Reprinted from Biochemistry, 1994,33.



# Influence of Charge, Charge Distribution, and Hydrophobicity on the Transport of Short Model Peptides into Liposomes in Response to Transmembrane pH Gradients?

Ajoy C. Chakrabarti,*,†,‖ Ian Clark-Lewis,§,‖ and Pieter R. Cullis‖

*Section of Molecular and Cellular Biology. Division of Biological Science, 2320 Storer Hall, University of California. Davis. California 95616. and Biomedical Research Center and Department of Biochemistry, University of British Columbia, Vancouver, British Columbia. Canada V6T 1Z3*

*Received September 9. 1993; Revised Manuscript Received May 16. 1994*®

ABSTRACT: Previous work [Chakrabarti et al. (1992) ***Biophys. J. 61,228-234]*** has shown that basic amino acids and peptides, in which the C-terminal carboxyl groups have been modified to form amides or methyl esters, can be rapidly and efficiently accumulated into large unilamellar vesicle (LUV) systems in response to transmembrane pH gradients (**ΔpH**, inside acidic). In this work, the ability of small (di and tri) peptides, composed exclusively of basic (lysine) and hydrophobic (tryptophan) amino acids, to accumulate into LUV systems in response to **ΔpH** has been investigated. In the case of the dipeptides Trp-Lys-amide and Lys-Trp-amide, remarkable differences in the rate constants associated with net transport were observed. In EPC:cholesterol LUV systems exhibiting a **ΔpH** of 3 units ($pH_i$ = 4.0; $pH_o$ = 7.0). for example, the rate constant for the uptake of Lys-Trp-amide is some $5 \times 10^3$ faster than for Trp-Lys-amide. Activation energies associated with the uptake also varied from 24 (Lys-Trp) to 29 kcal/mol (Trp-Lys). Related effects were observed for the tripeptides composed of one lysine and two tryptophan residues; however, the differences in rate constants were less sensitive to amino acid sequence. It is concluded that different charge distributions in short peptides of identical amino acid composition can strongly influence the ability of these groups to associate with and permeate across lipid bilayers. These observations may have relevance to the ability of basic peptides, such as signal sequences and peptide hormones, to translocate across biological membranes.

Translocation of weak acids and bases in response to transmembrane pH gradients has been previously demonstrated for amine uptake in chloroplasts (Crofts, 1967) and in liposomal systems for fluorescent amines used as pH indicators (Deamer et al., 1972). biogenic amines such as epinephrine (Schuldiner et al., 1978), various drugs (Madden et al., 1990) and acidic phospholipids (Hope et al., 1989; Redelmeier et al., 1990; Eastman et al., 1991). This phenomenon, which arises from rapid transbilayer movement of the neutral form of the weak acid or base, can result in large transbilayer concentration gradients. For example, a pH gradient (acidic interior) serves to produce a considerable inward net transport of weak bases by trapping the neutral membrane-permeable species in the charged (protonated)form after they traverse the bilayer. Thus, the weak base accumulates in the acidic compartment of the liposome. Raising the external pH will increase the proportion of exterior molecules in the neutral form and hence the rate and extent of transport. It is straightforward to show that, in the absence of membrane partitioning effects, lipophilic amines will be accumulated into **LUVs**[1] with an acidic interior to achieve inside/outside concentration ratios which correspond to the inside/outside concentration ratios of protons. Thus, a pH gradient of three units, for example, can result **in interior** concentrations of weak bases which are 1000 times larger than exterior values.

Previous work from this laboratory has shown that the presence of transmembrane pH gradients across lipid bilayers can cause rapid net transbilayer movement of amino acids and peptides in which the carboxyl functions were modified to create amides or methyl esters (Chakrabarti et al., 1992). It was further established that these compounds permeated as the neutral species. In this work, we extend these studies to di- and tripeptides composed exclusively of basic (lysine, Lys) and hydrophobic (tryptophan, Trp) amino acids. Six peptides were synthesized to determine the **influence of** charge, charge distribution, and hydrophobicity on transbiiyer movement into LUV systems. It is shown that while most of these peptides can be accumulated into EPC and EPC: cholesterol (55:45, mol:mol) LUVs with an acidic interior, the rates of transport are extremely sensitive to the number and location of basic functions within the molecule. Peptides with their charged groups concentrated at the N-terminal generally exhibited faster rates of t&sport and lower activation energies than peptides with their charged groups more equally distributed throughout the molecule.

## MATERIALS AND METHODS

*Materials* Egg phosphatidylcholine (EPC).was obtained from Avanti Polar Lipids (Birmingham, AL). [$^{14}$C] Methyl-

† This research was supported by the Medical Research Council (MRC) of Canada. A.C. is grateful to the University of British Columbia for a University Graduate Fellowship and to tbe Science Council of British Columbia for a GREAT Award.

• Author to whom correspondence should be addressed.
‡ Section of Molecular and Cellular Biology, University of California.
§ Biomedical Research Center. University of British Columbia.
‖ Department of Biochemist& University of British Columbia.

• Abstract published in Advance ACS *Abstracts,* June 15. 1994.

[1] Abbreviatons: EPC, egg phosphatidylcholine; HEPES, 4-(2-hydroxyetbyl)-1-pipcrazineethancsulfonic acid; LUV, large unilamellar vesicle; Lys, lysine; MES, 2-morpholinoethancsulfonic acid; MLV, multilamellar vesicle; TNBS, 2.4,6,-trinitrobcnzenesulfonic acid; Trp. tryptopban.

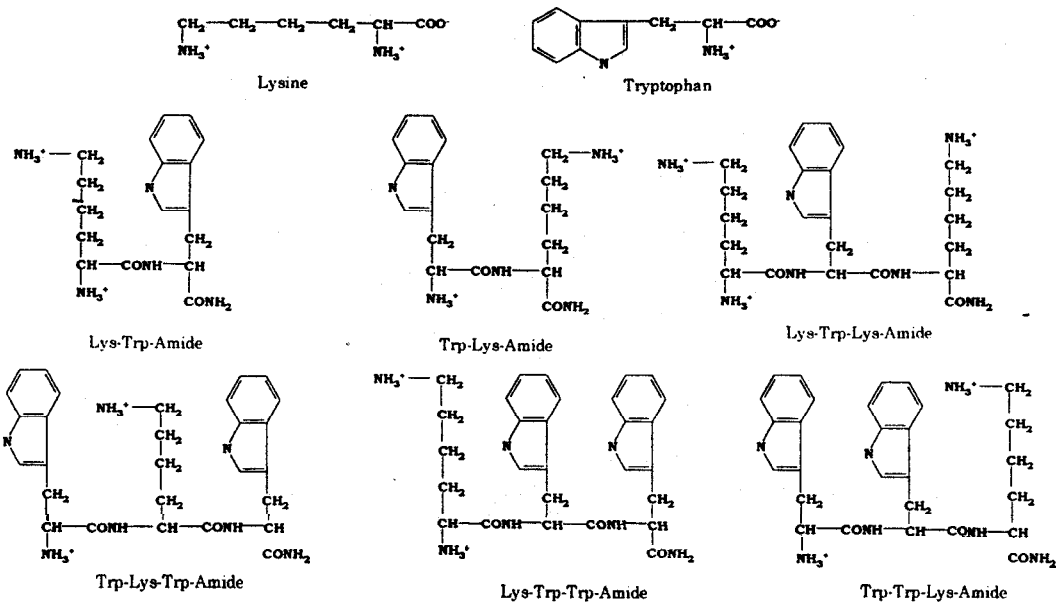


FIGURE 1: Structures of the model peptides that were synthesized. The + indicates the presence of a positively charged amino group.

amine was purchased from New England Nuclear. All other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO). The peptides were synthesized as carboxyl amides using solid-phase methods and purified by reverse-phase HPLC (Clark-Lewis & Kent, 1989). The structures of the peptida employed are given in Figure 1, with the location of charged residues being indicated by plus symbols.

***Preparation of Vesicles.*** Multilamellar vesicles (MLVs) were produced by hydrating 50 mg EPC in 1.0 mL of 300 mM citrate buffer (pH 4.0). The MLVs were frozen in liquid nitrogen and thawed at 60 °C (in water) for five freeze-thaw cycles. This treatment increases the trapped volume of the vesicles and promotes equilibrium transmembrane solute distributions (Mayer et al., 1985). Extrusion of the frozen and thawed MLVs through two stacked polycarbonate filters (Nuclepore; 100-nm pore size) was performed 10 times at 20 °C using an extrusion device obtained from Lipex Biomembranes Inc. (Vancouver, Canada) as described by Hope et al. (1985). The resulting large unilamellar vesicles (LUVs) were ca. 110 nm in diameter, as determined by quasielastic light scattering employing a NICOMP particle sizer.

EPC:cholesterol(55:45, mol:mol) vesicles were created by dissolving appropriate amounts of both compounds separately in chloroform, mixing the solutions, and drying down the resulting mixture using $N_2$ gas followed by incubation under reduced pressure for several hours. The 1 OO-nm vesicles were then prepared as described above.

Measurement ***of Peptide Translocation.*** In order to generate the transmembrane pH gradient, the LUVs in the pH 4.0 media were passed down a 10-cm Sephadex G-50 (G50-150) column previously equilibrated with 150 mM NaCl, 20 mM HEPES (pH 7.5) (HEPES buffered saline, HBS). Tricine (pH 8.5 and 9.0) and MES (pH 5.5) were also used for some experiments as the external buffer at a concentration of 20 mM. Uptake of the peptides was performed by first dissolving them in the HBS media (1 mL), to which the LUVs (0.25 mL, final lipid concentration of 1 to 5 mM) exhibiting a $\Delta pH$ ($pH_o$ = 7.5, $pH_i$ = 4.0, unless otherwise indicated) were added. Entrapment levels were monitored employing aliquots (0.1 mL) which were removed at selected times from this incubation mixture and passed through 1 .0-mL Sephadex G-50 columns (prespun) by centrifugation for 1 min at 2500 rpm to remove exterior (untrapped) material. All experiments were conducted at 20 °C unless otherwise indicated.

The amount of peptide trapped was quantified by measurement of lysine concentrations using TNBS (2,4,6-trinitrobenzenesulfonic acid) (Hope & Cullis, 1987) or by measuring tryptophan fluorescence (de Kroon et al., 1989). In the former method, TNBS is used to label the primary amino groups of lysine. The buffer used for the labeling was 100 mM $NaHCO_3$, 50 mM $H_3BO_3$ at pH 10.0. A reference cuvette containing 2.5 mL of buffer (pH 10.0) was placed in the reference beam. The sample cuvette contained 2.5 mL of buffer (pH 10.0) with 0.5 mM TNBS. Aliquots (50 μL) of vesicles containing lysine were then added. The resulting change in absorbance was measured at 420 nm after incubation for 1 h (in the dark). Triton X-100 (200 μL, 0.5%) was added to both cuvettes to solubilii the vesicles and thus expose all primary amino groups to the TNBS. The absorbance in the presence of detergent was taken to represent 100% labeling (Hope & Cullis, 1987).

The amount of peptide trapped was also quantified by measuring the tryptophan fluorescence ($\lambda_{ex}$ = 280 nm, $\lambda_{em}$ = 360 nm) employing a P&in-Elmer LS 50 luminescence spectrometer, in 0.5% (w/v) sodium cholate containing buffer (20 mM HEPES, 150 mM NaCl; pH 7.5). The sample volumes used were adjusted so that the resulting emission intensity was directly proportional to the amount of peptide present.

***Measurement of pH Gradients and Phospholipid Concentrations. The*** magnitude of the pH gradients present were measured using [$^{14}$C]methylamine as indicated elsewhere (Madden et al., 1990; Harrigan et al., 1992). The concentration used was 1 μCi/mL. The amount of probe accumulated was determined via liquid scintillation counting after removing untrapped label. Transmembrane pH gradients could then be calculated using the relationship $\Delta pH = \log\{[\text{methylamine}]_{in}/[\text{methylamine}]_{out}\}$ as indicated in Mayer et al. (1988).

Phospholipid concentrations were determined by a modificationofthemethodofFiskeandSubbarow(1925). Typical phospholipid concentrations were ca. 3 mM.

***Kinetic Analysis of Peptide Translocation.*** Under the assumption that only the neutral form of the peptide traverses the membrane (see, for example, Harrigan et al., 1993), it follows that

$$\frac{d[P]_o^{tot}}{dt} = \frac{-P_m A_m}{V_o}([P]_o^m - [P]_i^m) \quad (1)$$

where $[P]_o^{tot}$ is the total exterior concentration of the peptide (including charged, uncharged, free, and membrane-bound species), $P_m$ is the membrane permeability coefficient of the neutral form, A, is the area of the membrane, $V_o$ is the total external volume and $[P]_o^m$ and $[P]_i^m$ are the concentrations of the neutral forms of the peptide in the outer and inner monolayers, respectively.

As shown elsewhere (Harrigan et al., 1993) for a compound containing one amino function, the concentration $[P]_o^m$ in the outer monolayer can be written as

$$[P]_o^m = \frac{KK_d}{[H^+]_o}[P]_o^{tot} \quad (2)$$

where K is the membrane:water partition coefficient for the peptide, $K_d$ is the dissociation constant of the amino group, and $[H^+]_o$ is the exterior proton concentration. This analysis assumes that $[H^+]_o >> K_d$ and that $K << V_o/V_m$, where $V_m$ is the membrane volume. On substitution of eq 2 into eq 1 and assuming that $[P]_o^m >> [P]_i^m$, we can solve to obtain

$$[P(t)]_o = [P(0)]_o \exp(-kt) \quad (3)$$

where $[P(t)]_o$ is the total exterior concentration of the peptide at time $t$, $[P(0)]_o$ is the initial peptide exterior

$$k = \frac{P_m A_m K K_d}{V_o [H^+]_o} \quad (4)$$

peptide has two amino functions, assuming $[H^+]_o \gg K_{d1}$ and $K_{d2}$, and $K << V_m/V_o$, it can be shown that

$$k = \frac{P_m A_m K}{V_o} \frac{K_{d1} K_{d2}}{[H^+]_o^2} \quad (5)$$

where $K_{d1}$ and $K_{d2}$ are the dissociation constants for each of the two amino functions. Thii can be readily extended to a peptide containing three ammo groups where

$$k = \frac{P_m A_m K}{V_o} \frac{K_{d1} K_{d2} K_{d3}}{[H^+]_o^3} \quad (6)$$

The time dependence of the total interior peptide concentration $[P(t)]_i^{tot}$ can be expressed as

$$[P(t)]_i^{tot} = [P(eq)]_i^{tot}[1 - \exp(-kt)] \quad (7)$$

where $[P(eq)]_i^{tot}$ is the total interior peptide concentration at equilibrium The rate constant (k) was determined by applying a linear least-square analysis to a semilog plot of the uptake data using a commercially available plotting program (SigmaPlot, Jandel Scientific, 1986).

***Determination of the Membrane:Water Partition Coefficient, K.*** Partition coefficients were determined using an equilibrium filter centrifugation technique employing a Centrifree micropartition system (Amicon Div., W.R. Grace and Co., Danvers, MA). A 100-μL aliquot of vesicles (pH 7.5; 150 mM NaCl, 20 mM HEPES) was added to 900 μL of 2 mM peptide solution (pH 7.5. HBS) in the Centrifree apparatus. The mixture was vortexed and allowed to stand for 15 min. It was then centrifuged for 10 min at 3000 rpm The liposome containing 'supernatant" peptide solution and "pellet" (aqueous) peptide solution were collected and stored at 4 °C until utilized. Peptide concentrations for both solutions were obtained and phospholipid concentrations for the "supernatant" were calculated as outlined previously. The membrane:water partition coefficient (K) was then obtained using the relation

$$K = [\text{peptide}]_m/[\text{peptide}]_{aq} \quad (8)$$

where (peptide], is the concentration of the membrane-associated peptide and [peptide],, is the aqueous 'free" concentration of peptide determined from the concentration in the 'pellet" solution. The concentration of the membrane-associated peptide can be expressed as

$$[P]_m = ([P]_s - [P]_{aq})(v_s/v_m) \quad (9)$$

where [P] is the concentration of peptide, v is the volume, and the subscripts s and m refer to supernatant and membrane-associated peptide. The membrane:water partition coefficient (K) can then be written as

$$K = (v_s/v_m)([P]_s/[P]_{aq} - 1) \quad (10)$$

$v_m$ was calculated assuming a phospholipid molecular weight of 800 and a density of 1 g/mL.

Measurement of ***Octanol: Water and Olive Oil:Water Partition Coefficients.*** Octanol:water partition coefficients were measured by mixing 3 mL of 5 mM peptide (in aqueous solution) with 3 mL of octanol (Sigma Chemical Co.; organic phase) for ca. 1 h at 22 °C. The resulting mixture was allowed to settle for 24 h. The aqueous phase was then removed and stored (aqueous phase peptide concentration). An equal volume of "fresh" aqueous phase (containing no peptide) was then added to the octanol phase and mixed for 1 h at room temperature. The aqueous phase containing peptide extracted from the octanol phase (organic phase peptide concentration) was then removed after allowing the resulting mixture to settle for 24 h. Peptide concentrations present in both aqueous phases were then determined and K values calculated as described previously. This method assumes a low partition coefficient (established by early experiments); so the fresh aqueous phase can extract essentially all peptide present in the organic phase.

Olive oil:water partition coefficients were measured using a static equilibration technique. Preliminary experiments indicated very low partition coefficient values, so the aqueous extraction procedure described above was used with some modifications. A 1.0-mL sample of 32 mM peptide (in aqueous solution) was incubated with 2.0 mL of olive oil (Sigma Chemical Co.) at 22 °C for 72 h. No mixing of the solutions was done in order to avoid the formation of highly stable microemulsions. A 1 .0-mL aliquot of the olive oil was removed after this incubation and carefully added to 1 mL of 'fresh" aqueous phase. The two solutions were then incubated for 72 h. The olive oil phase was carefully removed, the peptide concentration of the aqueous phases was measured as described previously, and the $K$ values were determined.

***pH Titration of Peptides.*** Titrations were performed as described by Mauk et al. (1991). A Radiometer ABU93 Triburette equipped with three 1-mL burets and a SAM90 samplestation housed within an aluminum Faraday cage were used. Theautoburet was computer-controlled for the titration

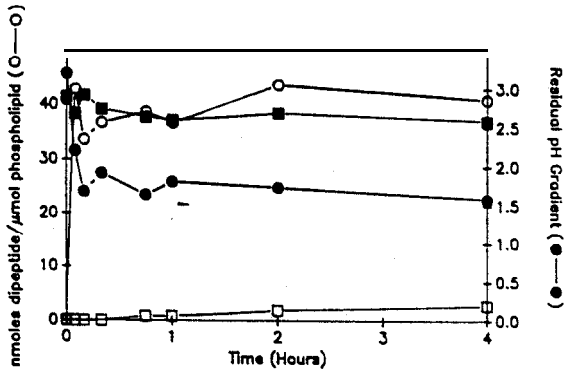


FIGURE 2: Time course of uptake of Lys-Trpamide (0) and Trp-Lys-amide (□) into 100-nm EPC vesicles (3.7 mM) exhibiting a pH gradient ($pH_i$ = 4.0, $pH_o$ = 7.5). Uptake studies were conducted at 20 °C and the initial external concentrations of the peptides were 0.16 mM (Lys-Trpamide) and 0.30 mM (Trp-Lys-amide). The residual pH gradients measured for the uptake of each peptide are represented by the filled symbols.

and data acquisition. For further details refer to the work of Mauk et al. (1991).

## RESULTS

The first set of experiments was aimed at exploring the transport properties of certain dipeptides with the same amino acid composition but where the amino acids are arranged in a different order. The two peptides synthesized were Lys-Trpamide and Trp-Lys-amide. Incubation of these peptides at 20 °C with EPC LUVs (100-nm diameter) exhibiting a transbilayer pH gradient (insideacidic; $pH_o$ = 7.5, $pH_i$ = 4.0) revealed markedly different uptake phenomena. As shown in Figure 2, Lys-Trpamide was accumulated into the LUVs effectively instantaneously, whereas Trp-Lys-amide exhibited much slower uptake kinetics. A corresponding decrease in the pH gradient, as measured by [$^{14}$C]methylamine, was observed after accumulation of Lys-Trp-amide (the gradient dropped from 3.2 to 1.6 pH units). This decrease, which may be attributed to protonation of the amino functions of the accumulated Lys-Trpamide and consequent consumption of internal protons, was not observed for Trp-Lys-amide, corresponding to the fact that little or no uptake was observed for this peptide under these conditions.

Three approaches were used to reduce the rate of uptake of Lys-Trp-amide so that the rate constants associated with the transbilayer movement of the two dipeptides could be compared quantitatively. The first of these measures involved lowering the external pH. As indicated in Materials and Methods, the rate constant of translocation for a compound such as Lys-Trp-amide, which contains two amino functions, will decrease as the square of the external proton concentration for pH values less than the pK of the amino groups. However, even at an external pH of 5.5, the uptake of Lys-Trp-amide was still too rapid to allow for an accurate analysis of rates (results not shown).

A second approach was to incorporate cholesterol into the LUVs, which would also be expected to reduce rates of transbilayer transport. As shown in Figure 3, the kinetics associated with the uptake of Lys-Trpamide into EPC: cholesterol (55:45, mol:mol) LUVs, at an exterior pH of 5.5 and temperatureof 21 °C can readily be measured. However, theuptakeof Trp-Lys-amide was much too slow to be measured under these conditions.

In order to increase the rate of uptake of Trp-Lys-amide for the EPC:cholesterol system, increased exterior pH values and temperatures were employed. As shown previously

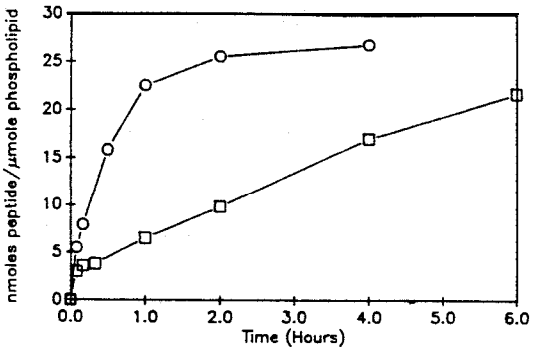


FIGURE 3: Time course of uptake of Lys-Trp-amide (0) and Trg Lys-amide (□) into 100-nm EPC:cholesterol vesicles (55:45, mol: mol; 1.7 mM for Lys-Trp-amide and 4.9 mM for Trp-Lys-amide) exhibiting a pH gradient (pHi = 3.0, $pH_o$ = 5.5, 21 °C for Lys-Trp-amide; $pH_i$ = 4.0. $pH_o$ = 8.0, 55 °C for Trp-Lys-amide). The initial external peptide concentration was 0.43 mM.

(Chakrabarti et al., 1992), the rate of translocation of lysine methyl ester is dramatically affected by changes in temperature. As shown in Figure 3, at an exterior pH of 8.0 and temperature of 55 °C the kinetics associated with the uptake of Trp-Lys-amide into EPC:cholesterol (55:45, mol:mol) vesicles could be readily determined.

In order to compare the rates of uptake of Trp-Lys-amide at 55 °C with that of Lys-Trp-amide at 21 °C, determination of the activation energies associated with transport are required. Related molecules can exhibit high activation energies, in the range of 31-36 kcal/mol (Chakrabarti et al., 1992). As shown in Figure 4A, the rate of uptake of Lys-Trp-amide increased considerably as the temperature was raised from 9.5 to 30.5 °C for an EPC:cholesterol (55:45, mol:mol) LUV system where $pH_o$ = 5.5 (20 mM MES and 150 mM NaCl) and $pH_i$ = 3.0. An analysis of the rate constants (Figure 4B) and subsequent calculation of the activation energy from the Arrhenius plot of Figure 4C revealed an activation energy of 23.7 kcal/mol. A set of data analogous to that obtained for Lys-Trpamide was also generated for Trp-Lys-amide in an EPC:cholesterol (55:45, mol:mol) system, where $pH_o$ = 8.0 (20 mM HEPES and 150 mM NaCl) and $pH_i$ = 4.0, over the temperature range of 40-55 °C (Figure 5). The resulting activation energy calculated for Trp-Lys-amide was 29.0 kcal/mol (Figure 5C).

Subsequent experiments revealed that the activation energy of translocation for Trp-Lys-amide in EPC vesicles was 26.8 kcal/mol at pH 7.5 (Table 1). It should also be noted that the activation energies were somewhat dependent upon the lipid system used, with EPC:cholesterol (55:45, mol:mol) vesicles giving higher activation energies than EPC alone (Table 1).

As indicated in Materials and Methods, the rate constant *k* associated with transport is directly proportional to the membrane:water partition coefficient of the peptide In order to determine whether such partitioning affects could explain the extremely large differences in uptake rates partition coefficients were determined for the dipeptides employing the centrifugation and octanol:water and olive oil:water phase separation methods outlined in Materials and Methods. The use of the centrifugation technique to determine membrane: water *K* values has been shown to correlate very well with membrane:water *K* values derived from other techniques (Harrigan et al., 1993). As shown in Table 1, the membrane: water partition coefficient and olive oil:water partition coefficient values obtained are quite similar for both Lys-Trp-amide and Trp-Lys-amide The octanol:water partition coefficient values are somewhat different, with Lys-Trpamide

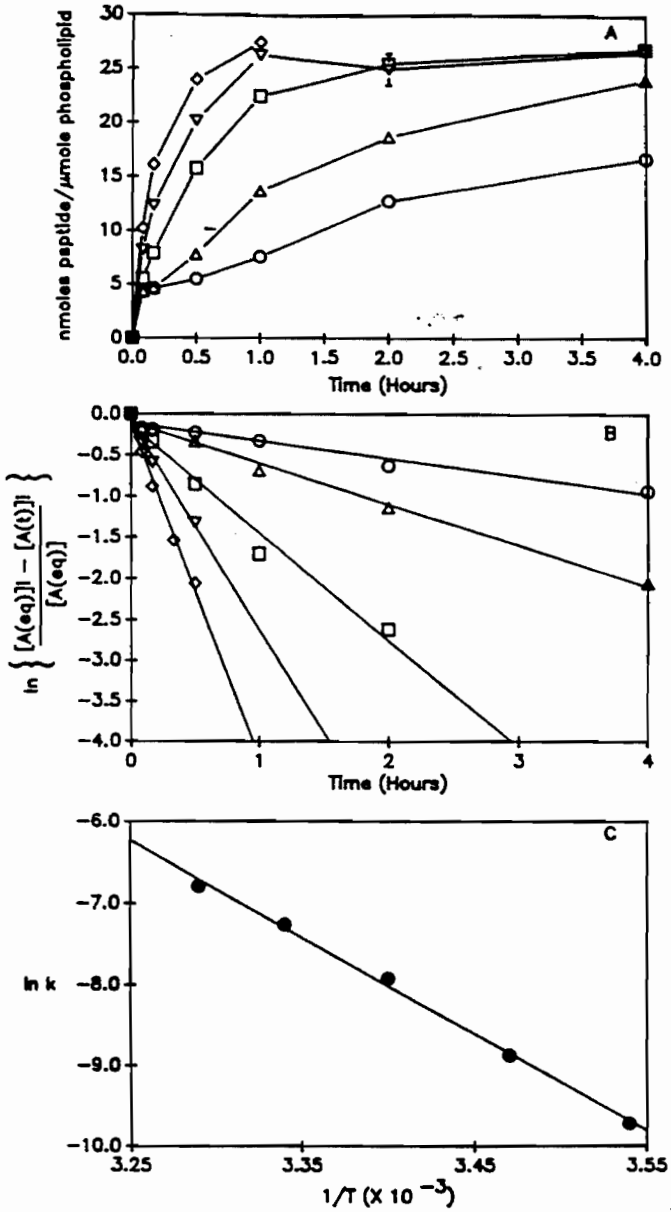


FIGURE 4: (A) Time course of uptake of Lys-Trp-amide into 100-nm EPC:cholesterol vesicles (55:45, mol:mol; 1.7 mM) bearing a 5.5/3.0 (external/internal) pH gradient. Uptake studies were conducted at 9.5 °C (○), 15 °C (△), 21 °C (□), 26 °C (▽), and 30.5 °C (◇). The external concentration of Lys-Trp-amide was 0.43 mM. (B) Plot of ln $\{([A(eq)]_i - [A(t)]_i)/[A(eq)]_i\}$ versus $t$, where $[A(t)]_i$ is the interior concentration of the accumulated amine at time $t$ and $[A(eq)]_i$ is the interior concentration at equilibrium. For details, see Materials and Methods. The slopes of the lines give the rate constants ($k$) for the transbilayer transport of Lys-Trp-amide. (C) Arrhenius plot of the rate constants ($k$) for Lys-Trp-amide uptake. The activation energy calculated from the slope of this plot is $E_a$ = 23.7 kcal/mol.

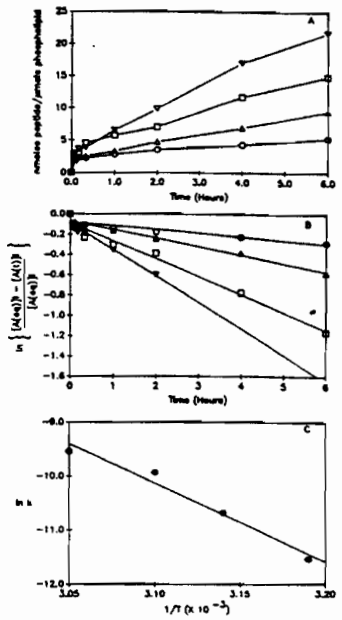

FIGURE 5: (A) Time course of uptake of Trp-Lys-amide into 100-nm EPC:cholesterol vesicles (55:45, mol:mol; 4.9 mM) bearing a 8.0/4.0 (external/internal) pH gradient. Uptake was conducted at 40 °C (○), 45 °C (△), 50 °C (□), and 55 °C (▽). The external concentration of Trp-Lys-amide was 0.42 mM. (B) Plot of ln $\{([A(eq)]_i - [A(t)]_i)/[A(eq)]_i\}$ versus $t$, where $[A(t)]_i$ and $[A(eq)]_i$ have the same meanings as indicated in the legend to Figure 4B. (C) Arrhenius plot of the rate constants ($k$) for Trp-Lys-amide uptake. The activation energy calculated from the slope of this plot is $E_a$ = 29.0 kcal/mol.

partitioning into the octanol phase to a 3-fold greater extent than Trp-Lys-amide (Table 1). The similarity of these values indicates that partitioning effects were not predominantly responsible for the differences observed in uptake rates.

The rate constants are also directly proportional to the dissociation constants associated with the two amino functions. It is possible that the proximity of the amino groups in Lys-Trp-amide might result in alterations of these p$K$ values, leading to very different uptake rates. In order to determine whether the p$K$ values of Lys-Trp-amide were significantly different from those of Trp-Lys-amide, pH titrations were performed on these two peptides. The titration curves for both peptides were essentially identical (data not shown). The p$K$ of the $NH_2$ terminus amino function was found to be 7.4, while that of the lysine side chain was 10.4 for both peptides.

Table 1: Peptide Partition Coefficients ($K$) and Activation Energies ($E_a$)[a]

| peptide | $K$ | $E_a$ (kcal/mol) |
|---|---|---|
| Lys-Trp-amide | 10.4 | (23.7) |
| octanol:water | $1.1 \times 10^{-3}$(±14%) | |
| olive oil:water | $1.6 \times 10^{-4}$ | |
| Trp-Lys-amide | 12.1 | 26.8 (29.0) |
| octanol:water | $4.0 \times 10^{-4}$(±15%) | |
| olive oil:water | $1.3 \times 10^{-4}$ | |
| Lys-Trp-Trp-amide | 15.1 | 19.6 |
| Trp-Trp-Lys-amide | 15.8 | 33.4 |
| Trp-Lys-Trp-amide | 11.9 | 14.9 |

[a] Partition coefficients and activation energies were calculated as described in the Materials and Methods. Activation energies given in parentheses are those calculated for EPC:cholesterol (55:45, mol:mol) vesicles. The activation energy for Lys-Trp-amide could not be determined in EPC LUVs (see Results). Standard errors of the mean for the octanol:water partition coefficient values are given in parentheses.

In order to further characterize the influence of charge distribution on the transbilayer movement of peptides, a series of tripeptides was synthesized based upon the Lys/Trp combinations. The peptides that were synthesized were Lys-Trp-Lys-amide, Trp-Lys-Trp-amide, Lys-Trp-Trp-amide, and Trp-Trp-Lys-amide (see Figure 1). The ability of these peptides to accumulate at 45 °C into EPC LUVs (100-nm diameter) experiencing a transbilayer pH gradient (inside acidic; $pH_o$ = 8.5, $pH_i$ = 5.0) are shown in Figure 6. Trp-Lys-Trp-amide ($k = 2.93 \times 10^{-4}$ s$^{-1}$) and Lys-Trp-Trp-amide ($k = 2.83 \times 10^{-4}$ s$^{-1}$) exhibited the most rapid uptake followed by Trp-Trp-Lys-amide ($k = 1.89 \times 10^{-4}$ s$^{-1}$). Lys-Trp-Lys-amide showed no appreciable accumulation, even after incubation for several hours at high temperature (50 °C) and high exterior pH ($pH_o$ = 10.0).

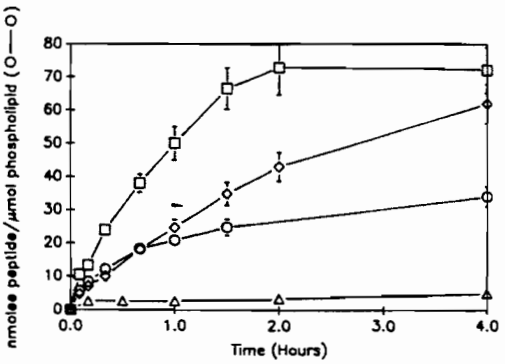


FIGURE 6: Time course of uptake of Lys-Trp-Trp-amide (○), Trp-Trp-Lys-amide (□), Trp-Lys-Trp-amide (△), and Lys-Trp-Lys-amide (◇) into 100-nm EPC vesicles (4.1 mM) exhibiting a pH gradient ($pH_i$ = 5.0, $pH_o$ = 8.5, except for the Lys-Trp-Lys-amide, where $pH_o$ = 10.0). Uptake was conducted at 45 °C and the initial external peptide concentration was 1.67 mM.

The three combinations of one lysine and two tryptophan residues revealed significantly different rates of accumulation, depending upon the distribution of the two charged residues (amino terminus and lysine side chain) within the molecule. Rate constants for the translocation of these three peptides were calculated from the resulting Arrhenius plots (Table 1). The activation energies for the transbilayer movement of these peptides ranged from 14.9 to 33.4 kcal/mol, remarkable differences for molecules that were composed of the same three amino acids. Partition coefficients were also determined for these three molecules and were found to range from 15–16 for Trp-Trp-Lys-amide and Lys-Trp-Trp-amide to ca. 12 for Trp-Lys-Trp-amide (Table 1).

## DISCUSSION

The results of this report indicate that basic peptides of identical amino acid composition which vary only in the amino acid sequence can show markedly different abilities to permeate through lipid bilayers. The primary focus concerns the influence of charge, charge distribution, and degree of hydrophobicity of the peptides on the transbilayer movement of these molecules.

As indicated in Materials and Methods, the ability of weak bases to translocate across lipid bilayers, which relies on permeation of the neutral form and can result in net accumulation into LUVs with an acidic interior, is strongly dependent on the external pH. In the case of a molecule containing two amino groups, for example, the rate constant associated with uptake is proportional to the inverse square of the external proton concentration. This dependence, together with the activation energy ($E_a$) associated with permeation of the neutral form, can be expressed in a generalized rate equation for the peptides containing two amino functions according to

$$k(T,\mathrm{pH}) = k(T_1,\mathrm{pH}_1) \times 10^{2(\mathrm{pH}-\mathrm{pH}_1)} \times \exp\{-E_a/RT_1(T_1/T-1)\} \quad (11)$$

where $k(T_1,\mathrm{pH}_1)$ is the rate constant measured at a particular temperature ($T_1$) and exterior pH ($\mathrm{pH}_1$). Employing the data of Figure 3 for EPC:cholesterol LUVs, we note that $k_{KW}$ (21 °C, pH 5.5) = $5.8 \times 10^{-4}$ $s^{-1}$, whereas $k_{WK}$ (55 °C, pH 8.0) = $6.4 \times 10^{-5}$ $s^{-1}$, where the subscripts KW and WK indicate Lys-Trp-amide and Trp-Lys-amide, respectively. Thus, given the activation energies $E_a^{KW}$ = 23.7 kcal/mol and $E_a^{WK}$ = 29.0 kcal/mol, we obtain the generalized rate equations for translocation of the Lys-Trp-amide and the Trp-Lys-amide dipeptides as

$$k_{KW}(T,\mathrm{pH}) = 5.8 \times 10^{-4} \times 10^{2(\mathrm{pH}-5.5)} \times \exp\{40.6(294/T) - 1)\} \quad (12)$$

$$k_{WK}(T,\mathrm{pH}) = 6.4 \times 10^{-5} \times 10^{2(\mathrm{pH}-8.0)} \times \exp\{44.5(328/T) - 1)\} \quad (13)$$

Thus at 20 °C and pH = 7.0, $k_{KW}$ = 0.67 $s^{-1}$ ($t_{1/2}$ = 1 s), whereas $k_{WK}$ = $1.3 \times 10^{-4}$ $s^{-1}$ ($t_{1/2}$ = 1.5 h).

The reasons for the very large differences in the rate constants associated with the uptake of Lys-Trp-amide as compared to Trp-Lys-amide are not clear. The partition coefficients for both molecules are comparable (Table 1) and could not account by themselves for a rate constant of Lys-Trp-amide which is $5 \times 10^3$ larger than that of Trp-Lys-amide. Similarly, no significant difference between the p$K$'s of the two amino groups associated with these molecules could be observed. Such differences could lead to large variations in $k$ through the relationship indicated in eq 4. A remaining possibility is that while the dissociation constants and abilities to partition into the lipid–water interface are similar, the ability of these molecules to enter the bilayer hydrocarbon are markedly different. Such differences, which could be related to conformational factors or perhaps to more favorable intramolecular hydrogen bonding for Lys-Trp-amide, may be reflected by the lower activation energy (23.7 kcal/mol) associated with the more rapidly translocated Lys-Trp-amide compared to Trp-Lys-amide (29 kcal/mol) in the EPC: cholesterol (55:45, mol:mol) system.

Activation energies associated with the transfer of molecules from aqueous media into the membrane have been estimated according to the number of hydrogen bonds that must be broken to enter the membrane less the number created once inside (Stein, 1967; Walter & Gutknecht, 1986). Calculation of activation energies in this manner becomes more difficult as the size of the molecule increases, given the problem of estimating formation of intramolecular hydrogen bonds once inside the membrane. Previous work has shown that lysine methyl ester exhibits a high activation energy, in the range of 36 kcal/mol (Chakrabarti et al., 1992). Addition of a tryptophan residue to the lysine molecule appears to lower the activation energy by at least 9 kcal/mol (Table 1), which may be attributed to the increased hydrophobicity of the resulting molecule. Further, peptides of identical amino acid composition exhibit different activation energies depending upon the distribution of charges within the molecule (Table 1), indicating that amino acid composition per se is insufficient to predict activation energies for insertion into the hydrocarbon. In the case of the tripeptides examined here, for example, the activation energies for transbilayer movement range from 15 to 33 kcal/mol, depending on the location of the lysine residue (at the amino terminus, in the middle, or at the amide-modified carboxyl terminus; Table 1). The peptides with the lowest activation energies had the lysine residue in the N-terminal or middle position. This may be suggested to cause increased amphipathic characteristics, increased partition coefficients, and lowered activation energies observed when compared to the Trp-Trp-Lys-amide peptide (see Table 1).

The inability of Lys-Trp-Lys-amide to accumulate may be attributed to the hydrophilic nature of this molecule, as well as the improbability of the formation of the fully deprotonated, neutral, membrane-permeable form. Use of the Kyte–Doolittle hydrophobicity scales (where positive values indicate