In Vitro Model for Transport of Solutes in Three-Phase System II: Experimental Considerations

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Abstract □ Key experiments were carried out that illustrated the main features of the three-phase transport theory presented earlier. These studies investigated the effects of partition coefficient and pH on transport rates and how these effects are altered by the aqueous diffusion layers adjacent to the lipid phase. The experimental setup incorporates significant improvements over previous *in vitro* systems: (*a*) stable lipid phases in which partition coefficients of solutes may be measured, and (*b*) a large aqueous–lipid phase volume ratio, enabling establishment of steady-state conditions. Experimental rates for a variety of conditions correlated reasonably well with computer predictions obtained by independent evaluation of physicochemical parameters in the system. Transfer rates leveled off with increasing (high) membrane permeability coefficients. Transport rates for these high permeability coefficient solutes were very dependent on stirring rates as well as on the pH of the receptor phase. The pH–transfer rate profiles generally paralleled solute titration curves, except where pH values created pseudo-two-phase conditions. The transport of a high permeability coefficient solute, *n*-amylpyridine, slowed the simultaneous transfer of pyridine through the system. Cumulative results indicate that the pH–partition theory is only a special case of a more general phenomenon.

Keyphrases □ Transport of solutes—three-phase (w/o/w) system, experimental considerations □ Solute transport—three-phase (w/o/w) system, experimental considerations □ Three-phase (water–oil–water) system—solute transport, experimental considerations □ Diffusional model—solute transport in three-phase (w/o/w) system, relationship to pH–partition theory □ pH–partition theory—effect on transport rates in three-phase (w/o/w) system □ Absorption, drug—three-phase (w/o/w) model investigated relative to pH–partition theory

In Part I (1), the three-phase diffusional model for transport of solutes was discussed. The significant inclusion of aqueous diffusion layers on both sides of the membrane led to transport behavior not predicted by the Brodie–Shore–Hogben pH–partition theory. This report outlines the development and results of key experiments, which establish the validity of the present transport model.

EXPERIMENTAL

Lipid Phases—Two lipid phases, oil–polymer mixtures incorporated into fiber glass filter disks[1], exhibited reasonably good stability when submerged in aqueous solutions of various pH values. These were a 2:1 by weight mixture of triolein[2] and acrylic resin[3] and a 3:1 mixture of heavy mineral oil[4] and the copolymer. The disks, with a nominal pore diameter of 0.9 μ and an average thickness of 500 μ, lent rigidity and geometrical reproducibility to the "membranes." The polylaurylmethacrylate copolymer increased the gross viscosity of the lipid phase, which, nevertheless, presented a fairly low viscosity to diffusing solutes at the molecular level.

The polymer, which comes as a 50% solution in "mineral oil," was separated out by repeated dilution with benzene, precipitation with acetone, and decantation of solvent. After the removal of the solvent by vacuum and heating at 50° in a dry air oven, the glassy residue was weighed, redissolved in benzene, and mixed with the appropriate amount of mineral oil or triolein. The benzene was once again removed by vacuum and moderate heating.

Table I—Principal Buffers (for 2 l. of 0.1 *M* Buffer)

pH 8.00	pH 6.50
0.7457 g. KH_2PO_4[a] 33.8815 g. K_2HPO_4[a] 58.44 g. NaCl[b]	12.512 g. KH_2PO_4[a] 18.822 g. K_2HPO_4[a] 58.44 g. NaCl[b]
pH 5.00	**pH 3.00**
8.658 g. KOH[c] 40.846 g. KHPhthalate[d] 58.44 g. NaCl[b] (or weigh out 0.2 mole of citric acid and titrate to pH 5.00 with potassium hydroxide)	20.778 g. $NaHC_2O_4$[d] 5.386 g. $Na_2C_2O_4$[d] 58.44 g. NaCl[b] (or weigh out 0.2 mole of citric acid and titrate to pH 3.00 with potassium hydroxide)

[a] J. T. Baker Chemical Co., Phillipsburg, N. J. [b] Mallinckrodt Chemical Works, St. Louis, Mo. [c] Baker & Adamson Reagent, Allied Chemical & Dye Corp., New York, N. Y. [d] Fisher Scientific Co., Fair Lawn, N. J.

Aqueous Buffers—Table I lists the buffer systems used for aqueous phases of various pH values. All systems included 0.5 *M* NaCl as a swamping electrolyte to buffer ionic strength changes. Polyprotic buffers were chosen, so that: (*a*) essentially two forms would be present at a given pH, thus providing input data for computer correlations, and (*b*) both species were ions. This latter requirement ruled out such systems as acetate and lactate. The free acid forms of these buffers could partition into, and diffuse through, the lipid membrane.

Solutes—A series of alkylated pyridines was studied, covering a two orders of magnitude range of partition coefficient values. The parent compound, reagent grade pyridine[5], was used without further purification. The alkylated derivatives[2], with the exception of the 4-*n*-amylpyridine, were redistilled under vacuum, the first fraction being discarded. All solutes were assayed by UV spectrophotometry[6].

Apparatus—Figure 1 depicts the apparatus used in the present studies. The center piece is of machined Teflon, reinforced on the outside with brass collars to minimize the deformation that occurs with this substance under pressure. The Teflon piece slides tightly onto ground-glass projections from the aqueous chambers and is adjustable for membranes of varying thicknesses. The available transport area is 7.89 cm.^{2}. The jacketed aqueous chambers maintain a constant 30° temperature from an external water bath.

Stirring of the aqueous phases was provided by 5.08 × 1.1-cm. (2 × 0.42-in.) magnetic stirrer bars driven by constant-speed high-torque motors[7,8]. The left or "source" chamber, when filled and sealed off, held about 330 ml.; the right chamber contained 300 ml. By using filter disks[1] with a diameter of 37 mm., a nominal pore size of 0.9 μ, an estimated porosity of 0.87, and a measured thickness of 500 μ, saturated with the oil–polymer mixture already described, an aqueous–lipid phase volume ratio of from 300 to 600:1 was realized. This ratio allowed steady-state transport conditions to be attained without significant depletion of the source compartment.

[1] Versapor filter disks 6429, Gelman Instrument Co., Ann Arbor, Mich.
[2] K&K Laboratories, Inc., Plainview, N. Y.
[3] Acryloid 917, Rohm & Haas, Philadelphia, Pa.
[4] White Oil No. 31, Standard Oil of Indiana.
[5] J. T. Baker Chemical Co., Phillipsburg, N. J.
[6] Perkin-Elmer–Hitachi 139, Hitachi, Ltd., Tokyo, Japan.
[7] Hurst Manufacturing Co., Princeton, Ind.
[8] Howard Electric Co., Detroit, Mich.

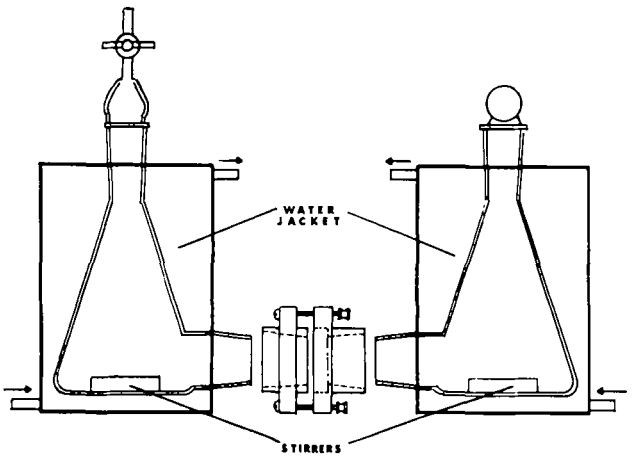

Figure 1—*Two-cell apparatus used in three-phase transport studies. The Teflon centerpiece slides onto ground-glass projections on cells. The stopcock seals off the left cell once it is completely filled. Sampling is from the right chamber. Cross-sectional transport area = 7.89 cm.*^{2}.

In an experiment, the source chamber was filled with 300 ml. of pyridine solution and the receiving chamber was simultaneously filled with 300 ml. of buffer solution. The right side was stoppered, and the source chamber was filled and sealed off (Fig. 1). Samples were removed periodically from the right side with replacement by buffer.

EVALUATION OF PARAMETERS

Quantitative correlation of experimental results with theoretical predictions by the model necessitated the determination of several parameters. Directly measurable quantities included: (*a*) aqueous phase pH values, (*b*) dissociation constants of solutes and buffers (at the ionic strength in the system), and (*c*) membrane thickness (500–510 μ).

Effective Aqueous Diffusion Layer Thicknesses (TH1 and TH3)—These parameters were determined in two different ways. The two-phase method of Howard (2) involved following the initial first-order disappearance of a high partition coefficient solute from an aqueous phase into the lipid membrane. The slope of an ln (C/C_0) *versus* time plot is inversely dependent on the diffusion layer thickness. The aqueous diffusion coefficient of the solute must be known. In the present three-phase system, separate uptake experiments were done on each side of the lipid-impregnated filter. Results for dinitrotoluene[9] (Fig. 2) and diethyl phthalate[9] uptake into a filter disk saturated with dibutyl sebacate[10] indicate an effective diffusion layer thickness of 60 ± 10 μ at 150 r.p.m. on either side of the membrane.

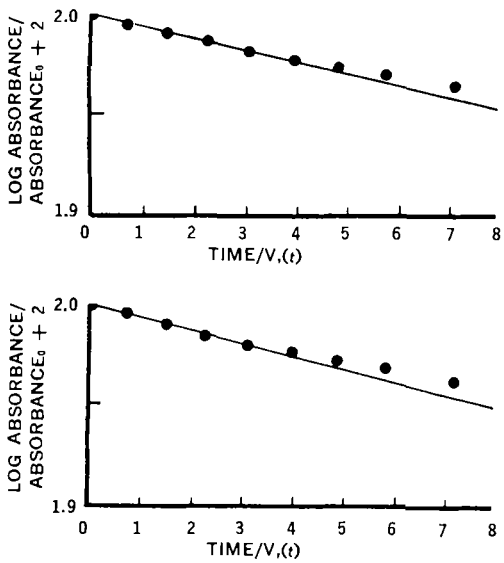

Figure 2—*First-order uptake plots of log (absorbance/absorbance$_0$) versus time/volume, (time) for two different initial (top = 0.01%; bottom = 0.001%) concentrations of dinitrotoluene. The ordinate is linear with the quantity:*

$$-\frac{A_{membrane}D_W}{V_{aq}\,TH_{aq}}$$

where A = *transport area. Volume is included in the abscissa to correct for sample removal.* D_W = 1.02×10^{-5} *cm.*^{2} *sec.*$^{-1}$.

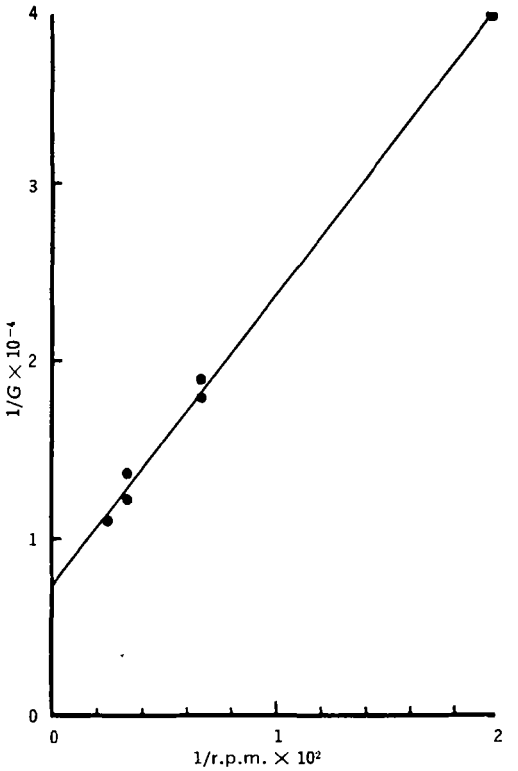

Figure 3—*1/G versus 1/r.p.m. for the series of benzoic acid diffusion runs through a silver filter. The intercept, representing the extrapolated rate at infinite stirring speed, is equal to* ($TH2/D_W' \cdot A \cdot \Delta C$), *where* D_W' = *porosity* × D_W ÷ *tortuosity, and* A = *transport area. This, with Eq. 8 of the previous paper (1), was used to give TH1 + TH3 = 150 μ at 150 r.p.m.*

The method of Ginzberg and Katchalsky (3) leads to the total diffusion layer thickness on both sides of the membrane. This involves running aqueous diffusion experiments with the same solute over a range of stirring speeds and plotting the reciprocal of the transport rate *versus* the reciprocal of the stirring speed, raised to some power such that the plot is linear. When $(r.p.m.)^{-n} = 0$ ("infinite stirring"), the contribution by the membrane may be obtained by extrapolation, making possible the calculation of the total diffusion layer thicknesses at the experimental stirring speeds.

The diffusion of benzoic acid[11] through silver filters[12] was studied in the transport apparatus. For the 50–400-r.p.m. range, $n = 1$ gave the best linear fit in the present system (Fig. 3). The total aqueous diffusion layer thickness (TH1 + TH3) was estimated in this manner to be 150 μ (2 × 75 μ) at 150 r.p.m.

At 150 r.p.m., 60 μ was used as the individual diffusion layer thickness; and in the light of the apparent inverse relation between stirring speed and diffusion barrier thickness in this particular apparatus, 180 μ was used for 50-r.p.m. stirring conditions.

Aqueous Diffusion Coefficients D_W (Table II)—The values of D_W for the various pyridines were found, using the method of Goldberg and Higuchi (4), with benzoic acid ($D_W = 1.11 \times 10^{-5}$) as a standard.

[9] Eastman Organic Chemicals, Rochester, N. Y.
[10] Aldrich Chemical Co., Inc., Milwaukee, Wis.
[11] Baker & Adamson Reagent, Allied Chemical & Dye Corp., New York, N. Y.
[12] Selas Flotronics, Spring House, Pa.

Table II—Pertinent Parameters Established for Solutes Studied

Solute	Molecular Weight	Partition Coefficient in Mineral Oil System	λ_{max} (pH)	pK'a	Diffusion Coefficient—Lipid	Diffusion Coefficient—Aqueous
Pyridine[a]	79.103	0.275	256.25 (8)	5.47	$2.2–2.3 \times 10^{-7}$	1.14×10^{-5}
4-Methylpyridine[b]	93.13	0.87	254.25 (8)	6.25	1.4×10^{-7}	1.08×10^{-5}
2-Ethylpyridine[b]	107.16	3.11	261.25 (8)	6.24	1.6×10^{-7}	9.78×10^{-6}
4-Ethylpyridine[b]	107.16	2.76	254.5 (8)	6.24	1.2×10^{-7}	1.00×10^{-5}
2-*n*-Propylpyridine[b]	121.18	22.9	261.75 (8)	6.19	$7.3–7.5 \times 10^{-8}$	8.84×10^{-6}
4-*n*-Propylpyridine[b]	121.18	19.9	254.75 (8)	6.25	$8.9–9.1 \times 10^{-8}$	1.00×10^{-5}
4-*tert*-Butylpyridine[b]	135.21	28.7	254.75 (8)	6.21	$9.0–9.5 \times 10^{-8}$	9.18×10^{-6}
4-*n*-Amylpyridine[b]	149.23	298	254.75 (8) 252 (2.1)	6.22	$6.9–7.8 \times 10^{-8}$	9.45×10^{-6}

[a] J. T. Baker Chemical Co., Phillipsburg, N. J. [b] K&K Laboratories, Plainview, N. Y.

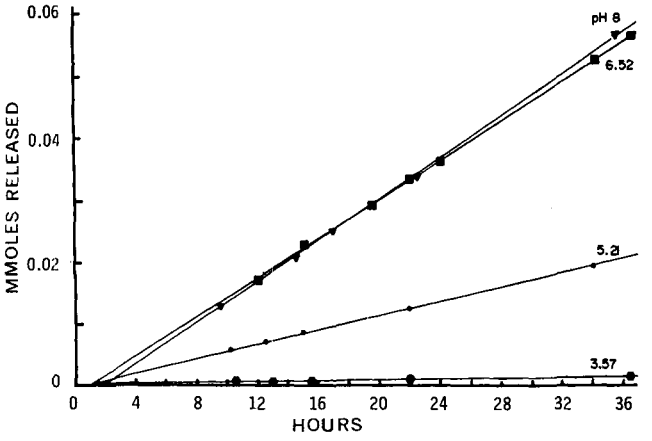

Figure 4—*Release data for pyridine (pK'a = 5.47) diffusing through disks saturated with a mineral oil–acrylic resin mixture. The pH of sampling chamber was 8.00. Numbers on lines indicate bulk pH of source chamber. For this batch of lipid mixture,* D_L *was calculated to be* 4.2×10^{-7}.

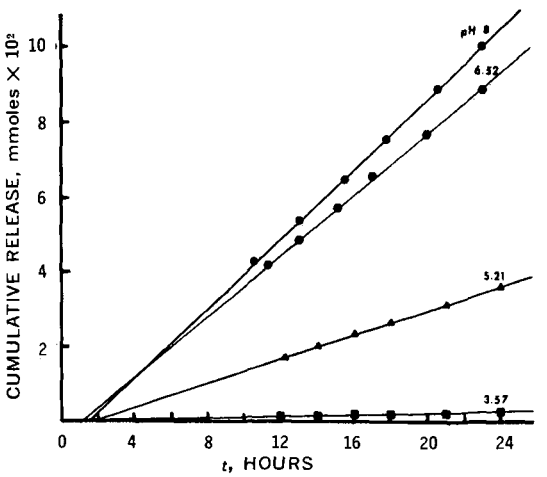

Figure 5—*Cumulative release data for pyridine transport through disks saturated with triolein–acrylic resin mixture.* $D_L = 5.6 \times 10^{-7}$ *cm.*^{2} *sec.*$^{-1}$.

Oil–Water Partition Coefficients (*KLH*) (Table II)—Partition coefficients were obtained by equilibrating an aqueous phase (pH 8) of the solute, at a concentration comparable to that used in the rate runs, with a suitable volume of the oil–polymer mixture. The aqueous phase was assayed before and after equilibration; the nature of the oil phase precluded its assay. The *KLH* values seemed to be reasonably independent of aqueous concentration.

Lipid Diffusion Coefficient (D_L)—This quantity was not directly measurable. A set of calibrating runs was done with the pyridines at pH 8, where they were essentially nonelectrolyte solutes. Equation 8 in the previous paper (1) was then used to solve for D_L. The diffusion coefficient could be obtained in more complex situations by computer fitting.

RESULTS

Figures 4 and 5 show raw transport data for pyridine through the mineral oil–polymer and triolein–polymer mixtures, respectively. Lag times were on the order of 2 hr. The D_L values calculated from this lag time value were an order of magnitude smaller than those obtained from steady-state rate data. However, the lag time decreased to about 15 min. if the lipid disks were presoaked in plain buffer solution before the run; this procedure did not visibly affect the steady-state rate. Figure 6 shows rate–pH profiles for pyridine (slopes of Figs. 4 and 5). The data indicate negligible transport of the pyridinium ion.

Figure 7 summarizes the results of runs for the uncharged pyridine derivatives diffusing through the lipid phase between aqueous phases of pH 8.00. All solutes were run at a concentration of 10^{-2} *M*, except pyridine (3×10^{-2} *M*) and the amyl derivative, which

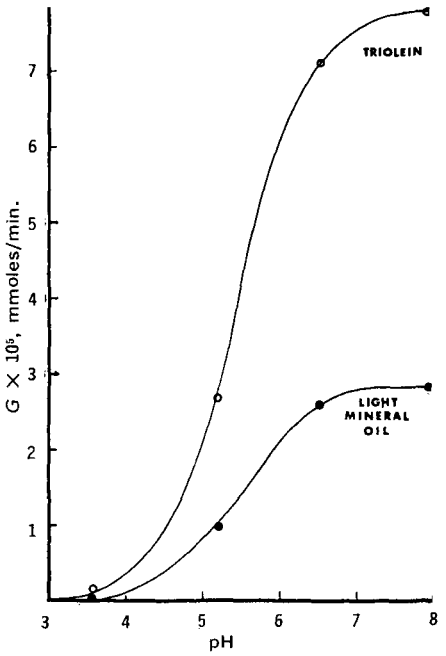

Figure 6—*Summary of rate data from Figs. 4 and 5 for pyridine. Solid lines are:* (a) *titration curves for pyridine (pK'a = 5.47) and/or* (b) *computer-generated curves for the respective diffusion coefficients in the two lipid phases.*

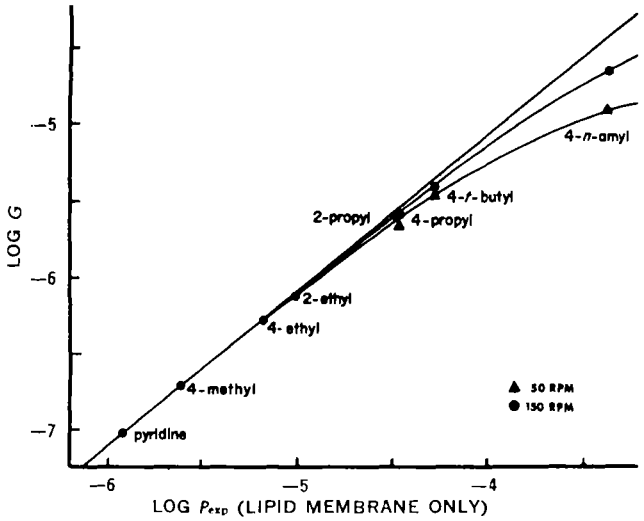

Figure 7—*Summary of data for transport of a series of alkylated pyridines through the mineral oil–acrylic resin system. Rates are normalized to correspond to a source compartment concentration of 0.01* M. *The abscissa is the permeability coefficient of the lipid membrane only and is equal to* $(KLH)(D_L)/TH2$. *The value of this quantity is calculated using Eq. 8 of the previous paper (1).*

had a solubility of 2.35×10^{-3} *M* at pH 8. Resulting rates for these latter two solutes were normalized to correspond to 10^{-2} *M* in the source compartment. Since the "experimental membrane permeability coefficient" in Fig. 7 includes the lipid diffusion coefficient D_L, obtained from the experimental rate and assuming the effect of the diffusion layers predicted by the model, the plot does not "prove" the model. What is directly apparent in Fig. 7, however, is the effect of stirring rate on transport of the high partition coefficient solutes. The lipid diffusion coefficients derived here were used in further studies.

Reasonable consistency was found in the data. For *n*-amylpyridine runs at 150 r.p.m., given a value of 60 μ for each of the aqueous diffusion layers (TH1 = TH3) and the experimental transport rate *G*, a value of 7.3×10^{-8} cm.^{2} sec.$^{-1}$ for D_L, the diffusion coefficient of the free base in the lipid phase, was calculated from Eq. 8 of the previous paper (1). When similar runs were made at 50 r.p.m., the same value of D_L (7.3×10^{-8}) and the experimental *G*, inserted into Eq. 8 (1), resulted in a calculated value of TH1 = TH3 ranging from 170 to 200 μ. The independently determined value of TH1 = TH3 at a stirring speed of 50 r.p.m. was 180–225 μ.

Figure 8 shows the effect of the sampling compartment pH in otherwise identical rate experiments with *n*-amylpyridine in a pH

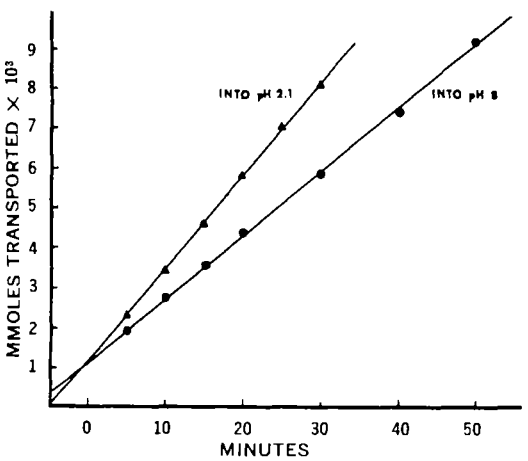

Figure 8—*Diffusion of amylpyridine* (C = 2.25×10^{-3} M) *through the mineral oil–acrylic resin system into aqueous phases of pH 8.00 and 2.10. An increase in rate for the latter case reflects the elimination of a significant portion of the total barrier (the right or serosal diffusion layer).*

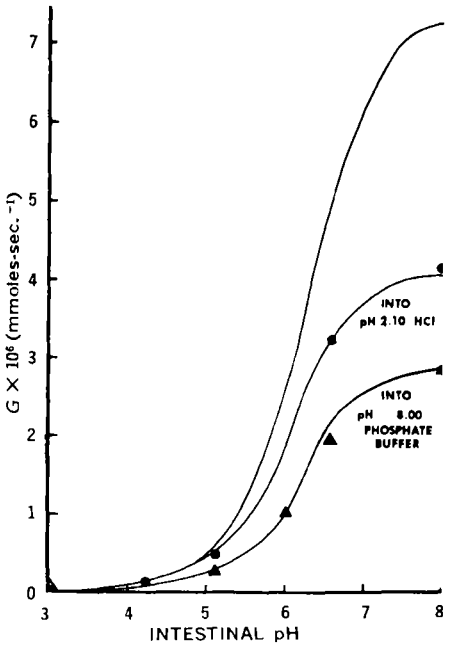

Figure 9—*The pH-rate profiles for amylpyridine diffusing through the mineral oil–acrylic resin mixture into aqueous phases of pH 8.00 and 2.10. Smooth curves are computer-generated lines for* $D_L = 7.3 \times 10^{-8}$ *cm.*^{2} *sec.*$^{-1}$. *Perfect overall fitting was not obtained due to the effect of the solute concentration on the lipid viscosity and, therefore, the diffusion coefficient. The top curve illustrates the predicted situation (Brodie-Shore-Hogben prediction) where* $D_L = 7.3 \times 10^{-8}$ *and the effect of aqueous diffusion layers is neglected.*

8.00 solution. As predicted by the model, the contribution of the diffusion layer on the sampling compartment side of the membrane is negated at the low pH, creating pseudo-two-phase conditions. Since the aqueous diffusion layers are a sizable part of the total barrier presented to amylpyridine, with a partition coefficient of 298, elimination of half of the total aqueous barrier increases the observed transport rate. About a 50% increase is seen in Fig. 8, indicating that the membrane still has an appreciable contribution to the total barrier.

Runs with amylpyridine (2.25×10^{-3} *M*) at various source compartment pH's, diffusing through the lipid phase into solutions of pH 8 and 2.10, respectively, gave the results shown in Fig. 9. The observed rates for transfer into pH 2.10 buffer are higher over essentially the entire source compartment pH range. Calculations for

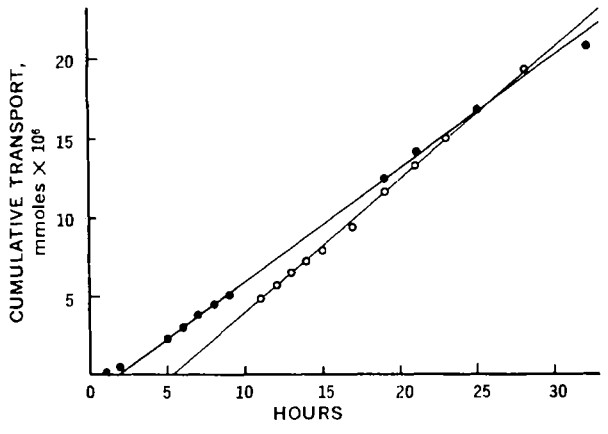

Figure 10—*Data for radiolabeled pyridine at* 8.0×10^{-5} M (○) *diffusing through the mineral oil-acrylic resin mixture from an aqueous phase of pH 5.10, and for pyridine at the same concentration* (●) *diffusing in the presence of simultaneous transport of amylpyridine* (2.10×10^{-3} M, *pH 5.10*). *The receptor phase in both instances was 0.01* M *HCl. The degree of lowering (14%) was not as great as predicted by the model.*

a system with very large buffer capacity, and therefore assuming that interfacial pH is equivalent to the bulk aqueous pH, show that all three curves in Fig. 9 would be identical for solutes with very low partition coefficient values. Under these experimental conditions, however, with bases having partition coefficients in the 10^2–10^3 range, calculations showed that the upper two curves in Fig. 9 (Brodie–Shore–Hogben prediction and the three-phase system with receptor phase of pH 2.10) should converge at low source phase pH and that the true three-barrier curve should always be below the other two curves. As the partition coefficient increases to very large values, the two-phase curve should fall below the Brodie–Shore–Hogben curve, which is based on a membrane barrier only.

An apparent shift toward lower pH values for the pH 2.10 curve as compared to the pH 8.00 curve would be visible if the solute partition coefficient were high enough (5). Upon normalizing both lower curves in Fig. 9 to the same maximum rate, a slight shift (~0.1 pH unit) may be visible. For this instance, the T factor of Suzuki *et al.* (5) would be:

$$T = \frac{(D_W)(TH2)}{(KLH)(TH1)(D_L)} = \frac{(9.45 \times 10^{-6})(5.08 \times 10^{-2})}{(298)(1.80 \times 10^{-2})(7.30 \times 10^{-8})} = 1.19 \quad \text{(Eq. 1)}$$

a value where a shift just became apparent in the two-phase model.

An 8.0×10^{-5} M solution of tritiated pyridine[13] in 0.7 M NaCl was adjusted to pH 5.10 with hydrochloric acid. Rate runs were made in the three-phase apparatus, through a disk containing the mineral oil–acrylic resin mixture, into pH 2.10 hydrochloric acid. One-milliliter samples were assayed using a liquid scintillation counter[14]. A toluene[15]–octoxynol[16]–2,5-diphenyloxazole[17] scintillation cocktail was used in the counting procedure. This particular mixture is intended for the counting of aqueous samples (6, 7).

A similar run was made with a mixture of 2.1×10^{-3} M *n*-amylpyridine (nonradioactive) and the 8.0×10^{-5} M tritiated pyridine, adjusted as before to pH 5.10. One set of data is shown in Fig. 10. As predicted by the model, the rate for pyridine is lowered in the presence of an excess of a base with a much higher partition coefficient. In actuality, the degree of rate lowering (~14%) was lower than that predicted by the model (~43%) when the appropriate parameters were fed into the computer program.

When a lipid-saturated disk was equilibrated in the apparatus with aqueous phases on both sides containing 2.10×10^{-3} M amylpyridine, however, and the 8.0×10^{-5} M radiolabeled pyridine then was injected into the source chamber, the observed rate for pyridine transfer was some 30% greater than that for the same experiment without preequilibration with amylpyridine in both aqueous phases. It is believed that the high concentration (~0.6 M) of the amyl derivative, when partitioned into the membrane, lowers the lipid's viscosity and results in a lipid diffusion coefficient of pyridine about 30% greater than the previously determined 2.24×10^{-7} cm.^{2} sec.$^{-1}$. With a D_L for pyridine of 3×10^{-7}, good agreement between model and experiment was obtained.

DISCUSSION

Two-phase *in vitro* systems were designed by Moore (8) and Ting *et al.* (9). Three-phase apparatus were used by Rosano *et al.* (10), Robertson and Bode (11), and Perrin (12) in *in vitro* transport studies. All systems shared one basic difficulty: instability of the interface. The V-shaped rocking apparatus, such as used by Reese *et al.* (13), was designed to minimize this turbulence, but it incorporates oil–water interfaces whose areas change with time. The lipid phases in such systems are unreasonably large in comparison to the aqueous phases. Steady-state conditions are probably never reached; the relatively large lipid phases have the potential, if unstirred, of storing most of the solute introduced into the source aqueous phase, especially those substances with high partition coefficients. These lipid phases are most generally stirred, making somewhat unclear the actual nature and magnitude of the lipoidal diffusion barrier. With rapid stirring of the lipid phase, the solute tends to "spill over" into the receptor aqueous phase. It is felt that this is not a realistic picture of the *in vivo* barrier. The present apparatus does away with these difficulties by providing a more realistic lipid–aqueous phase volume ratio and a hydrodynamically stable interface.

The experimental data obtained in this study outline the consequences of the existence of aqueous diffusion layers adjacent to the lipoidal membrane in the three-phase system. Without these additional barriers, the transport rate would be independent of stirring speed. Transfer rates, normalized to the same source compartment solute concentration, would increase indefinitely with the partition coefficient[18]. The pH of the receptor aqueous phase would not affect the transfer rates of high lipid permeability coefficient solutes. The simultaneous transport of a high permeability solute, such as amylpyridine, would not slow the rate for a lower permeability weak base. It is, moreover, significant that all these effects are observed only when the aqueous diffusion layers represent a significant portion of the total barrier presented to the diffusing solute.

How important a role aqueous diffusion barriers play in the *in vivo* state is yet to be determined. The buccal absorption data of Beckett and Moffat (14) provide some support, as do the toad bladder data of Rosen *et al.* (15). Additional verification and support of the theory by careful *in vivo* studies are anticipated.

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ACKNOWLEDGMENTS AND ADDRESSES

Received June 4, 1971, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48104*

Accepted for publication July 12, 1972.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Washington, D. C. meeting, April 1970.

Abstracted from a thesis submitted by R. G. Stehle to the University of Michigan in partial fulfillment of the Doctor of Philosophy degree requirements.

Supported by Grant GM-13368 and Predoctoral Fellowship 1-F1-GM-30,048-05 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, MD 20014

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[13] Obtained from Amersham-Searle, Des Plaines, Ill. Specific activity was 151 mc./mmole.

[14] Beckman LS 200, Beckman Instruments, Inc., Fullerton, Calif.

[15] J. T. Baker Chemical Co., Phillipsburg, N. J.

[16] Triton X-100, Rohm & Haas, Philadelphia, Pa.

[17] Packard Instrument Co., Inc., Downers Grove, Ill.

[18] The absolute transfer rate will ultimately be limited by the aqueous solubility in a homologous series of solutes.