholipid bilayer membranes. *J. Membrane Biol.* **9**:361

McLaughlin, S.G.A. 1973. Salicylates and phospholipid bilayer membranes. *Nature* **243**:234

Meech, R.W. 1972. Intracellular calcium injection causes increased potassium conductance in *Aplysia* nerve cells. *Comp. Biochem. Physiol.* **42A**:493

Meech, R.W. 1974*a*. Prolonged action potentials in *Aplysia* neurones injected with EGTA. *Comp. Biochem. Physiol.* **48A**:397

Meech, R.W. 1974*b*. The sensitivity of *Helix aspersa* neurones to injected calcium ions. *J. Physiol. (London)* **237**:259

Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev.* **41**:445

Mitchell, P., Moyle, J. 1967. Acid-base titration across the membrane system of rat-liver mitochondria. *Biochem. J.* **104**:588

Mitchell, P., Moyle, J. 1969. Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. *Europ. J. Biochem.* **7**:471

Omachi, A. 1964. Sulfate transport in human red cells: Inhibition by some uncouplers of oxidative phosphorylation. *Science* **145**:1449

Strickholm, A., Wallin, B.G. 1967. Relative ion permeabilities in the crayfish giant axon determined from rapid external ion changes. *J. Gen. Physiol.* **50**:1929

Vasington, F.D., Murphy, J.V. 1962. Ca^{++} uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. *J. Biol. Chem.* **237**:2670

Weast, R.C. (editor). 1971. Handbook of Chemistry and Physics. Chemical Rubber Co., Cleveland, Ohio

Webb, J.L., Hollander, P.B. 1956. Metabolic aspects of the relationship between the contractility and membrane potential of the rat atrium. *Circ. Res.* **4**:618