Diffusion of Weak Acids across the Toad Bladder

Influence of pH on non-ionic permeability coefficients

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ABSTRACT Studies have been carried out, using the toad bladder, to determine the influence of pH on the permeability coefficients (K_{trans}) of the non-ionic species of (*a*) a series of aliphatic acids ranging from propionic to octanoic and (*b*) the aromatic acids, benzoic and acetylsalicylic. The data demonstrate that as the acidity of the mucosal bathing solution is increased by changing pH from 6 to 4, the fluxes of propionic, butyric, and acetylsalicylic acids increase in direct proportion to the increase in the calculated non-ionic concentration; the permeability coefficients, therefore, remain constant. However, the fluxes of the six, seven, and eight carbon aliphatic acids and benzoic acid rise only slightly despite an almost tenfold increase in non-ionic concentration, the K_{trans} falling from approximately 20,000 $\times$ 10^{-7} cm sec.$^{-1}$ at pH 6 to approximately 2500 $\times$ 10^{-7} cm sec.$^{-1}$ at pH 4. It has been tentatively proposed that the common characteristic of the compounds exhibiting this anomalous behavior is their non-polarity and high degree of lipid solubility. Possible explanations for the differences observed between the more lipid-soluble and less lipid-soluble compounds have been considered.

INTRODUCTION

It is generally accepted that the movement of a weak electrolyte through living membranes occurs largely by diffusion of the lipid-soluble non-ionic moiety. This concept has been used to explain many of the experimental observations on the absorption, distribution, and excretion of weak electrolytes. The findings in systems such as the kidney and gastrointestinal tract have not, however, always agreed quantitatively with simple theoretical expectations (1–3). Because these *in vivo* systems are complex, involving the interaction of many factors, it has seemed desirable to characterize the kinetics of non-ionic diffusion under simpler experimental conditions.

The present study examines the permeability of the urinary bladder of the toad to the ionic and non-ionic species of a number of weak acids. The concen-

tration of each species was adjusted by varying the pH of the fluid bathing the mucosal surface of the bladder. In initial studies, observations on benzoic acid gave the unexpected finding that the permeability coefficient of the non-ionic species varied as a function of the pH of the mucosal medium. For this reason studies of a related cyclic compound, acetylsalicylic acid, were undertaken. This compound did not show the anomalous behavior of benzoic acid and the study was, therefore, extended to an homologous series of aliphatic carboxylic acids of three to eight carbon chain length. The data reveal an unsuspected effect of pH on the permeability coefficient for lipid-soluble, rapidly penetrating weak acids.

METHODS

General Design of Experiments The permeability of the urinary bladder of the toad (*Bufo marinus*) to the following C^{14}-labeled weak acids was examined: (*a*) the series of saturated aliphatic carboxylic acids ranging from propionic to octanoic, (*b*) the aromatic acids, benzoic and acetylsalicylic. The dissociation constants (pKa) range from 4.82 to 4.89 for the aliphatic acid, and are 3.49 and 4.19 for acetylsalicylic and benzoic acid respectively (4). The ionic permeability coefficients have been estimated by measurement of the flux across the bladder after the pH of the mucosal bathing solution was adjusted to a value of 8.0 or higher; in this pH range (at least 3 units above the pKa) the concentration of the non-ionic species is negligible. When the pH of the bathing solution is lowered to a value closer to the pKa, the concentration of the non-ionic species (HA) becomes appreciable and after appropriate correction for the ionic contribution to the flux, one can calculate a transepithelial permeability coefficient for HA.

Measurements of Flux The bladder was excised, rinsed in Ringer's solution, and each half mounted as a diaphragm between two halves of a lucite chamber with a cross-sectional area of 7.07 cm^2 (5). The composition of the Ringer's solution in mM was: Na 112, K 3.5, Ca 0.9, Cl 115, PO_4 1.2. Fluid to be added to the mucosal side was adjusted to the desired pH by titration with HCl or NaOH. Ringer's solution at pH 7.0 was used as the serosal medium. Fifteen ml of the appropriate solution was pipetted simultaneously into each half of the chamber. The second half-bladder was handled in the same manner as the first, with a delay of no longer than 10 minutes. Experiments were carried out with the membrane short-circuited (6) except that the short-circuit current (measured with a Weston DC microammeter, model 622) was briefly interrupted at 5 to 10 minute intervals in order to measure spontaneous transepithelial potentials (Keithley DC vacuum tube voltmeter, model 200B or General Radio electrometer, type 1230-A). Both bladder halves were discarded if either half did not promptly exhibit a spontaneous membrane potential of greater than 15 mv and a membrane "resistance" (calculated as the quotient of spontaneous potential and short-circuit current) of more than 200 ohms/7.07 cm^2. The majority of bladders exhibited a potential of over 30 mv and a resistance of over 300 ohms.

For measurement of non-ionic permeability coefficients, one half-bladder was studied at a mucosal pH of 4.0 and the paired half at pH 6.0. In the case of acetyl-

salicylic acid, with a pKa of 3.49, pH levels of 3.5 and 5.3 were employed. The experiments at pH 8, designed to measure permeability to the ionic species, were done on unpaired half-bladders.

Soon after the membranes were mounted the pH of the Ringer's solution bathing each side of the bladder was measured (Beckman model G pH meter with external electrodes). When the resistance had stabilized, 2.5 μc of the C^{14}-labeled weak acid was added to the mucosal bathing medium. This addition of weak electrolyte produced chemical concentrations in the mucosal medium ranging from 0.2 mM for hexanoic acid to 0.02 mM for benzoic acid. The system was allowed to equilibrate for 15 to 20 minutes and a final adjustment of pH was made, if necessary. Samples (0.1 ml) were then taken from the serosal and mucosal solutions. Fifteen and 30 minutes later additional samples were obtained and the pH of each bathing solution was measured once again.

In the initial experiments, which were done with benzoic acid, the experimental procedure was somewhat different. A single half-bladder was used and the permeability coefficient determined at several pH values in a random sequence. Following flux measurements at a given pH, the mucosal and serosal fluids were drained, the bladder rinsed, and Ringer's solution of appropriate acidity added for the next determinations.

Calculation of Permeability Coefficient The permeability coefficient, K_{trans}, is defined as the amount of the substance crossing 1 cm^2 of membrane surface per second for a driving force of unit concentration gradient, and has the dimension of centimeters per second (7). As mentioned earlier, the K_{trans} for the ionic species was calculated from fluxes at pH 8. The K_{trans} for the non-ionic species was calculated from the measurements at pH 6 and 4 in the following manner:

1. The mucosal radioactivity was partitioned between the ionic and non-ionic species using the pKa and measured pH of the mucosal medium.
2. The ionic flux was then calculated from the ionic concentration in the mucosal medium and the ionic K_{trans} previously determined at pH 8.
3. After the serosal radioactivity attributable to penetration by the ionic species was subtracted from the observed flux, the non-ionic K_{trans} was calculated.
4. Because the K_{trans} for the two successive flux periods agreed closely, the values for the two periods were averaged.

This simplified calculation ignores the back flux of both the ionic and non-ionic species. Only in the case of rapidly penetrating compounds does the accumulation in the serosal medium become sufficient to produce significant back diffusion. When corrections were made for back diffusion of heptanoic and octanoic acids, the resulting non-ionic K_{trans} was increased by 4 per cent or less at pH 6 and by 2 per cent or less at pH 4. In the case of benzoic and hexanoic acids, the error from these factors amounted to less than 2 per cent.

Evaluation of pKa' The dissociation constants used in the calculations were the thermodynamic constants (pKa). Actual titration of the acids in phosphate-free Ringer's solution gave pKa' values equal to or not more than 0.2 unit lower than these constants. As will be discussed below, use of the lower value (pKa') does not

significantly influence either the pattern of the results or the interpretation of the experimental findings.

Isotopic Techniques All compounds were labeled in the carboxyl group (New England Nuclear Corporation, Boston, Massachusetts). Isotopes were used at the specific activity at which they were supplied, ranging from 1.0 mc/mmole for hexanoic-1-C^{14} acid to 9.01 mc/mmole for benzoic-7-C^{14} acid. Samples were counted in a dioxane, naphthalene, 2,5-diphenyloxazole (PPO), 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) mixture (8) in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.).

Because of the small quantity of each compound which was used it was necessary to consider the possibility that adsorption of the isotope on the pipet or chamber might have affected the results. Therefore, adsorption was examined for a representative group of the compounds.

The following procedure was used: (*a*) Ten serial samples of a standard isotopic solution were pipetted with the same pipet. The radioactivity of the first and last samples was found not to differ. (*b*) A standard isotopic solution was placed in a chamber without a membrane and the solution was aerated as in the flux experiments. Samples of the solution were taken from the chamber at 5 to 10 minute intervals over a 1 hour period. There was no significant decrease in activity of the samples with time.

Measurement of Loss of Radioactivity In order to determine the possible loss of volatile radioactive material, the air issuing from the chamber was, for a 1 hour period, passed sequentially through two tubes each of which contained solutions of 0.5 N NaOH. Three ml portions of the NaOH solution were pipetted into vaccine bottles, along with 1 ml of 25 mM $NaHCO_3$. A vial containing 0.5 ml of *p*-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide (hydroxide of hyamine 10-X, Packard Instrument Co.) was inserted into the vaccine bottle and the bottle sealed with a rubber stopper. One ml of 6 N H_2SO_4 was then injected into the vaccine bottle and the bottle shaken for 1 hour. The hyamine was quantitatively transferred to a vial containing toluene scintillation mixture (5.0 gm/liter PPO and 0.4 gm/liter POPOP in toluene) and counted in the spectrometer. To evaluate the efficiency of this method, recovery of $C^{14}O_2$ from $NaHC^{14}O_3$ added to the chamber was determined subsequent to acidification of the medium; recoveries ranged from 70 to 90 per cent.

Chromatography Samples of bathing media obtained at the end of a flux experiment were examined by paper chromatography to determine whether significant alteration of the original isotopic species had occurred during the experiments. Using Whatman No. 1 paper, benzoic acid was chromatographed with *n*-butanol saturated with 1.5 N NH_4OH; acetylsalicylic acid with *n*-butanol, acetic acid, H_2O, (4:1:5); butyric acid with *n*-butanol saturated with H_2O; propionic acid with *n*-butanol, ethanol, 3 N NH_4OH, (4:1:5). Hexanoic, heptanoic, and octanoic acids were chromatographed with *n*-propanol, concentrated NH_4OH, (4:1) on paper previously washed with 2 N acetic acid, H_2O, and 10 N NH_4OH in succession (9). The strips were scanned in an automatic chromatogram scanner (Model 880 low-background autoscanner, Vanguard Instrument Co., La Grange, Illinois).

RESULTS

Permeability Coefficients of the Ionic Species The mean transepithelial permeability coefficients for the ionic species of the various weak acids are shown in Table I. Values are given ± the standard error. It is noteworthy that the short chain aliphatic acid ions have lower permeability coefficients than do the longer chain compounds; the values for the three, four, and five carbon fatty acids are significantly lower ($p < 0.01$) than those for the longer compounds. A similar difference is noted between acetylsalicylate and benzoate ($p < 0.01$).

TABLE I

PERMEABILITY OF THE TOAD BLADDER TO THE IONIC SPECIES OF VARIOUS WEAK ACIDS

Acid	Ionic K_{trans}* 10^{-7} cm sec.$^{-1}$	No. of experiments
Propionic	2.7±1.1	6
Butyric	6.6±0.8	7
Valeric	7.4±1.2	5
Hexanoic	22±4.2	6
Heptanoic	122±39	6
Octanoic	102±9	6
Acetylsalicylic	4.4±2.8	6
Benzoic	24±2.6	16

* Permeability coefficients are expressed as mean values of the ionic K_{trans} ± the standard error.

Permeability Coefficients of the Non-ionic Species Detailed data from a representative study on a short chain fatty acid, butyric, and a longer chain fatty acid, octanoic, are shown in Table II. The mean permeability coefficients at pH 6 and 4 for the non-ionic species of each of the weak acids are presented in Table III. It can be seen that the permeability coefficients at pH 6 of propionic, butyric, and acetylsalicylic acids are not significantly different from those observed in each instance at pH 4. By contrast, the remaining aliphatic acids and benzoic acid showed a marked and significantly greater K_{trans} at pH 6 than at pH 4. In the case of valeric acid a twofold difference in the permeability was observed, whereas with the longer aliphatic acids and benzoic acid an eight-to ninefold difference was found.

An additional nineteen experiments, not shown in Table III, were carried out with benzoic acid at the intermediate pH value of 5. The mean K_{trans} of the non-ionic species was 8230×10^{-7} cm sec.$^{-1}$, a value between those noted at pH 4 and 6.

It should be pointed out that the fall in non-ionic K_{trans} at pH 4 for the long chain acids resulted from a failure of flux to increase in proportion to the increase in concentration of HA in the mucosal medium. In fact, for the aliphatic acids of six or more carbon atoms (see example of octanoic acid in Table II) very little increase in non-ionic flux occurred between pH 6 and 4.

TABLE II
REPRESENTATIVE RESULTS OF FLUX EXPERIMENTS

Acid	Experiment No.*	Mucosal pH	Mucosal Activity	Mucosal [HA]	Mucosal [A⁻]	Flux Total	Flux Ionic	Flux Non-ionic	Non-ionic K_{trans}
			CPM/0.1 ml			*CPM/min./7.07 cm²*			10^{-7} *cm sec.*$^{-1}$
Butyric	127 B	6.00	17,251	1069	16,182	590	50	540	1190
		6.00	17,104	1060	16,044	720	50	670	1490
	127 A	4.00	15,908	13,833	2075	6360	10	6350	1080
		4.03	15,032	12,959	2073	5540	10	5530	1010
Octanoic	159 A	6.01	17,729	1250	16,479	10,870	720	10,150	19,200
		6.02	16,621	1147	15,474	8710	680	8030	16,520
	159 B	4.03	12,650	11,096	1554	12,630	70	12,560	2670
		4.08	10,845	9430	1415	9800	60	9740	2440

* Each experiment was carried out on paired bladder halves (A and B). The two values reported for each half-bladder are the results of successive 15 minute periods. The chemical concentration of each substance studied was 0.08 mM.

TABLE III
PERMEABILITY OF THE TOAD BLADDER TO THE NON-IONIC SPECIES OF VARIOUS WEAK ACIDS AT MUCOSAL pH VALUES OF 4 AND 6

Acid	Non-ionic K_{trans} (10^{-7} cm sec.$^{-1}$) pH 6	p	pH 4	p	No. of pairs	Average difference in K_{trans} (pH 6-4)	p
Propionic	580±110	<0.01*	670±63	<0.01*	6	−95±120	<0.5
Butyric	1570±272	<0.01	1130±24	<0.01	7	434±270	<0.2
Valeric	3410±535	<0.01	1440±93	<0.01	9	1980±520	<0.01
Hexanoic	18,200±1000	>0.9	2450±126	<0.8	6	15,700±960	<0.01
Heptanoic	17,900±3030	<0.5	2350±340	>0.9	6	15,500±2810	<0.01
Octanoic	20,400±1700		2380±285		6	18,000±1870	<0.01
Acetylsalicylic‡	360±230	<0.01	300±42	<0.01	6	63±189	<0.8
Benzoic	21,000±1320 (21)§		2420±138 (17)§		—	18,600±1330	<0.01

Values given are the mean ± standard error.
* Probability of the difference between the values for the two different adjacent acids being due to chance.
‡ pH 5.3 and 3.5 (for explanation see text).
§ The numbers in parentheses indicate the number of individual experiments at each pH value.

Factors Which Might Have Influenced the Estimated Values of the Non-ionic Permeability Coefficients

A. ASSUMED VALUE OF PKA′

Since the actual pKa' values might be as much as 0.2 unit lower than the pKa value used in the calculations (see Methods), several experiments were recalculated applying the lower figure. This recalculation has a larger absolute effect on the estimated non-ionic concentration at pH 4 than at pH 6 but the percentage change is greater at pH 6 than at 4. The result is, therefore, to increase the calculated K_{trans} at pH 6 more than at pH 4 and hence to accentuate any difference between the K_{trans} at pH 6 and 4. In the case of propionic, butyric, and acetylsalicylic acids, the effect on K_{trans} is not sufficient to produce a significant difference between the values at pH 4 and 6.

B. LOSS OF RADIOACTIVITY DURING EXPERIMENT

In order to assess the possibility that losses of volatile radioactive material may have contributed to the apparent paradoxical behavior of the longer chain aliphatic acids (five to eight carbon atoms) and of benzoic acid, measurements of such losses were carried out under the conditions of a flux experiment. When the permeability coefficients were recalculated for each compound, assuming that the entire amount of volatile material had come from the serosal side, the following results were obtained. For the long chain compounds there was a 5 to 10 per cent increase in K_{trans} at both pH 4 and 6. Thus, correction for possible losses served to widen even further the previously calculated difference between the permeability coefficients at the two pH levels since the K_{trans} was already about nine times higher at pH 6 than at pH 4. For benzoic acid the volatile losses were less than for the aliphatic acids and the effect on the permeability coefficients was negligible.

C. ALTERATIONS IN THE LABELED COMPOUND

The chromatograms demonstrated that for each compound the radioactivity in both mucosal and serosal media was located in the single peak representing the original isotope. It can be concluded, therefore, that significant conversion of the original compound to some other species did not occur.

Permeability in the Presence of CO_2 To determine whether use of a phosphate buffer rather than a carbonic acid–bicarbonate buffer might have influenced the experimental results, paired observations were made at the same mucosal pH value with one half-bladder bathed in Ringer's solution as described above, and the other half-bladder bathed in a phosphate-free bicarbonate Ringer's solution aerated with 4.1 per cent CO_2 in air. Three experiments were carried out with heptanoic acid using a mucosal solution at

pH 6.0 and three additional experiments at a pH of 4.0. At a given pH, there was no difference between the results obtained with the different buffering systems.

DISCUSSION

The data demonstrate that as the pH of the solution bathing the mucosal surface of the toad bladder is changed from 6 to 4, the fluxes of the non-ionic species of the short chain fatty acids remain proportional to the calculated concentration gradients and the permeability coefficients are, therefore, un-

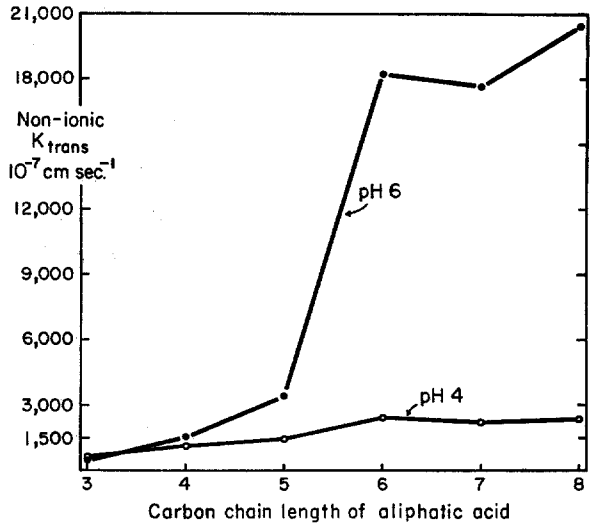

FIGURE 1. Relation between non-ionic permeability coefficient and chain length of an homologous series of aliphatic acids at pH 6 and 4.

affected. The longer chain acids exhibit an anomalous behavior in that the fluxes increase only slightly and the non-ionic permeability coefficients, therefore, fall as pH is lowered from 6 to 4. These differences are illustrated in Fig. 1. It can be seen that for the five carbon acid the value of the K_{trans} at pH 6 is approximately twice the value observed at pH 4; for the longer chain acids a much greater difference between the permeability coefficients exists at the two pH levels. Fig. 1 also shows the influence of carbon chain length on permeability at a given pH. At pH 6, as chain length is extended from three to five carbon atoms there is a small but significant, progressive increase in the rate of penetration (Table III). On advancing from the five to the six carbon acid a large further increase occurs but the seven and eight carbon acids show no additional significant change. This suggests that further extension of chain length would probably not increase the permeability coefficient above a value of approximately $20,000 \times 10^{-7}$ cm sec.$^{-1}$. A similar

though less pronounced pattern of increased permeability with increased chain length is seen at pH 4 (Table III).

The same behavior with respect to pH demonstrated by the longer chain fatty acids was also exhibited by the aromatic compound, benzoic acid. At pH 6 the permeability coefficient was 21,000 $\times$ 10^{-7} cm sec.$^{-1}$ while at pH 4 it fell to 2420 $\times$ 10^{-7} cm sec.$^{-1}$. By contrast, a closely related aromatic compound, acetylsalicylic acid, behaved in a fashion analogous to the shorter chain fatty acids, the permeability coefficient being unaffected by pH. The similarity in behavior of benzoic acid and the longer chain aliphatic acids on the one hand, and of acetylsalicylic acid and the shorter aliphatic acids on the other hand, suggests that polarity or lipid solubility, rather than molecular dimension, is the common determinant of the permeability differences.

Before discussing possible explanations for the striking influence of pH on the rate at which less polar compounds penetrate the bladder, it is necessary to consider several possible factors which might have affected the results:

(*a*) If the labeled molecule were metabolized by the tissue, either the C^{14} could have been rendered volatile and lost with aeration or the compound, without loss of label, could have been transformed into a new species with quite different transport properties. The first of these possibilities has been examined by assessment of losses of volatile radioactive material from the system and the potential error was found to be trivial. The possibility that significant transformation of the original compound may have taken place has been excluded by chromatography of both the serosal and mucosal media.

(*b*) If the pH change were to affect permeability of the tissue to the ionic species, an error would have been introduced into the estimate of non-ionic permeability. However, a non-specific change in ionic permeability of the tissue seems unlikely, since the permeability coefficients of the inorganic ions potassium and chloride were found to be unaffected by a change in pH over the range 3.0 to 8.6 (10). Furthermore, if one were to postulate a selective change in the permeability to organic ions, it must be assumed that the ionic permeability, which by measurement is low at pH 8, increased markedly at pH 6 to values of approximately 1500 $\times$ 10^{-7} cm sec.$^{-1}$; these values seem highly improbable for an ionic species. Such a postulation must also explain why pH does not similarly affect the permeability to the ionic species of the shorter aliphatic acids and of acetylsalicylic acid since these latter compounds do not behave in an anomalous fashion. The most reasonable interpretation of the present data would, therefore, seem to be that pH exerts a significant influence on the permeability of the non-ionic forms of the longer fatty acids and benzoic acid. Though the explanation for this phenomenon is not clear the possibilities seem to fall into three categories:

1. *The pH May Affect the State of the Solute in the Medium* If a reduction in pH were to cause the non-ionic species of the longer chain aliphatic acids and

benzoic acid to form aggregates or micelles, the effective concentration in solution would fall. Whether such aggregates do, in fact, occur in acid media at low concentrations of the solute has not been determined and the possible influence of this factor on the permeability coefficient cannot be evaluated at present.

2. *The Low pH May Affect the Membrane Directly so as to Alter Permeability to the Non-Ionic Species* Such an effect would have to be of importance in governing the movement of the more lipid-soluble substances but of little if any importance in influencing the diffusion of those compounds of lower lipid solubility. At the present time there are insufficient data to permit evaluation of this possibility.

3. *The Highly Lipid-Soluble Acids May Reach a Limiting Rate of Penetration* It is possible that as pH is lowered the non-ionic concentration in the mucosal medium reaches a critical value above which there is no further influence on non-ionic flux. The finding that the flux of the non-ionic species of the six to eight carbon chain fatty acids was virtually unaffected by a change of pH from 6 to 4 suggests some "saturation step" in the transport of these molecules across the bladder. According to this view only those molecules which moved most rapidly through the bladder approached the transport limit. To say this is, of course, only to describe the phenomenon observed and not to explain it.

Although it may be hazardous to draw an analogy between an *in vitro* study of amphibian tissue and mammalian physiology, it is of interest that the range of hydrogen ion concentration used in the present study is similar to that normally encountered in the nephron and in the gastrointestinal tract of the mammal. Hence, it seems likely that the present results may have important implications for the *in vivo* transport of weak electrolytes which are highly lipid-soluble. Unfortunately, available physiological evidence is insufficient to allow evaluation of this possibility. It would appear from the above considerations, however, that pH effects on permeability should be included among the other factors which may interfere with achievement of equilibrium for a weak electrolyte diffusing between plasma and luminal fluid.

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