Effect of pH on the *In Vitro* Absorption of Flufenamic Acid

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The effect of pH on the *in vitro* absorption of flufenamic acid is studied using the "everted sac" technique. The study reveals that the passage of this drug through the gut membrane is by passive diffusion. The amount diffusing through is dependent on the pH. Using Fick's diffusion law, a method is presented to calculate the apparent permeability constant at each pH value, and to determine the permeability constant. The relative surface area of the membrane through which diffusion of the drug takes place is estimated from the amount of "bound" drug.

AFTER ORAL administration, a drug to act systemically must be absorbed from the gastrointestinal tract. The rate of absorption is dependent on two independent processes: the rate of solution of the drug in the media and its rate of permeation through the gastric wall or intestinal lumen. For a relatively insoluble drug (less than 0.01 mg./ml.), the rate of solution becomes a fundamental factor affecting the rate of absorption. The reason is that unless the drug dissolves at a sufficiently rapid rate the necessary build-up of an effective concentration at the site of absorption will never occur. On the other hand, a relatively soluble drug will immediately saturate the system. In this case, the permeation rate becomes the important factor.

There have been many *in vitro* and *in vivo* studies (1, 2) dealing with the passage of drugs through the gut. However, as far as the authors have been able to determine, there has been no attempt to treat the data quantitatively as a diffusion phenomenon.

This study is concerned with the effect of pH on the *in vitro* absorption of flufenamic acid,[1] [*N*-(α,α,α-trifluoro-*m*-tolyl) anthranilic acid], with a pKa of 3.9 and solubility of 1 mg./ml. at pH 7.0. Based on Fick's law of diffusion, the apparent permeability constant at each pH value is calculated and the permeability constant is determined. A method is presented to estimate the relative surface area of the gut membrane through which diffusion of the drug takes place.

THEORETICAL CONSIDERATIONS

It has been known for many years (1) that cellular permeability to weak electrolytes may be dramatically affected by relatively small changes in the pH of the suspending medium. Although this phenomenon is of general significance in all cells, only recently were good examples described in the case of the intestinal absorption (3–8).

Absorption involves the transfer or permeability of materials from the intestinal lumen into the mucosal blood and lymph vessels. In the classification of permeability, a primary division is made between passive diffusion and special mechanisms such as active transport, facilitated diffusion, and pinocytosis. It has been shown that most drugs are absorbed by passive diffusion (3–5, 9).

Passive or simple diffusion describes the passage of a molecule across a barrier from a region of high to a region of low concentration. This phenomenon is quantitatively described by Fick's law (10), which states that the driving force which causes the transfer of a substance from regions of high to low concentrations is proportional to the concentration gradient or,

$$\frac{ds}{dt} = \frac{K\,(A)\,(C_0 - C_1)}{h} \qquad \text{(Eq. 1)}$$

where

$\frac{ds}{dt}$ = rate of movement of solute, s in mcg./min.,
A = area of the membrane in cm.^{2},
K = constant,
C_0 = amount of solute on the outside in mcg.,
C_1 = amount of solute on the inside in mcg.,
h = thickness of the membrane in cm.

When dealing with membranes, such as biological specimens, the thickness, h, of the membrane is not known. This factor is then commonly combined with the constant K to give a new constant P_1, the apparent permeability constant. Equation 1 then becomes

$$\frac{ds}{dt} = P_1(A)(C_0 - C_1) \qquad \text{(Eq. 2)}$$

If the permeability rate follows Eq. 2, a plot of amount *versus* time should be linear initially. The slope (l_1) of this line is equal to the change in amount with time, *i.e.*, to $\frac{ds}{dt}$.

Differentiating Eq. 2 with respect to area (A), we get

$$\frac{d^2s}{dt} = dA\,P_1(C_0 - C_1) \qquad \text{(Eq. 3)}$$

or

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[1] To be marketed as Arlef, Parke, Davis & Co., Detroit, Mich.

$$P_1 = \frac{d^2s}{dtdA(C_0 - C_1)} \qquad \text{(Eq. 4)}$$

The term $d^2s/dtdA$ can be evaluated by plotting the slope l_1 *versus* the area A and determining the slope l_2 of the resulting line. l_2 is then numerically equal to $d^2s/dtdA$. Knowing $C_0 - C_1$, P_1 can be calculated from Eq. 4.

If the permeation rate is measured at different pH values, P_1 can be calculated for each pH. Furthermore, the fraction of unionized moiety of the drug at different pH values can be determined theoretically, and assuming that it is only the unionized species that traverse the barrier, the permeation constant P can then be calculated, from the relationship

$$P = \frac{P_1}{U} \qquad \text{(Eq. 5)}$$

where U is the fraction of the unionized drug. P should then be a constant and independent of the pH.

The determination of the surface area of the membrane is difficult, particularly due to the presence of the villi on the surface of the intestinal wall. In this study, the concept of relative surface area is used. The apparent permeability constants derived are, therefore, relative; nevertheless, they are useful for comparing the transfer of the drug under different conditions.

Flufenamic acid is "bound"[2] to the intestine. At a particular concentration, the quantity "bound" is directly proportional to the surface area exposed or one can write

$$Q = kA \qquad \text{(Eq. 6)}$$

where Q = quantity of drug "bound" at a given concentration, k = constant, A = surface area.

If, then, for a series of experiments, at a particular concentration of the drug, one determines an average Q and assumes that A is 1, for the series, k can be calculated. This k can then be used to calculate other relative surface areas if the concentration of "bound" drug is known.

METHODOLOGY

To study adsorption of drugs both *in vivo* and *in vitro* procedures have been used. The selection of the method depends on the type of information desired. In this connection, Laster (11) points out that *in vivo* methods, *e.g.*, measuring the disappearance of a test substance from the gut or its appearance in the body fluids, tend to yield over-all results determined by a number of processes of which absorption is only one. On the other hand, he states that if excised intestinal tissue is studied *in vitro*, discrete absorption patterns may be established. However, it must be remembered that excised segments are deprived of their blood and lymph flows and depend on their oxygen supply on the diffusion of the gas across the epithelium.

From a number of *in vitro* methods available to study absorption, the authors used in these studies the everted-sac technique as modified by Crane and Wilson (12).

[2] The use of the term "bound" does not imply adsorption. The exact nature of physical and/or chemical interaction of flufenamic acid with the gut membrane is being investigated and will be the subject of a future presentation.

An excised segment (about 6 cm. in length) of the small intestine of a golden hamster is everted so that the mucosa faces outward. The segment is suspended in a glass tube, with a side arm, by tying to a cannula which in turn is supported by a rubber stopper. Forty-five milliliters of a buffered solution at the desired pH containing a known amount of the drug is added to the tube and bathes the mucosal surface of the intestine. A mixture of 95% oxygen and 5% carbon dioxide is bubbled through the solution through a longer cannula. Two milliliters of the buffer (without the drug) is placed inside the sac. The whole assembly is placed in a constant-temperature bath set at 37° ± 0.5°.

At known time intervals, the solution inside the intestine is removed for assay of flufenamic acid. Two milliliters of buffer is added to the inside of the intestine to rinse out any adhering drug and the solution is assayed. At the same time period, 1 ml. of the outside solution is removed for assay.

At the end of a run, the intestinal segment is taken out from the assembly, both surfaces washed 3 times with water, dried at 65°, powdered and extracted with 10 ml. of 0.1 N sodium hydroxide solu-

Table I.—Permeation of Flufenamic Acid at pH 7.2 (Concentration of Outside Solution, 20 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4
15	13.6	14.6	7.5	—
20	—	—	—	8.68
30	25.9	17.3	8.2	—
40	—	—	—	19.9
45	36.35	25.1	15.9	—
60	43.35	29.35	15.4	30.6

Table II.—"Bound" Drug, Relative Surface Area, and Rate at pH 7.2

Run	"Bound" Drug, mcg.	Relative Surface Area	Rate of Permeation, mcg./min.
1	152	2.47	0.725
2	61	1.0	0.4
3	29	0.48	0.25
4	87	1.42	0.55

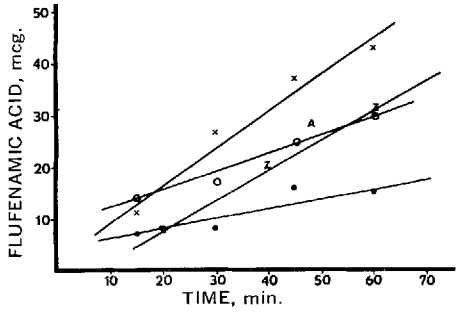

Fig. 1.—Plot of rate of permeation at pH 7.2 showing dependency on relative surface area. Key: Z, 1.42; ●, 0.48; O, 1.0; ×, 2.47.

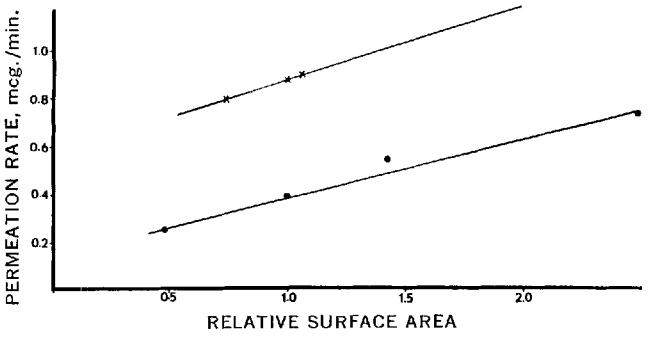

Fig. 2.—Plot of permeation rate *versus* relative surface area at pH 5.0 (×) and pH 7.0 (●).

TABLE III.—PERMEATION OF FLUFENAMIC ACID AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION 20 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3
15	10.88	15.5	—
20	—	—	13.95
30	27.0	—	—
50	—	49.05	—
60	52.0	—	31.0
75	—	—	56.0

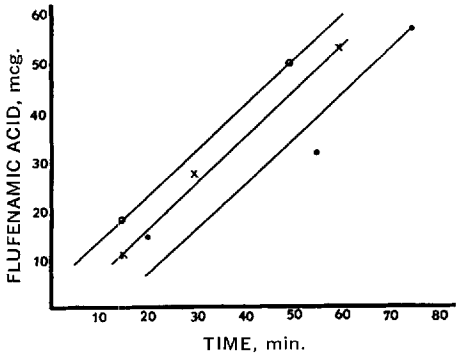

Fig. 3.—Plot of rate of absorption at pH 5.0 (three separate determinations). Initial concentration of outside solution 20 mcg./ml.

tion to remove any "bound" drug. It was necessary to follow this procedure to remove the "bound" drug since it adhered quite strongly to the gut membrane and could not be removed by simple washing with water or buffer solutions.

All samples are assayed using an Aminco Bowman spectrophotofluorometer, employing the assay procedure of Glazko and Dill (13).

The composition of the buffer used is:

	mmoles/L.
Sodium chloride	145
Potassium chloride	4.56
Calcium chloride	1.25
Sodium phosphate (dibasic)	1.33
Sodium phosphate (monobasic)	0.33
Distilled water	*q.s.*
pH of buffer 7.2	

The buffer is adjusted to pH 5.0, 3.9, and 2.5 by adding 20 mmoles of aspartic acid to the above solution and adjusting the pH with sodium hydroxide or hydrochloric acid solution.

RESULTS AND DISCUSSION

The quantity of drug which permeates through the intestine at different time intervals at pH 7.2 is given in Table I. This quantity represents the sum of the assays of the sample withdrawn from the inside of the intestinal segment, and the 2-ml. sample used to wash the inside. The initial concentration of the drug in the outside solution is 20 mcg. of flufenamic acid per ml. The results of four separate determinations are shown.

The quantity of "bound" drug for each of the runs and the relative surface area calculated from this are given in Table II. It is apparent from Table II that th rcelative surface area of the gut membranes, as gauged from the "bound" drug, varies considerably. This was due to the difference in thickness of the gut segments of the different animals used in this study.

The quantity of drug which permeates at pH 7.2 is plotted against time in Fig. 1. The slope of the

TABLE IV.—"BOUND" DRUG, RELATIVE SURFACE AREA, AND RATE AT pH 5.0

Run	"Bound" Drug, mcg.	Surface Area	Rate of Permeation, mcg./min.
1	168	1	0.875
2	179	1.06	0.9
3	130	0.774	0.8

TABLE V.—PERMEATION AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION, 15 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3
15	18.3	—	8.55
20	—	9.4	—
30	17.35	—	16.4
40	—	32.7	—
45	21.2	—	26.2
60	40.0	28.7	—
"Bound" drug, mcg.	91.3	105.6	142.8
Rate of permeation, mcg./min.	0.65	0.65	0.59

lines gives the rate of permeation in accordance with Eq. 2. These values are also included in Table II.

In Fig. 2, the rate of permeation is plotted against relative surface area. By determining the slope and using Eq. 4, the apparent permeability constant P_1 is calculated.

TABLE VI.—PERMEATION AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION, 5 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4
15	2.55	—	1.9	—
30	7.1	4.9	4.9	8.6
45	7.8	—	5.4	—
60	20.0	14.0	7.55	12.95
"Bound" drug, mcg.	38	24	36.7	47
Rate of permeation, mcg./min.	0.36	0.24	0.13	0.23

TABLE VII.—PERMEATION AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION, 2 mcg./ml.)

Time, min.	Run 1	Run 2
15	0.66	—
20	—	1.08
40	2.09	1.19
60	2.68	3.21
"Bound" drug, mcg.	6.93	11.6
Rate of permeation, mcg./min.	0.051	0.051

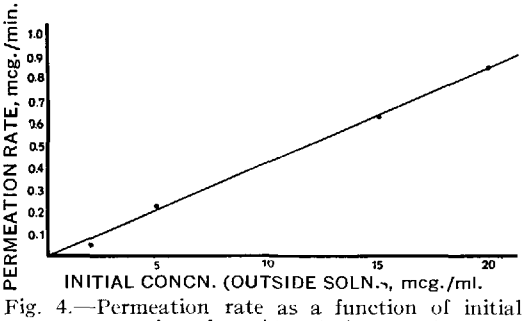

Fig. 4.—Permeation rate as a function of initial concentration of outside solution, pH 5.0.

Similarly, the quantity of drug which permeates at pH 5.0, when a concentration of 20 mcg./ml. is maintained on the outside, is given in Table III and shown in Fig. 3.

The quantity of "bound" drug, relative surface area, and rate of permeation at pH 5.0 are given in Table IV.

If Fick's law is applicable, the permeation rate should vary with the concentration of the drug in the outside solution, since the gradient across the membrane is proportional to the concentration. To test this hypothesis, permeation rates were determined at pH 5, using solutions containing 2, 5, and 15 mcg. of the acid per ml. The results are given in Tables V, VI, and VII. For convenience, the rate of permeation and concentration of "bound" drug are also included in these tables.

A plot of permeation rate *versus* concentration at pH 5.0 is shown in Fig. 4. It is apparent that a linear relationship exists between the rate and concentration of the outside solution. This is in agreement with Fick's law.

The quantity of drug "bound" in the membrane is also proportional to the concentration outside. Thus a plot of "bound" drug *versus* concentration should be linear, which is shown in Fig. 5. This is not surprising if one considers the nature of the membrane barrier. If one assumes that it is at least two to three cells thick, at a given time there is a certain amount of drug in the membrane. The quantity is proportional to the concentration gradient which in turn is determined by the concentration of the solution.

At low pH values, the solubility of flufenamic acid is limited; therefore, it is not possible to carry out studies using solutions containing 20 mcg./ml., as is done at pH 7.2 and 5.0. The studies at pH 2.5 and 3.9 are, therefore, carried out using solutions containing 2 mcg. of the drug per ml. In order to have a basis for comparison, the permeation rates for 20 mcg./ml. concentration are then computed from the value of 2 mcg./ml. It is felt that this procedure is valid since the concentration dependency should hold at pH 2.5 and 3.9 as was shown at pH 5.0.

The data are given in Tables VIII, IX, and X, and a plot of permeation rate *versus* relative surface area at these pH values is shown in Fig. 6.

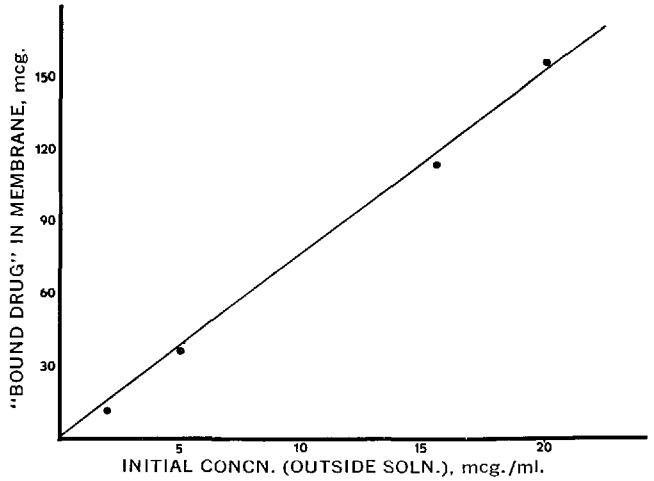

Fig. 5.—Plot of "bound drug" *versus* initial concentration of outside solution, pH 5.0.

TABLE VIII.—PERMEATION AT pH 3.9 (CONCENTRATION OF OUTSIDE SOLUTION, 2 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4	Run 5
15	4.22	1.82	1.90	1.7	1.20
30	—	2.35	1.40	5.70	—
45	4.82	2.67	2.20	6.45	3.32
60	8.1	6.3	3.75	9.8	—
"Bound" drug, mcg.	15.4	13	8.87	14.2	12.2
Relative surface area	1.19	1	0.68	1.09	0.94
Rate of permeation, mcg./min. computed to 20 mcg./ml. concn.	2.3	1.68	0.924	2.6	1.58

TABLE IX.—PERMEATION AT pH 2.5 (CONCENTRATION OF OUTSIDE SOLUTION, 2 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4
15	1.8	1.3	2.0	—
20	—	—	—	1.65
30	6.35	—	2.35	—
40	—	—	—	8.85
45	3.05	5.35	6.05	—
60	5.80	4.85	9.0	10.12
"Bound" drug, mcg.	29.25	27	33.75	36.98
Relative surface area	1	0.923	1.15	1.26
Rate of permeation, mcg./min. computed to 20 mcg./ml. concn.	1.56	1.56	2.32	2.85

TABLE X.—APPARENT PERMEABILITY CONSTANTS P_1 AND P

pH	Unionized Drug, %	$P_1 \times 10^{-4}$	$P \times 10^{-4}$
7.2	0.05	2.6	52
5.0	7.36	3.7	50
3.9	50.0	29.1	58
2.5	96.19	52.0	54

The apparent permeability constants P_1 at each pH value is calculated using Eq. 4. This is shown in Table X, together with the values for the permeability constant P, calculated from Eq. 5, and the per cent of unionized drug.

From Table X it is evident that the permeability constant P derived for measurements at each pH value is in excellent agreement. Furthermore, it is apparent that at pH 2.5 the permeation of flufenamic acid is 20 times faster than at pH 7.2. The study also shows that flufenamic acid follows the postulation (2–4, 8) that it is only the unionized moiety of the drug that passes through a cell, due to its lipoid solubility hypothesis. Since at pH 2.5 the drug is approximately 96% unionized, it is to be expected that the rate would be much faster than at pH 7.2, at which only 0.05% of the drug is in the unionized form.

In this study a procedure is described defining the permeation of flufenamic acid in terms of Fick's diffusion law. Perhaps this approach could be used in describing the passage of other drugs through the gut membrane, particularly those drugs which have an affinity for binding with the membrane. It is also suggested that in this type of measurement, the quantity of drug in the membrane should be determined. In some instances, as was found in this study, this can be considerable.

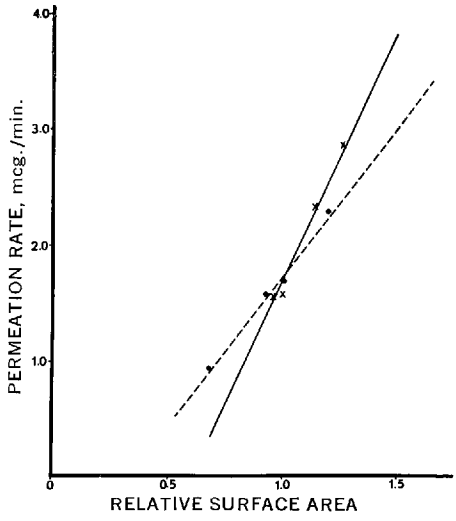

Fig. 6.—Plot of permeation rate *versus* relative surface area at pH 2.5 (×) and pH 3.9 (●).

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