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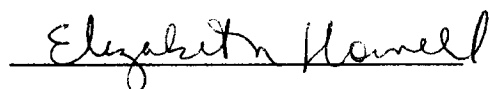
I am submitting herewith a dissertation written by Thomas Dale Bradrick entitled "Fluorometric Studies of the Interaction of Bee Venom Melittin with Model Biological Membranes." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

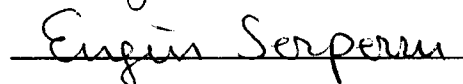


Solon Georgiou, Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate School

**Fluorometric Studies of the Interaction of
Bee Venom Melittin with Model Biological Membranes**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

Thomas Dale Bradrick

December, 1994

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Before all else, I would like to thank my research advisor, Dr. Solon Georghiou, for the guidance, patience and support he provided while the work described herein was being carried out. In addition, I would like to thank him for having given me the invaluable opportunity to combine in this research two things that I love: fluorescence and kinetics, as well as for having involved me in his research concerning the dynamic and excited-state properties of DNA. I cannot begin to enumerate all the things I have gained from having been a member of his research group. My debt to him is enormous and I can only hope to be able to live up to the "heritage" I have received. Ευχαριστώ πάρα πολύ, φίλε μου, και σέ χαιρετώ.

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Note: the work described in Part II of this dissertation was previously published in *Biochim. Biophys. Acta* (1987) 905, 494-498.

ABSTRACT

Stopped-flow fluorometric measurements were carried out in order to (i) measure the kinetics of melittin-induced fusion of dipalmitoylphosphatidylcholine small unilamellar vesicles at 51°C under conditions where melittin was either monomeric or tetrameric in solution; (ii) study the effects of melittin binding on the fluidity and permeability of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine small unilamellar vesicles for below, at and above their respective transition temperatures prior to the occurrence of lipid ordering effects due to melittin-induced fusion; and (iii) study the kinetics of melittin binding to dipalmitoylphosphatidylcholine small unilamellar vesicle over a range of temperatures. In the case of (i), it was found that the rate of melittin-induced fusion is strongly dependent on the state of aggregation of melittin in solution and, to a lesser extent ions in solution. This suggests that the fusion mechanisms are different for the different states of protein aggregation. In the case of (ii) it was found that for dimyristoylphosphatidylcholine small unilamellar vesicles, the effects of melittin binding on fluidity and permeability are maximal at the lipid transition temperature, in agreement with theory. The melittin-induced lipid perturbation was also found to be long range, rather than confined to a boundary annulus of lipids, and propagated rapidly through the bilayer. Interestingly, melittin binding to dipalmitoylphosphatidylcholine small unilamellar vesicles had little to no effect on membrane fluidity or permeability, which demonstrates the importance of acyl chain length in lipid stability. (iii) Kinetic data for the binding of melittin to dimyristoylphosphatidylcholine small unilamellar

vesicles was analyzed in the context of a more sophisticated model than hitherto used. There, it was found that the rates of melittin binding and disassociation decreased with decreasing temperature, as might be expected. In the case of binding, an Arrhenius analysis yielded an activation energy close to that for the melting of lipid acyl chains. The number of lipids required to bind a melittin molecule was found to decrease with decreasing temperature. Fluorescence polarization measurements analyzed using a model which considers statistical effects from the existence of multiple binding site may clarify future analyses.

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LIST OF ABBREVIATIONS

Differential Scanning Calorimetry	DSC
Dimyristoylphosphatidylcholine	DMPC
Dipalmitoylphosphatidylcholine	DPPC
Diphenylhexatriene	DPH
Ethylenediaminetetraacetic Acid	EDTA
Excimer-to-Monomer Fluorescence Intensity Ratio (of pyrene-PC)	E/M
Fused Unilamellar Vesicles	FUV
Lipid-to-Melittin Molar Ratio	R
Lipid Phase Transition (Melting) Temperature	T_m
1-palmitoyl-2-(10-pyrenyldecanoyl)- <i>sn</i> -glycero-3-phosphorylcholine	pyrene-PC
Small Unilamellar Vesicle	SUV
1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, p-toluenesulfonate	TMA-DPH

PART I

GENERAL INTRODUCTION

LITERATURE REVIEW

Melittin is a small peptide of molecular weight 2800 which consists of 26 amino acid residues and which constitutes approximately 50% of the dry weight of honey bee venom (Habermann, 1980). The peptide has amphipathic character, with amino acid residues 1 through 20 being predominantly hydrophobic and residues 21 through 26 being hydrophilic. (Its amino acid sequence is shown in Figure 1.) Because of its structure, melittin exists as a tetramer in crystals and in solutions at high protein and/or salt concentrations (Faucon *et al.*, 1979). (The crystal structure of the tetramer is shown in Figure 2.) At low protein and salt concentrations it assumes a largely random-coil conformation as a monomer in solution. The conformation and state of aggregation of membrane-bound melittin is uncertain, however. Several models for the former have been proposed, including the wedge (Dawson *et al.*, 1978 and Vogel *et al.*, 1983) (shown in Figure 3c), the trans-membrane α -helix (Vogel *et al.*, 1983) (shown in Figure 3a) and an α -helix with its axis parallel to the bilayer surface (Terwilliger *et al.*, 1982) (shown in Figure 4). These models all assume that the protein exists as a monomer when bound to the membrane. Conflicting conclusions have been reached, however, about the state of aggregation of melittin in lipid vesicles (Vogel and Jähnig, 1986; Hermetter and Lakowicz, 1986 and Talbot *et al.*, 1987), presumably due to varying effects on protein structure of the different types of labeling which were used to carry out resonance energy transfer studies. Bradrick *et al.* (1989) used differential scanning calorimetry in order to determine the state of aggregation of membrane-bound melittin using the unmodified

1	2	3	4	5	6	7	8	9	10	11	12
GLY	ILE	GLY	ALA	VAL	LEU	LYS	VAL	LEU	THR	THR	GLY
13	14	15	16	17	18	19	20	21	22	23	24
LEU	PRO	ALA	LEU	ILE	SER	TRP	ILE	LYS	ARG	LYS	ARG
25	26										
GLN	GLN										

Figure 1. Amino acid composition of melittin. (From The Honey Bee: *Apis Mellifera*) (Dayhoff, 1968)

Figure 2. Arrangement of the melittin α -helical monomers in the crystalline tetramer (From Terwilliger and Eisenberg, 1982.)

Figure 3. Models for the structure of a membrane-bound α -helical melittin monomer. Melittin consists of two helical segments (represented by the two rectangles) with a diameter of 10 Å (Terwilliger and Eisenberg, 1982) and an overall length (30 Å) that just spans the bilayer (Vogel and Jähnig, 1986). Helix 1 extends from residues 1 to 11 and helix 2 extends from residues 14 to 20. The bend occurs near the proline residue at position 14. The single positive charge at the N-terminus and the four positive charges at the hydrophilic amino terminal end (residues 21-26) are shown. The angle between the helix axes is about 120°. (A) Trans-membrane bent α -helix. (B) Partially-inserted α -helix. (C) "Wedge" model. (From Vogel *et al.*, 1983.)

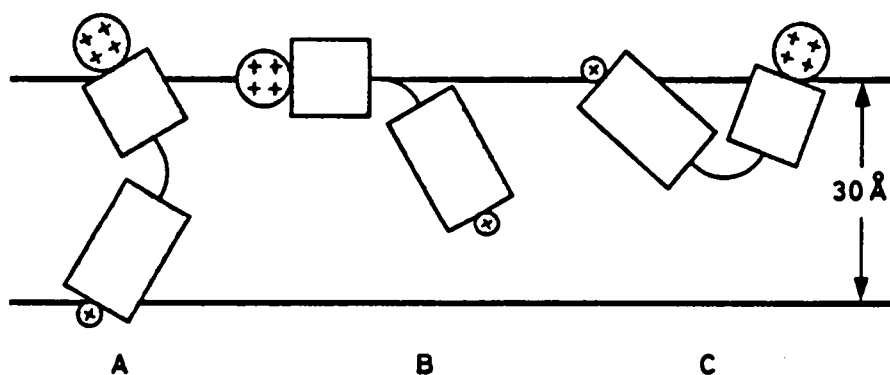
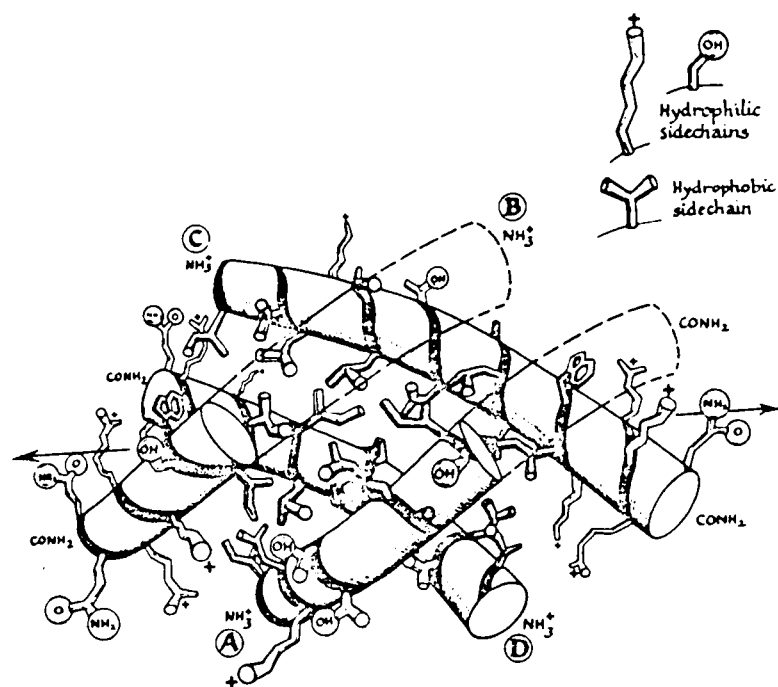
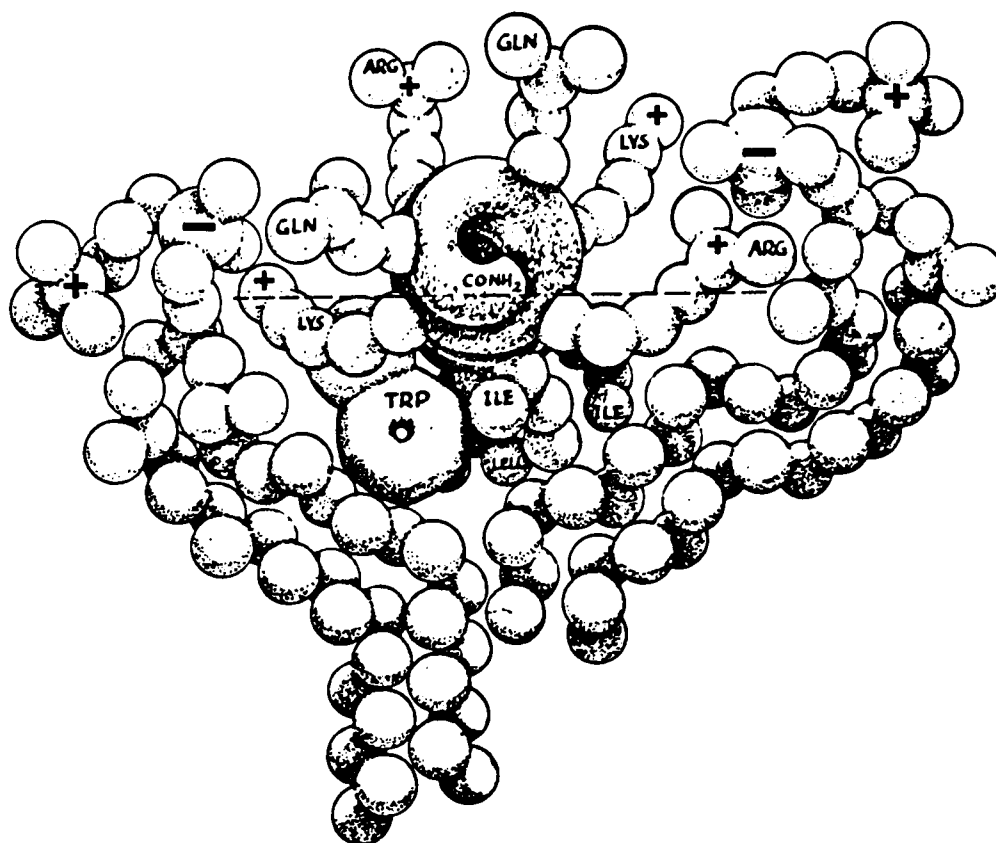
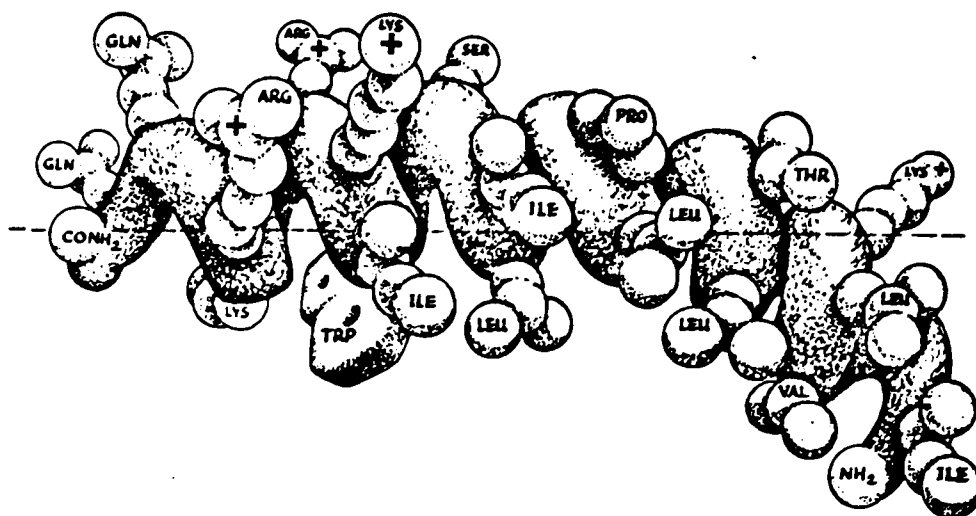


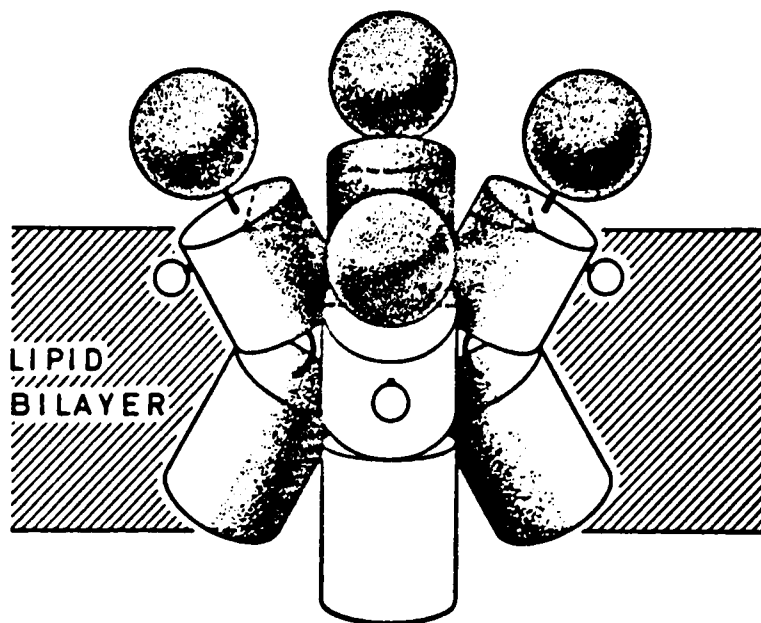
Figure 4. Longitudinal (upper) and axial (lower) views of the proposed space-filling model of a membrane-bound melittin monomer with its helix axis parallel to the bilayer surface and its nonpolar amino acid residues embedded into the membrane. The possible conformation of adjacent lipid molecules is shown in the lower figure to illustrate the effect that binding of such a melittin species might have on the lipid bilayer. (From Terwilliger *et al.*, 1982.)



protein. The model for a membrane-bound melittin tetramer as proposed by Vogel and Jähnig (1986) is shown in Figure 5. Knowledge of the structure of the protein in the membrane is necessary in order to gain an understanding of the effects of melittin binding on lipid bilayers. (That the different conformations might interact with and affect to different extents the lipid head groups and acyl chains is suggested by the figures.)

In terms of its physiological effects the protein has been found to enhance the activity of phospholipase A₂ (Vogt *et al.*, 1970; Mollay and Kreill, 1974; Mollay *et al.*, 1976 and Yunes *et al.*, 1977) by altering the structure of the membrane to which it binds, while at high concentrations it lyses model and natural membranes (Weissmann *et al.*, 1969; Sessa *et al.*, 1969 and Olson *et al.*, 1974). Other effects include the enhancement of the permeability of membranes to ions and solutes (Sessa *et al.*, 1969; Thelestam and Möllby, 1976; Tosteson and Tosteson, 1981; De Grado *et al.*, 1982; Kempf *et al.*, 1982 and Hanke *et al.*, 1983). It is found to alter the thermotropic properties of membranes (Mollay, 1976; Verma and Wallach, 1976; Lavialle *et al.*, 1980; Bernard *et al.*, 1982; Levin *et al.*, 1982; Prendergast *et al.*, 1982; Jähnig *et al.*, 1982; Posch *et al.*, 1983; Dasseux, 1983 and Dasseux *et al.*, 1984) and causes natural and artificial membranes to undergo fusion (Morgan *et al.*, 1983; Eytan and Almary, 1983 and Bradrick and Georghiou, 1987). The extent and character of its interactions with phospholipid membranes depend on the relative and absolute protein and lipid concentrations, on the type of membrane phospholipid under study and on the morphology of the liposomes to

Figure 5. Suggested model for the membrane-bound melittin tetramer. As in Figure 3, the spheres represent the nonhelical six residues at the C terminus. The two α -helical segments are represented by cylinders. The hydrophobic sides of the amphipathic α -helices face the bilayer lipids, while the hydrophilic sides face each other and form a membrane-spanning pore. The small circles show the positions of the tryptophan residues. (From Vogel and Jähnig, 1986.)



which it is being bound. [Dempsey (1990) contains a review of the recent literature concerning the structure of melittin, its actions on membranes and the result of physical studies regarding its interaction with model membranes.] The molecular origins of its interactions with lipids have yet to be completely understood, however. Nevertheless, there exists a significant level of interest in melittin, due both to its behavior as a toxin and to its potential, as a constituent of model systems, for helping to elucidate the way in which natural protein-membrane systems behave.

A considerable amount of work, using such experimental techniques as steady-state and time-resolved fluorometry (Mollay and Kreil, 1973; Talbot *et al.*, 1979; Bernard *et al.*, 1982; Georghiou *et al.*, 1982; Quay and Condie, 1983 and Bradrick *et al.*, 1987), NMR (Strom *et al.*, 1978; De Bony *et al.*, 1979; Brown *et al.*, 1982 and Stanislawski and Rüterjans, 1987), ESR (Williams and Bell, 1972 and Hegner *et al.*, 1973) and CD (Strom *et al.*, 1978; Drake and Hider, 1979; Knöppel *et al.*, 1979 and Vogel, 1981) has been performed in order to elucidate the effects which melittin has on phospholipid membranes. Of interest are the conformation and state of aggregation of the protein in membranes, as well as the effect that the protein has on the properties of the lipid acyl chains. Such information is essential for achieving an understanding of the activity of the protein as a natural toxin and may also be useful for gaining insights into structures and processes (such as signal peptides, membrane proteins, membrane fusion and pore formation: aspects of the model system which were mentioned above) which are of more general biological importance.

Below are described the results of three studies which utilized stopped-flow fluorometry in order to gain further insight into the interaction of melittin with model biological membranes. In the first study, a membrane fusion study is developed to give quantitative information about the rates of membrane fusion and is then used to study the kinetics of melittin-induced liposome fusion. It was found that there is a significant difference between the rates of vesicle fusion for monomeric and tetrameric melittin in solution. This is interpreted to indicate that the fusion mechanisms are different for these two species of melittin, and is discussed in the light of other results (Bradrick *et al.*, 1989) to suggest that in the case of monomeric melittin in solution, free melittin may play a role in the vesicle fusion mechanism.

In the second study, stopped-flow fluorometry is used to monitor changes in the fluorescence anisotropy and total fluorescence of a membrane-bound reporter molecule while melittin binding is taking place. There it is found that the perturbing effects of melittin on the membrane are propagated rapidly, are long range and, in the case of dimyristoylphosphatidylcholine small unilamellar vesicles, is maximal at the lipid transition temperature. The last two points are found to be in agreement with theory (Sperotto and Mouritsen, 1991). The protein is also found to have an affect on the permeability of the membrane to water.

The final study presents an attempt to determine the kinetics of melittin binding to lipid vesicles using stopped-flow fluorometry. The data are analyzed in the context of a model which is more realistic than those used in previous studies (Schwarz and

Beschiaschvili, 1989 and Sekharam *et al.*, 1991). The data are interpreted in light of the model and suggestions are made for improving the model and subsequent data analysis.

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PART II

KINETICS OF MELITTIN-INDUCED FUSION OF DIPALMITOYL- PHOSPHATIDYLCHOLINE SMALL UNILAMELLAR VESICLES

INTRODUCTION

The peptide melittin, which constitutes approximately 50% of the dry weight of honey bee venom, has for some time received considerable attention in the study of model protein-phospholipid interactions. It has been reported that the protein lyses model and natural membranes (Weissmann *et al.*, 1969; Sessa *et al.*, 1969 and Olson *et al.*, 1974) and fuses small unilamellar acidic (Eytan and Almary, 1983) and large unilamellar (Morgan *et al.*, 1983) and small unilamellar (Bradrick *et al.*, 1987) zwitterionic vesicles. The fusogenic properties of melittin are of considerable interest because of their relevance with respect to the protein's physiological effects, and because a knowledge of the mechanism involved may enhance our understanding of this important biological process.

Here, the results of a study of the kinetics of melittin-induced fusion of dipalmitoylphosphatidylcholine small unilamellar vesicles are presented. The fusion assay which was used to follow the kinetics exploits the change that takes place in the ratio of the excimer to monomer fluorescence intensities of pyrene, covalently attached to a phosphatidylcholine, when vesicles labeled with this modified lipid are induced to fuse with nonlabeled vesicles and the local pyrene concentration is thus caused to decrease. This property of pyrene was previously utilized to follow the kinetics of lipid vesicle fusion by serum albumin (Schenkman *et al.*, 1981) and the kinetics of fusion of Sendai virus particles with lipid vesicles (Amselem *et al.*, 1986). In the work reported here, this fusion assay was further developed so as to give quantitative information about the rate constants which are part of the mass-action model for lipid vesicle fusion.

It was found that, for a lipid-to-protein molar ratio of 60, the rate constant for the initial fusion process is affected by the state of aggregation of melittin in solution. Electrostatic interactions also appear to play a role.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC) of a purity greater than 99% was obtained from Avanti Polar Lipids (Alabaster, AL), while melittin was obtained from Sigma Chemical Co. (St. Louis, MO). Both were used without further purification. Chloroform, analytical grade, was from Mallinckrodt (Paris, KY). 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphorylcholine (pyrene-PC) was obtained from KSV-Chemicals Oy (Helsinki, Finland). Tris and ethylenediaminetetraacetic acid-disodium salt (EDTA) were analytical grade and obtained from Fisher (Pittsburgh, PA) and Sigma, respectively. The buffers used were 50 mM Tris, 1 mM EDTA, pH 7.6; 50 mM Tris, 5 mM EDTA, pH 7.6; and 50 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.6, all prepared in triply distilled water. 1 mM EDTA for low melittin concentrations and 5 mM EDTA for high melittin concentrations was included in the buffers in order to inactivate any residual phospholipase A₂ present in the sample. No detectable lipid degradation was found to take place under these conditions for the time span of the measurements (Dasseux *et al.*, 1984 and Bradrick and Georgiou, unpublished observations).

Each days experiments were performed with freshly-made samples of DPPC small unilamellar vesicles (SUVs), which were prepared in the following way. For labeled liposomes, pyrene-PC and DPPC were codissolved in chloroform, dried under a stream of nitrogen and vacuum desiccated for 24 hours. (All steps which involved pyrene-PC and/or labeled liposomes were carried out under cover of dim red light so as to avoid photodecomposition of the fluorescently-labeled lipid.) The dried lipids were then

hydrated at 48°C (10°C above their lipid phase transition temperature T_m) to obtain a dispersion which was then sonicated at 48°C until clearness in a Laboratory Supplies Co. (Hicksville, NY) bath sonicator and allowed to anneal for 30 minutes at 48°C. In order to remove any remaining multilamellar vesicles, the sample was centrifuged at 65000xg for 25 minutes at 45°C, after which the supernatant was removed and stored in a polystyrene screwcap tube at 33°C for subsequent use. [When stored above their lipid phase transition temperature, SUVs are stable (Schullery *et al.*, 1980).] Nonlabeled SUVs were prepared by hydrating at 48°C the desired amount of lyophilized DPPC and following that with sonication and centrifugation as described above. At the time of use, aliquots of the liposome solutions were removed and appropriately diluted to obtain the desired absolute lipid concentrations and ratio of nonlabeled-to-labeled vesicles for loading into the stopped-flow apparatus.

Melittin stock solutions were prepared by weighing the desired quantity of lyophilized melittin and dissolving it in 10 ml of buffer. The concentration of this stock solution was determined spectrophotometrically on a Cary 14 spectrophotometer given that $\epsilon_{280} = 5800 \text{ M}^{-1} \text{ cm}^{-1}$ (Berlman, 1971). Stock solutions were not used beyond five days after their formation.

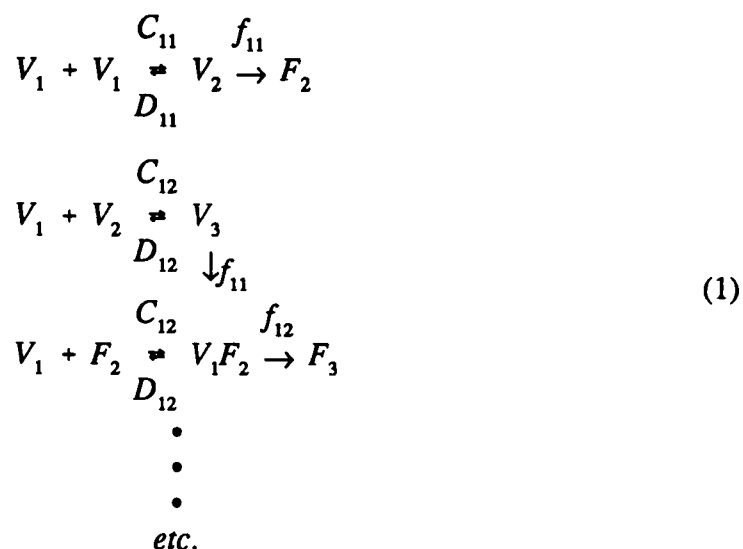
Stopped-flow fluorometric measurements were carried out on an Aminco DW-2 UV/Vis spectrophotometer equipped with a stopped-flow module which has been modified to allow fluorescence to be measured at right angles to excitation. Pyrene monomer fluorescence was viewed by an EMI 6256S photomultiplier through Corning 0-52 and 7-51 filters. Pyrene excimer emission was viewed through a Corning 3-72 filter. The

excitation monochromator of the stopped-flow spectrophotometer was set at 319 nm, with a 20 nm bandwidth. The stopped-flow sample cell and sample reservoirs were thermostated at the desired temperature with a circulating water bath. The photomultiplier output was recorded on a Houston Instruments model 100 x-y recorder and was digitized by hand for the subsequent computer analysis. For each "run" of (approximately) twelve stopped-flow "shots" or injections, melittin and lipid samples were loaded into the sample reservoirs and allowed to come to thermal equilibrium over a period of ten minutes. Three shots were then taken, the last of which was allowed to remain in the stopped-flow cell or cuvette for five minutes in order to allow its fluorescence to bring the photomultiplier response up to its steady-state level. Approximately six shots were then taken in which the pyrene monomer fluorescence intensity was recorded as a function of time. Emission filters were then changed and approximately six more shots were taken in which the pyrene excimer fluorescence intensity was recorded.

Baseline contributions to signal due to scattered light and buffer fluorescence were measured under the same conditions as for measuring pyrene fluorescence. The lipid sample consisted of nonlabeled vesicles only, however, at the same lipid concentration as the labeled plus nonlabeled samples used to follow fusion kinetics. Baselines were determined for both monomer and excimer signal by using the appropriate emission filters and the baselines were subtracted from their respective signals prior to calculating the pyrene excimer-to-monomer fluorescence ratio.

In order to obtain the rate constants for melittin-induced lipid vesicle fusion the experimental data were analyzed in the context of mass action kinetic theory (Bentz *et*

al., 1983) which views lipid vesicle fusion as proceeding according to the following kinetic scheme:



Here, the notation of Bentz *et al.* (1983) is followed. V_1 denotes the original (nonfused) vesicle monomer, V_i denotes an aggregate of $i \geq 2$ nonfused vesicles, F_2 denotes a fused doublet, F_i denotes the fusion product of $i \geq 3$ vesicles, and $V_1 F_2$ denotes an intermediate aggregate product of a monomer and a fused doublet. C_{11} is the rate constant for the aggregation of two vesicle monomers, D_{11} is their rate constant for dissociation, f_{11} is the rate constant for the fusion of an aggregate composed of two vesicle monomers, *etc.*

Over a sufficiently short period of time the fusion reaction should be well described by the first equation in (1). Also, the analysis of the kinetic data is greatly simplified if the dissociation step (with rate constant D_{11}) is omitted. (This approximation should also be valid at short times.) Then, the kinetic scheme is reduced to



for which the following differential equations obtain:

$$\frac{d[V_1]}{dt} = -2C[V_1]^2 \quad (3)$$

$$\frac{d[V_2]}{dt} = C[V_1]^2 - f[V_2] \quad (4)$$

$$\frac{d[F]}{dt} = f[V_2] \quad (5)$$

(Here, the square brackets denote concentration.) From the stoichiometry it follows that

$$X_o = [V_1] + 2[V_2] + 2[F] \quad (6)$$

where $X_o = [V_1]_o$. (The zero subscript denotes the initial or zero-time value.)

The solution to equation 3 is

$$[V_1] = \frac{X_o}{1 + 2CX_o t} \quad (7)$$

while equations 5 and 6 can be combined to yield

$$\frac{d[F]}{dt} = f \left(\frac{X_o}{2} - \frac{[V_1]}{2} - [F] \right) \quad (8)$$

or

$$\frac{d[F]}{dt} + f[F] = \frac{f}{2}(X_o - [V_1]) \quad (9)$$

Multiplying each side of equation 9 by the integrating factor $\exp(ft)$ and integrating, one obtains

$$\frac{[F]}{X_o} = \frac{f}{2} \exp(-ft) \int_0^t \exp(ft') \left(1 - \frac{[V_1]}{X_o} \right) dt' \quad (10)$$

as a solution to equation 9, or

$$\frac{[F]}{X_o} = \frac{f}{2} \exp(-ft) \int_0^t \exp(ft') \frac{2CX_o t'}{1 + 2CX_o t'} dt' \quad (11)$$

which cannot be further evaluated.

In order to analyze the experimental data and obtain values for the rate constants C and f for fusion, one must relate the change in $[F]$ with time to that of the observed excimer-to-monomer fluorescence intensity ratio of pyrene-PC in the sample. This was done in the following way. [It should be noted that in order to simplify the following derivation and the resulting analysis it is assumed that labelled and nonlabeled liposomes are all of a uniform size. It is also assumed that, with respect to the fusion process, labeling does not produce vesicles which have a preference for fusing with each other or with nonlabeled vesicles.]

In the case of low lipid concentrations (1.36 mM lipid prior to stopped-flow mixing) one part pyrene-PC labeled liposomes was mixed with ten parts nonlabeled liposomes. Upon mixing with melittin, liposome aggregation and fusion took place.

Three "types" of aggregation could thus occur: nonlabeled plus nonlabeled (most likely), nonlabeled plus labeled (less likely) and labeled plus labeled (least likely). The (relative) probabilities of these three events were obtained from the coefficients for the binomial expansion of a second-order reaction or combination:

$$Probability \propto \left(\frac{10}{11} + \frac{1}{11} \right) \left(\frac{10}{11} + \frac{1}{11} \right) = \frac{100}{121} + \frac{20}{121} + \frac{1}{121} \quad (12)$$

Here, 100/121 is the fraction of "events" in which two nonlabeled liposomes aggregate, 20/121 is the fraction in which a labelled and a nonlabeled liposome aggregate and 1/121 is the fraction which involves labeled plus labeled liposome aggregation. As opposed to the second and third types of "events", the first type clearly does not contribute to the fluorescence signal or its change. Of those events which involve labeled liposomes, one event in twenty-one will result in a fusion product in which the local pyrene-PC concentration remains unchanged. Twenty out of the twenty-one events will result in a fusion product in which the pyrene-PC concentration has been reduced by half. As was stated above, liposomes were labeled with 4 mole% pyrene-PC. Fusion of labeled with nonlabeled liposomes results in products which are labeled with 2 mole% pyrene-PC. Thus, as fusion proceeds, the observed sample excimer-to-monomer (E/M) fluorescence intensity ratio will be determined by the contributions these two populations make, along with that of the remaining labeled nonfused vesicles. Presumably the observed E/M ratio is a weighted average of E/M ratios for liposomes labeled at 4 mole% and 2 mole%, where the weighting factors are proportional to the relative concentration of liposomes with a particular per cent labelling, as well as the percentage of labeling. An expression

for this weighted average was derived and tested in the following way. Two samples of labeled liposomes were prepared in the manner described above, one with 4 mole% pyrene-PC, the other with 2 mole% pyrene-PC. Various amounts of these two samples were mixed together and the total E/M ratio under steady-state conditions (*i.e.* no melittin-induced vesicle) was measured. For these mixtures, the observed E/M ratio should be given by

$$\left\langle \frac{E}{M} \right\rangle = \frac{E_{4\%} + E_{2\%}}{M_{4\%} + M_{2\%}} \quad (13)$$

where $E_{4\%}$ and $E_{2\%}$ denote the total excimer fluorescence intensities from the 4 mole% and 2 mole% labeled liposomes, respectively. $M_{4\%}$ and $M_{2\%}$ denote the total monomer fluorescence intensities, similarly defined. Sample absorbances were kept low enough (≤ 0.05) that fluorescence intensity was proportional to fluorophore concentration. Then, for the mixture,

$$M_{4\%} = \frac{X[L]_{4\%}[\text{pyrene}]_{4\%}\epsilon q}{X + Y} \quad (14)$$

and

$$M_{2\%} = \frac{Y[L]_{2\%}[\text{pyrene}]_{2\%}\epsilon q}{X + Y} \quad (15)$$

where the mixture consists of X parts by volume of 4 mole% labeled liposomes and Y parts by volume of 2 mole% labeled liposomes. $[L]_{4\%}$ and $[L]_{2\%}$ are the lipid vesicle concentrations of the original stock solutions. ϵ is the molar extinction coefficient of pyrene-PC at the wavelength of excitation for unit intensity exciting light and q is its

fluorescence quantum yield. $[\text{pyrene}]_{4\%}$ and $[\text{pyrene}]_{2\%}$ are the local (i.e. number of fluorophore molecules per vesicle) pyrene-PC concentrations for labeling at 4 mole% and 2 mole%, respectively.

It is well known (Birks, 1970 and Galla and Sackmann, 1974) that

$$\begin{aligned} \frac{E}{M} &\propto \frac{\tau T}{\eta} [\text{pyrene}]_{\text{local}} \\ &= k [\text{pyrene}]_{\text{local}} \end{aligned} \quad (16)$$

where τ is the fluorophore lifetime, T is the absolute temperature and η is the solution viscosity. In writing the equality all the explicit and implicit constants have been subsumed under k . Therefore

$$E = kM [\text{pyrene}]_{\text{local}} \quad (17)$$

and, as the monomer fluorescence intensity is proportional to the pyrene concentration (equations 14 and 15), one obtains

$$\begin{aligned} E_{4\%} &= \frac{k[\text{pyrene}]_{4\%} X [L]_{4\%} [\text{pyrene}]_{4\%} \epsilon q}{X + Y} \\ &= \frac{k[\text{pyrene}]_{4\%}^2 X [L]_{4\%} \epsilon q}{X + Y} \end{aligned} \quad (18)$$

and

$$\begin{aligned} E_{2\%} &= \frac{k[\text{pyrene}]_{2\%} Y [L]_{2\%} [\text{pyrene}]_{2\%} \epsilon q}{X + Y} \\ &= \frac{k[\text{pyrene}]_{2\%}^2 Y [L]_{2\%} \epsilon q}{X + Y} \end{aligned} \quad (19)$$

Substituting equations 14, 15, 18 and 19 into equation 13, one obtains

$$\begin{aligned}
 \left\langle \frac{E}{M} \right\rangle &= \frac{k[\text{pyrene}]_{4\%}^2 X [L]_{4\%} + k[\text{pyrene}]_{2\%}^2 Y [L]_{2\%}}{X [L]_{4\%} [\text{pyrene}]_{4\%} + Y [L]_{2\%} [\text{pyrene}]_{2\%}} \\
 &= \frac{2X [\text{pyrene}]_{2\%} k [\text{pyrene}]_{4\%} [L]_{4\%} + Y k [\text{pyrene}]_{2\%}^2 [L]_{2\%}}{2X [\text{pyrene}]_{2\%} [L]_{4\%} + Y [\text{pyrene}]_{2\%} [L]_{2\%}} \quad (20) \\
 &= \frac{2X \left(\frac{E}{M} \right)_{4\%} [L]_{4\%} + Y \left(\frac{E}{M} \right)_{2\%} [L]_{2\%}}{2X [L]_{4\%} + Y [L]_{2\%}}
 \end{aligned}$$

as

$$\begin{aligned}
 \left(\frac{E}{M} \right)_{4\%} &= k [\text{pyrene}]_{4\%} \\
 \text{and} \\
 \left(\frac{E}{M} \right)_{2\%} &= k [\text{pyrene}]_{2\%}
 \end{aligned} \quad (21)$$

In light of the above result, the overall or average E/M ratio for the test mixtures of 4 mole% and 2 mole% labeled liposomes should then be given by

$$\left\langle \frac{E}{M} \right\rangle = \frac{2X \left(\frac{E}{M} \right)_{4\%} + Y \left(\frac{E}{M} \right)_{2\%}}{2X + Y} \quad (22)$$

as $[L]_{4\%} = [L]_{2\%}$ for the test cases. The presence in equation 22 of the factor of two coefficient for the volume fraction X takes into account the fact that the number of fluorophores in the liposomes labeled at 4 mole% is twice that of the liposomes labeled at 2 mole%.

In testing equation 21, two stock solutions of equal lipid concentration and labeled at 4 mole% and 2 mole% respectively, were made. It was found that $(E/M)_{4\%} = 1.49$ and $(E/M)_{2\%} = .739$. Using equation 22 it was predicted that a mixture of equal volumes from the two stock solutions should have an E/M ratio of 1.24. 1.22 was measured for this. Similarly, two parts by volume of 4 mole% and one part 2 mole% was predicted to have an E/M ratio of 1.34. 1.32 was obtained experimentally. Other measurements made were in equally good agreement with their predicted values which was taken to indicate that the above method of weighting is correct.

In light of the above results for weighting the contributions of lipid vesicles with different local pyrene-PC concentrations and using the results of equation 12 for the fractional populations of the different liposome species which result from the fusion process, it would then be expected that the observed sample E/M ratio would be given by

$$\left(\frac{E}{M}\right)_t = \frac{\frac{11}{121}[V_1]\left(\frac{E}{M}\right)_{4\%} + \frac{20}{121}[V_2]\left(\frac{E}{M}\right)_{4\%} + 2\frac{1}{121}[V_2]\left(\frac{E}{M}\right)_{4\%}}{\frac{11}{121}[V_1] + \frac{22}{121}[V_2] + \frac{22}{121}[F]} + \frac{\frac{20}{121}[F]\left(\frac{E}{M}\right)_{2\%} + 2\frac{1}{121}[F]\left(\frac{E}{M}\right)_{4\%}}{\frac{11}{121}[V_1] + \frac{22}{121}[V_2] + \frac{22}{121}[F]} \quad (23)$$

where the square bracketed quantities are the total (i.e. labeled plus nonlabeled) concentrations of the vesicle species. The first term on the right-hand side of equation 23 gives the contribution of the current (at time t) concentration of labeled single (nonfused) vesicles. The second and third terms contain the contributions of the

aggregation products. It should be noted that the third term is counted twice as it pertains to an aggregate of two labeled vesicles. The fourth and fifth terms give the contributions of the fusion products, the fourth for labeled fused with nonlabeled, and the fifth for labeled fused with labeled vesicles. (The latter is therefore also counted twice.) It should be noted that the normalization factor

$$\frac{11}{121}[V_1] + \frac{22}{121}[V_2] + \frac{22}{121}[F] \quad (24)$$

reduces to

$$\frac{11}{121}([V_1] + 2[V_2] + 2[F]) = \frac{11}{121} X_o \quad (25)$$

As $(E/M)_{4\%} = 2(E/M)_{2\%}$ and $(E/M)_o = (E/M)_{4\%}$ (where the zero subscript denotes the value at zero time), one may rewrite equation 23 to obtain

$$\begin{aligned} \frac{\left(\frac{E}{M}\right)_t}{\left(\frac{E}{M}\right)_o} &= \frac{\frac{11}{121}[V_1] + \frac{22}{121}[V_2] + \frac{12}{121}[F]}{\frac{11}{121} X_o} \\ &= \frac{11[V_1] + 22[V_2] + 22[F] - 10[F]}{11X_o} \\ &= 1 - \frac{10 [F]}{11 X_o} \end{aligned} \quad (26)$$

It may be noted that, as required, the final expression in equation 26 contains no dependence on $[V_2]$. (Aggregation by itself does not lead to a dilution of the local pyrene-PC concentration and so should not contribute to the change in the E/M ratio with time.) Also, at long times when $[F] \rightarrow X_o/2$ (in the context of the limited model,

equation 2) the normalized E/M ratio approaches $0.545 \approx 0.5$, as would be expected. [The presence of a small fraction (1 out of 21 or 4.76%) of the fused vesicles in which there was no pyrene-PC dilution causes the normalized E/M ratio to be slightly greater than 0.5 at long times. Indeed, on this basis one might have predicted that it would be about 9% greater, as indicated, since the contribution of such vesicles would have to be counted twice.]

The data were analyzed by using equation 26 to obtain from the E/M data experimental values for $[F]/X_o$. Equation 11 was then fitted to these values using a nonlinear least-squares program (Marquardt method) to obtain the best fitting values for the parameters f and C .

In the case of high lipid and melittin concentrations (60 mM and 1 mM, respectively), the mixture contained 1 part liposomes labeled at 4 mole% and 967 parts nonlabeled liposomes. (This reduction in the labeled to nonlabeled liposome ratio was necessary in order to maintain a pyrene-PC absorbance of 0.05 and proportionality between fluorescence intensity and fluorophore concentration.) For this case a derivation similar to that above was carried out in which the weighting factors were appropriately changed. In this case one then obtains

$$\frac{\left(\frac{E}{M}\right)_t}{\left(\frac{E}{M}\right)_o} = 1 - \frac{967}{968} \frac{[F]}{X_o} \quad (27)$$

Therefore, for the high lipid concentrations, equation 27 was used to obtain from the

normalized E/M data values for $[F]/X_0$ as a function of time. These values were then analyzed by fitting equation 11 to them.

The increased concentrations in the above case also made it necessary to record the excimer and monomer fluorescence intensities on a storage oscilloscope (Tektronix model 564), as the fusion reaction proceeded too rapidly for them to be recorded on an X-Y recorder as described above. Each trace was photographed on the oscilloscope screen with a Polaroid camera and was digitized by hand from the photograph.

As equation 16 indicates, the excimer-to-monomer (E/M) fluorescence intensity ratio has a dependence on the viscosity of the fluorophore's environment. As lipid vesicle fusion leads to the formation of larger structures in which the lipid bilayer is presumably more ordered and more rigid than before, it is possible that the viscosity of the fused vesicles could be different from that of the original nonfused vesicles. In that case, the change in the E/M ratio would be due to the change in bilayer viscosity as well as the dilution of the pyrene-PC molecules.

In the case of low lipid concentrations (1.36 mM lipid before stopped-flow mixing), the dependence of the E/M ratio on viscosity was examined by mixing in the stopped-flow device melittin with labeled vesicles only. Here the lipid and melittin concentrations were the same as for melittin mixed with one part labeled and ten parts nonlabeled liposomes. Since in this case vesicle fusion does not result in probe dilution, any observed change in the E/M ratio would be attributed to a change in viscosity [and/or pyrene fluorescence lifetime τ , (cf. equation 16)]. In general, the data from such an experiment would then be used to provide a correction to the data obtained from labeled

fusing with nonlabeled vesicles to get the true extent of pyrene-PC dilution in the case of the latter. The corrected data would be given by

$$\frac{\left(\frac{E}{M}\right)'_o \left(\frac{E}{M}\right)_i}{\left(\frac{E}{M}\right)'_i \left(\frac{E}{M}\right)_o} = \frac{[pyrene]_i}{[pyrene]_o} \quad (28)$$

In this equation the primed quantities refer to labeled-fusing-with-labeled vesicles and the unprimed quantities refer to labeled-fusing-with-nonlabeled vesicles. In the case of the lower lipid concentration used in the present study (1.36 mM lipid before stopped-flow mixing) no such correction was necessary; the E/M ratio for labeled-fusing-with-labeled vesicles was constant with time. (This was true in the case of low salt buffer in which melittin is monomeric in solution and in the presence of 2 M NaCl, for which melittin is tetrameric in solution.) No such correction could be determined, however, for the experiments in which the lipid concentration was 60 mM before mixing (and melittin was tetrameric in solution due to its concentration), as in that case a 60 mM solution of labeled liposomes only would have had a pyrene-PC absorbance greater than 4. That the data could not be so corrected is probably not a source of serious error, as can be inferred from the fact that none was needed for the lower lipid concentration in the presence of 2 M NaCl, in which case melittin is also tetrameric in solution.

RESULTS

Results of the study of melittin-induced fusion of DPPC SUVs, at 51°C for a lipid-to-melittin molar ratio of 60, are presented in Figures 1 and 2. In both cases the total lipid concentration was 1.36 mM and the melittin concentration was 22.5 μ M prior to stopped-flow mixing. Figure 1 shows how the E/M ratio, normalized to its value at zero time, changes with time for a low-salt solution in which melittin is monomeric in solution. As can be seen, at long times the E/M ratio approaches approximately one-third its initial value. This indicates that, on the average, fusion stops after the addition of a third vesicle to the original fused doublet. The insert in the figure shows how the experimental values for $[F]/X_0$ at early times, calculated using equation 26, and the least squares fit to the data. The latter yielded a value of $f = 0.006 \pm 0.001 \text{ s}^{-1}$ for the rate constant of fusion. Figure 2 shows the data in the presence of 2 M NaCl, in which case melittin is tetrameric in solution (Talbot *et al.*, 1974 and Georghiou *et al.*, 1982). As in Figure 1, the value that the normalized E/M ratio approaches at long time indicates that fusion stops after the addition of a third vesicle to the original fused doublet. Also, the insert shows the values calculated for $[F]/X_0$ and the least-squares fit to the data. Here, the best fit gave a value of $f = 0.12 \pm 0.01 \text{ s}^{-1}$ for the rate constant for fusion. It should be noted that in neither of these two cases was it found necessary to correct the data for changes in liposome viscosity as outlined in equation 28; in both cases it was found that for labeled fusing with labeled vesicles the E/M fluorescence intensity ratio was constant with time.

Figure 1. Plot of the excimer-to-monomer fluorescence intensity ratio E/M (normalized to its initial value) as a function of time for pyrene-PC (initially at 4 mole%) in DPPC SUVs in low salt buffer. The lipid concentration was 0.68 mM and the lipid-to-melittin molar ratio was 60. The temperature was 51°C. The buffer was 50 mM Tris, 1 mM EDTA, pH 7.6, in which melittin is monomeric in solution. The individual excimer and monomer fluorescence intensities were corrected for any contributions due to scattered light before the E/M ratio was calculated. The insert shows the experimental $[F]/X_0$ data as obtained from the normalized E/M ratio via equation 26, and the best fit of equation 11 to those data. This yielded a value of $f = 0.006 \text{ s}^{-1}$ for the rate constant for fusion.

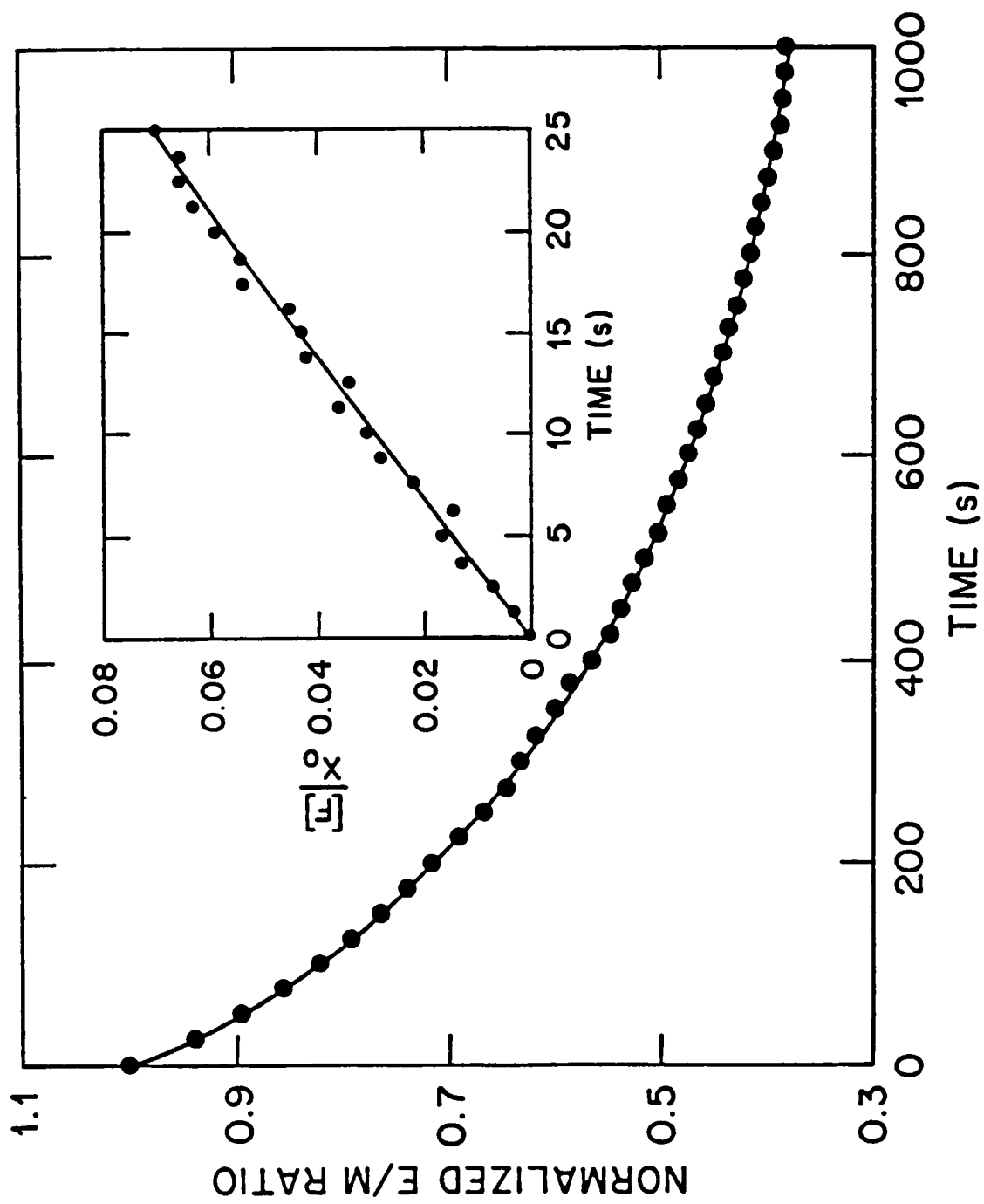
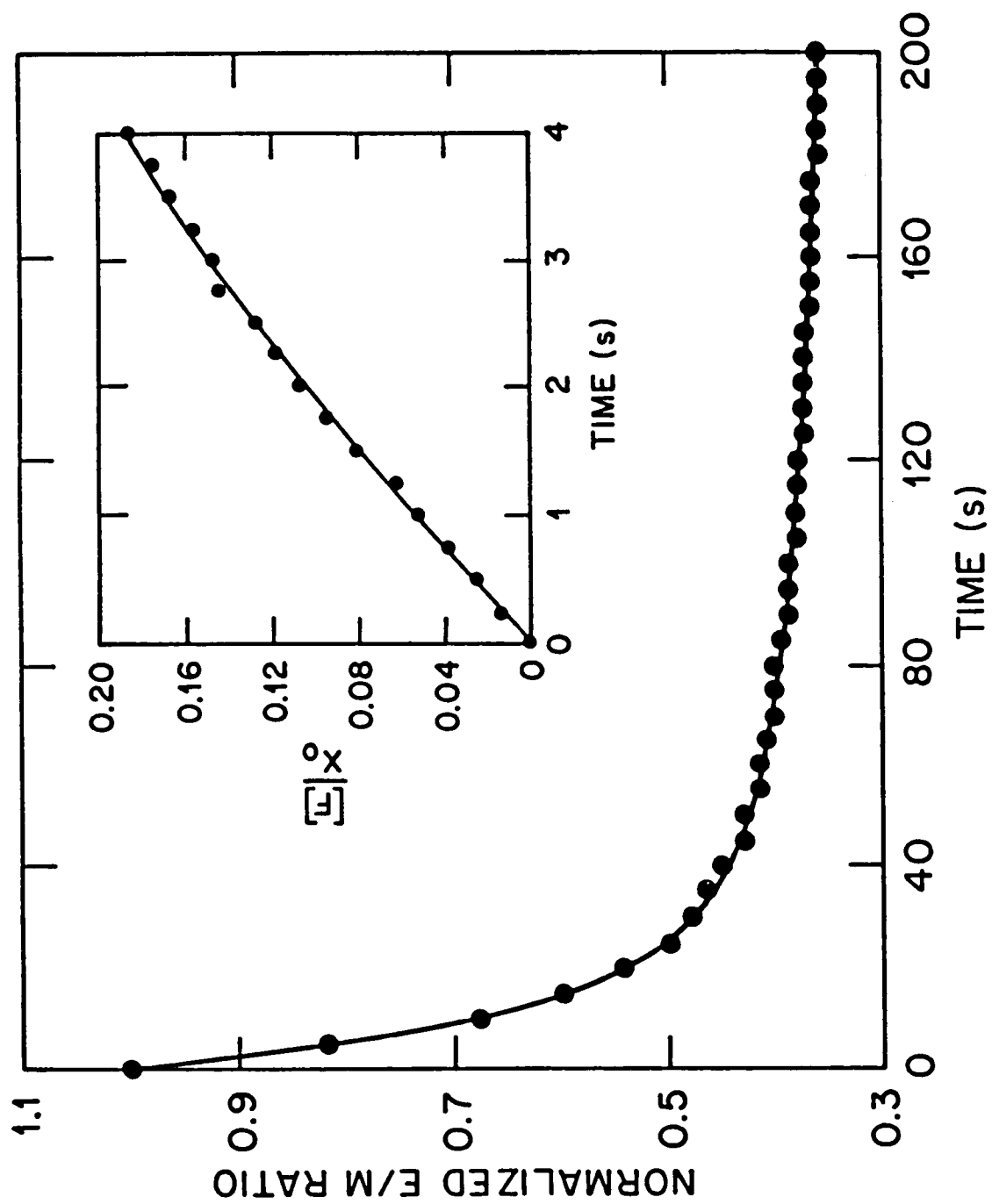


Figure 2. Plot of the excimer-to-monomer fluorescence intensity ratio E/M (normalized to its initial value) as a function of time for pyrene-PC (initially at 4 mole%) in DPPC SUVs in high salt buffer. The lipid concentration was 0.68 mM and the lipid-to-melittin molar ratio was 60. The temperature was 51°C. The buffer was 50 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.6, in which melittin is tetrameric in solution. The individual excimer and monomer fluorescence intensities were corrected for any contributions due to scattered light before the E/M ratio was calculated. The insert shows the experimental $[F]/X_0$ data as obtained from the normalized E/M ratio via equation 26, and the best fit of equation 11 to those data. This yielded a value of $f = 0.12 \text{ s}^{-1}$ for the rate constant for fusion.



Measurements were also made for a mixture of 60 mM lipid and 1 mM melittin (concentrations before mixing, data not shown). In this case melittin is tetrameric in solution. An analysis of the data, made using equation 27, yielded a value for $f = 0.06 \pm 0.006 \text{ s}^{-1}$. As was noted above in the Materials and Methods section of this part, no correction of the data for viscosity effects could be made in this case, though this should not be a serious source of error.

Because of the relatively high lipid concentrations used in the present study, the values for the rate constant for aggregation could not be accurately determined; this would require much lower lipid concentrations in order to reduce the rate at which aggregation takes place. The concomitant reduction in the pyrene-PC fluorescence which thus resulted made such measurements impractical at the time this work was carried out.

DISCUSSION

As was stated above in the Results section of this part, the rate constant k for fusion in the case of monomeric melittin in solution was found to be 0.006 s^{-1} , whereas that for melittin in 2 M NaCl (in which case the protein is tetrameric in solution) was 0.12 s^{-1} . That the rate constants differ by a factor of twenty strongly suggests that the mechanisms for monomeric and tetrameric melittin are different. The presence of a high concentration of salt ions in the case of tetrameric melittin, however, may have also played a role in increasing the rate of fusion in that case. Ions in solution may help shield charges on the protein and on the lipid headgroups and so aid in the dehydration of the space between two apposed vesicles in an aggregate. (The dehydration of the intervesicle space is a major step in the vesicle fusion process in terms of the energy required to carry out the former.) The presence of salt ions while the SUVs were being sonicated and annealed may also have resulted in the formation of vesicles which possess a somewhat different morphology from those formed in a low salt buffer. Therefore, in order to try to delineate the effects that ionic strength and melittin conformation have on the rate (and presumably the mechanism) of vesicle fusion, experiments were carried out in a low salt buffer and at a protein concentration for which melittin is tetrameric in solution due to its concentration alone. In that case, the rate constant for fusion was found to be 0.06 s^{-1} , *i.e.*, half that for tetrameric melittin in 2 M NaCl, and ten times that for monomeric melittin in solution. This result lends strong support to the interpretation that the enhancement of the fusion rate in the presence of 2 M NaCl is indeed due to a

combination of ionic effects and effects due to the protein's state of aggregation. Whether these effects are additive or synergistic cannot be said at this point.

A subsequent study (Bradrick *et al.*, 1989) which employed high-sensitivity differential scanning calorimetry (DSC) also obtained evidence that the fusion mechanisms are different for monomeric melittin in a low-salt solution and tetrameric melittin in 2 M NaCl. There, the lipid melting profiles for dipalmitoylphosphatidylcholine (DPPC) fused unilamellar vesicles (FUVs, 1000 Å diameter) were measured for a broad range of lipid-to-protein molar ratios R (from $R = \infty$ down to $R = 20$) for both low-salt and high-salt solutions. The percentages of melittin bound as a function of R were also determined for these two different salt concentrations.

In the case of the low salt solutions it was found that saturation binding occurs at about $R = 100$. Interestingly, however, it was seen that the lipid melting profile continued to change or evolve as one went to increasingly lower values of R below 100 (even though the amount of bound melittin did not change) to the extent that at $R = 20$ the melting profiles clearly included multiple peaks. These multiple peaks were interpreted as being due to a heterogeneous population of lipid structures that had been formed through melittin-induced vesicle fusion. The increase in total melittin added in going to lower values of R only resulted in an increase in the amount of free melittin in solution, however. Thus different amounts of free melittin seemed to coincide with fusion taking place to different extents and suggested that free melittin in solution plays a role in the fusion mechanism in the case of monomeric melittin. It was suggested (Bradrick *et al.*, 1989) that a plausible mechanism for the enhancement of fusion by free melittin might

be through the formation of protein bridges between apposed vesicle in an aggregate, in which the positively-charged N- and C-termini of the protein might become anchored on the charged headgroups of the outer surfaces of the two bilayers. Positioned as such, the protein could exclude water molecules from the intervesicle region and also perhaps perturb (along with any bound proteins) the two bilayers so that the adjacent bilayers could be disrupted and join together, possibly by "climbing" across the protein bridge. Bridge formation was previously suggested to explain how the Ca^{2+} -induced fusion of charged vesicles is facilitated by the adrenal medullary protein synexin (Hong *et al.*, (1981) and also how poly(L-lysine) causes the fusion of vesicles composed of dipalmitoylphosphatidylglycerol (Carrier and P  zolet, 1986).

In the case of tetrameric melittin in high-salt solution, the binding measurements indicated that the protein was virtually all bound over the whole range of R values examined. As for the case of monomeric melittin in solution, however, the DSC lipid melting profiles were found to evolve with increasingly lower values of R. As in the case of the low-salt solutions, these multiple peaks were interpreted as being indicative of a heterogeneous population of lipid structures formed through the fusogenic action of the protein. [It has been shown (Dufourcq *et al.*, 1986) through electron microscopy, light scattering and gel filtration that for zwitterionic small unilamellar and large multilamellar vesicles a heterogeneous population of vesicular and nonvesicular lipid structures is formed when $R < 30$.] The multiple peaks in the melting profiles were clearly different from those for the case of the low-salt solutions for (relatively) low R values. These results suggest that the fusion mechanism for monomeric and tetrameric melittin in

solution are different, in line with the results of the present kinetics study. As was stated above in the case of the present study, salt effects may have played a role in enhancing the fusion process in the case of the DSC study. In that case, however, such effects and those due to the state of protein aggregation could not be delineated as was attempted in the present study. The formation of a sample of DPPC FUVs at a high enough lipid concentration to have allowed the use of a high concentration of tetrameric melittin in a low-salt solution was not possible.

In order to gain a greater understanding of the role played by salt ions in the fusion kinetics reported here, it would presumably be helpful to examine liposome morphology using electron microscopy to determine whether the preparation of liposomes in the presence of 2 M NaCl results in the formation of structures which differ greatly from those prepared in the absence of salt. If the annealing of lipid vesicles in the presence of 2 M NaCl has an effect on subsequent liposome morphology, the vesicles could presumably be formed in a low salt buffer and then dialyzed above their lipid phase transition temperature T_m against the same buffer which contained 2 M NaCl. This would not, however, eliminate any effects on lipid packing density which might result from the shielding of lipid headgroup charges by ions in solution.

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PART III

EFFECTS OF MELITTIN BINDING ON THE FLUIDITY AND PERMEABILITY OF PHOSPHOLIPID SMALL UNILAMELLAR VESICLES

INTRODUCTION

In recent years the interaction of the peptide melittin from bee venom with phospholipid vesicles has attracted considerable attention for the following reasons: (i) there is compelling evidence that links this association with the physiological effects of the protein, and (ii) the results of experiments are of broader importance, as the active conformation of melittin in membranes [an amphipathic α -helix (Dawson *et al.*, 1978)] may pertain not only to cytotoxins but also to apolipoproteins and peptide hormones (Degrado *et al.*, 1982) as well as to signal peptides (Garnier *et al.*, 1980).

As was outlined above in the General Introduction, melittin possesses a broad range of physiological effects and a number of experimental techniques have been employed in order to try to obtain information about the effects the protein has on the order and fluidity of the lipid acyl chains, and on the lipid head groups. All of these techniques, however, involve making measurements on samples which, of necessity, have been previously incubated with melittin and have thus undergone melittin-induced vesicle fusion. Consequently, any changes observed in these bilayer properties as measured by these techniques are presumably a combination or convolution of effects due to the insertion of the protein into the bilayer and effects due to an increase in vesicle size (and thus presumably lipid order) brought about by fusion. Therefore, in order for the effects of the protein to be understood at the molecular level, the primary events in the melittin-phospholipid interaction must be identified prior to the occurrence of melittin-induced fusion.

In the present study the question is addressed as to what is the effect of melittin on the fluidity of phospholipid bilayers, a property which is thought to be essential to their function. The measurements reported herein involved following as a function of time the effects of melittin on the fluorescence anisotropy and the total fluorescence of TMA-DPH incorporated into the bilayer as a fluorescent probe, following stopped-flow mixing of the lipid with melittin. TMA-DPH is a cationic analog of the fluorescent probe DPH (diphenylhexatriene) which, unlike the latter, is an oriented probe that anchors itself to the bilayer-water interface and aligns itself parallel to the normal to the bilayer. There, it reports on the order and dynamics of the glycerol backbone and the uppermost region of the bilayer. Moreover, because the fluorescence of the probe is enhanced upon going from a more polar to a less polar environment (Prendergast *et al.*, 1981) TMA-DPH can give us important information about the accessibility of water to the interior of the bilayer. Since melittin binding takes place on the millisecond time scale (Schwarz and Beschiaschvili, 1989, Sekharam *et al.*, 1991) whereas melittin-induced vesicle fusion takes place on the seconds to minutes time scales (Bradrick and Georghiou, 1987) stopped-flow fluorometry can be used to measure the effect of melittin binding on TMA-DPH in the absence of the complications that arise from fusion.

In the present study, measurements were made for DMPC and DPPC SUVs for temperatures below, at and above the respective thermal transition temperatures of these lipids in order to explore what effect acyl chain length and the physical state of the bilayer has on the extent of melittin-induced changes in the bilayers. Measurements were

also made in order to try to determine whether or not the perturbing effects of melittin are long range in the lipid bilayer.

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) of a purity greater than 99% were obtained from Avanti Polar Lipids (Alabaster, AL); melittin was obtained from Sigma Chemical Co. (St. Louis, MO). All three were used without further purification. 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, p-toluenesulfonate (TMA-DPH) was a product of Molecular Probes (Eugene, OR). Tris and ethylenediaminetetraacetic acid-disodium salt (EDTA) were analytical grade and obtained from Fisher (Pittsburgh, PA) and Sigma, respectively. N,N-dimethylformamide was from Mallinckrodt (Paris, KY). The buffer used was 50 mM Tris, 1 mM EDTA, pH 7.6 prepared in triply distilled water. For the melittin concentrations used in these experiments, EDTA at a concentration of 1 mM is sufficient to eliminate the activity of any residual phospholipase A₂ in the sample (Dasseux *et al.*, 1984 and Bradrick and Georgiou, unpublished observations).

Each day's experiments were performed with a freshly-prepared sample of DMPC or DPPC small unilamellar vesicles (SUVs) which was made in the following way. For those experiments in which the change in the fluorescence anisotropy of TMA-DPH was monitored during melittin binding to the liposomes, a stock lipid dispersion with a concentration of 5.9 mM was formed by hydrating the appropriate amount of lipid in 10 ml of buffer at a temperature 10°C above the phase transition temperature T_m of that particular lipid. (*i.e.*, hydration was done at 33°C in the case of DMPC and at 51°C in the case of DPPC.) To this dispersion was added 120 μ l of a 1 mM solution of

TMA-DPH in N,N-dimethylformamide. The resulting lipid:fluorescent probe ratio was about 500:1; this ratio was chosen so as to minimize perturbation or distortion of the bilayer by the probe molecule. The lipid dispersion was then stirred for 30 minutes at 10°C above T_m in order to allow incorporation of the fluorescent probe into the lipid bilayers. (The formation of the TMA-DPH stock solution in N,N-dimethylformamide as well as all subsequent steps which involved the formation and use of the TMA-DPH-labeled liposomes, were carried out under cover of dim red light so as to avoid photobleaching the probe.) The liposomes were then sonicated in a Laboratory Supplies Co. (Hicksville, NY) bath sonicator at either 33°C (for DMPC) or 45°C (for DPPC) for two twenty-minute periods separated by a five minute rest. This was followed by another 30 minute rest during which time the resulting small unilamellar vesicles (SUVs) were allowed to anneal. Finally, the sample was centrifuged at either 33°C or 45°C for 25 minutes in order to pellet any remaining multilamellar vesicles. After centrifugation, the supernatant was removed (under cover of darkness) and stored in a screwcap polystyrene tube for subsequent use. At that time, aliquots of the lipid sample were removed and diluted by a factor of two to give a lipid concentration of 2.95 mM. This sample was then loaded into the stopped-flow device where, upon mixing with an equal volume of a melittin solution, the final lipid concentration in the stopped-flow cell or cuvette was reduced to 1.485 mM. The resulting concentration of TMA-DPH in the cell was then such that it had an absorbance of about 0.06 at the excitation wavelength of 365 nm. For this absorbance, fluorescence intensity is proportional to probe concentration.

Lipid samples for the measurement of the baseline scattered light solution to TMA-DPH fluorescence anisotropy, or for the measurement of the kinetics of melittin binding (based on the resulting change in melittin tryptophan fluorescence) were prepared as described above with the exception that they were not labelled with TMA-DPH. Some (purely) lipid samples were made, however, with the addition of the appropriate amount of the TMA-DPH solvent N,N-dimethylformamide in order to determine whether its presence had a measurable effect on the behavior or characteristics of the liposomes and the kinetics of melittin binding to them. It was found that there was no difference in sonication time, amount of scattered light or the kinetics of melittin binding, between solutions which did contain N,N-dimethylformamide and those which did not. Therefore, all subsequent measurements which involved nonlabeled liposomes were carried out on samples which did not contain the TMA-DPH solvent.

Initially, the baseline contributions of scattered light to polarized measurements of TMA-DPH fluorescence were made by using a nonlabeled lipid solution as described above. After a number of such measurements, however, sufficient information had been obtained to determine that for vertically polarized exciting light and the horizontal component of fluorescence emission, the ratio of scattered light (baseline) to fluorescence signal was 3.4% in the case of DMPC SUVs and 12.4% in the case of DPPC SUVs. (In the case of both DMPC and DPPC SUVs, the horizontal component of TMA-DPH fluorescence was flat over the time during which change was observed in the vertical component. When they were taken, the baselines were of course also flat. Consequently, these percentages are based on the simple averages of the appropriate data files. It was

also found that the ratio of the vertical (emission) baseline to that of the horizontal was 1.22 in the case of DMPC and 2.01 in the case of DPPC. Therefore, for most experiments, baselines due to scattered light were not measured but were instead calculated using the above percentages and the average values of the (flat) horizontal components of the TMA-DPH fluorescence for that particular experiment.

Melittin stock solutions were prepared by weighing a quantity of lyophilized melittin (typically between 10 and 15 mg) and dissolving it in 10 ml of buffer. The concentration of this stock solution was determined spectrophotometrically on a Shimadzu UV-265 spectrophotometer given that $\epsilon_{280} = 5800 \text{ M}^{-1} \text{ cm}^{-1}$ (Berlman, 1971). Melittin solutions of the desired concentration were formed by diluting aliquots of this stock solution by the appropriate amount. Stock solutions were not used beyond five days after their formation.

Stopped-flow fluorometric measurements were carried out on an Aminco DW-2 UV/Vis spectrophotometer equipped with a stopped-flow module which had been modified to allow fluorescence to be measured at right angles to excitation. Sample temperature was controlled with a circulating water bath. The exciting light was provided by a Spectral Energy (Westwood, NJ) 600 W Xe-Hg arc lamp. In the case of measurements made of the polarized fluorescence of TMA-DPH, the lamp output was first attenuated by positioning neutral density filters in the beam. (This was necessary in order to avoid photobleaching the fluorophore. The extent to which the beam needed to be attenuated was determined by observing the time course of the fluorescence intensity of TMA-DPH-labelled liposomes mixed with buffer in the stopped-flow. Since this signal

should be flat, the intensity of the exciting light was reduced until this fluorescence signal remained constant over the same time scale during which kinetics was observed when labelled liposomes were mixed with melittin.) The exciting beam then passed through a Corion 365 nm (7 nm bandwidth) interference filter to select the excitation wavelength, and a Karl Lambrecht Corp. (Chicago, IL) SQWO depolarizer. The presence of the latter (as well as that of a Karl Lambrecht Corp. SQLO depolarizer on the emission side) removed any polarization bias that the stopped-flow's optical system might otherwise have had, see below. Finally, the light passed through a DaLite (Cincinnati, OH) 105UV polarizer which was used to select either vertically or horizontally polarized light for exciting the sample. The sample fluorescence was viewed by an EMI 6256S photomultiplier through a Schott KV418 cut-off filter (with 50% transmittance at 418 nm) after having first passed through the Karl Lambrecht Corp. SQLO depolarizer mentioned above. The photomultiplier output was digitized by an IBM PC equipped with an Analog Devices (Norwood, MA) RTI-815 A/D converter.

For each "run" of (approximately) twelve stopped-flow "shots" or injections, melittin and lipid samples of the desired concentration were loaded into the sample reservoirs and allowed to come to thermal equilibrium over a period of ten minutes. Three shots were then taken, the last of which was allowed to remain in the stopped-flow cell or cuvette for five minutes in order to allow its fluorescence to bring the photomultiplier response up to its steady-state level. Vertical and horizontal fluorescence components were measured separately. For each loading of samples into the stopped-flow device, data for about six shots were then taken with the analyzer first oriented vertically.

(Photomultiplier voltage was adjusted so as to give the maximum signal possible within the range of the A/D converter.) The analyzer was then rotated to the horizontal position, after which approximately six more shots were taken. This procedure was also followed when nonlabeled liposomes and buffer were loaded into the stopped-flow device for making baseline measurements.

In the case of measurements made of the kinetics of melittin binding to DMPC and DPPC SUVs, lipid samples were prepared as described above with the exception that they were not labelled with TMA-DPH. Stopped-flow kinetic measurements were also made as described above. In this case, however, the DaLite polarizer and analyzer were removed. Also, the exciting-light wavelength was chosen by placing a 280 nm (10 nm bandwidth) interference in the lamp beam. Fluorescence was viewed through a Corning 0-51 glass cut-off filter. Measurements were made at the same temperature and protein and lipid concentrations as for TMA-DPH fluorescence anisotropy measurements.

Experiments were also carried out to determine whether energy transfer could take place between excited tryptophan residues of membrane-bound melittin molecules (the donors) and TMA-DPH (the acceptors). TMA-DPH-labelled liposomes were prepared as described above, as were melittin stock solutions. Stopped-flow kinetic measurements were also made as described above, except that neither a polarizer nor an analyzer was included in the system optics. Melittin was excited using a 280 nm interference filter in the exciting light beam. Fluorescence was viewed through a Schott KV418 cut-off filter.

The change in polarized fluorescence of membrane-bound TMA-DPH upon melittin binding was analyzed in the following way. As was stated above, the horizontal

fluorescence component was invariably found to be flat. Individual averages were found for each of the (approximately) six data files that were collected for the horizontal component. These individual averages were in turn averaged together and the individual file with an average closest to that of the group was then taken as being representative of the horizontal fluorescence. Horizontal and vertical baseline components were then calculated (as described above) based on the group average. The time-dependent experimental fluorescence anisotropy of TMA-DPH was then calculated as follows. First, the data file for the representative file for the horizontal fluorescence component was read into the nonlinear least-squares fitting program, at which time the average horizontal background component was subtracted out. Then, each of the six data files for the vertical fluorescence component was read into the program individually, at which point the calculated vertically background average was subtracted out. As this operation was performed for each of the vertical data file, the time-dependent steady-state fluorescence anisotropy was calculated using the equation

$$r = \frac{I_{VV} - \left(\frac{I_{HV}}{I_{HH}} \right) I_{VH}}{I_{VV} + 2 \left(\frac{I_{HV}}{I_{HH}} \right) I_{VH}} \quad (1)$$

Here, r denotes the time-dependent fluorescence anisotropy and I denotes the fluorescence intensity. The subscripts V and H denote vertical and horizontal polarization orientation, respectively, where, the first subscript pertains to the polarization orientation of the exciting light and the second subscript pertains to the orientation of the fluorescence

component. The presence of the ratio I_{HV}/I_{HH} is to account for any polarization bias that the detection system might have. The presence of depolarizers in the stopped-flow system's optical system removed any such bias; for these data I_{HV}/I_{HH} was found to equal unity. After the time-dependent fluorescence anisotropy was calculated for each of the six vertical fluorescence component data files, these six time-dependent steady-state anisotropy vectors were simultaneously fitted with the equation

$$r(t) = (r_o - r_\infty)\exp(-k_1 t) + r_\infty \quad (2)$$

Here, r_o is the fluorescence anisotropy at zero time, r_∞ is the fluorescence anisotropy at long times and k_1 is the observed or effective rate constant for change. (In all cases it was found that the anisotropies were well described by a decaying exponential plus a constant, as judged from the random shape of the residuals for the fits.) For each temperature and lipid-to-protein molar ratio R , five separate experiments, made using five separately-prepared samples, were typically performed. The individual values for r_o , r_∞ and k_1 were averaged, and it is these averages which are reported below.

The time-dependence of the total fluorescence of TMA-DPH was also analyzed. Data for the horizontal and vertical fluorescence components were read into a nonlinear least-squares fitting program and corrected for scattered light as described above for the fluorescence anisotropy. In this case, the total fluorescence intensity was calculated for each of the six I_{VV} files from

$$F(t) = I_{VV} + 2I_{VH} \quad (3)$$

The six total fluorescence intensity vectors thus calculated were then simultaneously fitted

with the equation

$$F(t) = A \exp(-k_2 t) + B \quad (4)$$

As for the case of fluorescence anisotropy (above), the results for k_2 from five separate experiments were averaged and reported below.

Data for the kinetics of melittin binding (observed via the change in tryptophan fluorescence intensity that results from binding) were analyzed by simultaneously fitting equation 4 to the (typically) twelve data files which resulted from each loading of sample into the stopped-flow device for a particular temperature. As in the case for TMA-DPH polarized fluorescence, measurements for a particular lipid-to-protein molar ratio R and temperature were typically carried out on five separately-prepared lipid samples, the results from which were averaged.

Energy transfer from the tryptophan residue of melittin to TMA-DPH was measured by exciting melittin at 280 nm and looking for either a loss in melittin fluorescence (as compared to that in the absence of TMA-DPH labeling) or else fluorescence from TMA-DPH itself. R_0 , the critical distance for energy transfer, was calculated to be about 35 Å. Published spectra for the donor and acceptor (Berlman, 1971) were used to calculate the overlap integral between the donor fluorescence spectrum and the acceptor absorption spectrum as described by Birks (1970).

RESULTS

As was stated above in the Introduction to this section, the purpose of this study was to determine the effects that melittin binding has on phospholipid vesicles prior to the occurrence of melittin-induced vesicle fusion. Experiments were carried out for two different lipids at temperatures below, at and above their respective phase transition temperatures T_m in order to explore what effect such lipid parameter as acyl chain length and degree of flexibility have on the extent of melittin-induced changes in the bilayer. Typical results from such an experiment are shown in Figures 1 and 2. In Figure 1, the upper panel shows a plot of the fluorescence anisotropy r (for steady-state excitation) of TMA-DPH as a function of time for melittin binding to DMPC SUVs at 23°C (T_m) for a lipid-to-melittin molar ratio of 90. The upper panel shows the values for r , calculated from the individually-measured vertical I_{VV} and horizontal I_{VH} fluorescence components using equation 1. The smooth line through the data is the best fit to the data of equation 2, which yielded values of 0.213, 0.242 and 11.8 s⁻¹ for the initial (r_0) and final (r_∞) anisotropies and the rate of change k_1 or r , respectively. In Figure 2, the upper panel shows a plot of the total fluorescence intensity F (also for steady-state excitation) of TMA-DPH as a function of time, calculated (using equation 3) from the same fluorescence intensity components I_{VV} and I_{VH} that went into calculating the fluorescence anisotropy r above. In this case, equation 4 was fit to the data; this fit is shown by the smooth line in the upper panel.

Figure 1. Effect of melittin binding as a function of time on the steady-state fluorescence anisotropy r of TMA-DPH incorporated into DMPC small unilamellar vesicles. The buffer was 50 mM Tris, 1 mM EDTA, pH 7.6. The lipid concentration was 1.485 mM, the lipid-to-melittin molar ratio was 90 and the sample temperature was 23°C. The upper panel shows the fluorescence anisotropy r , calculated from the individual vertical and horizontal fluorescence components using equation 1 and the best fit to the data (smooth line) of equation 2. This analysis yielded values of 0.213, 0.242 and 11.8 s⁻¹ for the initial (r_0) and final (r_∞) anisotropies and the rate of change k_1 , respectively. The lower panel shows the residuals of the fit. That the residuals are random is taken to indicate that the fit is good.

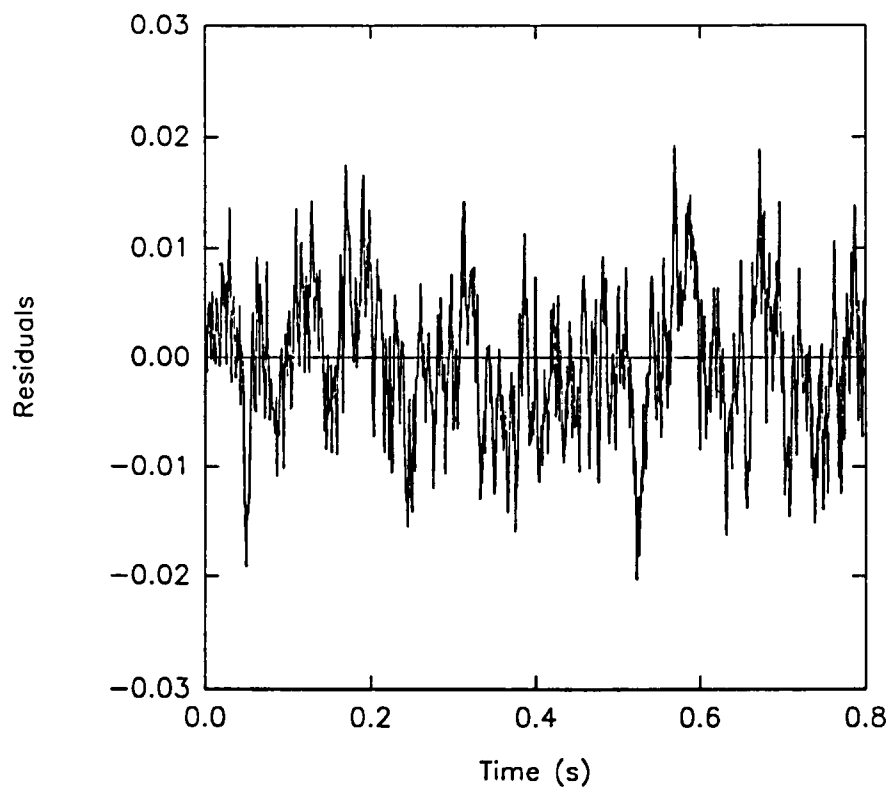
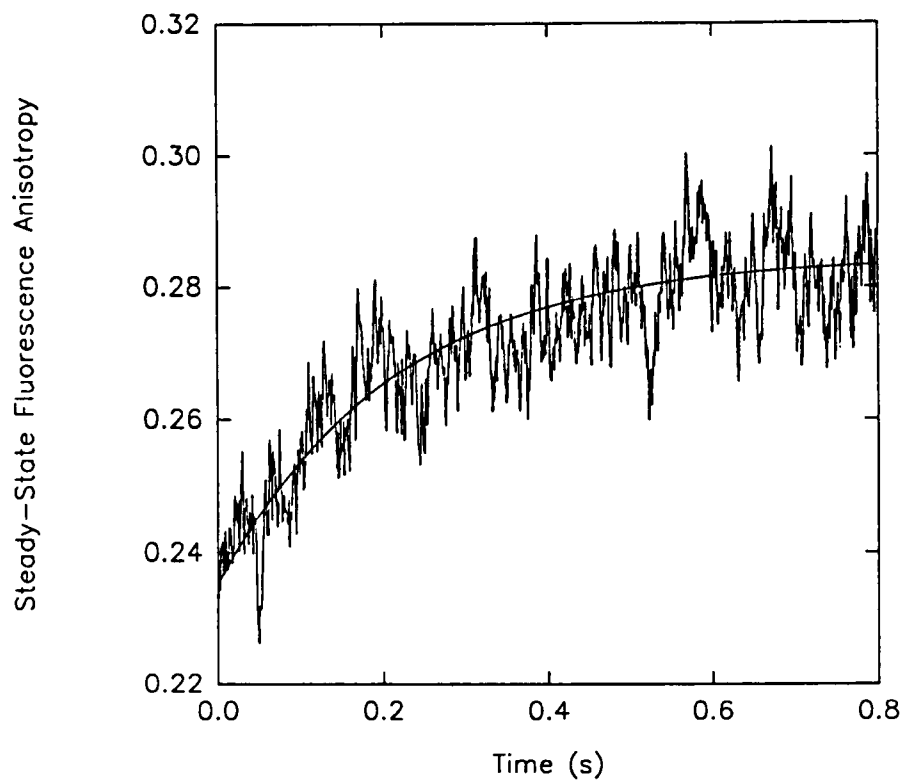
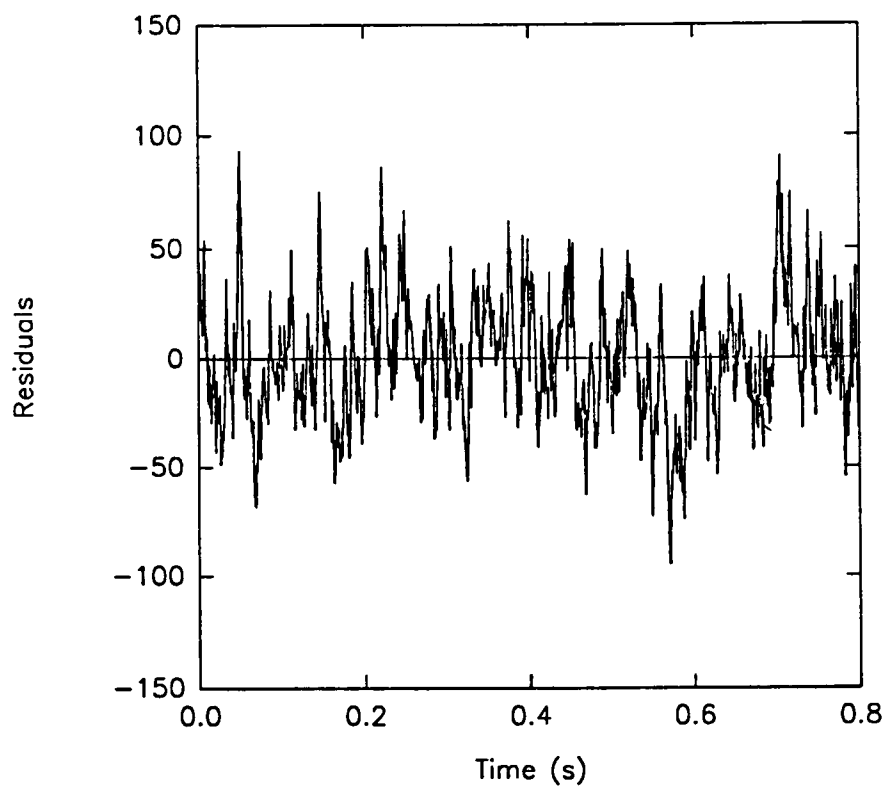
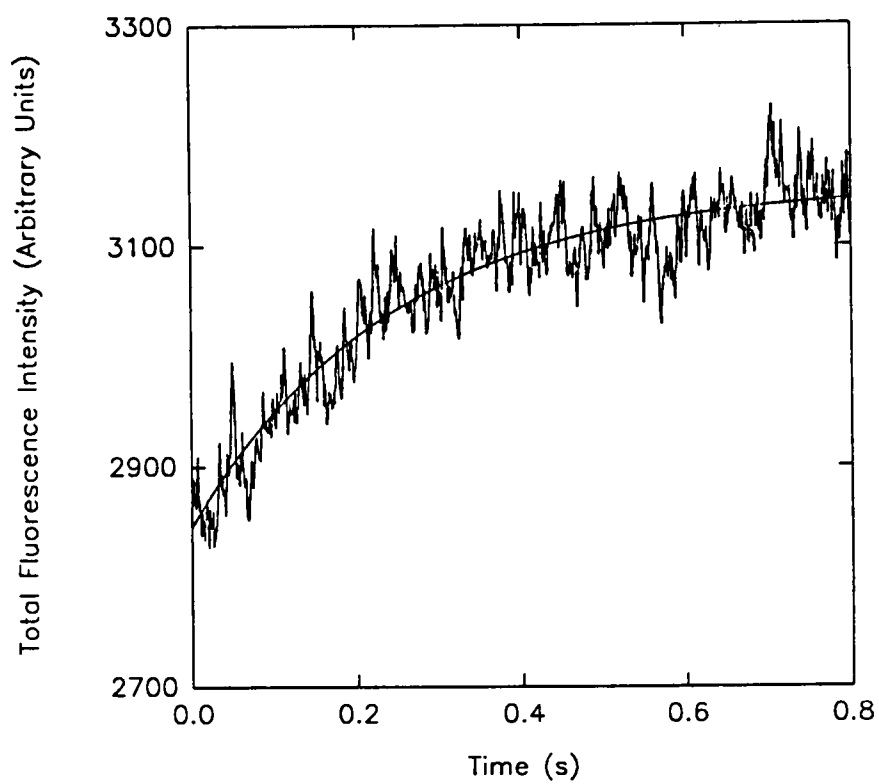


Figure 2. Effect of melittin binding as a function of time on the steady-state fluorescence intensity F of TMA-DPH incorporated into DMPC small unilamellar vesicles. The buffer was 50 mM Tris, 1 mM EDTA, pH 7.6 and the sample temperature was 23°C. The upper panel shows the total fluorescence intensity F , calculated from the individual vertical and horizontal fluorescence components using equation 3 and the best fit to the data (smooth line) of equation 4. This analysis yields a value of 11 s^{-1} for the rate of change k_2 of $I_V + 2I_H$. The lower panel shows the residuals from this fit. That the residuals are random is taken to indicate that the fit is good.



The results of the measurements of the melittin-induced changes in TMA-DPH anisotropy and total fluorescence are summarized in Table 1. There it will be noted that for each temperature, measurements were made for four different lipid-to-protein molar ratios, R (specifically, $R = 60, 90, 135$ and 180). This was done in order to explore the effects of varying amounts of bound melittin on bilayer fluidity and permeability.

Table 2 summarizes the results of measurements of the kinetics of melittin binding, for the same temperatures and absolute protein and lipid concentrations as used in the measurements reported in Table 1. These measurements were carried out by following the decrease in tryptophan fluorescence due to the change in environment as the protein inserts itself into the bilayer. In the case of melittin binding to DPPC SUVs, the results analogous to those for DMPC are presented in Table 3.

For the case of DMPC SUVs at 21°C , it can be seen from Table 1 that for all four lipid-to-protein molar ratios R , the fluorescence anisotropy r of TMA-DPH exhibits an overall increase of about 14% upon melittin binding, which signifies a reduction in the fluidity of the bilayer in the vicinity of the fluorescent probe, *i.e.* at the interfacial region and the uppermost region of the bilayer. Another quantity of interest is the total fluorescence intensity $I_v + 2I_H$ of the fluorescent probe. This quantity has been found to increase with time in the presence of melittin with a rate k_2 that is within the experimental error identical to that obtained from the increase in r with time. The overall increase in this quantity was found to be about 8%, 7%, 7% and 6% for $R = 60, 90, 135$ and 180 , respectively. It should be noted that the initial values of $I_v + 2I_H$ and of r for these experiments were found to be the same as those in the absence of melittin for all four

TABLE 1

Effects of melittin on the kinetics of the fluorescence anisotropy r and the total fluorescence $I_V + 2I_H$ of TMA-DPH in DMPC small unilamellar vesicles.

Lipid/melittin molar ratio R	k_1 (s ⁻¹)*	21°C§		33°C k_2 (s ⁻¹)†
		r_o	r_∞	
60	10.1±3.5	0.220±0.006	0.255±0.012	26±2
90	7.3±1.4	0.213±0.006	0.242±0.007	25±3
135	4.8±0.7	0.212±0.004	0.242±0.008	17±3
180	3.4±0.7	0.220±0.005	0.245±0.008	0 (flat)

*Rate of increase of the fluorescence anisotropy r , which was found within experimental error to be identical to the rate k_2 of increase of the total fluorescence intensity. r_o and r_∞ are the initial and final values of r .

§Phase transition (melting) temperature T_m of DMPC SUVs.

†Rate of increase of the total fluorescence intensity. The fluorescence anisotropy at 33°C was found to be independent of time on the millisecond time scale.

TABLE 2

Rate of melittin binding (s^{-1}) to DMPC small unilamellar vesicles as determined by measuring the rate of decrease of melittin fluorescence while binding takes place.

Lipid/melittin molar ratio R	16°C	21°C (T_m)	33°C
60	3.1±0.2	8.1±1.4	42±4
90	5.5±0.3	6.8±1.0	35±4
135	5.0±0.3	7.3±1.0	30±4
180	9.0±1.0	7.4±1.0	30±3

values of R . This observation implies that no changes are induced in the bilayer by the protein within the deadtime of the stopped-flow fluorometer (about 3 ms).

As can be seen by comparing the values in Table 1 and Table 2, the rate of increase in TMA-DPH anisotropy k_1 (Table 1) and the rate of decrease in melittin fluorescence (Table 2) at 21°C are in agreement to within their uncertainties for all four lipid-to-protein molar ratios except $R = 180$ and $R = 135$.

It was found that at 33°C, for all four values of R , the TMA-DPH fluorescence anisotropy was practically independent of time. This implies that the rate k_1 is very small. This is in contrast to the increase with time that was observed in the total fluorescence $I_V + 2I_H$ of the probe. This increase was found to be 7%, 6%, 4% and 0% for $R = 60, 90, 135$ and 180 , respectively. The values of the rate k_2 obtained from such plots are listed in Table 1.

At 16°C, no changes in either r or $I_V + 2I_H$ were detected within the time scale of 4 s. As was the case at 21°C and at 33°C, changes in these parameters were detected on the much longer time scales over which melittin-induced fusion of vesicles has been reported (Bradrick and Georgiou, 1987). Concerning the rate of decrease in melittin fluorescence with binding at this temperature (Table 2), the observed trend with R values is the reverse of what would be expected: the rate of binding decreases as lower values of R (and higher concentrations of melittin) are attained.

Measurements similar to those described above were carried out for DPPC SUVs (Table 3). It was found that there was virtually no change in TMA-DPH fluorescence intensity or anisotropy at 25, 37 (the lipid phase transition temperature T_m) or 51°C for

TABLE 3

Rate of melittin binding (s^{-1}) to DPPC small unilamellar vesicles as determined by measuring the rate of decrease of melittin fluorescence while binding takes place.

Lipid/melittin molar ratio R	25°C	37°C (T_m)	51°C
60	27.6±3.6	38±7	25±4
90	40.9±6.2	42±6	24±7
135	79.4±13.6	82±14	99±32
180	124.5±10.8	123±25	99±20

the four lipid-to-protein molar ratios R of 180, 135, 90 and 60. (Only in the total fluorescence intensity at 51°C for the two lowest R values of 90 and 60 was any change observed with melittin binding, for which rate constants of $10.9 \pm 1.9 \text{ s}^{-1}$ and $19.8 \pm 3.7 \text{ s}^{-1}$ and increases of about 10% and 15% obtained, respectively.) At this temperature and for these two ratios there is disagreement between the rates of increase in TMA-DPH fluorescence and decrease in protein fluorescence. For both ratios, the former is less than the latter (11 versus 24 s^{-1} for $R = 90$ and 20 versus 25 s^{-1} for $R = 60$, which differences are outside the experimental errors).

In the case of the measurements for energy transfer from melittin to TMA-DPH, we found virtually no difference in the fluorescence signals of either melittin or TMA-DPH. This indicates that the average donor-acceptor distance is greater than the value of $35 \text{ \AA}$ that was calculated for R_0 , the critical distance for energy transfer.

DISCUSSION

As was pointed out above, melittin has its greatest effect on DMPC SUVs at 21°C, the lipid phase transition temperature T_m . This finding is in agreement with the results of a recent theoretical study (Sperotto and Mouritsen, 1991) which concluded that membrane perturbation by a protein is maximal there. At T_m , both the fluorescence anisotropy and the total fluorescence of TMA-DPH increase with time. The former indicates that the protein has caused the membrane to become more ordered. The latter indicates that concomitantly with that, water is excluded from the interfacial region of the bilayer to a greater extent. [TMA-DPH is known to be sensitive to the presence of water in its environment (Prendergast *et al.*, 1981).] This may be a consequence of the fact that the order of the lipid acyl chains has increased and so their packing density is decreased, which may also allow an increase in packing density near the lipid headgroups. Little is known, however, about the relationship between these two parameters.

As was noted above, for the protein-to-lipid molar ratio R of 180 there is disagreement between the rates of change of fluorescence anisotropy of TMA-DPH and melittin fluorescence (Tables 1 and 2). For that ratio, TMA-DPH anisotropy decreases more slowly ($\sim 3 \text{ s}^{-1}$) than the rate at which protein insertion takes place ($\sim 7 \text{ s}^{-1}$). Perhaps at this ratio (for which the least amount of melittin had been added to the lipid sample) the average probe-to-bound protein distance is large enough that the melittin-induced perturbation of the bilayer is felt by fewer of the TMA-DPH molecules as protein insertion into the bilayer takes place. Nevertheless, that a 33% increase in the amount

of added melittin (to $R = 135$) is enough to almost mitigate this effect seems surprising and at this point cannot be explained (Tables 1 and 2).

The experimental results also indicate that the melittin-induced perturbation of the bilayer is felt virtually instantaneously by the probe molecule as judged from the fact that the rate of melittin binding (measured by the change in its fluorescence upon going from water into the bilayer) is the same as the rate of change in TMA-DPH fluorescence anisotropy. If the melittin-induced membrane perturbation is viewed as a compressional wave which travels outward from the point of protein insertion, the speed of propagation of this wave would be about 1500 m s^{-1} , given a typical lipid bulk modulus of $2 - 3 \times 10^9 \text{ N m}^{-2}$ and a lipid molar density of about 640 g cm^{-3} (Marsh, 1990). That the perturbation is long range can be judged from the fact that we observed no melittin-to-TMA-DPH energy transfer, for which we calculated a value of $35 \text{ \AA}$ for the critical distance for energy transfer (the distance at which the efficiency of energy transfer is 50%.) This result is also in agreement with the theoretical predictions of Sperotto and Mouritsen (1991). [Concerning the range of the melittin-induced bilayer perturbation reported by TMA-DPH, an approximate lower limit of $65 \text{ \AA}$ can be calculated in the following way. For a $300 \text{ \AA}$ -diameter SUV (with surface area of approximately $2.38 \times 10^5 \text{ \AA}^2$), assuming a value of $5 \text{ \AA}$ for the radius of the surface area associated with a lipid headgroup (Lis *et al.*, 1982 and Marsh, 1990), it follows that there are about 3600 lipid molecules in the outer leaflet of an SUV bilayer. For a lipid-to-probe (*i.e.* TMA-DPH) molar ratio of 500 as used here, this corresponds to having about 7 TMA-DPH molecules in the outer leaflet of the bilayer. If the probe molecules are considered to be uniformly distributed across

the membrane surface, then each probe molecule has a surface area associated with it which is equal to about $39,000 \text{ \AA}^2$. (If viewed as a square, this surface would be about $200 \text{ \AA}$ on each side.) For the case of the minimum lipid-to-protein molar ratio used here ($R = 60$) this translates into there being about 8 protein molecules associated with each TMA-DPH molecule in a bilayer leaflet. If one subdivides the $200 \text{ \AA} \times 200 \text{ \AA}$ square into nine units, each $66 \text{ \AA} \times 66 \text{ \AA}$, and places the TMA-DPH molecule into the center subdivision and the melittin molecules in the remaining eight subdivisions (this again assumes a homogeneous distribution of molecules), then the distance between the probe molecule and its nearest neighboring protein molecules would be about $66 \text{ \AA}$.]

As was stated above, at 33°C TMA-DPH fluorescence anisotropy is flat, although its total fluorescence does experience a general increase. Concerning the former, perhaps the bilayer is already sufficiently disordered above the phase transition temperature that the binding of melittin can have no further effect on it in terms of the fluidity of the lipid acyl chains. That the total fluorescence increases suggests, however, that the protein does have an effect at this temperature on the packing density of the bilayer so that water is further excluded from the bilayer. That for all four R values, k_2 is much larger at 33°C than at 21°C suggests that the accessibility of the interfacial region of the bilayer to water is somewhat reduced, although, unlike the situation at 21°C , the fluidity of that region is not significantly affected by the binding of melittin. At this temperature (33°C), the rates of increase of TMA-DPH fluorescence (Table 1) are similar to or somewhat larger than (depending on the value of R) the rates of decrease in melittin fluorescence (Table 2).

The results at 16°C for DMPC SUVs indicate that melittin binding has no effect on the acyl chain order. Little is known about the binding of melittin to lipid vesicles which are in the gel phase. Perhaps the protein is associated with the surface of the membrane loosely enough that it little perturbs either the acyl chains or the headgroups. Bound as such, its fluorescence properties might be anomalous, which may explain why the trend in the rate of binding based on the decrease in protein fluorescence is the opposite of what one would expect.

As was stated above, in the case of DPPC SUVs melittin binding has virtually no effect on TMA-DPH fluorescence anisotropy or fluorescence intensity (the exception being at 51°C for $R = 90$ and 60). These results along with those for the binding of melittin to DMPC SUVs (Table 1) indicate that the effect melittin has on the fluidity of lipid bilayers greatly depends on the lipid acyl chain length. As was perhaps the case for DMPC at 33°C, melittin induced perturbations may propagate more slowly through the bilayer owing to the more disordered state of the lipid molecules above the transition temperature. For all three temperatures, however, the trend in the rate of decrease of melittin fluorescence with R is the opposite of what would be expected: as the melittin concentration is increased the observed rate of binding should also increase. This behavior cannot be explained at this time.

As was mentioned in the introduction to this section, previous studies which used the steady-state or time-resolved fluorescence of membrane-bound probe molecules to examine the effects of melittin binding on membrane order and fluidity were carried out on lipid samples which had been incubated with melittin and undergone melittin-induced

fusion. Therefore, their results presumably reflected both the effects of the bound protein on the membrane and the (presumably) ordering effects of fusion. For purposes of comparison, it would have been instructive to again measure the fluorescence anisotropy and total fluorescence intensity of TMA-DPH in the samples after a sufficient amount of time in order to allow for fusion to take place (about 15 minutes, judging from the results reported in Part II). (As TMA-DPH is photosensitive the lipid sample would have to be shielded from exciting light during this time.) Based on the results of such measurements made before and after fusion had taken place one could most likely determine whether fusion itself increases or decreases the fluidity and permeability of the lipid bilayer.

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PART IV

**KINETICS OF MELITTIN BINDING TO DIMYRISTOYL-
PHOSPHATIDYLCHOLINE SMALL UNILAMELLAR VESICLES**

INTRODUCTION

The interaction of melittin, a major protein component of bee venom (Habermann, 1980), with phospholipid vesicles has been used in several studies as a model for protein-lipid interactions. The protein fuses lipid vesicles (Eytan and Almary, 1983, Morgan *et al.*, 1983 and Bradrick and Georghiou, 1987) and exerts profound effects on the conformation and motions of the lipid acyl chains (Vogel and Jähnig, 1985, Bradrick *et al.*, 1987, Faucon and Lakowicz, 1987). In almost all of the studies published to date, however, melittin was incubated with the lipid vesicles for some time before measurements were made, and consequently, their results pertain to the interaction of the protein with fused vesicles. Of the two published reports that have appeared to date concerning the interaction of melittin with lipid vesicles which involved measurements on the millisecond time scale prior to an appreciable amount of fusion, one study (Schwarz and Beschiaschvili, 1989) analyzed their binding data in the context of pseudo-first order kinetics, for which there was little indication that the basic assumptions apply. The other study (Sekharam *et al.*, 1991) presented an essentially phenomenological description of the data. In the present work, the kinetics of melittin binding were studied using stopped-flow fluorometry. Data were collected for a number of different concentrations at five different temperatures in order to carry out a global analysis of the data in the context of a more realistic binding model. The results are discussed in terms of the apparent consistency with the model and suggestions are made for making future improvements in the analysis of the data.

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC) of a purity greater than 99% was obtained from Avanti Polar Lipids (Alabaster, AL), while melittin was obtained from Sigma Chemical Co. (St. Louis, MO). Both were used without further purification. Tris and ethylenediaminetetraacetic acid-disodium salt (EDTA) were analytical grade and obtained from Fisher (Pittsburgh, PA) and Sigma, respectively. The buffer used was 50 mM Tris, 1 mM EDTA, pH 7.6 prepared in triply distilled water. For the melittin concentrations used in these experiments, EDTA at a concentration of 1 mM is sufficient to eliminate the activity of any residual phospholipase A₂ in the sample (Dasseux *et al.*, 1984 and Bradrick and Georgiou, unpublished observations).

Each day's experiments were performed with a freshly-made sample of DMPC small unilamellar vesicles (SUVs), which was prepared in the following way. A stock lipid dispersion of 11.8 mM (or 23.6 mM or 35.4 mM, depending on the desired final sample concentration) was formed by hydrating the appropriate amount of lipid in 5 ml of buffer at 33°C (10°C above the lipid phase transition temperature of 23°C). This lipid dispersion was then sonicated with a Laboratory Supplies Co. (Hicksville, NY) bath sonicator at 33°C for two 20-minute periods, separated by a 5 minute rest (or until clearness) and allowed to anneal for 30 minutes at 33°C. In order to remove any remaining multilamellar vesicles, the sample was centrifuged at 65000xg for 25 minutes at 33°C, after which the supernatant was removed and stored in a polystyrene screwcap tube at 33°C for subsequent use. [When stored above their lipid phase transition

temperature, SUVs are stable (Schullery *et al.*, 1980).] At the time of use, aliquots of the liposome solution were removed and appropriately diluted to obtain the desired lipid concentration for loading into the stopped-flow apparatus.

Melittin stock solutions were prepared by weighing a quantity of lyophilized melittin (typically 10 to 15 mg) and dissolving it in 10 ml of buffer. The concentration of this stock solution was determined spectrophotometrically on a Shimadzu UV-265 spectrophotometer given that $\epsilon_{280} = 5800 \text{ M}^{-1} \text{ cm}^{-1}$ (Berlman, 1971). Melittin solutions of the desired concentration were formed by diluting aliquots of the stock solution by the appropriate amount. Stock solutions were not used beyond five days after their formation.

Stopped-flow fluorometric measurements were carried out on an Aminco DW-2 UV/Vis spectrophotometer equipped with a stopped-flow module which has been modified to allow fluorescence to be measured at right angles to excitation. Fluorescence was viewed by an EMI 6256S photomultiplier through a Corning 0-51 glass cut-off filter. Excitation light was provided by a Spectral Energy (Westwood, NJ) 600 W Ze-Hg arc lamp whose output was passed through a 280 nm interference filter which had a bandwidth of 10 nm full-width at half-maximum. The stopped-flow sample cell and sample reservoirs were thermostated at the desired temperature with a circulating water bath. The photomultiplier output was digitized by an IBM PC equipped with an Analog Devices (Norwood, MA) RTI-815 A/D converter. For each "run" of (approximately) twelve stopped-flow "shots" or injections, melittin and lipid samples were loaded into the sample reservoirs and allowed to come to thermal equilibrium over a period of ten minutes. Three shots were then taken, the last of which was allowed to remain in the

stopped-flow cell for five minutes in order to allow its fluorescence to bring the photomultiplier response up to its steady-state level.

The stopped-flow data were analyzed according to the following kinetic model:



where P denotes a free protein (melittin) molecule, nL denotes a lipid binding site which is composed of n lipid molecules and P•nL denotes the resulting bound protein-lipid binding site complex. k_f is the forward rate constant (rate constant for binding) and k_r is the reverse rate constant (rate constant for dissociation). The rate at which the concentration of the protein-lipid complex P•nL changes with time is governed by

$$\frac{d[P \cdot nL]}{dt} = -k_r[P \cdot nL] + k_f[P][nL] \quad (2)$$

where the square brackets denote the concentrations of the different species. From the stoichiometry, one obtains

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

and

$$[nL]_t = [nL]_o = [nL](t) + [P \cdot nL](t) \quad (4)$$

where the subscripts t and o denote total and initial (*i.e.* at zero time) concentrations, respectively. Combining equations (3) and (4) with (2) yields

$$\int \frac{d[P \bullet nL]}{a[P \bullet nL]^2 + b[P \bullet nL] + c} = -\int dt \quad (5)$$

where

$$\begin{aligned} a &= -k_f \\ b &= k_r + k_f([nL]_o + [P]_o) \\ c &= -k_f[P]_o[nL]_o \end{aligned} \quad (6)$$

The integral on the left-hand-side of equation 5 is of the form

$$\int \frac{dx}{ax^2 + bx + c} \quad (7)$$

and has the solution

$$\frac{1}{\sqrt{b^2 - 4ac}} \ln \left(\frac{2a[P \bullet nL](t) + b - \sqrt{b^2 - 4ac}}{2a[P \bullet nL](t) + b + \sqrt{b^2 - 4ac}} \right) \quad (8)$$

Therefore, applying the initial condition that $[P \bullet nL]_o = 0$ and rearranging to solve for $[P \bullet nL](t)$, one obtains

$$[P \bullet nL](t) = \frac{b - k_{eff}}{2k_f} \cdot \frac{1 - \exp(-k_{eff}t)}{1 - \left(\frac{b - k_{eff}}{b + k_{eff}} \right) \exp(-k_{eff}t)} \quad (9)$$

where

$$k_{eff}^2 = b^2 - 4ac \quad (10)$$

For all experiments $[P]_o$ was kept low enough (≤ 0.059 mM) that sample absorbance was

0.05 or less over the 2 mm cuvette path length from which fluorescence was collected. In that case, fluorescence intensity is proportional to fluorophore concentration. Therefore, according to the above kinetic model, the sample fluorescence as a function of time should be given by

$$F(t) = I\gamma_f\epsilon_fq_f[P](t) + I\gamma_b\epsilon_bq_b[P\bullet nL](t) \quad (11)$$

Here, γ is the emission filter transmittance, ϵ is the molar extinction coefficient at the wavelength of excitation and q is the fluorescence quantum yield for a particular melittin species (free or bound as denoted by the subscripts f and b , respectively). $[P](t)$ and $[P\bullet nL](t)$ are the free and bound melittin concentrations, respectively, as a function of time (given by equations 3 and 9 above) and I denotes the (relative) intensity of the exciting light. Experimental values for k_r , k_f and n were determined by performing stopped-flow kinetic measurements at a particular temperature for a number of different absolute lipid and melittin concentrations. A global analysis, made using the Simplex optimization method, was carried out by fitting equation 11 to these stopped-flow data at various concentrations, with the constraint that the same values for k_r , k_f and n be used to fit all the data. [By "global" analysis is meant a simultaneous analysis of a number of data sets for which a "global" variable(s) associated with the fitting parameters (such as reactant concentrations, as here) has different values for the different data sets. A "correct" choice of values for the global variable can provide the necessary constraints for obtaining unique values for the fitting parameters, where otherwise the problem would be inadequately specified and the parameters indeterminate.]

It should be noted that equation 11 can be written in matrix form as

$$\begin{bmatrix} F(t_1) \\ F(t_2) \\ \cdot \\ \cdot \\ \cdot \end{bmatrix} = \begin{bmatrix} I[P](t_1) & I[P \cdot nL](t_1) \\ I[P](t_2) & I[P \cdot nL](t_2) \\ \cdot & \cdot \\ \cdot & \cdot \\ \cdot & \cdot \end{bmatrix} \begin{bmatrix} \gamma_f \epsilon_f q_f \\ \gamma_b \epsilon_b q_b \end{bmatrix} \quad (12)$$

or

$$F = CA \quad (13)$$

Here, **F** is the fluorescence intensity matrix, **C** is the species concentration matrix ("rescaled" by the relative lamp intensities) and **A** is the matrix which contains the "effective" species absorbances (or fluorescence coefficients) $\gamma_f \epsilon_f q_f$ and $\gamma_b \epsilon_b q_b$. It should be understood that these matrices "extend" over or encompass all the (multiple) data sets which are being globally analyzed for a particular temperature. As the two products $\gamma_f \epsilon_f q_f$ and $\gamma_b \epsilon_b q_b$ enter into the model (equations 11 and 12) in a linear way, they are not independent parameters, but are instead determined by the fluorescence data (the experimental values for **F**) and the calculated values for the free- and bound-protein concentrations, and are given by the equation

$$(C^T C)^{-1} (C^T F) = A \quad (14)$$

Here, ⁻¹ and ^T denote the matrix inverse and transpose, respectively. This elimination of the fluorescence coefficients as independent parameters thus reduces from five to three the dimension of the parameter space which must be searched by the nonlinear least-

squares fitting program. This in turn reduces convergence time and uncertainty in the parameter values. Therefore, $\gamma_r \epsilon_r q_r$ and $\gamma_b \epsilon_b q_b$ were determined by using equation 14.

For each experiment performed for a particular combination of protein and lipid concentrations, four "runs" each of about twelve stopped-flow "shots" or injections were performed. These runs, in order, consisted of (i) protein vs. buffer, (ii) protein vs. lipid, (iii) buffer vs. buffer and (iv) lipid vs. buffer. (iii) should contain the contribution that scattered light and buffer fluorescence makes to (i). Likewise, (iv) should contain the same type of contribution to (ii). (iii) was used to adjust the lamp intensity so as to maximize the melittin fluorescence signal. [Protein concentrations in (i) and (ii) were the same.] This was necessary in order to obtain the maximum resolution from the stopped-flow system's A/D converter and also to try to obtain equal weighting of the data from the different experiments which were included in each global computer analysis. (Total protein concentrations could differ by up to a factor of five between different experiments.) (i) minus (iii) also provided the values for the relative lamp intensity I in equations 11 and 12. For any particular global analysis, the first data file read into the computer was assigned a relative lamp intensity of unity. Subsequently-read data files were assigned relative lamp intensities scaled to that of the first file through the equation

$$I_j = I_1 \cdot \frac{c_{fl}}{c_{ff}} \cdot \frac{signal_j}{signal_1} \quad (15)$$

Here, I_j is the relative lamp intensity being assigned to the j th data file in the global analysis, I_1 is the relative lamp intensity for the first data file that was read in (set equal to unity as stated above) and "signal" denotes the fluorescence intensity for protein vs.

buffer, less its buffer background, as determined by the A/D converter (subscripts j and l as for I). c_{fi} denotes the molar concentration of free melittin for the i th data file. From the data for the first file being read in for a particular global analysis, it was also possible to calculate a value for $\gamma_f \epsilon_f q_f$ from the equation

$$signal_1 = I_1 \gamma_f \epsilon_f q_f c_{f1} \quad (16)$$

(Here, symbols and subscripts have the same meaning as for equation 15.) This value for $\gamma_f \epsilon_f q_f$ was compared with that calculated independently through equation 14 as an additional check of the appropriateness of the model in describing the data.

As was stated above, twelve stopped-flow shots were typically obtained with each filling of the reservoirs. In the cases of (i), (ii) and (iv) (*i.e.* melittin vs. buffer, buffer vs. buffer and lipid vs. buffer), the resulting traces were flat. For these cases, the values for the free melittin fluorescence signal as well as the values for the buffer and lipid baselines were obtained by simply averaging the values from all twelve respective data files. In the case of melittin mixed with lipid, a representative file for use in the global analysis was chosen by first performing an initial analysis in which the equation

$$F(t) = A + B \exp(-kt) \quad (17)$$

was simultaneously fitted to all twelve fluorescence data file. (It was found that in all cases the decrease in melittin fluorescence with time upon binding could be described quite well by a decaying exponential plus a constant.) That file which was best fit, as judged by the shape of the residuals, by the resulting parameter values from this

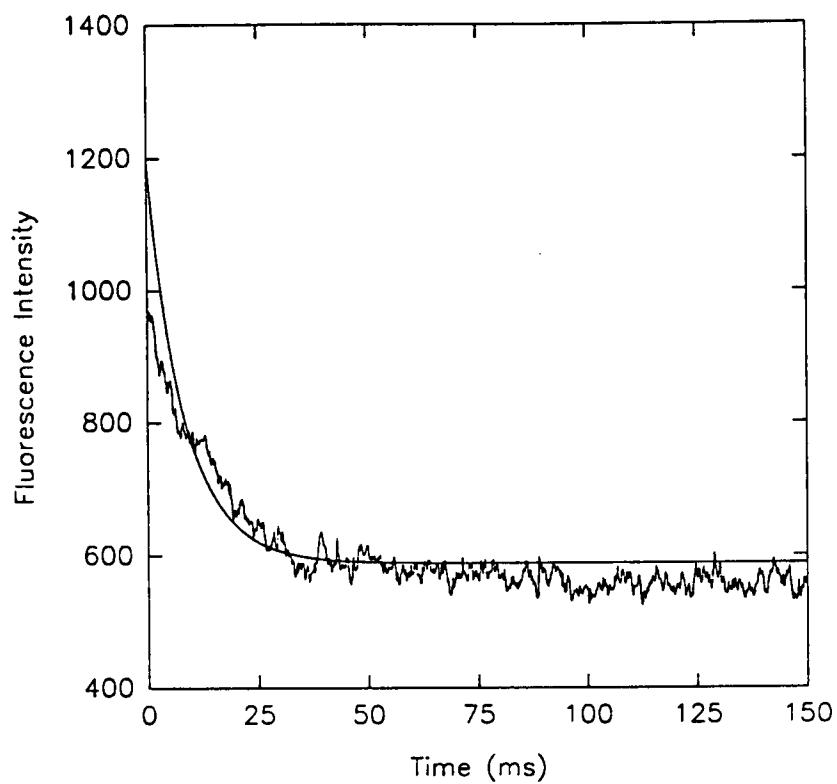
simultaneous analysis was the one chosen for inclusion in the global analysis of different concentrations.

RESULTS

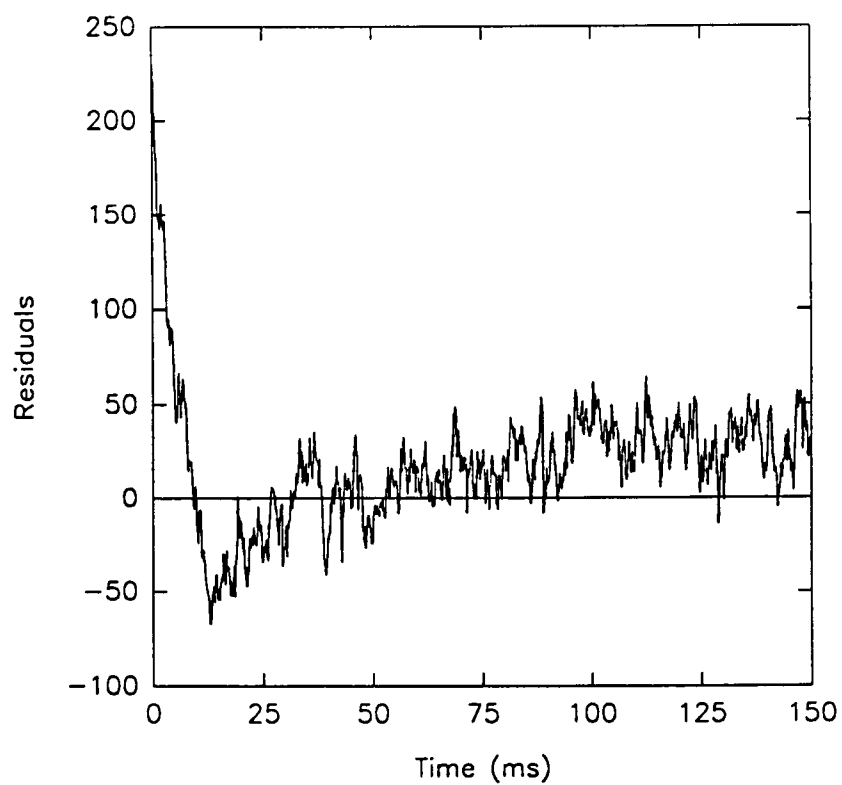
Representative results from the measurement of the kinetics of melittin binding are shown in Figures 1A through 1P. For these data the sample temperature was 27°C. The figures also show the results of the global analysis of the data in which equation 11 was simultaneously fitted to all the data with the constraint that the values for the parameters k_p , k_r and n be the same for all the different sample concentrations. The data set included six files (shown in Figures 1A through 1F) for which the lipid-to-protein molar ratio R was equal to 180. Three of these files (shown in Figures 1A through 1C) were for a lipid concentration of 10.62 mM and a melittin concentration of 59 μ M; the other three files (shown in Figures 1D through 1F) were for a lipid concentration of 2.95 mM and a melittin concentration of 16.38 μ M. (The two sets of three files each differed in the lipid and melittin concentrations by a factor of 3.6.) Six other files (shown in Figures 1G through 1L) were for $R = 120$. This group of six consisted of three files (shown in Figures 1G through 1I) for which the lipid and melittin concentrations were 7.08 mM and 59 μ M, respectively, while the other three files (shown in Figures 1J through 1L) were for lipid and melittin concentrations of 1.967 mM and 16.38 μ M, respectively. (These two sets of three files also differed in the lipid and melittin concentrations by a factor of 3.6.) Figures 1M and 1N are for $R = 90$ ([lipid] = 1.475 mM and [melittin] = 16.38 μ M) and Figures 1O and 1P were for $R = 60$ ([lipid] = 3.54 mM and [melittin] = 59 μ M). In each figure the upper panel shows the experimental data and the fit (smooth line) derived from the global analysis. The lower panel shows the residuals resulting from that fit. As

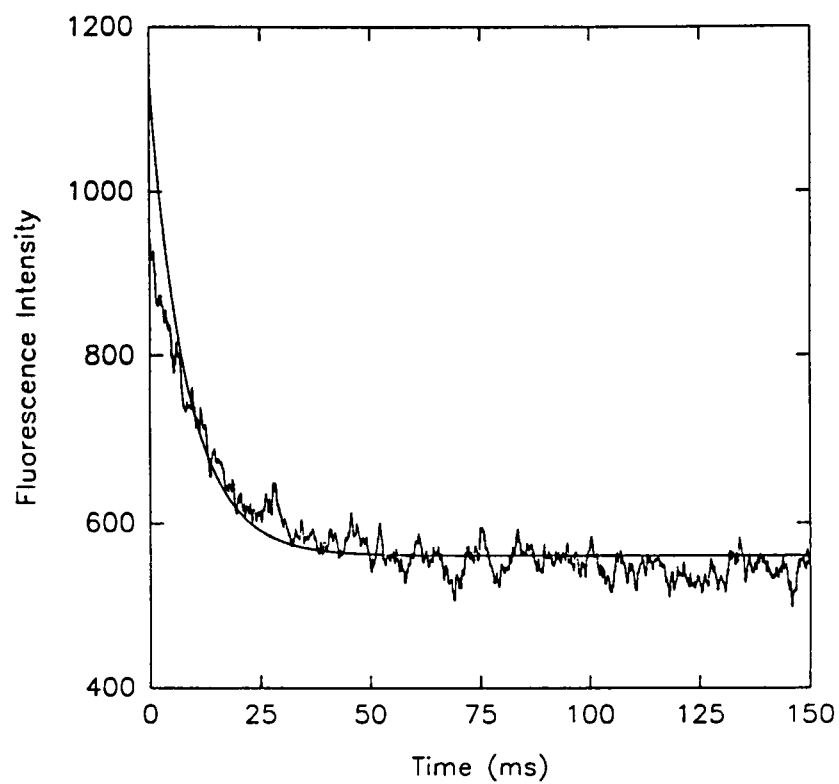
Figures
1A-1P.

Results of the global analysis for the binding of melittin to DMPC small unilamellar vesicles at 27°C. Figures 1A-1C are for a lipid concentration of 10.62 mM and a melittin concentration of 59 μ M and a lipid-to-protein molar ratio R of 180. Figures 1D-1F are for a lipid concentration of 2.95 mM and a melittin concentration of 16.38 μ M and R = 180. Figures 1G-1I are for a lipid concentration of 7.08 mM and a melittin concentration of 59 μ M and R = 120. Figures 1J-1L are for a lipid concentration of 1.967 mM and a melittin concentration of 16.38 μ M and R = 120. Figures 1M and 1N are for a lipid concentration of 1.475 mM and a melittin concentration of 16.38 μ M and R = 90. Figures 1O and 1P are for a lipid concentration of 3.54 mM and a melittin concentration of 59 μ M and R = 60. (All concentrations are for after stopped-flow mixing.) The data for each figure were obtained from different samples and were subjected to a global analysis in which equation 11 was simultaneously fitted to all the data. This analysis yielded values of $4.98 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for k_r , 3.40 s^{-1} for k_f and 37 lipid molecules per melittin binding site. The upper panel in each figure shows the experimental data and the fit from the global analysis (smooth line). The lower panel shows the residuals from the fit to the data.

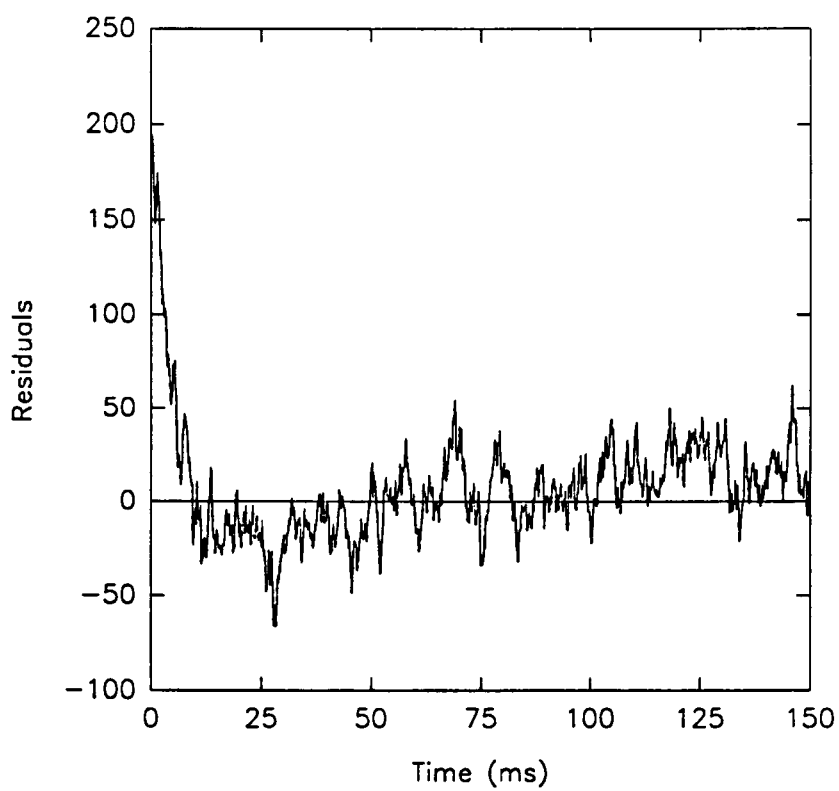


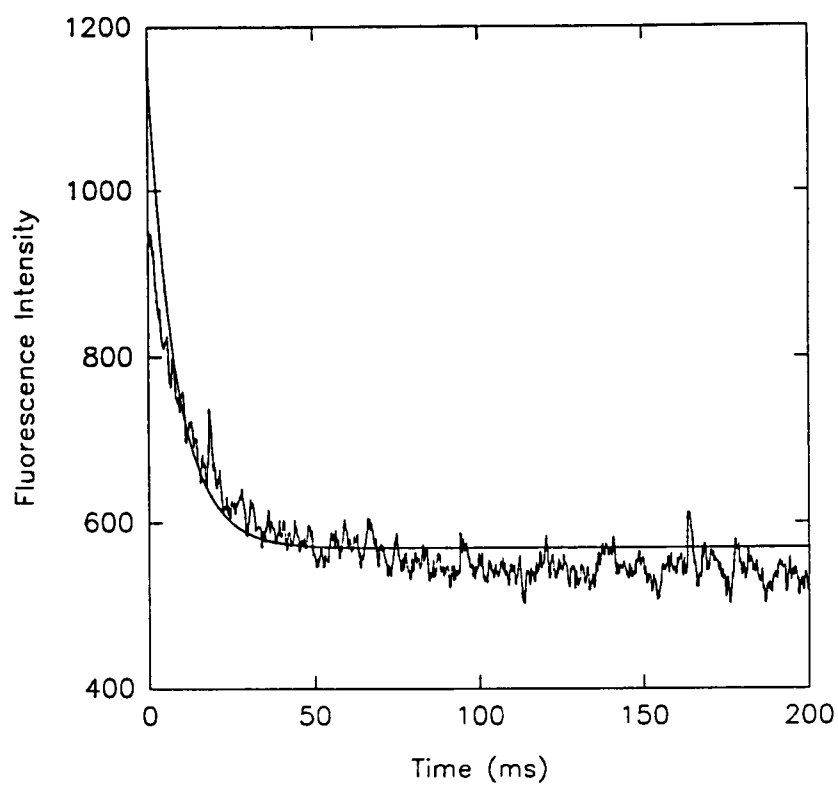
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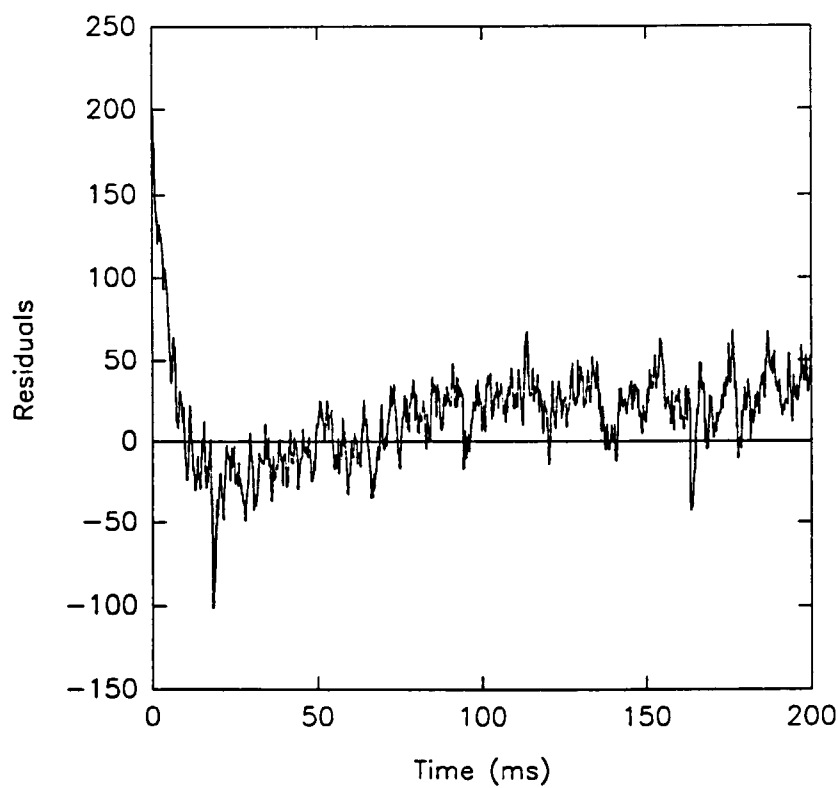


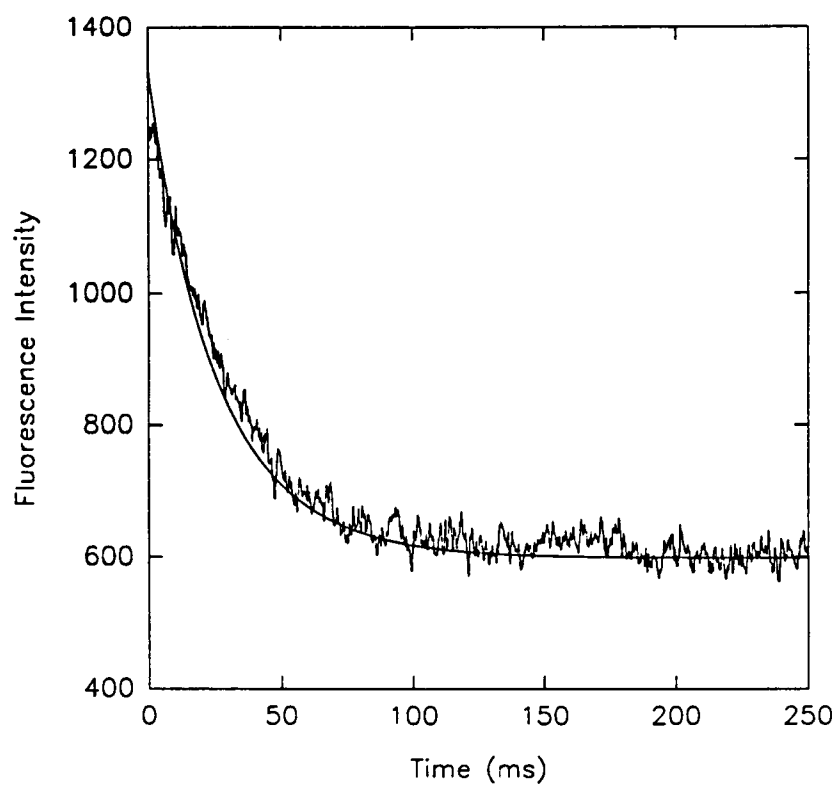
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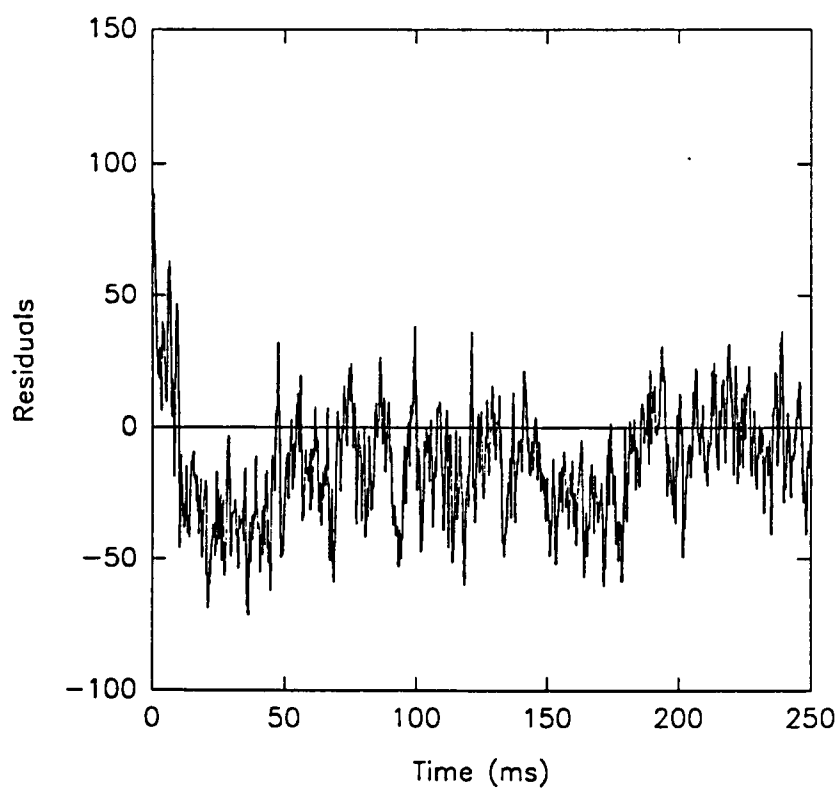


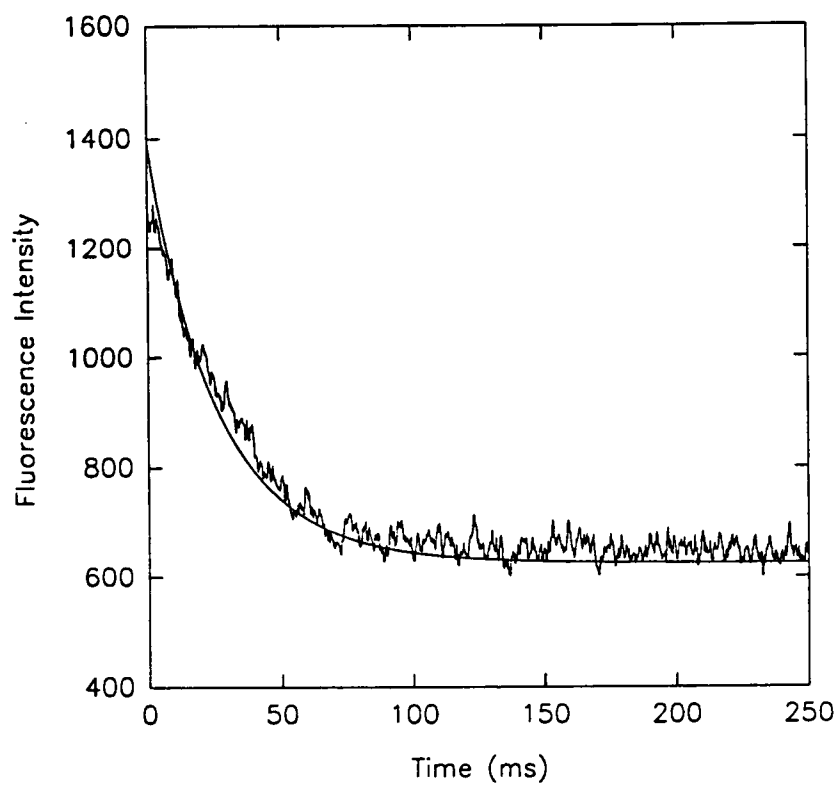
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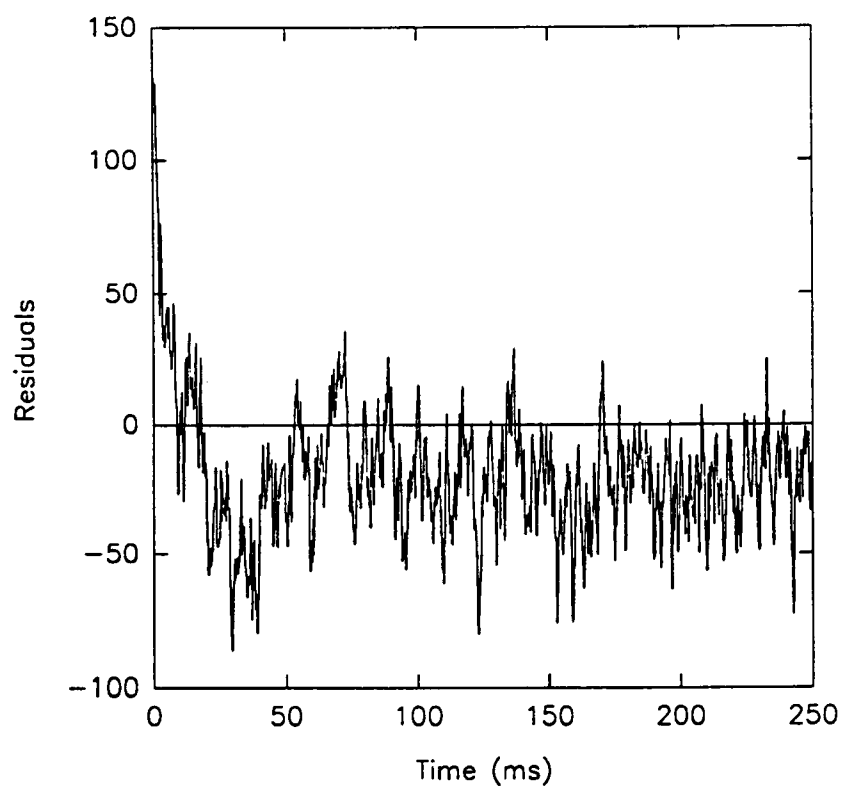


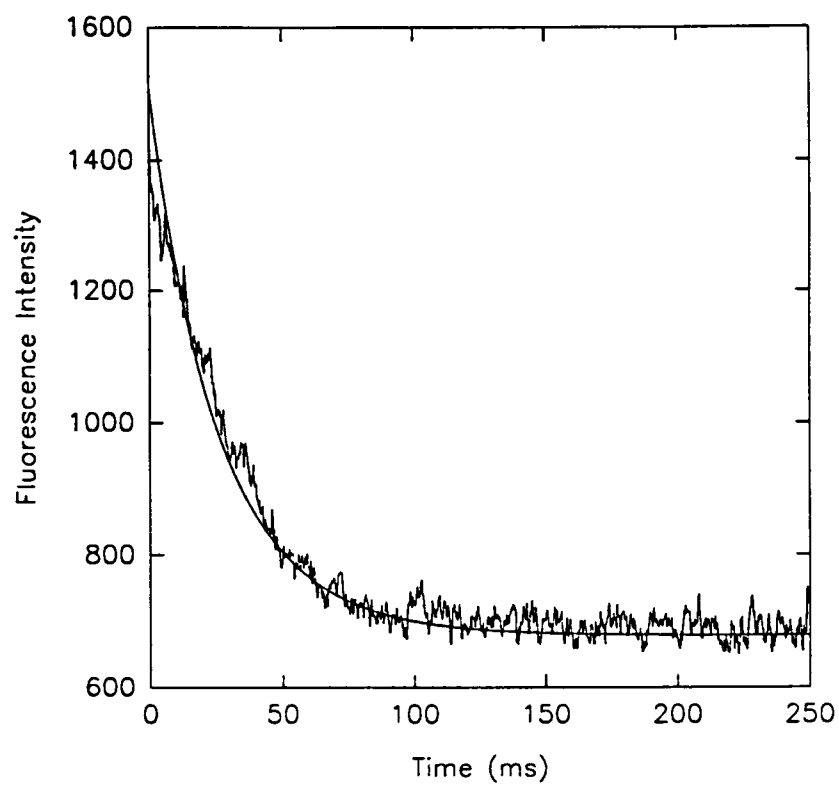
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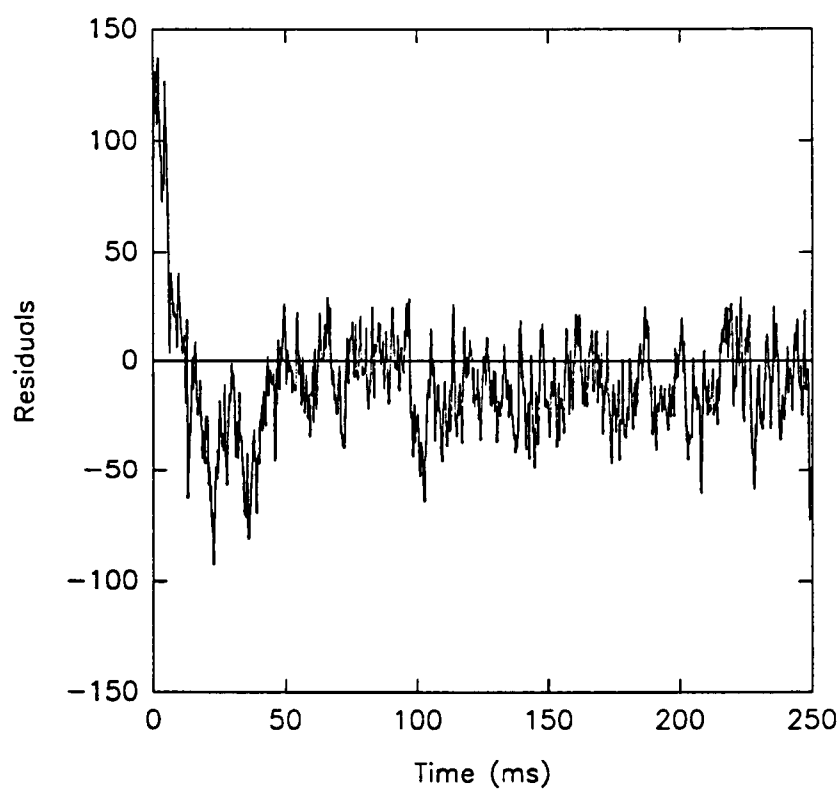


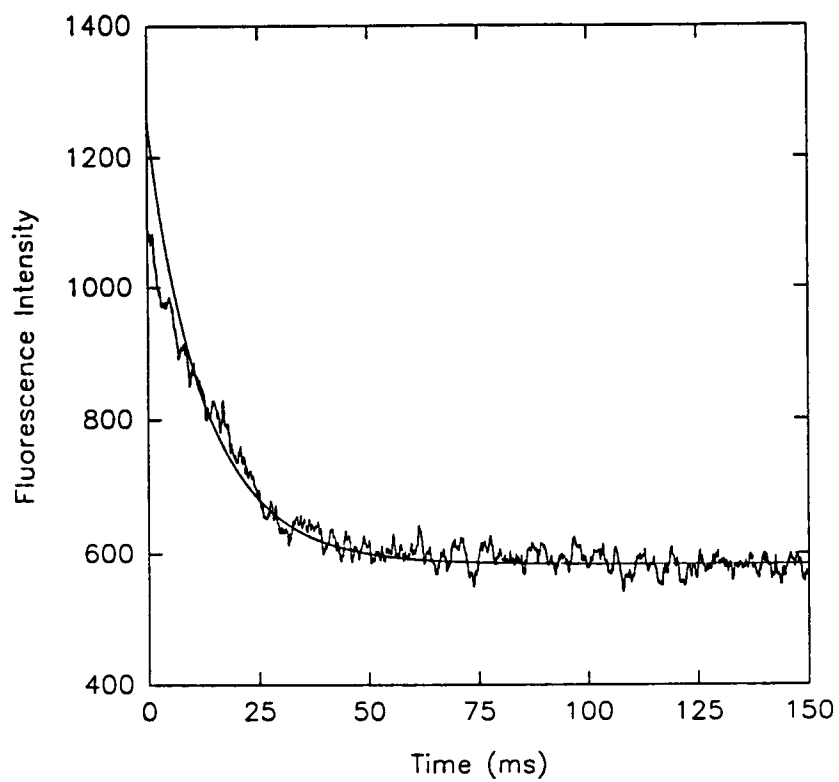
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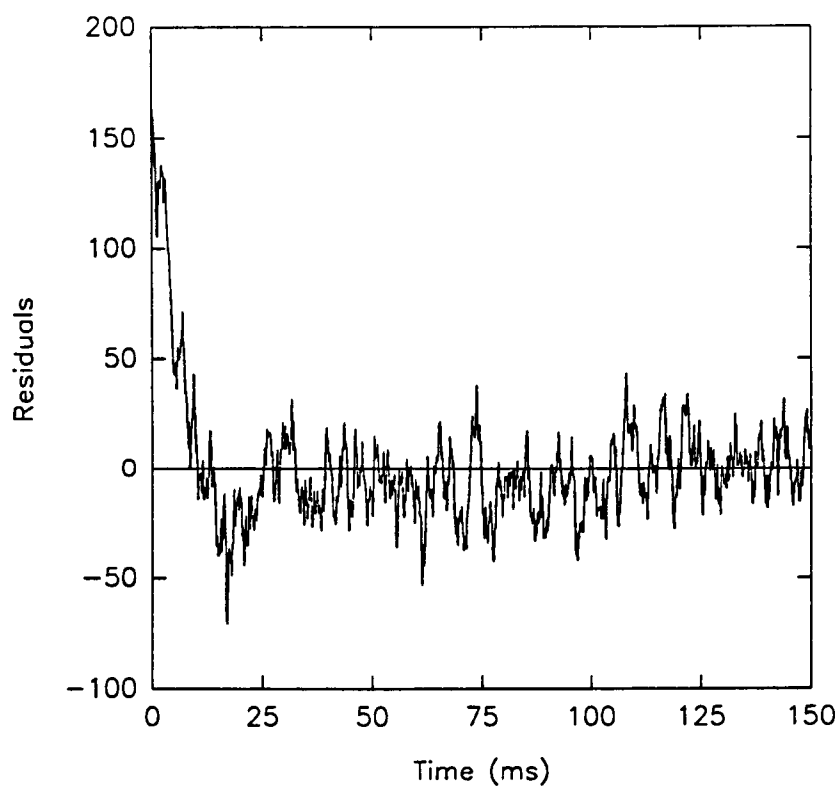


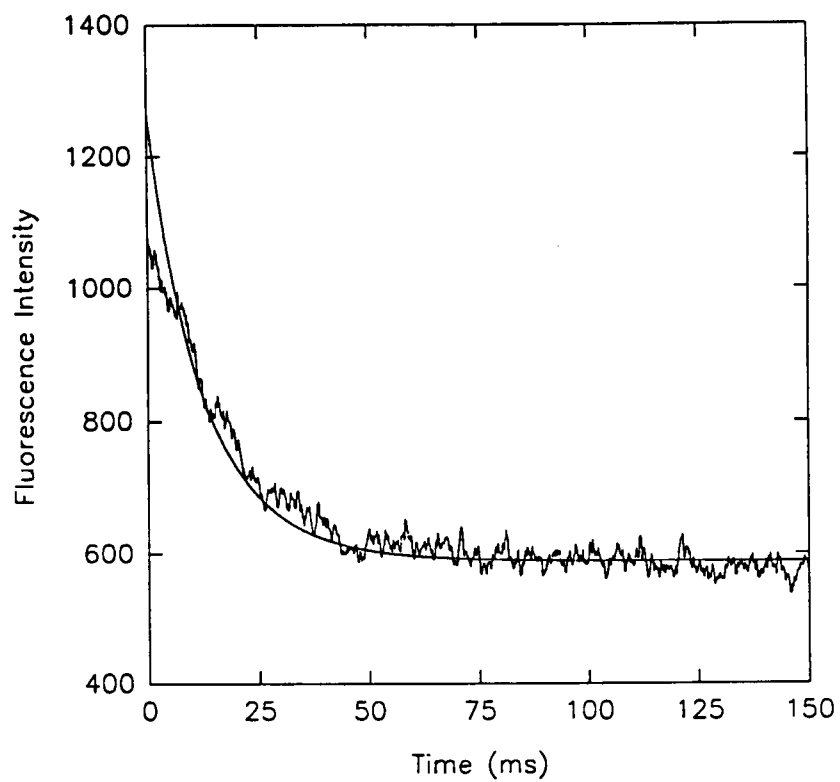
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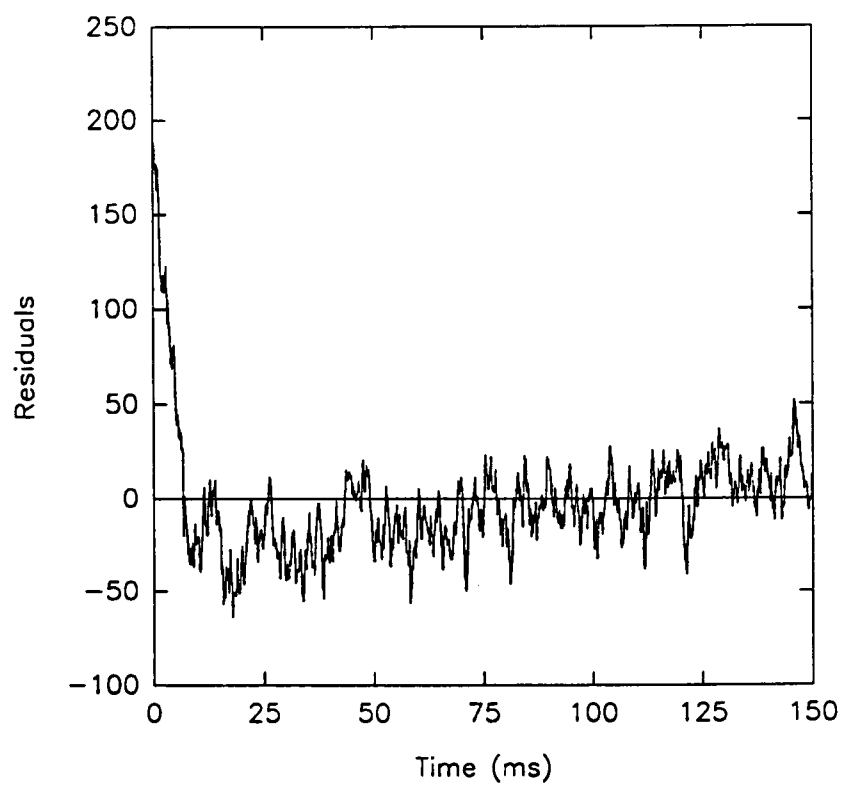


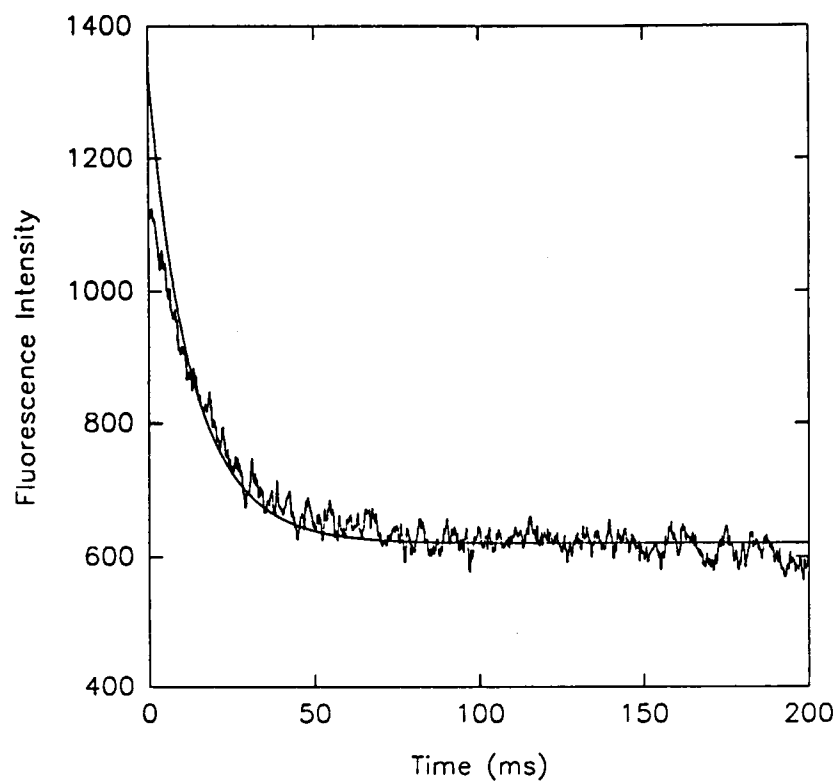
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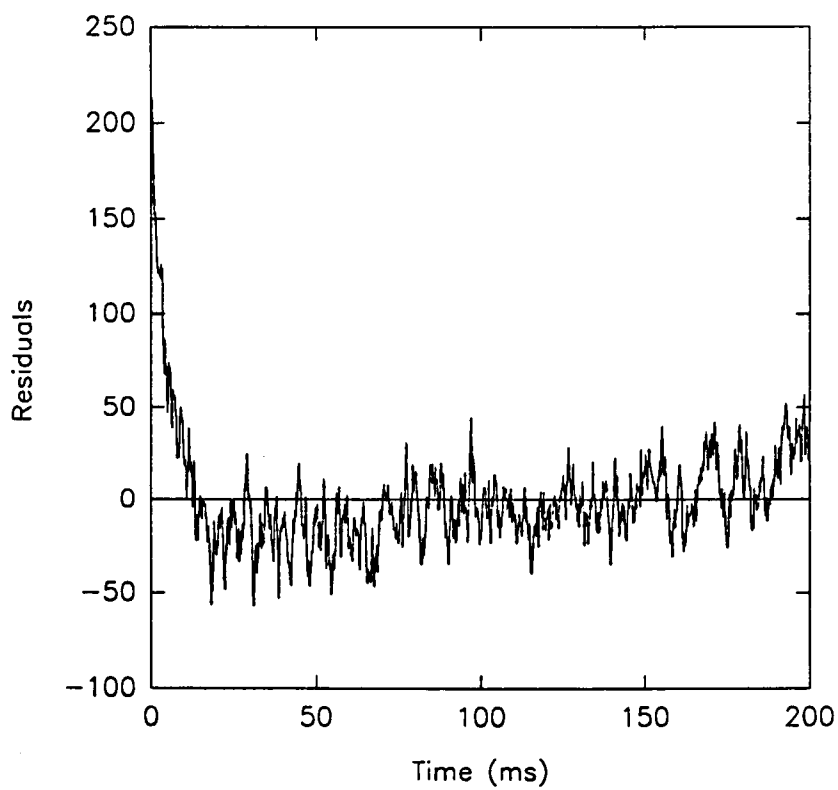


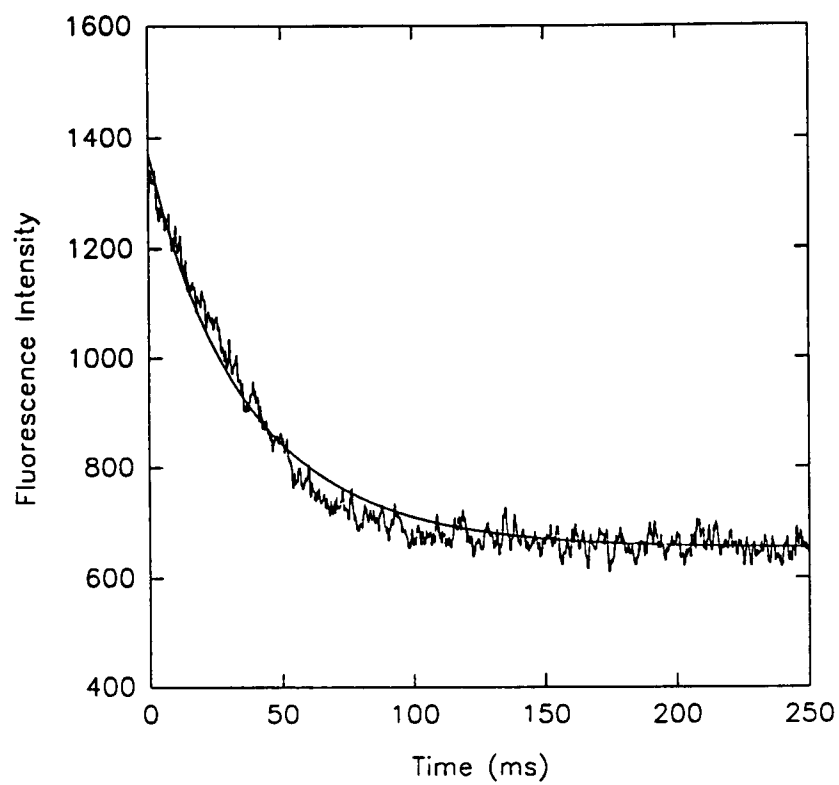
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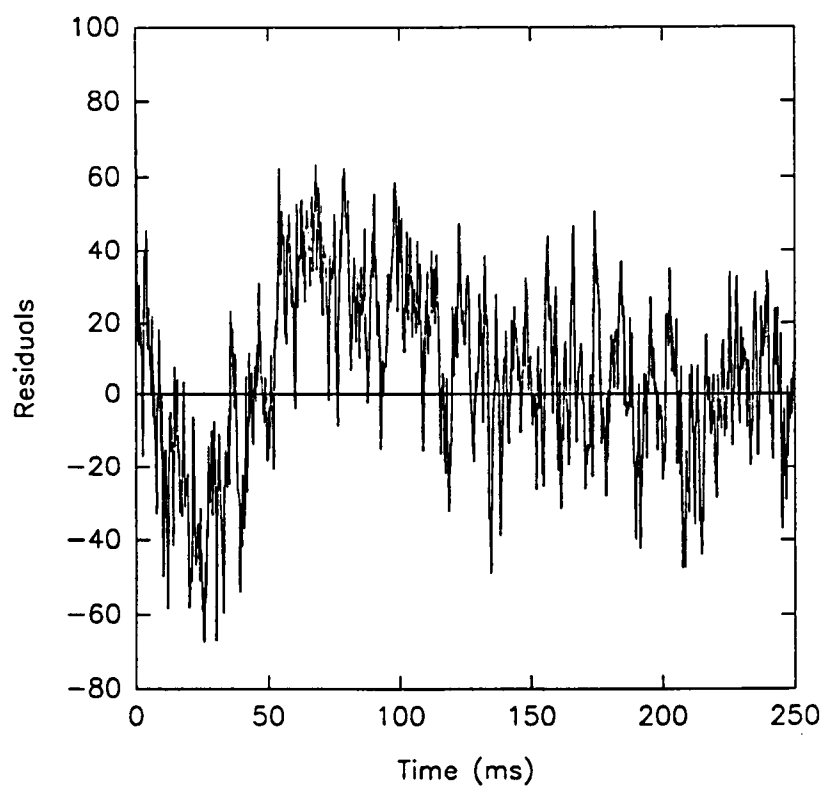


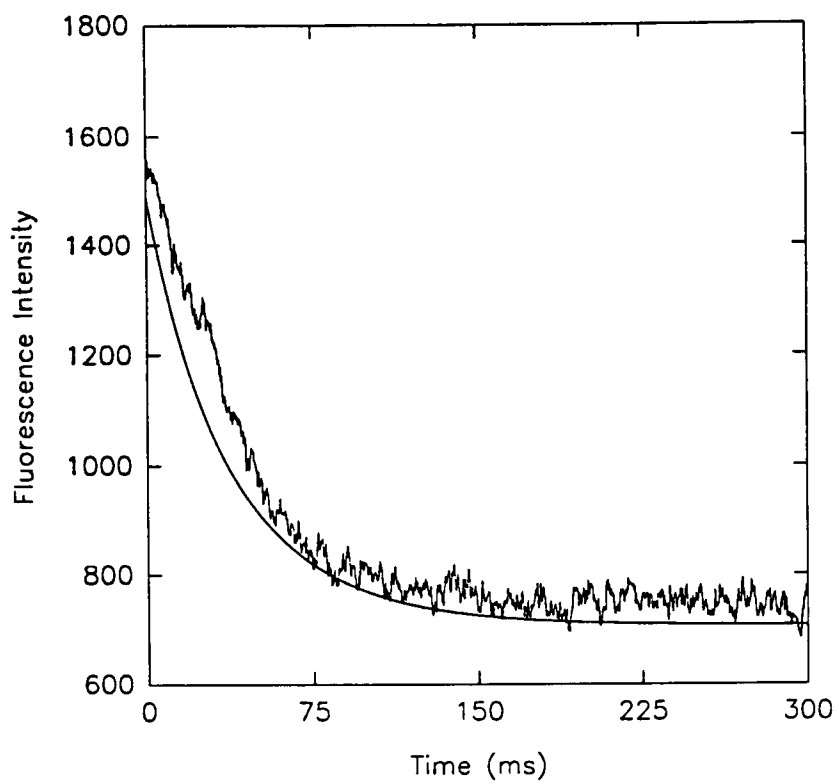
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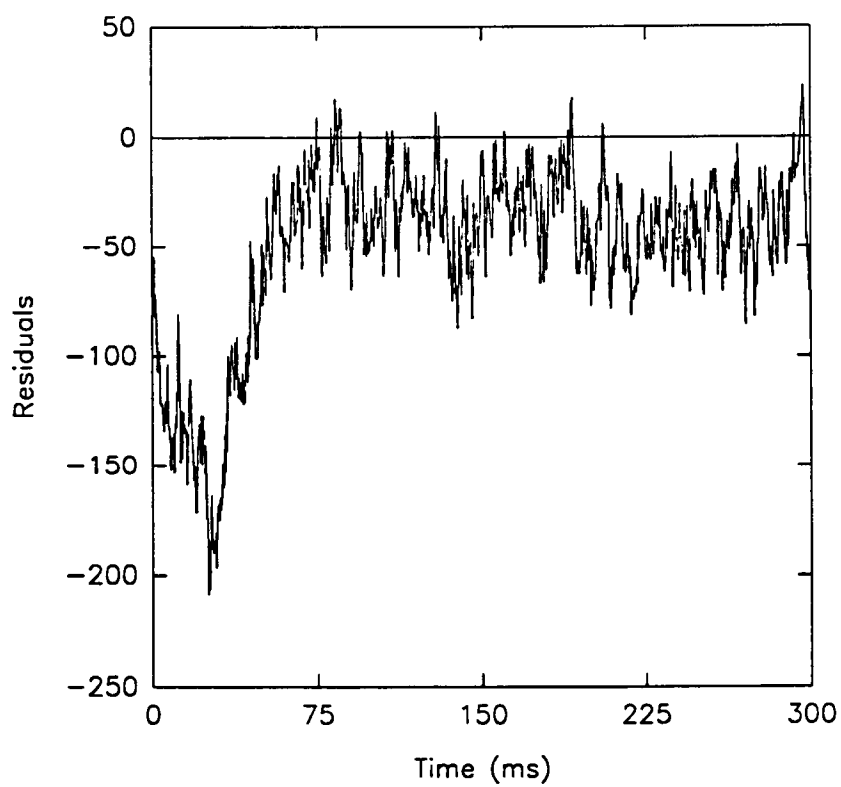


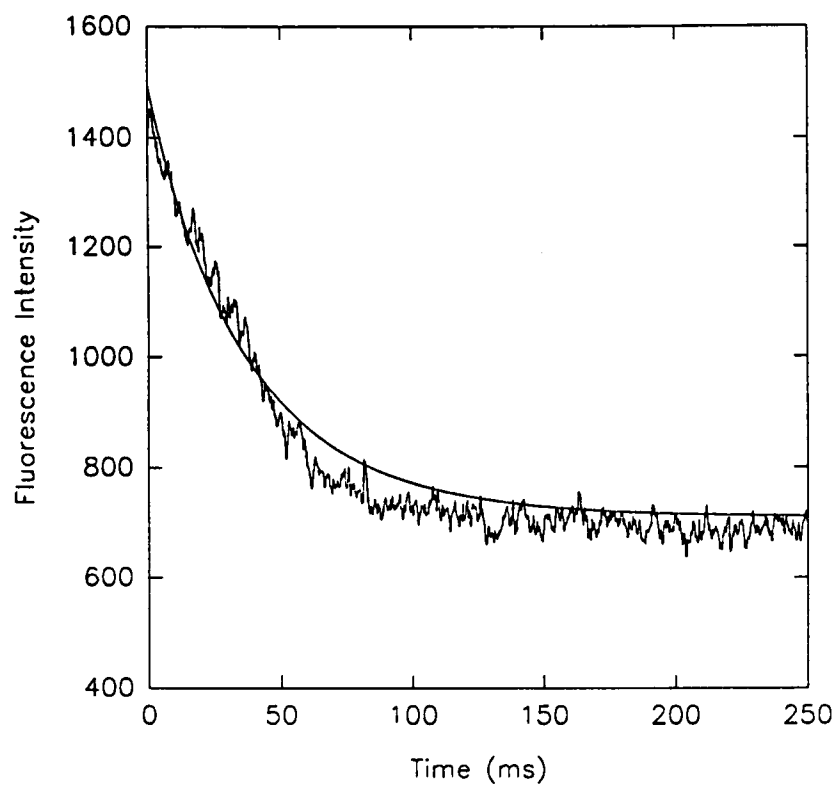
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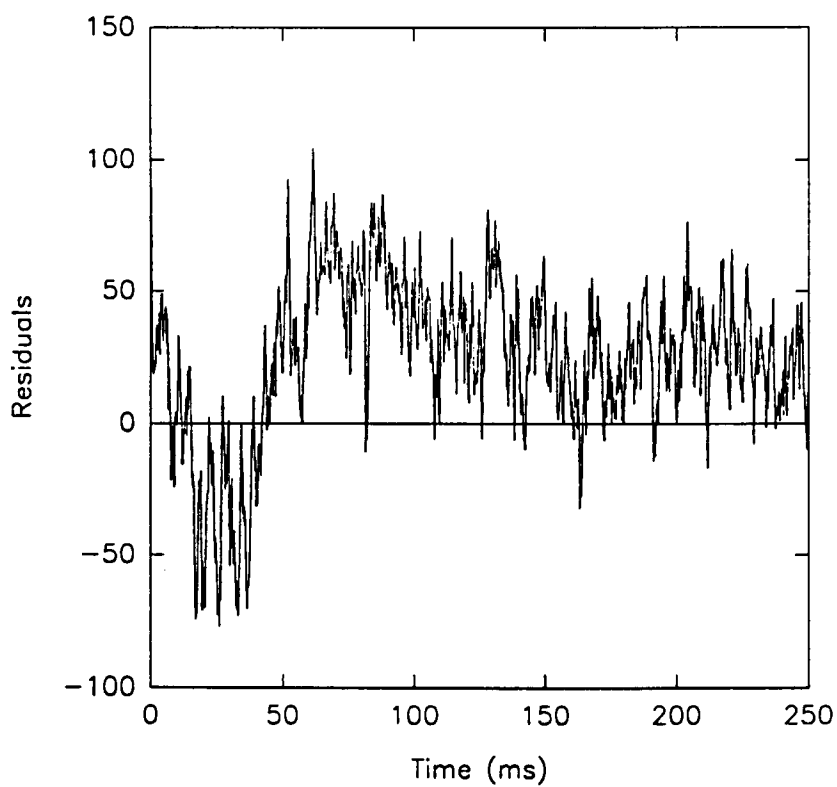


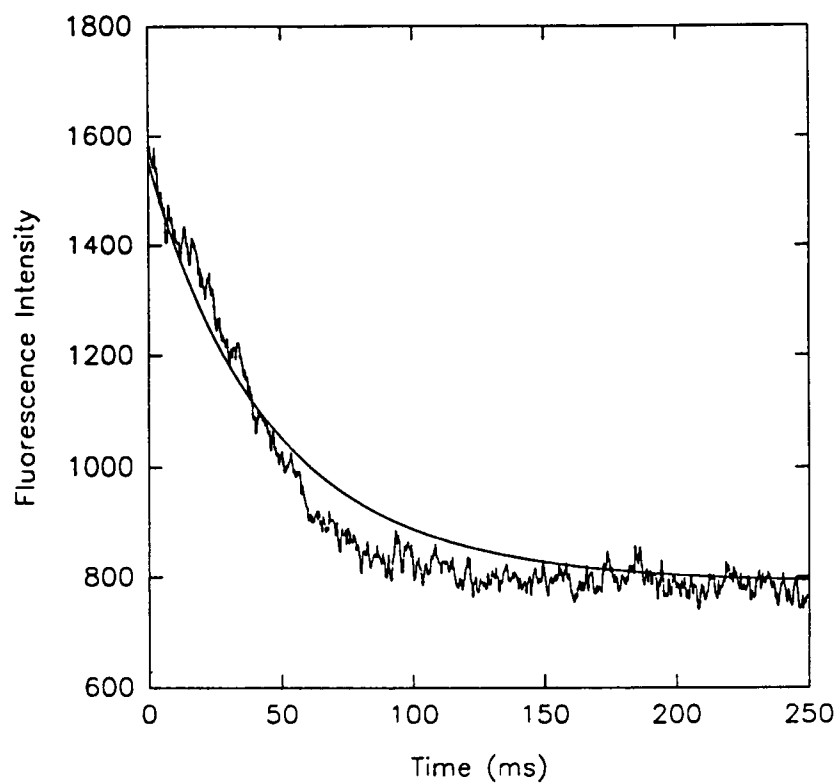
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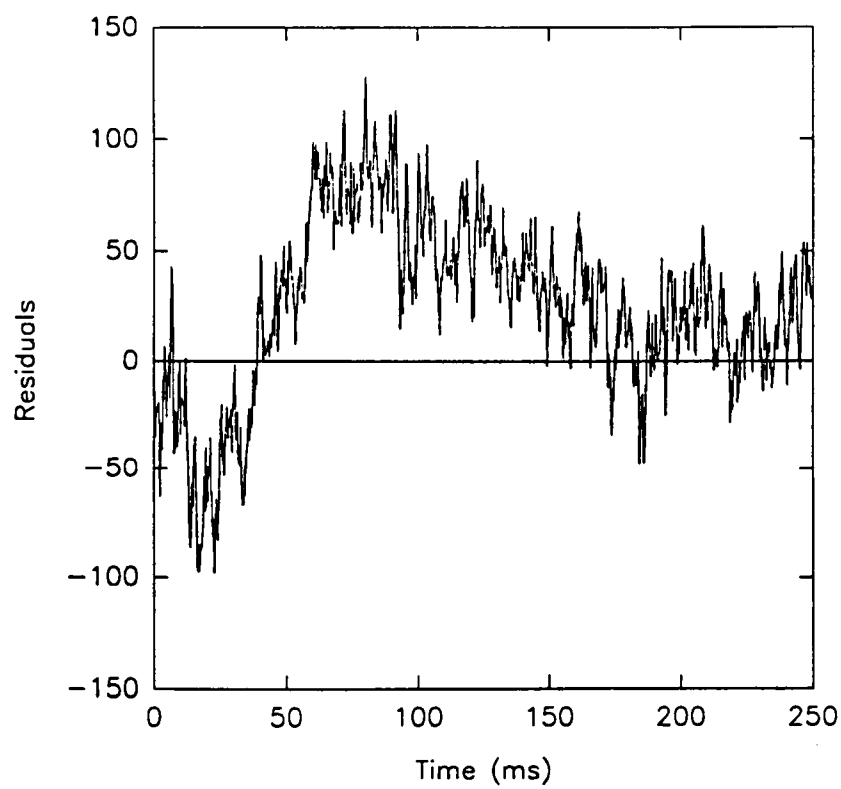


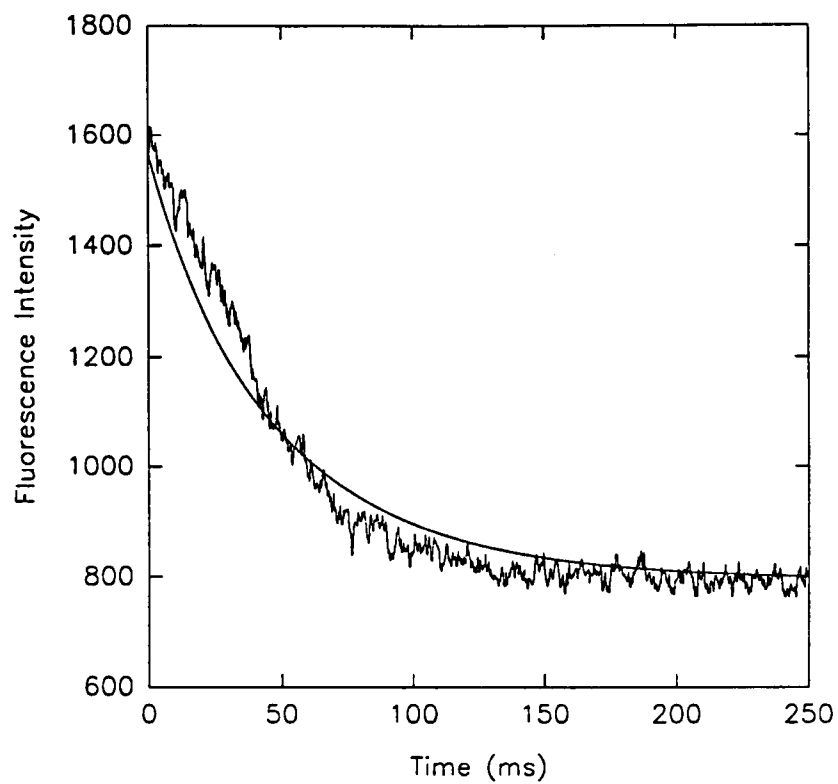
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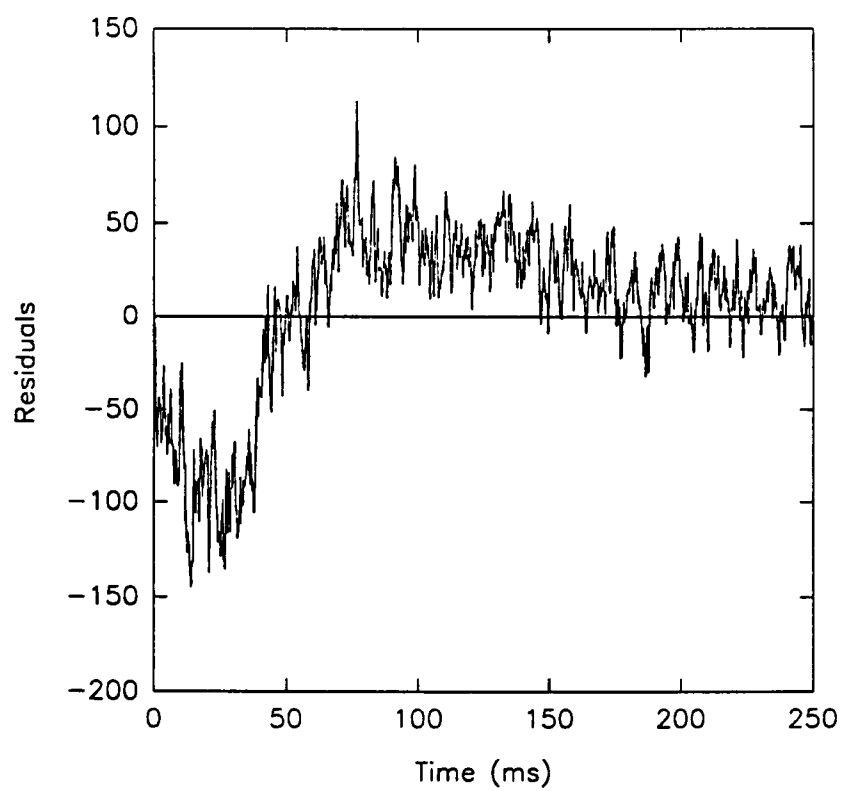


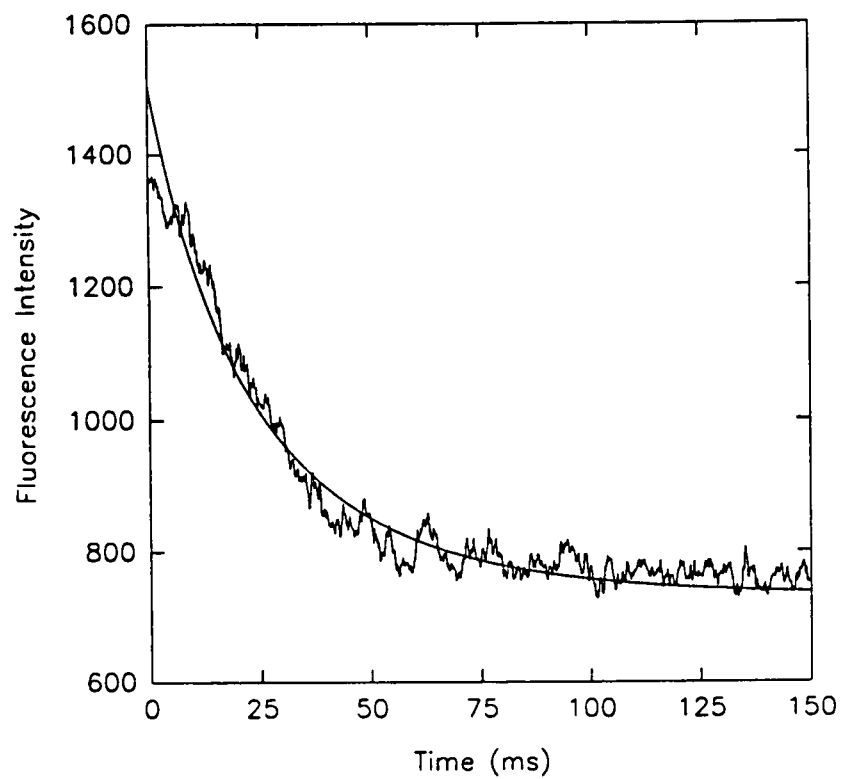
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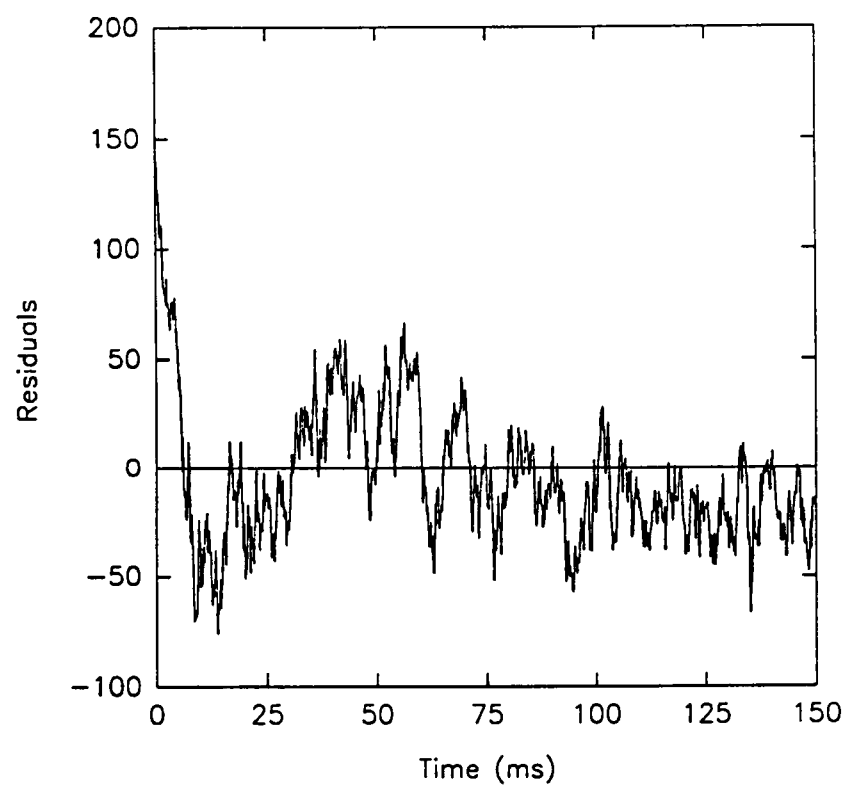


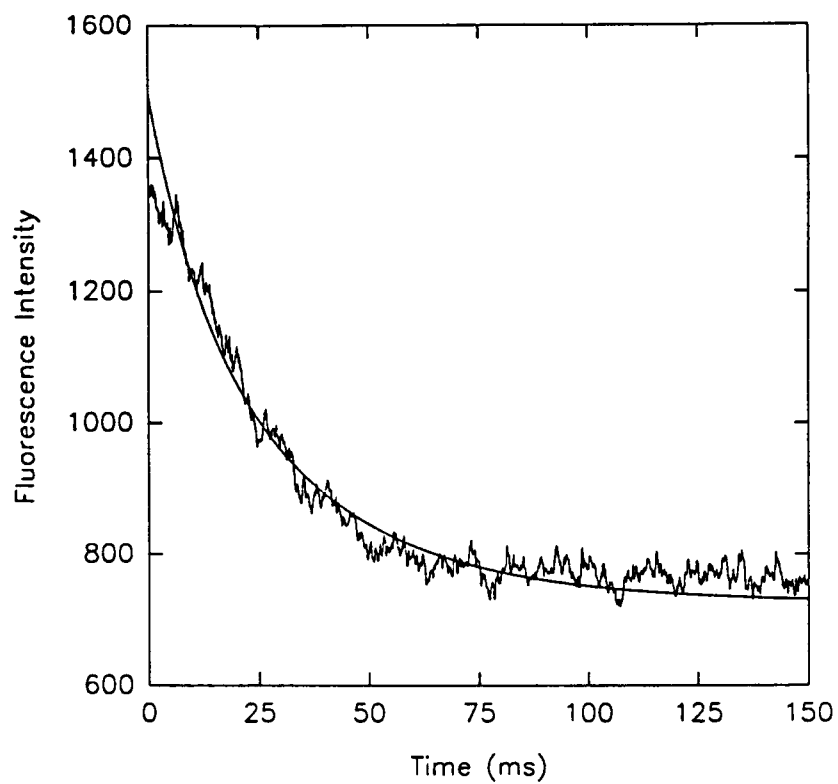
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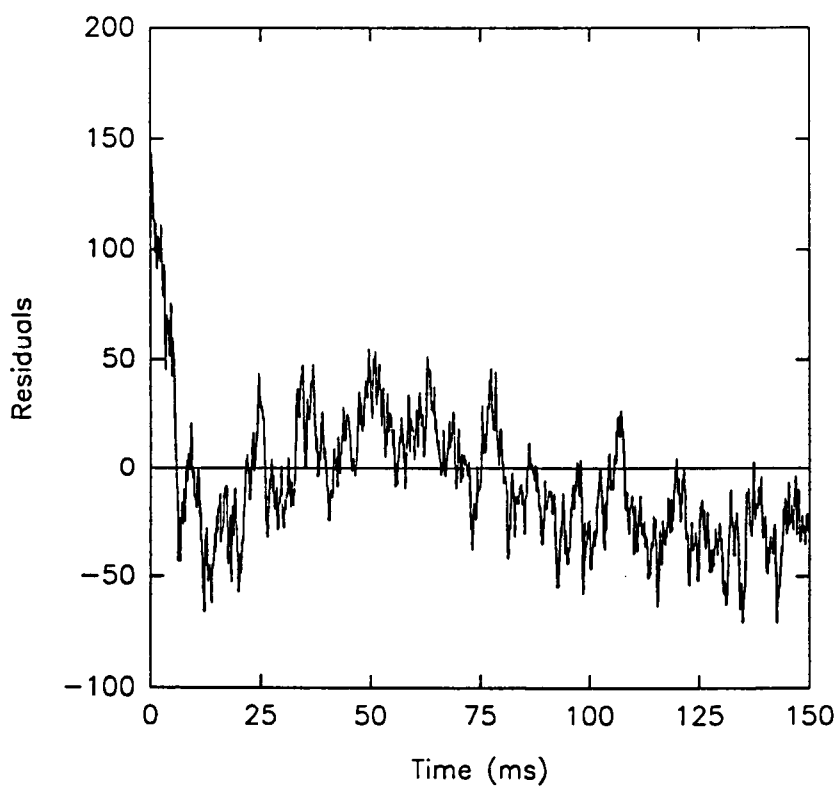


O





P



can be seen, the data are fitted rather well by the model (equation 11). Within each group of files for which the lipid and melittin concentrations were the same, there does not appear to be any consistent over- or under-estimation of any significance.

The results from the global analyses for all the temperatures examined (33°C, 30°C, 27°C, 24°C and 21°C) in terms of the values for the parameters k_f , k_r and the number of lipids per binding site (n^{-1}) are summarized in Table 1. As can be seen from that table, both k_f and k_r (generally) decrease with temperature as might be expected, although the values for rate constants appear to be somewhat anomalous at 30°C (in the case of k_f) and 27°C (in the case of k_r) (*cf.* Fig.2). Presumably these values are somewhat in error, as is also suggested by the values the fluorescence coefficients $\gamma_f \epsilon_f q_f$ and $\gamma_b \epsilon_b q_b$ (see below). Except for the value at 30°C, the number of lipids per binding site also decrease with temperature. As can be seen from the table, the standard errors for the parameters as determined from the computer analysis are rather large compared to the values for the parameters themselves. (The standard errors for k_f , k_r and the number of lipids per binding site were determined from the results of analyzing various combinations of the available data files for the lipid-to-protein molar ratios collected for a particular temperature.)

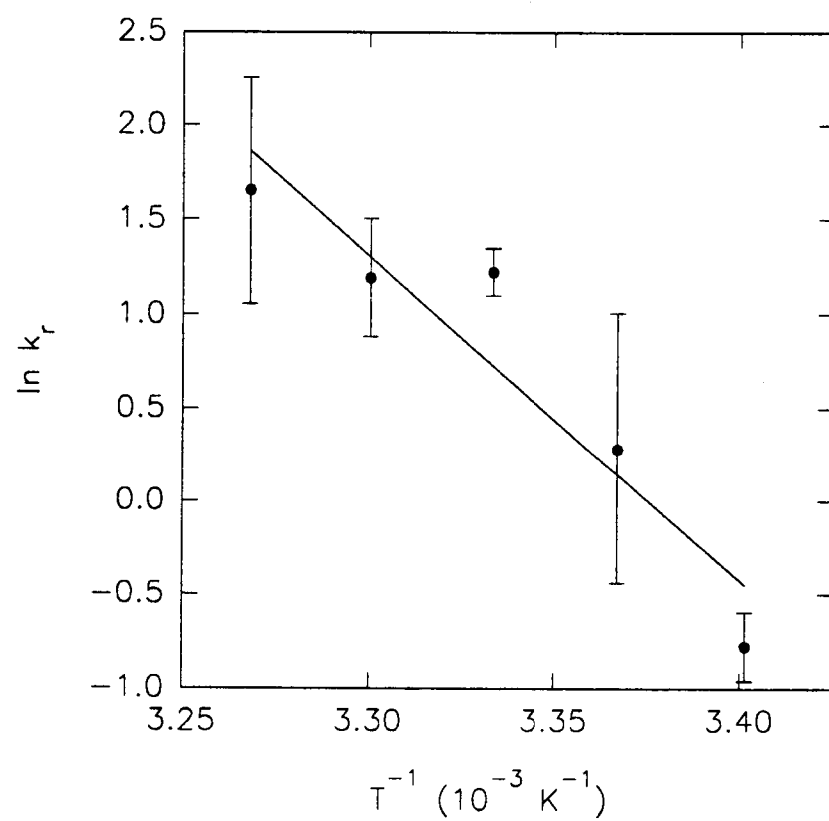
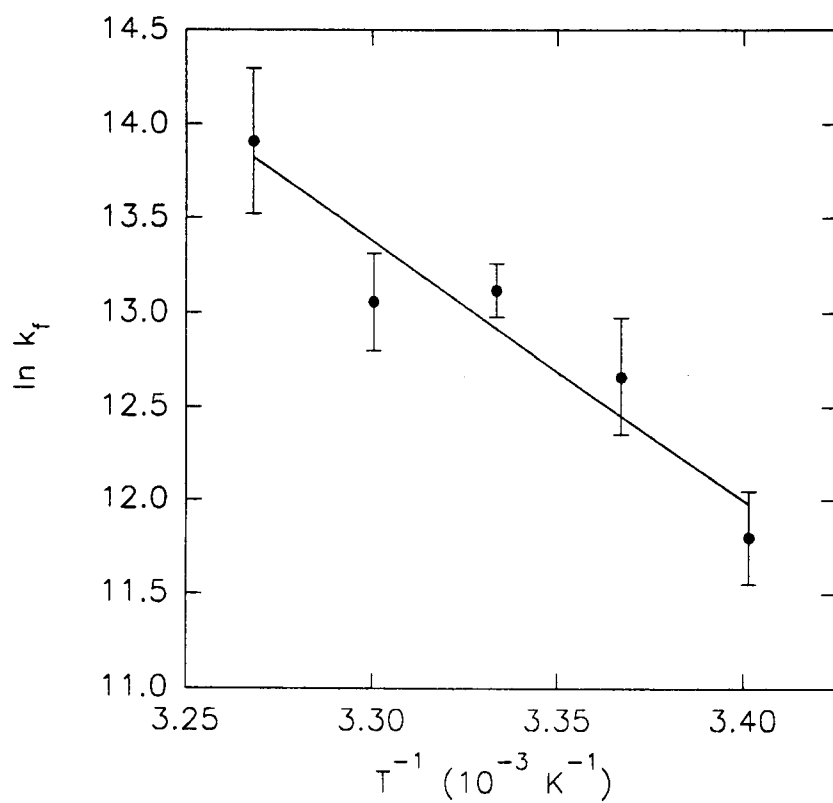
Figure 2 shows the results of an Arrhenius analysis of the values for the forward (k_f) and reverse (k_r) rate constants in Table 1. The upper panel shows a plot of $\ln k_f$ versus the inverse absolute temperature and the least-squares fit to the data, which yielded a value for the activation energy of 13.8 kcal mol⁻¹. The lower panel shows a similar plot for the case of k_r . There, a value of 17.3 kcal mol⁻¹ was obtained for the activation

TABLE 1

Results of measurements made of the binding of melittin to DMPC small unilamellar vesicles as determined by following the decrease in melittin fluorescence with binding of the protein to the membrane. The standard deviations as obtained from the computer analysis of the data are also given for each value of k_f , k_r and the number of lipids per binding site (n^{-1}).

Temperature	k_f ($10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_r (s^{-1})	Number of Lipids per Binding Site (n^{-1})
33°C	10.95 ± 4.24	5.23 ± 3.13	41.78 ± 19.78
30°C	4.69 ± 1.20	3.30 ± 1.06	19.00 ± 5.80
27°C	4.98 ± 0.70	3.40 ± 0.42	37.31 ± 4.34
24°C	3.16 ± 0.98	1.33 ± 0.96	30.32 ± 8.35
21°C	1.33 ± 0.33	0.46 ± 0.08	25.06 ± 5.18

Figure 2. Arrhenius plots of $\ln k_f$ versus T^{-1} (K^{-1}) (upper panel) and $\ln k_r$ versus T^{-1} (K^{-1}) (lower panel) for the data in Table 1. The smooth lines are the least-squares fits to the data, which gave values for the activation energies of $13.8 \text{ kcal mol}^{-1}$ and $17.3 \text{ kcal mol}^{-1}$ for the forward and reverse processes, respectively.



energy. These graphs suggest that the data for 30°C (in the case of k_r) are understated and that those for 27°C (in the case of k_r) are overstated, as was suggested above. The exclusion of the point for 27°C from the fit was found to make no significant difference in the value obtained for the activation energy for dissociation. Exclusion of the point for 30°C in the case of k_r would increase the activation energy for binding from 13.8 to 15.2 kcal mol⁻¹.

Table 2 summarizes the results of the global analysis with respect to the values for the fluorescence coefficients or effective quantum yields $\gamma_r \epsilon_r q_r$ and $\gamma_b \epsilon_b q_b$. As was stated above in the Materials and Methods section, the data file that was read in first for a particular global analysis was assigned an exciting light intensity of unity. Based on the concentration of the melittin solution that was used to calibrate the light source for that file and the solution's fluorescence intensity as recorded by the A/D converter, a value for $\gamma_r \epsilon_r q_r$ could be calculated for that particular analysis using equation 16. The value thus calculated is listed in column two of the table. The remaining files in that particular analysis were assigned exciting light intensities relative to that of the first based on the fluorescence intensity of their respective melittin calibration solutions (equation 15). Independently of this value for $\gamma_r \epsilon_r q_r$, the program also calculated $\gamma_r \epsilon_r q_r$ and $\gamma_b \epsilon_b q_b$ from equation 14. These values are listed in columns 3 and 4 of Table 2. Column 5 contains the values for $\gamma_b \epsilon_b q_b$ in column 4 but rescaled for the same exciting light intensity as at 33°C in order to enable comparison. As can be seen from the table, for each temperature examined, the two values for $\gamma_r \epsilon_r q_r$ are in good agreement with each

TABLE 2

Results of the computer analysis for the fluorescence coefficients or effective quantum yields, $\gamma_b e_b q_b$ and $\gamma_f e_f q_f$. No uncertainties were determined for these values.

Temperature	$\gamma_f e_f q_f^*$	$\gamma_f e_f q_f^{\ddagger}$	$\gamma_b e_b q_b^{\dagger}$	$\gamma_b e_b q_b^{\ddagger}$
33°C	9.36×10^4	8.87×10^4	3.80×10^4	3.80×10^4
30°C	2.78×10^4	2.94×10^4	1.20×10^4	4.04×10^4
27°C	2.74×10^4	2.75×10^4	9.41×10^3	3.21×10^4
24°C	9.51×10^4	9.41×10^4	3.84×10^4	3.78×10^4
21°C	2.89×10^4	2.88×10^4	1.20×10^4	3.89×10^4

*Fluorescence coefficient or effective absorbance for free melittin in solution as determined using equation 16.

$\ddagger$ Fluorescence coefficient or effective absorbance for free melittin in solution as determined from equation 14.

$\dagger$ Fluorescence coefficient or effective absorbance for bound melittin as determined from equation 14.

$\ddagger$ Fluorescence coefficient or effective absorbance for bound melittin as determined from equation 14 for exciting light intensity normalized to that used at 33°C.

other. Also, the rescaled values for $\gamma_b \epsilon_b q_b$ (column 5) are in good agreement amongst the different temperatures. (30°C and 27°C are again somewhat anomalous.)

DISCUSSION

Implicit in the model that was used to analyze the data presented above is that the sample lipid molecules are organized into distinct, unconnected, physically separate units which are diffusing freely through the solution. Each cluster of lipid molecules contains just the requisite number of lipid molecules to form a protein-lipid assembly. This is obviously not the case. To the extent that a protein molecule can be thought of as "binding" to a lipid vesicle, the latter should presumably be thought of as a much larger object which possesses multiple binding sites. As is well known (Cantor and Schimmel, 1980), the binding of a ligand to a macromolecule (or macromolecular assembly in this case) which possesses multiple binding sites is not simple, even if there is no cooperative interaction between the sites and they have identical affinities for the ligand (*i.e.*, the sites are identical and independent). With respect to analyzing the binding of a ligand in such a case, the kinetics would be governed by as many coupled differential equations as there are binding sites on the macromolecule. Justifiably, in light of the above one might not expect an analysis of the type carried out here to adequately describe the binding of melittin to phospholipid vesicles, although the model expressed in equation 1 would seem to be an improvement over those that have been used before (Schwarz and Beschiaschvili, 1989 and Sekharam *et al.*, 1991).

The evidence presented here suggests that the model is adequate for describing the binding of melittin to (at least) DMPC SUVs. Previously, it was found that the steady-state binding constant which would be obtained for the reaction in equation 1 fit the

isothermal titration calorimetric data for the binding of the leader sequence of ornithine transcarbamylase to phospholipid vesicles (Myers *et al.*, 1987) as well as the binding of melittin to DMPC SUVs and DPPC fused unilamellar vesicles (Bradrick, Freire and Georghiou, unpublished data). For these cases the above objection apply as well. In the present case, the lipid-to-protein molar ratios that were used were chosen to be relatively high in the hope of largely avoiding (or reducing) the statistical effects that appear in the case of multiple binding sites. The lipid-to-protein molar ratios used often included a value of $R = 45$, however, which is close to the number of lipids per binding site. Nevertheless, the data are fit rather well by the model, as can be seen from Fig. 1. Other aspects of the system, such as the diffusion of the binding sites over the membrane surface on the microsecond time scale and perhaps solute entrapment, in which the protein molecule would be prevented from rapidly diffusing away from the liposome, might serve to reduce the significance of the statistical effects in that they would alter the likelihood of an incoming protein molecule for finding a vacant binding site. Also, for all five temperatures, the association constant (k_f/k_r) is between 1 and $2 \times 10^5 \text{ M}^{-1}$. This means, for example, that at 33°C for the highest lipid and protein concentrations used (10.62 mM and 59 μM , respectively, which would most favor association), only one in four binding sites is filled at equilibrium. For these same maximum lipid and protein concentrations, at 21°C and 25 lipids per binding site, only about 14% of the sites would be occupied when equilibrium is reached. Given the values obtained for k_f and k_r , these results suggest that statistical effects may have had little influence over the binding kinetics observed in these experiments.

The temperature range over which binding was measured in this work extended from slightly below the lipid phase transition temperature T_m (23°C) to a temperature (33°C) at which the lipid sample had completely passed through its phase transition. Thus, over this temperature range the samples contained varying proportions of gel and liquid crystalline domains. It is known that in the case of large unilamellar vesicles composed of zwitterionic phospholipids (which are highly ordered and have a very narrow transition profile), melittin will not bind to liposomes when added to a sample that has a temperature lower than T_m (Dasseux *et al.*, 1984). If for SUVs it were also the case that melittin will not bind to domains that are in the gel phase, then presumably one could expect that for these data, the binding rate k_f and the dissociation rate k_r would experience little change as the temperature was varied. Presumably only n would change appreciably, as the number of liquid crystalline domains would decrease with temperature. For example, one might expect n at T_m to be twice that of n at 33°C as the number of liquid crystalline domains at T_m would be half that at a temperature at which the lipids are completely melted. (This argument of course ignores temperature effects on solvent viscosity and solute kinetic energy and how these would affect the binding constants.) This is apparently not the case here, which may suggest that binding to both phases (with presumably different affinities) is taking place and that in the context of the limited model employed here, the differences are being averaged out. One might assume that if binding to both gel and liquid crystalline phases is taking place that the conformation, orientation and/or degree of insertion of melittin into the bilayer might be different for the two phases. The present results (Table 2) can be interpreted to suggest, however, that this is

not the case as the effective quantum yield of the bound species ($\gamma_b \epsilon_b q_b$) is the same for all the temperatures. Quantum yield and peak emission wavelength (here correlated with γ , the filter transmittance) are sensitive indicators of protein conformation.

In light of the above it is difficult to interpret the results of the Arrhenius analysis. In the case of the activation energy for binding ($13.8 \text{ kcal mol}^{-1}$), this value is about twice that of the transition enthalpy ($5.9 \text{ kcal mol}^{-1}$) for phospholipids with fourteen acyl chain carbons (Marsh, 1990). This might be taken to indicate that with respect to binding (and presumably insertion) of the protein, more than the melting of the lipid acyl chains is required in order to allow protein binding. The activation energy of $17.3 \text{ kcal mol}^{-1}$ for the dissociation process is even more difficult to interpret.

It can be seen that in a number of the graphs (Fig. 1) there is a greater discrepancy between data and fit at early times than at later times. This may be due to the presence of a very fast initial step in the binding process. Such a process might involve electrostatic interactions between the positively-charged protein and local charges on the membrane surface. Also, structural changes may be taking place in the protein as it makes its way through the vesicle hydration layer. These changes could alter the environment of the protein's tryptophan residue and lead to a change in protein fluorescence. As the model used to analyze the data does not include such a fast initial process, this could explain why it does not fit the data as well at early times. Alternatively, the difference may simply be due to an instrumental artifact. Ongoing work (M. Lipkin, personal communication) indicates that mixing may introduce artifacts in the signal over the first 15 ms.

That the simple model expressed by equation 1 (which assumes only one type of "binding site") is apparently adequate to describe what is presumably a more complicated kinetic scheme (*e.g.* binding to both gel and liquid crystalline domains with different affinities) might be due to the fact that fluorescence intensity measurements alone are not always sufficient for gaining a complete description of the kinetics. Beechem *et al.* (J.M. Beechem, private communication) have shown, using synthetic data, that in the case of protein folding, nonpolarized fluorescence intensity data may be fitted by a model that is simpler than that for which the data were generated. In the case of the present system, it may take polarized fluorescence intensity measurements to gain enough information to delineate the binding to different domains, if that is indeed what is taking place. In that case, vertical and horizontal fluorescence would be collected for a number of different protein and lipid concentrations, as was done here. For associative fluorescence anisotropy decay one would then have

$$\begin{aligned}
 I_V(t) &= I \sum_i \gamma_i \epsilon_i q_i [c_i(t)] \left(\frac{1 + 2r_i}{3} \right) \\
 I_H(t) &= I \sum_i \gamma_i \epsilon_i q_i [c_i(t)] \left(\frac{1 - r_i}{3} \right)
 \end{aligned}
 \tag{18}$$

Here, the sums are over the *i* fluorescent species in the sample. *I* is the relative intensity of the exciting light, and $[c_i]$ and r_i are the concentration and fluorescence anisotropy of the *i*th species, respectively. The model could be further improved by treating the binding process as involving vesicles which possess multiple binding sites. This would involve the numerical solution of the relevant coupled differential equations within the context of a

nonlinear least-squares optimization. As the number of binding sites per vesicle would not be known *a priori*, one would presumably need to vary the number of differential equations being included in the analysis until an adequate fit is achieved. Additional data at lower protein-to-lipid molar ratios (where statistical effect would probably become more important) could be added in order to better specify the problem.

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PART V

SUMMARY

CONCLUSIONS

The above results indicate that the protein melittin can exert a strong influence on the behavior of phospholipid membranes. For some time, melittin has been known to possess fusogenic properties. The first part of this work describes the development of a fusion assay based on the dilution of the fluorescently-labeled phospholipid which provides quantitative information about the rate constants which describe vesicle fusion. When applied to the melittin-phospholipid systems, the fusion assay gave results which indicate the rates of fusion for monomeric and tetrameric melittin are greatly different. This suggests that the fusion mechanisms may be different for two states of melittin aggregation. The results also suggest that ions in solution may affect the fusion process, perhaps through the shielding of charges on the protein and/or lipids. The second part provides evidence that melittin has an ordering effect on lipid membranes when it binds in that it brings about a reduction of membrane fluidity and permeability. In the case of dimyristoylphosphatidylcholine small unilamellar vesicles, this effect is greatest at the lipid phase transition temperature. The latter result is found to be in agreement with recent theory. The melittin-induced perturbation of the bilayer is found to be virtually instantaneous on the time scale of these measurements, which is what would be expected based on the physical properties of the bilayer. That the perturbation is long range would seem to disagree with the boundary lipid hypothesis, which considers the lipid perturbation to be confined to a layer of lipid molecules immediately surrounding the protein. Lipid acyl chain length was also found to be an important physical parameter

as the addition of two additional carbon atoms (to obtain dipalmitoylphosphatidylcholine) results in lipid structures that experience little perturbation of the bilayer upon melittin binding, perhaps due to the increased intrinsic vesicle stability. Data for the binding of melittin to dimyristoylphosphatidylcholine small unilamellar vesicles were analyzed using a model which considers the lipid molecules to constitute independent, physically separate binding sites. For lipid-to-protein molar ratios for which this appears to be a good approximation, values for the forward and reverse rate constants were obtained which seem reasonable. The present model macroscopically describes the kinetics for the melittin-phospholipid system for which there are most likely two different populations of binding sites and which presumably have different affinities for melittin. Additional work involving fluorescence anisotropy may be necessary in order to obtain information about the kinetics of melittin binding to these two different types of binding sites.

VITA

Thomas Dale Bradrick was born in Shelby, Ohio and attended primary and secondary schools nearby. In 1978 he received a Bachelor of Arts degree in physics from Taylor University and in 1980 he received a Masters of Science degree in general physics from Case Western Reserve University. He then entered the graduate program of the Department of Physics of the University of Tennessee, Knoxville, from which he received a Master of Science degree in August, 1986. He was then admitted to the graduate program of the Department of Biochemistry of the University of Tennessee, Knoxville, from which he received a Master of Science degree in August, 1989, and a Doctor of Philosophy degree in December, 1994.