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Permeability of lipid bilayers to amino acids and phosphate

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Permeability coefficients for amino acid classes, including neutral, polar, hydrophobic, and charged species, were measured and compared with values for other ionic solutes such as phosphate. The rates of efflux of glycine, lysine, phenylalanine, serine and tryptophan were determined after they were passively entrapped in large unilamellar vesicles (LUVs) composed of egg phosphatidylcholine (EPC) or dimyristoylphosphatidylcholine (DMPC). The following permeability coefficients were obtained for: glycine, $5.7 \cdot 10^{-12}$ cm s^{-1} (EPC), $2.0 \cdot 10^{-11}$ cm s^{-1} (DMPC); serine, $5.5 \cdot 10^{-12}$ cm s^{-1} (EPC), $1.6 \cdot 10^{-11}$ cm s^{-1} (DMPC); lysine, $5.1 \cdot 10^{-12}$ cm s^{-1} (EPC), $1.9 \cdot 10^{-11}$ cm s^{-1} (DMPC); tryptophan, $4.1 \cdot 10^{-10}$ cm s^{-1} (EPC); and phenylalanine, $2.5 \cdot 10^{-10}$ cm s^{-1} (EPC). Decreasing lipid chain length increased permeability slightly, while variations in pH had only minor effects on the permeability coefficients of the amino acids tested. Phosphate permeability was in the range of 10^{-12}–10^{-13} cm s^{-1} depending on the pH of the medium. The values for the polar and charged amino acids were surprisingly similar to those previously measured for monovalent cations such as sodium and potassium, which are in the range of 10^{-12}–10^{-13} cm s^{-1}, depending on conditions and the lipid species used. This observation suggests that the permeation rates for the neutral, polar and charged amino acids are controlled by bilayer fluctuations and transient defects, rather than partition coefficients and Born energy barriers. The results are relevant to the permeation of certain peptides into lipid bilayers during protein translocation and membrane biogenesis.

Introduction

A universal characteristic of cells is the membrane boundary that separates the interior cytoplasm from the exterior environment. The lipid bilayer moiety provides a barrier to the free diffusion of solutes, while specialized integral proteins permit specific solutes to pass, either by passive diffusion (channels and carriers) or enzyme-catalyzed active transport. The general barrier properties of the bilayer have been well-established by previous studies, particularly with respect to ionic solutes (sodium, potassium, chloride, hydrogen ion), polar solutes (water, glucose) and small neutral molecules (glycerol, urea) [1]. Surprisingly little work has been done on more complex ionic solutes such as the amino acids and phosphate, clearly two of the more important solute species involved in cell function.

In the present report, we will describe both direct and indirect permeability measurements for several amino acids and phosphate. In particular, we have addressed the following questions:

(1) What are the permeability coefficients for the primary amino acid classes, including neutral, polar, hydrophobic, and charged species?

(2) What is the effect of pH and phospholipid chain length on permeation rates?

(3) How do the permeabilities compare with measurements for other ionic solutes, particularly phosphate, potassium and chloride?

(4) What mechanism best explains the measured permeation rates? That is, are the permeation rates controlled by standard models involving partitioning and Born energy barriers, or are they better explained in terms of bilayer fluctuations and transient defects? These concerns are directly relevant to the permeation of lipid bilayers by certain peptides, for example during insertion and translocation of signal sequences [2].

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; EPC, egg phosphatidylcholine; Hepes, 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicle; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MLV, multilamellar vesicle; Tricine, (*N*-tris[hydroxymethyl]methylglycine).

Materials and Methods

Materials

All amino acids, chemicals and buffers used were obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC) and dimyristoylphosphatidylcholine (DMPC) were obtained from Avanti Polar-Lipids (Birmingham, AL).

Preparation of lipid vesicles

Multilamellar vesicles (MLVs) were produced by hydrating either 40 mg EPC or 35 mg DMPC in 2.0 ml of buffer (containing 50 mM amino acid). These MLVs were frozen in liquid nitrogen and thawed at approx. 50°C in water for five freeze-thaw cycles. This treatment increased the trapped volume of the vesicles and promotes equilibration of transmembrane solute distributions [3]. Extrusion of the frozen and thawed MLVs through two stacked polycarbonate filters (Nuclepore) (200 nm pore size) was performed ten times at 20°C using a custom-built extrusion device modeled upon one produced by Lipex Biomembranes (Vancouver, Canada) as described by Hope et al. (1985) [4].

Measuring efflux of amino acids from the liposomes

To remove external solutes the LUVs containing the passively-trapped amino acid were then passed down a 10 cm Sephadex G-50 (G50-150) column previously equilibrated with buffer of the appropriate pH. The composition of the buffers used was 50 mM buffer and 150 mM NaCl. The following buffers were used: citrate (pH 2–4); Mes (pH 5–6); Hepes (pH 7–8) and Tricine (pH 9).

The amino acid-containing LUVs (1.5 ml) were subsequently placed in 16 mm dialysis tubing (Spectrapor™). This tubing has a molecular weight cut-off of approx. 12000–14000, so free amino acids lost from the liposomes were able to diffuse out of the tubing while the liposomes remained inside. The loaded dialysis tubing was then placed in a beaker containing 100 ml of buffer solution of the appropriate pH which was stirred continuously. Samples (1.0 ml) were taken at appropriate time points from the external buffer solution to monitor amino acid efflux from the liposomes. Control experiments with free amino acid solutions (50 mM) entrapped in dialysis tubing revealed that essentially all the amino acid diffused out of the tubing within 10 min. With the exception of the hydrophobic amino acids, half-times of amino acid permeation were in the range of one hour, so that the diffusion barrier of the dialysis membrane was negligible. All experiments were conducted at 20–22°C for the EPC liposomes and 30–32°C for the DMPC liposomes to ensure that both types of vesicles were in the liquid-crystalline phase. Samples were stored at 4°C until amino acid concentrations were determined.

Fluorescamine labeling of amino acids

Fluorescamine (Sigma, St. Louis, MO) was used to fluorescently label the primary amino groups of the amino acids for quantitative analyses. A 0.5-ml aliquot of the amino acid-containing sample to be measured was added to 1.0 ml of sodium borate buffer (200 mM, pH 9.0). The sample was vortexed vigorously while 0.5 ml of fluorescamine (at a concentration of 20 mg/100 ml in acetone) was added. Mixing was continued for several seconds following fluorescamine addition to ensure labeling of all amino acid present.

The concentrations of the resulting fluorescently-labeled amino acids were determined by measuring their fluorescence (excitation wavelength = 390 nm; emission wavelength = 480 nm) using an SLM 8000C Spectrofluorimeter. Standard curves were created for each amino acid (generally in the range of 0–25 nmoles). It was established under these conditions that the emission intensity was directly proportional to the amount of amino acid present.

Measurement of hydrophobic amino acid efflux

Preliminary experiments revealed that the efflux of tryptophan and phenylalanine from the liposomes was too rapid to be measured using the dialysis tubing protocol. A modification of a technique using KI as an aqueous quencher was employed to allow for the on-line monitoring of tryptophan efflux [5]. A 50-μl aliquot of vesicles was added to 2.0 ml of 1.0 M KI (containing 0.25 mM $Na_2S_2O_3$ as an antioxidant) in a cuvette. The vesicles were prepared in a solution containing 50 mM tryptophan, 50 mM buffer and 900 mM NaCl. The vesicles were used immediately after the extrusion procedure was completed and were not passed down a column. Tryptophan fluorescence (excitation wavelength = 280 nm; emission wavelength = 360 nm) was monitored continuously for a period of 5 min following addition of the vesicles. Any free tryptophan present at the start of the experiment was immediately quenched by the KI. The efflux of tryptophan was then measured as a decrease in the fluorescence intensity due to quenching of the inherent tryptophan fluorescence upon exposure to the KI present in the external solution. Control experiments measuring the efflux of [^{14}C]glucose established that 1 M KI had no significant effects on membrane permeability over time courses of up to 3 h in the EPC systems used.

Phenylalanine permeability was measured using light scattering changes related to osmotically-driven water flux [6]. Liposomes were initially prepared in a low osmotic strength buffer (2.0 mM buffer, 6.0 mM NaCl). A 200-μl aliquot of these vesicles was then added to 1.8 ml of high osmotic strength buffer containing 100 mM phenylalanine, 50 mM buffer and 150 mM NaCl. Vesicle volumes were monitored by measuring the light scattering at 550 nm over a 5 minute interval. An initial

increase in scattering was observed, corresponding to osmotic shrinkage of the liposomes in response to the initial osmotic gradient. A point of minimum volume was reached, followed by a decrease in scattering as influx of the amino acid occurred. The maximum rate of light scattering decrease following the point of minimum volume was used as a measure of the rate of influx of phenylalanine [6]. Control experiments indicated that osmotic gradients of the magnitude employed here did not result in increased membrane permeability to solutes, such as [^{14}C]glucose.

Calculation of rate constants and permeability coefficients

The kinetics of the efflux process were calculated using an initial rate analysis, which assumes that the concentration of amino acid inside the liposome is much greater than that on the outside (except for phenylalanine). As developed more completely elsewhere (Cullis et al., 1992, manuscript in preparation) the efflux process should obey the relation

$$A(t)_{ex} = A(eq)_{ex}(1 - e^{-kt})$$

where $A(t)_{ex}$ is the exterior concentration of amino acid at time t, $A(eq)_{ex}$ is the equilibrium exterior concentration at t = infinity and k is the rate constant associated with the efflux process. Thus, a plot of $\ln\{([A(eq)]_{ex} - [A(t)]_{ex})/A[(eq)]_{ex}\}$ versus t should give a slope of $-k$, the rate constant. Rate constants were determined by applying a linear least-square analysis to efflux data using a commercially available graphing program (Cricket Graph Version 1.2, Malvern, PA).

Permeability coefficients (P) were calculated using the expression

$$P = (V_0/A_m)k$$

where P is expressed in cm s^{-1}, k is the rate constant, V_0 is the aqueous volume of the lipid dispersion and A_m is the area of the LUV membrane. This expression can be readily simplified to:

$$P = (r/3)k$$

where r is the radius of the vesicle. This was assumed to be 100 nm for the 200 nm vesicles used in this work.

Measurement of phosphate permeability

Quantitative analyses of phosphate were insufficiently sensitive to be carried out by the dialysis method used for amino acids. Furthermore, we wished to determine the effect of pH and pH gradients on phosphate flux. Therefore it was more convenient to use influx of phosphate into liposomes with buffered interior volumes, followed by gel separation and phosphate analysis by standard molybdate assays. Liposomes (15 ml) were prepared from egg phosphatidylcholine (20 mM) as described before, containing 0.5 M sodium chloride, 50 mM sodium borate buffer (pH 9.0), and 5 mM sodium citrate. Following preparation, sodium phosphate was added to a final concentration of 50 mM, and the external pH was adjusted with sulfuric acid to values ranging from near 1 to near 5. The liposomes were then incubated for periods up to 6 h. During this time 1-ml aliquots were run through short Sephadex G-50 gel columns, and phosphate was determined by the Lowry method [13].

Results

Permeability to amino acids

Several representative amino acids were chosen to be studied. These amino acids were: glycine (no side chain); serine (aliphatic hydroxyl side chain); lysine (charged (basic) side chain); phenylalanine and tryptophan (aromatic (hydrophobic) side chains). The first set of experiments was designed to determine whether pH had an effect on the permeability coefficient of the amino acids. Fig. 1 illustrates a typical set of efflux data obtained for serine from 200 nm DMPC vesicles. Fig. 1A shows that the time course of amino acid efflux from the liposomes was exponential and reached equilibrium after approximately 2 h. Fig. 1B reveals how rate constants for the efflux process were derived (see Materials and Methods for further details) from a linear transformation of the data in Fig. 1A. Table I shows that pH had only a minor effect on the rate constants and permeability coefficients of the charged and aliphatic amino acids, with all rate constants being in the range of 10^{-4} s^{-1} and with the permeability coefficients being in the range of 10^{-12} cm s^{-1}.

The next set of experiments was aimed at finding out whether altering lipid chain length would have an effect on the permeability of amino acids for liposomes of the same size (approx. 200 nm). Previous work has shown that liposomes made from C_{12} and larger amphiphiles have reduced membrane permeability [14]. DMPC (14 C) was chosen as the lipid for comparison with EPC (18 C) since it was the shortest chain lipid that still formed stable liposomes (provided a sufficient permeability barrier for these studies to be undertaken; unpublished results). Control experiments established that the differences in temperature between the EPC and DMPC systems (approx. 10°C) would only account for a 1.5-fold increase in permeation rates. Table II shows that permeability coefficients and rate constants for efflux of the charged and aliphatic amino acids for the DMPC liposomes were in the same range as those observed for the EPC liposomes (approx. 10^{-11} cm s^{-1} and 10^{-4} s^{-1}).

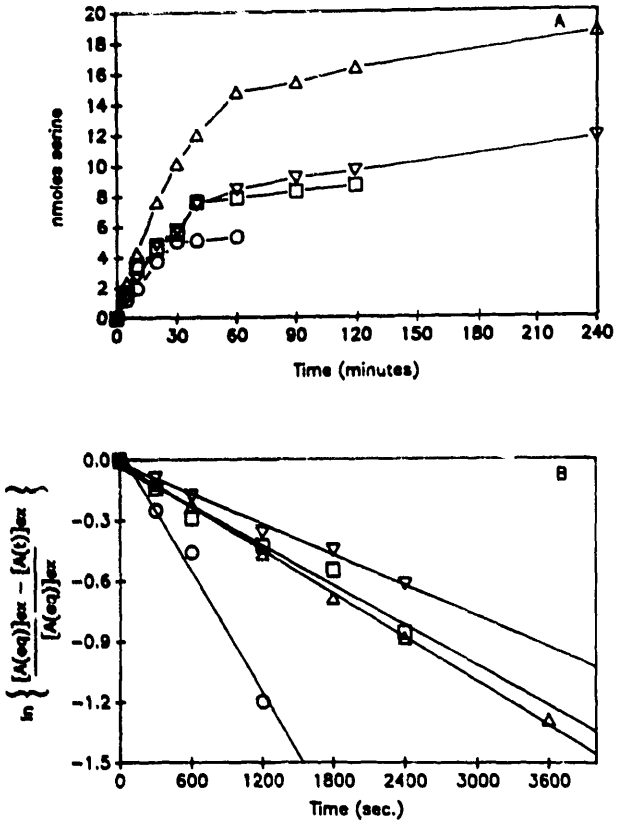

Fig. 1. (A) Time course of release of serine from 200 nm DMPC vesicles at various pH values. The pH values of the samples were 2.0 (○), 4.0 (△), 6.0 (□) and 8.0 (▽). The experiment was conducted at 31°C and the initial serine concentration was 50 mM. (B) Plot of $\ln(([A(eq)]_{ex} - [A(t)]_{ex})/[A(eq)]_{ex})$ versus time; where $[A(eq)]_{ex}$ is the exterior concentration of amino acid at equilibrium and $[A(t)]_{ex}$ is the exterior concentration of amino acid at time t. For details see Materials and Methods. The slopes of the lines gives the rate constant (k) for the transbilayer diffusion of serine.

A key observation of the previous experiments was that the hydrophobic amino acids (phenylalanine and tryptophan) both had much higher rates of efflux than any of the other types of amino acid examined. Both of these amino acids exhibited transbilayer movement which was too rapid to be monitored using the dialysis tubing protocol. The permeability coefficients of phenylalanine and tryptophan were therefore determined via the use of on-line fluorescence methods as outlined in the materials and methods section. Figs. 2A and 2B show typical time courses for tryptophan and phenylalanine efflux from EPC vesicles at pH 6.0 as monitored by changes in fluorescence quenching (tryptophan) or light scattering (phenylalanine). Permeability coefficients calculated from this data were $4.1 \cdot 10^{-10}$ cm s^{-1} for tryptophan and $2.5 \cdot 10^{-10}$ cm s^{-1} for phenylalanine, two orders of magnitude greater than those observed for the other amino acids (average permeability coefficient of $5.4 \cdot 10^{-12}$ cm s^{-1}).

TABLE I

Permeability coefficients (P) and rate constants (k) for the efflux of glycine, lysine and serine from 200 nm EPC vesicles at various pH values

pH	P (10^{-12} cm s^{-1})	k (10^{-4} s^{-1})
Glycine		
3	7.3	2.2
4	8.3	2.5
5	4.0	1.2
6	5.0	1.5
7	5.3	1.6
8	5.0	1.5
9	4.7	1.4
Lysine		
2	9.0	2.7
4	2.0	0.6
7	3.7	1.1
8	6.7	2.0
9	4.0	1.2
Serine		
2	0.7	0.2
3	1.3	0.4
4	4.7	1.4
5	8.3	2.5
6	10.7	3.2
7	3.3	1.0
8	4.3	1.3
9	4.0	1.2

Permeability to phosphate

Typical results for phosphate entry are shown in Fig. 3. There was a modest effect of pH on influx, which was approximately six times more rapid at pH near 1, where most of the phosphate was in the neutral form, than at pH near 4 where most of the phosphate existed

TABLE II

Permeability coefficients (P) and rate constants (k) for the efflux of glycine, lysine and serine from 200 nm DMPC vesicles at various pH values

pH	P (10^{-11} cm s^{-1})	k (10^{-4} s^{-1})
Glycine		
3	2.6	7.9
4	1.9	5.7
6	1.6	4.7
8	1.7	5.2
Lysine		
2	2.3	7.0
4	1.8	5.4
6	1.8	5.3
8	1.7	5.2
Serine		
2	3.3	10.0
4	1.2	3.6
6	1.1	3.3
8	0.8	2.5

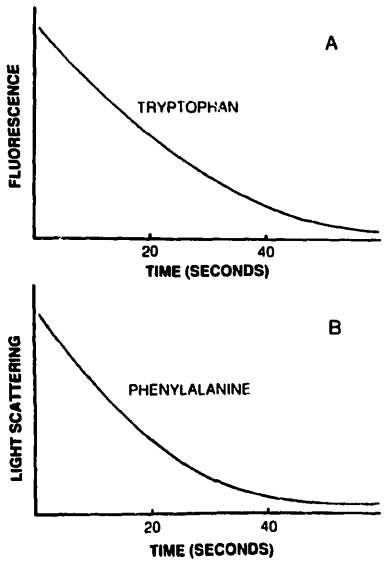

Fig. 2. (A) Time course of the efflux of tryptophan from 200 nm EPC liposomes at pH 6.0 measured by the quenching of tryptophan fluorescence (arbitrary fluoresence units; excitation wavelength, 280 nm; emission wavelength, 360 nm). The experiment was conducted at 22°C and the initial tryptophan concentration was 50 mM. (B) Time course of influx of phenylalanine into 200 nm EPC liposomes at pH 6.0 monitored by changes in light scattering (excitation and emission wavelengths, 550 nm). The experiment was conducted at 22°C and the initial phenylalanine concentration was 90 mM. The light scattering starts from the maximum point of vesicle shrinkage.

as the monoanion. Permeability of the neutral species was $3 \cdot 10^{-11}$ cm s^{-1}, and for the monoanion was $5 \cdot 10^{-12}$ cm s^{-1}.

Discussion

The results of this report establish that amino acid permeation of lipid bilayers is independent of the pH. The length of the lipid chain did have a modest effect on permeability, with shorter chain lipids (DMPC) being slightly more permeable, as might be expected. Lipid bilayers were observed to be much more permeable to hydrophobic amino acids under the conditions used here. Earlier research by Naoi et al. (1977) concluded that lipid composition modulated amino acid permeability [7]. However, rather than comparing pure lipid systems of different chain lengths, that paper used a variety of lipids mixed with egg phosphatidylcholine. Therefore longer chain lipids were always present and it was difficult to draw conclusions about the effect of varying hydrocarbon chain length on amino acid permeability. Our results indicate that decreasing hydrocarbon chain length from 18 to 14 carbons increased amino acid permeation rates by approx. 5-fold. This increase in permeability may also be attributed to differences in chain kinking between the EPC and DMPC vesicles (presence of one or more double bonds in the EPC vesicles) and the heterogeneity in chain length of the EPC vesicles.

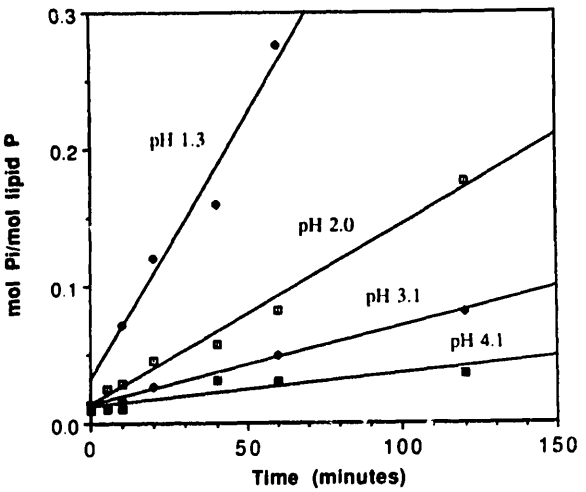

Fig. 3. Phosphate flux across 200 nm EPC liposome membranes. Liposome preparation and flux measurements are described in the Materials and Methods. The amount of permeating phosphate is expressed as moles phosphate per mole lipid.

Previous research measured the permeation rates of certain modified amino acids and peptides in response to transmembrane pH gradients [8]. Lysine methyl ester was used, because it would be neutral at higher pH values and positively charged at lower pH values. Hence, the effect of ionic charge on permeation rates could be determined. It was demonstrated that it was the neutral form of the modified amino acids and peptides that was translocated across the bilayer. This translocation was very rapid ($P = 2.1 \cdot 10^{-2}$ cm s^{-1}) for the lysine methyl ester [8]. In the work presented here, the transbilayer movement for the unaltered amino acid lysine is many orders of magnitude slower (average $P = 5.1 \cdot 10^{-12}$ cm s^{-1}) than that of the lysine methyl ester. Our results also indicate that amino acid flux is essentially independent of the pH, which contrasts with the dramatic pH effects observed for transport of lysine methyl ester [8]. This would be expected for a zwitterion, which would remain predominantly in the charged form regardless of pH. Previous research has established that the presence of charged groups restricts transbilayer diffusion, when compared to the neutral form [8,9].

The differences in permeability coefficients for the neutral, polar and charged amino acids, together with the observation that transport is unaffected by pH, indicates that a mechanism other than partitioning is likely responsible for the observed efflux. It seems probable that the efflux of amino acids occurs because of transient defects or 'leaks' in the membrane [10]. These transient defects were first described by Nagle and Scott as cavities which allow small ions to enter and subsequently pass through the bilayer without having to overcome the Born energy required for a charged molecule to 'dissolve' in the low dielectric membrane interior [11]. This conclusion is supported by the fact

TABLE III

Partition coefficients for some of the amino acids studied (from Leo et al. [12])

Amino acid	Log *P* octanol
Tryptophan	−1.04
Phenylalanine	−1.43
Lysine	−2.82
Glycine	−2.26 (average)

that the permeability coefficients for monovalent cations such as Na^+ and K^+ are in the range of 10^{-12}–10^{-13} cm s^{-1}, remarkably similar to those presented here for several amino acids and phosphate. Transient hydrated defects have been implicated as the mechanism by which these monovalent cations permeate lipid bilayers [1,10].

Our results confirm the observation by Naoi et al. that permeability is dependent on the structure of the amino acid [7]. They observed that bilayers were approximately 25–30-times more permeable to hydrophobic amino acids than charged amino acids such as lysine. However, their technique involved the indirect determination of amino acid flux through measurement of oxidation rates via the use of passively entrapped D-amino acid oxidase [7]. Results presented here using a more direct approach indicate that bilayer permeability to hydrophobic amino acids may be up to 100-times higher when compared with other amino acids. The permeability differences between amino acids appears to be related to differences in their partition coefficients between the lipid and water phases. Table III gives previously published partition coefficient data for most of the amino acids that were examined in this study [12]. Both phenylalanine and tryptophan partition into the organic phase (roughly equivalent to the lipid bilayer) 10–100-times more readily than any of the aliphatic or charged amino acids. It follows that the translocation of hydrophobic amino acids may involve a modest amount of partitioning into the membrane interior, as well as translocation through transient defects. For comparison, a truly neutral solute such as lysine methyl ester translocates entirely by partitioning, and can therefore permeate at rates up to 10^8 faster than the hydrophobic amino acids. The latter must bring charged groups into the hydrophobic phase and therefore still faces a formidable Born energy barrier.

Another possible explanation for the permeability coefficients presented here is that the neutral form of the amino acids is translocating across the bilayer. Only a very small proportion of the amino acids present would be in the neutral form (the doubly charged form [+/−] is in equilibrium with the single charged forms [+ or −], which in turn are also in equilibrium with the neutral form). The small size of the neutral population (theoretically 10^6–10^8-fold less when compared to the charged forms of an average amino acid) could account for the much slower permeation rates observed when compared to modified amino acids, such as lysine methyl ester (which are predominantly in the neutral form at basic pH values [8]). This possibility is currently under investigation.

This work is also pertinent to the mechanism by which peptides insert into and translocate across lipid bilayers in vivo. Simple diffusion of amino acids is clearly too slow to permit sufficient protein translocation rates to allow for cell growth. It follows that a different mechanism must be in place so that potentially charged amino acid side chains are able to overcome the Born energy barrier. One possibility is that transmembrane pH gradients are involved, so that peptide signal sequences would be produced on the alkaline side of the membrane. These would then be able to permeate as the neutral form, with a net accumulation on the acidic side of the membrane (for a general review regarding the mechanism of transbilayer transport in response to transmembrane pH gradients see Cullis et al. (1991) [15]).

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