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Entry of Organic Acid Anions into Roots

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Abstract. Entry of formate, acetate, succinate, and salts of several other organic acids into barley roots takes place chiefly as ions from solutions at pH 5 to 7. The results are in accord with entry of the organic acid predominantly at pH 5 as ions through hydrophylic regions of the limiting membranes rather than by distribution of the undissociated acid to lipid-rich regions, as has previously been held. These results with a plant tissue parallel observations of others indicative of similar hydrophylic properties of membranes from animals relative to transfer of ions and small nonelectrolytes.

Introduction. Early studies of Overton and Collander, and recent ones of others[1, 2] show that permeation of many nonelectrolytes into cells is correlated with lipoidal solubility. Such permeation is one basis for the well-supported Davson-Danielli model of many cellular membranes as double lamella containing lipids and proteins.[3] Weak acids are also thought to enter plant tissues chiefly in the unionized form by nonmediated distribution to the lipid of the membranes.[4, 5] The unionized weak acids are known to have physiologic, plasmolytic, or toxic effects consistent with such a distribution. These effects, which often bear only indirectly on the question of entry of a compound, are a basis for the widely held impression that the acid anion enters plant tissue to a negligible extent compared with the unionized form.[5, 6] In contrast to these views, we find by direct measurements of uptake of a number of isotopically labeled acids by barley roots (Table 1), that small organic anions are the dominant entrant form in acidity ranges tolerated by roots.

The fact that the unionized acid molecules have physiologic effects, which could arise from distribution to lipid, while the acid anion is the dominant entrant form can be interpreted by a current elaboration of the Davson-Danielli membrane model. The modified model assumes predominant lipid-protein lamellar regions which include minor hydrophylic areas where mediated transfer of ions is likely, as well as some transfer of small nonpolar molecules.[1] The model is in harmony with electron micrographs of plant tissues (reviewed in ref. 7) which show both a double lamellar membrane structure and "substructural complexity" of particles on or within the lamella. Our measurements of entry of organic acid anions into barley roots indicate "equivalent pore"[8] or hydrophylic region[1] "diameters" of the order of 8A. These "diameters" are near those deduced for several animal tissues from measurements of reflection coefficients for nonelectrolytes.

Materials and Methods.—The roots used were from 6-day-old barley seedlings which had been dark-grown in aerated 2×10^{-4} *M* $CaSO_4$, pH 5.6, at 25°.[9] In the uptake experiments, 0.5 gm washed excised roots were held for 0.25 to 24 hr in large volumes (100–1000 ml) of aerated ^{14}C-organic acid solutions to minimize changes in concentration and pH. The pH was adjusted with dilute NaOH or KOH as needed on aliquots, which were discarded. The ^{14}C contents of the roots after treatment and rinsing were measured by scintillation counting with close attention to quenching. A sample was placed in 15 ml of the scintillation medium and allowed to equilibrate overnight. Counts of ^{14}C in the medium were made after removal of the roots. The residual ^{14}C in the roots was obtained by comparison following the same procedure with roots of known ^{14}C-content of the given acid. The known contents were established by measurements of ^{14}C disappearing from solutions with 0.05 root weight/solution volume ratio.

The roots were recombined with the counting medium evaporated to dryness, and heated to remove naphthalene derived from the scintillation medium. K^+, Na^+, Ca^{2+}, and Cl^- were extracted from the residue by refluxing with H_2O and finally by standing in a HNO_3-acetic acid solution. The extracts were combined and diluted to 25 ml with adjustment to 10% acetic and 0.1 *N* nitric acids. Aliquots were analyzed for Cl^- conductimetrically, and for K^+, Na^+, and Ca^{2+} by flame or atomic absorption photometry. Treatment solutions were also analyzed to assure against appreciable concentration changes. $^{14}CO_2$ evolved from the roots during treatments was trapped in 0.1 NaOH, aliquots of which were measured in the scintillation medium neutralized by addition of glacial acetic acid. Uptake is defined as the ^{14}C content of the roots plus the amount of ^{14}C evolved.

Buffering capacities were determined by grinding duplicate 2-gm root samples in water immediately after treatment with final dilution to 25 ml. One of the homogenates was rapidly titrated to pH 2.5 with standardized HCl and the other to pH 11.5 with KOH. The particular pH values were selected to be well outside the range where dissociation of the weak acids and bases involved were changing appreciably. Water blanks were titrated similarly and buffering capacity was calculated from differences between the water and sample titrations. Volume changes during the titration were less than 25%. Equality of back titrations with KOH and with HCl indicated absence of appreciable bicarbonate.

Results. Monocarboxylic acids: Roots in 10^{-2} eq/liter of 2-^{14}C acetate or ^{14}C formate at pH 5 (Fig. 1) show an initially rapid increase in ^{14}C. A 20 per cent free space in the roots would contain 2 μeq/gm and such retention would be reduced by rinsing to a negligible value in this, as well as in the other experiments. Evolution of $^{14}CO_2$ is at a low steady rate for > six hours (Fig. 1) despite a decrease in ^{14}C content of the roots after two hours. Comparable results were obtained with 10^{-2} eq/liter ring-labeled ^{14}C-benzoate, and 2-^{14}C propionate at pH 5 (Table 1), with little difference in amounts of the acids ultimately retained by the roots.

Initial ^{14}C accumulation rates for the several acids are 20–25 per cent lower at pH 7 than at pH 5 (Table 1). The rates, however, diminish comparatively little with time at pH 7, so that the roots ultimately accumulate much more ^{14}C than at pH 5, where accumulation is limited because of injury induced by the particular unionized acid (Fig. 1).

The effect of pH on uptake of the monocarboxylic acids was studied also at salt concentrations $<10^{-2}$ eq/liter, which avoids evident injury. Typical results are shown by formate uptake rates from 10^{-3} eq/liter sodium formate (Fig. 2). The treatments were for six hours, during which time formate and Na^+ uptake rates were constant. Evolution of $^{14}CO_2$ decreases by <2 per cent as the

pH is changed from 5.0 to 7.0. Losses of Ca^{2+} (1.4–1.9 μeq/gm from 3.8 μeq/gm present initially), and of K^+ (1.0–1.8 μeq/gm from an initial 14 μeq/gm) are independent of pH and are near the values for Cl^- salt solutions. Sodium uptake rates increased 20–30 per cent (15.0–20.7 μeq/gm taken up in 6 hr) at pH's above 5.5, an increase which is similar to that in treatments with chloride salts.

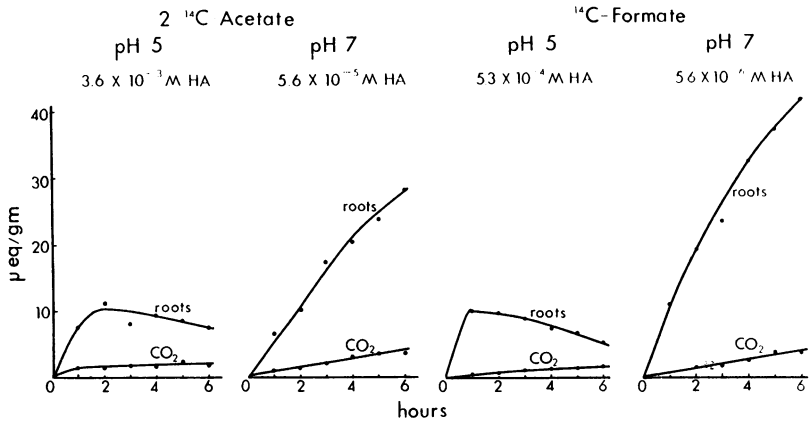

FIG. 1.—Entry 2-^{14}C-acetate and ^{14}C-formate into barley roots at pH 5 and 7 from solutions of 10^{-2} eq/liter of the sodium salts. The calculated concentrations of the undissociated acid, HA, and the observed evolution of $^{14}CO_2$ are also shown.

Decreases in initial rates of formate uptake at pH 6.4 compared to the rates at pH 5 are the same over a wide range of formate concentrations (Table 2). The inhibition is about 29 per cent irrespective of concentration. The formate uptake rates were calculated from linear time course data for each treatment. They were constant for 6 hours in solutions from 10^{-4} eq/liter to 10^{-3} eq/liter formate and for 2 to 3 hours at higher concentrations.

Uptake of amino acids and citrate: The roots take up D,L-alanine, aspartate, and glutamate, as well as citrate, at both pH 5 and 7, without evident injury,

TABLE 1. *Comparison of initial uptake rates of chloride and several organic and amino acids by barley roots.**

	Approximate Molecular Dimensions (A°)			Initial Uptake Rates (mμmoles/min-gm) pH 5	pH 7	pH 7/pH 5 (%)
Chloride	3.6	3.6	3.6	170	175	103
Formate	3.4	5.4	3.4	183	139	76
Acetate	3.4	5.4	5.2	134	110	81
Propionate	3.4	5.4	6.8	95	70	73
Benzoate	3.4	5.4	8.3	52	40	77
Alanine	4.7	5.4	6.8	228	193	85
Aspartate	4.7	5.4	8.6	128	108	84
Glutamate	4.7	5.4	9.9	107	89	83
Succinate	3.4	5.4	8.6	113	71	63
Citrate	8.0	5.6	9.9	73	62	84

* The solutions are 10^{-2} *M* Na^+ or K^+ salts. Rates were measured over periods of 1 to 6 hr. Molecular dimensions (diameters) are those of principle axes of a dimensional ellipsoid estimated from relative measurements of molecule scale models based on intermolecular distances in crystals. The amino acid salts were unresolved with respect to optical form (D,L).

at rates which are constant for six hours or longer. Uptake of the amino acids eventually exceeds Cl^- accumulation although Cl^- uptake is faster initially than uptake of any of these compounds except alanine. Uptake of the amino acids or citrate decrease by less than 20 per cent with the pH change from 5 to 7 (Table 1).

Succinate uptake: Roots in 10^{-2} eq/liter of 2,3 ^{14}C-succinate at pH 5.0 and 7.0 show a constant rate of uptake for >6 hours (Fig. 3). Decrease in the rates from 10^{-3} eq/liter solutions of the Na^+ salt with increasing pH closely follows the relative concentration of the monobasic anionic species, HA^- (Fig. 4). As with formate, Na^+ uptake from succinate solutions, increases 30 per cent over the pH range similar to Na^+ uptake from chloride salts. The pH has no effect on the slight Ca^{2+} loss from the roots (1.3–1.7 μeq/gm loss in 6 hr from an initial content of 5.6 μeq/gm). There is no K^+ efflux (a measure of injury) at any pH over the six hours of treatment. Thus, greater decrease in uptake of succinate than in any of the other acids with increasing pH is primarily an effect on the monobasic succinate anion concentration.

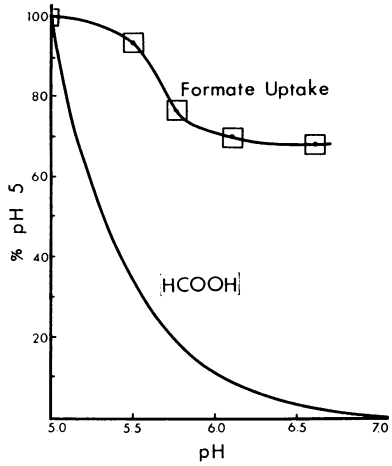

FIG. 2.—The relative effects of pH changes on the undissociated formic acid concentrations and on ^{14}C formate uptake rates from solutions of 10^{-3} eq/liter of the sodium salt. Uptake was measured over 6-hr periods.

Possibility of a succinate ion species effect was further tested by measurements of uptake rates over a range of succinate concentrations from 10^{-4} to 10^{-2} *M* at pH 5 and 6.7 (Table 3). The higher pH markedly inhibits uptake at low concentrations and the inhibition lessens as the succinate concentration of the external solution increases. The observed increases in pH 6.7/pH 5 uptake ratios follow the trend of calculated ratios of HA^- uptake; however, the observed percentage effects are all greater than expected if HA^- were the only species involved and the pH effect solely due to the lower HA^- concentration at pH 6.7. These differences are in accord with uptake of divalent succinate, A^{2-}, the concentration of which is about nine times the HA^- concentration at pH 6.7.

Buffering capacity measurements: The ^{14}C-organic and ^{14}C-amino acid retentions can be as large as the total organic and free amino acid contents of the roots, which total about 60 to 80 μeq/gm. Such increases would be expected to result in greater buffering capacity of the roots. Furthermore, relative content of titratable base (A^-) and acid (H^+ or HA) could reflect relative amounts of weak acid and weak acid anions accumulated. This expectation was realized (Table 4).

Buffering capacity of roots maintained in water for six hours does not change. Total buffering capacity ($A^- + H^+$) of roots maintained in sulfate (Table 4) also does not change appreciably from the initial value of 160 μeq/gm. Decrease in titratable acid (H^+) is balanced by an increase in titratable base (A^-), as ex-

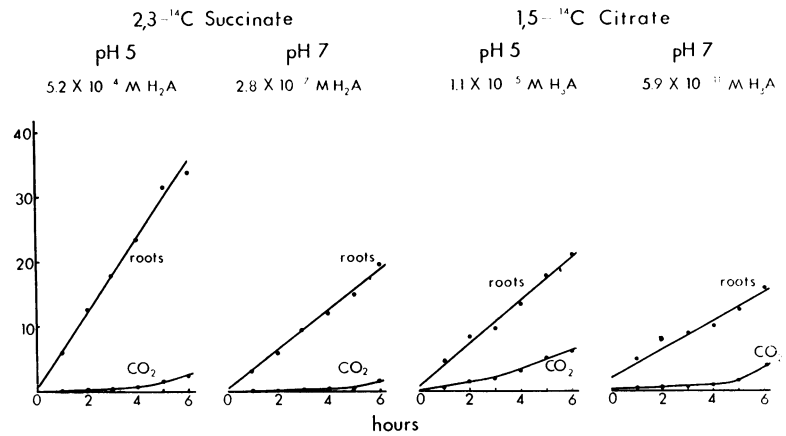

FIG. 3.—Entry of 2,3-^{14}C-succinate and 1,5-^{14}C-citrate into barley roots at pH 5 and 7 from solutions of 10^{-2} eq/liter of the sodium salts. The calculated concentrations of the undissociated acid, H_2A and H_3A, and the observed evolution of CO_2 are shown.

pected from cation uptake in excess of sulfate uptake, in exchange for H^+[9]. In succinate at pH 5, buffering capacity increases by an amount that is equal to the measured uptake of 37.8 μeq/gm succinate in the roots. A^- and H^+ increase by the same amount, consistent with uptake of monobasic succinate anions, HA^-; i.e., for every mole of succinate taken up, one mole of H^+ also appears. No loss of titratable H^+ would be expected from excess cation -H^+ exchange because cation accumulation and ^{14}C- succinate uptake in the roots are essentially equal at pH 5. At pH 7, the titratable H^+ decrease (-16 μeq/gm) is very close to the cation accumulation in excess of succinate (36.4–17.7 = 18.7 μeq/gm) and the titratable anion increase is equal to the amount of succinate taken up plus the H^+ loss. Thus, most of the succinate taken up at pH 7 appears to be the divalent anion species. H^+ taken up as monobasic succinate seems not to be appreciably more than the 2.7 μeq/gm difference (if real) between excess cation accumulation and the H^+ titration decrease (18.7–16.0 μeq/gm).

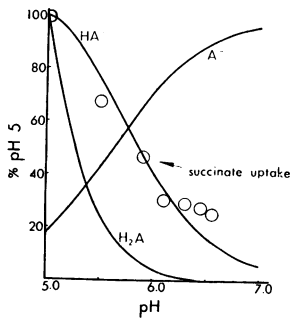

FIG. 4.—The relative effects of pH changes on succinate species, A^{--}, HA^-, and H_2A concentrations and ^{14}C-succinate uptake rates (○) by barley roots from solutions of 10^{-3} eq/liter of the sodium salt.

Total buffering capacity of roots in acetate at pH 7 increases by 26 μeq/gm; which is close to the amount of acetate entering the roots (Table 4). Acetate taken up appears predominantly as an increase in titratable anions, with the accompanying excess cation uptake reflected by a H^+ decrease balanced by part of the anion increase.

Discussion. Uptake of the monobasic acid anion, A^-, greatly exceeds that of the undissociated acid, HA, at pH 6.4. This is evident for formate from re-

sults listed in Tables 1 and 2. An 83-fold increase in formate concentration at pH 5.0 or 6.4 increases uptakes by about 5-fold (Table 2). The $[A^-]/[HA]$ ratio is, of course, constant as the concentration is shifted at constant pH. If the pH is decreased at constant concentration, however, [HA] increases in about the same ratio as the acidity (Fig. 2). Thus, [HA] increases by 95-fold when the pH is decreased from 7.0 to 5.0. Such a change increases uptake by only 1.3-fold (Table 1). Accordingly, HA uptake, at pH 6.4 cannot be more than a minor part of the total uptake.

The small increase in formate uptake with decrease in pH at constant concentration might arise from or be influenced by any of three factors; namely, the increase in [HA], competition of A^- uptake with H^+ or OH^-, and modification of the membranes involved. Involvement of the last of these is shown by the de-

TABLE 2. *Formate uptake by barley roots as a function of sodium formate concentration.*

Solution concentrations (eq/liter)	Initial Formate Uptake Rates		
	pH 5.0	pH 6.4	pH 6.4/pH 5.0
	(mμeq/min-gm)		(%)
1.2×10^{-4}	36	26	71
3.1×10^{-4}	52	33	62
6.0×10^{-4}	83	59	72
1.0×10^{-3}	112	82	74
2.8×10^{-3}	144	100	69
5.3×10^{-3}	162	128	79
1.0×10^{-2}	185	135	73

TABLE 3. *Succinate uptake by barley roots as a function of sodium succinate concentration.**

Solution concentration (eq/liter)	Succinate Uptake Rates			
	pH 5.0	pH 6.7	pH 6.7/pH 5.0	
	(mμeq/min-gm)		Observed	Calculated*
			(%)	
5.9×10^{-4}	13.4	2.8	21	12
1.3×10^{-3}	24.0	6.6	28	13
2.4×10^{-3}	38.1	12.8	34	15
6.4×10^{-3}	65.5	20.4	42	23
1.2×10^{-2}	92.2	42.6	47	32

* Calculated pH 6.7/pH 5.0 uptake ratios are from the uptake expected at pH 6.7, calculated on the basis that only singly charged succinate anions are taken up at either pH, from two Michaelis-Menten equations which fit the data at pH 5.0.

TABLE 4. *Comparison of buffering capacity changes in barley root homogenates with anion and cation changes.**

Treatment	ΔA^- (HCl titration)	ΔH^+ (KOH titration)	Δ Anions	$\Delta\Sigma$ ($K^+ + Na^+ + Ca^{2+}$)
	(μeq/gm roots)			
Sulfate				
pH 5	+9	−12	+12.0	+21.9
pH 7	+14	−8	+8.0	+22.8
Succinate				
pH 5	+20	+19	+37.8	+35.5
pH 7	+33	−16	+17.7	+36.4
Acetate				
pH 7	+39	−13	+28.1	+45.5

* Roots were in 10^{-2} eq/liter K^+ or Na^+ salts for 6 hr.
Initial contents (μeq/gm) were: 54 A^-, 106 H^+, 160 $\Sigma(A^- + H^+)$, 20.6 $\Sigma(K^+ + Na^+ + Ca^{2+})$ μeq/gm roots.

tail in the change of formate uptake over the pH range of 6.6 to 5.0 (Fig. 2). About two-thirds of the total change takes place between pH 6.0 and 5.5. This indicates operation of some factor other than one of the first two, both of which would change monotonously with change in $[H^+]$. In the case of formate, $[A^-]$ decreases only slightly (<5%) between pH 7 and 5. This apparently is reflected by the approximate constant uptake of formate over the pH range of 6.6 to 6.05 (Fig. 2).

The kinetics (see refs. 10 and 11 for treatments of kinetics of salt uptake) of formate uptake indicates that the uptake of A^- also greatly exceeds HA at pH 5.0. The uptake rates (v) at pH 5.0 can be fitted by two Michaelis-Menten type linear equations each of the form, $v = {}^-vK_m/[\text{formate}] + V_{max}$ as shown by the uppermost curve in Figure 5. Two other equations give the intermediate curve in accord with uptake rates at pH 6.4. The curves are parallel in the polar diagram for concentration (Fig. 5). This situation indicates that values of K_m do not change appreciably with pH. The concentration of the species taken up can be expressed as [Formate] X (a constant) over the pH range 6.4–5.0. The value of [HA] changes by 19-fold between pH 6.4 and 5.0 while $[A^-]$ changes by only 5 per cent. The results accordingly indicate that A^- is the dominant species taken up at both pH 5.0 and 6.4. Absence of any appreciable uptake of HA is also shown in another way. The parameters K_m and V_{max} for the equations at pH 5.0 and values for [HA] are used to calculate uptake at pH 6.4 assuming that only HA is taken up. The curve calculated on this basis is the lowest one in Figure 5, which is not at all close to the observed values.

The increase in formate uptake with decrease in pH might suggest, at first sight, that it is a result of increasing HA uptake. The kinetics, however, show that V_{max} values change while K_m values are constant, corresponding to uncompetitive inhibition of A^- uptake. The other acids are similar to formate in this respect as indicated by the approximate constancy of the initial uptake ratios for pH 7.0 and 5.0.

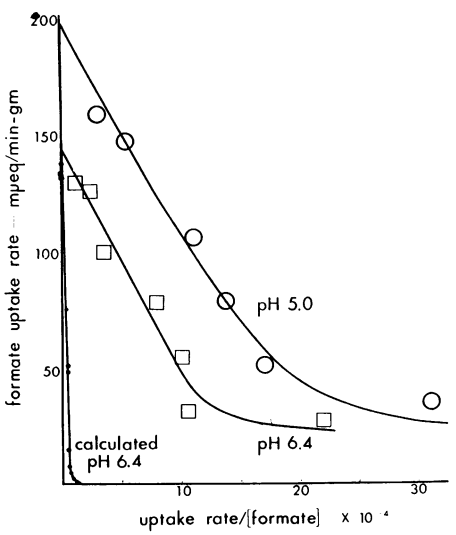

FIG. 5.—Michaelis-Menten representation of rates of formate uptake at pH 5.0 and 6.4 by barley roots. Calculated pH 6.4 values were based on the parameters, K_m and V_{max}, at pH 5, and the assumption that only the undissociated acid HA is taken up.

Evidence that the undissociated acid does not contribute appreciably to uptake in the region of pH 5 to 7 is also clear-cut for the dibasic acid, succinic. Results in Table 3 show that the succinate uptake at a given pH is approximately proportional to the concentration below 1.3×10^{-3} eq/liter. The decrease of succinate uptake at 1.0×10^{-3} eq/liter for various pH's relative to that at pH 5.0 (Fig. 4) closely follows the relative change in $[HA^-]$ between pH 5 and 6, but deviates greatly from the relative changes in $[H_2A]$.

This indicates that uptake of HA^- greatly exceeds H_2A. With an increase of pH above 6, the succinate uptake deviates upward from the [HA^-] curve (Fig. 4) as would be required if uptake of A^{--} is becoming evident.

Changes in buffering capacity agree with entries of the acid anions. Three possibilities need to be considered; (1) HA in, (2) HA in, H^+ out, cation$^+$ in, and (3) A^- in, cation$^+$ in. The observed uptake of cations equivalent to acetate eliminates (1). Possibilities (2) and (3) lead to the same final internal compositions. In (2), however, entry of HA must be accompanied by loss of H^+ equivalent to HA uptake. If this is valid, entry of an unionized acid would have to cause equivalent loss of H^+. That this does not happen is shown by uptake of succinate, HA^-, where H^+ equivalent to HA^- is retained.

Uptake rates of the organic acid anions by barley roots decrease with decrease in the hydrophylic character of the anion and with increase in molecular size (or weight) in a homologous series of compounds (Table 1). Entrance falls off rapidly over the range of dimensions bracketed by formate and propionate. The results (Table 1) are in qualitative accord with some hydrophylic properties of the limiting membranes adequate to account for entry of ions having diameters of <10 A. These findings with barley roots parallel ones obtained for diffusion exchange of similar anions with Cl^- ions in erythrocytes at pH 7.4.[12] They are in accord with currently developing concepts of membrane structure. These concepts are figured by Branton[7] as a double lamellar lipid-protein membrane, of the Davson-Danielli type, with protein elements or hydrophylic aggregates distributed between the lamella. His suggestions are based on the presence of particle-like features on electron micrographs of surface replicas of freeze etched and fractured plant tissues. Similar concepts are discussed by Wright and Diamond[1] in explaining the anomalously high permeation of small nonelectrolytes through epithelial tissues of rabbit gall bladders. They state: "According to this picture... the anomalous permeability component should depend mainly on size and not on partition coefficient..." The functional aspect of the model are much the same as implied by the "equivalent pore" model of others[8, 13–15] in dealing with transport of water and small nonelectrolytes having moderate solubility in water. The terms "equivalent pores" and "hydrophylic" region are essentially synonymous. The terms merely recognize the hydrophylic functioning of predominantly hydrophobic membranes.

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