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Salicylates and Proton Transport through Lipid Bilayer Membranes: A Model for Salicylate-Induced Uncoupling and Swelling in Mitochondria

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Summary. Mechanisms of proton transport were investigated in phospholipid bilayer membranes exposed to salicylates and benzoates. Membranes were formed from diphytanoyl phosphatidylcholine in decane plus chlorodecane (50% vol/vol). Proton and anion conductances (G_H and G_A) were calculated from the total conductances and the H^+ or A diffusion potentials produced by transmembrane H^+ or A gradients. At low pH salicylate caused a G_H which was proportional to the square of the total weak acid concentration, and G_H was maximum when pH = pK. At neutral to alkaline pH salicylate caused a G_A which was proportional to the first power of the salicylate concentration, and G_A was independent of pH. Both G_H and G_A were inhibited by phloretin. The results suggest that salicylate acts as an HA_2-type proton carrier at low pH and as a lipid-soluble anion at neutral pH. Salicylate has been implicated as a causal factor in Reye's syndrome, as well as in aspirin poisoning, and salicylate has been reported to increase the proton conductance of inner mitochondrial membranes. The present results suggest that in mitochondria salicylate increases passive proton uptake by a combination of HA influx (driven by the concentration gradient) and A efflux (driven by the voltage and concentration gradients). Model calculations suggest that over the range of therapeutic to toxic concentrations, salicylate causes net H^+ influx sufficient to explain the reported "loose coupling," uncoupling and swelling of mitochondria. The relative ineffectiveness of aspirin and benzoate can be explained by their low A permeabilities, whereas the ineffectiveness of 2,6-dihydroxybenzoate can be explained by its low pK.

Key Words salicylate · proton transport · phospholipid bilayer membrane · mitochondria · aspirin · Reye's syndrome

Introduction

Consumption of salicylates (mainly aspirin) in the United States is more than 10,000 tons per year, and reported cases of salicylate poisoning occur at a rate of about 10,000 per year [9, 37]. Salicylate ingestion is also associated with gastric mucosal injury [21, 32] and with Reye's syndrome [10, 31], a disease characterized by impaired energy metabolism and mitochondrial swelling, especially in liver and brain [2, 16, 35, 40]. During and following its absorption from the gastrointestinal tract, aspirin is hydrolyzed rapidly ($t_{1/2} \simeq 20$ min) to form salicylic and acetic acids [9, 21, 32]. Thus, many of the side effects, as well as the therapeutic effects, of aspirin are due to salicylate and/or salicylic acid [9, 21, 37].

Previous studies have shown that salicylate causes dose-dependent "loose coupling" [16], uncoupling [4, 39], and swelling [40] in isolated mitochondria. However, acetylsalicylate (aspirin), benzoate, and 2,6-dihydroxybenzoate are either ineffective or less effective than salicylate [4, 39, 40]. The uncoupling caused by salicylate is qualitatively similar to that caused by 2,4-dinitrophenol [4, 39], suggesting that salicylate acts as a "proton ionophore" [16]. The reasons for the relative ineffectiveness of the other benzoates and aspirin are not clear [4, 39, 40].

The objectives of this study were, first, to characterize the proton and anion conductances caused by salicylates and benzoates in phospholipid bilayer membranes and, second, to predict the effects of salicylates and benzoates on mitochondria. The results suggest that at low pH salicylate acts as an HA_2^--type proton carrier, but at neutral pH salicylate behaves as a lipid-soluble anion moving in parallel with salicylic acid, which crosses the membrane by nonionic diffusion. Collectively, the data provide a simple explanation for the mitochondrial uncoupling and swelling which occurs in salicylate poisoning and possibly also in Reye's syndrome. The lesser effectiveness of benzoate and aspirin can be explained by their low anionic permeabilities, whereas the ineffectiveness of 2,6-dihydroxyben-

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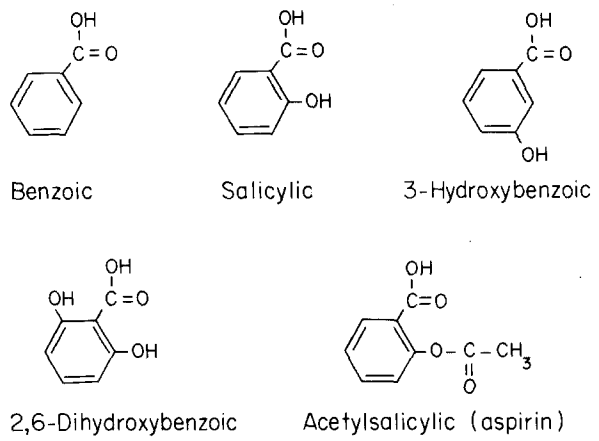

Fig. 1. Structures of salicylates and benzoates used in this study: benzoic acid (pK = 4.1), salicylic acid (pK = 3.0), 3-hydroxybenzoic acid (pK = 4.1), 2,6-dihydroxybenzoic acid (pK = 1.1) and acetylsalicylic acid (aspirin) (pK = 3.5). The pK's are from [34]

zoate can be explained by its low pK. A preliminary account of this work has been published [14].

Materials and Methods

Materials

Diphytanoyl phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL). Decane (99.9%) was obtained from Wiley Organics (Columbus, OH), and 1-chlorodecane (95%) was obtained from Aldrich (Milwaukee, WI). Buffers were obtained from Research Organics (Cleveland, OH) or Sigma (St. Louis, MO). Salicylic acid, benzoic acid, 3-hydroxybenzoic acid, acetylsalicylic acid (aspirin) and phloretin were obtained from Sigma. 2,6-dihydroxybenzoic acid was obtained from Eastman Kodak (Rochester, NY). The structures of the salicylates and benzoates are shown in Fig. 1.

The decane and chlorodecane were passed through an aluminum oxide column to remove polar impurities. Water was deionized and then doubly distilled. In some experiments the water was HPLC grade obtained from Burdick and Jackson (Muskegon, MI).

Methods

Planar bilayer membranes (1.4 mm^2) were formed from diphytanoyl PC (31 mg/ml or 37 mM) dissolved in *n*-decane plus 1-chlorodecane (50%, vol/vol). Membranes were formed in an open chamber designed so that both the front and rear solutions could be modified by perfusion and/or injection [13, 38]. The front and rear solution volumes were 1.2 ml each, and both solutions were stirred magnetically. The temperature was 24 ± 2°C.

The chlorodecane was added to slightly increase the dielectric constant of the nonpolar region of the membrane [7, 26]. Dilger et al. [7] showed that bilayers containing chlorodecane rather than decane have ionic, e.g., ClO_4^- and SCN^-, permeabilities similar to those of inner mitochondrial membranes, which contain large amounts of integral membrane proteins. Bilayers containing 50% chlorodecane solvent have ionic permeabilities about an order of magnitude lower than bilayers made with 100% chlorodecane, but the 50% mixture provides better mechanical stability [13].

Salicylates or benzoates were prepared in concentrated stock solutions (0.5–1.0 M) as either free acids in ethanol or sodium salts in water. After the membrane was formed and the baseline conductance was measured, the salicylates or benzoates were injected by microsyringe to give final concentrations ranging from 0.1 to 30 mM. Aqueous stock solutions of sodium acetylsalicylate deteriorated within several hours, giving artifactually high conductances which were probably due to salicylate. However, stock solutions of acetylsalicylic acid in 100% ethanol were stable for at least a month at 4°C.

The method of measuring proton conductance is described elsewhere [11–13]. In brief, the membranes were exposed to small (0.3–0.7 unit) pH gradients produced by mixtures of weakly acidic and weakly basic buffers, e.g., HEPES plus Tris, MES plus Bis-Tris, TAPS plus Bis-Tris propane, etc. In most experiments the front and rear solutions contained similar concentrations of all ions except H^+ and OH^-. The H^+ diffusion potential produced by the pH gradient was measured with a high impedance electrometer and two calomel-KCl electrodes.

The transference number for H^+ (T_H) was calculated from the relation, $T_H = V/E_H$, where V is the measured diffusion potential and E_H is the Nernst equilibrium potential for H^+. The H^+ conductance (G_H) was calculated from the relation, $G_H = T_H G$, where G is the total steady-state conductance, measured by applying a small voltage pulse across the membrane.

Anion conductances (G_A) were measured by imposing an A^- concentration gradient under conditions of symmetrical and well-buffered pH. The A^- diffusion potential and the total conductance were measured, and G_A was calculated in a manner similar to that described for G_H.

Since $E_H = E_{OH}$, it is impossible to distinguish between H^+ and OH^- conductances without a prior knowledge of the transport mechanism. However, in this study we will be dealing with weak acids which are expected to act as H^+, not OH^-, carriers. For this reason, as well as for convenience, I will use the term "proton conductance" rather than "proton/hydroxide conductance."

Results

Salicylate-Induced Conductance as a Function of pH

Figure 2 shows the membrane conductance as a function of pH at a constant total salicylate ($[A^-] + [HA]$) of 2 mM. When pH was near pK, the conductance was maximum and the membrane behaved as a pH electrode, giving H^+ diffusion potentials of 56 to 59 mV per pH unit. At neutral to alkaline pH the conductance was lower and independent of pH, and the membrane behaved as a salicylate electrode, giving A^- diffusion potentials of 50 to 56 mV per 10-fold concentration gradient. "Background" conductances due to Na^+, Cl^- and buffer ions com-

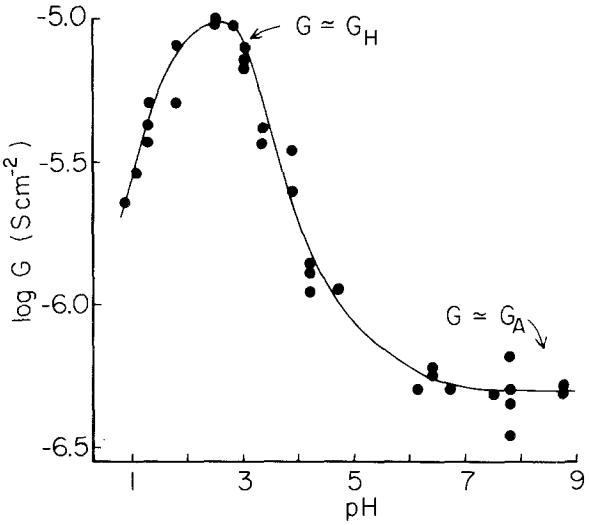

Fig. 2. Membrane conductance (G) as a function of pH at a constant total salicylate of 2.0 mM. Solutions were buffered with HCl (pH 0.9–1.7), glycine plus HCl or citric acid (pH 2.4–3.0), β-alanine plus HCl or gluconic acid (pH 3.0–4.1), citric acid plus NaOH (pH 4.1–4.7), MES plus Bis-Tris (pH 6.1–6.8), HEPES plus Tris (pH 7.4–8.2) and TAPS plus Bis-Tris propane (pH 8.6–9.2). Total buffer concentrations ranged from 100 to 240 mM (except for HCl at pH 1.3–1.7), and ionic strengths ranged from 0.04 to 0.12. Each point represents a single membrane

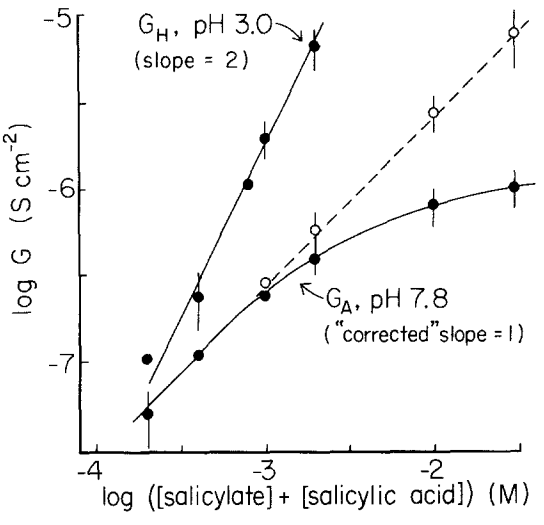

Fig. 3. Proton conductance (G_H) and salicylate conductance (G_A) as a function of total salicylate concentration at constant pH. At pH 3.0 the buffer was 120 mM glycine plus 60 mM citric acid or 100 mM β-alanine plus 80 mM HCl. At pH 7.8 the buffer was 80 mM HEPES plus 80 mM Tris. The dashed line is G_A "corrected" for surface charge as described in the text. Vertical bars indicate the standard deviations of 3–5 membranes

prised <5% of the total conductance at pH 1 to 5 and <15% of the total conductance at pH 6 to 9.

Salicylate Concentration Dependence of G_H and G_A

Figure 3 shows G_H and G_A as functions of the salicylate concentration at constant pH. At pH 3.0, when $G \simeq G_H$, the conductance was proportional to the second power of the salicylate concentration, giving a slope of 2.0 on the double-log plot. Similar results were obtained at pH's 1.3, 2.5 and 3.6 (*data not shown*). At pH 7.8, when $G \simeq G_A$, the conductance was proportional to the first power of the salicylate concentration at low concentrations, but G_A tended to "saturate" at high concentrations.

McLaughlin [25] showed that salicylate adsorbs to neutral (phosphatidylcholine or phosphatidylethanolamine) bilayers at pH 7, producing negative surface potentials which increase from 0 to 100 mV as the salicylate concentration increases from 1 to 120 mM. Thus, I used his estimates of surface potential to "correct" the salicylate conductances over the range of 1 to 30 mM from the relation,

$$G'_A = G_A \exp(-F\Psi/RT) \quad (1)$$

where G'_A is the "corrected" conductance, Ψ is the surface potential and R, T and F have their usual meanings. The calculated G'_A (dashed line in Fig. 3) has a slope of 1.0, suggesting that the saturation of G_A at high concentrations is due to salicylate adsorption to the membrane.

Conductances and Permeabilities of Benzoates and Salicylates

The Table compares the conductances caused by salicylate, benzoate, aspirin, 3-hydroxybenzoate and 2,6-dihydroxybenzoate. Benzoate, aspirin and 3-hydroxybenzoate were much less effective than salicylate, and 2,6-dihydroxybenzoate was much more effective than salicylate in producing G_H and G_A. None of the weak acids produced significant G_H at pH 7.8.

The salicylate permeability coefficient (P_A) was estimated from the relation,

$$P_A = G_A RT/F^2[A^-] \quad (2)$$

which yields a value of about 7×10^{-8} cm sec^{-1} at 1 mM salicylate. At higher salicylate concentrations P_A decreased due to the sublinear relationship between G_A and $[A^-]$ (Fig. 3). For comparison, the

Table. Proton and anion conductances (G_H and G_A) caused by salicylates and benzoates in phospholipid bilayer membranes[a] (the total concentration of each weak acid was 2.0 mM)

Weak acid	pK[b]	G_H when pH = pK (nS cm^{-2})	G_A when pH = 7.8 (nS cm^{-2})
Salicylic	3.0	9,300 ± 1,200	480 ± 112
Benzoic	4.1	18 ± 8	11 ± 4
Acetylsalicylic	3.5	16 ± 9	9 ± 5
3-Hydroxybenzoic	4.1	<1	<1
2,6-Dihydroxybenzoic	1.1	>500,000	4,500 ± 800

[a] Membrane-forming solution contained diphytanoyl phosphatidylcholine (31 mg/ml) in decane plus chlorodecane (50%, vol/vol).
[b] pK's are from Serjeant and Dempsey [34].

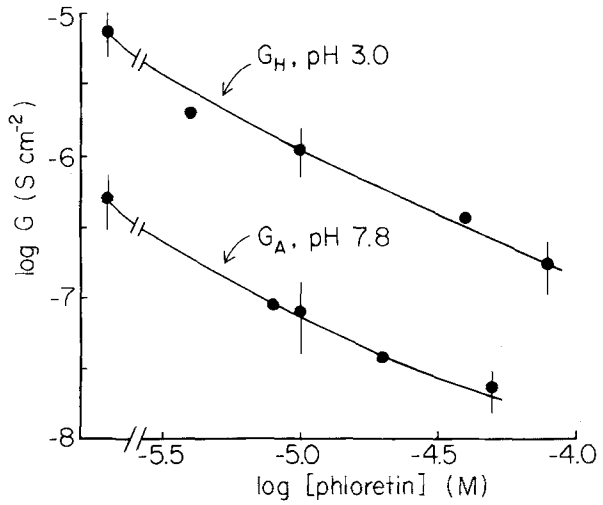

Fig. 4. Inhibition of salicylate-induced G_H and G_A by phloretin. The total salicylate concentration ([A$^-$] + [HA]) was 2.0 mM, and the solutions were buffered as described in the legend of Fig. 3. Phloretin (in ethanol) was injected either before or after membrane formation. Error bars indicate standard deviations of 2–4 membranes

permeability coefficient of salicylic acid (P_{HA}) is about 0.7 cm sec^{-1} [15], seven orders of magnitude higher than P_A.

Inhibition of G_H and G_A by Phloretin

Phloretin inhibited the salicylate-induced G_H and G_A, causing about 90% inhibition at 20 μM phloretin (Fig. 4). The primary effect of phloretin on bilayers is to decrease the membrane dipole potential, thus increasing cation conductance and decreasing anion conductance [1, 30]. Thus, the inhibition of both G_H and G_A by phloretin suggests that both H^+ and A^- transport are rate limited by the translocation of an anion. Phloretin also inhibits the conductances caused by other weak acids, e.g., 2,4-dinitrophenol [30], CCCP (carbonylcyanide *m*-chlorophenylhydrazone) [1, 30] and long-chain fatty acids [13].

Conductance-Voltage Relationships

Figure 5 shows the salicylate-induced conductance as a function of voltage for several different pH values. The conductances were normalized by taking the ratio of the conductance at voltage, V, to the conductance at 40 mV. Then G_V/G_{40} was plotted against membrane voltage. The results were compared to the predictions of a trapezoidal energy-barrier model, i.e.,

$$G_V/G_{40} = b \sinh(u/2)/\sinh(bu/2) \quad (3)$$

where b is the fraction of the membrane spanned by the minor base of the trapezoid and $u = FV/RT$ [17, 18].

At pH 1.8 to 3.8, when $G \simeq G_H$, the conductance-voltage curves were superlinear, falling in the range of $b = 0.6$ to 0.7, similar to some other weak acid proton carriers [3, 20]. However, at pH 6.4 to 7.8, when $G \simeq G_A$, the conductance was nearly constant up to at least 120 mV. Unfortunately, at pH > 7 salicylate caused the membranes to rupture at <140 mV. Nevertheless, the data suggest that G_A is less voltage sensitive than G_H.

Discussion

Mechanisms of H^+ and A^- Conductances Caused by Salicylates and Benzoates

Exposure of phospholipid bilayers to salicylate and salicylic acid at low pH causes a large increase in G_H, and G_H is maximum when pH is near pK (Fig. 2). G_H shows a quadratic dependence upon the total salicylate concentration (Fig. 3), and G_H is inhibited

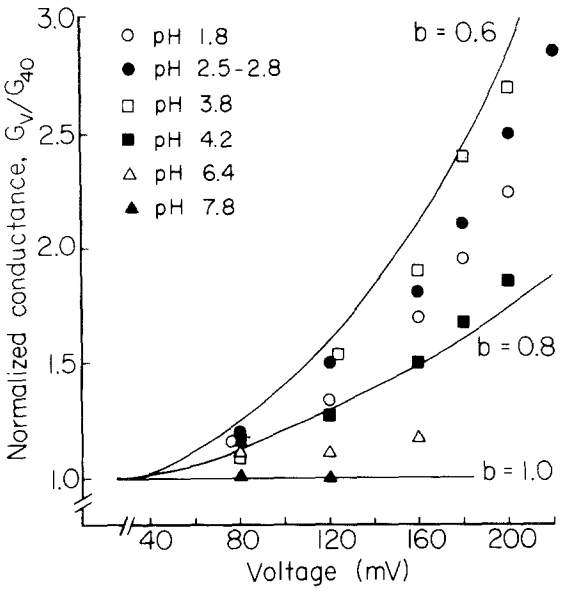

Fig. 5. Voltage dependence of salicylate-induced conductance at several pH values. Total salicylate concentrations ranged from 0.6 to 6 mM, and the solutions wre buffered as described in the legend of Fig. 2. The solid lines were calculated from Eq. (3), where b is the fraction of the membrane spanned by the minor base of the trapezoidal energy barrier. Each point is the average of measurements from 2–3 membranes. A few data points have been shifted slightly to prevent overlap

by phloretin (Fig. 4). At neutral to alkaline pH the membranes are A^- rather than H^+ selective, but G_A is lower than G_H, and G_A is independent of pH (Fig. 2). Finally, G_A is proportional to the first power of the salicylate concentration (Fig. 3) and is inhibited by phloretin (Fig. 4).

The characteristics of G_H are those expected for an HA_2^--type proton carrier, as exemplified previously by 2,4-dinitrophenol and several other weak acid "protonophores" [8, 22, 24, 26]. According to the HA_2^- model, reviewed by McLaughlin and Dilger [26], the rate limiting step in H^+ transport is the translocation of an anionic dimer, HA_2^-, formed by a combination of A^- and HA on or near the membrane surface. The HA_2^- model provides a simple explanation for the maximum G_H when pH = pK (Fig. 2) and the quadratic dependence of G_H on total salicylate concentration (Fig. 3). The inhibition of G_H by phloretin (Fig. 4) and the voltage dependence of G_H (Fig. 5) are also consistent with the HA_2^- model. The slight shift of the maximum G_H to the acid side of the pK (Fig. 2) can be explained by the titration of the phosphate groups at pH < 3 [36].

The characteristics of G_A are also consistent with the HA_2^- model, because when pH ≫ pK the only significant charge carrier is salicylate, a slightly lipid-soluble anion. Thus, G_A is lower than G_H (Fig. 2), independent of pH (Fig. 2), proportional to $[A^-]$ (Fig. 3) and inhibited by phloretin (Fig. 4).

The ability of salicylate to produce G_H and G_A is orders of magnitude greater than benzoate and aspirin (Table). Usually the addition of a hydroxyl group to a molecule decreases its membrane permeability due to the formation of additional hydrogen bonds with water, e.g., 3-hydroxybenzoate compared to benzoate (Table) [19]. However, in salicylate the location of the hydroxyl adjacent to the carboxyl group permits the formation of an internal hydrogen bond [29] which delocalizes the negative charge, reduces the pK from 4.1 to 3.0, and increases G_A by about 40-fold (Table). In 2,6-dihydroxybenzoate each hydroxyl group forms an internal hydrogen bond with an adjacent carboxyl oxygen [29], thus creating a "super-salicylate" which has a pK 2 units lower and a G_A 10-fold higher than salicylate (Table). In acetylsalicylate the internal hydrogen bond is absent and G_A is low, about the same as benzoate (Table). Thus, the permeabilities of the salicylates and benzoates are determined by both the nature and location of the substituent groups on the benzene ring.

Effects of Salicylates and Benzoates on Mitochondria

Salicylate acts as an uncoupler of oxidative phosphorylation [4, 16, 39] and causes rapid swelling of isolated mitochondria [40]. The ability of salicylate to cause mitochondrial uncoupling and/or swelling can explain some of the symptoms of salicylate poisoning [4, 9, 40], gastric mucosal injury [21] and Reye's syndrome, a "mitochondrial disease" characterized by impaired energy metabolism and swollen mitochondria [2, 16, 35, 40]. Benzoate, aspirin, 3-hydroxybenzoate and 2,6-dihydroxybenzoate are either less effective or ineffective in causing mitochondrial uncoupling or swelling [4, 39, 40].

Figure 6 shows a model for salicylate-induced uncoupling and swelling in mitochondria. The present results suggest that salicylate causes A^- conductance but not H^+ conductance at pH 7 to 8. However, the salicylic acid (HA) permeability is so high (0.7 cm sec^{-1}) [15] that HA rapidly equilibrates across the inner mitochondrial membrane even at pH 7.4[1]. Salicylate accumulates in the matrix due to

[1] If salicylate transport occurs primarily by HA diffusion and follows first-order kinetics, then the half time ($t_{1/2}$) for salicylate turnover in mitochondria will be less than 0.1 sec, as estimated from the relation, $t_{1/2} = 0.693V/AP_t$, where V/A is the ratio of matrix volume to inner membrane surface area (approx. 10^{-6} cm) [33] and P_t is the total salicylate permeability (approximately 10^{-5} cm sec^{-1}). i.e., P_{HA} multiplied by the fraction of HA at pH 7.4.

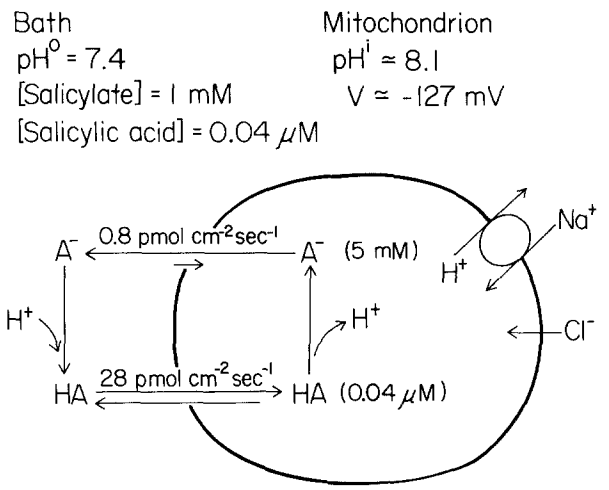

Fig. 6. Model for salicylate-induced uncoupling and swelling in mitochondria. Net H^+ uptake results from HA influx (driven by the [HA] gradient) and A^- efflux (driven by the voltage and $[A^-]$ gradients). Thus, the electrochemical H^+ gradient (protonmotive force) decreases, resulting in "loose coupling" at 0.5–1.0 mM salicylate [16] and complete uncoupling at 2–4 mM salicylate [4, 39]. Values of pH^o, pH^i, and V are those reported by Haas et al. [16] for isolated mitochondria exposed to 1 mM salicylate under state-4 conditions. In the absence of salicylate the pH^i and V were about 8.1 and −157 mV [16]. Osmotic swelling (due to NaCl uptake) occurs at >1.6 mM salicylate [40]. One-way HA fluxes were calculated from: $J_{HA} = P_{HA}[HA]$, assuming $P_{HA} = 0.7$ cm sec^{-1} [15]. The net efflux of A^- was calculated from: $J_A = G_A V/F$, assuming $G_A = 600$ nS cm^{-2} (Fig. 3) and a linear current/voltage relation at pH 7.4–8.1 (Fig. 5). The approximately equal bidirectional arrows for HA indicate near equilibrium conditions. The unequal arrows for A^- indicate that salicylate is far from equilibrium. Thus, salicylate efflux is the rate-limiting step in net H^+ uptake

the more alkaline pH, but salicylate is also driven out by the negative voltage and the $[A^-]$ gradient. Thus, the overall result is a net H^+ influx that is rate limited by A^- efflux across the inner mitochondrial membrane.

In Fig. 6 the values shown for pH^o, pH^i and V are those reported by Haas et al. [16] for state-4 mitochondria exposed to 1 mM salicylate. In addition to partially depolarizing the membrane, 1 mM salicylate causes a twofold increase in state-4 oxygen consumption and a fourfold increase in "H^+ conductance"[2], thus decreasing the efficiency of ATP production, a condition described as "loose coupling" [16]. Higher salicylate concentrations cause complete uncoupling of oxidative phosphorylation [4, 39]. Previously, McLaughlin and Dilger [26] described the "minimal uncoupling concentration" of a proton ionophore to be that producing about a twofold increase in state-4 respiration and a net H^+ uptake of about 0.4 pmol cm^{-2} sec^{-1}, in reasonable agreement with Haas et al. [16] as well as with the calculated net H^+ flux shown in Fig. 6. For comparison, therapeutic concentrations of free (unbound) salicylate in plasma usually range from 0.1 to 0.7 mM (total serum salicylate about 1.0 to 2.5 mM) [5, 9, 40]. In salicylate poisoning the plasma concentrations usually range from 3 to 8 mM [2, 5, 9, 32].

Salicylate also causes rapid osmotic swelling of isolated mitochondria [40]. This probably results from both the net H^+ uptake, which promotes H^+/Na^+ exchange, and the depolarization of the membrane, which promotes net Cl^- uptake [6]. Swelling and uncoupling do not occur with benzoate [4, 39, 40], apparently because G_A is too low (Table) to allow significant H^+ uptake via the mechanism shown in Fig. 6. Alternatively, the benzoic acid (HA) permeability might be too low to allow significant HA influx. However, this alternative is ruled out by the P_{HA} of 0.5 cm sec^{-1} [38], combined with the pK of 4.1, from which we predict that benzoic acid will equilibrate with mitochondria even faster than salicylic acid. Thus, the ineffectiveness of benzoate as both a swelling agent [40] and an uncoupler [4, 39] is due to its low P_A rather than its low P_{HA}. The relative ineffectiveness of aspirin [39] may have the same explanation (Table), although P_{HA} has not been measured.

The ineffectiveness of 2,6-dihydroxybenzoate probably has a different explanation. Although G_A produced by 2,6-dihydroxybenzoate is 10-fold higher than G_A produced by salicylate (Table), the pK is almost 2 units lower, which means that the HA concentration will be almost 100-fold lower at pH 7.4. Based on its partition coefficient into organic solvents [23], we expect the P_{HA} of 2,6-dihyroxybenzoic acid to be similar to that of salicylic acid. Thus, under the conditions shown in Fig. 6, 2,6-dihydroxybenzoic acid has a predicted HA influx of about 0.1 pmol cm^{-2} sec^{-1}, consistent with the observation that 2,6-dihydroxybenzoate is about 10% as effective as salicylate in uncoupling mitochondria [4]. A similar argument was invoked by McLaughlin and Dilger [26] to explain why picrate (2,4,6-trinitrophenolate) does not uncouple mitochondria.

In conclusion, salicylate behaves as an HA_2^--type proton carrier at low pH and as a lipid-soluble anion at neutral to alkaline pH. The salicylate con-

[2] The "H^+ conductance" defined by Haas et al. [16] (*see also* refs. 27 and 28) is the state-4 rate of oxygen consumption divided by the protonmotive force. For rat liver mitochondria the control (salicylate-free) value was about 10 nmol H^+ (mg protein)$^{-1}$ sec^{-1} $volt^{-1}$ [16], which corresponds to a specific conductance of about 0.5 μS cm^{-2} [27]. The "H^+ conductance" measured by all these workers includes both electroneutral and "electrogenic" H^+ uptake. Thus, the model shown in Fig. 6 is consistent with the reported "increase in H^+ conductance" caused by salicylate [16], even though the model shows no H^+ conductance *per se*.

ductance, operating in parallel with salicylic acid diffusion, can explain the concentration-dependent loose coupling, uncoupling and swelling caused by salicylate in mitochondria. The relative ineffectiveness of benzoate and aspirin can be explained by their low A^- permeabilities, and the ineffectiveness of 2,6-dihydroxybenzoate can be explained by its low pK. Since ingested aspirin breaks down rapidly to form salicylic acid, the results of this study should help to clarify the mechanisms of aspirin toxicity in humans.

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References

1. Andersen, O.S., Finkelstein, A., Katz, I., Cass, A. 1976. Effect of phloretin on the permeability of thin lipid membranes. *J. Gen. Physiol.* **67**:749–771
2. Aprille, J.R. 1977. Reye's syndrome: Patient serum alters mitochondrial function and morphology in vitro. *Science* **197**:908–910
3. Benz, R., McLaughlin, S. 1983. The molecular mechanism of action of the proton ionophore FCCP (carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone). *Biophys. J.* **41**:381–398
4. Brody, T.M. 1956. Action of sodium salicylate and related compounds on tissue metabolism in vitro. *J. Pharmacol.* **117**:39–51
5. Crandall, E.D., Winter, H.I., Schaeffer, J.D., Bidani, A. 1982. Effects of salicylate on HCO_3^-/Cl^- exchange across the human erythrocyte membrane. *J. Membrane Biol.* **65**:139–145
6. Cunarro, J., Weiner, M.W. 1975. Mechanism of action of agents which uncouple oxidative phosphorylation: Direct correlation between proton-carrying and respiratory-releasing properties using rat liver mitochondria. *Biochim. Biophys. Acta* **387**:234–240
7. Dilger, J.P., McLaughlin, J.G.A., McIntosh, T.J., Simon, S.A. 1979. The dielectric constant of phospholipid bilayers and the permeability of membranes to ions. *Science* **206**:1196–1198
8. Finkelstein, A. 1970. Weak-acid uncouplers of oxidative phosphorylation. Mechanism of action on thin lipid membranes. *Biochim. Biophys. Acta* **205**:1–6
9. Flower, R.J., Moncada, S., Vane, J.R. 1985. Analgesic-antipyretics and anti-inflammatory agents. *In*: The Pharmacological Basis of Therapeutics. (7th Ed.) A.G. Gillman, L.S. Goodman, T.W. Rall, and F. Murad, editors. pp. 674–715. Macmillan, New York
10. Forsyth, B.W., Horwitz, R.I., Acampora, D., Shapiro, E.D., Viscoli, C.M., Feinstein, A.R., Henner, R., Holabird, N.B., Jones, B.A., Karabelas, A.D.E., Kramer, M.S., Miclette, M., Wells, J.A. 1989. New epidemiologic evidence confirming that bias does not explain the aspirin/Reye's syndrome association. *JAMA* **261**:2517–2524
11. Gutknecht, J. 1987. Proton conductance through phospholipid bilayers: Water wires or weak acids? *J. Bioenerg. Biomembr.* **19**:427–442
12. Gutknecht, J. 1987. Proton/hydroxide conductance through phospholipid bilayer membranes: Effects of phytanic acid. *Biochim. Biophys. Acta* **898**:97–108
13. Gutknecht, J. 1988. Proton conductance caused by long-chain fatty acids in phospholipid bilayer membranes. *J. Membrane Biol.* **106**:83–93
14. Gutknecht, J. 1989. Proton transport caused by salicylates in phospholipid bilayer membranes: A model for salicylate-induced "loose coupling" in mitochondria. *Biophys. J.* **55**:568*a*
15. Gutknecht, J., Tosteson, D.C. 1973. Diffusion of weak acids through lipid bilayer membranes: Effects of chemical reactions in the aqueous unstirred layers. *Science* **182**:1258–1261
16. Haas, R., Parker, W.D., Stumpf, D., Eguren, L.A. 1985. Salicylate-induced loose coupling: Protonmotive force measurements. *Biochem. Pharmacol.* **34**:900–902
17. Hall, J.E., Mead, C.A., Szabo, G. 1973. A barrier model for current flow in lipid bilayer membranes. *J. Membrane Biol.* **11**:75–97
18. Hladky, S.B. 1974. The energy barriers to ion transport by nonactin across thin lipid membranes. *Biochim. Biophys. Acta* **352**:71–85
19. Joy, M.M., Cutler, D.J. 1987. On the mechanism of transport of salicylate and *p*-hydroxybenzoic acid across human red cell membranes. *J. Pharm. Pharmacol.* **39**:266–271
20. Kasianowicz, J., Benz, R., McLaughlin, S. 1984. The kinetic mechanism by which CCCP (carbonylcyanide *m*-chlorophenylhydrazone) transports protons across membranes. *J. Membrane Biol.* **82**:179–190
21. Kauffman, G. 1989. Aspirin-induced gastric mucosal injury: Lessons learned from animal models. *Gastroenterology* **96**:606–614
22. Lea, E.J.A., Croghan, P.C. 1969. The effect of 2,4-dinitrophenol on the properties of thin lipid films. *J. Membrane Biol.* **1**:225–237
23. Leo, A., Hansch, C., Elkins, D. 1971. Partition coefficients and their uses. *Chem. Rev.* **71**:525–616
24. McLaughlin, S. 1972. The mechanism of action of DNP on phospholipid bilayer membranes. *J. Membrane Biol.* **9**:361–371
25. McLaughlin, S. 1973. Salicylates and phospholipid bilayer membranes. *Nature (London)* **243**:234–236
26. McLaughlin, S.G.A., Dilger, J.P. 1980. Transport of protons across membranes by weak acids. *Physiol. Rev.* **60**:825–863
27. Mitchell, P., Moyle, J. 1967. Acid-base titration across the membrane system of rat-liver mitochondria. *Biochem. J.* **104**:588–600
28. Nicholls, D.G. 1982. Bioenergetics: An Introduction to Chemiosmotic Theory. Academic, New York
29. Pauling, L. 1960. The Nature of the Chemical Bond. (3rd Ed.) Cornell University Press, Ithaca, New York
30. Perkins, W., Cafiso, D.S. 1987. Procedure using voltage-sensitive spin-labels to monitor dipole potential changes in phospholipid vesicles: The estimation of phloretin-induced conductance changes in vesicles. *J. Membrane Biol.* **96**:165–173
31. Pinsky, P.F., Hurwitz, E.S., Schonberger, L.B., Gunn, W.J. 1988. Reye's syndrome and aspirin: Evidence for a dose-response effect. *JAMA* **260**:657–661
32. Rainsford, K.D. 1984. Aspirin and the Salicylates. Butterworths, New York
33. Scarpa, A. 1978. Transport across mitochondrial membranes. *In*: Membrane Transport in Biology. G. Giebisch, D.C., Tosteson, and H.H. Ussing, editors. Vol. 2, pp. 263–355. Springer-Verlag, New York
34. Serjeant, E.P., Dempsey, B. 1979. Ionisation Constants of Organic Acids in Aqueous Solution. Pergamon, New York
35. Stumpf, D.A. 1979. Mitochondrial multisystem disorders: Clinical, biochemical and morphologic features. *In*: Current Neurology. H.R. Tyler and D.M. Dawson, editors. Vol. 2, pp. 117–149. Mifflin Professional Publishers, Boston

36. Szabo, G., Eisenman, G., McLaughlin, S.G.A., Krasne, S. 1972. Ionic probes of membrane structures. *Ann. NY Acad. Sci.* **195:**273–290
37. Vane, J., Botting, R. 1987. Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB J.* **1:**89–96
38. Walter, A., Gutknecht, J. 1984. Monocarboyxlic acid permeation through lipid bilayer membranes. *J. Membrane Biol.* **77:**255–264
39. Whitehouse, M.W. 1964. Biochemical properties of anti-inflammatory drugs: III. Uncoupling of oxidative phosphorylation in a connective tissue (cartilage) and liver mitochondria by salicylate analogues. *Biochem. Pharmacol.* **13:**319–336
40. You, K. 1983. Salicylate and mitochondrial injury in Reye's syndrome. *Science* **221:**163–165

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