A New Model of Weak Acid Permeation through Membranes Revisited: Does Overton Still Rule?

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ABSTRACT According to a recent publication by Thomae, A. V., H. Wunderli-Allenspach, and S. D. Krämer (2005. *Biophys. J.* 89:1802–1811), membrane bilayers are well-permeable to the charged species of aromatic carboxylic acids. At physiological pH, the anions were claimed to be the major diffusing species. In contrast, calculation of the Born energy barrier predicts a 10^5-fold higher permeability for the uncharged (protonated) form. To test the new model, we now have measured both the current carried by the salicylate anion through solvent-free planar membranes and the amount of protons transported by the neutral species. The corresponding membrane permeabilities of the charged and protonated forms were 4×10^{-7} cm/s and 1.2 cm/s. These data are in perfect agreement with literature data gathered in the last three decades (compare, e.g., Gutknecht, J., and D. C. Tosteson. 1973. *Science.* 182:1258–1261). They indicate that the report by Thomae at al. represents an experimental artifact. The well-documented role of neutral species in the permeation process of weak acids and bases across artificial and natural membranes is not in question. Overton still rules.

Weak acids and bases constitute a class of pharmacologically important substances. Since most of them have an intracellular target, investigation of their membrane transport has attracted much attention in the past. At physiological pH most weak acids are anions, A^-, while their neutral form, AH, is much more membrane-permeable. Consequently, proton-transfer reactions in the immediate membrane vicinity play an important role in the permeation process (1,2). They include several steps: 1), the diffusion of A^- to the membrane; 2), proton uptake; 3), permeation of AH across the membrane; 4), dissociation; and 5), diffusion of A^- into the bulk.

Belonging to the class of weak acids as well, protonophores (uncouplers of oxidative phosphorylation) permeate membranes by essentially the same mechanism (3). Kinetic modeling always revealed that the rate constant for the backward movement of the anionic form is several orders-of-magnitude smaller than the rate constant of the neutral form. For example, the respective numbers for carbonyl cyanide *m*-chlorophenylhydrazone amount to 175 s^{-1} and $12,000 \text{ s}^{-1}$ in chlorodecane-containing membranes (4). Without the increase of membrane dielectric permittivity, ε_m, mediated by the polar solvent, the difference in the permeabilities would have been even larger. In the case of fatty acids the difference rate constants of AH and A^- is extremely large. They translocate through lipid bilayers exclusively in their protonated form because of the low anion permeability (5,6).

The difference in the transport rates for AH and A^- has been attributed to the energetic barrier by which any membrane opposes the movement of charged entities. In addition to the neutral energy term that is equal for AH and A^-, the interaction of A^- with lipid bilayers is characterized by the electrical Born, image, and dipole contributions (7).

The Born energy barrier is a function of ion radius, a, and ion charge, q,

$$\Delta G_{\text{Born}} = -\frac{q^2}{8\pi\varepsilon_0 a}\left(\frac{1}{\varepsilon_m} - \frac{1}{\varepsilon_w}\right), \quad (1)$$

where ε_w is the dielectric constant of water. Thus, the partition of the salicylate anion with $a = 1.25$ nm (8) into the membrane costs 6.7 kcal mol^{-1}. Since the partition coefficients are by far the major determinants of the relative magnitudes of the permeability coefficients (9), the ratio, r, of AH and A^- permeabilities, $P_{m,\text{AH}}$ and $P_{m,A-}$, can be estimated using the Boltzmann distribution:

$$r = P_{m,\text{AH}}/P_{m,A^-} = e^{\Delta G/kT} = 73,000. \quad (2)$$

In contrast to the prediction made by Eq. 2, Thomae et al. (10) claimed $r = 66$ for salicylic acid (SA). Theoretically, the divergence may be attributed to image and dipole contributions, which are ignored in Eq. 2. However, for an uncharged lipophilic substance, the reported $P_{m,\text{AH}}$ of 3.5×10^{-7} cm/s is unreasonably low. It violates the Overton rule. The SA octanol/water partition coefficient of 300 (10) suggests $P_{m,\text{AH}} \sim 1$ cm/s. This is in line with $P_{m,\text{AH}} = 0.7$ cm/s determined earlier (11).

Strikingly enough, the ratio of the two experimental $P_{m,AH}$ values ($0.7 : 3.5 \times 10^{-7}$) is close to r from Eq. 2. Even more surprising is that the $P_{m,AH}$ reported by Thomae et al. (10) corresponds to the membrane permeability one would expect for a charged species according to the Overton rule. A possible explanation for this coincidence is offered by the fact that Thomae et al. (10) have monitored SA transport into buffer-free vesicles. The accompanying proton transport is likely to lower intravesicular pH. In turn, the resulting pH gradient opposes acid diffusion. This phenomenon is very well known and the reverse effect has widely been used to load vesicles (12,13).

Exceptions to the Overton rule are rare (14). They are mostly due to proteinaceous transporters (15) or membrane phase transitions (16). To clarify whether Thomae et al. (10) have discovered a new exception or whether Overton still rules, we simultaneously monitored AH and A^- permeations across solvent-free planar lipid bilayers (17) by electrochemical microscopy and current measurements under voltage-clamp conditions. The former method makes use of scanning pH-sensitive microelectrodes (18,19). Small changes in proton concentration close to the membrane surface are detected that accompany weak acid transport. As expected, the pH shift adjacent to the membrane depended on bulk pH (Fig. 1). Calculation of hydrogen ions flux, j_p, were performed according to the equation (20,21)

$$j_p = j_H + j_{OH} + j_B$$
$$= D_H \frac{\Delta[H^+]}{\delta_H} + D_{OH} \frac{\Delta[OH^-]}{\delta_{OH}} + D_b \frac{b \times \Delta[H^+]}{\delta_b}, \quad (3)$$
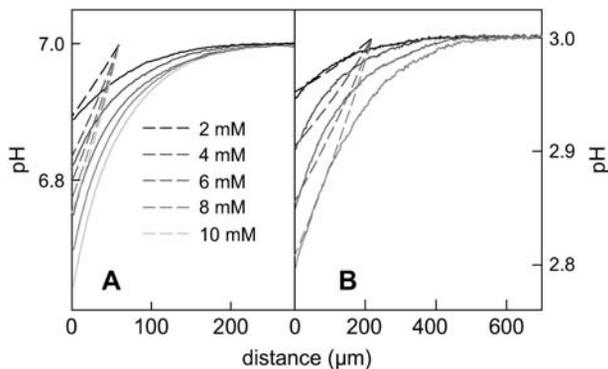
FIGURE 1 Acidification of the *trans* aqueous layers in the immediate vicinity of a planar membrane at different concentrations of sodium salicylate added at the indicated concentrations to the *cis* compartment at pH 7 (*A*) and at pH 3 (*B*). Dotted lines correspond to theoretical calculations with the following parameters: $P_{m,AH} = 1.2$ cm/s, $pK_{a,salicylate} = 2.75$, $pK_{a,HEPES} = 7.5$, $D_{salicylate} = 5.5 \times 10^{-6}$ cm^2 s^{-1}, and $D_{HEPES} = 5 \times 10^{-6}$ cm^2 s^{-1}, with other parameters as in Antonenko et al. (20). Planar bilayer lipid membranes were folded from diphytanoyl-phosphatidyl-choline (Avanti Polar Lipids, Alabaster, AL) monolayers. They were spread across a circular hole (0.15 mm in diameter) in a diaphragm separating two aqueous phases of a Teflon chamber. The aqueous 0.2 M NaCl solutions contained 3 mM HEPES, 3 mM β-alanine, and 0.1 mM sodium salicylate.

where D_H, D_{OH}, D_b, δ_H, δ_{OH}, and δ_b denoted the aqueous diffusion coefficients and individual unstirred fluid layer thicknesses of H^+, OH^-, and buffer, respectively. The value b was the buffer capacity of aqueous solutions. At pH 7, the contribution of the two first terms can be neglected. The value j_p amounts to 2×10^{-11} mol cm^{-2} s^{-1} at 2 mM SA.

Assuming that all permeating acid molecules are protonated at one side of the membrane and deprotonated at the other, j_p allows determination of the lower $P_{m,AH}$ limit:

$$P_{m,AH} = \frac{j_p}{[AH]} = 0.2 \, \text{cm s}^{-1}. \quad (4)$$

$P_{m,A-}$ is very low, as revealed by the lack of an increment in electrical conductivity after addition of salicylic acid at pH 7 (Fig. 2). The $pK_a = 2.75$ indicates that at pH $= 3$, the transmembrane concentration gradient of A^- exceeds that of AH nearly twofold. Consequently, Eq. 2 predicts a j_{AH}/j_{A-} ratio of 36,500. Based on $j_p = 10^{-9}$ mol cm^{-2} s^{-1} measured at 2 mM SA, $j_{m,A-} = 2.8 \times 10^{-14}$ mol cm^{-2} s^{-1} is expected. From the experimentally observed increase in current, I, an anion flux, $j_{m,A-}$, of

$$j_A = \frac{RT}{z^2 F^2} G = 5 \times 10^{-14} \, \text{mol cm}^{-2} \text{s}^{-1} \quad (5)$$

is derived, where z and F have their usual meanings. Thus, the rough estimate based solely on Born energy works reasonably well. The absolute value of $P_{m,A-}$ amounts to 4×10^{-7} cm s^{-1}. Thus, our $P_{m,AH}/P_{m,A-}$ ratio obtained for salicylic acid is comparable to experimental data available for other weak acids. For example, the flip-flop rate constants of long-chain fatty acid anions vary between 1×10^{-6} and 49×10^{-6} s^{-1} (22), whereas the protonated forms are transported at rates between 0.1 s^{-1} (23) and 15 s^{-1} (24). The increase in the salicylate-mediated conductivity at low pH is consistent with previous data (25).
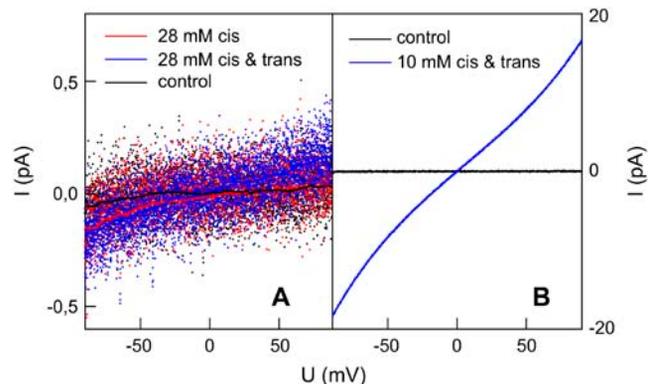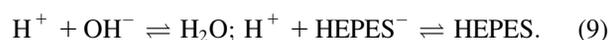

FIGURE 2 I-V curves of diphytanoylphosphatidylcholine membranes at pH 7 (*A*) and at pH 3 (*B*) at different concentrations of sodium salicylate added at the indicated concentrations.

Due to the small $P_{m,A}$ value, the anion flux may be neglected. For pH < 8, the experimental results are analyzed precisely by solving the complete system of differential equations which takes into account all relevant chemical reactions in the immediate membrane vicinity (18,19),

$$J_i = -D_i dc_i/dx_i, \ i = 1, \ldots, 6, \tag{6}$$

$$dJ_i/dx = R_i(c), \ c = (c_1, \ldots, c_6), \tag{7}$$

where J_i, D_i, and $c_i(x)$ denote, respectively, the flux, diffusion coefficient, and concentration of the ith species, and where $1 = H^+$; $2 = C_6H_4OHCOOH$; $3 = C_6H_4OHCOO^-$; $4 = OH^-$; $5 = T^-$; and $6 = TH$ (T is the buffer molecule). $R_i(c)$ is the specific local rate of expenditure of the ith species in the chemical reactions:

$$H^+ + C_6H_4OHCOO^- \rightleftharpoons C_6H_4OHCOOH, \tag{8}$$

$$H^+ + OH^- \rightleftharpoons H_2O; \ H^+ + HEPES^- \rightleftharpoons HEPES. \tag{9}$$

At the membrane-water interface, the fluxes of all species are required to be equal to zero, except for J_2, so that

$$J_1 = J_3 = J_4 = \ldots = J_8 = 0; \quad J_2 = J. \tag{10}$$

The numerical solutions are derived, assuming that the rates of chemical reactions (like dissociation/recombination of water, buffer, and SA) are very high compared to the rate of diffusion through the unstirred fluid layer, so that the local chemical equilibrium is maintained (18,19). The best agreement between model and experiment was achieved for $P_{m,AH}$ = 1.2 cm s^{-1} (Fig. 1). It should be noted that setting $P_{m,AH}$ to 3.5×10^{-7} cm/s as reported by Thomae et al. (10) resulted in the lack of a pH shift in the membrane vicinity. The discrepancy with the experiment indicated that the result of Thomae et al. is not correct.

Thus, salicylate transport across membranes is well described by the pH-partition mechanism assuming that only the protonated species diffuses through the membrane. At physiological pH, lipid bilayer permeation of the acid anion is negligible. The result agrees well with negligible partitioning of the charged species into *n*-octanol. Overton still rules. The energetic barrier imposed by partitioning of a charged species into the membrane still holds—in contrast to the report by Thomae et al. (10).

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