Nonelectrolyte Diffusion across Lipid Bilayer Systems

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ABSTRACT The permeability coefficients of a homologous series of amides from formamide through valeramide have been measured in spherical bilayers prepared by the method described by Jung. They do not depend directly on the water:ether partition coefficient which increases regularly with chain length. Instead there is a minimum at acetamide. This has been ascribed to the effect of steric hindrance on diffusion within the bilayer which increases with solute molar volume. This factor is of the same magnitude, though opposite in sign to the effect of lipid solubility, thus accounting for the minimum. The resistance to passage across the interface has been compared to the resistance to diffusion within the membrane. As the solute chain length increases the interface resistance becomes more important, until for valeramide it comprises about 90% of the total resistance. Interface resistance is also important in urea permeation, causing urea to permeate much more slowly than an amide of comparable size, after allowance is made for the difference in the water:ether partition coefficient. Amide permeation coefficients have been compared with relative liposome permeation data measured by the rate of liposome swelling. The ratios of the two measures of permeation vary between 3 and 16 for the homologous amides. The apparent enthalpy of liposome permeation has been measured and found to be in the neighborhood of 12 kcal mol⁻¹ essentially independent of chain length. Comparison of the bilayer permeability coefficients with those of red cells shows that red cell permeation by the lipophilic solutes resembles that of the bilayers, whereas permeation by the hydrophilic solutes differs significantly.
though not quantitatively, related to the relative permeability of liposomes to these same amides, which we have also measured by the rate of swelling.

There are three important factors that govern nonelectrolyte permeability: the rate constant for entrance into the membrane from the aqueous environment, the diffusion coefficient within the membrane, and the partition coefficient. The present results illustrate the role of each process in nonelectrolyte transport across an egg lecithin bilayer.

**METHODS AND MATERIALS**

**Bilayer Membranes**

Spherical bilayer membranes were prepared according to the techniques of Jung (9) by using a micrometer-driven microsyringe to blow thin lipid bubbles at the end of a glass capillary or polyethylene tube. All experiments used egg lecithin purified either by the method of silicic acid chromatography (10) or alumina chromatography (11). The lecithin was dissolved at a concentration of 2–3 g% in 5:3 chloroform:tetradecane (reagent grade, Eastman-Kodak, Rochester, N. Y.). Membranes were formed in a temperature-jacketed 2.5-ml cell at 25°C, except where otherwise specified. The solutions on either side of the membrane were identical, containing in mM: 125 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 2 TES (N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid) at pH 7.4. The buffer also contained 20 mM of the test solute. The bubbles were blown to a diameter of about 3 mm and the thinning time ranged from 20–60 min. The solution was stirred with a magnetic bar at ~200 rpm during and after the thinning process. It was important that the density of the membrane-forming solution remain constant to avoid too rapid thinning. If the solution becomes too dense, a solvent lens may form, tearing the membrane.

Permeability measurements were made in the following manner. After thinning had occurred, 20 μl of ¹⁴C radioactively labeled test solute (∼2 μCi) was added to the external solution. The labeled compounds were obtained from New England Nuclear (Boston, Mass.) except for [¹⁴C]formamide obtained from Mallinckrodt Chemical Works (St. Louis, Mo.) and [¹⁴C]valeramide obtained from International Chemical and Nuclear Corp. (Irvine, Calif.). After a given time period (2–10 min), the bubble was gently retracted into the capillary using the micrometer syringe. The capillary was rinsed in distilled water, and then all the radioactivity in the capillary tube was ejected into a solution of liquid scintillation counting medium (Bray's solution). The sample was counted in a Nuclear Chicago (Des Plaines, Ill.) model 724 liquid scintillation counter. A sample of the external solution was also counted and the permeability coefficient was calculated using the simple Fick equation and computing the area, A, of the truncated sphere from the standard formula \( A = \frac{1}{2} \pi h r^2 \) in which \( r \) is the radius in centimeters and \( h \) the truncated height in centimeters. Areas ranged from 0.064 to 0.315 cm² with an average of 0.168 cm².

Alternatively, permeability coefficients could be obtained using Jung's technique of preloading the spheres with label and measuring the efflux of label into the external medium. Because of label efflux during the thinning process the Jung technique produced a higher initial radioactivity in the external medium and thus caused larger errors.

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1 It was confusing to us at first sight, and has also puzzled one referee that the formula for the area of a truncated sphere is the same as that for a cylinder with open ends. However the validity of the equation for the truncated sphere can readily be shown and it is given in the 45th edition of the Handbook of Chemistry and Physics, The Chemical Rubber Company, Cleveland, Ohio, 1964, A-168.
The electrical resistance of several bilayers were measured and found to be between $1.5 \times 10^7$ and $2.0 \times 10^8$ $\Omega$cm$^2$ which is higher than the value of $1 \times 10^6$ $\Omega$cm$^2$ given by Miyamoto and Thompson (12) for egg lecithin planar bilayers made with chloroform, methanol, and tetradecane. Once the membrane had thinned, the resistance remained relatively stable for the duration of the experiment.

**Liposome Permeability Measurements**

Liposomes were prepared according to the technique of Bangham et al. (13) by making a “hand-shaken” dispersion from either a mixture of pure egg lecithin prepared as for the spherical bilayers (96%) and phosphatidic acid (4%), or from a total lipid extract from red blood cells according to the method of Bruckdorfer et al. (14). The liposomes were prepared in 50 mM KCl with a total lipid concentration of 10 mM.

Permeation was measured by the method of Cohen and Bangham (15). 0.2 ml of the lipid dispersion was rapidly mixed with 2.5 ml of a 150 mM solution of the test solute. All solutions were preincubated at the desired temperature, and the optical density of the suspension was measured in a water-jacketed glass cuvette at 450 nm in a Perkin-Elmer 402 spectrophotometer (Norwalk, Conn.). The time required for mixing was about 3 s and changes in optical density were observed from 5 to 120 s. According to the Cohen and Bangham (15) technique, the liposomes first shrink and then swell; the relative permeability is measured by determining the initial rate of the swelling phase from the changes in optical density. This technique was checked by measuring the swelling rate of the liposomes in isotonic solutions of the various test solutes according to the method of de Gier et al. (2) and virtually identical relative permeability results were obtained. Controls were performed on each set of liposomes to determine that the liposomes were osmotically active, stable, and that the volume changes could be ascribed only to the test solute.

When the effect of phloretin on liposome permeability was to be studied, $2.5 \times 10^{-4}$ M phloretin (Fluka, A. G., Buchs, Switzerland) was added to the lipid dispersion and incubated at room temperature for at least 30 min before measuring the relative permeability.

**RESULTS AND DISCUSSION**

**Thickness of Unstirred Layer**

In order to determine the thickness of the unstirred layer, we measured the diffusion of tritiated water across the spherical bilayer and compared this permeability coefficient with the hydraulic conductivity of the membrane under an osmotic pressure gradient. Cass and Finkelstein (16) have shown that in the limiting case of zero unstirred layer, these two coefficients are equal for a mixed lipid planar bilayer, and this conclusion has been confirmed by Everitt et al. (17) for an egg phosphatidyl choline and cholesterol planar bilayer. We have used the method of Cass and Finkelstein (16) to measure the hydraulic conductivity of our spherical bilayer under a sucrose osmotic pressure gradient of 200–400 mosM. Trace amounts of phenol red were added to the bathing medium to make the membrane visible in the microscope. In five experiments, carried out at 24°C, we obtained a value of $1.3 \pm 0.5 \times 10^{-3}$ cm s$^{-1}$ for the hydraulic conductivity of our membranes. This agrees reasonably with the values of $0.78 \times 10^{-3}$ cm s$^{-1}$ given by Cass and Finkelstein and $1.9 \times 10^{-3}$ cm s$^{-1}$ given by Hanai and Haydon (18) for their membranes.
The permeability coefficient was measured in two experiments at 24°C by adding THO (New England Nuclear) to the outside buffer and making measurements as described for amide bilayer permeation, except that samples were taken at 1.5 and 3 min. The average value obtained, $0.69 \times 10^{-3}$ cm s$^{-1}$ ($0.76 \times 10^{-3}$ and $0.62 \times 10^{-3}$ cm s$^{-1}$) agrees reasonably with the values of $0.62 \times 10^{-3}$ to $1.06 \times 10^{-3}$ cm s$^{-1}$ given by Cass and Finkelstein for THO diffusion in their system. The ratio of two permeability coefficients, (hydraulic conductivity/THO permeability coefficient) which is 1.9, may be used to estimate the thickness of the unstirred layer, assuming that the value of the hydraulic conductivity is a measure of the “true” permeability coefficient as discussed, for example, by Cass and Finkelstein.

In order to determine the magnitude of the unstirred layer correction for spherical bilayers, we have used the equation derived by Milgram which is given in the Appendix. The true permeability coefficient, $P_{d,true}$, is given by

$$1/P_{d,true} = 1/P_{d,obs} - (t_1/D) \left( 1 - \frac{t_1}{r} \right) - (t_2/D) \left( 1 + \frac{t_2}{r} \right),$$

in which the subscript obs means observed, $t_{1,2}$ are the thicknesses of the unstirred layer on the outside and the inside of the spherical bilayer, respectively, and $r$ is the radius of the sphere. In the limit, if $t_1 = t_2$, Eq. 1 reduces to the planar solution

$$1/P_{d,true} = 1/P_{d,obs} - 2t_1/D.$$  (2)

Using Eq. 2 and taking the THO permeability coefficient as $P_{d,obs}$, we can calculate that the total unstirred layer thickness is 177 µm for the sum of the layers on both sides of the membrane at 24°C.

In order to use Eq. 1, we need to estimate the thickness of the unstirred layer on the outside of the spherical bilayer. Everitt et al. (17) give data for unstirred layer thickness for planar bilayers. In the absence of stirring on either side of the planar bilayer, the average thickness of the unstirred layer was 250 µm. With the most vigorous stirring it could be reduced to 33 µm. These authors provide evidence (with Everitt and Haydon [19]) that the thickness of the unstirred layer is controlled by natural convection. Ginzburg and Katchalsky (20) have concluded that, even with the most vigorous stirring, the unstirred layer cannot be brought below a “limiting value” of 25 µm, in a much more robust system with cellophane membranes. If we take 35 µm as an estimate of the unstirred layer thickness on the outside of the spherical bilayer under our stirring conditions, Eq. 1 leads to a value of 128 µm for the unstirred layer thickness on the inside of the membrane. Hence the correction for the spherical geometry is relatively unimportant, since the sum of both layer thicknesses is 163 µm, relatively close to the total of 177 µm for planar geometry. In view of the assumptions involved in estimating the thickness of the outer unstirred layer, and the simplicity of Eq. 2, we have used Eq. 2 and a total thickness of 177 µm to correct our data for the unstirred layer.2 The correction is most important for the most permeable

2 In the course of discussion after the manuscript was submitted, Finkelstein (private communication) suggested that measurement of the unstirred layer by butanol permeation as described by Holz...
solute, valeramide, and in this case, the ratio of the true permeability coefficient to the observed one is 1.34.

Comparison of Permeation across Spherical Bilayers with Planar Bilayer Data

The permeability coefficients for a homologous series of monoamides and urea across egg lecithin spherical bilayers are given in Table I. Our values agree very well with those obtained by others for the permeability coefficient of urea. Vreeman (4) obtained a value of \(0.42 \times 10^{-5}\) cm s\(^{-1}\) for \(P_{d,\text{urea}}\) at 20°C and Gallucci et al. (5) give \(0.37 \times 10^{-5}\) cm s\(^{-1}\) for \(P_{d,\text{urea}}\) at 28°C. Both agree reasonably with our figure of \(0.41 \times 10^{-5}\) cm s\(^{-1}\) at 25°C.

However, our experimental data for the permeation coefficients of formamide and acetamide are not in agreement with those of Gallucci et al. (5); the disagreement increases with lipid solubility. For formamide, Gallucci et al. give \(P_{d} = 16 \times 10^{-5}\) cm s\(^{-1}\), about twice as large as our value; for acetamide, their value is \(14 \times 10^{-5}\) cm s\(^{-1}\) about six times greater than our value. These disagreements are unexpected in view of the good agreement for urea and water permeation. In a subsequent section of this paper, evidence is presented that urea permeation depends significantly on the rate constant for transfer from the aqueous solution to the membrane, so that the difference in the permeability coefficients of the somewhat more lipophilic amides may reside in the lipid properties of the membrane.

The two experimental systems differ in several significant aspects, particularly in the solvent system from which the bilayers were made; Gallucci et al. used decane and we used 5:3 chloroform:tetradecane. Fettiplace et al. (23) have measured the thickness and composition of the hydrocarbon portion of black lipid membranes made from egg phosphatidyl choline dispersed in various solvents. When membranes are made from decane dispersions, the thickness of the hydrocarbon region is 48 Å and the volume fraction of the phosphatidyl choline hydrocarbon chains is 0.69. For similar membranes made from tetradecane dispersions, the hydrocarbon thickness is 37 Å and the volume fraction is 0.86. It seems quite likely that membrane composition and thickness are both important determinants of the permeation process. For example, Toyoshima and Thompson (24) have measured Cl exchange flux in membranes of diphytanoylphosphatidyl choline prepared from a decane solution and found them to be some two orders of magnitude smaller than Cl exchange fluxes reported earlier by Pagano and Thompson (25) in egg phosphatidyl choline membranes pre-
<table>
<thead>
<tr>
<th>Solute</th>
<th>T</th>
<th>Permeability coefficient*</th>
<th>Partition coefficient, § k_{eter}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( Pa \times 10^6 )</td>
<td>( \omega \times 10^{11} )</td>
</tr>
<tr>
<td>Formamide</td>
<td>25</td>
<td>7.8 ± 0.5 (6)</td>
<td>3.1</td>
</tr>
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<td>Acetamide</td>
<td>25</td>
<td>2.4 ± 0.4 (5)</td>
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<td>Propionamide</td>
<td>25</td>
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<td>Valeramide</td>
<td>25</td>
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<tr>
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</tr>
<tr>
<td>Urea</td>
<td>20</td>
<td>0.23 ± 0.04 (5)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Mean ± SE; number of experiments in parenthesis \( \omega = P_d/RT \).

† The results obtained in preliminary experiments by one of us (P. C. W.) in which the radioactivity was initially placed inside the bubble and the time-course of efflux was measured as discussed in the text, are for \( P_d \) in cm s\(^{-1}\): acetamide, 3.4 ± 0.7 \( \times 10^{-5} \); propionamide, 3.0 ± 1 \( \times 10^{-5} \); and urea, 0.30 ± 0.05 \( \times 10^{-5} \). These are not corrected for unstirred layer thickness, since the correction is less than 3% for a permeability coefficient of 2.5 \( \times 10^{-5} \) cm s\(^{-1}\).

§ The values for \( k_{eter} \) have been taken from Collander (21) except for valeramide. Collander gives \( k_{eter} \) for isovaleramide of 0.17 and we have increased this by a factor of 1.35 which Lange et al. (22) determined as the ratio of partition coefficients of valeramide to isovaleramide in several bulk solvents as well as in egg lecithin vesicular particles.

∥ Two of these experiments were carried out at 24°C.

pared from a chloroform-methanol-tetradecane solvent system. The electrical resistance of the Toyoshima and Thompson bilayers was about 10\(^9\) \( \Omega \) cm\(^2\), as compared with 3 \( \times 10^8 \) \( \Omega \) cm\(^2\) given by Pagano and Thompson. The mean electrical resistance of our spherical bilayers was about 10\(^8\) \( \Omega \) cm\(^2\). Gallucci et al. do not specify the electrical resistance of their membranes, but refer to a paper by Läuger et al. (26) who give a range of resistances of 10\(^6\) to 10\(^8\) \( \Omega \) cm\(^2\) for their membranes. Since the lower limit is more than an order of magnitude lower than our lowest measured value of 1.5 \( \times 10^7\) \( \Omega \) cm\(^2\), it is possible that the resistances of the membranes used by Gallucci et al. were less than ours.

There may also be differences in the contribution of permeation through the torus which normally comprises about 1–2% of the membrane area of the spherical bilayers and is never greater than 6%. Läuger et al. give a figure of about 10% for their planar membranes. There is also a difference in solute concentration since our experiments were carried out with a 20 mM amide concentration, and we estimate from the Gallucci et al. data that their concentrations were probably an order of magnitude lower. This difference may be of significance in view of Solomon’s (27) suggestion that amide dimerization may play a role in lipid bilayer permeation.

A possible systematic error in our measurements should also be considered. In the measurements reported in Table I, we measured the flux into the spherical bubble. Only one sample could be taken in each experiment since the discharge of the sample destroyed the bubble. Our calculations were made using the simple diffusion equation and neglecting backflux. If the radioactivity in the
bubble at the time the experiment was terminated was significant, this procedure would lead to an underestimate of the flux, because backflux had been neglected. It is clear that the radioactivity was dissolved in a volume larger than that of the spherical bubble alone and must have penetrated some distance up the tube which supported the bubble due to some combination of stirring and convection. It was not possible to determine the total volume in which the solute was dispersed and we relied instead on measurements of the unstirred layer to determine the contribution of mixing within the spherical bubble. THO is the most permeable of the solutes we have studied and the fact that its permeability coefficient agrees reasonably with that of Cass and Finkelstein (16) supports the validity of our procedure.

There are two other lines of evidence that should be taken into account. Valeramide is the most permeable of the amides we have studied and two series of experiments were made on it. In the first series at 25°C, sampling times were 5.5 and 7 min and the permeability coefficients (uncorrected for unstirred layer) were $12.8 \times 10^{-5}$ and $14.1 \times 10^{-5}$ cm s$^{-1}$. In the second series, at 24°C, the sampling times were 2 and 3 min and the permeability coefficients were $15.7 \times 10^{-5}$ and $12.1 \times 10^{-5}$ cm s$^{-1}$, so there does not seem to be a consistent source of error in the valeramide data due to sampling time problems.

As far as the other solutes are concerned, one of us (P. C. W.) carried out a preliminary set of experiments in which permeation was measured in the opposite direction, the radioactivity being placed initially in the bubble with samples taken from the external medium. In this way it was possible to take several samples and determine that the flux was linear with time. Since radioactivity escapes from the bubble during the thinning period, there was a high initial background and we concluded that the method used for Table I was preferable. However, we have presented these preliminary data in the second footnote to Table I to show how well the two methods agree. The agreement is not unreasonable, considering the large standard errors of the mean and there is no systematic progression in difference which increases either with lipid solubility or with permeability coefficient.

**Rate-Governing Steps for Permeation across Spherical Bilayers**

At first sight it seems strange that the permeability coefficients shown in Fig. 1 do not increase uniformly with chain length in accordance with Overton's rule since the water:ether partition coefficients, $k_{\text{ether}}$, increase with chain length, as shown in Table I. Instead there is a minimum at acetamide, formamide permeating three times faster than acetamide despite its lower value of $k_{\text{ether}}$. Overton's rule implicitly assumed that the sole rate-determining step is partition, but Table I shows that this cannot be the case, at least for the smallest solute, formamide. The presence of a minimum means that there must be at least two controlling processes governing lipid bilayer permeation. These can be identified as partition between bathing solution and bilayer, and diffusion through the bilayer, as illustrated in Fig. 2. The bottom figure shows that $\ln k_{\text{ether}}$ increases linearly with the addition of each methylene group. In the top figure $\ln(P_{\text{ether}}/k_{\text{ether}})$, which is a measure of diffusion through the bilayer, is seen to decrease linearly with increased solute molar volume.
FIGURE 1. Permeability coefficient for spherical egg lecithin bilayer as a function of solute molar volume. The symbols are: F, formamide, A, acetamide, P, propionamide, and V, valeramide.

FIGURE 2. (Top) Dependence of \( \ln(P_{d}/k_{ether}) \) on solute molar volume. (Bottom) Dependence of \( \ln(k_{ether}) \) for the linear alkane monoamides on total number of carbons in chain.

The relation between these two processes can be illuminated by the following equation of Zwolinski et al. (28),

\[
P_{m} = A'e^{-\Delta G^{*}/RT},
\]

in which \( P_{m} \) is the permeability coefficient within the bilayer in cm s\(^{-1}\); \( A' = (K\lambda^{3/2}/\delta)(kT/h) \); \( \Delta G^{*} \) is the free energy of activation for diffusion in the bilayer; \( k \) is Boltzmann's constant, \( h \), Planck's constant, and \( R \), the gas constant; \( T \) is absolute temperature, \( \lambda \) is the diffusion jump distance, and \( \delta \) is the membrane thickness; \( K \) is the partition coefficient between bathing solution and the membrane. Since \( K \) is not, in general, known whereas \( K_{ether} \) has been measured for the solutes we have used, the following transform is useful. By introducing \( A = A'k_{ether}K \), \( P_{m} \) can be expressed as

\[
P_{m} = A e^{-\Delta G^{*}/RT},
\]

in which \( \Delta G_{m}^{*} \) is defined as
\[ \Delta G_m^* = \Delta G^\ddagger - RT \ln \left( \frac{A'}{A} \right). \] (5)

In the subsequent treatment the variations between members of the homologous series of monoamides will be considered to be reflected only in \( \Delta G_m^* \), which is equivalent to assuming that the ratio of \( k_{\text{ether}}/K \) is the same for all members of the series. The validity of such a relationship for \( k_{\text{octanol}}/K \) has been demonstrated by Roth and Seeman (29) who showed that the water:octanol partition coefficient of the straight chain monohydric alcohols was linearly related to the buffer:red cell membrane partition coefficient. \( k_{\text{octanol}} \) was uniformly five times greater than \( k_{\text{red cell membrane}} \) for alcohols from pentanol through decanol. Diamond and Katz (30) have found a similar relationship between partition coefficients of dimyristoyl lecithin liposomes and isobutanol. The nature of the relationship depends upon the solvent, and the properties of isobutanol as a solvent are very much closer to dimyristoyl lecithin than those of ether. However dimyristoyl lecithin has a sharp phase transition at 25°C, whereas egg phosphatidyl choline has a broad phase transition centered about -10°C so that the properties of these two phosphatides as solvents may well differ substantially.

The next question is how to relate \( P_m \) to the measured permeability coefficient \( P_d \) which includes the terms for entering and leaving the bilayer in addition to \( P_m \). The Zwolinski et al. equation has been used by Galey et al. (31) in the form

\[ \frac{1}{P_d} = 2 \frac{D_m k_{\text{ether}}}{D_m k_{\text{ether}} + K}, \] (6)

in which \( D \) is the diffusion coefficient: across the interface, \( D_m \), within the membrane. The linearity of this equation, as Zwolinski et al. point out, depends upon the variations in \( D_m \) being small as one progresses through a homologous series of solutes. Eq. 6 illustrates clearly the major terms governing solute permeation through a membrane treated as a homogeneous barrier. \( 2D_m \) is the resistance of the interface to solute crossing. \( D_m/\lambda^2 \) is \( k_m \), the rate constant for crossing the interface and entering the membrane. \( K \), the steady-state partition coefficient is a ratio of two rate constants, \( k_m/k_{\text{ether}} \), in which \( k_m \) is the rate constant for leaving the membrane and returning to the bathing medium. \( K \) is included in \( (D_m k_{\text{ether}}) \) which is also related to the rate constant for permeation within the membrane, since \( D_m/\lambda^2 = k_m \). Hence permeation is controlled by two rates (\( k_m \) and \( k_{\text{ether}} \)) and one distribution coefficient (\( K = k_m/k_{\text{ether}} \)). Galey et al. (31) have shown that Eq. 6 is linear with \( k_{\text{ether}} \) for the permeation of the higher lipophilic members of the series (propionamide, butyramide, and valeramide) across human red cell membranes at 25°C (see Fig. 3). These authors have pointed out that a linear relationship is also observed for permeation of the same solutes across Chara membranes, as well as for the analogous members of the monocarboxylic acid series across human red cell membranes.

There is no such linear relationship for spherical bilayers as shown in the upper line in Fig. 3 in which the data from Table I are plotted according to Eq. 6. The nonlinearity can be attributed in part to the assumption in Eq. 6 that diffusion within the membrane can be approximated by a single value of \( D_m \) independent of solute size. This is not the case even for diffusion in bulk water since the diffusion coefficients of these same solutes in water decreases as their...
Figure 3. Inverse permeability coefficient ($P_a^{-1}$) as a function of the inverse partition coefficient ($k_{ether}$) for monoamide permeation of spherical lipid bilayers (upper) and lipophilic monoamide permeation of human red cells at 25°C (lower). The additional symbol is B, butyramide.

 volume increases. The difference between the red cell results and the present ones may be also related to the inclusion of the shorter chain nonlipophilic amides in the present data, since the shorter the chain, the greater the fractional increase in volume per methylene added.

Nonetheless, Fig. 3 illustrates that the $y$ intercept of $P_a^{-1}$ has a positive value much greater than that for the red cell membrane though the slopes are similar. As the partition coefficients increase, the relative importance of passage across the interface increases until, for valeramide, it is very nearly the rate-controlling step. This is a consequence of the fact that the concentration of solute within the bilayer increases with $k_{ether}$; the increased concentration gradient causes diffusion within the membrane to go much faster. Hence, the overall rate, $P_a$, becomes increasingly dependent upon the interface step.

The following relation (31) replaces the permeability terms in Eq. 6 with relative membrane resistances

$$R_d = R_{sm} + R_m,$$

in which $R$ is the resistance to permeation ($=P^{-1}$); the subscript $d$ refers to the resistance of the entire system, $sm$ to the interface, and $m$ to the resistance within the membrane. The values of $R_m/R_d$ given in Table II have been computed from Eq. 7 and Fig. 3 using an extrapolated value of $0.49 \times 10^4$ s cm$^{-1}$ for $R_{sm}$. $\Delta G_m^*$
TABLE II

APPARENT ACTIVATION ENERGIES FOR PERMEATION ACROSS LIPID BILAYERS

<table>
<thead>
<tr>
<th>Solute</th>
<th>$R = \frac{R_m}{\bar{V}}$</th>
<th>$\Delta G_m^*$</th>
<th>$\bar{V}$</th>
<th>$\Delta G_d^*$</th>
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</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>0.62</td>
<td>7,150</td>
<td>40.0</td>
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<tr>
<td>Acetamide</td>
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<td>8,650</td>
</tr>
<tr>
<td>Propionamide</td>
<td>0.70</td>
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<td>70.1</td>
<td>9,100</td>
</tr>
<tr>
<td>Valeramide</td>
<td>0.10</td>
<td>9,000</td>
<td>98.9</td>
<td>10,300</td>
</tr>
<tr>
<td>Urea</td>
<td>--</td>
<td>--</td>
<td>45.0</td>
<td>8,600</td>
</tr>
</tbody>
</table>

may be calculated from $R_m$ according to Eq. 4, using $\lambda^3 = \bar{V}/N_Ae$ as suggested by Glasstone et al. (32) with $\delta$ equal to the bilayer thickness of 62 Å, as given by Cherry and Chapman (33). $\bar{V}$ is partial molar volume and $N_Ae$, Avogadro's number. The results are given in column 3 of Table II. In order to examine the difference between $\Delta G_m^*$ and $\Delta G_d^*$, the value of the $RT \ln (A'/A)$ term in Eq. 5 may be roughly estimated, assuming that $K/k_{ether}$ is the same as $K/k_{octanol}$ and taking Roth and Seaman's figure of 0.2 for this ratio. This leads to a value of $-1,000$ cal mol$^{-1}$ for $RT \ln (A'/A)$.

$\Delta G_m^*$ has been plotted against solute partial molar volume in Fig. 4. The partial molar volume has been used as an index of solute size because solute diffusion in bulk water solution, through small aqueous pores in red cells and across antibiotic-treated lipid bilayers, has been found to depend smoothly upon this parameter (1, 34). As the solute molar volume increases, $\Delta G_m^*$ approaches a maximum. This is a consequence of the fact that crossing the interface is rapidly becoming the rate-determining step; as Table II shows, the interface provides 90% of the resistance to valeramide permeation across the bilayer.

In many systems there are not enough data to separate passage across the interface from diffusion within the membrane, and it is useful to be able to define an apparent activation energy for passage across the entire system, which may be done by defining $\Delta G_d^*$ by

$$P_d = Ae^{-\Delta G_d^*/RT},$$

the subscript $d$ denoting that $\Delta G_d^*$ refers to diffusion from the solution on one side of the membrane to the solution on the other side. Eq. 8 differs from that given by Zwolinski et al. (28)

$$P_d = (kT/h)(\lambda^3/\delta)e^{-\Delta G'/RT}.$$
FIGURE 4. Apparent activation energies for spherical bilayer permeation, $\Delta G_m^*$ and $\Delta G_s^*$, as a function of solute molar volume, $V_s$.

FIGURE 5. $\Delta G_d^*$ for spherical lipid bilayers (upper curve) compared with $\Delta G_d^*$ for red cell membrane (lower curve) as a function of solute molar volume. The errors for the lower curve are less than $\pm 0.07$ kcal mol$^{-1}$ for each of the solutes.

average 550 cal mol$^{-1}$ for the succeeding methylene groups which produce a progressively smaller proportional increase in solute volume.

Similarly successive methylene groups increase the water:ether partition coefficient of the monoamide. Each succeeding methylene group decreases $\Delta G^o$ for partition, by 790 cal mol$^{-1}$ (computed from the data of Collander [21] using $\Delta G^o = -RT \ln K$). Seeman et al. (35) have measured the partition of monohydric alcohols into the red cell membrane and have found a decrease of 695 cal mol$^{-1}$ per methylene group. As indicated in Fig. 2 changes in $\Delta G^o$ are of the same order of magnitude, though opposite in sign to the increments in $\Delta G_s^*$, thus accounting for the minimum in the permeability coefficient in the monoamide series.

Table II gives the value of $\Delta G_d^*$ for urea as 8,600 cal mol$^{-1}$. On the assumption that the ratio, $k_{ether}/K$, for urea is the same as that for the monoamides, the upper curve in Fig. 4 can be used to separate steric hindrance to urea permeation from other hindrances, arising principally from hydrogen-bonding effects. The value of $\Delta G_d^*$ for a monoamide of molar volume 45.0 cm$^3$ mol$^{-1}$, that of urea, is 700 cal mol$^{-1}$ less than $\Delta G_d^*$ for urea. This difference probably measures the additional hindrance offered by the hydrogen bonding of one amide group, essentially the energetic cost of traversing the two highly ordered polar interfaces on either side of the lipid bilayer.

The contribution of the partition coefficient to permeation is virtually excluded from $\Delta G_d^*$ since $k_{ether}$ is specifically included in the coefficient $A$ in Eq. 8. $\Delta G_d^*$ represents the apparent activation energy for a lumped set of rate processes. Though they are different parameters, it is instructive to compare differences in $\Delta G_d^*$ with differences in equilibrium distribution represented by $\Delta G^o$ for partition. $\Delta G^o$ for water:ether partition for urea is $-4,500$ cal mol$^{-1}$, some 600 cal mol$^{-1}$ less than the comparable $\Delta G^o$ for formamide partition ($-3,900$ cal
mol$^{-1}$). In other words, if we lump all the nonstERIC restraints for the permeation of a single amide group into a single difference in the apparent activation energy, this difference (700 cal mol$^{-1}$) is about the same as the difference in energy required to extract that same amide group from an aqueous environment and place it in a nonpolar solvent such as ether (600 cal mol$^{-1}$).

**Studies on Liposomes**

Cohen and Bangham (15) used the rate of liposome swelling to measure the relative permeability coefficients of monoamides in liposomes which contained cholesterol. Although they found that the relative permeability of formamide was greater than that of acetamide, the importance of this finding was obscured by the general conclusion they reached that permeation across liposomes was generally in accord with Overton's rule. Our results with spherical bilayers indicate that the process is significantly more complex.

There are many uncertainties in using the liposome swelling rates to measure relative permeabilities, primarily as a result of the multilayered nature of this preparation. It is difficult to establish the role that diffusion in the aqueous region between the layers plays in determining the overall swelling rate. This theoretical difficulty is balanced by the experimental ease in making liposome swelling measurements and we have also used this technique primarily to study the temperature dependence of amide permeation. Our results for egg lecithin liposomes (96% egg lecithin, 4% phosphatidic acid) and also for liposomes made from extracts of total red cell lipids are given in Table III. Column 2 shows that the apparent ratio of permeation (formamide/acetamide) determined from initial rates of swelling in the egg lecithin liposomes preparation is 1.3, in fair agreement with the formamide/acetamide ratio calculated from the Cohen and Bangham graph (15) of 1.5, but very much less than the 3.3 ratio of $P_4$'s for the spherical bilayer. Thus the results obtained with the two systems, though qualitatively similar, are quantitatively different. This is illustrated in the fourth column of Table III which shows that the ratios of permeabilities determined by the two methods are quite variable.

Lelievre and Rich (36) have computed the permeability coefficients from determinations of the reflection coefficient in liposomes of lecithin with 3.6% phosphatidic acid. They also found that acetamide, as well as propionamide, permeated more slowly than formamide. Their formamide/acetamide ratio is 1.8, greater than the ratios either of Cohen and Bangham (15) and ourselves.

The apparent enthalpy of permeation of red cell lipid liposomes, $\Delta H'$, has been determined by plotting $\ln$ (relative swelling rate/T) against $T^{-1}$ for urea, glycerol, and the homologous amides. The slope of this line is $(-\Delta H'/R)$ and the resultant values for $\Delta H'$ are given in column 5 of Table III. $\Delta H'$ for urea in red cell lipid liposomes is 12.1 kcal mol$^{-1}$ which agrees reasonably with the figure of 14.3 kcal mol$^{-1}$ we have obtained in spherical bilayers from the data in Table I. In view of this agreement, we have considered the figures in Table III to be reasonable indices of the apparent activation enthalpies for lipid bilayer permeation. Our value of $\Delta H'$ for glycerol is 18 kcal mol$^{-1}$ in agreement with the figure of 18 kcal mol$^{-1}$ given by de Gier et al. (2) for liposomes prepared from egg...
TABLE III

<table>
<thead>
<tr>
<th>Solute</th>
<th>Relative swelling rates</th>
<th>Apparent activation enthalpy: kcal mol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg lecithin liposomes (15°C)</td>
<td>Red cell lipid liposomes (15°C)</td>
</tr>
<tr>
<td>Formamide</td>
<td>1.0</td>
<td>1.00±0.01 (8)</td>
</tr>
<tr>
<td>Acetamide</td>
<td>0.78</td>
<td>0.94±0.01 (4)</td>
</tr>
<tr>
<td>Propionamide</td>
<td>0.82</td>
<td>1.05±0.02 (3)</td>
</tr>
<tr>
<td>Butyramide</td>
<td>0.94</td>
<td>1.25±0.02 (3)</td>
</tr>
<tr>
<td>n-Valeramide</td>
<td>1.15</td>
<td>1.51±0.02 (8)</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Obtained in duplicate experiments. The average difference between duplicates was 2%.
† For red cell liposomes.

lecithin containing 30 mol % of cholesterol.

Table III shows that ΔH' is insensitive to chain length. A similar observation was made by de Gier et al. (2) for liposome permeation by alkane diols. They found ΔH' to be essentially independent of chain length from ethane-1,2-diol through pentane-1,5-diol. An explanation for the independence of ΔH' of chain length can be found by writing Eq. 8 in the following form

\[
(P_a/T) = k_{ether}(\Delta S/k_b)\exp(-\Delta G^*/R),
\]

and considering the temperature dependence of \(k_{ether}\). Differentiating Eq. 10 with \(1/T\), we obtain

\[
\frac{d(ln P_a/T)}{d(1/T)} = \frac{d(ln k_{ether})}{d(1/T)} - \frac{\Delta H_d^*}{R} = -\frac{\Delta H'}{R},
\]

since \(\Delta S_d^*\) is considered to be independent of temperature. For partition, \(\Delta G^o = -RT \ln k_{ether}\), so

\[
\frac{d(ln k_{ether})}{d(1/T)} = -\frac{\Delta H^o}{R},
\]

and

\[
\Delta H' = \Delta H^o + \Delta H_d^*.
\]

A similar relation holds for \(\Delta G_d^*\) and \(\Delta S_d^*\).

The fact that ΔH' is independent of chain length may probably be ascribed to the balance between the decrease of ΔH^o with chain length, and the concomitant increase of ΔH_d^*. Tanford (37) reports that ΔH^o for transfer of ethane from water to pure liquid hydrocarbon is 2.5 kcal mol⁻¹ and that ΔH^o decreases by 800 cal mol⁻¹ for propane and another 900 cal mol⁻¹ for butane. Diamond and Katz (30) have measured the change of ΔH^o per methylene group for the partition of two pairs of solutes from water to dimyristoyl lecithin liposomes and find that
\( \Delta H^\circ \) decreases by 1.6 kcal mol\(^{-1} \) going from methanol to ethanol and an additional 1.25 kcal mol\(^{-1} \) going from ethanol to n-propanol. Table III shows that \( \Delta G_d^* \) increases by 1.2 kcal mol\(^{-1} \) going from formamide to acetamide and an average of 600 cal mol\(^{-1} \) for each additional methylene group from acetamide to valeramide. Since \( \Delta H_d^* = \Delta G_d^* + T \Delta S_d^* \), \( \Delta H_d^* \) will increase with \( \Delta G_d^* \) as long as \( \Delta S_d^* \) is either positive or zero. \( \Delta S^\circ \) for partition between water and pure liquid hydrocarbon increases by 1 eu per methylene group between ethane and butane (36). Since larger molecules require larger holes to diffuse into, we would also expect \( \Delta S_d^* \) to increase with chain length. As a consequence, \( \Delta H_d^* \) would be expected to increase with the increase in \( \Delta G_d^* \) and thus, to compensate for the decrease of \( \Delta H^\circ \). Hence, those thermodynamic parameters of the permeation process that have so far been measured appear to fit in a self-consistent framework.

**Comparison with Red Cell Permeation**

Sha'afi et al. (1) have measured the permeability coefficients of the human red cell for the same homologous series of amides and have found a minimum at propionamide. They also interpreted the minimum as evidence of two separate rate-controlling processes which were identified as permeation in two separate pathways, one hydrophilic and the other lipophilic. We have observed a minimum in lipid bilayer permeation, at acetamide rather than propionamide, and have also interpreted the minimum in terms of two rate-controlling processes, one partition into the membrane, and the other diffusion across it. The question that this raises is whether the demonstration of the latter mechanism in bilayers casts doubt on the conclusions previously advanced for red cells.

The most convincing evidence that this is not the case is given in Table IV which shows the effect of phloretin on liposome permeation. When phloretin at a concentration of \( 2.5 \times 10^{-4} \) M is added to either egg lecithin or red cell lipid liposomes, the relative rate of swelling is increased by about 75% for 75% for both hydrophilic and lipophilic amides and by more than a factor of 2 for urea. Cass et al. (38) have also observed that phloretin doubles the permeability of lecithin-

<table>
<thead>
<tr>
<th>Solute</th>
<th>Relative swelling rate phloretin-treated/control</th>
<th>Relative permeability coefficient phloretin-treated/control, Red cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg lecithin liposomes (15°C)</td>
<td>Red cell lipid liposomes (10°C)</td>
</tr>
<tr>
<td>Formamide</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Acetamide</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Propionamide</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>Butyramide</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>n-Valeramide</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Urea</td>
<td><strong>2.5</strong></td>
<td></td>
</tr>
</tbody>
</table>

* Average of two values, one at 16°C (2.3) and one at 12°C (2.7).
cholesterol bilayers to acetamide. In the case of the red cell, however, Owen and Solomon (39) have shown that phloretin at 2.5 \times 10^{-4} \text{ M} inhibits the passage of the hydrophilic solutes whereas it stimulates the permeation of the lipophilic solutes, the degree of stimulation being proportional to \(k_{\text{ether}}\). These latter results were confirmed by Owen et al. (40) for phloretin concentrations above \(10^{-4} \text{ M}\), who found phloretin to inhibit the permeation of formamide, acetamide, and urea whereas it increased the flux of propionamide, and many other lipophilic solutes. Thus, with respect to the effect of phloretin, the permeation process of lipid bilayers has properties similar to the lipophilic path in red cell membranes.

Further insight into the difference between red cell membranes and lipid bilayer can be obtained by the use of \(AG^*_d\), which increases linearly with \(\bar{V}_s\) in the lipid bilayer (Fig. 4). In other words, after making allowance for the partition coefficient, the lipid bilayer discriminates among the monoamides strictly according to size. This is not the case for the red cell membrane. \(AG^*_d\) has been computed for the permeation of monoamides across the human red cell membrane using the data of Sha’afi et al. (1) and Eq. 8. The values are plotted in Fig. 5 together with comparable data from Fig. 4. In lipid bilayers \(AG^*_d\) depends linearly on molar volume as we have seen, whereas in the red cell the dependence of \(AG^*_d\) on molar volume, is biphasic. This shows that the explanation we have given to account for the minimum in lipid bilayer permeation does not suffice to explain the red cell case. \(AG^*_d\) depends more sharply on \(\bar{V}_s\) for the two smaller solutes in the red cell than it does in lipid bilayers because these solutes enter the red cell through an aqueous pathway. For such solutes the size of the aqueous channel discriminates effectively among solutes according to size, the smaller the molecule, the more important the aqueous pathway. It is interesting that the larger lipophilic solutes also permeate the red cell membrane faster than lipid bilayers. This may be attributed in part to differences in crossing the interface. Fig. 3 shows that, \(R_m\), the resistance for crossing the interface, is about 10 times greater for spherical bilayers than for human urea cells.

It is also instructive to compare urea permeation across lipid bilayers and red cells. Urea permeates bilayers very much more slowly than does formamide so that the ratio \(P_{d,\text{urea}}/P_{d,\text{formamide}}\) is 0.053 (from Table I). As has already been stated, this difference can be accounted for: in part by differences in \(k_{\text{ether}}\), in part by steric factors, and in part by hindrance to urea diffusion through the polar regions on either face of the lipid bilayer as a result of hydrogen bond formation. By contrast, Sha’afi et al. (1) have found \(P_{d,\text{urea}}/P_{d,\text{formamide}}\) to be 0.83 in human red cells, 14 times larger than the figure for the lipid bilayer. There is an even more striking difference when urea permeation is compared with that of water. Urea permeates lipid bilayers much more slowly than water, the ratio \(P_{d,\text{urea}}/P_{d,\text{H}_2\text{O}}\) being 0.0081, more than an order of magnitude smaller than the 0.11 ratio for \(P_{d,\text{urea}}/P_{d,\text{H}_2\text{O}}\) in human red cells (1). To obtain the lipid bilayer ratio, we have used the hydraulic conductivity of the lipid bilayers, rather than the THO permeability coefficient in order to correct for the unstirred layer as already discussed.

It has been suggested (41, 42) that carrier mechanisms may be involved in the transport of urea across the red cell membrane. However, the red cell data fit
quantitatively to expectations for passive diffusion through narrow aqueous channels. Urea diffusion depends upon molar volume in exactly the same way as other hydrophilic solutes such as ethylene glycol and acetamide. Such narrow channels have been induced in lipid bilayers (3, 43) by the action of the antibiotics, nystatin, and amphotericin B. Holz and Finkelstein (43) have shown that the ratio $P_{d.urea}/P_{d.water}$ is 0.11 in amphotericin B-treated planar bilayers, in remarkable agreement with the 0.11 ratio for human red cell membranes. Furthermore, on the basis of independent evidence, de Kruijff and Demel (44) have proposed that amphotericin B and cholesterol are complexed in lipid bilayers to form an aqueous pore of 4-Å radius in good agreement with the 4.3-4.5-Å radius proposed for the equivalent pore in human red cells (45). This suggests that if there is a carrier mechanism for urea transport in human red cells, its properties should be similar to those of an amphotericin B pore. Thus the passage of urea across human red cell membranes differs strikingly from diffusion across an unmodified lipid bilayer, but is consistent with passage through small aqueous channels.

APPENDIX

Unstirred Layer Corrections for Thin Spherical Membranes

J. M. MILGRAM

The problem considered here is the steady-state diffusion of a substance through a composite spherical shell made up of a membrane and unstirred layers on both sides of the membrane. Fig. 6 shows the geometry of the system. The concentration of the diffusing substance is called $C$. $C$ satisfies the spherically symmetric Laplace equation

$$\frac{1}{r^2} \frac{d}{dr} \left( r^2 \frac{dC}{dr} \right) = 0. \tag{1a}$$

The solution to this equation in any homogeneous domain is:

$$C = \frac{a}{r} + b. \tag{2a}$$

Thus, in region 1,

$$C = \frac{A}{r} + B, \tag{3a}$$

and in region 2,

$$C = \frac{C}{r} + H. \tag{4a}$$

$A,B,G,$, and $H$ are constants to be determined from the boundary and continuity conditions. The boundary conditions are: at $r = R$ in region 1,

$$C = C_{m1} = \frac{A}{R} + B, \tag{5a}$$

and
FIGURE 6. Geometry for a spherical vesicle with unstirred layers inside and outside. $D_1$ and $D_2$ are diffusion coefficients, $C_1$ and $C_2$ are concentrations at the edges of the unstirred layers furthest from the membrane and $C_{m1}$ and $C_{m2}$ are concentrations at the membrane.

\[
\frac{dC}{dr} = -\frac{A}{R^2}, \quad (6a)
\]

at $r = R$ in region 2

\[
C = C_{m2} = \frac{G}{R} + H, \quad (7a)
\]

and

\[
\frac{dC}{dr} = \frac{G}{R^2}, \quad (8a)
\]

at $r = R + t_1$

\[
C = C_1 = \frac{A}{R + t_1} + B, \quad (9a)
\]

and at $r = R - t_2$

\[
C = C_2 = \frac{G}{R - t_2} + H. \quad (10a)
\]

The flow rate of the diffusing substance is called $Q$ and is given by Fick's Law as:

\[
Q = -4\pi R^2 D \frac{dc}{dr}, \quad (11a)
\]

where $D$ is the diffusion coefficient. The continuity condition at the membrane is that the flow rates on each side must be equal. Thus,
where $D_1$ and $D_2$ are the diffusion coefficients in region 1 and region 2, respectively. The definition of the membrane permeability coefficient, $P_{d, true}$ is:

$$P_{d, true} = \frac{Q}{4\pi R^2 (C_{m_1} - C_{m_2})} \quad (13a)$$

However, in an experiment $C_{m_1}$ and $C_{m_2}$ are not measured, but instead, $C_1$ and $C_2$, the concentrations just outside the unstirred layers, are known. The apparent permeability coefficient for the system of unstirred layers and membrane is called $P_{d, obs}$ and is defined by:

$$P_{d, obs} = \frac{Q}{4\pi R^2 (C_2 - C_1)} \quad (14a)$$

The purpose of this appendix is to give the relationship between $P_{d, true}$ and $P_{d, obs}$. From Eqs. 13a and 14a,

$$\frac{1}{P_{d, true}} = \frac{1}{P_{d, obs}} \frac{C_{m_1} - C_{m_2}}{C_2 - C_1} \quad (15a)$$

It is necessary to determine $C_{m_1} - C_{m_2}$ in terms of $C_2 - C_1$. From Eqs. 5a and 7a,

$$C_{m_1} - C_{m_2} = \frac{G - A}{R} + H - B \quad (16a)$$

$A, B, G,$ and $H$ can be determined from Eqs. 9a, 10a, 12a, and 14a as:

$$A = \frac{R}{D_1} P_d(C_2 - C_1), \quad (17a)$$

$$B = C_1 - \frac{R_2}{D_1} \frac{P_{d, obs}(C_2 - C_1)}{(R + t_1)}, \quad (18a)$$

$$G = \frac{R^2}{D_2} P_{d, obs}(C_2 - C_1), \quad (19a)$$

$$H = C_2 - \frac{R^2}{D_2} \frac{P_{d, obs}(C_2 - C_1)}{(R - t_2)} \quad (20a)$$

Using Eqs. 17 through 20 in Eq. 16 then gives

$$C_{m_1} - C_{m_2} = (C_2 - C_1) \left[ 1 - P_{d, obs} \frac{D_{d,1} \left(1 - \frac{t_1}{R}\right) + D_{d,2} \left(1 + \frac{t_1}{R}\right)}{D_{d,1} D_{d,2} \left(1 + \frac{t_1}{R}\right) \left(1 - \frac{t_2}{R}\right)} \right] \quad (21a)$$

Then from Eqs. 15 and 21,

$$\frac{1}{P_{d, true}} = \frac{1}{P_{d, obs}} \frac{D_{d,1} \left(1 - \frac{t_2}{R}\right) + D_{d,2} \left(1 + \frac{t_1}{R}\right)}{D_{d,1} D_{d,2} \left(1 + \frac{t_1}{R}\right) \left(1 - \frac{t_2}{R}\right)} \quad (22a)$$
Eq. 22 is exact for the problem solved here. A simpler approximate result is obtained by expanding Eq. 22 in powers of \( t_1/R \) and \( t_2/R \) and retaining terms up to order \( t_1/R \) and \( t_2/R \) only.

\[
\frac{1}{P_{d, true}} \approx \frac{1}{P_{d, obs}} - \frac{t_1}{D_1} \left( 1 - \frac{t_1}{R} \right) - \frac{t_2}{D_2} \left( 1 + \frac{t_2}{R} \right). \tag{23a}
\]

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