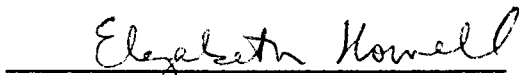


To the Graduate Council:

I am submitting herewith a thesis written by Angela K. Payongayong entitled "A Study of the Interaction of Bee Venom Melittin with Model Biological Membranes Using Both Total and Polarized Fluorescence Measurements." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Physics.


Solon Georghiou, Ph.D., Major Professor

We have read this thesis and
recommend its acceptance:


Elizabeth Howell, Ph.D.


Engin Serpersu, Ph.D.

Accepted for the Council:


Associate Vice Chancellor and
Dean of the Graduate School

**A Study of the Interaction of Bee Venom Melittin
with Model Biological Membranes
Using Both Total and Polarized Fluorescence Measurements**

**A Thesis
Presented for the Degree of
Master of Science
The University of Tennessee, Knoxville**

**Angela K. Payongayong
December 1995**

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ABSTRACT

Polarized fluorescence intensity measurements consisting of vertical and horizontal components were taken using a stopped-flow fluorometer in order to study the kinetics of melittin binding to dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles and dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles over a range of temperatures. The data were analyzed within the framework of a sophisticated model proposed by Bradrick (1994). The results for DMPC small unilamellar vesicles showed that the rates of melittin-vesicle association and dissociation increased with increasing temperature, as expected. The number of lipid molecules per binding site did not show any noticeable trend although at the transition temperature of 21 °C, it was lower. An Arrhenius plot gave an activation energy greater than that required for melting the acyl chains, showing that more than the melting of the acyl chains is required for binding to take place. Most probably, some contribution goes into an ordering/disordering of the membrane so as to accommodate the melittin molecule. To determine whether there may exist more than one species of bound melittin, the effective quantum yield and the anisotropy of bound melittin were taken as indicators. A constant effective quantum yield for bound melittin at different temperatures was obtained which by itself may not be conclusive since it is possible for say, two different modes of binding to take place and yet result in effective quantum yields that may not be distinguishable. The anisotropy, a sensitive indicator of rotational dynamics, was then used to further explore the question of whether there may be more than one species of

bound melittin. The resulting anisotropies for bound melittin did not show any variation at the different temperatures used in the study, indicating that for these temperatures binding took place in one class of binding site.

For DPPC small unilamellar vesicles, the results of the analysis are less conclusive due to the fact that the change in the fluorescence intensity for some concentrations was too small and could not be read by the numerical technique employed in the analysis. These concentrations were, thus, excluded from the analysis. The results, which may not be the unique solutions being sought in the global analysis, seem to indicate a tighter binding which, if true, agree with observations by Bradrick et al. (1995).

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INTRODUCTION

A REVIEW OF LITERATURE

The melittin utilized in the study is a major protein component of bee venom with a molecular weight of 2800. It has 26 amino acid residues where residues 1 to 20 are known to be predominantly hydrophobic and residues 21 to 26, being predominantly hydrophilic. (The amino acid composition of melittin is shown in Figure 1.) It is known to contain one tryptophan residue at position 19 that is fluorescent. As such, the tryptophan in melittin is being employed conveniently in the present study as an intrinsic fluorescent probe in order to provide some information concerning the nature of the interaction when the protein binds itself to a lipid membrane.

The conformation of melittin as a whole has been an area of interest for workers in this field. For low concentrations in a low-ionic strength buffer that has been used in this study, free melittin exists as a monomer and is said to exhibit a random-coil structure in solution (Lauterwein et al., 1980). It exists as a tetramer when in solution at high concentration or when in the presence of high salt concentration (Faucon et al., 1979).

In the state bound to a membrane, it has been proposed (Terwilliger et al., 1982) that it exists as a monomer and consists of two helical components that make a bent with respect to each other at an angle of about 120° near the residue at position 14 (proline). There is one positive charge near the hydrophobic N-terminal sequence and four positive charges in the

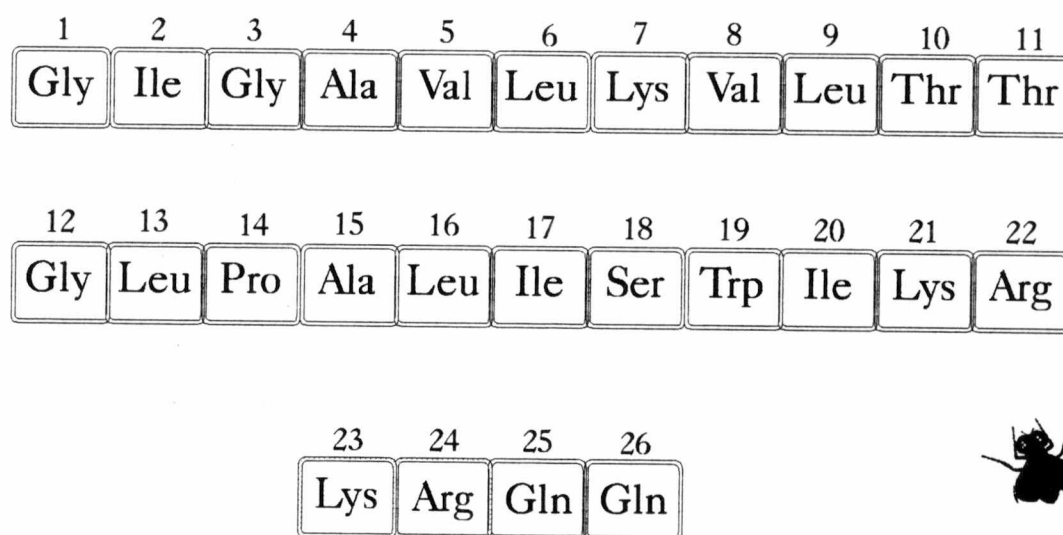


Figure 1. Amino acid sequence of melittin. (Dempsey, 1990)

hydrophilic C-terminal tetrapeptide sequence Lys-Arg-Lys-Arg. (This is shown in Figure 2A.) Different models have been proposed to describe the manner in which melittin attaches to the lipid membrane (survey by Dempsey, 1990). One, the wedge model, indicates that the two positively charged termini are anchored to the membrane surface with the two embedded helical components forming a wedge (Dawson et al., 1978; Vogel et al., 1983). (This is shown in Figure 2B.) This is conceivable when one considers that the positively charged termini of melittin, through electrostatic attraction, might prefer to lodge themselves near the negative region of the polar headgroups (arranged on the surface) of the zwitterionic lipid membranes. Another plausible model describes a partially embedded α -helix (Terwilliger et al., 1982) with the axis lying parallel to the bilayer surface. (This is shown in Figure 2C.) This is quite reasonable since one side of the helix of melittin is hydrophobic and would tend to exclude itself from the aqueous environment and position itself into the inner region of the bilayer. Figure 3 shows a melittin monomer partially inserted in a bilayer (Terwilliger et al., 1982).

Numerous articles have reported the effects that melittin exerts on the membrane to which it binds. Earlier studies indicated that the protein lyses natural and artificial membranes (Weissmann et al, 1969; Sessa et al., 1969; Olson et al., 1974) and fuses lipid vesicles (Eytan and Almany, 1983; Morgan et al., 1983; Bradrick and Georgiou, 1987). Its presence causes a decrease in the fluidity of DMPC (dimyristoylphosphatidylcholine) small unilamellar

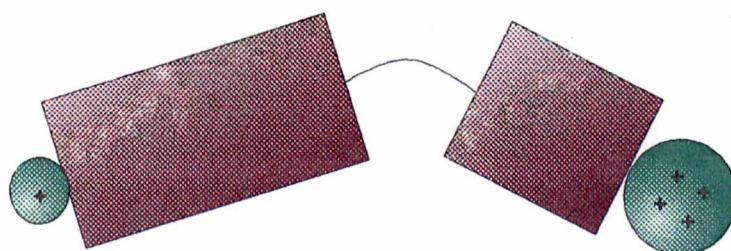


Figure 2A. Membrane-bound melittin monomer is shown as consisting of two helical segments represented by two rectangles here. Helix 1 extends from residues 1 to 11 and helix 2 extends from residues 14 to 20. A bent of about 120 degrees occurs near the proline residue at position 14. (Terwilliger et al., 1982)

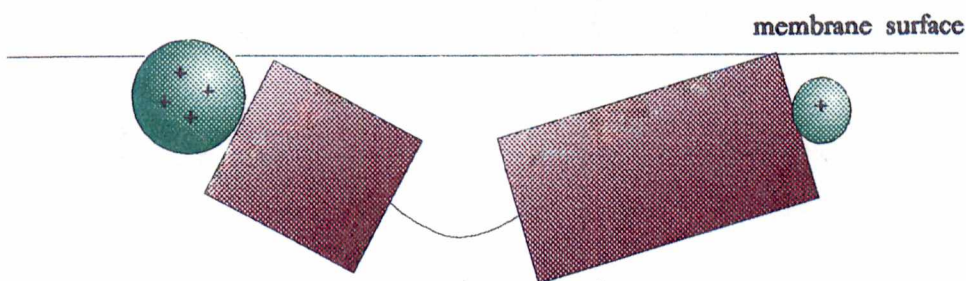


Figure 2B. The wedge model. (Dawson, et al., 1978; Vogel et al., 1983)

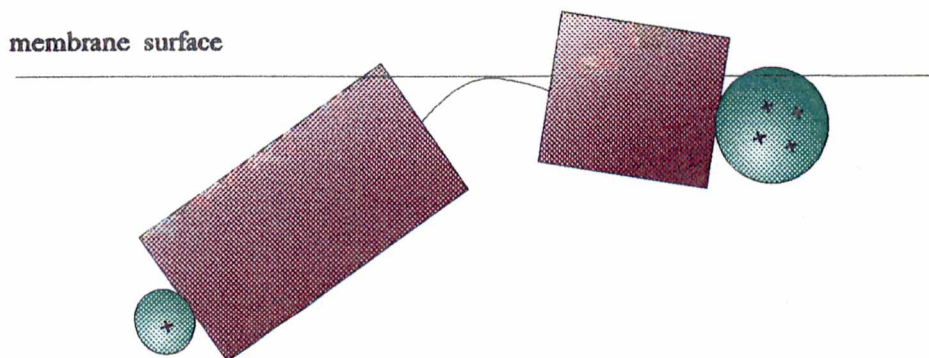


Figure 2C. The partially-inserted alpha-helix. (Terwilliger et al., 1982)

Figure 2. Structure of bound melittin.

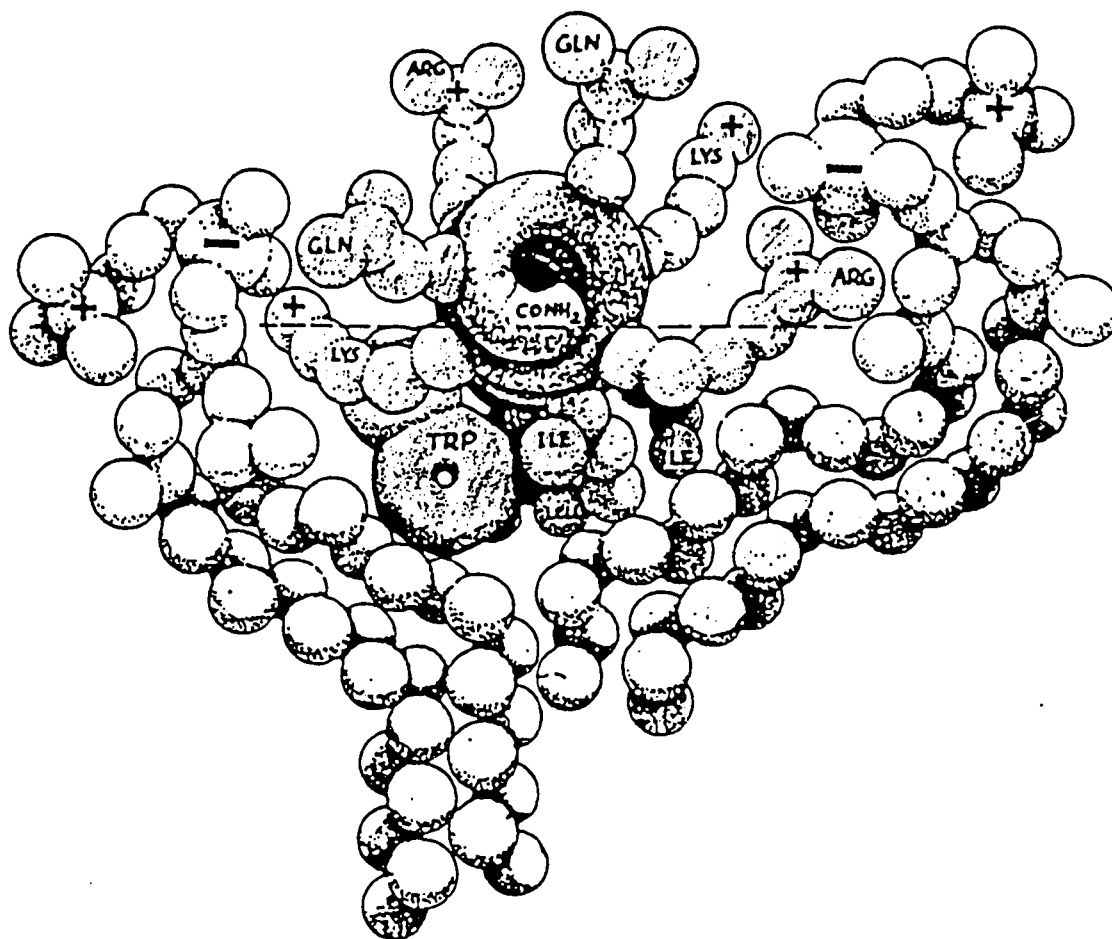


Figure 3. A melittin monomer in a bilayer. (From Terwilliger et al., 1982)

vesicles that is brought about by a greater ordering of the membrane's acyl chains (Bradrick et al., 1995). This study reported that the perturbation is maximal at the phase transition temperature of the bilayer. In the dissertation of Bradrick (1994), the disturbance has been viewed as a compressional wave which travels outward from the point of protein insertion with a speed of about 1500 m s^{-1} . Given that, and the molecular dimensions involved, the disturbance so induced is reported to be felt almost instantaneously and is long-range. The effect of the length of the lipid acyl chains on the disturbance was also reported (Bradrick et al., 1995). Concerning the latter, the hypothesis (Mouritsen and Bloom, 1984; Sperotto and Mouritsen, 1991) that invokes a length mismatch between the hydrophobic part of the inserted protein and the acyl chains of the lipid vesicles is fascinating. There, it is proposed that a greater mismatch would tend to disturb the bilayer more as the mismatched acyl chains attempt to accommodate the foreign body that just went in. This hypothesis was applied to the melittin-vesicle interaction and experimentally confirmed for the first time by Bradrick et al. (1995) where melittin binding to DMPC small unilamellar vesicles (with acyl chains 14 carbons long) and to DPPC small unilamellar vesicles (with acyl chains 16 carbons long) were investigated and compared. It was concluded in that study that melittin disturbed the DMPC bilayer more than the DPPC bilayer owing to a greater mismatch between the shorter acyl chains of DMPC and the inserted protein.

In the dissertation of Bradrick (1995), a kinetic model had been employed that systematically followed the interaction at a molecular level and on a time scale (in the

millisecond range) before fusion of the lipid vesicles took place. That study made use of nonlinear least squares fitting of measured total fluorescence data. Although the fitting was good and the model appeared to yield reasonable results for the rates of melittin-membrane association and dissociation, the author suggested that perhaps the model could be improved by extending it to include simultaneous applications to both total as well as polarized fluorescence intensity measurements. A specific question addressed in that study was whether more than one class of binding site were available for the incoming melittin; e. g., binding to both the gel phase domains and the liquid crystalline domains. The effective quantum yields of bound melittin (a resulting parameter in the study) at different temperatures were compared as a way of checking for the presence of more than one class of binding sites. The values of this parameter in that study at different temperatures, however, did not significantly change to allow detection of different sites. In the case of binding to two different sites, it is conceivable that the bound melittin molecules will exhibit different mobilities. Measuring polarized fluorescence data provides, in addition to the effective quantum yield, the anisotropy of the bound melittin. This parameter measures the rotational dynamics of the molecule being investigated and may be a more sensitive indicator of the presence of different bound melittin species that possess different rotational mobilities. The present study is made in response to the suggestion of the author and seeks to determine whether, in fact, a more complicated kinetic scheme needs to be employed in describing the melittin-membrane interaction than was previously used.

DEFINITION OF THE PROBLEM

When melittin interacts with a lipid membrane, it could result in a two-state melittin after the interaction: one that is free and the other, bound. It could possibly produce more than two species. For instance, there may result a free melittin, a surface-bound melittin and a partially-inserted melittin. These are a total of three species. There may be other binding schemes such as binding to the gel phase domains and to the liquid crystalline domains of the membrane. Just which of these different possibilities occurs when melittin interacts with lipid membranes is yet to be established. The study by Bradrick et al. (1995) appears to support a two-state process. However, the authors cautioned that total fluorescence measurements alone might not be sufficient to establish whether the process is a two-state or not, and that both total fluorescence and polarized fluorescence data are needed to resolve the problem at hand. Another paper (Otto et al., 1994) showed that the unfolding of yeast phosphoglycerate kinase (PGK) in guanidine hydrochloride (GuHCL) would appear as a two-state reaction instead of the actual three-state when either total fluorescence data or polarized fluorescence data are analyzed in isolation. These authors made the same suggestion that one would need to take both total fluorescence and polarized fluorescence measurements to be able to make a better resolution.

The problem in this study is to make use of both total fluorescence data and polarized fluorescence data to establish the kinetics of the interaction when melittin binds to lipid membranes.

MATERIALS AND METHODS

The bee venom melittin utilized in the study was supplied by SIGMA Chemical Co. (St.Louis, MO). The lipids used were the model membranes dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) supplied by Avanti Polar Lipids (Alabaster, AL). The buffer solution used in the experiments was 50 mM Tris, 1 mM EDTA prepared in triply distilled water with pH set at 7.6. EDTA (ethylenediamine tetraacetic acid-disodium salt) was added to the buffer solution to inactivate the enzyme phospholipase A₂ that may still be present in the melittin sample.

Melittin stock solutions were prepared by hydrating the appropriate amount of melittin in 10 ml buffer. This was done in dim red light to avoid photobleaching the melittin fluorophore. Melittin stock solutions used in the experiments were not kept beyond five days after they were prepared.

The stock solution of either DMPC or DPPC for each experiment was prepared by hydrating the appropriate amount of DMPC or DPPC in 5 ml buffer at a temperature of either 33 °C or 51 °C, respectively. These temperatures are about 10 °C above either DMPC's phase transition temperature of 21 °C or 37 °C for that of DPPC. At such temperatures, the respective vesicles are known to be very stable (Shullery et al., 1980). Throughout the sample preparation and just prior to loading into the stopped-flow machine, the lipid was kept at either 33 °C or 51 °C. The lipid sample was then sonicated using a Laboratory Supplies Co. (Hicksville, NY) bath sonicator for 20 minutes, then kept at rest for 5 minutes after which

it was again sonicated for another 20 minutes. Next, it was left alone to anneal for 30 minutes. At the end of this period, the sample was centrifuged at 30000 rpm for 30 minutes. The supernatant was removed and placed in a screwcap polystyrene tube. This constituted the sample solution of either DMPC or DPPC small unilamellar vesicles (SUV's) ready for use. The desired concentration was obtained by appropriately diluting an aliquot of the sample stock solution. Only freshly prepared sample was used. Any leftover or unused DMPC or DPPC stock solution after each experiment was discarded.

The stopped-flow experimental set-up consisted of a modified Aminco DW-2 UV/Vis spectrophotometer. (This is shown in Figure 4.) This had been modified to enable observations of fluorescence to be made in a direction perpendicular to the path of the excitation light beam. The excitation light came from a Spectral Energy (Westwood, NJ) 600 W Xe-Hg arc lamp. For excitation, a 297-nm interference filter was used. The fluorescence emitted by the sample was viewed by an EMI 6256S photomultiplier through a Corning 0-51 glass cut-off filter and the corresponding output was digitized by an Analog Devices (Norwood, MA) RTI-815 A/D converter before being sent to an IBM PC computer for initial processing. The temperature of both the sample cell and the sample reservoirs was controlled by means of a circulating water bath. Each of the two DaLite polarizers (Cincinnati, OH) used, one on the excitation side and the other on the emission side, was equipped with a lever switch that allows a selection of either the vertical or horizontal orientation. In the experiments, with the excitation light polarized vertically, both the vertical and horizontal components of the emitted fluorescence were measured while melittin binding

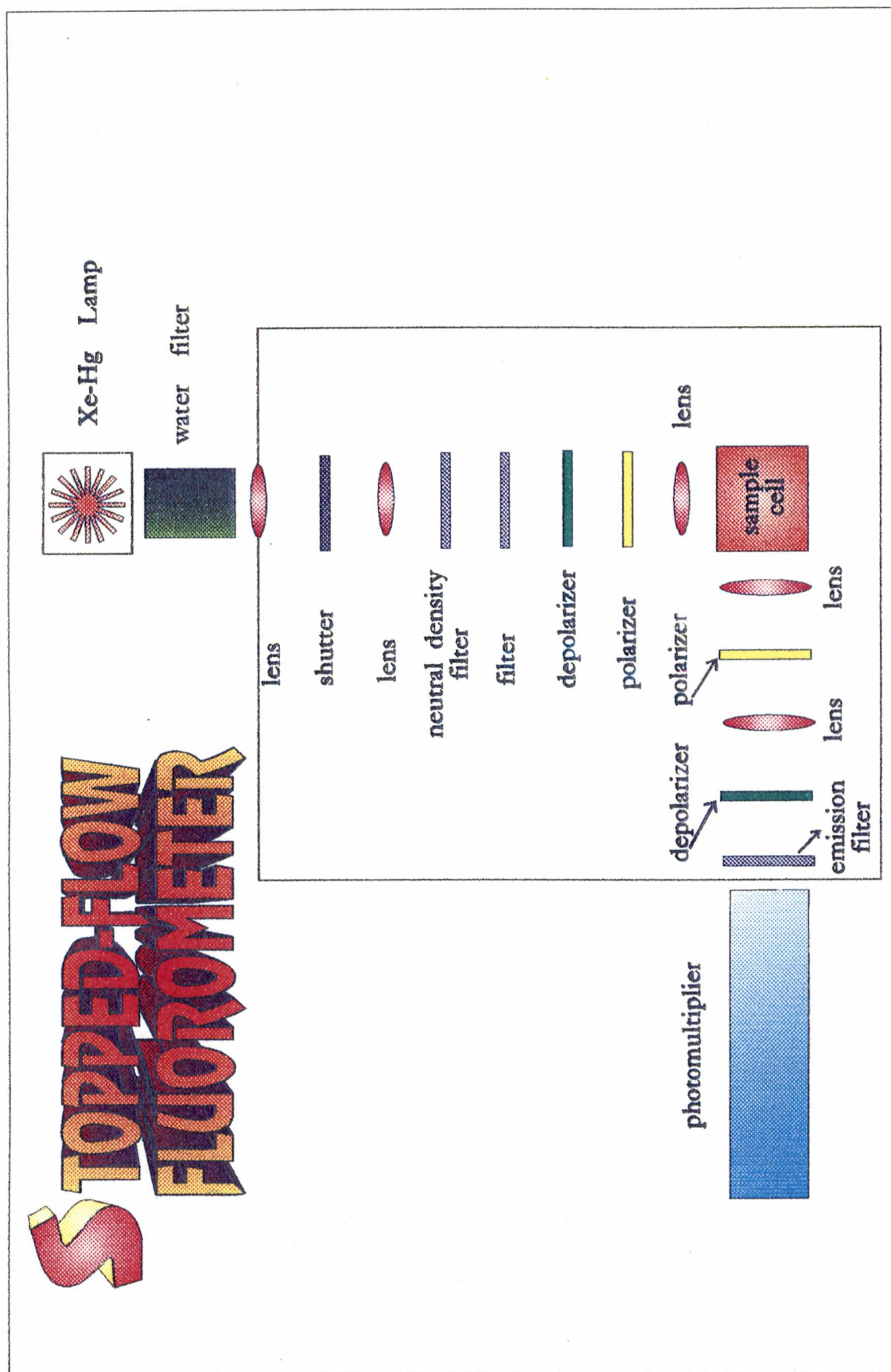


Figure 4. Schematic diagram of stopped-flow fluorometer.

to the lipid took place.

Measurements were made as follows. Melittin and DMPC or DPPC sample solutions of the desired concentration were loaded into the machine in their respective reservoirs, then allowed to come to thermal equilibrium at the set temperature for 10 minutes. Three shots were initially made with the last shot left to stay in the sample cell for five minutes so as to bring the photomultiplier response to steady state level. With the excitation light vertically polarized, the vertical component of the fluorescence was measured in six shots, followed by six subsequent shots for the horizontal component. The data files for all twelve shots were saved by the computer for later analysis. The same procedure was followed in taking baseline measurements of DMPC or DPPC vs. buffer and buffer vs. buffer. The lamp intensity for free melittin was measured in the same manner using melittin vs. buffer.

The interaction of melittin with either lipid (DMPC or DPPC) was modeled as a bimolecular process as originally done by Bradrick (1994). The work was extended here to include two sets of polarized fluorescence data together with their corresponding equations. This also meant that instead of fitting one set of functions, a simultaneous fitting of two functions had to be done subject to a greater constraint that the two sets of data share the same set of parameters. The model being adopted here could be described by



P represents a free protein molecule; nL represents a binding site consisting of m molecules of the lipid (where $m = n^{-1}$); P·nL represents the bound protein-lipid assembly. k_f is the forward rate constant whereas k_r is the reverse rate constant. Stoichiometric calculations give the concentrations of [P] and [P·nL] as functions of time as

$$[P]_0 = [P](t) + [P \cdot nL](t) \quad (2)$$

$$[nL]_0 = [nL](t) + [P \cdot nL](t) \quad (3)$$

The subscript 0 refers to the initial value of the concentration. Model fitting of the experimental data, as will be shown in what follows, requires that both the concentrations [P] and [P·nL] as functions of time (while melittin binds itself to DMPC or DPPC) be known. Since these concentrations are not independent of each other, one may determine the concentration of [P·nL] only, for example, then use equation (2) to find the corresponding concentration of [P] as a function of time. The derivation of the closed-form expression for [P·nL](t) may be found in the dissertation by Bradrick (1994) and has the result

$$[P \cdot nL](t) = \frac{b - k_{eff}}{2k_f} \cdot \frac{1 - \exp(-k_{eff}t)}{1 - \left[\frac{b - k_{eff}}{b + k_{eff}} \right] \exp(-k_{eff}t)} \quad (4)$$

where

$$a = -k_r$$

$$b = k_r + k_r([nL]_0 + [P]_0)$$

$$c = -k_r[P]_0[nL]_0$$

$$k_{eff}^2 = b^2 - 4ac$$

The equations that were used to determine the effective quantum yield and the anisotropy are presented in the following. The effective quantum yield (γ_{eq} 's) may be determined using the total fluorescence function given by

$$F(t) = I\gamma_f\epsilon_fq_f[P](t) + I\gamma_b\epsilon_bq_b[Pn\cdot L](t) \quad (5)$$

The subscript f refers to the free melittin species after binding, while b refers to the bound melittin. γ is the emission filter transmittance, ϵ is the molar extinction coefficient at the wavelength of excitation, while q is the fluorescence quantum yield. $[P](t)$, as mentioned earlier, refers to the free melittin concentration as a function of time. I refers to the relative intensity of the excitation light beam. It may be mentioned at this point that the total fluorescence $F(t)$ does not have to be measured separately in this study since it may be obtained using the vertical and horizontal components of polarized fluorescence data by the relation,

$$F(t) = (I_v + 2I_h) \quad (6)$$

Equations (5) and (6) are matrix equations and can be combined and recast to show as such by

$$\begin{bmatrix} (I_V + 2I_H) \\ \cdot \\ \cdot \\ \cdot \end{bmatrix} = \begin{bmatrix} I[P](t) & I[P \cdot nL](t) \\ \cdot & \cdot \\ \cdot & \cdot \\ \cdot & \cdot \end{bmatrix} \begin{bmatrix} \gamma_f \epsilon_f q_f \\ \gamma_b \epsilon_b q_b \end{bmatrix} \quad (7)$$

Or, in simple matrix notation

$$\mathbf{F} = \mathbf{CA} \quad (8)$$

where $\mathbf{F}$ is the 1-column total fluorescence data vector, $\mathbf{C}$ is the two-column concentration matrix and $\mathbf{A}$ is the two-dimensional vector for the $\gamma \epsilon q$'s. Since we are looking for the $\gamma \epsilon q$'s, we have

$$\mathbf{C}^T \mathbf{F} = \mathbf{C}^T \mathbf{CA}$$

$$\mathbf{A} = [\mathbf{C}^T \mathbf{C}]^{-1} \mathbf{C}^T \mathbf{F} \quad (9)$$

-1 denotes the matrix inverse and T indicates the transpose.

Thus, if we know the concentration matrix C and, of course, we have the total fluorescence data F , then we can determine $\gamma_f \epsilon_f q_f$ and $\gamma_b \epsilon_b q_b$ contained in the vector A . The computer does this by doing the required numerical matrix inversion as specified by equation (9).

The anisotropies r 's may be determined from the standard anisotropy function

$$r = \frac{I_V - I_H}{I_V + 2I_H} \quad (10)$$

Rewriting this equation gives

$$I_V - I_H = (I_V + 2I_H) r \quad (11)$$

Again $(I_V - I_H)$, $(I_V + 2I_H)$ and r may be represented as matrices, and using the form for $(I_V + 2I_H)$ in equation (7), one gets

$$\begin{bmatrix} (I_V - I_H) \\ \cdot \\ \cdot \\ \cdot \end{bmatrix} = \begin{bmatrix} I\gamma_f \epsilon_f q_f [P](t) & I\gamma_b \epsilon_b q_b [P \cdot nL](t) \\ \cdot & \cdot \\ \cdot & \cdot \\ \cdot & \cdot \end{bmatrix} \begin{bmatrix} r_f \\ r_b \end{bmatrix} \quad (12)$$

In symbolic matrix notation, this may be written as

$$\mathbf{D} = \mathbf{G}\mathbf{R} \quad (13)$$

$\mathbf{D}$ stands for the $(I_v - I_h)$ vector, $\mathbf{G}$ is a two-column matrix containing the products of $\gamma\epsilon q$'s and concentrations as indicated by equation (12), and $\mathbf{R}$ is the two-dimensional vector of anisotropies. Since $\mathbf{R}$ is the unknown, rewriting equation (13) gives

$$\mathbf{R} = [\mathbf{G}^T\mathbf{G}]^{-1}\mathbf{G}^T\mathbf{D} \quad (14)$$

Again, the computer software used in this study has been designed to do the required numerical matrix inversion to calculate r_v and r_h contained in the vector $\mathbf{R}$.

Data fitting was done simultaneously on both the vertical and the horizontal components of the fluorescence intensity data taken while melittin was binding to DMPC or DPPC. The fitting functions are presented in what follows. Combining equations (5) and (6) leads to

$$(I_v + 2I_h) = I\gamma_v\epsilon_v q_v [P](t) + I\gamma_h\epsilon_h q_h [P \cdot nL](t) \quad (15)$$

Equation (12) may be written as a function, as

$$(I_v - I_h) = I\gamma_v\epsilon_v q_v r_v [P](t) + I\gamma_h\epsilon_h q_h r_h [P \cdot nL](t) \quad (16)$$

Equations (15) and (16) may be solved for $I_v(t)$ and $I_H(t)$ to obtain

$$I_v(t) = I\gamma_f\epsilon_f q_f [P](t) \left(\frac{1+2r_f}{3} \right) + I\gamma_b\epsilon_b q_b [P \cdot nL](t) \left(\frac{1+2r_b}{3} \right) \quad (17)$$

$$I_H(t) = I\gamma_f\epsilon_f q_f [P](t) \left(\frac{1-r_f}{3} \right) + I\gamma_b\epsilon_b q_b [P \cdot nL](t) \left(\frac{1-r_b}{3} \right) \quad (18)$$

To fit these functions, one has to determine the concentrations $[P](t)$ and $[P \cdot nL](t)$. The latter two, in turn, require the values k_p , k_r and n . These three constants make up the parameter space being searched by the nonlinear least squares fitting software used in the study. (For this study, a substantial amount of time was spent in modifying an existing computer software in order to accommodate the vertical and horizontal fluorescence data files and the relevant equations, and do a simultaneous data fitting on both files.)

As mentioned earlier, twelve stopped-flow shots were taken each time the two sample reservoirs were filled (six shots for the vertical component and six shots for the horizontal component of the fluorescence intensity while melittin was binding to the membrane). These are a total of six vertical-horizontal pairs. Before a global analysis was done, the six files for each of the horizontal and vertical components were initially fitted simultaneously by an approximate function of the form

$$F(t) = A + B \cdot \exp(-kt) \quad (19)$$

The four best fitting files, as judged by the randomness of the residuals, were selected for inclusion in the global analysis of the different concentrations. Each set of data submitted for a global analysis contained sixteen vertical-horizontal data file pairs with different DMPC(or DPPC)-to-melittin molar ratios of 180, 120, 90, and 60. The first pair of data files read into the computer was assigned a relative intensity of one. The other pairs of data files had their lamp intensities scaled to that of the first pair by the relation

$$I_j = I_1 \cdot \frac{c_f \text{ signal}_j}{c_f \text{ signal}_1} \quad (20)$$

The subscript 1 refers to the first pair of files, j refers to the file being evaluated, and f stands for the free melittin species. I is for the relative lamp intensity, c denotes the molar concentration of the free melittin sample, and signal refers to the vertically polarized fluorescence intensity of melittin vs. buffer when excited by the vertically polarized excitation light.

The parameter $\gamma_f \epsilon_f c_f$ for free melittin may be obtained by direct measurement using the relation

$$\text{signal}_1 = I_1 \gamma_f \epsilon_f c_f \quad (21)$$

In equation (21), signal_1 is given by $(I_v + 2I_H)$ of the melittin vs. buffer measurements, less the buffer background for the first pair of data files.

The anisotropy for free melittin may also be directly measured using the relation,

$$\text{signal}_1 = I_1 \gamma_f \epsilon_A c_f r_f . \quad (22)$$

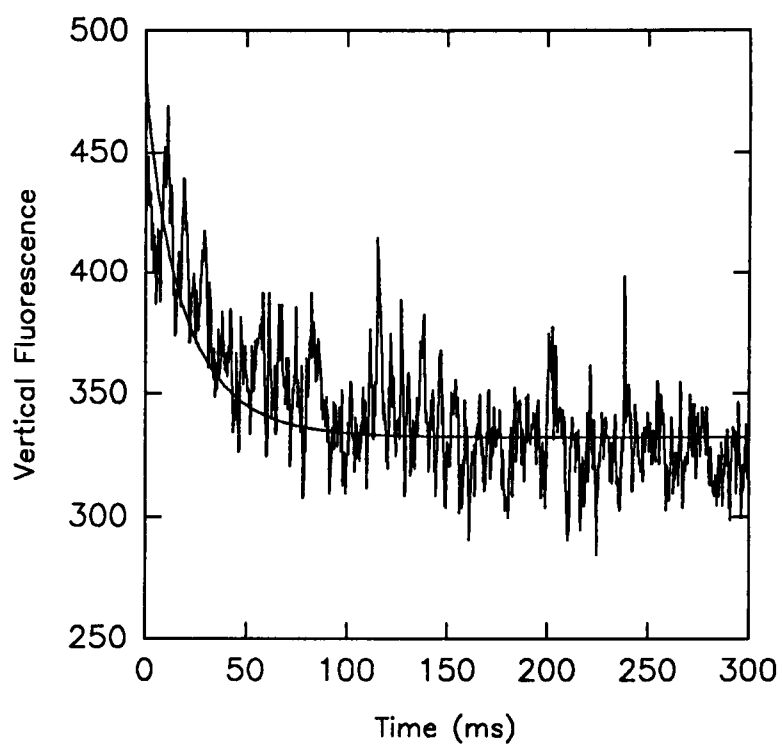
Here, signal_1 is obtained by $(I_v - I_H)$ of the melittin vs. buffer measurements, less the buffer background for the first pair of data files. These two measured values of the effective quantum yield and the anisotropy of free melittin could then be compared with the corresponding values obtained from the model, i.e., using equations (9) and (14), and the extent of the agreement would be one criterion in judging the appropriateness of the model given by equation (1) in describing the interaction. (This is true, in general, except in a special case where the value of the quantity, say r_f , is too small.)

RESULTS

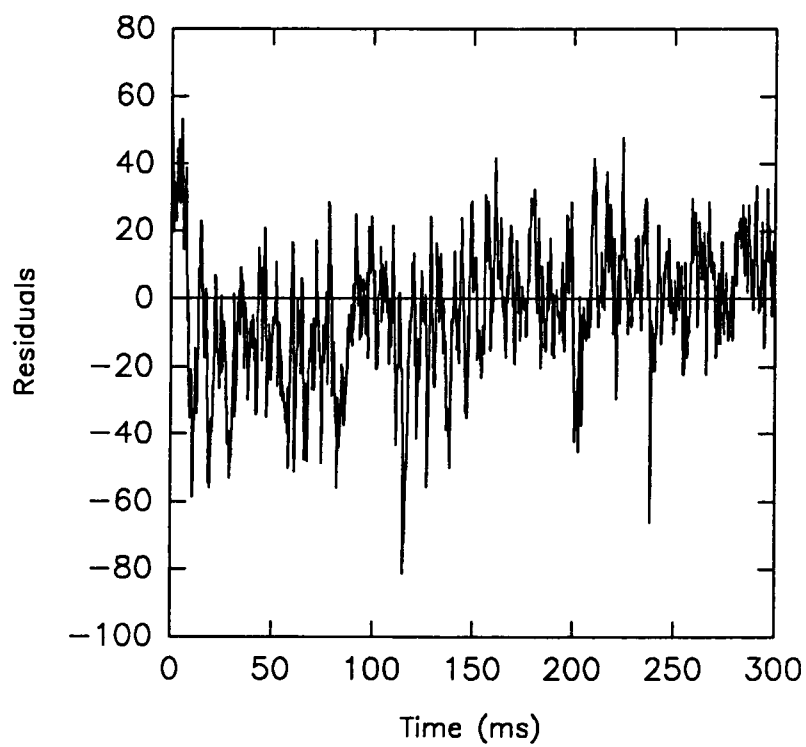
Typical results of the curve fitting of the measured data taken while melittin binds to DMPC are shown in Figures 5A through 5H for a temperature of 27 °C. Equations (17) and (18) were used in simultaneously fitting these data. For Figures 5A through 5D, the upper plots show the experimental data as well as the smooth-curve fitting function of equation (17) for the vertical component of the fluorescence intensity for lipid-to-melittin molar ratios of 180, 120, 90, and 60, respectively. The lower plots give the corresponding residuals from these fits to the data. The randomness of the residuals signifies a good fit. Figures 5E through 5H give the corresponding plots for the horizontal component. The concentrations used were as follows: For a molar ratio of 180, [DMPC]=18.0 mM, [melittin]=0.1 mM. For a molar ratio of 120, [DMPC]=12.0 mM, [melittin]=0.1mM. For a molar ratio of 90, [DMPC]=9.0 mM, [melittin]=0.1 mM. Finally, for a molar ratio of 60, [DMPC]=6.0 mM, [melittin]=0.1 mM. It may be noted that the polarized fluorescence intensities were quite low (about 1/3 the output of total fluorescence intensity) so the concentration of melittin had to be maximized so as to be able to obtain readings that are not masked by the baseline caused by scattered light. 0.1 mM is the highest concentration that melittin may have without tetramerizing in solution (Faucon et al., 1979).

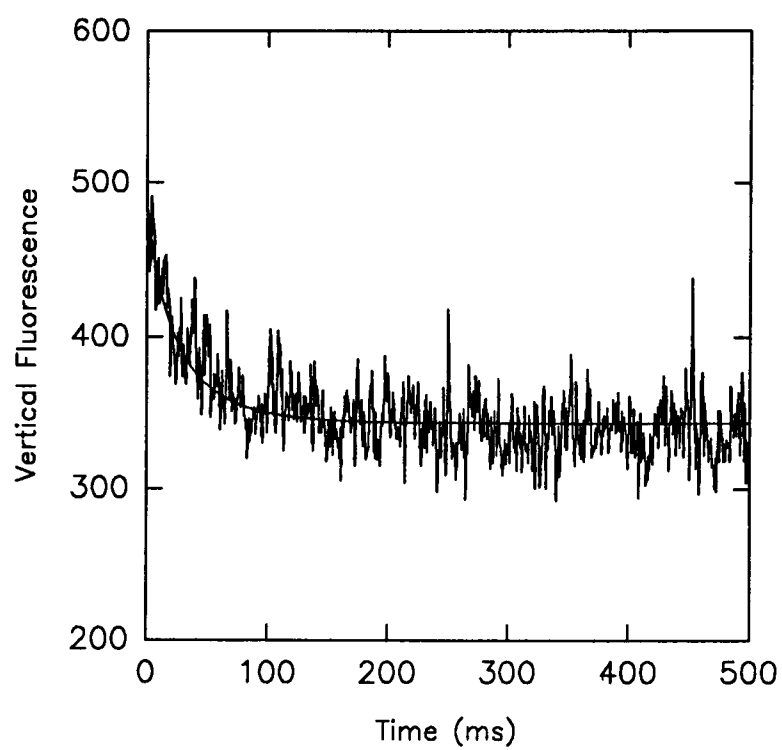
Figures
5A-5H.

Results of the global analysis for the binding of melittin to DMPC small unilamellar vesicles at 27 °C. Figures 5A through 5D are for the vertical component of the fluorescence intensity. Figure 5A is for a lipid concentration of 18 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 180. Figure 5B is for a lipid concentration of 12 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 120. Figure 5C is for a lipid concentration of 9 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 90. Figure 5D is for a lipid concentration of 6 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 60. Figures 5E through 5H are for the horizontal component of the fluorescence intensity. Figure 5E is for a lipid concentration of 18 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 180. Figure 5F is for a lipid concentration of 12 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 120. Figure 5G is for a lipid concentration of 9 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 90. Figure 5H is for a lipid concentration of 6 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 60. In these figures, the upper plot shows the experimental data and the fit obtained in the global analysis. The lower plot shows the residuals from the fit to the data.

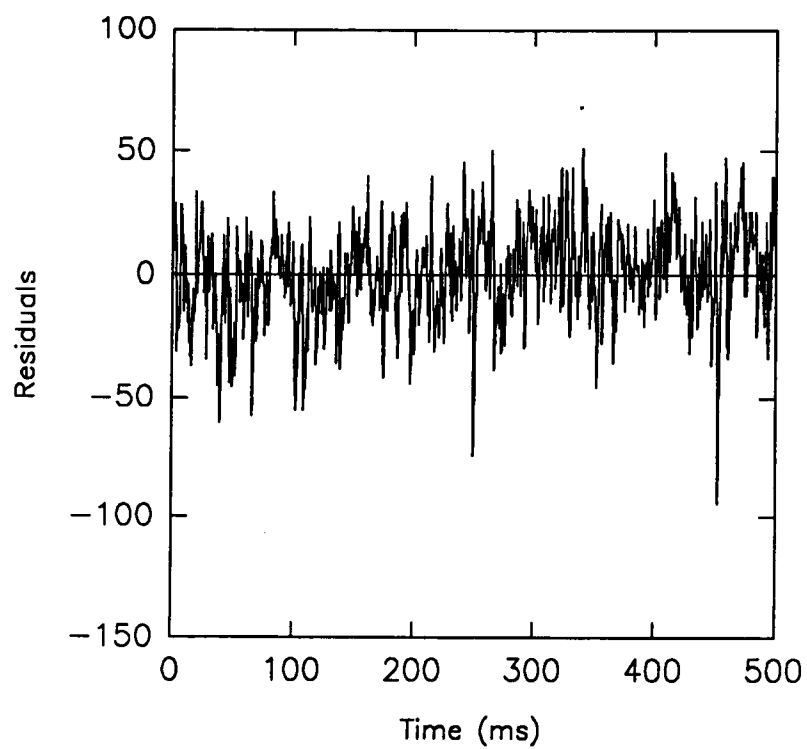


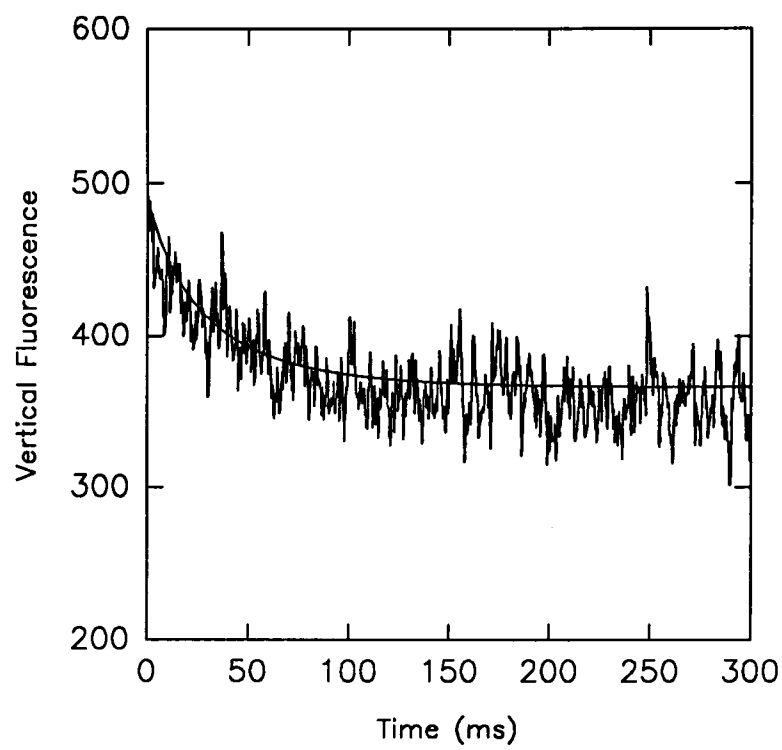
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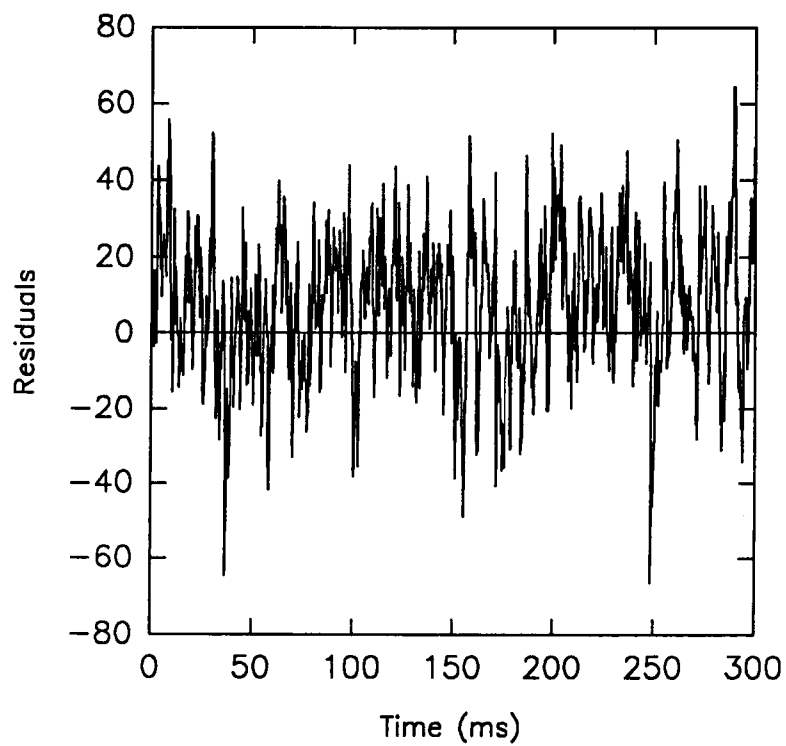


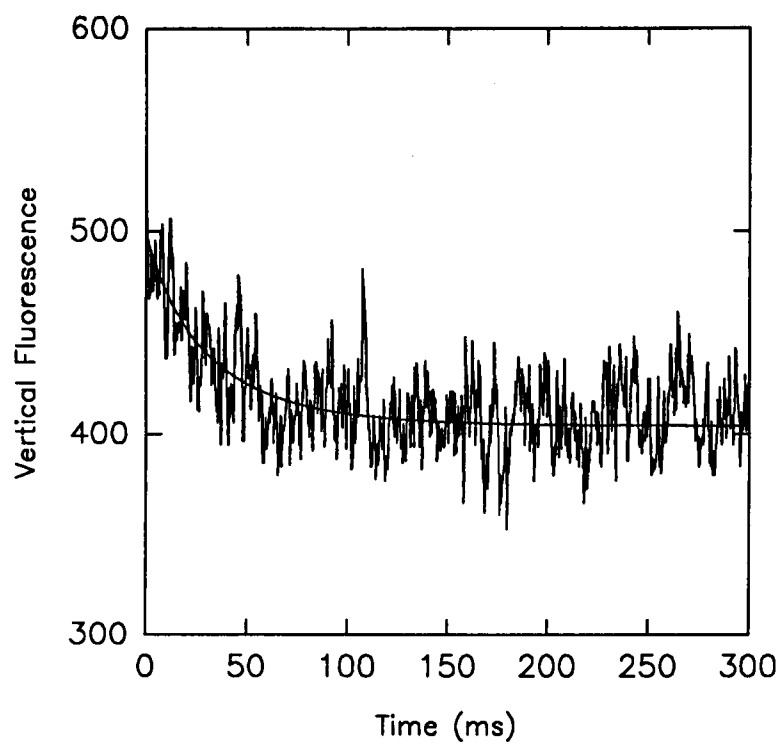
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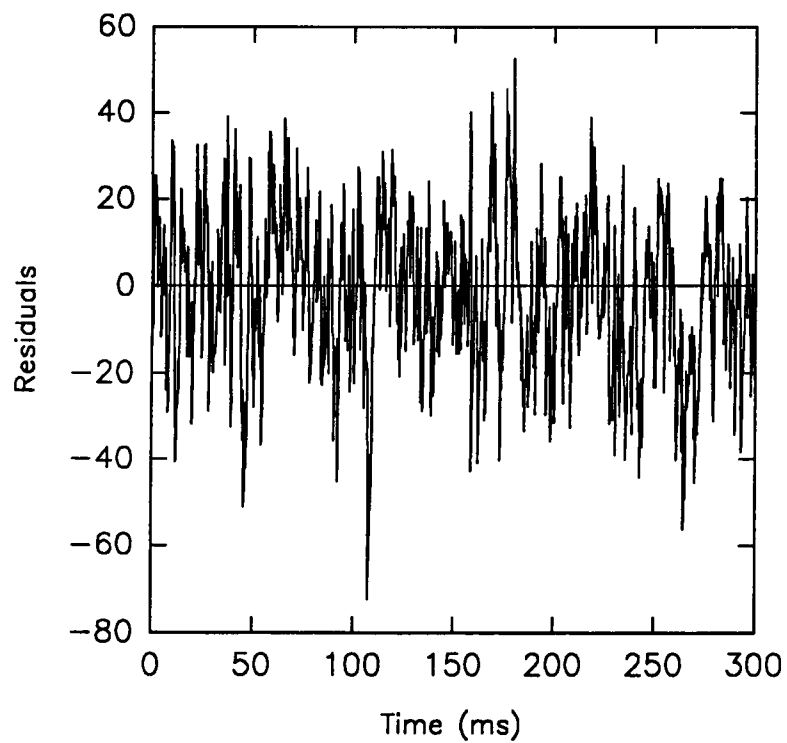


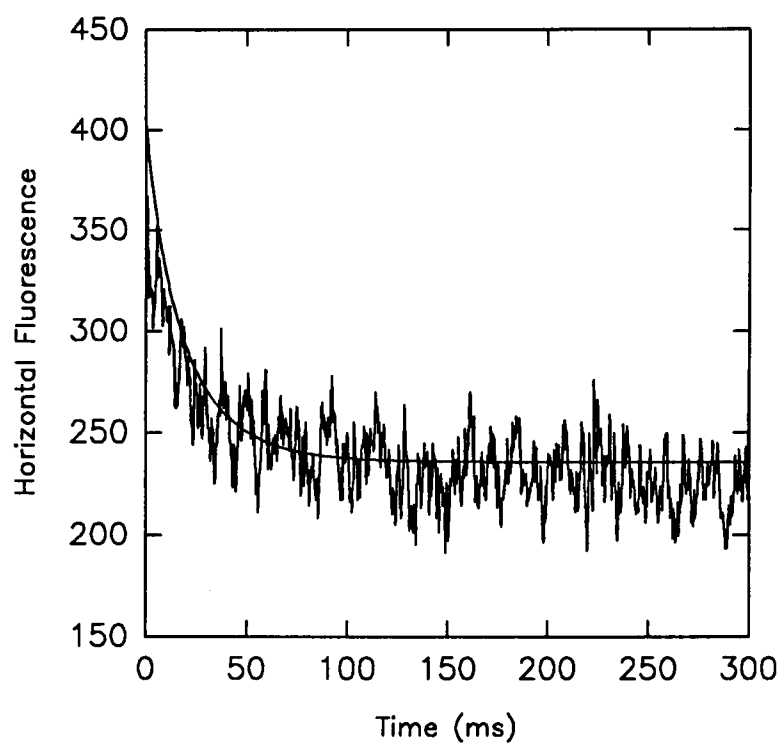
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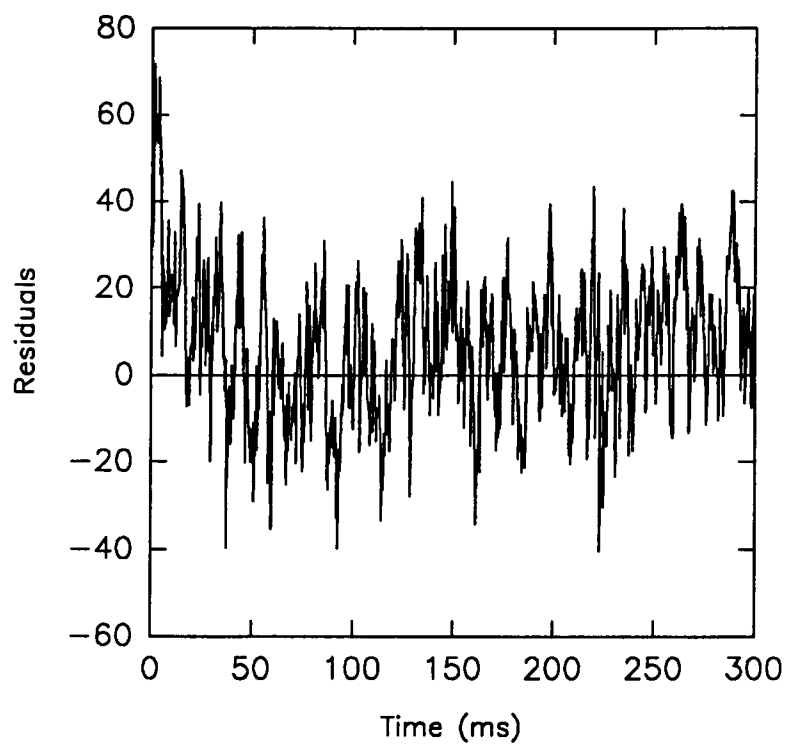


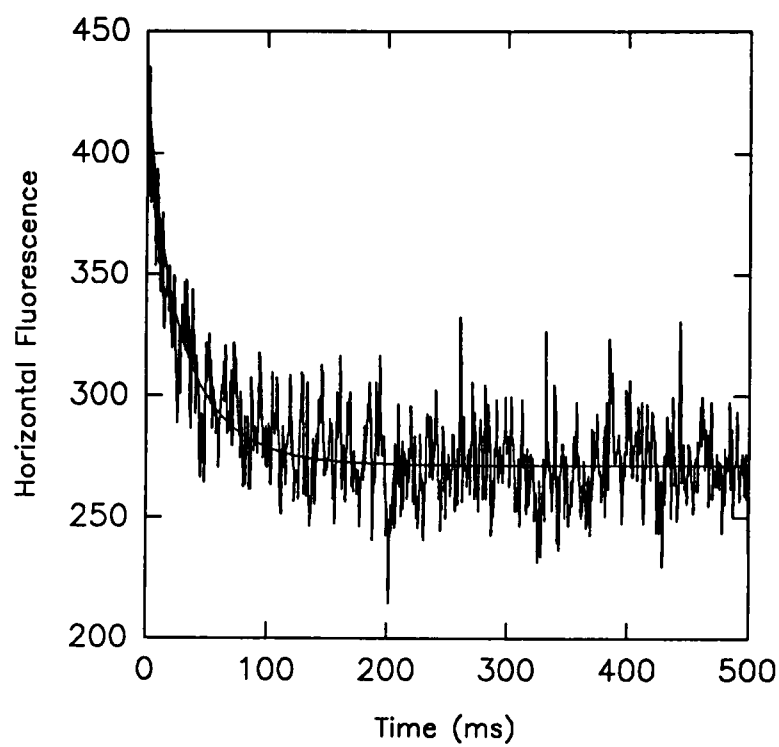
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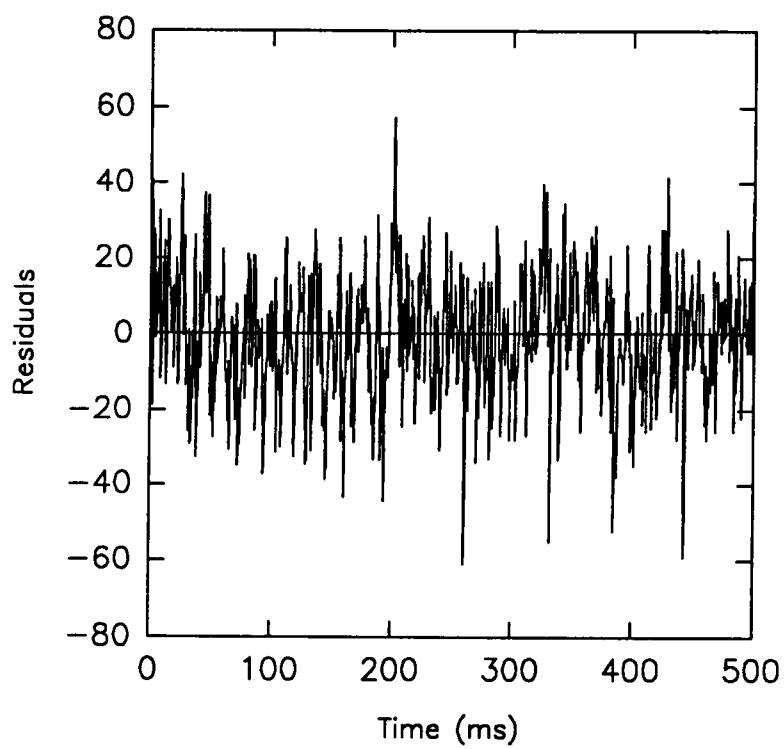


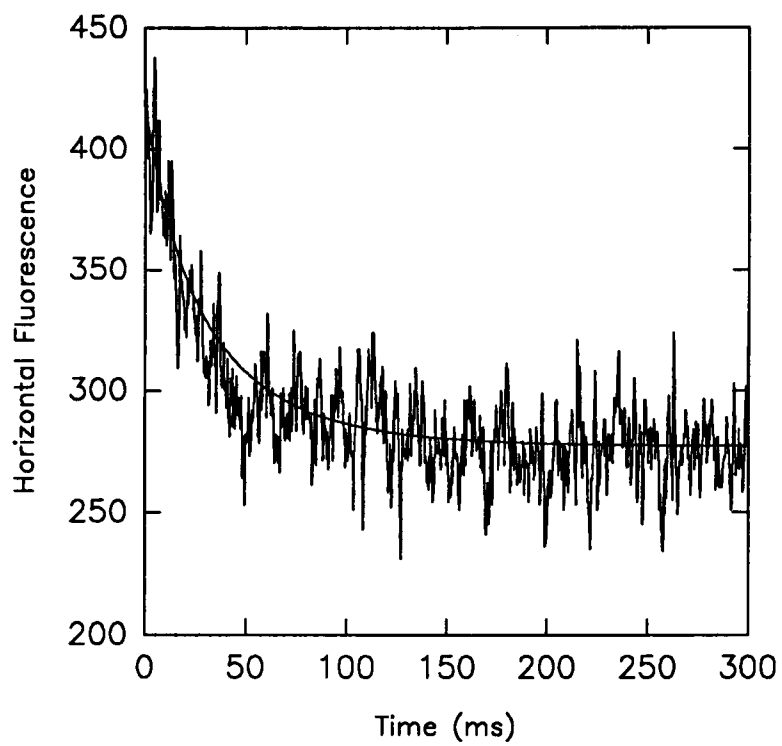
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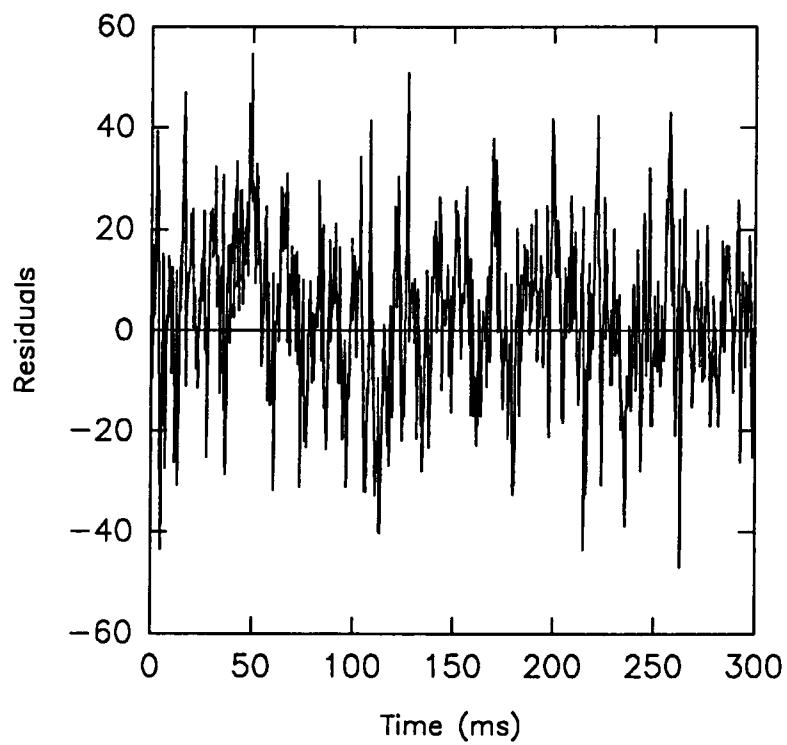


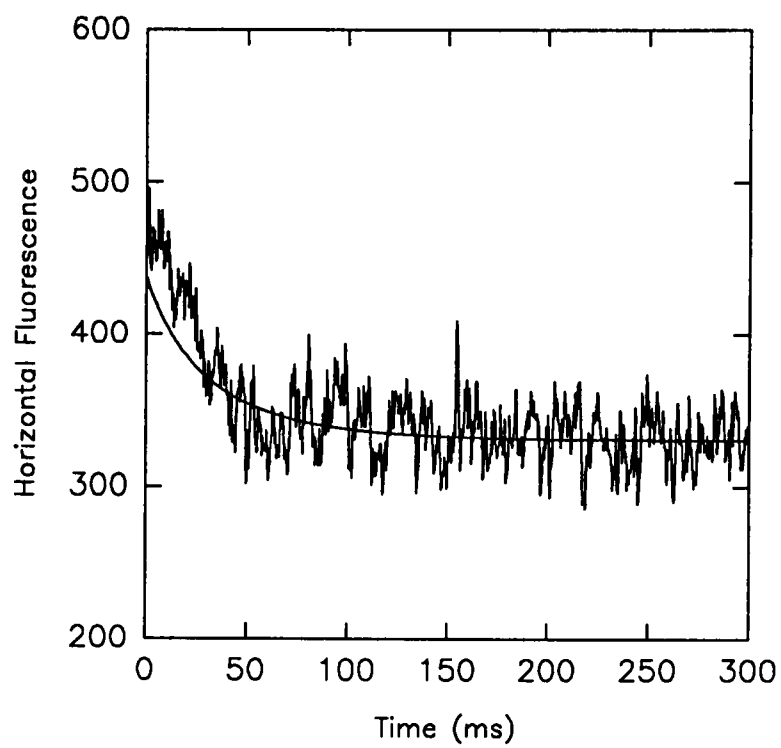
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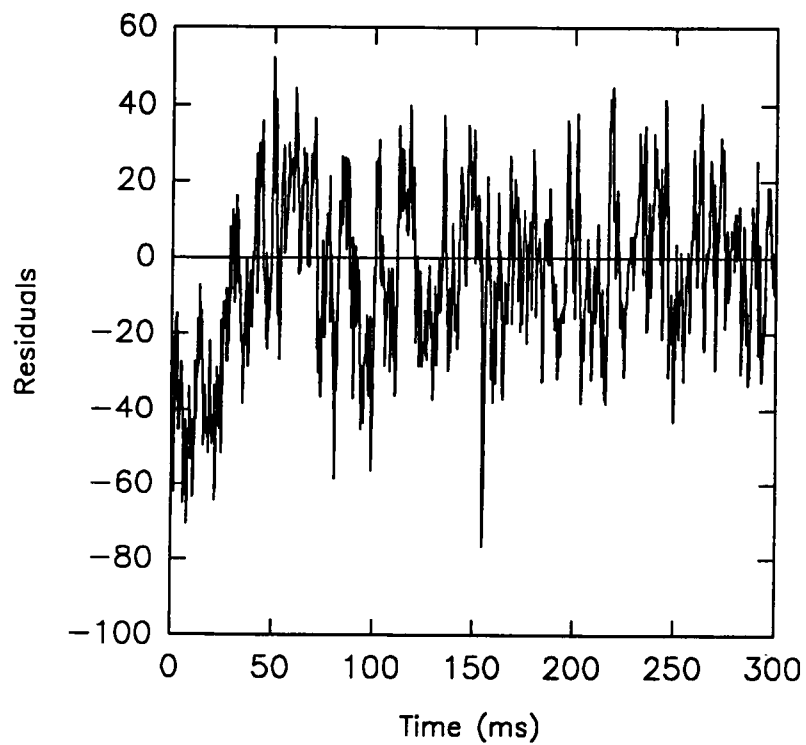


5G





5H



The values of the parameters k_f , k_r and the number of lipids per binding site m ($=1/n$) resulting from the global analysis for the different temperatures used are given in Table 1. These values show that both k_f and k_r decrease with decreasing temperature, as might be expected. For a temperature of 30 °C, however, k_r appears to be rather high. The number of lipids per binding assembly does not appear to exhibit any trend although it may be noted at this point that the value of 53 at the phase transition temperature of 21 °C is relatively low. Except for what have just been noted, the trend in the rest of the values is apparently what would have been expected.

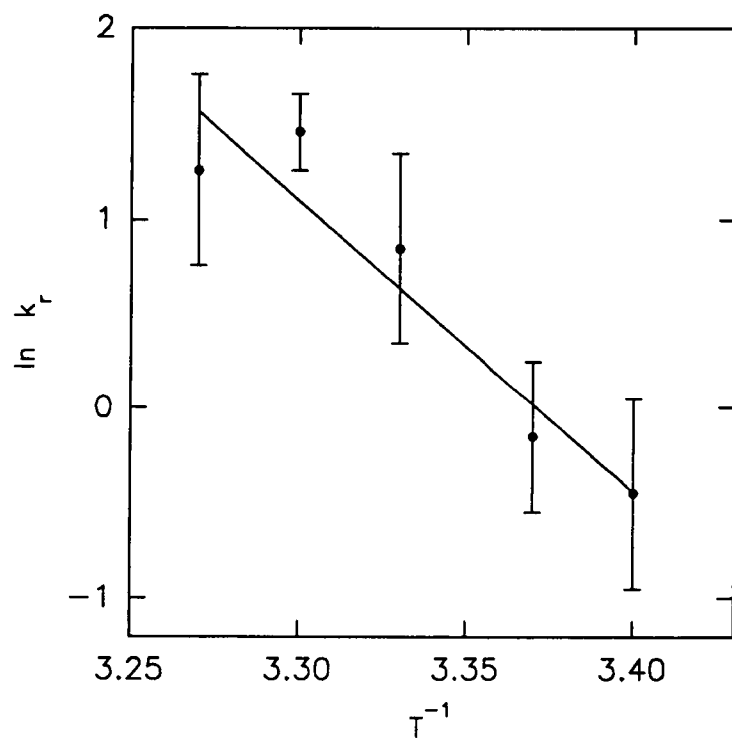
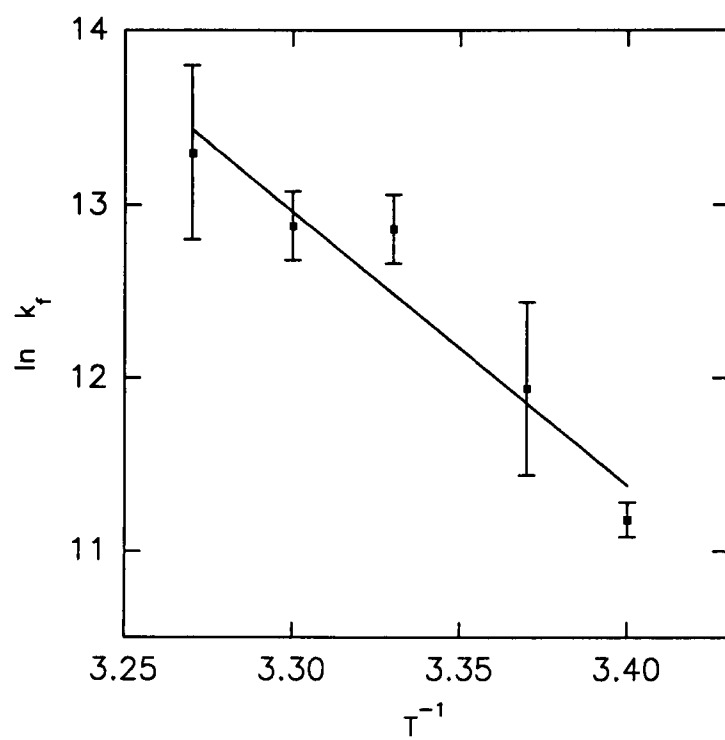
Arrhenius plots are given in Figure 6 using the resulting k_f and k_r values from the global analysis. The upper figure shows the plot of $\ln k_f$ vs. T^{-1} (inverse of the absolute temperature). For this figure, the smooth line is the linear regression line which gives a slope of -15.8×10^3 . This slope yields an activation energy of 31.3 kcal mol⁻¹. The lower figure gives a similar plot using k_r . There, the slope is -15.4×10^3 with an activation energy of 30.6 kcal mol⁻¹.

The resulting γ_{eq} 's from the global analysis for different temperatures are tabulated in Table 2. The second column gives the values of the effective quantum yield for free melittin in solution as determined by equation (21). The third and fourth columns give the effective quantum yield for free and bound melittin, respectively, using equation (9) in the global analysis. Lastly, to enable comparison, the effective quantum yield for bound melittin has been rescaled to that at 33 °C in column five. It is seen that the effective quantum yield

Temperature °C	k_f ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_r (s^{-1})	No. of Lipids per Binding Site
33	6.0 ± 2.8	3.5 ± 1.9	80 ± 6
30	4.0 ± 1.0	4.2 ± 1.0	95 ± 5
27	3.9 ± 1.0	2.3 ± 1.2	86 ± 11
24	1.5 ± 0.7	0.9 ± 0.4	97 ± 7
21	0.7 ± 0.1	0.6 ± 0.3	53 ± 8

TABLE 1. Forward and reverse rate constants, and the number of lipid molecules per binding site as determined by the model applied to the polarized fluorescence data taken while melittin binds to DMPC small unilamellar vesicles .

Figure 6. Arrhenius plots of $\ln k_f$ versus T^{-1} (K^{-1}) (upper figure) and $\ln k_r$ versus T^{-1} (K^{-1}) (lower figure) using the data in Table 1. The smooth lines are the linear least-squares fits to the data. These lines give activation energies of $31.3 \text{ kcal mol}^{-1}$ and $30.6 \text{ kcal mol}^{-1}$ for the forward and reverse processes, respectively.



Temperature °C	$\gamma_f \epsilon_f q_f^{\dagger}$ ($\times 10^4$)	$\gamma_f \epsilon_f q_f^{\ddagger}$ ($\times 10^4$)	$\gamma_b \epsilon_b q_b^{\ddagger}$ ($\times 10^4$)	$\gamma_b \epsilon_b q_b^{\S}$ ($\times 10^4$)
33	1.60 ± 0.25	1.53 ± 0.27	0.88 ± 0.20	0.88 ± 0.20
30	1.64 ± 0.11	1.49 ± 0.20	0.81 ± 0.12	0.79 ± 0.11
27	1.61 ± 0.10	1.46 ± 0.16	0.80 ± 0.12	0.80 ± 0.12
24	1.72 ± 0.13	1.40 ± 0.17	0.80 ± 0.10	0.74 ± 0.12
21	1.64 ± 0.20	1.52 ± 0.14	0.90 ± 0.10	0.88 ± 0.13

TABLE 2. The measured fluorescence coefficients (or effective quantum yields) for the DMPC-melittin interaction.

[†] Fluorescence coefficient for free melittin in solution as determined by equation (21).

[‡] Fluorescence coefficient for free melittin in solution as determined by equation (9).

[‡] Fluorescence coefficient for bound melittin as determined by equation (9).

[§] Fluorescence coefficient for bound melittin as determined by equation (9) for exciting light intensity normalized to that used at 33 °C.

at different temperatures appears to be constant. This is rather surprising as one might expect a decrease of the quantum yield as the temperature is raised. (At higher temperatures, the probability for radiationless transitions to take place becomes higher. Since radiationless transitions compete with fluorescence emission, the quantum yield decreases.) This discrepancy may be attributed to the fact that the temperature interval involved is small.

Column 2 of Table 3 shows the resulting anisotropies for bound melittin as obtained from the global analysis using equation (14). Column 3 gives the anisotropies for free melittin as obtained from direct measurements using equation (22). The anisotropy is an added parameter provided by the polarized fluorescence data. At a given temperature, it serves to indicate how the hydrodynamic properties of the surrounding environment change when melittin makes its way into the inner region of the bilayer. The values of the anisotropy r_b for bound melittin show virtually no temperature dependence. It may be added at this point that equation (14) as used in the global analysis also determines the anisotropy of free melittin (and that for bound melittin) that may then be compared with the anisotropy of free melittin (column 3) taken from direct measurement. This should have been one criterion in determining the appropriateness of the model given by equation (1). It turned out that since the values involved were so small, the computer program had difficulty extracting these values. The values of the former were almost always about four times the values obtained from direct measurement. In fitting the functions given by equations (17) and (18), the

Temperature °C	$r_b^{\S}$	$r_f^{\dagger}$
33	0.09 ± 0.003	0.009 ± 0.001
30	0.12 ± 0.003	0.013 ± 0.003
27	0.11 ± 0.008	0.012 ± 0.001
24	0.11 ± 0.002	0.013 ± 0.003
21	0.12 ± 0.002	0.012 ± 0.001

TABLE 3. Measured fluorescence anisotropies for the DMPC-melittin interaction.

§ Fluorescence anisotropy for bound melittin as determined by equation (14).

† Fluorescence anisotropy for free melittin as determined by equation (22).

anisotropy of free melittin as obtained from direct measurement was used and was one of the input parameters in the global analysis.

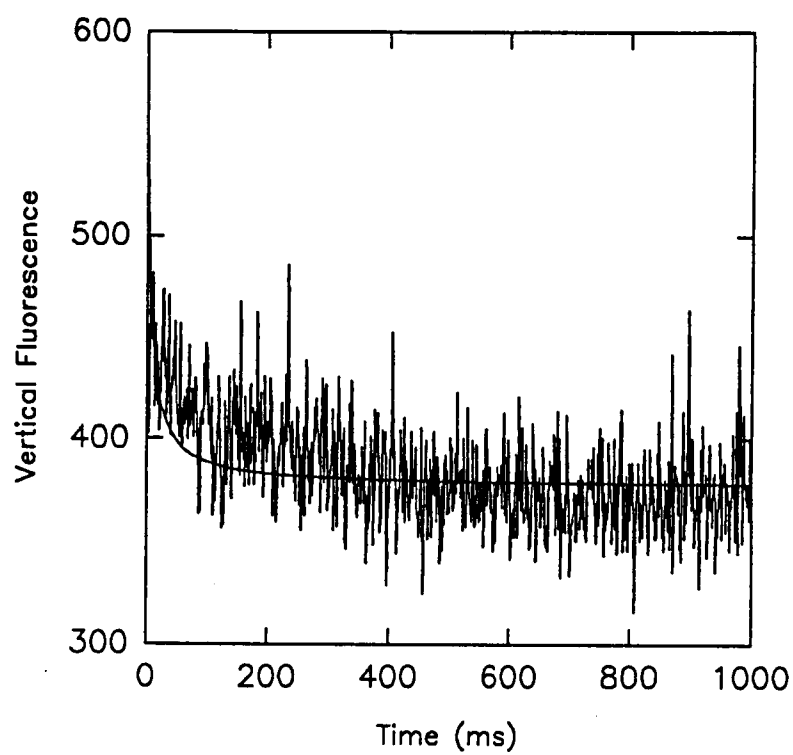
Measurements for DPPC taken in the same manner as for DMPC had been initially made for the temperatures 51 °C, 44 °C and 37 °C. Whereas DMPC small unilamellar vesicles have a phase transition temperature of 21 °C, for DPPC small unilamellar vesicles the phase transition temperature is 37 °C.

For DPPC, it was noted that the relative drop in the fluorescence intensity level after melittin binding took place was much smaller than that for DMPC. This drop was about one third that for DMPC. As a consequence to this, the kinetic fluorescence profile looks much flatter. In fact, some of the data files were so flat that the data fitting program used in the analysis could not read the drop. These data files were subsequently excluded from the analysis for having reached the limit of resolution of the numerical technique employed in the analysis. This led to fewer concentrations that could be included in the global analysis. And because not enough different concentrations could be included, it was surmised that the results so obtained might not be the unique solution that the global analysis expected to pin down. However, the results were being presented here so as to serve as an index of the order of magnitude for the different parameters that could be expected for DPPC.

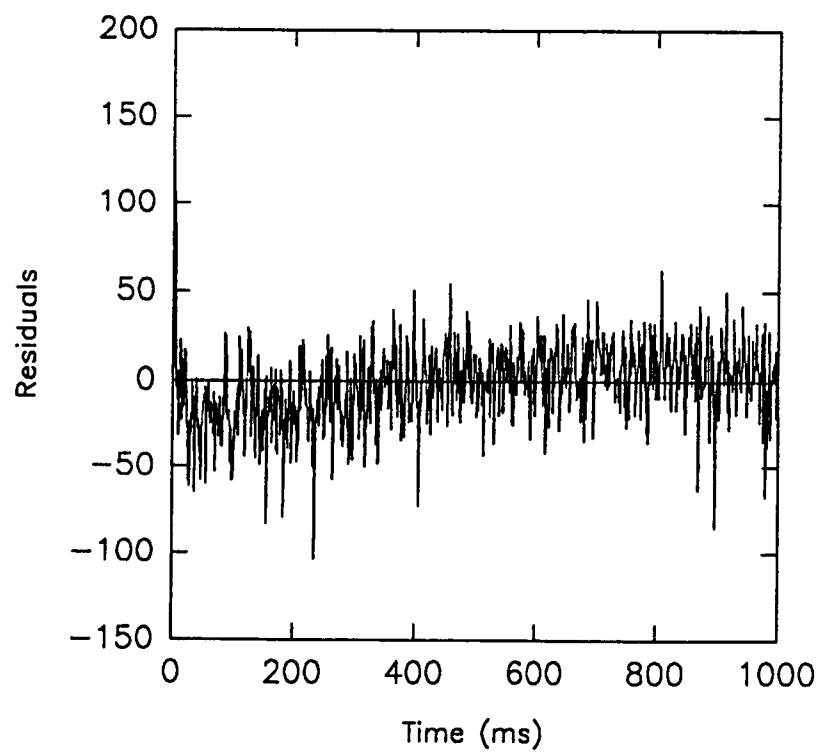
Typical results of the analysis for DPPC for a temperature of 44 °C are presented in Figures 7A through 7F. These are for molar ratios of 120, 90 and 60. The first three

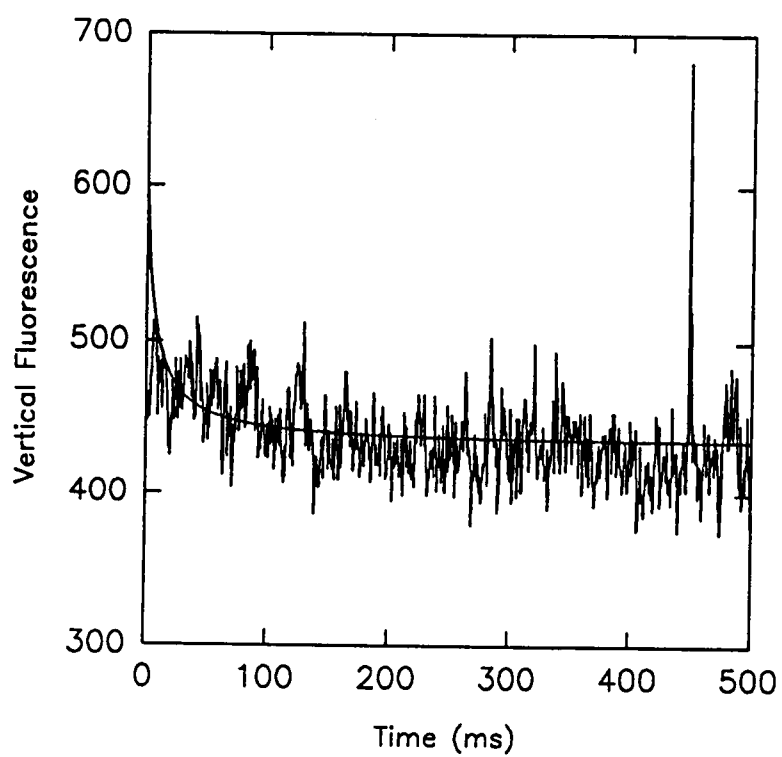
Figures
7A-7F.

Results of the global analysis for the binding of melittin to DPPC small unilamellar vesicles at 44 °C. Figures 7A through 7F are for the vertical component of the fluorescence intensity. Figure 7A is for a lipid concentration of 12 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 120. Figure 7B is for a lipid concentration of 9 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 90. Figure 7C is for a lipid concentration of 6 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 60. Figures 7D through 7F are for the horizontal component of the fluorescence intensity. Figure 7D is for a lipid concentration of 12 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 120. Figure 7E is for a lipid concentration of 9 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 90. Figure 7F is for a lipid concentration of 6 mM and a melittin concentration of 0.1 mM and a lipid-to-melittin molar ratio of 60. In these figures, the upper plot shows the experimental data and the fit obtained in the global analysis. The lower plot shows the residuals from the fit to the data.

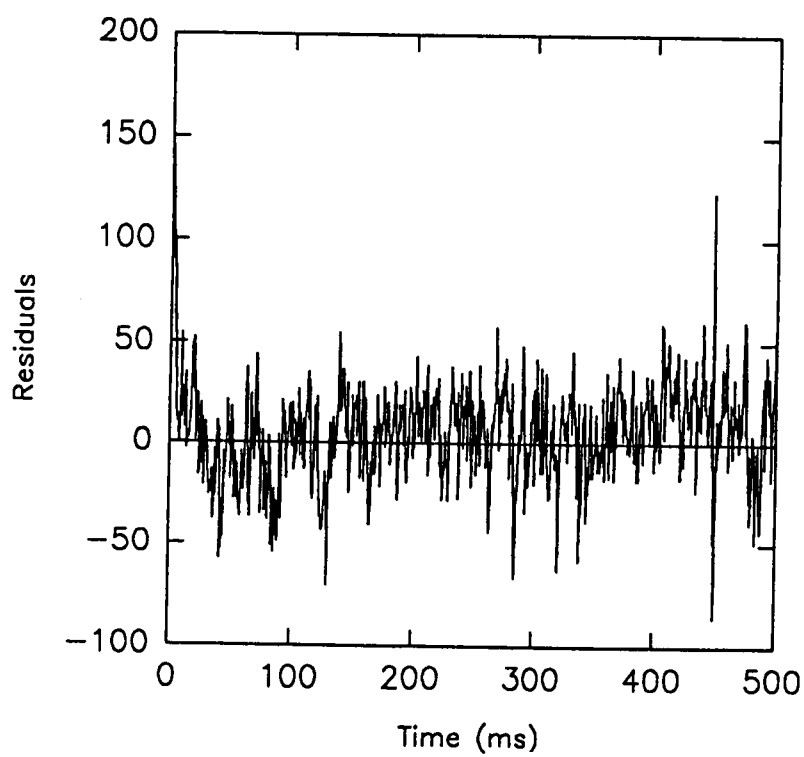


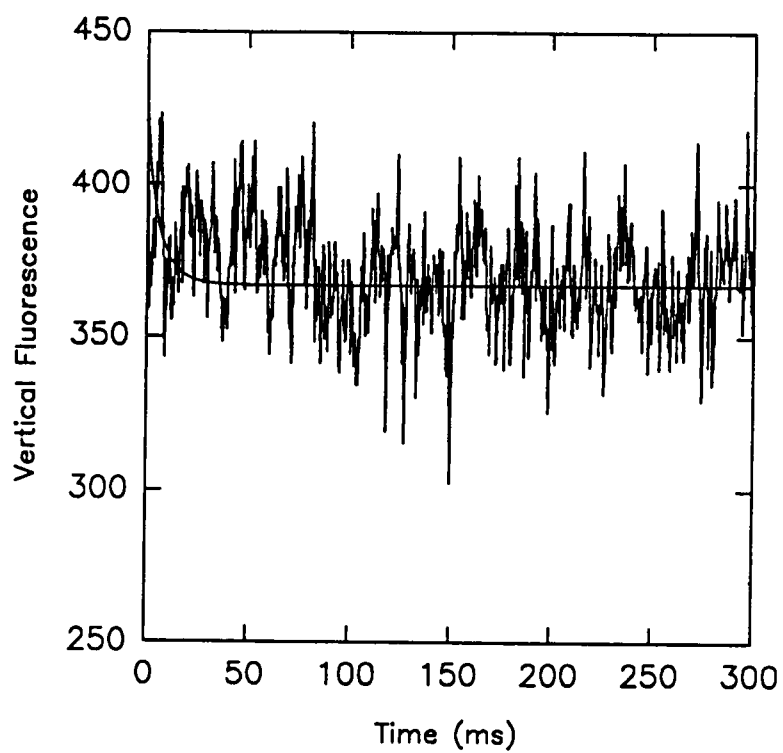
7A



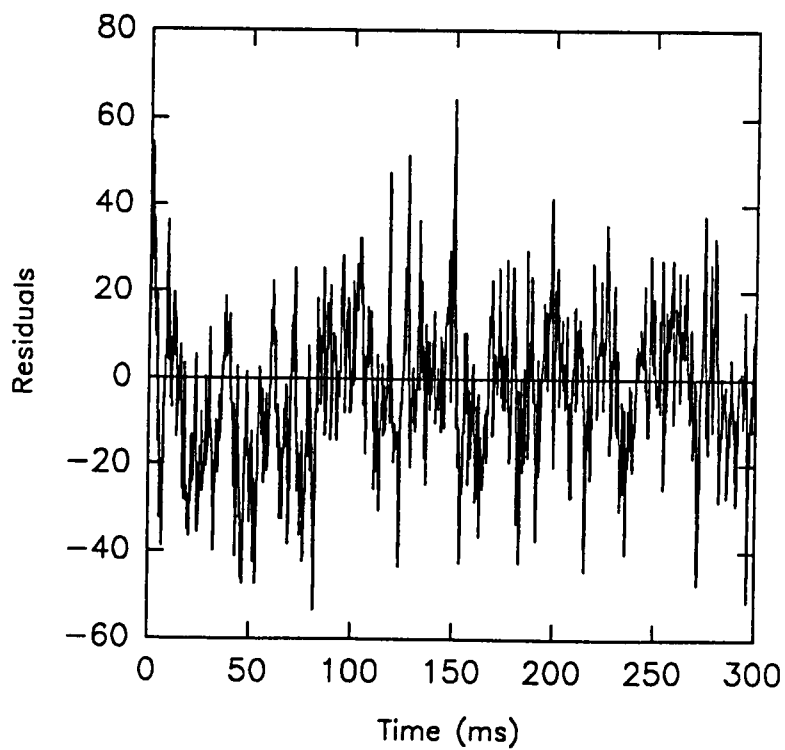


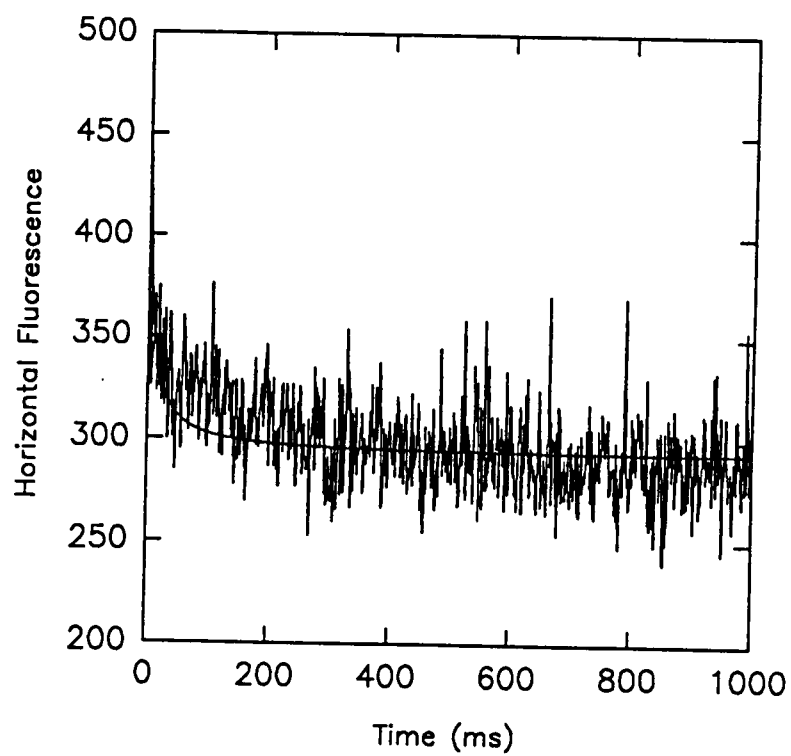
7B



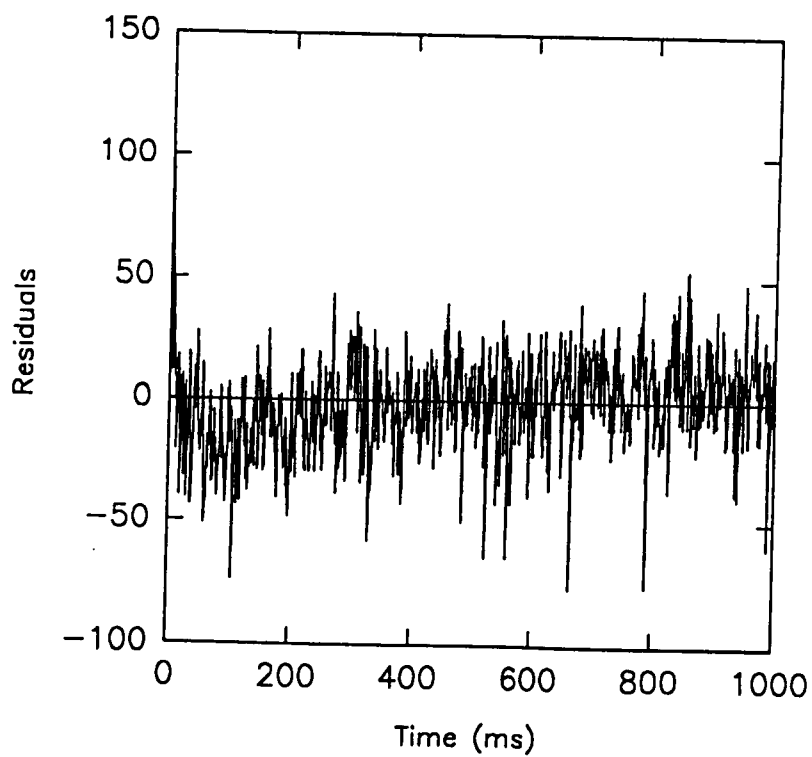


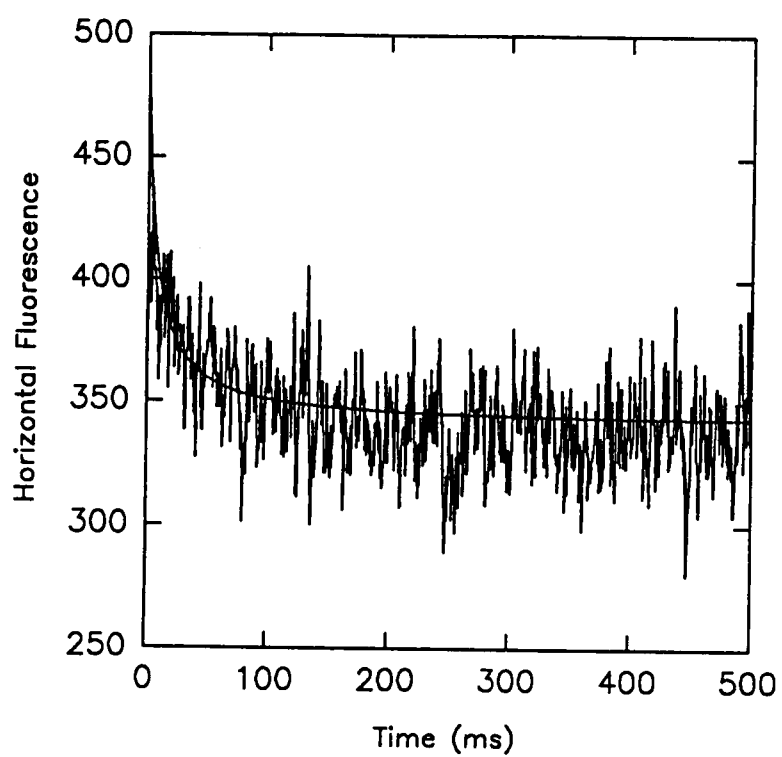
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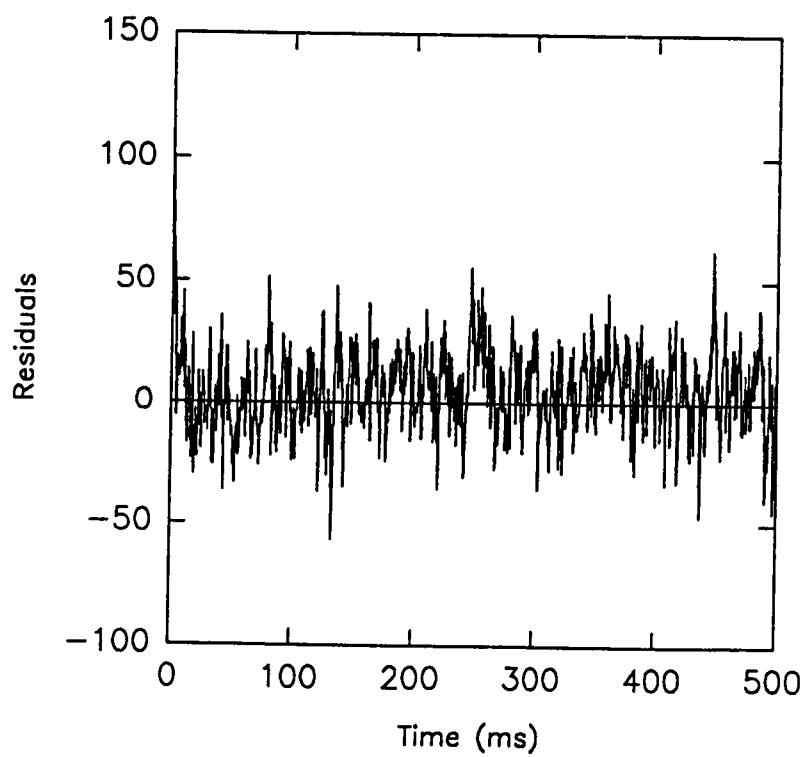


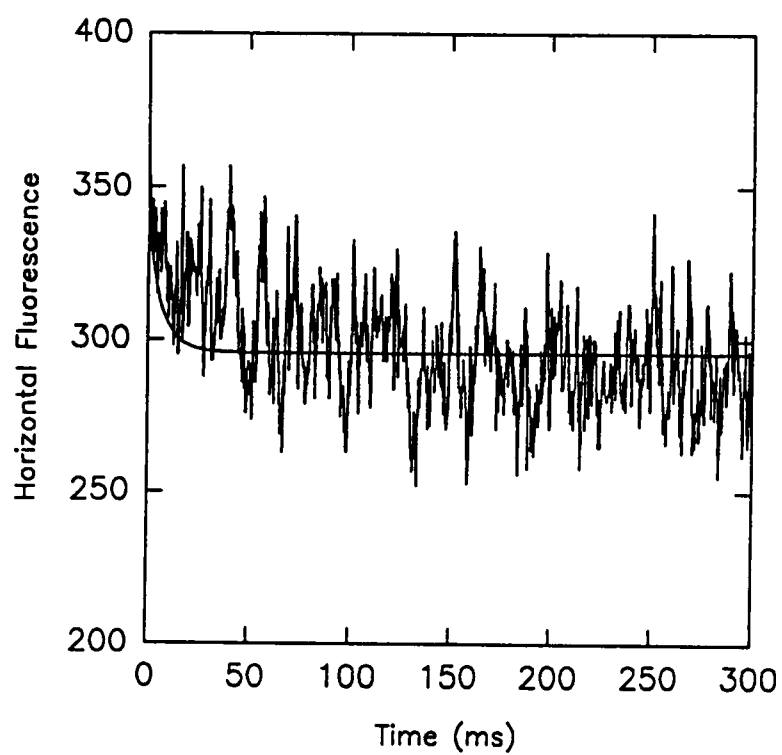
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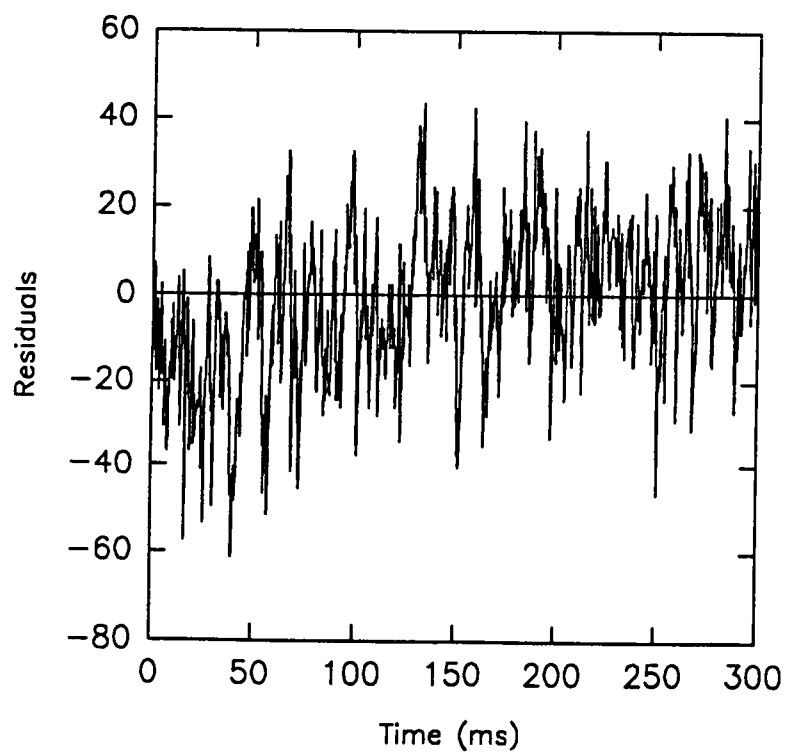


7E





7F



figures give the vertical component of the fluorescence intensity for these ratios and the latter three figures are for the horizontal component for the same ratios.

Table 4 shows the values of the parameters k_p , k_r and the number of lipids per binding site. The missing values were either too small or negative and may be indicative of the insufficient number of different concentrations and were, thus, left out. As mentioned earlier, these results are to be taken only as an index of the order of magnitude that may be expected and may not be the precise unique solutions being sought.

Tables 5 and 6 give the rest of the parameter values.

Temperature °C	k_f ($\times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_r (s^{-1})	No. of Lipids per Binding Site
51	—	—	173.5
44	31.4	—	93.2
37	7.78	—	68.3

TABLE 4. Forward and reverse rate constants, and the number of lipid molecules per binding site as determined by the model applied to the polarized fluorescence data taken while melittin binds to DPPC small unilamellar vesicles.

Temperature °C	$\gamma_f \epsilon_f q_f$ [†] ($\times 10^4$)	$\gamma_f \epsilon_f q_f$ [†] ($\times 10^4$)	$\gamma_b \epsilon_b q_b$ [‡] ($\times 10^4$)	$\gamma_b \epsilon_b q_b$ [§] ($\times 10^4$)
51	1.42	1.15	1.00	1.00
44	1.67	1.45	0.77	0.65
37	1.76	1.00	0.84	0.68

TABLE 5. The measured fluorescence coefficients (or effective quantum yields) for the DPPC-melittin interaction.

[†] Fluorescence coefficient for free melittin in solution as determined by equation (21).

[†] Fluorescence coefficient for free melittin in solution as determined by equation (9).

[‡] Fluorescence coefficient for bound melittin as determined by equation (9).

[§] Fluorescence coefficient for bound melittin as determined by equation (9) for exciting light intensity normalized to that used at 51 °C.

Temperature °C	r_b [§]	r_f [†]
51	0.052	0.014
44	0.12	0.011
37	0.076	0.012

TABLE 6. Measured fluorescence anisotropies for the DPPC-melittin interaction.

[§] Fluorescence anisotropy for bound melittin as determined by equation (14).

[†] Fluorescence anisotropy for free melittin as determined by equation (22).

DISCUSSION AND CONCLUSION

In the analysis done for DMPC, it has been noted that there is some signal loss in the first few milliseconds during which kinetics was observed. (The stopped-flow fluorometer has a deadtime of about 3 ms.) This may possibly be due to a fast initial process which may include fast electrostatic interaction between the charged side chains of the protein and the negative region of the polar headgroup of the bilayer. On the other hand, this could also be caused by a mechanical action of pushing down the sample pistons on the machine during the mixing process. Because such loss was not taken into account in the model described by equation (1), the curve fitting was not as good at earlier times as that at longer times.

Both the forward and reverse rate constants presented in Table 1 for DMPC small unilamellar vesicles show that these parameters increase with increasing temperature. The value of 4.2 s^{-1} observed for a temperature of 30°C , however, seems a little bit high and may be the effect of statistical fluctuations. Otherwise, the observed trend is what would have been expected.

An early study (Dasseux et al, 1984) showed that melittin does not bind to lipid samples kept at a temperature below the phase transition temperature in the case of large unilamellar vesicles. At such temperature, the liposome contains only domains in the gel phase. If this also applies to small unilamellar vesicles, it would be expected that the number of lipids per binding site would decrease with temperature since as the phase transition

temperature is approached (in going from higher temperatures towards 21 °C in the case of DMPC), the proportion of the gel phase domains increases. This behavior was not observed here since the number of lipid molecules per binding site was found to be the same (about 90) at all temperatures except at 21 °C where it dipped to a value of 53. It could then be argued that probably the gel phase domains do not participate significantly in the binding process and as long as the requisite number of liquid crystalline domains are present, binding will take place. If the requisite number of lipids per binding site could not be satisfied, a more loosely formed complex would probably result. The present data apparently agree with this argument. Except for the slight anomaly cited earlier at a temperature of 30 °C, the binding constant (k_b/k_f) at the temperatures of 33 °C, 27 °C, and 24 °C is about $1.7 \times 10^5 \text{ M}^{-1}$ and decreases to $1.2 \times 10^5 \text{ M}^{-1}$ for a temperature of 21 °C. That binding may be limited to domains of one type may be further seen in the invariance of the γ_{eq} 's for bound melittin at different temperatures seen in Table 2 (column 5). (This is in agreement with the results of Bradrick, 1994.) This argument is based on the fact that the conformation and orientation of melittin when it binds to different domains would be different. Additionally, if the proportion of these species change with temperature as is conceivable, then the measured q 's (which would be the average for the different species) should vary with temperature. The argument is further strengthened by the observed anisotropies for bound melittin (again, a measure of the average values for whatever bound species that may be present) seen in Table 3 (column 2). The anisotropy gives a measure of the rotational dynamics of the molecule

under investigation. As such, it gives information about the rigidity of the microenvironment surrounding it. If there are different species of bound melittin, such as binding to different binding sites, and these species vary with temperature, then the resulting anisotropies should exhibit some variation at different temperatures. The values presented in Table 3 do not exhibit such variation. There, the anisotropy for bound melittin is constant at 0.11. In relation to this, it has been noted that a large decrease was not seen in the quantum yield in going from 21 °C to 33 °C as expected (Table 2 column 5). However, it is possible that such a decrease took place within experimental error. That would result in a shortening of the lifetime of the excited state and therefore in an increase in the anisotropy at 33 °C. This may explain the observation (Table 3, column 2) that the anisotropy did not decrease at 33 °C despite the increased mobility of the protein at 33 °C. The increase in anisotropy due to a shorter lifetime apparently compensated its decrease due to increased mobility at higher temperature. All these results appear to complement each other well. First, the γ_{eq} 's for bound melittin should be able to note differences in the conformations and/or orientations of the different species of bound melittin, if they exist. Second, the anisotropies should be able to also report on the presence of these different classes of bound melittin which are assumed to possess different rotational dynamics. In both cases, the data apparently reveal that only one class of bound melittin is present.

An activation energy for the association process of 31.3 kcal mol⁻¹ is seen to be about five times that of the transition enthalpy of 5.9 kcal mol⁻¹ reported for phospholipids

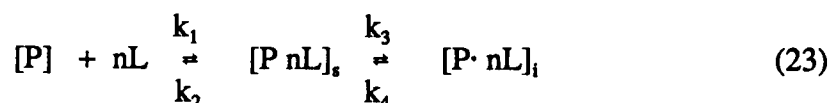
with fourteen carbons in the acyl chain (Marsh, 1990). This may be interpreted to mean that part of the energy goes into enthalpic effect and the rest goes into entropic effect. Or, that for binding to occur, melting of the acyl chains and some ordering/disordering of the membrane are both required. The same thing may be said of the activation energy of $30.6 \text{ kcal mol}^{-1}$ for the dissociation process. (These values, again, are quite close to those obtained by Bradrick, 1994.)

Overall, the resulting parameters appear to be consistent with the model and indicate that the melittin-DMPC interaction is a two-state process where binding occurs only to one class of binding sites.

For DPPC, due to the limitations cited in the previous section, the results are less conclusive. The change in the anisotropy, as melittin binds to DPPC small unilamellar vesicles, was small and difficult to analyze. Therefore, the total fluorescence intensity was analyzed. The justification for doing this is that, in the case of DMPC, the results of the analysis of anisotropy data as presented here are very similar to those previously reported by Bradrick in his dissertation (1994) for total fluorescence decay of DMPC data. From the present analysis of the total fluorescence data for DPPC, the forward rate constant for the temperature of 37°C was $48 \pm 10 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and that for 44°C was $56 \pm 15 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The errors were estimates. For the temperature of 51°C , no significant result was obtained and additional data may have to be taken. The resulting reverse reaction rates have been found to be too small for the computer program to determine. The number of lipids per binding site

was 105 ± 25 for both temperatures. The results thus far obtained are encouraging since they indicate (through the binding constant, k_t/k_r) that the DPPC-melittin complex is much more stable than the corresponding one formed by DMPC, in agreement with the findings of Bradrick et al. (1995) that the longer acyl chains of DPPC offer a better match to the hydrophobic part of the inserted melittin.

For the melittin-DMPC interaction, it is concluded that the process follows a two-state model which results in free melittin and one class of bound melittin as indicated by the fact that the γ_{eq} 's for bound melittin stay the same for different temperatures and that the anisotropies for bound melittin at different temperatures also do not change. When the requisite number of liquid crystalline domains is not present, the resulting complex appears to be weaker. A final test on the appropriateness of a two-state process described by equation (1) would be to extend the model to include a third state and described by



The subscript s is for the surface bound species and the subscript i is for the partially inserted species. Based on such a model, a similar analysis as carried out here could be performed. Since a closed-form expression for the different concentrations as functions of time may not be readily obtained, the concentrations will have to be determined by numerically solving the associated differential equations. The residuals of each fit could then be compared in

deciding whether a two-state or a three-state model will be more appropriate in describing the interaction. Although such an analysis is being proposed here, the results of the current measurements do not appear to suggest that such analysis is necessary.

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