Temperature Dependence of Nonelectrolyte Permeation across Red Cell Membranes

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ABSTRACT The temperature dependence of permeation across human red cell membranes has been determined for a series of hydrophilic and lipophilic solutes, including urea and two methyl substituted derivatives, all the straight-chain amides from formamide through valeramide and the two isomers, isobutyramide and isovaleramide. The temperature coefficient for permeation by all the hydrophilic solutes is 12 kcal mol^{-1} or less, whereas that for all the lipophilic solutes is 19 kcal mol^{-1} or greater. This difference is consonant with the view that hydrophilic molecules cross the membrane by a path different from that taken by the lipophilic ones. The thermodynamic parameters associated with lipophile permeation have been studied in detail. ΔG is negative for adsorption of lipophilic amides onto an oil-water interface, whereas it is positive for transfer of the polar head from the aqueous medium to bulk lipid solvent. Application of absolute reaction rate theory makes it possible to make a clear distinction between diffusion across the water-red cell membrane interface and diffusion within the membrane. Diffusion coefficients and apparent activation enthalpies and entropies have been computed for each process. Transfer of the polar head from the solvent into the interface is characterized by $\Delta G^{\ddagger} = 0$ kcal mol^{-1} and $\Delta S^{\ddagger}$ negative, whereas both of these parameters have large positive values for diffusion within the membrane. Diffusion within the membrane is similar to what is expected for diffusion through a highly associated viscous fluid.

Permeability coefficients for the passage of small hydrophilic and lipophilic solutes across red cell membranes have been determined by Sha'afi et al. (1) who concluded that physical chemical interactions between solute and membrane were the basic determinants of red cell permeability for both classes of solutes. We have measured the temperature dependence of permeation for typical members of these hydrophilic and lipophilic classes of solutes in order to gain further insight into the nature of the interactions with the red cell membrane structure.

The temperature coefficients for permeation by hydrophilic solutes are significantly lower than those for lipophilic ones, thus providing an additional

physical chemical parameter which may be used to differentiate membrane permeation by these two classes of solutes. A detailed physical chemical analysis, using Eyring's absolute rate theory (2, 3) has enabled us to distinguish and separate two steps in lipophilic permeation. The coefficient for diffusion across the solute-membrane interface has been computed separately from that for diffusion within the fabric of the membrane. Apparent entropies and enthalpies of activation have also been obtained for each diffusional step. Diffusion within the membrane is characterized by properties similar to those expected for an associated oil with a viscosity of the order of 10 P.

EXPERIMENTAL METHOD

The temperature dependence of the permeability coefficient, ω, was determined for human red cells by the minimum method of Sha'afi et al. (4). The units for ω are mol $\text{dyn}^{-1}\,\text{s}^{-1}$ and it is related to the permeability coefficient P (cm s^{-1}) by $P = \omega RT$, in which R is the gas constant and T, the absolute temperature. When a suspension of red cells is rapidly mixed with a hyperosmolar concentration of a permeant solute, water leaves the cells so that they shrink initially. Subsequently the rate of water efflux is balanced by the rate of solute influx and the volume reaches a minimum value after which the cells swell as both permeant molecules and water enter the cells. The swelling process is terminated when the activity gradients of both the permeant solute and water have reached zero.

The time-course of red cell volume was measured using the stop-flow apparatus as described by Sha'afi et al. (5). ω can be determined from the ratio of the minimum volume to the original volume of the cells together with the derivative of the rate of volume change at the minimum volume, $(d^2V/dt^2)_{\text{min}}$, as previously described (4).

The entire stop-flow apparatus was enclosed in an environmental control box which allowed the temperature of syringes, tubing, stop-cock, and mixing chamber to be set at the desired temperature $\pm$ 1 °C. The temperature of the solutions and suspensions were kept constant to $\pm$ 0.1 °C in a separate temperature bath outside the environmental chamber and portions were removed immediately before the experimental run. The temperatures of the mixed permeant solute and red cell suspension were measured with thermistor probes inserted in the entrance and exit tubes leading to and from the mixing chamber, and found to agree with the solution temperatures to better than 0.5 °C. Therefore when thermistor measurements were not available, the bath temperature was used.

Human blood obtained by venipuncture of healthy donors was heparinized (10,000 U/ml; 0.2 ml/0.1 liter of blood) and suspended in a buffered solution to give a 3 % suspension (by volume of whole blood). The buffer solution used to suspend the red cells consisted of (in millimoles/liter): 108 NaCl, 4.00 KCl, 1.54 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 24.9 NaHCO_3, 1.27 CaCl_2, and 1.19 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. pH was adjusted to approximately 7.4 by aerating with 5 % CO_2, 95 % air. The resulting osmolality was about 270 mosmol, as measured with a Fiske G 62 osmometer (Fiske Associates, Inc., Uxbridge, Mass.).

Permeability measurements were made on three homologous series of compounds: (*a*) the straight-chain amides from formamide to valeramide; (*b*) the branched-chain amides, isobutyramide and isovaleramide; and (*c*) urea and two methyl substituted ureas. All solutes were reagent grade from Eastman Kodak Co., Rochester, N. Y. The concentration of the nonelectrolytes in their buffer (whose composition was otherwise the same as the red cell buffer) was about 0.75 M except for valeramide and isovaleramide in which solubility considerations limited the concentration to about 0.5 M. Consequently, the experiments with valeramide and isovaleramide were carried out with a smaller osmotic perturbation. As a result there was increased scatter in these experimental curves. Otherwise, the permeability coefficients appeared to be relatively independent of concentration.

The experiments were long and several molecules were studied in each experiment. In our normal procedure, the 30°C experiments were carried out in the morning, the 25°C experiments in the early afternoon, and the 20°C experiments in the late afternoon or early evening. The total procedure took about 9 h and there was some shift in buffer pH over this long period—a rise of about 0.2 pH U to pH 7.4–7.6.

Data Processing Procedure

In the original experimental plan we intended to study the temperature range from 15° to 35°C and began by a comprehensive series of preliminary experiments. The computer least squares program was designed to determine the curvature at the minimum volume $[(d^2V/dt^2)_{min}]$ by fitting the data to the sum of two exponentials. The fitting procedure was developed to cover a limited time scale around the minimum. The equation governing the volume change is more complex than a simple sum of two exponentials and diverges from the exponential fit particularly during the shrinking phase.[1]

At the lowest temperature, 15°C, the computer least squares fit had problems which we could not resolve when a time sweep of 4 s was used. When the time sweep was reduced to 2 s, the minimum was very shallow and broad. Visual examination of the printout left us with no assurance that the computer fit was definitive. Another problem confronted us at 35°C because at this temperature, the noise, possibly due to the higher ambient temperature of the photomultiplier, was so large that the standard deviation was at least as great as the permeability coefficient. In consequence, we limited our experiments to the narrower temperature range of 20°–30°C.

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[1] Some details of the curve fitting procedure should be mentioned. Originally we picked the minimum by eye from the computer printout and instructed the computer to find the best fit in the neighborhood of this point by least squares. In this procedure, the time range which the computer selected could be varied, and we learned, by trial and error, that the permeability coefficient varied with the time range. We therefore specified a time range from 10 to 800 ms, a procedure which worked well for most solutes. The only exception was 1,3-dimethylurea, the least permeant solute, for which we extended the maximum time to 1100 ms. We also learned that more reliability could be achieved if we instructed the computer to look for the minimum between 100 and 120 ms. This was so far away from the actual minimum that the computer worked very hard and succeeded in picking the true minimum as verified both by visual examination and the lowest standard deviation. In some instances the scatter was so great that the computer could not pick a minimum, so we determined it from visual examination of the graph.

RESULTS AND DISCUSSION

Comparison between Hydrophilic and Lipophilic Solutes

Table I presents the values for ω at 25°C as compared with values previously determined by Sha'afi et al. (1) at room temperature. The agreement is unexpectedly good (except for urea)[2] particularly when one bears in mind that room temperature must have varied by several degrees in the earlier experiments. The laboratory is normally warm and the air conditioning is sporadic at best. Table II gives the values of ω at 20° and 30°C.

TABLE I

PERMEABILITY COEFFICIENTS OF NONELECTROLYTES AT 25°C

Molecule	Present study* $\omega \times 10^{15}$	Previous results $\omega \times 10^{15}$	k_{ether}§
	$mol\ dyn^{-1}s^{-1}$	$mol\ dyn^{-1}s^{-1}$	
Urea	11±1 (4)	15±1 (6)	0.00047
Methylurea	3.2±1.1 (2)	2.0±0.3 (2)	0.0012
1,3-Dimethylurea	1.5±0.2 (3)	1.1±0.2 (2)	0.0031
Formamide	14±3 (4)	18±1 (2)	0.0014
Acetamide	5.1±0.7 (4)	5.0±0.5 (4)	0.0025
Propionamide	2.9±0.4 (4)	4.0±0.5 (3)	0.013
Butyramide	10±2 (5)	14±1 (3)	0.058
Isobutyramide	3.3±0.5 (7)	5±1 (3)	[0.058]
Valeramide	24±10 (3)	27±2 (2)	[0.17]
Isovaleramide	20±13 (2)	7.2±0.5 (2)	0.17

* The number of experiments is indicated in parenthesis. Errors are standard errors of the mean.
§ Taken from Collander (6). The k_{ether} values for the isomers enclosed in brackets are taken to be the same as for the other member of the isomer pair.

When the logarithm of $P(P = \omega RT)$ is plotted against $T(A°)^{-1}$ over the range from 20° to 30°C the three points generally can be described by a straight line; Fig. 1 shows the results for acetamide in three experiments. If a logarithmic relationship is observed over a wide temperature range, an Arrhenius activation energy may be obtained from the slope of the line. However the present observations are confined to a limited temperature range and we will present evidence below that lipophilic permeation involves at least two separate steps with different activation energies. Hence it is more appropriate to describe the temperature dependence, obtained by a graphical procedure as in Fig. 1, in terms of temperature coefficients rather than apparent activation energies. These coefficients are given in units of kcal mol^{-1} computed

[2] The apparent disagreement in the case of isovaleramide is not significant because of the large standard error. In view of this large error we have not used the isovaleramide data either in discussion or for further calculation.

TABLE II

PERMEABILITY COEFFICIENTS OF NONELECTROLYTES
AT 20° AND 30°C

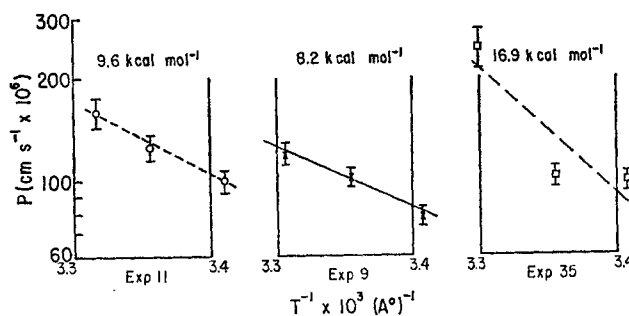| Molecule	20°C $\omega \times 10^{15}$		30°C $\omega \times 10^{15}$	
	mol $dyn^{-1}s^{-1}$		*mol* $dyn^{-1}s^{-1}$	
Urea	9.5±1	(4)	15.9±0.8	(4)
Methylurea	3±1	(2)	2.4	(1)
1,3-Dimethylurea	1.2±0.1	(3)	1.3±0.6	(3)
Formamide	9±4	(3)	22±6	(4)
Acetamide	4.1±0.3	(5)	7.4±0.9	(5)
Propionamide	2.1±0.2	(6)	6.1±0.9	(6)
Butyramide	3.5±0.4	(6)	17±3	(6)
Isobutyramide	3.1±0.2	(6)	8.6±1.2	(8)
Valeramide	12±7	(2)	40±10	(2)
Isovaleramide	8±5	(2)	50±13	(3)

FIGURE 1. Typical data for the temperature coefficient of human red cell permeation by acetamide as determined in three experiments.

from the slope of the line, a procedure which gives a shorthand description of temperature effects that is useful in comparison with other data, and meaningful when the comparison is restricted to similar processes, similar molecules, and similar temperatures.

Table III gives the temperature coefficients of all the solutes. Variation between experiments has been minimized by constructing a graph similar to those in Fig. 1 for each experiment and averaging all the slopes for each solute. Nonetheless considerable variability remains which reflects our experimental difficulties.

Fig. 2 relates the temperature coefficients of all the homologous amides from Table III to the permeability coefficients at 25°C from Table I and to the values of the ether:water partition coefficient, k_{ether}, also given in Table I. The value of k_{ether} for water[3] of 0.003 provides a demarcation between hydrophilic and lipophilic molecules which is indicated by a vertical line in Fig. 2.

[3] C. M. Gary-Bobo. Personal communication.

TABLE III
TEMPERATURE COEFFICIENTS OF HUMAN RED
CELL PERMEATION

Molecule	Temperature coefficient
	kcal mol^{-1}
Urea	11±2 (5)
Methylurea	4±0 (2)
1,3-Dimethylurea	10±3 (4)
Formamide	12±5 (4)
Acetamide	12±2 (5)
Propionamide	19±3 (6)
Butyramide	26±2 (6)
Isobutyramide	22±4 (8)
Valeramide	38±4 (3)
Isovaleramide	48±16 (3)
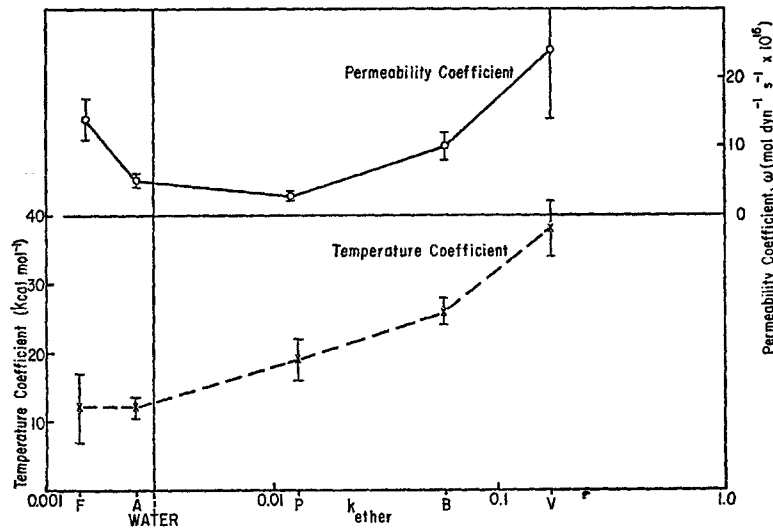
FIGURE 2. Human red cell permeability to homologous amides. The top portion of the figure shows the relation of the permeability coefficient of the homologous amides (Table I) formamide (F) acetamide (A), propionamide (P), butyramide (B), and valeramide (V) to k_{ether} and the bottom shows the relation of the temperature coefficients (Table III) of permeation to k_{ether}.

To the left of the line, the permeability coefficient decreases with increasing ether solubility and the temperature coefficients are about 12 kcal mol^{-1}, independent of k_{ether}. To the right of the line, the permeability coefficients increase with increasing lipid solubility as do the temperature coefficients which start out at 19 kcal mol^{-1} and progressively increase. Thus the differences between the processes of hydrophilic permeation and lipophilic permeation are clearly apparent, entirely consonant with our view that hydrophilic molecules cross the membrane by a different path than that taken by lipophilic solutes.

Hydrophilic Solutes

Within the hydrophilic group, the temperature coefficients in Table III range from 10 to 12 kcal mol^{-1} except for methylurea whose coefficient is significantly less than that of the others. The reason for this difference is not clear, though it may possibly be an artifact arising from the very small dispersion in these two experiments. The figures of 10–12 kcal mol^{-1} may be compared with the activation energy for free diffusion of urea in water of 4.4 kcal mol^{-1} (7) and that of acetamide of 6.3 kcal mol^{-1} (8) both over the range from 4° to 24°C. This difference is indicative of some interaction between the hydrophilic solutes and the walls of the aqueous channel, presumably mediated by hydrogen bonding. There is evidence for such bonding from the studies of water diffusion across the human red cell membrane since the apparent activation energy (9) for this process of 6.0 kcal mol^{-1} is greater than the 4.5 kcal mol^{-1} for free diffusion of water (10). The hydrophilic amides and the ureas we have studied are larger than water and hence they may be expected to spend a larger fraction of time in direct contact with the walls of the narrow hydrophilic channels.

The possibility that the temperature coefficients reflect a temperature-induced change in properties of the hydrophilic channel was investigated by Vieira, Sha'afi, and Solomon (9). They measured the temperature dependence of the hydraulic permeability of human red cells over the range from 5° to 39°C. If the temperature had no effect on the geometry of the aqueous pores, the hydraulic conductivity should be inversely proportional to the bulk viscosity of water. The agreement of their data with this relationship supported the conclusion that the dimensions of the aqueous channel were essentially independent of temperature between 5° and 39°C.

Temperature Coefficients of Lipophilic Permeation

The introduction of successive methylene groups in the lipophilic straight-chain amides causes increasingly large changes in temperature coefficients, the average increment being 8 kcal mol^{-1} per methylene group (Table III). No such dependence on size or k_{ether} is found for the hydrophilic permeants. The distinction between the two classes of solute agrees with the findings of Smulders and Wright (11) who measured the apparent activation energies of solute permeation across gallbladder epithelia in the rabbit, another tissue in which there is evidence for separate pathways for hydrophilic and lipophilic solutes. They found the apparent activation energies for the hydrophilic solutes, urea and acetamide, to lie between 5 and 7.5 kcal mol^{-1}, whereas those for the lipophilic solutes, 1,4-butanediol and antipyrine, are about twice as great, between 12 and 15 kcal mol^{-1}. Smulders and Wright contrasted this

behavior with that observed in goldfish gallbladder in which there is no separate pathway for small polar solutes. In this gallbladder the apparent activation energy of the hydrophilic solute, acetamide, does not differ significantly from that of the lipophilic solute, 1,4-butanediol.

Free Energy Changes in Amide Partition between Water and Nonpolar Solvents

Two steps are involved when a nonelectrolyte is partitioned between water and a nonpolar solvent. The first is adsorption from the aqueous phase onto the interface and the second is transfer from the interface into the bulk nonpolar solvent. For the short chain aliphatic amides it can easily be shown that the only step which is up a free energy gradient is the transfer of the amide group from the interface into the nonpolar solvent, which reflects the cost of breaking the hydrogen bonds that link the amide group to the water.

Wang et al. (12) have measured the free energy of adsorption,[4] $\Delta G_{w/i}$, at the interface between water and decane for straight-chain aliphatic amides. Their data is shown in the lower (negative) half of Fig. 3 in which the free energy of adsorption is plotted against the number of carbons in the chain. These data led Wang et al. to conclude that the total free energy change on adsorption comprises two components: -400 cal mol^{-1} for the free energy of adsorption of the polar group plus -820 cal mol^{-1} for each carbon in the chain. The latter figure is in good agreement with comparable data of Haydon and Taylor (13) who also find -830 cal mol^{-1} per $-CH_2-$ group for short-chain alcohols. Since these free energy changes have a negative sign, it is energetically favorable for the amides to move from bulk water to the interface. Although there are attractive van der Waals' forces for the $-CH_2-$ groups in the oil phase, the most important interactions are those in the aqueous phase. An important consideration is the large entropy gain due to change in water structure as a result of removing the $-CH_2-$ group from the aqueous domain, as Diamond and Wright (14) and others have pointed out.

The free energy change for partition can be computed from the relation $k = e^{-\Delta G_{w/o}/RT}$ in which k is the partition coefficient and $\Delta G_{w/o}$ represents the standard free energy change between bulk water and bulk nonpolar solvent. Since the partition coefficients for the short-chain amides given in Table I are less than unity, $\Delta G_{w/o}$ is positive, and free energy would be required for transport from bulk water to bulk nonpolar solvent at unit activity in each solvent. The partition free energy changes are plotted in the upper (positive) half of Fig. 3 as a function of chain length using the values of k_{ether} in Table I and $k_{olive\ oil}$ as determined by Collander and Bärlund (15). The partition coefficient lines are analogous to those for surface adsorption and yield intercepts $\Delta G_{w/o(CONH_2)}$ of 4900 cal mol^{-1} when ether is the solvent and 5050 cal

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[4] It is important to note that $\Delta G_{w/i}$ is used to denote adsorption free energy (water/interface) which is not to be confused with $\Delta G_{w/o}$, the free energy for partition (water/oil).

mol^{-1} for olive oil, for an average of 4980 cal mol^{-1}. These are in excellent agreement with values of 4900 cal mol^{-1} for ether and 4800 cal mol^{-1} for olive oil obtained by Diamond and Wright (16) by a different method. The intercepts of the two lines are virtually the same in spite of the difference in the slope which would confirm that for relatively nonpolar solvents such as ether and olive oil, $\Delta G_{w/o}$ for the $CONH_2$ group is a reflection primarily of interactions in the water phase.

As previously discussed, transfer of the $-CH_2-$ group to the nonpolar phase is energetically favorable and the values of $\Delta G_{w/o(-CH_2-)}$ obtained from the
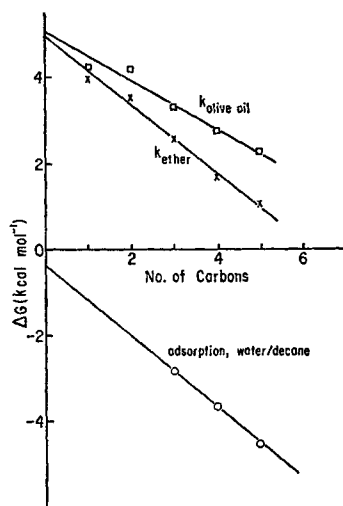

FIGURE 3. Free energy changes associated with partition as related to the number of carbons in homologous amides. The top two lines give the partition coefficients, $k_{olive\ oil}$ and k_{ether}. The bottom line gives the free energy of adsorption at a water/decane interface.

slopes are -610 cal mol^{-1} for olive oil and -790 cal mol^{-1} for ether. These small differences in $\Delta G_{w/o(-CH_2-)}$ may be taken as an index of the characteristics of the solute. The fact that $\Delta G_{w/i(-CH_2-)}$ is -820 cal mol^{-1} for decane is consistent with the view that there is no significant energy difference in the environment of the methylene group whether it is just below the interface or dispersed in the bulk of the nonpolar phase. The only transfer up a standard free energy gradient is the transfer of the head group from the interface to the bulk nonpolar solute. The standard free energy required for this step is the difference $[\Delta G_{w/o} - \Delta G_{w/i}]$ which is equal[5] to 5380 cal mol^{-1}.

The broad view that emerges is that the similarities which have often been

[5] As Wang et al. (12) point out, this figure differs slightly from the true value because $-(\Delta G_{w/i})_{true} = -\Delta G_{w/i} + RT$.

remarked in the distribution coefficient in nonpolar solvents may be attributed to the similarities in the energetic cost of moving the head group from a nonpolar environment to a polar one, and the fine structure that discriminates between solvents of similar polarity is based on small differences in $\Delta G_{(-CH_2-)}$.

Comparison of Interface Permeability with That of Membrane Fabric

The effective barrier for lipophilic permeation may be either: (a) adsorption from the solution at the external membrane face, (b) passage through the membrane, (c) desorption at the interior face, or (d) some combination of these. Zwolinski, Eyring, and Reese (3) have used absolute reaction rate theory to make a penetrating analysis of the diffusion process which they have separated into two components. D_m is the generalized diffusion coefficient that applies within the membrane fabric. Zwolinsky, Eyring, and Reese do not discriminate between interface diffusion at the inner and outer face, but use the same generalized diffusion coefficient, D_{sm}, for both faces. Their equation is

$$1/P = 2\lambda/D_{sm} + \delta/D_m \, k_{mem} \qquad (1)$$

in which P is the permeability coefficient ($P = \omega RT$), λ is the jump distance between successive equilibrium positions for the solute, δ is the membrane thickness ($\delta = m\lambda$, in which m is the number of jumps across the membrane), and k_{mem} is the partition coefficient for the solute between water and membrane. Zwolinski, Eyring, and Reese used a generalized partition coefficient, K, but we prefer to specify k_{mem} in order to illustrate the dependence of Eq. 1 on the actual partition coefficient used to represent k_{mem}, in our case, k_{ether}. Thus Eq. 1 becomes

$$1/P = 2\lambda/D_{sm} + \delta/D_m \, k_{ether} \, (k_{mem}/k_{ether}). \qquad (2)$$

We shall make the reasonable assumption that the temperature dependence of (k_{mem}/k_{ether}) is slight over the temperature range of 20°–30°C used in these experiments. Zwolinski, Eyring, and Reese point out that Eq. 2 may be used to separate the resistance to passage across the interface from that within the membrane by plotting P^{-1} against k_{ether}^{-1}. The intercept is $2\lambda/D_{sm}$ and the slope, $\delta/D_m(k_{mem}/k_{ether})$.

The validity of Eq. 1 depends upon the variation in D_{sm} and D_m among the solutes being small with respect to the dependence upon k_{mem}. Zwolinski, Eyring, and Reese (3) state that it is likely that these conditions will be fulfilled by the lower members of homologous series. The generalized diffusion coefficients, D_{sm} and D_m, are not specific coefficients for a single solute in the usual sense, but rather describe the average diffusion of the homologous solutes across the interface and within the membrane fabric. In Fig. 4, we
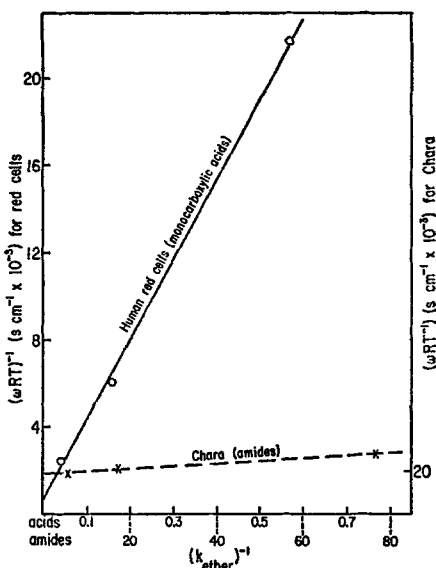
FIGURE 4.   The relation between permeability coefficients and k_{ether} plotted according to Eq. 2 for propionic acid, butyric acid, and valeric acid in human red cells and propionamide, butyramide, and isovaleramide in Chara.

have plotted the data of Collander and Bärlund (15) on the permeability of Chara to propionamide, butyramide, and isovaleramide according to Eq. 2 and it will be seen that the equation is satisfied. Green (17) has determined the permeability of the human red cell membrane to a homologous series of monocarboxylic acids. We have computed the permeability coefficients from the initial slope of his curves relating optical density to time and obtained values of ωRT for propionic, butyric, and valeric acids. Fig. 4 shows that these data also fit Eq. 2.

Fig. 5 shows a plot of $(\omega RT)^{-1}$ as a function of k_{ether} for propionamide, butyramide, and valeramide at 20°, 25°, and 30°C using the values for ω given in Tables I and II. The values for k_{ether} as a function of temperature have been computed from the relation, $k_{ether} = e^{-\Delta G_{w/o}/RT}$. $\Delta G_{w/o}$ is obtained from k_{ether} at the temperatures reported (6) by Collander (23° and 24°C). If $\Delta G_{w/o}$ were independent of temperature, k_{ether} could be determined simply as a function of temperature, and this computation has been made. However, it is unlikely that $\Delta G_{w/o}$ is temperature independent since Johnson and Bangham (18) have shown that k_{ether} for butanol increases by a factor of 2.5 as the temperature is raised from 20° to 50°C. No data are available for the temperature dependence of k_{ether} for the amides so it has not been possible to take account of this factor. Collander measured k_{ether} for isovaleramide, but not that for valeramide. However, Lange[6] has measured
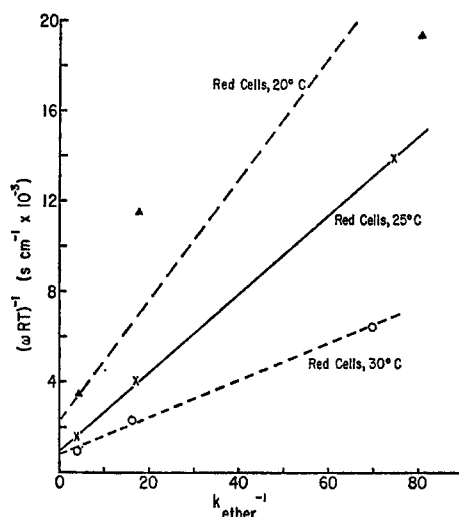
[6] Y. Lange. Personal communication.

FIGURE 5.   The relation between permeability coefficient and k_{ether} according to Eq. 2 for propionamide, butyramide, and valeramide at 20°, 25°, and 30°C.

the partition of isovaleramide and valeramide in a number of solvents, though not in ether. The ratio of these partition coefficients is essentially solvent independent and we have used it to compute k_{ether} for valeramide as 0.23 at 24°C from Collander's value for isovaleramide. The three points at 25° and 30°C in Fig. 5 fall very nicely on a straight line, whereas those at 20°C do not, so that Eq. 2 is satisfied by the monoamides at the two higher temperatures.

Fig. 5 shows that the intercept and, hence, D_{sm}, is virtually independent of temperature between 25° and 30°C. This value for the intercept of the mono-amides is also close to that for the monocarboxylic acids shown in Fig. 4. It is interesting that the free energy of adsorption at the decane/water interface is also virtually temperature independent between 20° to 50°C (Haydon and Taylor [13]).

Diffusion within the membrane, on the other hand, is sharply dependent upon temperature, the slope having values of 12.3×10^{-3} cm s^{-1} at 30°C and 5.7×10^{-3} cm s^{-1} at 25°C, more than a twofold difference over this 5°C temperature range. We have computed a generalized temperature coefficient of 27 kcal mol^{-1} from these data, making the reasonable assumption that membrane thickness is temperature independent, in accordance with the conclusions of Vieira et al. (9). This figure of 27 kcal mol^{-1} is large compared to the temperature coefficient of the diffusion of other solutes in nonpolar solvents. From Thovert's (19) data on the diffusion of phenol in benzene, the activation energy for diffusion of this solute may be calculated as 3.2 kcal mol^{-1} over the temperature range from 5° to 25°C. If we take

this as representative of the effect of temperature on diffusion in bulk low viscosity lipid solute, the major portion of the generalized temperature coefficient, some 24 kcal mol^{-1}, may be attributed to factors specific to the membrane fabric which points to a high degree of organization within the membrane.

We may next determine the relative importance of the interface as compared to the membrane interior in controlling membrane permeation. Since resistances to flow are additive and inversely proportional to permeabilities, Eq. 1 is equivalent to the statement

$$R_{tot} = R_{sm} + R_{mem} \qquad (3)$$

R_{tot} is the total transmembrane resistance equal to $(\omega RT)^{-1}$. R_{sm} is twice the resistance at one interface $= 2(\lambda/D_{sm})$, and R_{mem} is the membrane resistance given by m times the resistance per jump of $(\lambda/D_m k_{mem})$. R_{sm} is 0.9×10^3 s cm^{-1}, the value of $(\omega RT)^{-1}$ at the intercept in Fig. 5. R_{mem} may be obtained using the values of ω given in Tables I and II.

The results in Table IV make it clear that both barriers are important in establishing the permeability coefficient as can be seen from the ratio R_{mem}/R_{tot}. Thus for butyramide at 25°C, 78% of the resistance is offered by the membrane, whereas for valeramide at 30°C, 91% of the resistance is provided by the interface. Diamond and Wright (16) have pointed out the dilemma in assigning the rate limiting step in membrane permeation. Their clear discussion shows that the observed permeability coefficients may be used equally well to support the view of Stein (20) that diffusion is controlled primarily by the water:membrane interface just as well as their own assumption that the rate limiting step is diffusion through the membrane interior. In fact, as shown in Table IV, neither barrier controls permeation uniquely and the role of each must be considered separately.

The separate diffusion coefficients may now be computed. D_{sm} may be obtained in a straightforward manner from the intercept in Fig. 5 which, according to Eq. 1, is equal to $2\lambda/D_{sm}$. For the jump distance, λ, we have taken

TABLE IV

CONTRIBUTION OF MEMBRANE RESISTANCE TO AMIDE PERMEATION

	R_{tot}	R_{mem}	$\dfrac{R_{mem}}{R_{tot}}$	D_m	R_{tot}	R_{mem}	$\dfrac{R_{mem}}{R_{tot}}$	D_m
		(25°C)				(30°C)		
	$s\,cm^{-1} \times 10^{-3}$			$cm^2\,s^{-1} \times 10^9$	$s\,cm^{-1} \times 10^{-3}$			$cm^2\,s^{-1} \times 10^9$
Propionamide	13.9	13.0	0.94	2.9	6.5	5.6	0.86	6.3
Butyramide	4.1	3.2	0.78	2.8	2.3	1.4	0.61	5.8
Valeramide	1.7	0.8	0.47	2.8	0.99	0.09	0.09	—
Isobutyramide	12.2	11.3	0.93	0.76	4.7	3.8	0.81	2.1

(molar vol/$N_{Avogadro}$)$^{1/3}$ as suggested by Glasstone, Laidler, and Eyring (2) which equals 5.2 Å for butyramide. This leads to a value of D_{sm} = 11.6 × 10^{-11} cm^2 s^{-1}. D_m for butyramide may be computed as 2.8 × 10^{-9} cm^2 s^{-1} at 25°C using 50 Å for δ and the value of k_{ether} in Table I. This value and those for the other amides are given in Table IV. The uncertainties in the computation of these diffusion coefficients are surely less than a factor of two for D_{sm} and about two orders of magnitude for D_m. λ will surely be greater than 2.5 Å and less than 10 Å. The uncertainty in D_m resides in the factor (k_{mem}/k_{ether}). Lange[7] has measured the partition of valeramide in a number of solvent systems, including lipid vesicles. The greatest partition coefficient she found was less than an order of magnitude greater than k_{ether} and the lowest about two orders of magnitude smaller. Even taking these uncertainties into effect both diffusion coefficients are at least two orders of magnitude lower than Thovert's value of 1.84 × 10^{-5} cm^2 s^{-1} for the diffusion coefficient of phenol in benzene at 25°C. If lipophilic diffusion were limited to 10% of the membrane area, both D_{sm} and D_m would increase by an order of magnitude since the entire red cell membrane area is used in the computation for ω on which these diffusion coefficients are based. Even so, diffusion both into and through the membrane would be greatly hindered as compared to bulk nonpolar solvents like benzene or decane. The low value of D_{sm} is consistent with a high degree of organization at the interface and that of D_m with diffusion through a medium whose viscosity is some orders of magnitude greater than that of benzene. This is not an impossible specification, since at 25°C the viscosity (21) of benzene is 0.6 × 10^{-2} P, whereas that of castor oil is 7.2 P and glycerol is 9.5 P. Such values are entirely consistent with what may be expected in the interior of biological membranes, where Cone (22) has calculated a viscosity of about 2 P for the site in which rhodopsin is embedded in the visual receptor membrane.

Viscosities greater than 1 P in nonpolar solvents are the exception rather than the rule. The viscosity of hexadecane at 20°C is 0.033 P, just three times greater than water. Oleic acid has a viscosity of 0.26 P at 30°C and olive oil, which has 84% oleic acid in its triglyceride (23) has a viscosity of 0.84 P at 20°C. Detailed molecular requirements are quite important in specifying viscosity. Castor oil differs from olive oil primarily by replacement of oleic acid with its 12-hydroxy derivative, ricinoleic acid, which comprises 87% of the triglyceride (23) in castor oil, the remaining composition being roughly similar. Yet this change appears to be responsible for a 10-fold increase in viscosity, to 9.9 P at 20°C.

Enthalpy and Entropy of Permeation Process

The apparent enthalpy and entropy of activation for diffusion in the membrane may be computed in a straightforward manner by using the following

equation given by Glasstone, Laidler, and Eyring (2):

$$D_m = (\lambda^2 k T/h)\ e^{\Delta S\ddagger/R} e^{-\Delta H\ddagger/RT}.$$ (4)

D_m is taken from Table IV. λ is computed from the molar volume as previously described. k is Boltzmann's constant; T, the absolute temperature; h, Planck's constant; and R, the gas constant. $\Delta S\ddagger$ is the entropy and $\Delta H\ddagger$ the enthalpy of activation for diffusion. We may compute $\Delta H\ddagger$ from the ratio

$$D'_m/D''_m = (T'/T'')\ e^{-(\Delta H\ddagger/R)(T'^{-1}-T''^{-1})}.$$ (5)

It is important to stress that Eq. 5 does not depend on the use of k_{ether} to represent k_{mem} provided that the ratio $(k_{\text{mem}}/k_{\text{ether}})$ is temperature independent between 25° and 30°C, as already discussed. Since the principal uncertainty is in the value of k_{ether} we are in the somewhat paradoxical position of only being able to specify D_m to about two orders of magnitude whereas the value for $\Delta H\ddagger$ for this process is quite accurate.

The values for $\Delta H\ddagger$ are 28 kcal mol^{-1} for propionamide and 26 kcal mol^{-1} for butyramide using the data for D_m in Table IV. We have not made a computation for valeramide because only 9% of the impedance is located within the membrane and the subtraction process involved in computing D_m from the difference of two large numbers introduces great error. Since there does not appear to be any temperature dependence of D_{sm}, the apparent $\Delta H\ddagger$ for diffusion across the interface is 0 kcal mol^{-1} between 25° and 30°c.

Our values for $\Delta H\ddagger$ may be compared with those for $\Delta H'$ computed by Zwolinski, Eyring, and Reese (3) for the permeability of unfertilized Arbacia eggs using the data of Jacobs and Stewart (24). The comparison is, at best, qualitative since $\Delta H'$ refers to the entire permeation process including diffusion both across the interface and through the membrane. $\Delta H'$ is 21.6 kcal mol^{-1} for propionamide and 22.8 kcal mol^{-1} for butyramide, in general agreement with our results. It is interesting that Dalmark and Wieth (25) have also found a high value of 33 kcal mol^{-1} for $\Delta H'$ for chloride transport across human red cell membranes. They suggest that chloride ions may be transported through an apolar region of the red cell membrane by a lipid-soluble carrier and our studies show that this high activation energy is consistent with transport through such a region.

Although no data are available on the temperature dependence of amide permeation in model systems, De Gier et al. (26) have studied the permeation of alcohols through liposomes. The apparent activation energy for erythritol and glycerol diffusion into and across egg lecithin liposomes is 18–21 kcal mol^{-1} and values as high as 50 kcal mol^{-1} are found in liposomes containing saturated phosphatides such as dipalmitoyl lecithin. Though these apparent activation

energies are in the same neighborhood as ours, diffusion across liposomes is very different from red cell permeation. The apparent activation energy of diol passage through egg lecithin (with 30 mol % of cholesterol) is independent of chain length between 1,2-ethanediol and 1,5-pentanediol. This makes a very striking contrast with red cells in which the temperature coefficient clearly depends on chain length (Fig. 2).

The most valid comparison for $\Delta H^{\ddagger}$ is with the activation energy for viscous flow since diffusion across the membrane is inversely proportional to membrane viscosity. Glasstone, Laidler, and Eyring (2) have pointed out that high diffusion coefficients are characterized by low activation energies; conversely, high viscosities are characterized by high activation energies for viscous flow, E_{vis}. In benzene, whose viscosity at 20°C is 0.008 P, the activation energy for phenol diffusion is 3.2 kcal mol^{-1} and E_{vis} is 2.2 kcal mol^{-1}. In castor oil, the viscosity at 20°C is 9.9 P and E_{vis} is 13.9 kcal mol^{-1}. Cone (22) has measured the transient dichroism of rhodopsin in the visual receptor membrane and obtained a viscosity of 2 P and an apparent E_{vis} of 19.5 kcal mol^{-1}. Thus our data are entirely consistent, both with respect to D_m and $\Delta H^{\ddagger}$, with a red cell membrane whose viscosity is within an order of magnitude of 10 P. The high value of $\Delta H^{\ddagger}$ may be ascribed to the difficulty of making holes in the highly associated interior of the membrane. The fluidity of the membrane, like that of other fluids with similar high viscosity, increases markedly with temperature even over the 5°C range between 25° and 30°C.

Recently, Rudy and Gitler (27) have measured the viscosity in the membrane interior of human red cell ghosts from the fluorescence depolarization of perylene (peri-dinaphthalene, mol wt 252.30). Their viscosity of 1.3 P is in agreement with our expectation. However their activation energy of 3.9 kcal mol^{-1} is much lower than we would expect. It is also much lower than that found in other membranes, not only the visual receptor membrane studied by Cone (22), but also the tissue culture cell membranes in which Frye and Edidin observed translational diffusion of fluorescent antibodies attached to surface antigens (28). Cone interpreted Frye and Ediden's data to indicate viscosities in these cells of 1–10 P with a Q_{10} in the range of 2–8 which corresponds to E_{vis} of 12–37 kcal mol^{-1}. These discrepancies in E_{vis} may be accounted for by the inhomogeneity in the cell membrane. Our measurements are concerned with transverse diffusion and are thus an average of all the environments traversed as the solute moves across the membrane fabric. Both Cone's results and those of Frye and Edidin are measurements made on proteins which are large molecules. The rotation of the one and the translational diffusion of the other must reflect average viscosities over an appreciable fraction of the membrane's thickness. Perylene, however, is a much smaller molecule and Rudy and Gitler surmise that it is located in the vicinity of the terminal portion of the phospholipid chains.

Values for $\Delta S^\ddagger$ may also be computed from Eq. 4. There is much more inherent inaccuracy in these computations than those for $\Delta H^\ddagger$ because they depend directly upon the assumptions made about k_{mem}, δ, and λ. Fortunately these terms are all in the coefficient of the exponential, to which $\Delta S^\ddagger$ is relatively insensitive since two orders of magnitude difference between k_{mem} and k_{ether} would only introduce an error of about 10 entropy units (eU). Since the value of $\Delta S^\ddagger$ is 61 eU for propionamide and 55 eU for butyramide, the conclusion that diffusion through the membrane is accompanied by a large positive apparent entropy of activation is essentially independent of the exact value of k_{mem}. These large values are consonant with the disruption introduced into the highly organized viscous membrane by the holes required for the passage of the permeant solute.

On the other hand, the apparent entropy of activation across the interface is large and negative, having a value of -34 eU. In this case the uncertainties are much less since $D_{sm} = 2\lambda/R_{sm}$ and the value of λ will not be very much different from 5 Å. The negative apparent entropy of activation at the interface would fit with the view that removal of the polar group from its aqueous environment increases order in that solvent. Reactions particular to the interface would also be expected to contribute to the large negative value of $\Delta S^\ddagger$.

Data for the apparent entropy of activation of permeation in other biological membranes are sparse. Zwolinski, Eyring, and Reese (3) computed values of $\Delta S'$ in unfertilized Arbacia eggs of 34 eU for propionamide and 40 eU for butyramide, but these values include both the interface contribution and that of the membrane. Nonetheless it seems clear that a positive apparent entropy of activation is also a feature of amide diffusion through the Chara membrane.

Discrimination between Butyramide and Isobutyramide

One of the initial objectives of this study was an investigation into the mechanism by which the cell membrane discriminates between isomeric pairs such as butyramide and isobutyramide. Sha'afi et al. (1) had found that at room temperature the permeability coefficient for isobutyramide was about one-third that of butyramide and that a similar factor related isovaleramide to valeramide. As shown in Table I, we have confirmed their observation in the case of butyramide though the errors arising due to the lower solubility of valeramide are too great to allow for a meaningful comparison in this isomer pair. In order to obtain information about the site of isomer discrimination, Wang et al. (12) compared the free energy of adsorption of the isomer pairs at a decane/water interface and found no measurable difference between isomers. They therefore suggested that the difference in permeability coefficient might be attributed to differences in the diffusion rate across the membrane. We had initially hoped to find a reflection of this differentiation in the temperature coefficient of the permeation process, but Table III shows that there is no significant difference

between the raw temperature coefficients of permeation of butyramide and isobutyramide.

However, much more information can be obtained when D_m is computed for isobutyramide as it has been for butyramide. We have already shown that D_{im}, the interface diffusion coefficient, is independent of chain length between propionamide and valeramide at 25° and 30°C, so it is reasonable to extend this argument to isobutyramide at these temperatures, particularly in view of Wang et al.'s (12) observations on the free energy of adsorption. On this basis we have computed R_{mem} and D_m for isobutyramide at 25° and 30°C. As shown in the bottom line of Table IV, D_m is about three times smaller for isobutyramide than for butyramide.

Interestingly, the difference between the isomer pairs is not apparent at 20°C, the same temperature at which the relation between the permeability coefficient and k_{ether} is aberrant as shown in Fig. 5. Thus it is possible that the state of organization within the membrane is different at this lower temperature.

Nonetheless we can compute the apparent enthalpy and entropy for the activated state within the membrane, using only the data at 25° and 30°C as was done for the straight-chain amides. This process leads to a value of 36 kcal mol^{-1} for $\Delta H^{\ddagger}$ for isobutyramide as compared to 26 kcal mol^{-1} for $\Delta H^{\ddagger}$ for butyramide. Similarly the value for the apparent entropy of activation is higher, $\Delta S^{\ddagger}$ being 83 eU for isobutyramide as compared with 55 eU for butyramide. These data support the view of a highly organized structure within the membrane fabric. The cylindrical radius of isobutyramide is 2.97 Å as compared to 2.68 Å for butyramide (1). Hence the hole required for the diffusing solute must be significantly larger and the higher value of $\Delta H^{\ddagger}$ is a measure of the energetic expense required. Similarly the high value of $\Delta S^{\ddagger}$ is a measure of the consequent increase in disorder within the membrane.

Glasstone, Laidler, and Eyring (2) point out the "striking fact" that the free energy of activation for flow of associated liquids such as water and glycerol is not abnormally high because in these instances the high value of $\Delta H^{\ddagger}$ is compensated by high positive values of $\Delta S^{\ddagger}$. This is remarkably illustrated by the red cell membrane, since $\Delta G^{\ddagger}$ for isobutyramide is 11 kcal mol^{-1}, virtually unchanged from the 10 kcal mol^{-1} value for butyramide, notwithstanding the very large changes in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

The picture of the membrane that emerges is consistent with observations from many sources. The permeation process is shown schematically in Fig. 6. Adsorption of lipophilic solutes is energetically favorable and leads to a configuration in which the nonpolar chain is within the membrane phase, leaving the polar head group in contact with the bathing solution. As the length of the carbon chain is increased, the free energy of adsorption continues to increase. This accounts for Overton's rule as Diamond and Wright (16) have pointed

out. Transfer of the polar head group from its aqueous environment into the membrane fabric requires energy, 5.4 kcal mol^{-1} for the amide group, as previously discussed. The special properties of the interface are such that the formation of the activated complex is accompanied by an increase in order, as a result of removing the polar head from an aqueous environment and inserting it into the membrane. Once within the membrane, the solute passes through a region that is highly ordered as reflected in the large positive values both for the apparent enthalpy and the apparent entropy of the activated

aqueous | membrane

ΔG negative

ΔG positive
$\Delta H^{\ddagger}$ zero
$\Delta S^{\ddagger}$ negative

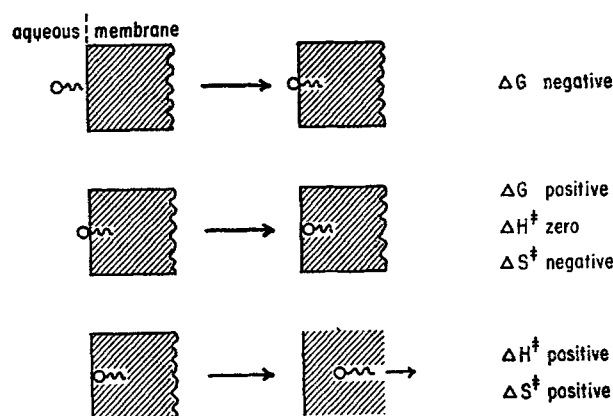$\Delta H^{\ddagger}$ positive
$\Delta S^{\ddagger}$ positive

FIGURE 6. Membrane permeation by lipophilic amides. Schematic drawing showing the thermodynamic parameters for the steps in the permeation process.

complex. This is consonant with diffusion through a highly organized membrane characterized by a viscosity of the order of 10 P, similar to that of castor oil. It is this viscous region which enables the membrane to discriminate according to the size and shape of the permeant solute.

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