# Mechanism of Leakage of Phospholipid Vesicle Contents Induced by the Peptide GALA†

Roberta A. Parente,‡ Shlomo Nir,§ and Francis C. Szoka, Jr.*,‡

*Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143, and Seagram Center for Soil and Water Sciences, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel*

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ABSTRACT: The synthetic, amphipathic peptide GALA undergoes a pH-dependent conformational change and induces leakage of contents from large unilamellar phosphatidylcholine vesicles when in a helical conformation. The kinetics of this process have been investigated over a wide range of pH and lipid and peptide concentrations. Leakage from lipid vesicles is rapidly initiated (within 2 s) when the pH is lowered below 6 and is rapidly terminated when the pH is raised to 7.5. The leakage shows a selectivity to the size of the entrapped molecules and occurs by an all or none mechanism; vesicles either leak or retain all of their contents. Using this experimental data, we have developed a mathematical description of the kinetics of leakage induced by GALA. This model assumes that GALA becomes incorporated into the vesicle bilayer and aggregates to form a pore. Leakage occurs when a critical number of peptides assemble into a supramolecular aggregate in the bilayer. Leakage curves generated at lipid/peptide ratios ranging from 500/1 to 30000/1 can be well described by this formalism. On the basis of the results and the model, we suggest that GALA forms a transbilayer channel composed of 8–12 monomers. The channel diameter ranges from 5 to 10 Å. To the best of our knowledge, this is the first model that can predict the leakage kinetics of solutes entrapped in lipid vesicles induced by a pore-forming peptide. The analysis may be of general use in defining the kinetics and state of aggregation of similarly acting peptides and proteins which form multimeric assemblies in membranes.

The understanding of the interrelationships among dynamics, structure, and function of membrane-interacting peptide segments has been greatly advanced by the study of cytolytic peptides such as gramicidin, alamethicin, and melittin. The emphasis to date has been on describing the structure and dynamics of the peptide in the bilayer and its interaction with lipids (Bernheimer et al., 1986; Spach et al., 1989). Ideally, one would like to know the effect of varying individual amino acids on structure and function. This can be accomplished through mutagenesis or alternatively through the design and synthesis of membrane-interactive peptides (Lear et al., 1988; DeGrado et al., 1989).

We have followed the latter strategy and designed a synthetic peptide designated GALA to explore structure–function relationships in an amphipathic peptide. GALA is a 30 amino acid peptide with the sequence W-E-A-A-L-A-E-A-L-A-E-A-L-A-E-H-L-A-E-A-L-A-E-A-L-E-A-L-A-A that is water soluble at pH 7.5 but interacts with and destabilizes lipid bilayers at a pH less than 6.0. At high lipid to peptide ratios leakage of large unilamellar vesicles occurs while at low lipid to peptide ratios both leakage and fusion of small unilamellar vesicles are observed (Subbarao et al., 1987; Parente et al., 1988). In addition, phospholipid acyl chain composition influences the GALA–membrane interaction (Subbarao et al., 1988).

The pH dependence of the membrane binding (Parente et al., 1990b) and leakage induced by GALA permits a careful study of the kinetics of leakage. When oligomerization of a membrane-bound monomer is involved in the leakage, the kinetics can assist in determining the number of monomers forming the leaking unit, information which casts some light on the possible structure of the pore or channel. Compared to other aspects of peptide–membrane interactions, the kinetics of channel formation has received only modest attention (Schwarz, 1989; Belmonte et al., 1987) although such information would aid the understanding of the structure and mechanism of membrane-damaging molecules such as complement or staphylococcal α-toxin (Bhakdi & Tranum-Jensen, 1983; Bernheimer & Rudy, 1986).

In this paper we use a fluorescence leakage assay and a mathematical model to analyze the kinetics of solute leakage from lipid vesicles induced by GALA. In addition, we show that leakage is an all or none event restricted to solutes with radii below 6 Å. The leakage data are consistent with the formation of an oligomeric channel in the bilayer containing between 8 and 12 GALA monomers. The analysis described in this paper may be applicable to kinetic data on other channel-forming peptides and proteins.

## Materials and Methods

*Materials.* Egg phosphatidylcholine (egg PC)[1] in chloroform was purchased from Avanti Polar Lipids Inc. (Pelham, AL). 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and

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* Address correspondence to this author at the Department of Pharmacy, University of California.

‡ University of California.

§ The Hebrew University of Jerusalem.

[1] Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; $Ap_5A$, diadenosine pentaphosphate; DPX, *p*-xylylenebis[pyridinium bromide]; egg PC, egg phosphatidylcholine; NADH, nicotinamide adenine dinucleotide; REV, reverse-phase evaporation vesicles; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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*p*-xylylenebis[pyridinium bromide] (DPX) were obtained from Molecular Probes (Eugene, OR). Diadenosine pentaphosphate ($AP_5A$), acid blue 9 (erioglaucine), and nicotinamide adenine dinucleotide (NADH) were purchased from Sigma (St. Louis, MO), and [$^{14}$C]inulin was obtained from American Radiolabelled Chemicals Inc. (lot 86822).

*Vesicle Preparation.* Reverse-phase evaporation vesicles (REV) were prepared as described (Szoka & Papahadjopoulos, 1978) in 5 mM TES and 100 mM KCl or in 12.5 mM ANTS, 45 mM DPX, 5 mM TES, and 20 mM KCl at pH 7.5 and routinely extruded three times through a 0.1-$\mu$m polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) at 100 psi $N_2$ pressure. For a larger vesicle population, vesicles were extruded once through a 0.2-$\mu$m membrane and for smaller vesicles were extruded three times through a 0.05-$\mu$m membrane. A quick freeze–thaw step was included after the first extrusion at 0.05 $\mu$m. When necessary, a Sephadex G-75 column (1 × 20 cm) was used to separate vesicles from unencapsulated material with 5 mM TES–100 mM KCl, pH 7.5, as the elution buffer. Lipid phosphorus was determined by a modification of the Bartlett (1959) method.

*Peptide Synthesis.* GALA was synthesized by the Biomolecular Resource Center (UCSF) with an automatic synthesizer and Merrifield resin. Details of the synthesis and purification have been described (Subbarao et al., 1987). Briefly, purification was carried out by reverse-phase HPLC on a Waters Associates instrument equipped with a Dynamax $C_{18}$ column. GALA was eluted with a linear gradient of acetonitrile and water containing 0.1% trifluoroacetic acid. Analysis of purified material on a Vydac analytical $C_{18}$ column showed that the final peptide fractions were greater than 99% pure.

*Fluorescence Measurements.* Measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source.

*Leakage.* The ANTS/DPX assay (Ellens et al., 1984) was used to monitor leakage induced by peptide interaction with vesicles. The fluorescence signal resulting from the dequenching of ANTS released into the medium was observed through a Schott GG 435-nm cutoff filter (50% transmittance at 435 nm) while samples were irradiated at 360 nm; 90° light scattering was simultaneously recorded through a monochrometer at 360 nm. Data points were recorded at 0.5-s intervals over a 10-min period after which vesicles were lysed with the detergent dodecyloctaethylene glycol monoether (Calbiochem, La Jolla, CA) to obtain the maximal fluorescence value, which was set to 100% leakage. Fluorescence from intact vesicles in buffer was set to 0% leakage, and results were normalized according to this scale. Unless otherwise stated, peptide was added to stirred vesicle suspensions (100 $\mu$M) at 20 °C to begin an experiment. Reaction buffers consisted of 5 mM TES–100 mM KCl, pH 7.5, 5 mM MES–100 mM KCl, pH 6, or 5 mM acetate–100 mM KCl, pH 5 or 4.5. At pH values between 5 and 6, the MES-containing buffer was used and adjusted to the desired pH with acetate.

*Leakage Mechanism.* To differentiate if leakage of contents was an all or none event (some of the vesicles release all of their contents) as opposed to a graded event (all of the vesicles release some of their contents), we employed a modification of the fluorescence dequenching method of Weinstein et al. (1981), encapsulating the fluorophore/quencher pair ANTS/DPX in place of carboxyfluorescein in the original report. Two sets of experiments are prerequisite for this study. First, the extent of leakage of ANTS/DPX induced by GALA was determined at various lipid to peptide ratios. Second, a quench curve for vesicles containing varying concentrations of ANTS and DPX was constructed. ANTS concentrations ranged from 0.07 to 12.5 mM while DPX concentrations ranged from 2.5 to 45 mM. The quencher and fluorophore were always encapsulated at a 3.6/1 mole ratio.

Vesicles prepared for the fluorescence dequenching experiments contained 6.25 mM ANTS and 22.5 mM DPX. From the quench curve these vesicles are 81% quenched or have 19% associated fluorescence. This starting condition was chosen at a point on the quench curve such that a small change in ANTS concentration due to vesicle leakage would give rise to a significant change in fluorescence. Vesicles were incubated with GALA at ratios varying from 3500/1 to 8500/1 for 15 min. The lipid concentration was kept constant at 100 $\mu$M while the peptide concentration was varied from 12 to 28 nM in the 0.25-mL incubation volume. Samples were then applied to a Sephadex G-75 column (0.5 cm × 7 cm) and eluted with 5 mM TES–100 mM KCl, pH 7.5, to separate the vesicle fraction from ANTS and DPX which leaked from vesicles during incubation. Fluorescence of the eluted vesicle fraction was measured before and after detergent addition as described by Weinstein et al. (1981), and the percent fluorescence remaining with the vesicles was calculated.

One could predict the percent fluorescence that would be vesicle associated for an all or none or graded response by making use of the results from the prerequisite experiments. For example, at 5000/1 there is approximately 80% leakage after 10 min. If this means that all vesicles leak 80% of their contents (graded release), then the ANTS/DPX concentration remaining inside would be 1.25 and 4.5, respectively. From our quench curve this corresponds to 46% quenching or 54% fluorescence remaining with the vesicles. However, if 80% of the vesicles leak all of their contents (all or none release), the ANTS and DPX concentrations inside the intact vesicles would be unchanged from the starting condition (81% quenched or 19% associated fluorescence). These predicted results were used to interpret the observed values.

*Size Selectivity of GALA-Induced Leakage.* Liposomes (REV) were made in the presence of acid blue 9, NADH, $AP_5A$, or [$^{14}$C]inulin in 5 mM TES–100 mM KCl, pH 7.5. After elution on a Sephadex G-75 column (0.7 × 20 cm) to remove unencapsulated material, an aliquot of known lipid concentration was incubated with GALA for 15 min at a 500/1 or 2500/1 mole ratio in pH 5 or 7.5 buffer. The mixture was then placed on the same column and eluted with buffer at pH 7.5. Absorbance or radioactivity of each fraction was determined along with lipid phosphorus. When absorbance was used to quantitate release, the amount of encapsulated material in each fraction was determined from a standard curve after correction for the absorbance of lipid at the appropriate wavelength (630 nm for acid blue 9, 339 nm for NADH, and 260 nm for $AP_5A$). Vesicles without encapsulated material were incubated with each compound and passed through the column to determine if any correction was necessary for binding of these compounds to the lipid. In all cases, no correction was necessary.

*Theoretical Analysis of Leakage Kinetics.* The model assumes that (1) the peptides (GALA in our case) bind and become incorporated into the bilayer of the vesicles and (2) within the membranes peptide aggregation occurs. When an aggregate within a membrane has reached a critical size, a channel or a pore is created within the membrane, and leakage of encapsulated molecules can occur. The size of the pore dictates the upper bound on the size (and shape) of molecules

that can leak. The size of the pore or channel depends on the number of peptides forming it.

The assumption that a critical number of peptides in a vesicle is required to cause leakage has been introduced to account for two of our findings. (i) In those instances where the final extent of leakage was less than 100%, the vesicles were divided into two sharply defined groups, those that leaked all their contents and those that leaked practically none of their contents (see results in Table I). (ii) In systems where the final extent of leakage (e.g., after 10 min) was not complete and the rate of leakage had practically become zero, additional leakage could be induced by elevating the pH to neutral and reducing it again to pH 5. When the pH was elevated, the peptides dissociated from the membranes [see Parente et al. (1990b) and Figure 2B], and when the pH was again reduced, there was a probability that vesicles that had not leaked in the first round would incorporate a sufficient number of peptides to undergo leakage in the second round. This argument is substantiated further under Results and Discussion.

*Final Extents.* According to the model, the final extent of leakage is due to the leakage of contents from all the vesicles containing $M$ or more peptides, where $M$ is the critical number of peptides in an aggregate. For a completely homogeneous population of vesicles, the final extent of leakage $L \equiv L(\infty)$ is given by

$$L = \sum_{i=M}^{N} A_i/G_0 \tag{1}$$

in which $N$ is the largest number of peptides that can be bound to a single vesicle, $G_0$ is the molar concentration of vesicles computed on the basis of a surface area of 60 Å$^2$ for PC, and $A_i$ is the concentration of vesicles containing $i$ incorporated peptides. The dimensionless quantities $\bar{A}_i = A_i/G_0$ represent the distribution function, satisfying the relation

$$\sum_{i=0}^{N} A_i/G_0 = \sum_{i=0}^{N} \bar{A}_i = 1 \tag{2}$$

The procedure for calculating the functions $A_i$ was derived by Nir et al. (1986), who calculated the kinetics and extent of virus binding to and fusing with cells. The reduction of these equations to the case of no fusion yields the kinetics and extent of binding according to

$$dA_i/dt = -CPA_i(N - i)/N + DA_{i+1}(i + 1) + CPA_{i-1}(N - i + 1)/N - DA_i i \tag{3}$$

in which $P(t)$ is the peptide molar concentration in solution at time = $t$, $C$ (units = $M^{-1}$ $s^{-1}$) is the forward rate constant of binding, and $D$ (units = $s^{-1}$) is the rate constant of dissociation. At equilibrium $dA_i/dt = 0$, and the solutions $A_i$ yield the equilibrium distribution. Bentz et al. (1988) derived closed-form solutions:

$$A_i/G_0 = \binom{N}{i} T^i (1 - T)^{N-i} \tag{4}$$

where

$$T = (P_0 - P)/(NG_0) \tag{5}$$

$$\binom{N}{i} = N!/[i!(N - i)!]$$

and $P_0$ is the total molar concentration of peptide. Computationally it was more convenient to calculate $A_i$ from the recursion equation:

$$A_i = A_{i-1} PK[N - (i - 1)]/(Ni) \tag{6}$$

$$K = C/D$$

which can be easily derived from eq 3 by mathematical induction. For instance, setting $i = 0$, $dA_0/dt = 0$ yields

$$CPA_0 = DA_1 \text{ or } A_1 = A_0 PK, \text{ etc.}$$

The quantity $K$ was calculated from the amount bound. For a certain range of peptide and lipid concentrations the binding of peptides to the lipid satisfied a partition relation, where for a lipid concentration of 100 $\mu$M the fraction of peptides bound ($B$) is 0.645 irrespective of peptide concentration. This corresponds to an apparent bilayer/aqueous molar partition coefficient = $1.0 \times 10^6$ as calculated according to Pownall et al. (1984). For lipid/peptide mole ratios of 2000–30 000 this partition relation (0.645) was satisfied quite well with a 1–4% deviation from the value computed according to

$$(P_0 - P)/P_0 = \sum_{i=1}^{N} iA_i/P_0 \tag{7}$$

By comparing the experimental value of $L$, the final extent fraction that has leaked (within 10 min), to the calculated fraction according to eq 1 for each ratio of lipid/peptide, we aimed at finding a range of $M$ values that could yield the critical number of peptides in a vesicle for which complete leakage occurred. As it turned out, we could explain (and predict) quite satisfactorily all the experimental results by assuming $M = 10$ for all cases. We accounted for the inhomogeneity of the vesicle diameters (when the vesicles were extruded through 0.1-$\mu$m polycarbonate membranes) by representing them by two diameters, of radii 50 and 100 nm, at number ratios of 24/1, respectively. The encapsulated volume fractions of these populations are 0.735 and 0.265, and the total lipid fractions are 0.852 and 0.148, respectively. This representation is in accord with our measurement of dynamic light scattering and with the previous electron microscopic measurements (Szoka et al., 1980). However, we recognize that the largest experimental uncertainty is in the determination of the vesicle size distribution, despite our current efforts to measure this accurately. Further improvements to the fit might arise if we could experimentally reduce the uncertainties in defining vesicle sizes. Finally, we assumed the same binding affinity of peptides to both small and large vesicles. Thus, the fraction of total peptide bound to small liposomes was 0.85.

*Kinetics of Leakage.* In calculating the kinetics of leakage, we assumed that the process of peptide incorporation in the vesicle membranes was rapid (Parente et al., 1990a) and that once a channel (or a pore) has been formed in a vesicle all its contents would leak rather fast, within less than 1 s (Lewis & McConnell, 1978). Thus, the kinetics of leakage was equated to that of formation of aggregates consisting of $M$ or more peptides in the bilayer. Extensive treatments of the problem of dynamical aggregation are given in Bentz and Nir (1981) and Nir et al. (1983). The scheme is

$$\begin{aligned} X_1 + X_1 &\underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} X_2 \\ X_1 + X_2 &\underset{D_{12}}{\overset{C_{12}}{\rightleftharpoons}} X_3 \\ X_1 + X_i &\underset{D_{1i}}{\overset{C_{1i}}{\rightleftharpoons}} X_{i+1} \\ X_2 + X_2 &\underset{D_{22}}{\overset{C_{22}}{\rightleftharpoons}} X_4 \\ &\vdots \end{aligned} \tag{8}$$

where $X_i$ are molar concentrations of aggregates of order $i$. Here we have used the same scheme, but in our case $X_i$ denotes surface concentrations. In these calculations we have introduced the computational simplifications that aggregate dissociation could be ignored and, furthermore, that the Smoluchowski (1917) treatment that assumed $C_{ij} = C$ could be

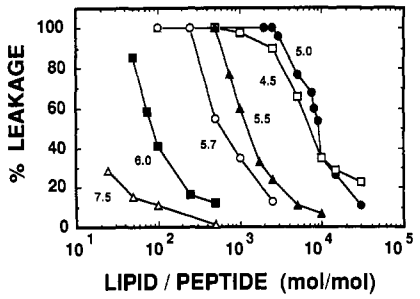


FIGURE 1: Percent leakage as a function of lipid/peptide ratio from pH 4.5 to pH 7.5. The extent of leakage of ANTS and DPX was recorded 10 min after the addition of GALA to stirred phospholipid vesicle suspensions. The final extents of leakage at a given lipid/peptide ratio and pH varied no more than ±2% when the extents of leakage were above 20% and no more than 10% of the measured value below 20% leakage. The lipid concentration was kept constant at 100 μM. Curves are drawn through individual points at pH 7.5 (△), pH 6.0 (■), pH 5.7 (○), pH 5.5 (▲), pH 5.0 (●), and pH 4.5 (□).

employed. A consequence of setting $D$ to zero is that $M$ will be an overestimate. However, the satisfactory fit to the data achieved by this approach suggests that this is an acceptable assumption. It has been shown (Bentz & Nir, 1981) that in many cases the initial kinetics of aggregation as calculated by the model of dynamical aggregation, which assumes a finite value of $N$, were in agreement with the Smoluchowski equation, which has no upper bound on $N$. Since we were merely interested in finding the fraction of vesicles containing peptide aggregates of order $M = 10$ and above, the finite size of the vesicles should not be an obstacle in using the Smoluchowski approach. In calculating (for a given vesicle at time $t$) the fraction, $F_M(t)$, of peptides in aggregates composed of $M$ or more molecules, we employed an equation derived from the Smoluchowski distribution (Nir et al., 1983):

$$F_M(t) = \left(\frac{M+\tau}{1+\tau}\right)\left(\frac{\tau}{1+\tau}\right)^{M-1} \quad (9)$$

In eq 9, $\tau = \bar{C}it$, where $t$ is the time (s), $i$ is proportional to the surface concentration of peptides (units = 1/area), and $\bar{C}$ is a forward rate of aggregation. If $i$ has a unit of inverse area, then $\bar{C}$ has a unit of area/time and $\tau$ is a dimensionless quantity. We chose here $i$ as a number, so that $\bar{C}$ has a unit of $s^{-1}$.

For a vesicle population consisting of two sizes, the fraction of encapsulated material that has leaked at time $t$ is given by

$$L(t) = \sum_{j=1}^{2}\sum_{i=M}^{N} \bar{A}_{ij}F_{Mj}(t)f_j \quad (10)$$

in which $f_j$ is the fraction of encapsulated volume in vesicles of type $j$. In our calculations we assumed that the big and small vesicles have the same surface properties, irrespective of the number of peptides bound, which means that the same value of $\bar{C}$ was employed for all cases. However, for the same value of $i$ the surface density of peptides is larger for the small vesicles. This was taken into account in the calculations. The value of $\bar{C}$ corresponds to the outer surface area of a 100 nm radius vesicle.

## Results and Discussion

*Leakage.* GALA has been shown to induce leakage of contents from egg PC REV in a pH-dependent manner (Subbarao et al., 1987). The extent of ANTS/DPX leakage from egg PC REV induced by GALA as a function of lipid/peptide ratio is given in Figure 1 over three pH units. Leakage was measured 10 min after GALA addition at a

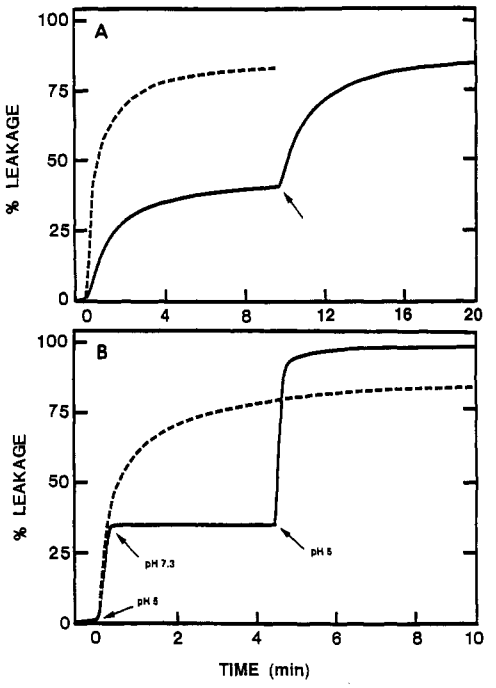


FIGURE 2: (A) Percent leakage as a function of time and peptide concentration at pH 5. GALA was injected into egg PC vesicles containing 12.5 mM ANTS and 45 mM DPX at a 10000/1 lipid/peptide ratio. After 10 min, an equivalent aliquot of peptide was added to give a final lipid/peptide ratio of 5000/1. The dashed line is the leakage curve obtained when GALA is added at a 5000/1 lipid/peptide ratio at time zero. (B) Percent leakage as a function of time and pH. Leakage was arrested by the injection of a concentrated TES solution, which raised the pH to 7.3. A final injection of acetate lowered the pH back to 5, and leakage resumed. Injection points are indicated by arrows. The dashed line shows the leakage obtained for a 5000/1 lipid/peptide ratio at pH 5 without interruption. In both panels the results presented are from a single representative experiment. Replicate experiments showed no more than a ±2% variation in the final extents of leakage.

constant lipid concentration of 100 μM. GALA-induced leakage is facilitated to the greatest extent at a pH around 5. As the pH is increased, both the rate (not shown) and the extent decrease. In order to induce the same extent of leakage at pH 6 as seen at pH 5, the peptide concentration must be increased over 10-fold. At pH 7.5, the extent of leakage never becomes greater than 30% even at a 25/1 lipid/peptide ratio.

Figure 2A (dashed line) shows a typical leakage curve obtained under these conditions (100 μM egg PC) when GALA was added to a vesicle suspension at pH 5 to give a 5000/1 lipid/peptide mole ratio. The solid line is the leakage pattern obtained when initial conditions were set for a 10000/1 lipid/peptide ratio and an equal aliquot of peptide was injected (after 10 min, see arrow) to give a final ratio of 5000/1. The two segments of the curve are superimposable, and the initial rates of leakage after each peptide addition are identical. The final extent of leakage is the same at a given ratio whether GALA is added in one or more aliquots. This shows that GALA acts reproducibly, and the solid curve suggests that GALA does not act on the entire vesicle population at this ratio.

The reversible pH dependency of GALA's activity is clearly illustrated in Figure 2B. Vesicles were placed into suspension at pH 5, and GALA was added to give a final mole ratio of 5000/1. After 0.75 min a concentrated TES solution was added to bring the pH to 7.3, and at 4.5 min an aliquot of concentrated acetate was added to return the pH to 5. Raising the pH dramatically stopped further leakage, while returning the pH to 5 caused an immediate increase in the rate of

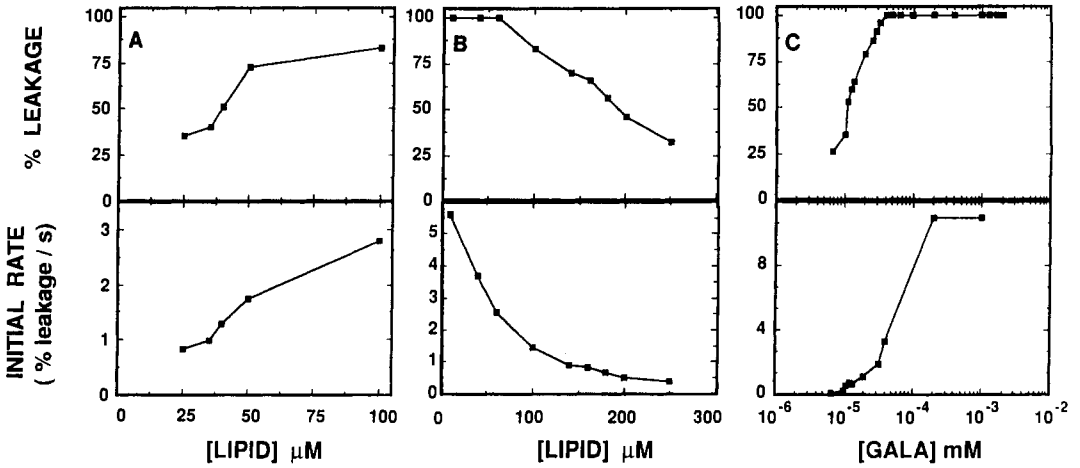


FIGURE 3: (A) Percent leakage after 10 min and initial rate as a function of lipid concentration at pH 5. The amount of lipid and GALA was varied, a constant mole ratio of 5000/1 being kept. (B) Percent leakage after 10 min and initial rate as a function of lipid concentration at pH 5 when the peptide concentration was held constant at 0.02 μM. The lipid concentration was varied to obtain the desired lipid/GALA ratio. (C) Percent leakage after 10 min and initial rate as a function of GALA concentration at pH 5. The lipid concentration was kept constant at 100 μM while the peptide concentration was varied to obtain the desired ratio. In all cases the extent of ANTS/DPX leakage was recorded after 10 min while the initial rate was calculated as the slope of a tangent line drawn to the individual leakage curves. Data points are the average of at least two to four experiments which agreed to within ±2% in the final extents of leakage and to within 10% for the initial rate values.

leakage (vs the initial rate). The dotted line shows the leakage curve obtained at pH 5 without interruption at the same lipid/peptide ratio. We believe that leakage begins as the peptide enters into the vesicles in its helical form at pH 5 and is halted at pH 7 as the peptide changes conformation and disassociates from the membrane. When the pH is returned to 5, GALA can redistribute among the vesicles and induce leakage of those previously left intact. The overshoot to 100% leakage that is observed when the pH is cycled back to 5 vs uninterrupted can be explained by such a mechanism.

Leakage has been studied as a function of a constant lipid/GALA ratio, GALA concentration (keeping lipid concentration constant), and lipid concentration (keeping GALA concentration constant) (Figure 3). At a constant lipid/GALA ratio of 5000/1 the extent of leakage at 10 min and the initial rate decrease as the lipid concentrations decrease (Figure 3A). This is expected on the basis of the partitioning of the peptide between the bilayer and the aqueous phase; as the lipid concentration is lowered the fraction of the peptide that becomes vesicle associated decreases. When the GALA concentration is held constant (Figure 3B), increasing the lipid concentration leads to a decrease in the rate and extent of leakage. Since increasing the lipid concentration increases the number of vesicles in suspension, these results are in accord with the hypothesis of a defined number of peptides required for formation of a peptide pore in the bilayer. These data also indicate that once in the bilayer the peptide does not rapidly redistribute between different vesicles. Finally, at constant lipid (Figure 3C), increasing the peptide concentration induces a greater extent and a more rapid leakage until a maximum value for both is reached. The initial leakage rate becomes very sensitive to the GALA concentration. This result is consistent with a monomer–multimer assembly process being involved in the mechanism of leakage. In all cases, the lipid/peptide ratios were determined from the amount of vesicles and GALA initially added to the cuvette. The effective ratio will depend on the lipid/water partition coefficient of GALA.

In spite of similar structural features at pH 5, the modified peptide, LAGA (see preceding paper), was unable to induce leakage of egg PC large vesicles even at lipid/peptide ratios of 50/1 (a 50-fold greater amount of peptide than needed for 100% leakage by GALA). This result illustrates the importance of the amphipathic nature of GALA in its interaction with membranes.

Table I: Release Mechanism of GALA at pH 5[a]

| lipid/peptide (mol/mol) | % fluorescence remaining with vesicles: predicted[b] all or none release | predicted[b] partial release | observed[c] |
|---|---|---|---|
| 3500/1 | 19 | 67 | 23 ± 3 |
| 5000/1 | 19 | 54 | 19 ± 2 |
| 6000/1 | 19 | 45 | 20 ± 2 |
| 8500/1 | 19 | 26 | 17 ± 1 |

[a] Details of how both the predicted and observed values were obtained are given under materials and Methods. [b] Predicted value is from a quench curve of ANTS/DPX fluorescence and has an error of ±2%. [c] Observed value is an average of at least three measurements at each lipid/peptide ratio.

*Mechanism of Leakage.* The phenomenon of incomplete leakage was intriguing; we focused on it in order to explain the mechanism of GALA-induced leakage. The following question arises: is the leakage a result of all vesicles releasing on the average a certain portion of their contents, or does a fraction of the vesicles leak completely while the remainder retain their contents? The answer to this question has broad implications with regard to how the peptide acts. For several lipid/peptide ratios, Table I gives the percent fluorescence remaining with vesicles after being incubated and subsequently separated from peptide. The second and third columns are the predicted results (see Materials and Methods) for the two types of release mechanisms while the last column contains the experimentally obtained values. From a comparison of the predicted and observed results, it is quite evident that the leakage induced by GALA occurs as an all or none event. This implies that the number of peptide molecules needed to induce complete leakage from a single vesicle must exceed a certain critical value. At the lipid/peptide ratios where partial leakage occurs, one can assume that on average there is a certain percentage of vesicles which do not have the critical number of peptide molecules associated and/or properly arranged in the bilayer to cause leakage.

*Pore Formation versus Vesicle Disruption.* As a result of leakage being an all or none event, a mechanism involving

Table II: Size Dependence of Marker Retention in Vesicles[a]

| contents entrapped | MW | % contents remaining with vesicle fraction pH 5 | pH 7.5 |
|---|---|---|---|
| ANTS | 445 | 0 | 100 |
| NADH | 660 | 26 | 95 |
| acid blue 9 | 795 | 14 | 100 |
| $AP_5A$ | 915 | 100 | 100 |
| inulin | 5200 | 87 | 100 |

[a] Vesicles were incubated for 15 min with GALA prior to separation of vesicle fractions from released material on a Sephadex G-75 coloumn. Lipid to peptide ratios were 2500/1 and below.

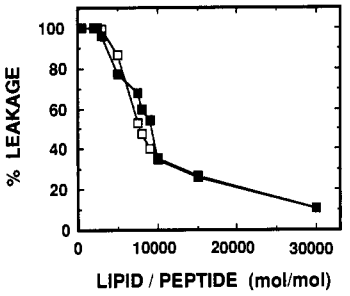


FIGURE 4: Comparison of experimental (■) and predicted (□) final extents of leakage as a function of lipid/peptide mole ratio at pH 5 when the lipid concentration is held constant at 100 $\mu$M. Below 2500/1 the experimental and calculated curves overlap at 100%. The curves converge again at ratios greater than 10000/1. The reproducibility in the experimental extents of leakage is excellent in replicates from the same experiment (±2%) and varies no more than ±5% between experiments. A value of $M = 10$ was used for predicting the final extents of leakage.

assembly of peptides into a pore in the bilayer seems most plausible although a detergent-like action of the peptide can also be envisioned as a means to disrupt vesicles. It was reasoned that if a pore formed, then there would be a size/molecular weight dependence on the leakage of initially encapsulated compounds, while size should not be a factor if peptides could act in a detergent-like fashion.

Compounds ranging in size from MW 445 to 5200 were encapsulated in vesicles and incubated with GALA at pH 5 or 7.5 prior to determination of how much material leaked out. At pH 7.5 none of the compounds leaked significantly in 15 min (Table II), while at pH 5 only leakage of molecules greater than MW 800 was reduced (Table II). The size dependence of leakage supports the idea of GALA forming a channel or pore in the vesicle bilayer.

*Results of Theoretical Model.* In developing a model to describe GALA-induced leakage of vesicle contents, the final extents of leakage at 10 min were first used to determine the critical number of peptides ($M$) needed to assemble to form a pore. The 10-min time period was selected since leakage was essentially complete by this time. When the extent of leakage was measured at 1 h, it was found to be unchanged over the value at 10 min. The value for $M$ was required to satisfy the leakage over the range of lipid to peptide ratios studied. This determination was independent of the kinetics of the leakage. In order to compute $M$, we chose an experimental estimate of the bilayer/aqueous partition coefficient of GALA of $1.0 \times 10^6$ (see preceding paper). The observed and calculated extents of leakage as a function of lipid to peptide mole ratio at a constant lipid concentration are shown in Figure 4. The open squares are the calculated extents of leakage, 10 being used as the critical peptide unit. The value of $M = 10$ provides a resonable fit to the final extent leakage data over a wide range of lipid/peptide ratios. We have carried out a statistical test for the goodness of fit using the bootstrap

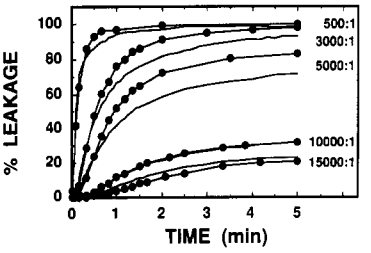


FIGURE 5: Extents of leakage as a function of time after GALA addition at pH 5. Lipid concentration was kept constant at 100 $\mu$M while the lipid/peptide ratio was varied. The solid lines are the experimental data recorded at the five lipid/peptide ratios indicated at the right of the figure. The reproducibility in the experimental extents of leakage is excellent in replicates from the same experiment (±2%) and varies no more than ±5% between experiments. The filled circles represent the best fit to the data at these ratios with $B = 0.645$ $\bar{C} = 0.002$ $s^{-1}$, and $M = 10$ as described in the text.

estimate (Efron, 1982) for the standard deviation of $M$, SD($M$), according to

$$\mathrm{SD}(M) = \sum_{j=1}^{P}[(10 - M_j)^2/P]^{1/2} \qquad (11)$$

with $P = 121$ (11 points) in which $M_j$ are other estimates chosen to yield the best fit upon omission of two points at random. The application of eq 11 yielded SD($M$) = 1.2. The calculation of $R^2$ gave 0.94. These indicies indicate a good statistical fit of the calculated to experimental values for the final extents of leakage and a small variance in the estimate of $M$.

After having fixed the critical number of peptides in a vesicle to be $M = 10$, from the results of final extents of leakage, we tested the ability of the model to explain and predict the kinetics of leakage. The leakage kinetics induced by GALA were calculated as described (theoretical analysis) and give rise to the curves represented by the closed circles in Figure 5. These are plotted with the experimental curves (solid lines) for representative lipid to peptide ratios. The calculated curves are in close agreement with the observed data especially within the first minute after peptide addition. Agreement is critical at these early time points since the onset of leakage lags behind the addition of peptide. This is clearly evident at the higher lipid to peptide ratios. It is noteworthy that a single parameter, $\bar{C}$, which is proportional to the forward rate constant of surface aggregation, can explain and predict the kinetics of leakage for the five curves in Figure 5. The value of $R^2$ for the fit of the kinetic results was 0.98, and the standard deviation of the parameter $\bar{C}$ was 0.0004 $s^{-1}$.

However, despite the fact that our choice of the parameters gave a good fit, there exists a possibility that parameter values might be somewhat biased due to an experimental error in the determination of the parameter $B$ that measures the fraction of peptide bound. Our calculations indicate that increasing $B$ by 15% would still enable one to fit the data, but with the value of $M = 12$, and vice versa, a 15% decrease would require setting $M = 8$. For larger changes in $B$ (e.g., 30%) the fit becomes poor. Hence, in view of the uncertainty in $B$ (±30%), we can set $M = 10 \pm 3$.

As an illustration of the choice of the values for the adjustable parameters in the model ($\bar{C}$, $M$) and the determination of the experimental value ($B$), we have varied each independently to define boundary conditions for the three parameters. This enables one to view the effect of each parameter on the overall fit. The 5000/1 condition was picked to illustrate this point since this is where the current model shows the greatest deviation from the experimental curve. The results of these alterations are represented in panels A–C of Figure

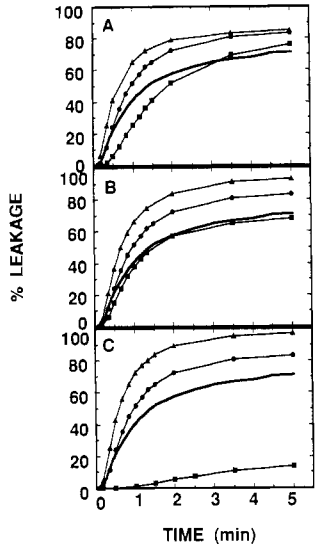


FIGURE 6: Effect of variations in $\bar{C}$, $M$, and $B$ on the predicted leakage as a function of time at a 5000/1 lipid/peptide ratio. In each panel, the experimental curve is depicted as a solid line and the current model by the filled circles ($B = 0.645$, $\bar{C} = 0.002$ s$^{-1}$, $M = 10$). (A) $M$ and $B$ are held constant while $\bar{C}$ is 0.003 (▲), 0.002 (●), and 0.001 s$^{-1}$ (■). (B) $B$ and $\bar{C}$ are held constant while M is 8 (▲), 10 (●), and 12 (■). (C) $M$ and $\bar{C}$ are held constant while $B$ is 0.95 (▲), 0.645, (●), and 0.15 (■).

6 where we have varied $B$, $M$, and $\bar{C}$, respectively. As a reference, the experimental leakage curve is plotted in each case as a solid line, and the current model ($B = 0.645$, $M = 10$, $\bar{C} = 0.002$ s$^{-1}$) is depicted by the filled circles. In viewing these curves it is necessary to keep in mind that the final choice of adjustable parameters was based on the fit to the complete data set not just one condition, e.g., the 5000/1 lipid/peptide ratio.

The lipid/peptide ratio is actually a convenient experimental way to set the vesicle to peptide ratio. This assumes a defined number of vesicles per mole of lipid. However, for unilamellar vesicles the number of vesicles per mole of lipid depends upon the vesicle diameter (Szoka & Paphadjopoulos, 1980). By changing the diameter of the vesicles, the peptide to vesicle ratio can be altered. A prediction of the model is that for a constant amount of lipid and a given lipid/peptide ratio the extent of leakage should decrease as the vesicle diameter decreases. To test this, a single vesicle preparation was fractionated into different size populations by sequential extrusion through 0.2-, 0.1-, and 0.05-μm polycarbonate membranes. The predicted trend was observed when leakage from these vesicles was measured 10 min after GALA addition. The experimental leakage values at a 5000/1 and 15000/1 lipid/GALA ratio are given in Table III along with the calculated values. Again, a two size distribution of vesicle diameters was used in the calculations to describe each sample.

Table III: Effect of Vesicle Size on Experimental and Calculated Extents of Leakage

| lipid/peptide (mol/mol) | extrusion limit[a] (μm) | % leakage exptl | % leakage calcd |
|---|---|---|---|
| 5000/1 | 0.05 | 54 | 48 |
| 5000/1 | 0.1 | 77 | 86 |
| 5000/1 | 0.2 | 100 | 100 |
| 15000/1 | 0.05 | 11 | 17 |
| 15000/1 | 0.1 | 26 | 27 |
| 15000/1 | 0.2 | 36 | 33 |

[a] For the purpose of the calculation each vesicle preparation was assumed to be composed of two sizes in a 24/1 ratio. Vesicle radii selected to represent the resulting sample populations were as follows: for vesicles extruded through 0.05 μm, 40 and 80 nm in radius; for 0.1 μm, 50 and 100 nm; for 0.2 μm, 60 and 120 nm.

## General Discussion

A detailed picture is beginning to emerge on the leakage mechanism and structure of the leaking unit formed by GALA in egg PC bilayers. The model is based upon the kinetic studies and physical measurements made on the GALA–lipid complex. Leakage of contents from phosphatidylcholine vesicles induced by GALA is a sensitive function of pH. This corresponds with the pH-dependent random coil to α-helical transition in the secondary structure of GALA observed by circular dichroism (Subbarao et al., 1987) as glutamic acid residues become protonated. We have shown here that leakage is an all or none event. This finding supports the idea that a critical number of peptides must interact with a vesicle before it can become leaky. In conjunction with this, the size dependence of molecules which can be released from the vesicles supports the idea that peptides organize to form a pore.

A major thrust of this present work has been to develop a kinetic model of vesicle leakage to incorporate these findings. The model assumes rapid association of peptides with the membrane and self-association of peptides to form a multimeric unit in the membrane. At the peptide concentrations used in the leakage studies (<1 μM), we believe aggregation of GALA in solution at pH 5.0 is minimal so that competition between membrane insertion and self-aggregation is not a significant factor. Once such a pore or channel assembles, leakage will be rapid and complete. It is possible that the channel could assemble by the stepwise addition of monomers or through a concerted association of multimeric aggregates. In either case leakage only occurs when the channel becomes large enough to permit diffusion of the entrapped solute. Therefore, the observed leakage kinetics will also depend upon the characteristics of the leaking solute.

Previous work on the kinetics of peptide association with membranes has been described for melittin (DeGrado et al., 1982; Schwarz & Beschiaschvili, 1989) and alamethicin [Schwarz et al., 1986, 1987; reviewed in Schwarz (1989)]. The kinetics of melittin-induced hemolysis of erythrocytes exhibited a transient rapid phase ($t_{1/2} < 1.2$ min) followed by a slower phase (DeGrado et al., 1982). The rapid phase was attributed to the initial rapid association of melittin with the membrane. Stopped-flow kinetic studies have shown that melittin association with PC bilayers is a fast single-step process that is not fully diffusion controlled (Schwarz & Beschiaschvili, 1989). Alamethicin also incorporates into PC bilayers in a rapid one-step mechanism ($t_{1/2} < 50$ ms; Schwarz et al., 1986, 1987). On the basis of the absence of a second relaxation step in the stopped-flow experiments at the time scales examined and a computed diffusion-controlled encounter of two incorporated peptides of less than 10 μs, the aggregation of alamethicin in the bilayer has also been postulated to be practically instantaneous (Schwarz et al., 1987; Schwarz, 1989).

However, in the case of GALA, at the given lipid concentrations used here, the observed kinetics of contents release indicate that the assembly of a leaking pore in the membrane, due to aggregation of GALA monomers, must be slower than the association of the monomers with the bilayer. By use of the experimentally determined estimate for the bilayer/aqueous partition coefficient, 10 ± 3 GALA molecules are needed to aggregate to predict the observed final extents of leakage. The calculated final extents should be similar for all solutes that can freely diffuse through the channel. On the basis of the various assumptions and measurements described above, the calculated curves for the kinetics of leakage agree quite well with the experimental observations. The most im-

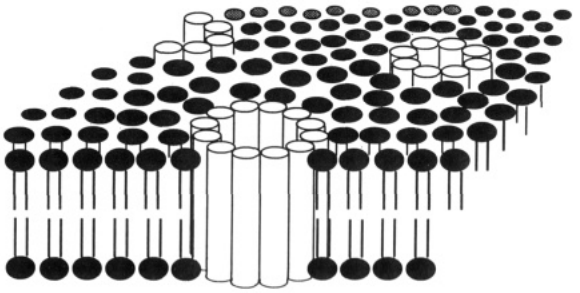

FIGURE 7: Schematic drawing of how GALA peptides would organize to form a transbilayer pore in a membrane at low pH. Individual GALA helices are depicted as cylinders.

portant source for the discrepancies between the curves probably arises from our inability to experimentally define (and thus mathematically describe) the size distribution of the vesicles. However, below 2 min, the model is able to reasonably predict the observed lag in the onset of leakage which is especially noticeable at high lipid/peptide ratios. We hypothesize that this lag time arises from the molecular assembly of monomeric peptides to form an aggregate in the plane of the membrane. The lag time is similar to what has been observed in pore formation of *Staphylococcus aureus* $\alpha$-toxin, where an initial delay is followed by a nonlinear leakage curve (Belmonte et al., 1987). The toxin binds to the membrane as a monomer which then assembles into a hexameric channel (Reichwein et al., 1987; Harshman et al., 1989). This hypothesis concerning the lag time observed with GALA is being tested by examining the fast kinetics of GALA–bilayer interactions and leakage; the results will be used to refine our model (Parente et al., 1990a).

The structural aspects of the model are quite similar to those of the model for the alamethicin pore originally put forth by Fox and Richards (1982). We propose that when the pH is reduced from 7.5 to 5, GALA rapidly partitions into the membrane and then assembles into an oligomeric complex containing between 8 and 12 GALA monomers. In the complex, individual GALA molecules have a predominantly $\alpha$-helical secondary structure and the helix axis is aligned perpendicular to the bilayer surface (Figure 7). When in a helix, a 30 amino acid peptide would have an end to end length of 45 Å sufficient to span an egg PC bilayer with a phosphate to phosphate distance of 40 Å (White & King, 1985).

The notion that GALA is predominantly $\alpha$-helical in the membrane is indirectly supported from X-ray diffraction studies with other synthetic peptides such as $Lys_2$-Gly-$Leu_{24}$-$Lys_2$-Ala-amide (Huschilt et al., 1989) in oriented multilayers. This peptide forms $\alpha$-helices, which orient with the helical axis perpendicular to the lipid bilayer. The proposed orientation and extent of helical structure of GALA in the bilayer receive direct support from polarized FTIR measurements on GALA in oriented multilayers (Goormaghtigh et al., personal communication).

Theoretical studies have shown that $\alpha$-helices composed of Ala or $Ala_3$-Leu repeat units can pack into transmembrane bundles with an antiparallel orientation and to a lesser extent with a parallel orientation through nonbonding interactions (Furois-Corbin & Pullman, 1987). In these studies five helix bundles could be packed in various stable configurations. In the case of alamethicin, the voltage dependence of the channel favors the parallel orientation of adjacent monomers (Fox & Richards, 1982; Hall et al., 1984; Cascio & Wallace, 1988; Schwarz, 1989). These model cases along with other suggestions concerning the assembly of helices to form channels (Fox & Richards, 1982; Lear et al., 1988; Spach et al., 1989) can by analogy lead to a proposed structure of GALA in the membrane. The quenching of the tryptophan fluorescence when GALA incorporates into bilayers at low pH suggests that tryptophan residues from adjacent monomers can interact. This would lend support to a parallel orientation of the monomers when they assemble into a pore.

Complexes containing 8 or 12 GALA subunits were symmetrically arranged to form a circular pore by interactive computer graphics. The starting point for these studies was to place GALA in an ideal $\alpha$-helix. Pairs of GALA units were positioned in a parallel orientation (for reasons given above) to minimize contacts between adjacent monomers. Pairs were then transposed in a symmetrical arrangement such that glutamic acid residues were oriented to line the water-filled channel while the leucine face was oriented to interact with the hydrocarbon chains of the bilayer. As observed for the model of the alamethicin channel (Fox & Richards, 1982) and the model channel-forming peptides of Lear and colleagues (1988), there is little steric overlap of the solvent-accessible surfaces (Connolly, 1983) between helices.

The presence of the glutamic acid residues on the hydrophilic face of GALA necessitates that the channel core increase in diameter, compared to the $Ala_3$-Leu helices (Furois-Corbin & Pullman, 1987), to accommodate differences in steric and charge characteristics of the partially protonated glutamate residues. To estimate the pore diameters of GALA assemblies, we calculated the minimum distance between the van der Waals surfaces of apposing glutamate residues lining the pore. An eight-helix bundle of GALA molecules would have a radius of about 8 Å and would permit the diffusion of molecules with a cross-sectional area of 200 $Å^2$. If we assume that the interior of the channel is lined by a single-shell hydration layer, then the channel radius becomes 5 Å and the cross-sectional area would be about 80 $Å^2$. A channel composed of 12-GALA helices arranged in a similar fashion and lined by a single hydration layer would have a radius of 10 Å and a cross-sectional area of 310 $Å^2$. The size dependence of leakage induced by GALA is consistent with a channel diameter between 5 and 10 Å since NADH (MW 660) has a Stokes radius of about 6 Å (Peters, 1986) and is permeant while fluorescein dextran (MW 4100) and inulin (MW 5200) have Stokes radii of 12 and 13 Å, respectively (Peters, 1986) and are not permeant. The molecular weight cutoff for release of solutes and the computed channel diameter are similar to those described for the staphyloccal $\alpha$-toxin (Krasilnikov et al., 1988). The similarities of the channel properties of the membrane lytic protein staphyloccal $\alpha$-toxin to those formed by GALA reinforce the concept that simple peptide sequences can mimic the actions of more complicated biological proteins (DeGrado et al., 1989).

The detailed kinetic analysis of solute leakage induced by GALA at low pH has permitted the construction of a model of the organization of GALA in the bilayer. Important aspects of this analysis are the use of a fluorescently quenched solute which allows rapid analysis of the leakage and a mathematical model to describe the results. The mathematical model may be of general use in studies of the mechanism of leakage observed in other peptides or proteins that form multimeric bilayer channels or pores.

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**Registry No.** GALA, 107658-43-5.

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