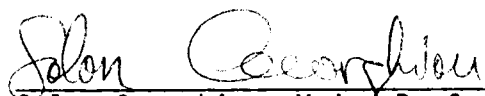


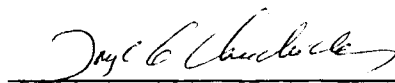
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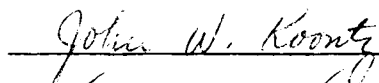
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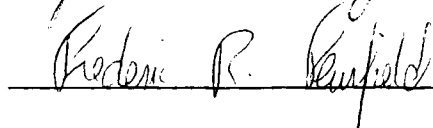
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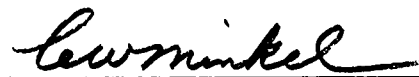
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EFFECTS OF BEE VENOM MELITTIN ON THE ORDER AND DYNAMICS OF
DIMYRISTOYLPHOSPHATIDYLCHOLINE UNILAMELLAR
AND MULTILAMELLAR VESICLES

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

Thomas Dale Bradrick

August 1986

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ABSTRACT

Nanosecond emission anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe, the enhancement of the 0-0 vibronic transition in the fluorescence spectrum of pyrene (which serves as a measure of the microenvironment of the probe), and a membrane fusion assay employing chlorophyll a and chlorophyll b as indicators of the mixing of membrane contents, have been used to investigate the effects of bee venom melittin on dimyristoylphosphatidylcholine (DMPC) unilamellar and multilamellar liposomes.

Nanosecond emission anisotropy studies performed on DMPC unilamellar vesicles at 16°C, 33°C and at the transition temperature, in the absence of melittin and in the presence of melittin at a protein to lipid molar ratio of $\sim 1/60$ have shown that at all three temperatures melittin brings about an increase in the order of the membrane's acyl chains and a reduction in the rate of diffusion of the probe molecule. This effect is most noticeable below the transition temperature. The same measurements made on multilamellar vesicles have shown that the protein causes a decrease in order only at the transition temperature, and does not affect the rate of diffusion at any of the temperatures investigated.

Measurements of the enhancement of the forbidden 0-0 vibrational transition in pyrene were performed for pyrene in unilamellar and multilamellar vesicles, in the absence of the protein and in its presence at a relative concentration of $\sim 1/60$, over the temperature

range from approximately 5°C to 35°C. For sonicated samples it was found that melittin enhanced the 0-0 vibronic transition of pyrene at 16°C, 33°C and at the lipid transition temperature. This indicates that the protein brought about an increase in the order of the membrane and a decrease in its fluidity which allowed the probe molecule to migrate closer to the glycerol backbone of the lipid molecules. This interpretation is consistent with the nanosecond emission anisotropy data. It was also found that melittin induced a sharpening of the gel-to-liquid crystalline transition, implying that the protein increased the cooperativity of the lipid molecules. Similar experiments performed on multilamellar vesicles under the same conditions showed that melittin caused little change in the enhancement of the 0-0 vibrational transition at 16°C and 33°C. In the vicinity of the transition temperature, however, melittin was found to greatly decrease the strength of the enhancement, implying that the membrane's order had decreased, which allowed the probe to migrate further towards the nonpolar center of the bilayer. Results of the nanosecond emission anisotropy and the 0-0 vibrational enhancement measurements were again found to be consistent. The measurements using pyrene also indicated that melittin broadens the gel-to-liquid crystalline transition in multilamellar vesicles, thus reducing the cooperativity of the lipid molecules.

Finally, a membrane fusion assay, which measures energy transfer efficiency between chlorophyll a and chlorophyll b incorporated into the membrane, was used to determine that, at the protein-to-lipid

molar ratio used, melittin caused the fusion of approximately three unilamellar liposomes when the latter were incubated with melittin at 33°C. No such fusion was found to occur in the case of the multi-lamellar vesicles.

The results of these studies show that melittin exerts its greatest effect on the unilamellar vesicles, where it alters the order and fluidity of the membranes, and presumably their morphology as well. The changes in the order should be viewed as the collective effect of a number of different melittin-induced processes which have taken place in the unilamellar vesicles.

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CHAPTER I

INTRODUCTION

Melittin is a small peptide which consists of 26 amino acid residues and which constitutes approximately 50% of the dry weight of honey bee venom (Habermann, 1972). The protein's amino acid sequence is shown in Figure 1. It has amphipathic character, with amino acid residues 1-20 being predominantly hydrophobic and residues 21-26 being hydrophilic. Because of its structure it exists as a tetramer in crystals and in solutions at high concentrations (Faucon et al., 1979). At low concentrations the protein has been found to enhance the activity of phospholipase A_2 (Vogt et al., 1970; Mollay and Kreil, 1974; Mollay et al., 1976; Yunes et al., 1977) by altering the structure of the membrane to which it binds, and at high concentrations it lyses model and natural membranes (Weissmann et al., 1969; Sessa et al., 1969; Olson et al., 1974). 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 which it is being bound. The origins of its interactions with lipids have yet to be completely determined, however. Nevertheless there exists a significant amount 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 work. It is the purpose of this study to help

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

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

further the understanding of the way in which this protein interacts with phospholipid membranes.

One of the techniques by which information can be obtained on the behavior of these model systems is the use of intrinsic and extrinsic fluorescent probes. Such probes report on the fluidity and effective polarity of their immediate environment, and give information about the structural changes undergone by the lipids during their phase transition, the effect of the binding of proteins, the perturbations wrought by cholesterol, ions and drugs, and also the accessibility of their binding sites to molecules that can quench their fluorescence, the proximity of the functional sites of proteins embedded in lipid bilayers, and the existence of membrane potentials and vesicle fusion.

In the present study we have employed the following techniques for delineating the effects of mellitin on the structure and dynamics of dimyristoylphosphatidylcholine (DMPC) unilamellar and multilamellar vesicles: (i) nanosecond emission anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe; (ii) enhancement of the weakly allowed 0-0 vibronic transition in the fluorescence spectrum of pyrene, which is the result of ground-state complexation with polar groups; and (iii) a fusion assay which measures the reduction in energy transfer efficiency between membrane-bound chlorophyll a and chlorophyll b molecules, which takes place through membrane dilution when pigmented and nonpigmented vesicles are caused to fuse together.

1. Nanosecond Emission Anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH)

DPH is a rod-shaped molecule, about $13 \text{ \AA}$ long, whose absorption and emission dipole moments lie along the molecule's long axis of symmetry. DPH is nonpolar and insoluble in water. Its emission spectrum is only slightly sensitive to solvent polarity, viscosity and temperature and in membranes it partitions itself into the hydrophobic region of the bilayer. Also, its radiative lifetimes in aromatic and aliphatic solvents differ greatly, and are found to increase with temperature. The fluorescence decay of DPH in organic solvents has always been reported as being single exponential, although a double exponential decay has been observed in ethanol at -25°C (Birch and Imhof, 1977). Because of its size, shape and fluorescence properties it has found wide use as a fluorescent probe in phospholipid membranes.

The theory of fluorescence anisotropy may be summarized as follows. After excitation with polarized light, fluorescent molecules undergo electronic and rotational relaxation, so that, whereas the molecule's fluorescence is initially highly polarized (anisotropic), it becomes increasingly depolarized with time as the molecule precesses. For the case of completely unrestricted motion (e.g., a freely rotating spherical molecule in an isotropic system) the fluorescence eventually becomes completely depolarized so that the fluorescence components parallel and perpendicular to excitation are equal. The anisotropy decreases with time as a single exponential.

The rate at which the molecule rotates and decreases its fluorescence anisotropy is related to the microviscosity of the molecule's environment and to the molecule's volume.

Complications enter into the time decay of the anisotropy when the molecule is freely rotating but is nonspherical. Then the molecule's motion will depend on the three different rates of rotational diffusion it has about its three principal axes of symmetry. Furthermore, if the molecule is not freely rotating but has its motion somehow restricted by its environment, the fluorescence anisotropy will be prevented from decaying to zero. DPH imbedded in a lipid bilayer is an example of the latter type of system. Because it positions itself along the lipid acyl chains it moves in a restrictive environment. The extent to which its fluorescence is allowed to become isotropic will depend on the degree to which the lipid acyl chains allow it to move, i.e., on their order and flexibility. Under these conditions its diffusive motion is confined to a cone, through which it wobbles while it fluoresces; the size of the cone is determined by the degree to which the acyl chains restrict its motion. Measurements of the fluorescence anisotropy's time decay give information about the size of this cone and the mobility of the probe molecule. As the DPH molecule is quite similar to the lipid acyl chains in terms of its size and polarity, it is reasonable to expect that the information gained about its motion applies to the acyl chains as well (Lakowicz, 1983; Yguerabide and Foster; 1981; Zannoni et al., 1983).

2. Enhancement of the 0-0 Vibronic Transition of Pyrene

The 0-0 vibronic transition in the fluorescence spectrum of pyrene (at about 373 nm), which is very weak in inert solvent (e.g., heptane), is enhanced considerably when pyrene is dissolved in polar solvents such as butanol, methanol and water (Lianos and Georghiou, 1979a; Nakajima, 1971). By employing IR and nanosecond fluorometric techniques it was demonstrated (Lianos and Georghiou, 1979a; Lianos and Georghiou, 1979b) that the enhancement effect is the result of ground state molecular complex formation between pyrene and polar solvent groups. Such complexation reduces the symmetry of the molecule and renders the forbidden 0-0 vibronic transition more allowed. These studies also established that the degree of enhancement is a measure of the effective polarity of the microenvironment of the fluorophore. This method of obtaining information on the polarity of the probe's microenvironment offers an advantage over determining the same through fluorescence spectral shifts in that the latter can be adversely affected by restricted relaxations of nearby polar groups during the lifetime of the probe's excited state. Pyrene suffers from no such uncertainty as the emission is from a probe-polar group complex, formed in the ground electronic state of the molecule, whose association constant does not change appreciably upon excitation. Previous work from this laboratory (Lianos et al., 1980) compared the enhancements in various phospholipids with those in different solvents; the results clearly indicated that the fluorescent probe does not penetrate deeply into the bilayer, but is instead located in the

vicinity of the semipolar lipid glycerol backbone. This finding is particularly important in light of the necessity of having accurate information concerning the location of the probe in order to use it effectively. Finally, it was demonstrated that the enhancement effect offers a method for determining phase transition temperatures in phospholipids (Georghiou and Mukhopadhyay, 1981).

In the present work we have employed this phenomenon to determine the phase transition temperature of the bilayer, the width of the phase transition profile and the effect of melittin on the degree of enhancement to draw conclusions from them about membrane fluidity.

3. The Use of Chlorophylls a and b in the Membrane Fusion Assay

When present together in close proximity, chlorophyll b molecules will transfer energy which they have absorbed, to nearby chlorophyll a molecules. The efficiency of this process depends on the relative and absolute concentrations of the two molecules, i.e., on how many donors there are for every acceptor and on how close the donors and acceptors are to each other. For a mixture of the two chlorophylls excited at 468 nm (where chlorophyll a has zero absorbance but chlorophyll b does not), the fluorescence spectrum of the sample will depend on the above two parameters. If molecules of the two species are so far apart that energy transfer is unlikely, then the fluorescence peak will be at 658 nm, where b has its maximum. Increasing the concentrations will move the molecules closer together, however, so that energy transfer will become more likely. In that

case the fluorescence of chlorophyll a (peak at 677 nm) will make an increasingly substantial contribution to the total fluorescence of the sample. Thus one could crudely define the efficiency of energy transfer as being the ratio of the fluorescence intensities at 677 nm to that at 658 nm, with normalization made with respect to the fluorescence intensity at some third wavelength where the intensity of b is low. Such a definition of energy transfer efficiency is albeit crude, but if calibration is made using samples with known chlorophyll concentrations, then one has an easy and effective means of determining the chlorophyll concentration in an unknown sample (as long as the relative concentrations of a and b are the same as in the calibration samples). When applied to lipid vesicles which fuse, such concentration determinations can be used to measure the degree to which membrane contents have been diluted. This in turn would allow one to estimate the amount of fusion which has taken place (Gad and Eytan, 1983).

CHAPTER II

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPH), chromatographically pure, ethylenediaminetetraacetic acid (EDTA)-disodium salt, and chlorophyll a and chlorophyll b were purchased from Sigma Chemical Co., and were used without further purification. 1,6-diphenyl-1,3,5-hexatriene (DPH) and zone refined pyrene, both scintillation grade, were obtained from K&K Laboratories and P. Pauric, Philadelphia, respectively. Hydrochloric acid, Tris and scintanализed dioxane were from Fisher. Lyophilized melittin was purchased from ICN and was used without further purification, while triply distilled water was purchased from Carolina Biological Supply. Reagent grade diethyl ether was obtained from American Scientific Products.

All measurements were made on samples prepared in Tris buffer. The 50 mM Tris, pH 7 buffer, which contained EDTA at a concentration of 1 mM, was prepared as follows. A 0.5 M Tris solution was prepared by dissolving 3.028 g of Tris in 50 ml of triply distilled water. Then a 0.5 M HCl solution was made by diluting 2.075 ml of concentrated hydrochloric acid to 50 ml using triply distilled water. Next 26 ml of the Tris solution was added to 24 ml of the HCl solution to give 50 ml of 0.5 M Tris buffer. This, with 0.168 g of EDTA-disodium salt was diluted to 500 ml with triply distilled water to yield a 50 mM Tris, 1 mM EDTA buffer solution, whose pH was adjusted to 7 using sodium hydroxide, and checked with a Fisher pH meter.

Fresh samples of liposomes were made prior to each experiment. To prepare small unilamellar vesicles (SUVs), 10 mg of lipid were hydrated in 10 ml of 50 mM Tris, 1 mM EDTA, pH 7 buffer at 33°C, and were then stirred at 33°C for half an hour. (The lipid concentration was 1.48 mM.) The sample was then split into two portions of 5 ml each. To one portion was added the appropriate volume of a fluorescent probe dissolved in solvent (see below). This solution of fluorescently-labeled liposomes was stirred for an additional half hour to ensure complete incorporation of the probe molecules. The other 5 ml portion remained unlabeled, and after sonication and centrifugation was used to obtain the fluorescence experiment's baseline due to scattering. Both samples were sonicated for 25 minutes (or to clearness) at 33°C in a Laboratory Supplies bath sonicator. Finally, the samples were centrifuged (to remove remaining nonsonicated MLVs) at 65000xg for 25 minutes at 33°C using a Ti50 rotor in a Beckman L8-80 model ultracentrifuge. The supernatant was then extracted and held at 33°C until needed.

Multilamellar vesicles (MLVs) were prepared by hydrating 2.5 mg of DMPC in 25 ml of 50 mM Tris, 1 mM EDTA, pH 7 buffer at 33°C, and were then stirred for half an hour. (Here the lipid concentration was one-tenth that for SUVs, i.e., 0.148 mM. This reduced concentration was required in order to carry out the fluorescence measurements; higher concentrations caused the lipid to settle out of the solution over the course of the experiment.) To a 10 ml portion of the dispersion was added the appropriate volume of fluorescent probe

dissolved in solvent (see below). This solution of fluorescently-labeled liposomes was stirred for an additional 25 minutes to ensure complete incorporation of the fluorescent probe. Labeled and non-labeled lipid solutions were held at 33°C until needed.

A 1 mM solution of pyrene (M.W.=202) was prepared by dissolving 2 mg of pyrene in 10 ml of dioxane. Twenty-five μ l of this solution was added to 5 ml of the 1.48 mM lipid dispersion when forming SUVs. Alternately, 5 μ l of the probe solution was added to 10 ml of the 0.148 mM lipid dispersion when forming MLVs. A fresh solution was made for each experiment.

For DPH (M.W.=232) a solution of 1 mM was prepared by dissolving 2.3 mg of DPH in 10 ml of dioxane. Fifteen μ l of this solution was added to 5 ml of the 1.48 mM lipid dispersion for SUVs. For MLVs, 3 μ l of the DPH stock solution was added to 10 ml of the 0.148 mM lipid dispersion. In both cases a lipid to DPH molar ratio of $\sim$ 500 was maintained to minimize any distortion of the lipid bilayer by the probe molecule. A fresh stock solution was made prior to each experiment.

Solutions of chlorophyll a and chlorophyll b were made by dissolving 1 mg of either in 1 ml of diethyl ether. To lipid samples destined for the addition of melittin, 100 μ l of the chlorophyll a stock and 50 μ l of the chlorophyll b stock were added to 5 ml of the 1.48 mM dispersions for SUVs. By weight this gave a chlorophyll concentration of 2.4%. Other lipid solutions containing 80 μ l of a and 40 μ l of b (1.9%), 50 μ l of a and 25 μ l of b (1.2%), and 20 μ l of a

and 10 μ l of b (0.48%) were formed in the same way, and along with a sample of the 2.4% solution (without melittin) served as standards to calibrate the fusion assay. A nonchlorophyll-labeled lipid solution was also made (1.48 mM) for mixing with the labeled liposomes, as required in the assay. As indicated, all labeled liposomes contained chlorophyll a and chlorophyll b in a 2:1 ratio, similar to that found in plants. This was done to optimize the sensitivity of the assay.

Fresh samples of melittin were made prior to each experiment by dissolving 7 mg of the lyophilized powder in 1 ml of 50 mM Tris, 1 mM EDTA, pH 7 buffer. Given a molecular weight of 2800 for melittin, this would yield a stock with a concentration of 2.5 mM. ICN melittin contains some salt however, so that actual stock concentrations were 2 mM as determined by measuring the absorbance at 280 nm (extinction coefficient = $5800 \text{ M}^{-1} \text{ cm}^{-1}$) on a Cary 14 spectrophotometer. To 4 ml of the sonicated and centrifuged SUVs (both fluorescence and baseline samples) was added 50 μ l of the melittin stock. To 4 ml of the MLV dispersions (again, both fluorescence and baseline samples) was added 5 μ l of the melittin stock. In all experiments a melittin to lipid molar ratio of $\sim 1/60$ was obtained. After the addition of the protein the lipid solutions were incubated for 30 minutes at 33°C to reach the equilibrium binding of melittin and to insure the completion of all morphological changes brought about by the protein's addition.

All commercial melittin contains phospholipase A₂ contamination so that it was necessary to carry out all experiments in buffer containing EDTA. For the absolute concentrations of melittin in liposomes

used here (100 μ M for SUVs and 10 μ M for MLVs) EDTA at a concentration of 1 mM was found to be adequate for inactivating the enzyme.

The steady-state spectral measurements of pyrene and chlorophyll fluorescence were made on a spectrofluorometer which employs a 1 kW Xe-Hg light source, a sample holder equipped with a Cryo-Tip device for controlling sample temperature, two 0.5 m Bausch and Lomb monochromators for selecting excitation and emission wavelengths, a thermoelectrically cooled 9558 QB EMI photomultiplier, a PAR lock-in amplifier, and an X-Y recorder. The fluorescence, observed at a right angle to the exciting light beam (wavelength selected by the first monochromator) passes through the second monochromator and is collected by the photomultiplier. The output of the photomultiplier is directed to the lock-in amplifier, which is triggered by the chopper that modulates the exciting light beam. The output voltage of the lock-in amplifier drives the vertical displacement of the recorder pen. The motor which advances the wavelength of the emission monochromator can also drive the horizontal displacement of the recorder pen. A block diagram of the spectrofluorometer is shown in Figure 2.

A nanosecond fluorometer, previously described (Badea and Georgiou, 1976) was used to perform the nanosecond emission anisotropy measurements. The fluorometer employs a high pressure (200 psi) N_2 discharge lamp that provides light pulses at a repetition rate of 10 kHz, a Cryo-Tip device for controlling sample temperature, an Amperes 56 TUVP photomultiplier and a 162 PAR boxcar averager. The excitation wavelength of 334 nm was chosen by placing a Corion 3-34

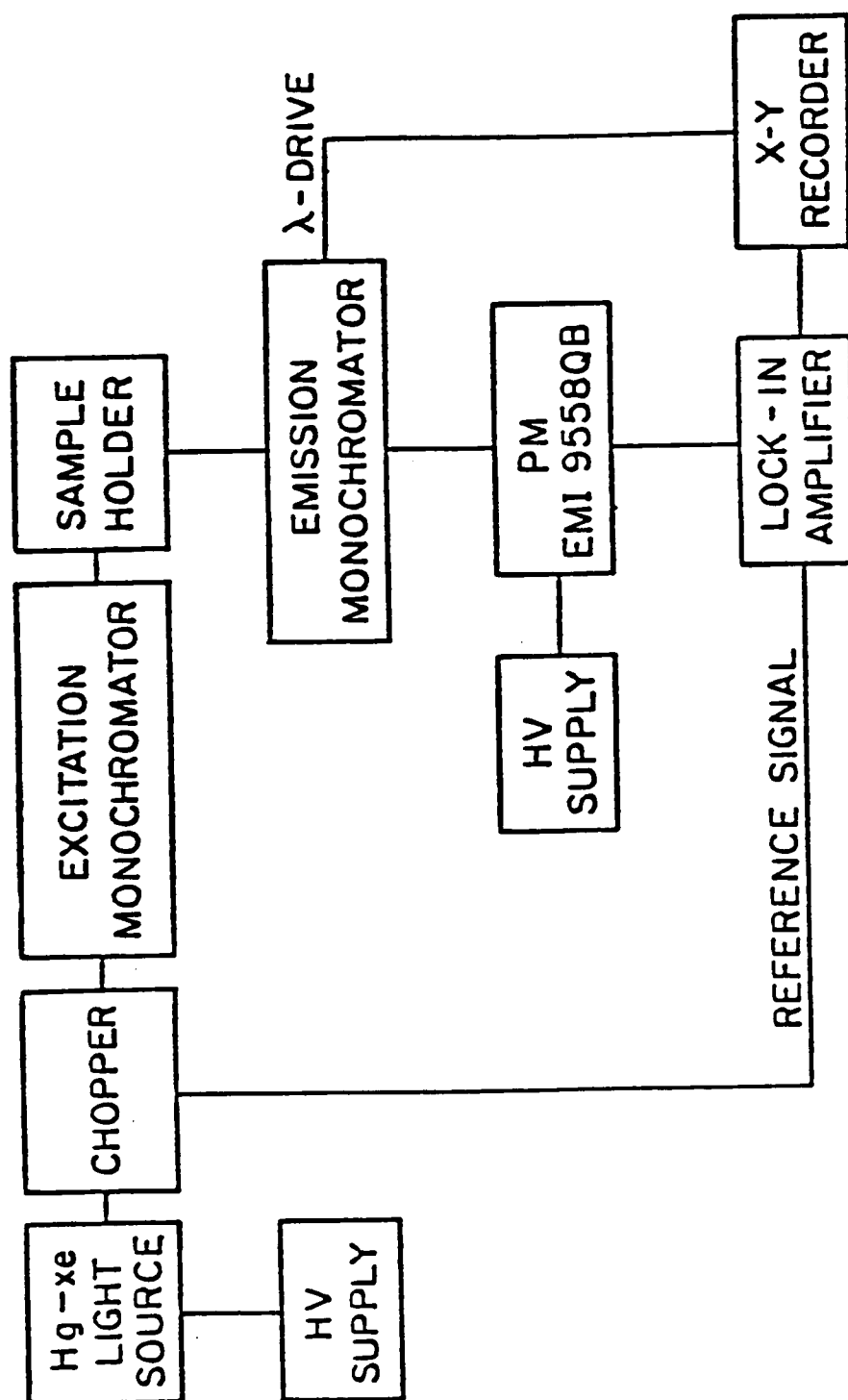


Figure 2. Block diagram of the steady-state fluorometer.

interference filter in the excitation light path. A 3M 105-UV-WRMR polarizer came next to expose the sample to vertically polarized exciting light. Fluorescence was viewed at a right angle to excitation through a Polaroid HNP'B polarizer which was used to select either the vertical or horizontal fluorescence components for analysis. This light passed through a Corning 3-72 color filter and was collected by the photomultiplier, which sent its signal to the boxcar averager. The total number of channels and the sampling frequency were controlled by an interface which also transmitted the fluorescence intensity for each channel to an IBM PC for storage. Simultaneously, the boxcar averager drove an X-Y recorder which plotted fluorescence intensity vs. time. A block diagram of the fluorometer is shown in Figure 3.

For each experiment the horizontal and vertical components of the fluorescence and baseline intensities were collected, as well as the lamp and lamp baseline intensities. Then, a 20 ns delay cable was added to the path between the photomultiplier and the boxcar averager, and the lamp profile was rerun on the X-Y recorder. The consequent 20 ns delay in the arrival of the signal was used to calibrate the time-axis of the fluorescence decay profiles.

For both fluorometers sample temperature was measured with a potentiometer connected to a copper-constantan thermocouple immersed directly into the sample in the cuvette.

The wavelengths at which all fluorescence measurements were made were such that no correction had to be made to the spectra due to

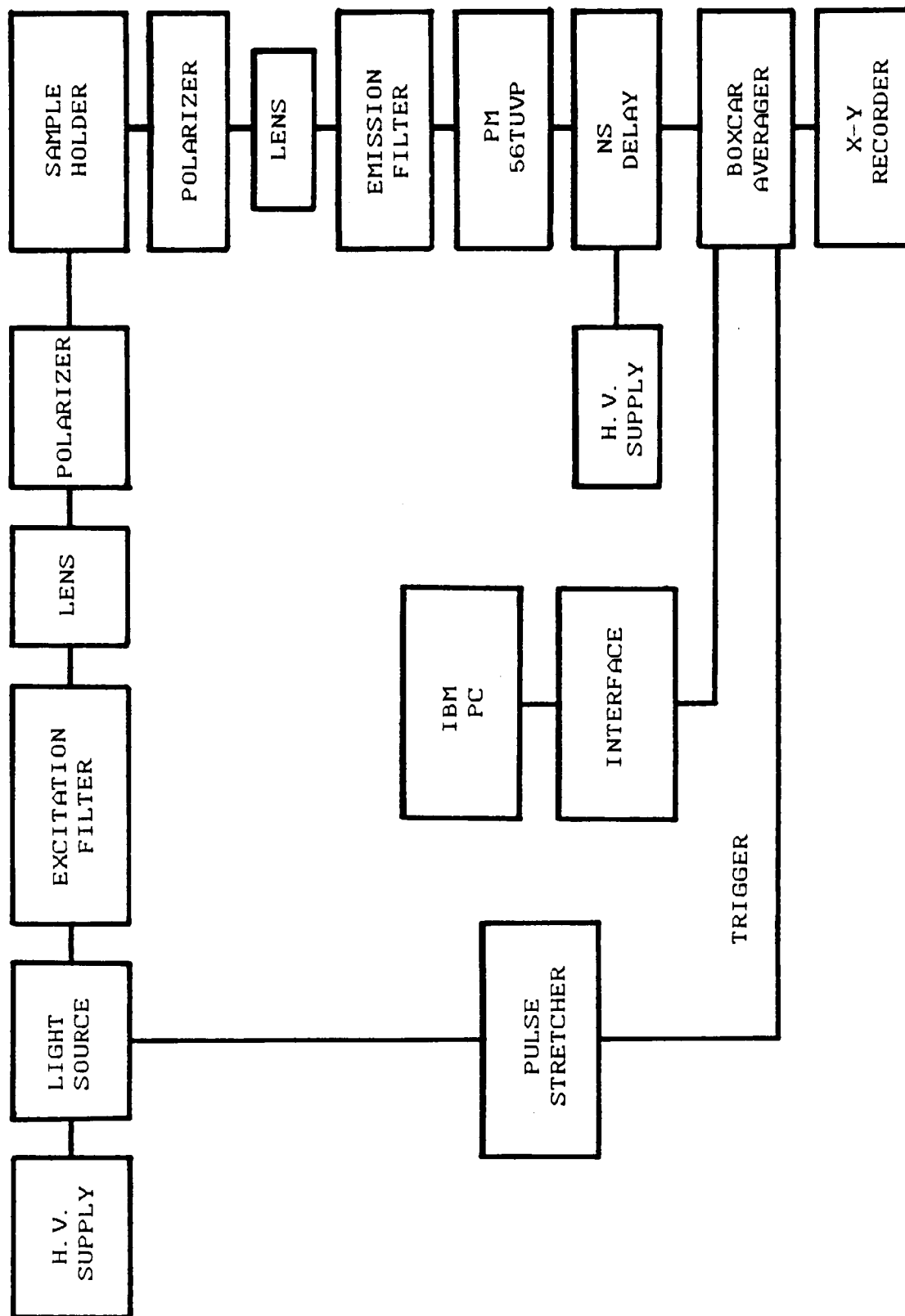


Figure 3. Block diagram of the nanosecond fluorometer.

any wavelength-dependent sensitivity of the photomultipliers and/or monochromators used.

It has been established that the morphology and behavior of liposomes bound with melittin generally depend on the past history of the sample (Dassuex et al., 1984). For this reason strict control was maintained over the temperature of lipid solutions. All solutions were formed at 33°C, and it was at this temperature that all fluorescent probes were incorporated into the bilayers. Sonification and centrifugation were also carried out at 33°C and all solutions awaiting their next step in sample preparation were maintained at 33°C as well. After centrifugation the recovered supernatant was kept at 33°C and the incubation after melittin addition was carried out at this temperature as well. For those measurements made at or below T_M , the sample temperature was not lowered until just prior to when the measurement was made. Repeated heating and cooling of the samples was not allowed to take place.

In order to test for possible quenching of the DPH fluorescence due to oxygen dissolved in the lipid bilayer or buffer, emission anisotropy measurements were also made on samples which had been deoxygenated, through bubbling with oxygen-free N_2 for half an hour, after the centrifugation step. This test was especially important in light of the fact that we consistently obtained a double exponential time decay of the total fluorescence of DPH in liposomes. Normally DPH possesses a single lifetime when it is free in solution. It is thought, however, that its two phenyl groups may behave as independent

fluorophores, and we were concerned that oxygen in the buffer outside the liposomes might quench the fluorescence of the phenyl group nearest the surface of the bilayer. No change in the fluorescence or the anisotropy was seen, though. Previous work (Georgiades, M.S. thesis) showed that deoxygenation of the pyrene-containing liposomes was not necessary either.

As was indicated above, there existed a factor of ten difference in the lipid and protein concentrations used for the sonicated and nonsonicated samples. As melittin undergoes aggregation with increased concentration (Faucon et al., 1979) it was thought desirable to establish that possibly differing amounts of protein aggregation did not in some way contribute to the differences in anisotropy results obtained for SUVs and MLVs. To this end emission anisotropy measurements were carried out on sonicated samples (SUVs) having a lipid concentration of 0.148 mM (i.e., that of the MLVs) and a protein to lipid molar ratio of 1/60. No difference in results was seen for the two concentrations of SUVs, which led us to conclude that melittin exists in the same degree of aggregation in the experiments on SUVs and MLVs described here.

For the pyrene 0-0 enhancement studies carried out in this study it is important to note that pyrene-in-lipid concentrations were made low enough so that intermolecular excimer formation by the probe was negligible. This was verified by examining the fluorescence intensities at 470 nm (where the peak in excimer fluorescence occurs) of the samples' spectra. This point is important as pyrene at high

enough a concentration to give much excimer fluorescence would seriously distort the membrane.

Finally, for the steady-state fluorometric measurements made to determine the pyrene 0-0 fluorescence enhancement, the slits of the emission monochromator were adjusted to give a resolution of 1.3 nm, as the fluorescence peaks are quite sharp. Also, for each measurement made at each temperature the emission monochromator was readjusted to find the peak intensity in fluorescence. This, as opposed to simply resetting the monochromator to a particular wavelength each time, was necessary as the peak wavelengths shift somewhat as the sample temperature is changed. Once the monochromator was set at the desired wavelength the fluctuations in fluorescence intensity were recorded for whatever amount of time was necessary in order to obtain the average intensity.

CHAPTER III

RESULTS

Measurement of the nanosecond emission anisotropy of the fluorescent probe DPH was one of the techniques used in this study to examine the effect of melittin on lipid bilayers. In the membrane DPH is excited with vertically polarized light, and the vertical and horizontal fluorescence components are measured as a function of time. The emission anisotropy, $r(t)$, is defined as

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

where $I_V(t)$ and $I_H(t)$ are the vertical and horizontal fluorescence intensities, respectively. For a rod-shaped molecule like DPH undergoing restricted motion in a lipid bilayer, the emission anisotropy is well approximated by the following relation from the wobble-in-cone model (Kinosita et al., 1977; Kawato et al., 1977; Stubs et al., 1981; Kinosita et al., 1981; Konosita and Ikegami, 1984):

$$r(t) = (r_0 - r_\infty)e^{-t/\phi} + r_\infty.$$

Here, r_0 is the initial anisotropy, r_∞ is the limiting anisotropy, and ϕ is the rotational correlation time. The order parameter S , which reflects the angular amplitude of the probe's motion, is given by (Jähnig, 1979 and Heyn, 1979):

$$S = \left(\frac{r_{\infty}}{r_0} \right)^{1/2} .$$

The cone's half angle θ is related to S through (Kinosita et al., 1977)

$$S = \frac{1}{2} \cos \theta (1 + \cos \theta) .$$

We note that ϕ alone does not completely specify the probe's rate of diffusion, as the latter quantity will depend not only on ϕ but on the size (i.e., half-angle) of the cone as well. The rate of diffusion D_W , is given by

$$D_W = \frac{\langle \sigma(S) \rangle}{\phi}$$

where $\langle \sigma(S) \rangle$ is a unitless function of S that expresses the averaged rates of diffusion an arbitrarily shaped molecule has about its different axes. Figure 3 in Kinosita et al., 1977, shows $\langle \sigma \rangle$ as a function of S for the case of a rod-shaped molecule, and it was from this source that the appropriate values for $\langle \sigma \rangle$ were obtained. That graph is reproduced here as Figure 4. Since the rod-shaped DPH molecule resembles the lipid acyl chains and does not show a preference for the gel or the liquid-crystalline states of the bilayer (Jähnig et al., 1982 and Lentz et al., 1976), it reports rather faithfully on the structure and on the molecular motions of the bilayer.

The nanosecond emission anisotropy analysis was carried out in the following way. First a theoretical expression was fit to the total

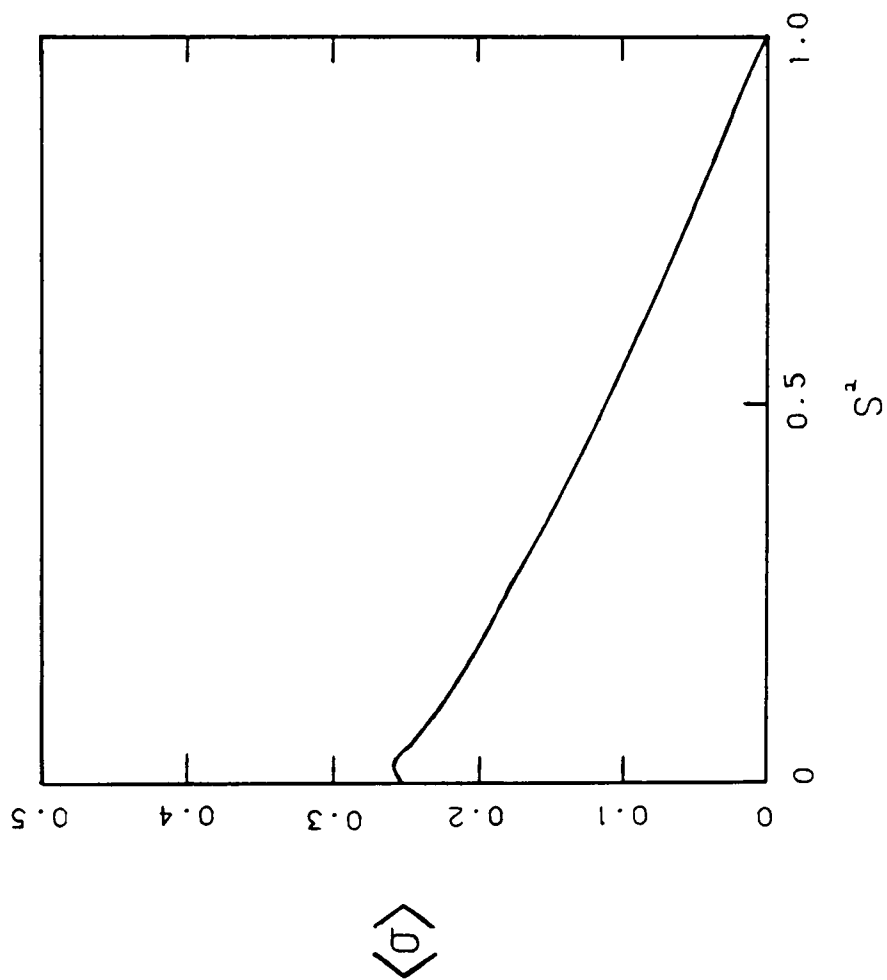


Figure 4. Plot of $\langle \sigma \rangle$ as a function of the square of the order parameter. For $0.1 \leq S^2 \leq 1.0$ we have found that $\langle \sigma \rangle = 0.2571 - 0.3161 S^2 + 0.0541 S^4 + 0.0043 S^6$ fits the curve quite well.

fluorescence, $I_V(t) + 2I_H(t)$, as obtained by calculating the sum of I_V and $2 \times I_H$ for each channel. (Figure 5 shows a plot of I_V and I_H vs. channel number for a typical experiment.) For each experiment, both single and double exponential fits to the total fluorescence were attempted and it was always found that a double exponential best described the fluorescence decay. In making this judgement the residuals and the autocorrelation function of the residuals were used as criteria for goodness of fit.

After having obtained a double exponential fit to the experimental total fluorescence,

$$I_V(t) + 2I_H(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

the anisotropy parameters were extracted, using the best fit to the total fluorescence, by varying r_0 , r_∞ and ϕ in the following equation to fit the experimental $I_V(t) - I_H(t)$ data:

$$(A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2})[(r_0 - r_\infty)e^{-t/\phi} + r_\infty] = I_V(t) - I_H(t) .$$

Here $I_V(t) - I_H(t)$ was obtained by taking the difference between the two fluorescence components, channel per channel.

Both steps in this process were carried out on The University of Tennessee Computing Center's IBM 3081 computer using standard non-linear least-squares fitting routines. The exciting light pulse profile for each experiment was used by the programs to deconvolve the data.

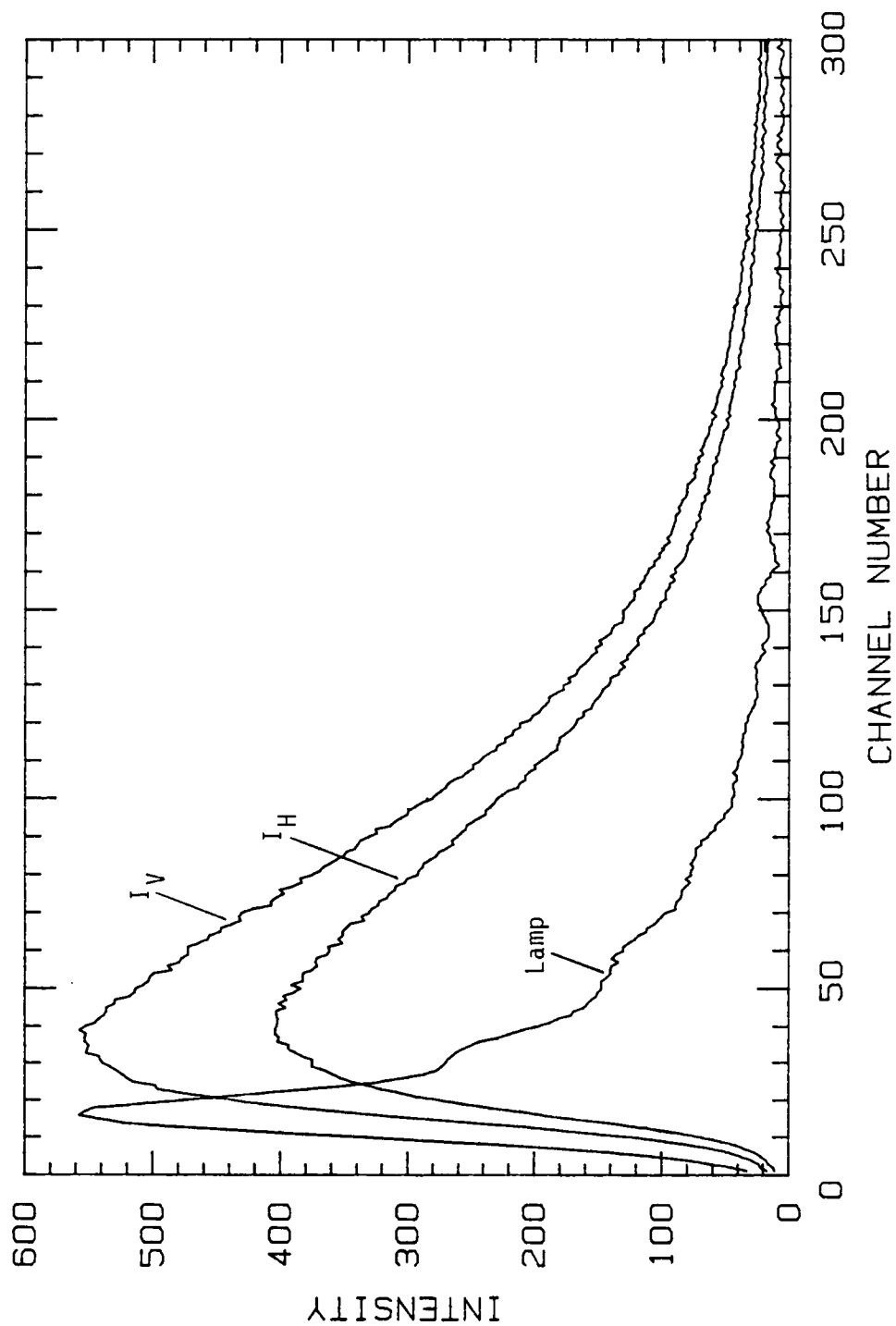


Figure 5. Plot of lamp, vertical and horizontal fluorescence component intensities vs. channel number for a typical experiment.

Experiments were performed at 16°C, 33°C and at the transition temperatures for both unilamellar and multilamellar vesicles, in the absence and in the presence of melittin at a protein to lipid molar ratio of $\sim 1/60$. Each experiment was performed at least four times in order to obtain good statistics. (This is important as ϕ is a somewhat difficult parameter to extract from the data and can show some variability from experiment to experiment.)

Results of the lifetime determinations are shown in Table I; the fitted emission anisotropy parameters are shown in Table II. Estimates of the uncertainties of the lifetimes and anisotropy parameters are included with the tables.

Figures 6 through 69 show the results of the computer-generated fits for the following sets of experimental conditions: Figures 6 through 13, sonicated DMPC at 16°C with no melittin; Figures 14 through 21, sonicated DMPC at 16°C with melittin at a protein to lipid molar ratio of $\sim 1/60$; Figures 22 through 29, sonicated DMPC at 33°C with no melittin; Figures 30 through 37, sonicated DMPC at 33°C with $\sim 1/60$ melittin; Figures 38 through 45, nonsonicated DMPC at 16°C with no melittin; Figures 46 through 53, nonsonicated DMPC at 16°C with $\sim 1/60$ melittin; Figures 54 through 61, nonsonicated DMPC at 33°C with no melittin; and Figures 62 through 69, nonsonicated DMPC at 33°C with $\sim 1/60$ melittin. Each eight figure group contains graphs in the following order: lamp and total fluorescence intensity decays, residuals and autocorrelation for a single exponential fit to the total fluorescence, residuals and autocorrelation for a double exponential fit to the total

TABLE I
FLUORESCENCE DECAY TIMES OF DPH IN DMPC VESICLES

Sample Conditions	Unilamellar Vesicles				Multilamellar Vesicles			
	τ_1	τ_2	A_1/A_2	$\langle\tau\rangle^a$	τ_1	τ_2	A_1/A_2	$\langle\tau\rangle^a$
16°C, No Melittin	10.68	5.60	2.13	9.68	11.45	7.36	0.36	8.83
16°C, 1/60 Melittin	13.92	8.06	0.79	11.44	13.15	7.21	0.42	9.80
T_M^b , No Melittin	13.79	8.17	0.24	9.80	12.40	6.64	0.44	9.24
T_M^b , 1/60 Melittin	13.39	7.76	0.92	11.21	12.71	5.92	0.62	9.81
33°C, No Melittin	10.17	6.90	0.47	8.24	10.32	6.68	0.33	7.92
33°C, 1/60 Melittin	10.29	6.31	1.49	9.13	10.59	6.02	0.41	7.92

^aThe average decay time $\langle\tau\rangle$ was calculated from: $\langle\tau\rangle = (A_1 \tau_1^2 + A_2 \tau_2^2)/(A_1 \tau_1 + A_2 \tau_2)$.

^bThe measurements were made at 20.5°C and 23°C for unilamellar and multilamellar vesicles, respectively, which are close to their transition temperatures in the absence of melittin.

Note: Decay times τ are in ns; the standard deviation in their values is typically ± 0.05 ns. A_1/A_2 is the ratio of the amplitudes of the two decay components. These parameters were used for the analysis of the nanosecond emission anisotropy data (see Table II).

TABLE II
EFFECTS OF MELITTIN ON THE EMISSION ANISOTROPY PARAMETERS OF DPH VESICLES

Sample Conditions	Unilamellar Vesicles				Multilamellar Vesicles					
	S ^a	φ(ns)	<σ>	D _w ^b (ns ⁻¹)	r ₀	S ^a	φ(ns)	<σ>	D _w ^b (ns ⁻¹)	r ₀
16°C, No Melittin	0.85	2.2	0.059	0.027	0.333	0.93	2.6	0.026	0.010	0.316
16°C, 1/60 Melittin	0.93	2.2	0.028	0.013	0.328	0.92	2.7	0.032	0.012	0.340
T _M ^C , No Melittin	0.76	3.0	0.093	0.031	0.327	0.92	2.5	0.033	0.013	0.289
T _M ^C , 1/60 Melittin	0.90	2.0	0.040	0.020	0.334	0.86	4.1	0.053	0.013	0.307
33°C, No Melittin	0.39	2.0	0.21	0.11	0.295	0.42	1.9	0.20	0.11	0.270
33°C, 1/60 Melittin	0.46	2.1	0.19	0.090	0.271	0.41	2.1	0.21	0.10	0.278

^aThe standard deviation in S is ~2% for both types of vesicles. For unilamellar vesicles, the changes in S introduced by melittin are outside the experimental error for all three temperatures, whereas for multilamellar vesicles that is the case only at T_t (for which the actual standard deviations are ~1% and 2% in the absence and in the presence of melittin, respectively).

^bThe standard deviation in D_w is in the range of ~10-20% for unilamellar vesicles; for multilamellar vesicles, the probable error is also in that range except at T_t where it is ~30%.

^cThe measurements were made at 20.5°C and at 23°C for unilamellar and multilamellar vesicles, respectively, which are close to their transition temperatures in the absence of melittin.

Note: S is the order parameter and ϕ is the rotational correlation time. $\langle\sigma\rangle$, which is a function of S, is obtained from a graphical presentation. D_w is the diffusion rate and r₀ is the initial anisotropy.

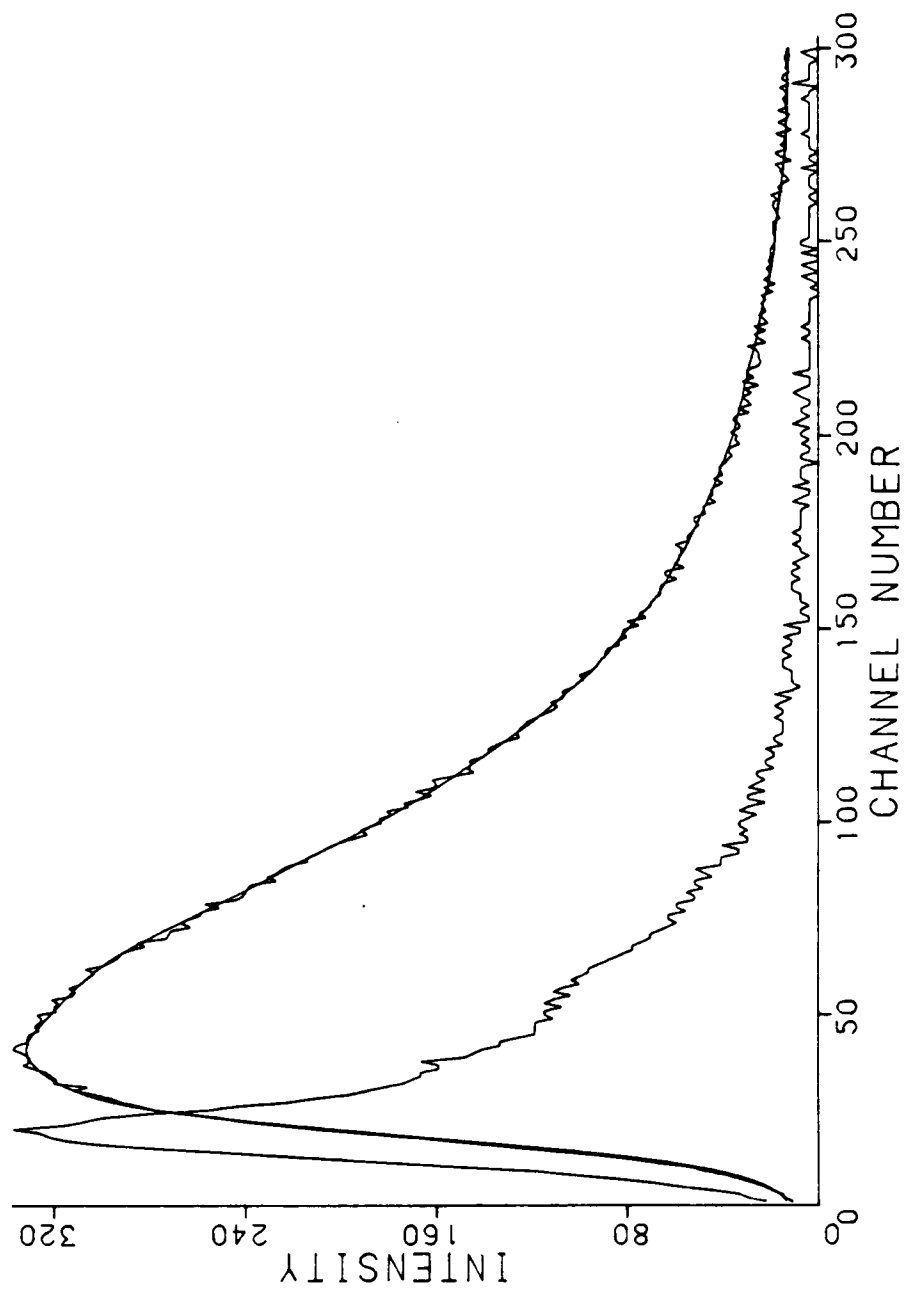


Figure 6. Plot of lamp and total fluorescence intensities for DPH in sonicated DMPC at 16°C in the absence of melittin. Channel width = 0.257 ns.

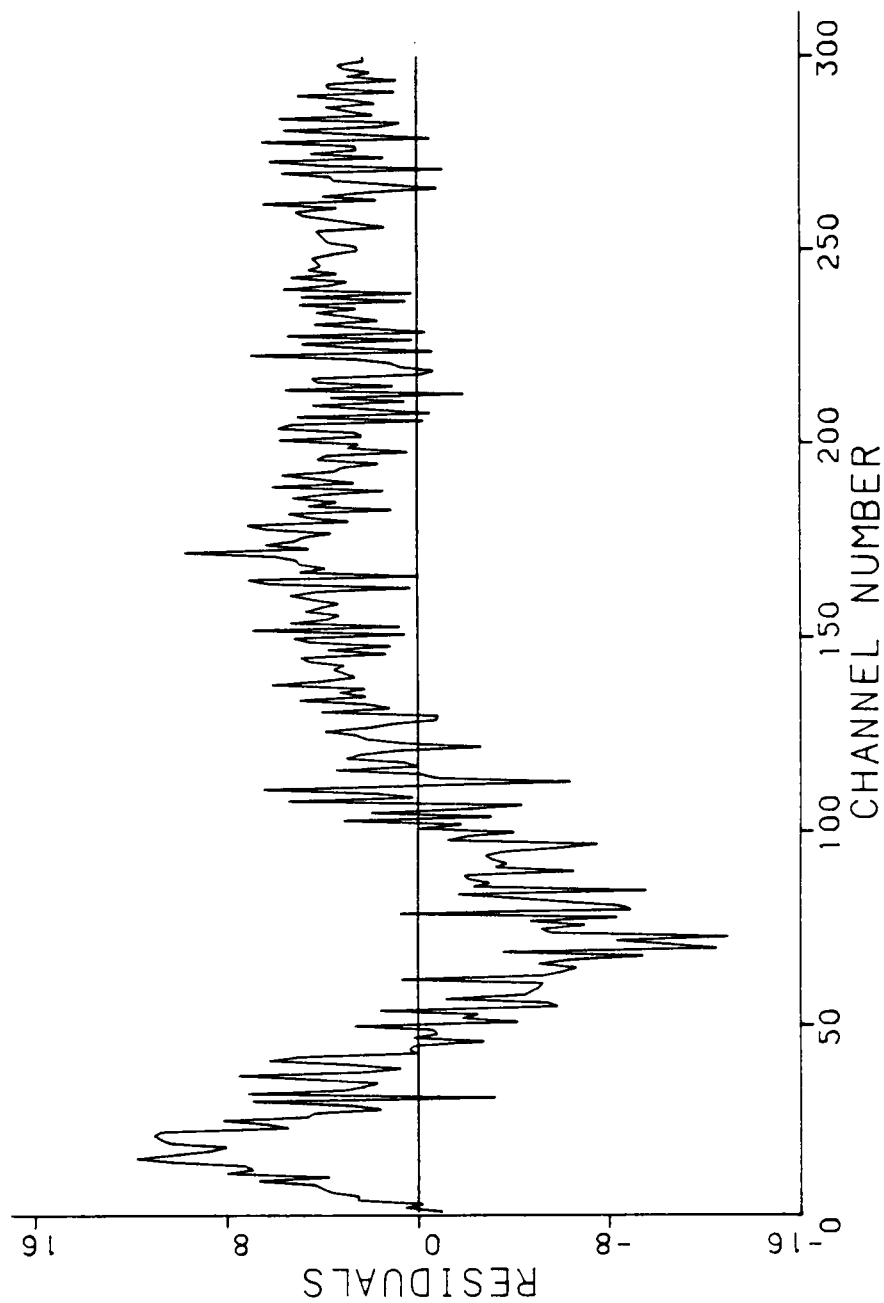


Figure 7. Plot of residuals for single exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C in the absence of melittin.

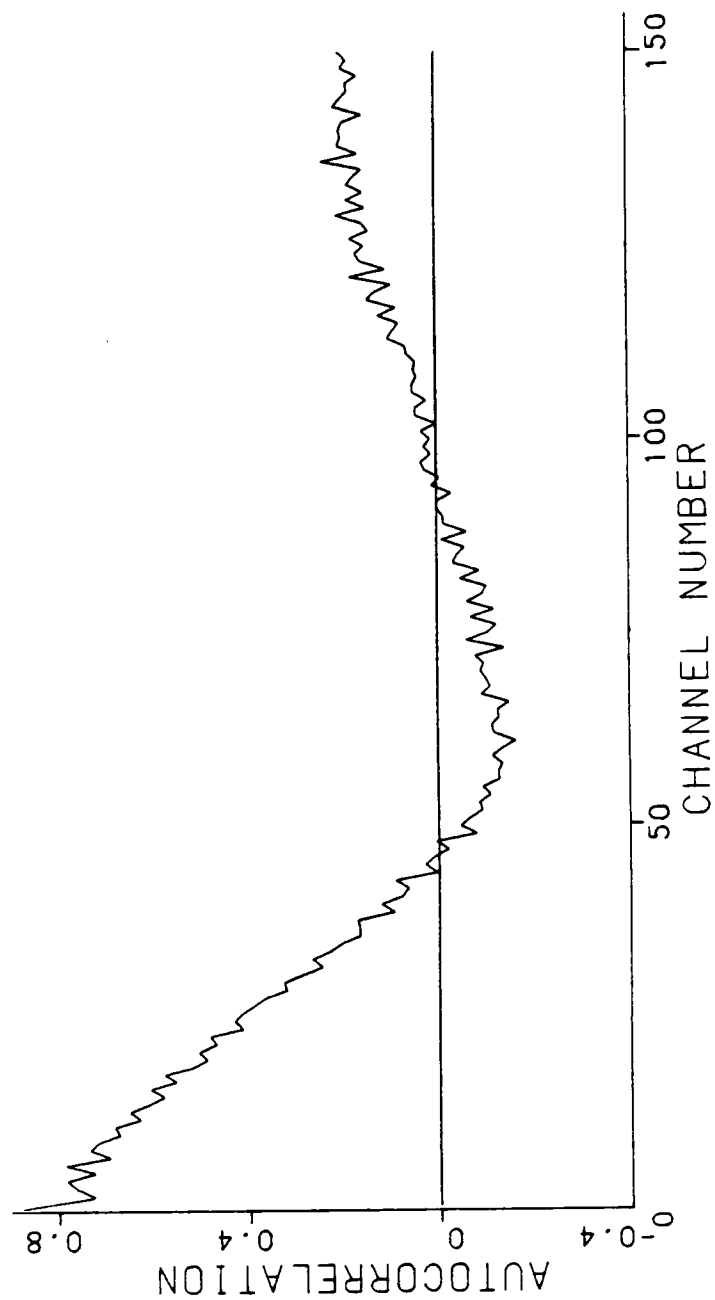


Figure 8. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C in the absence of melittin.

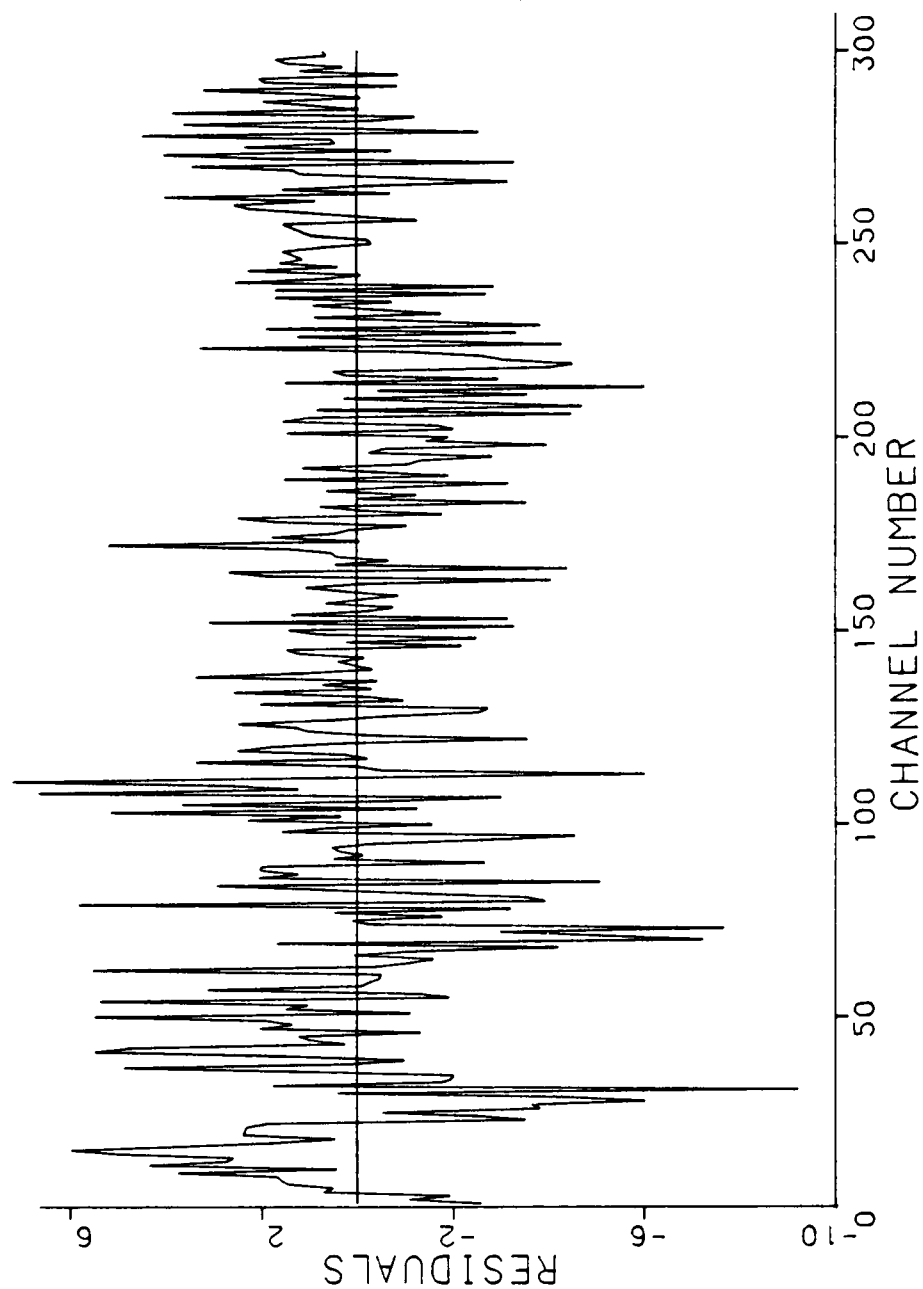


Figure 9. Plot of residuals for double exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C in the absence of melittin.

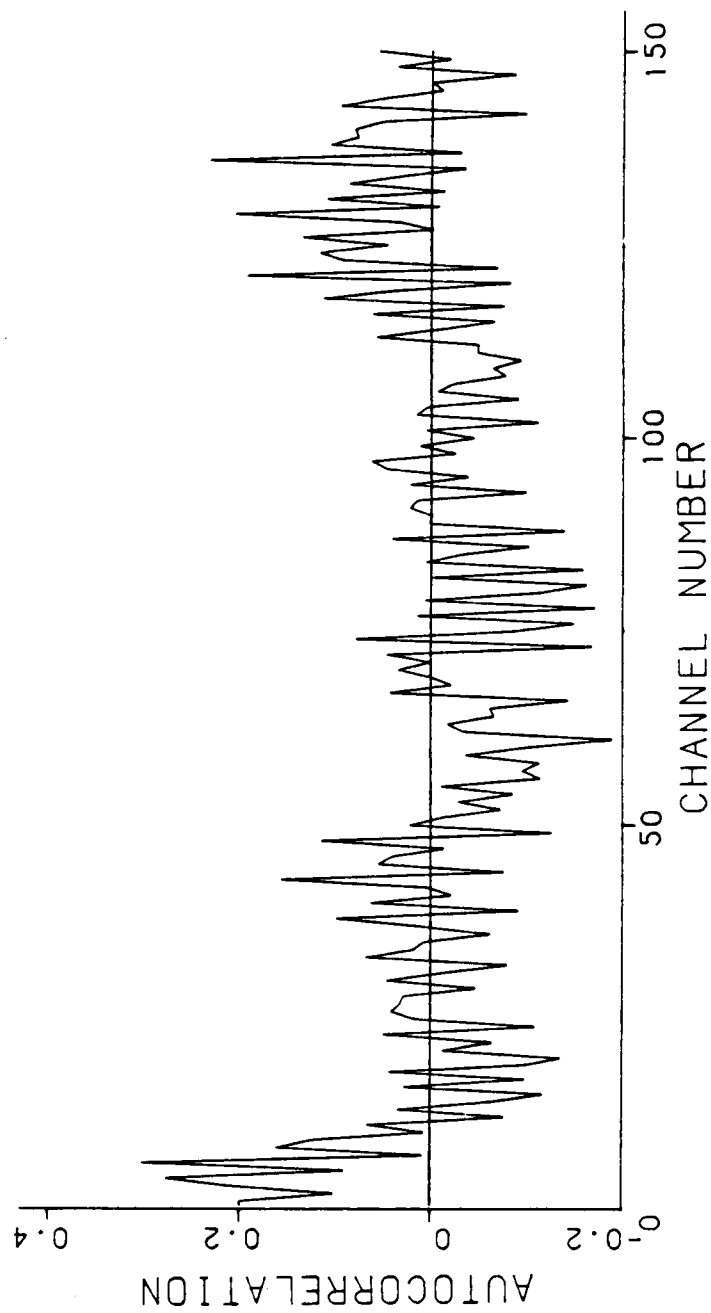


Figure 10. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C in the absence of melittin.

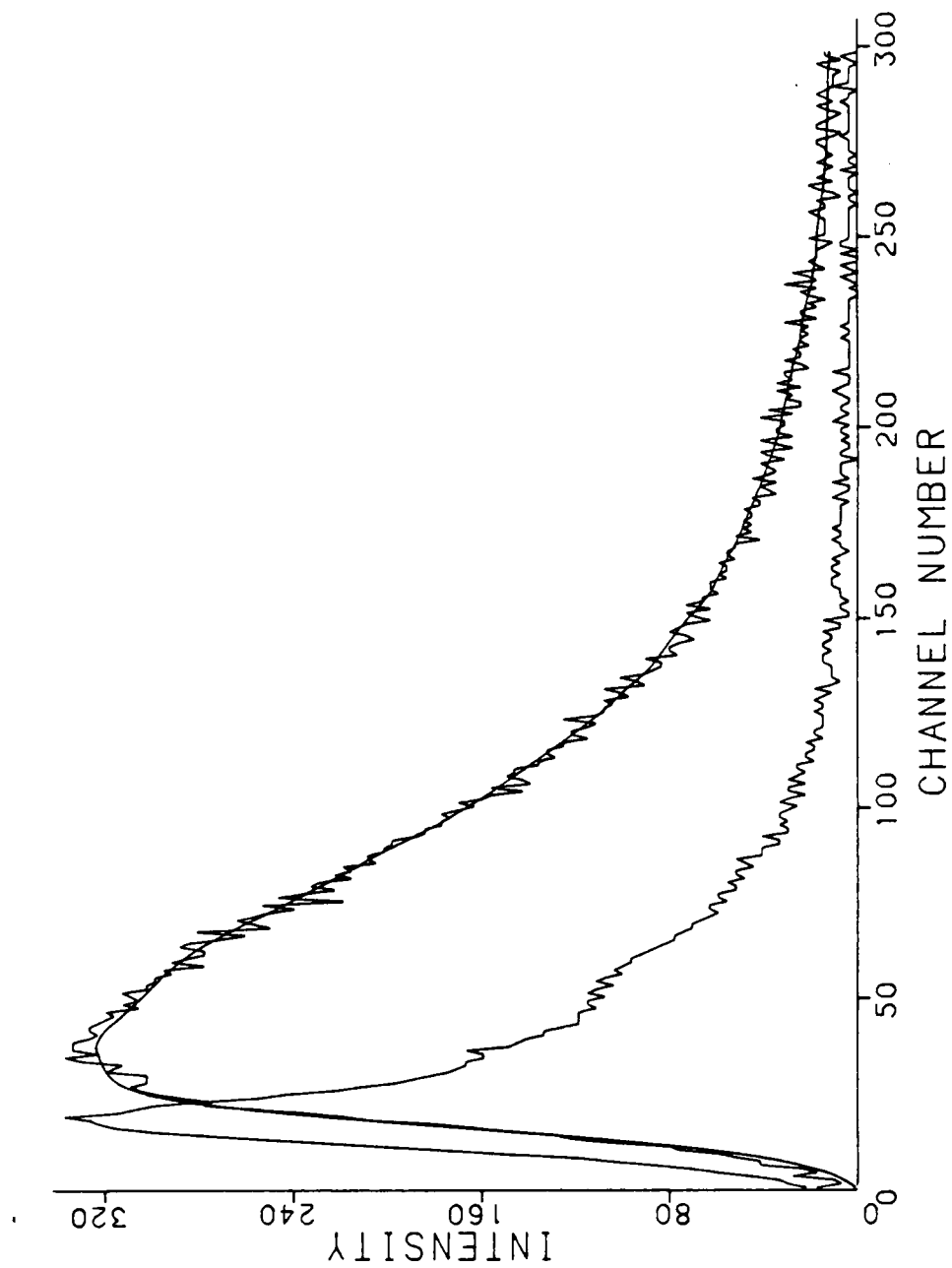


Figure 11. Plot of lamp and I_V-I_H intensities for DPH in sonicated DMPC at 16°C in the absence of melittin.

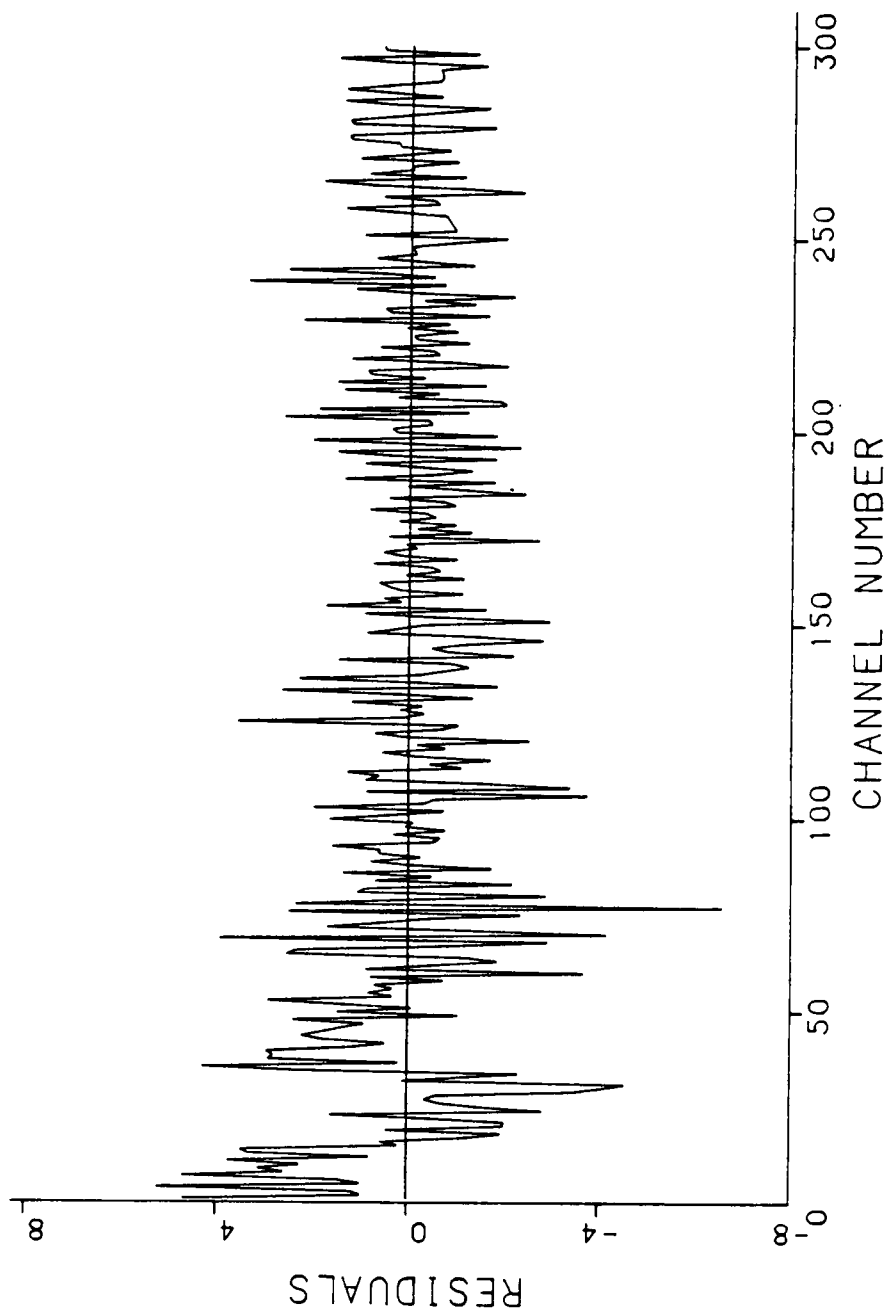


Figure 12. Plot of residuals for fit to $I_V - I_H$ for DPH in sonicated DMPC at 16°C in the absence of melittin.

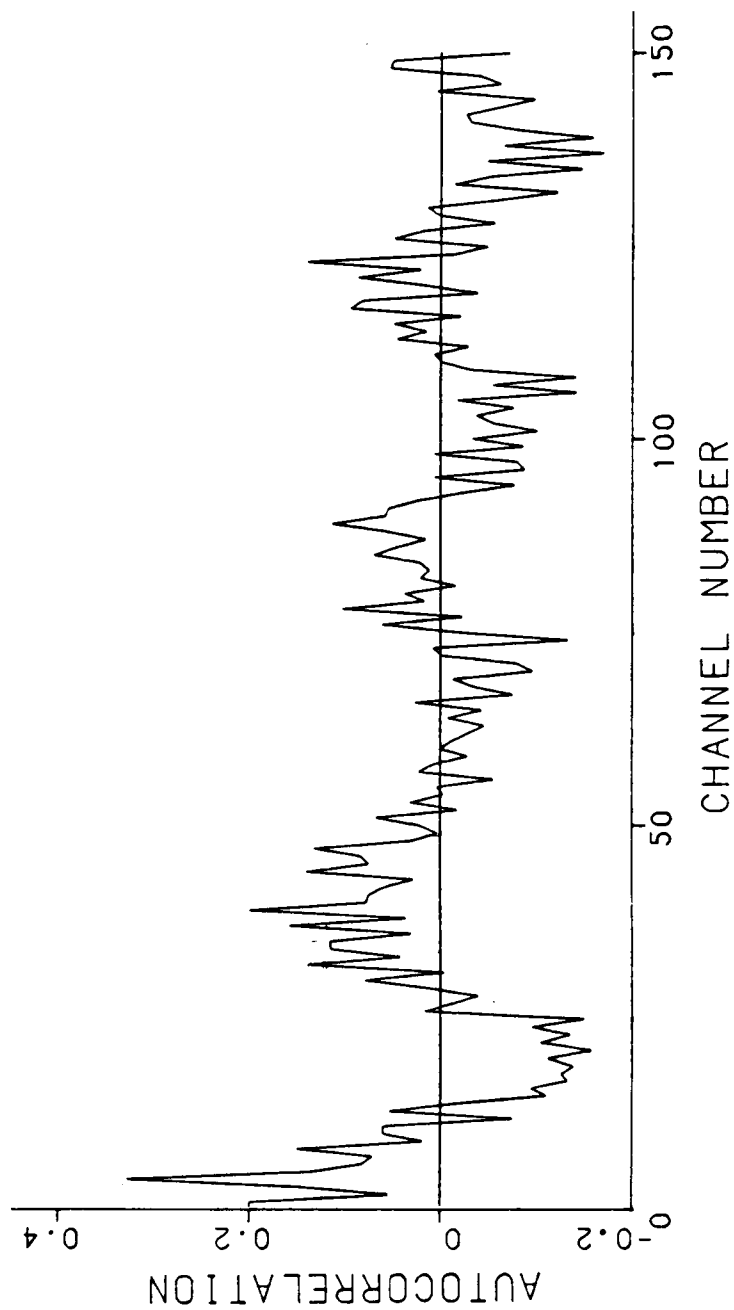


Figure 13. Plot of autocorrelation function for fit to $I_V - I_H$ for DPH in sonicated DMPC at 16°C in the absence of melittin.

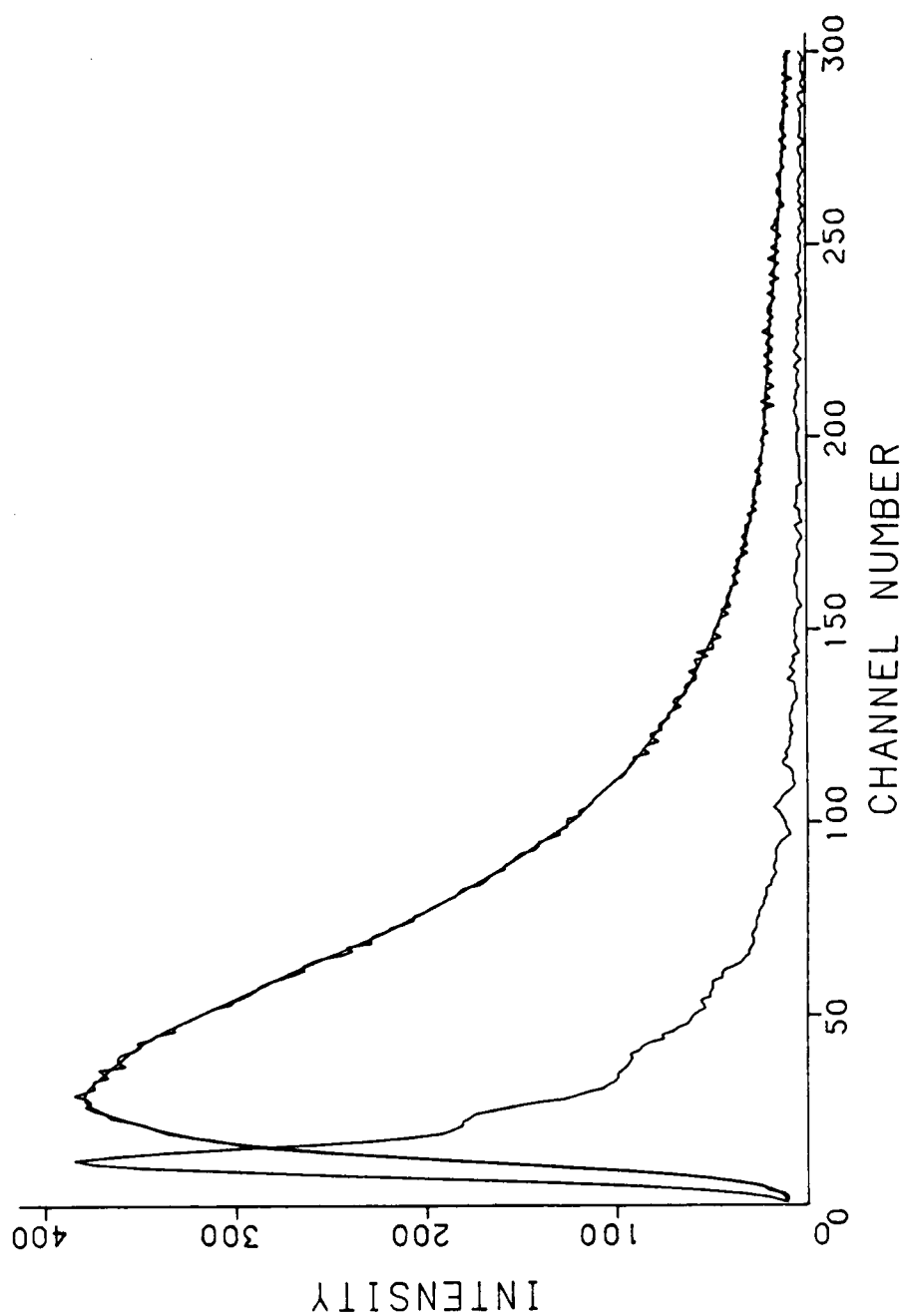


Figure 14. Plot of lamp and total fluorescence intensities for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$. Channel width = 0.351 ns.

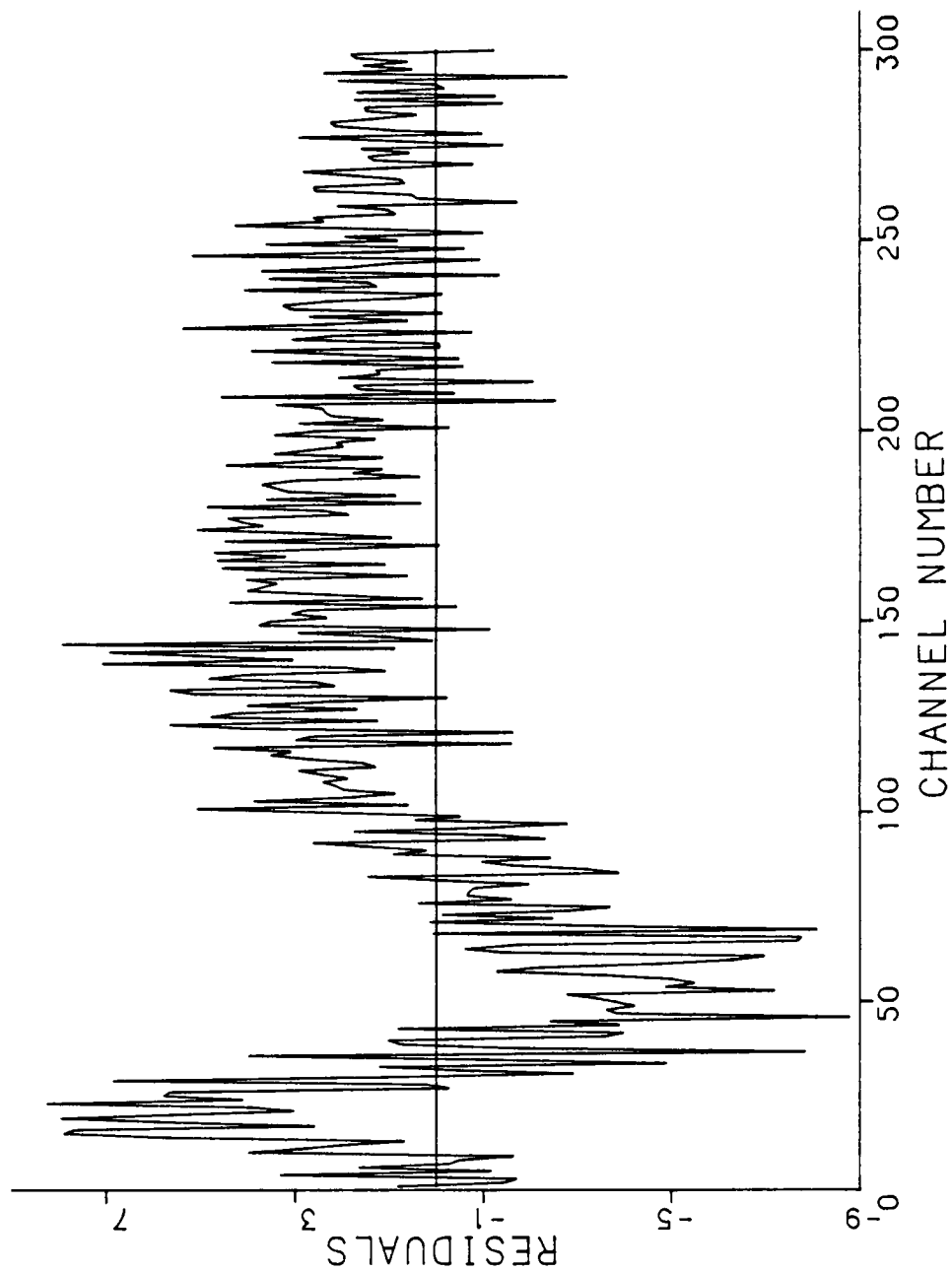


Figure 15. Plot of residuals for single exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

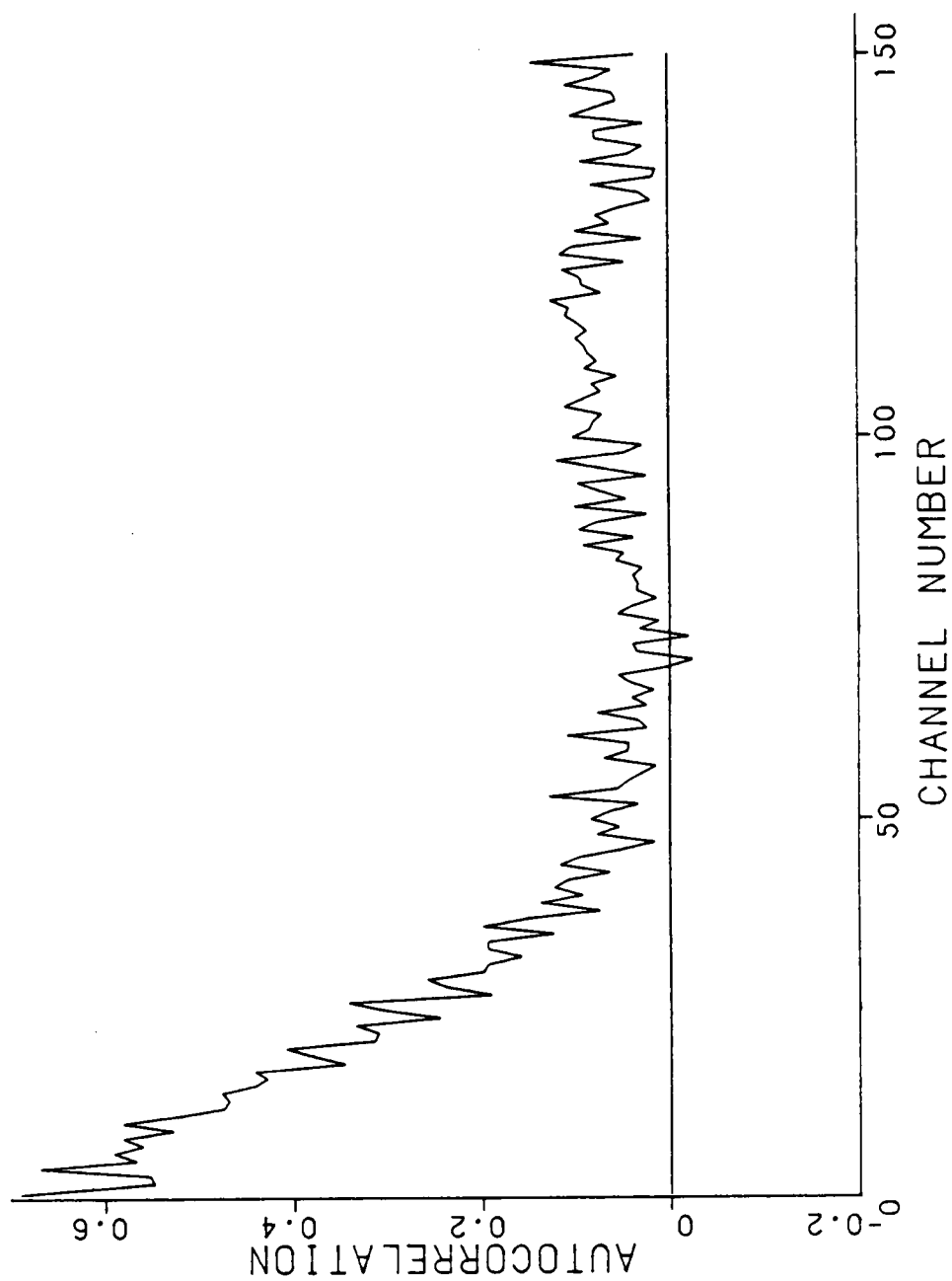


Figure 16. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

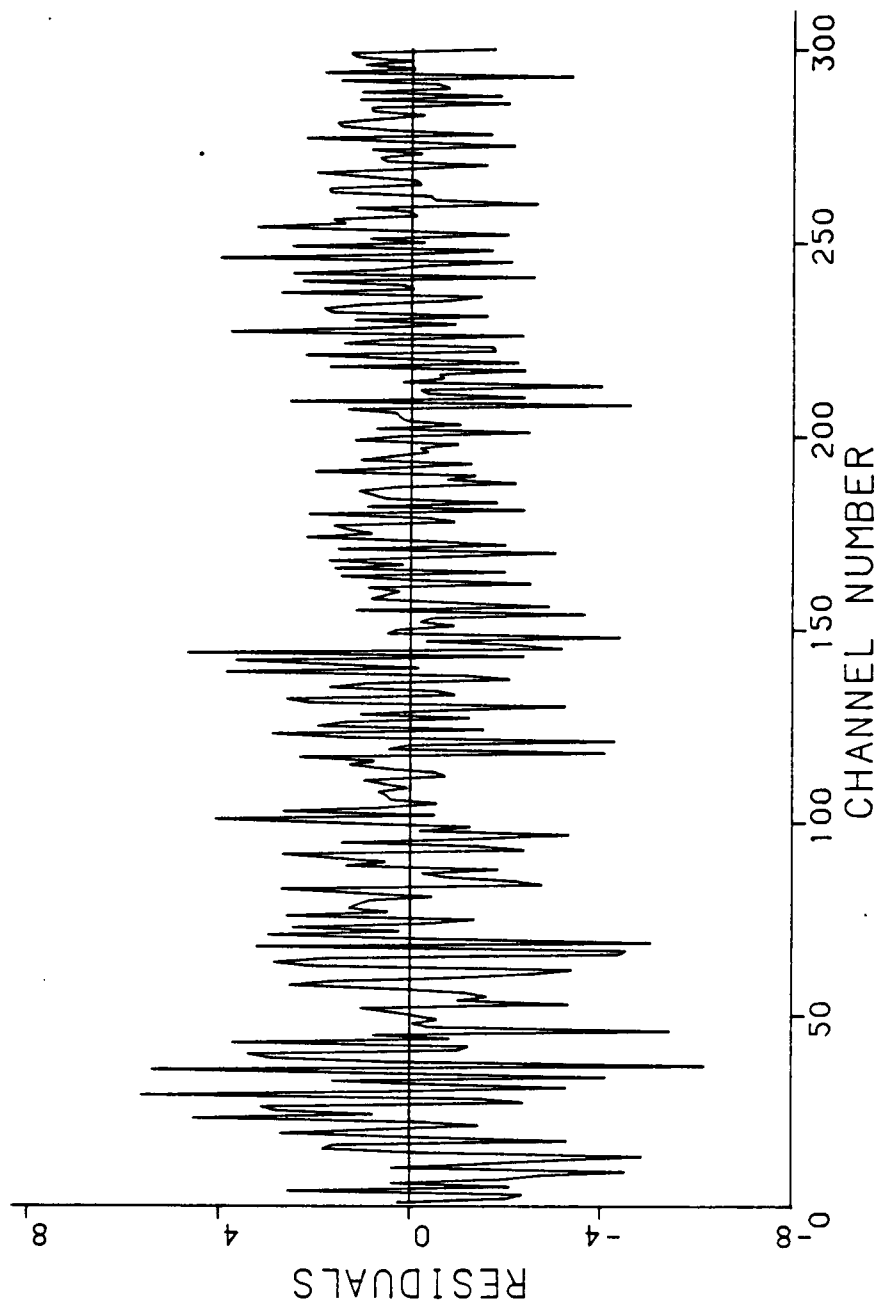


Figure 17. Plot of residuals for double exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

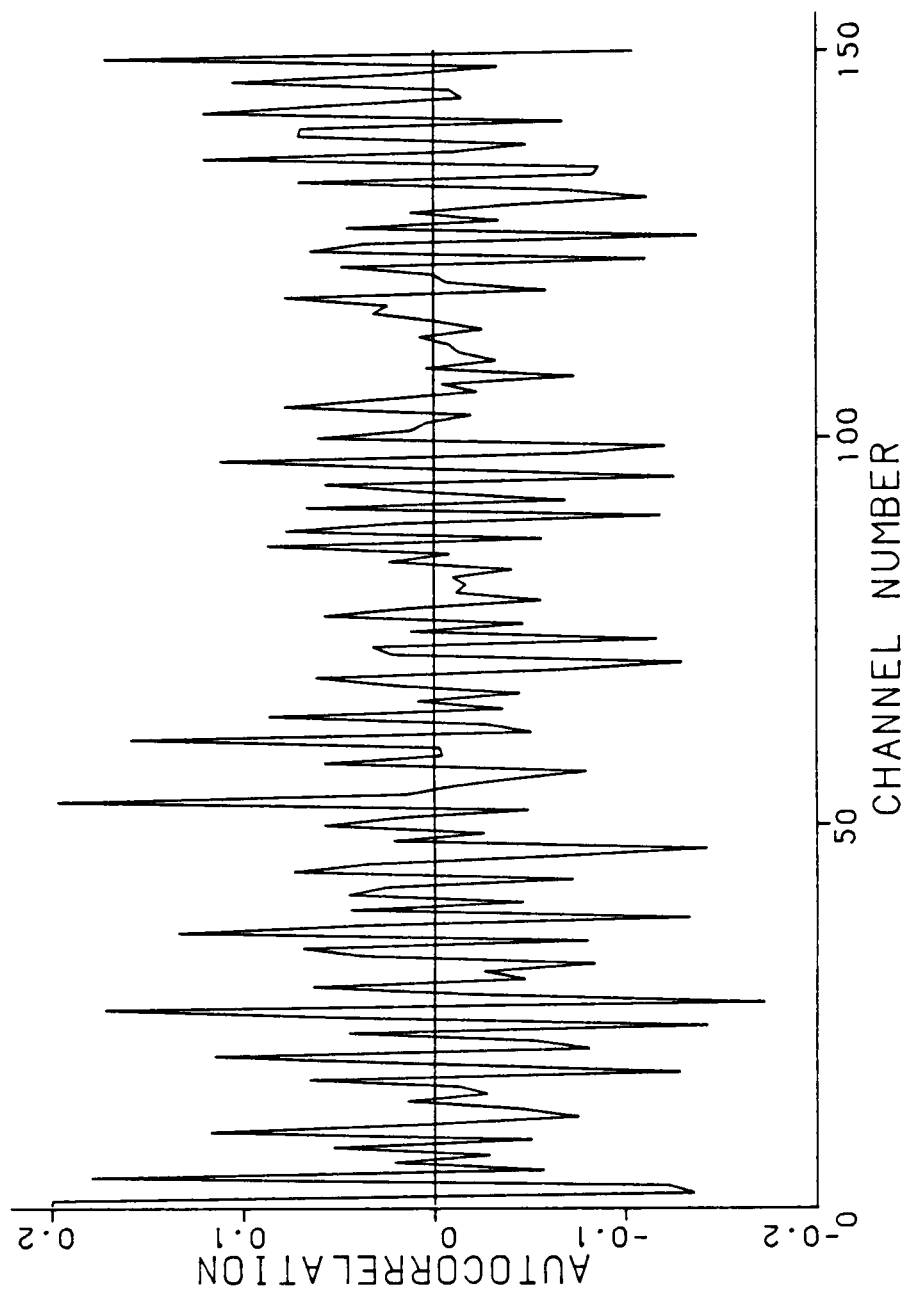


Figure 18. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

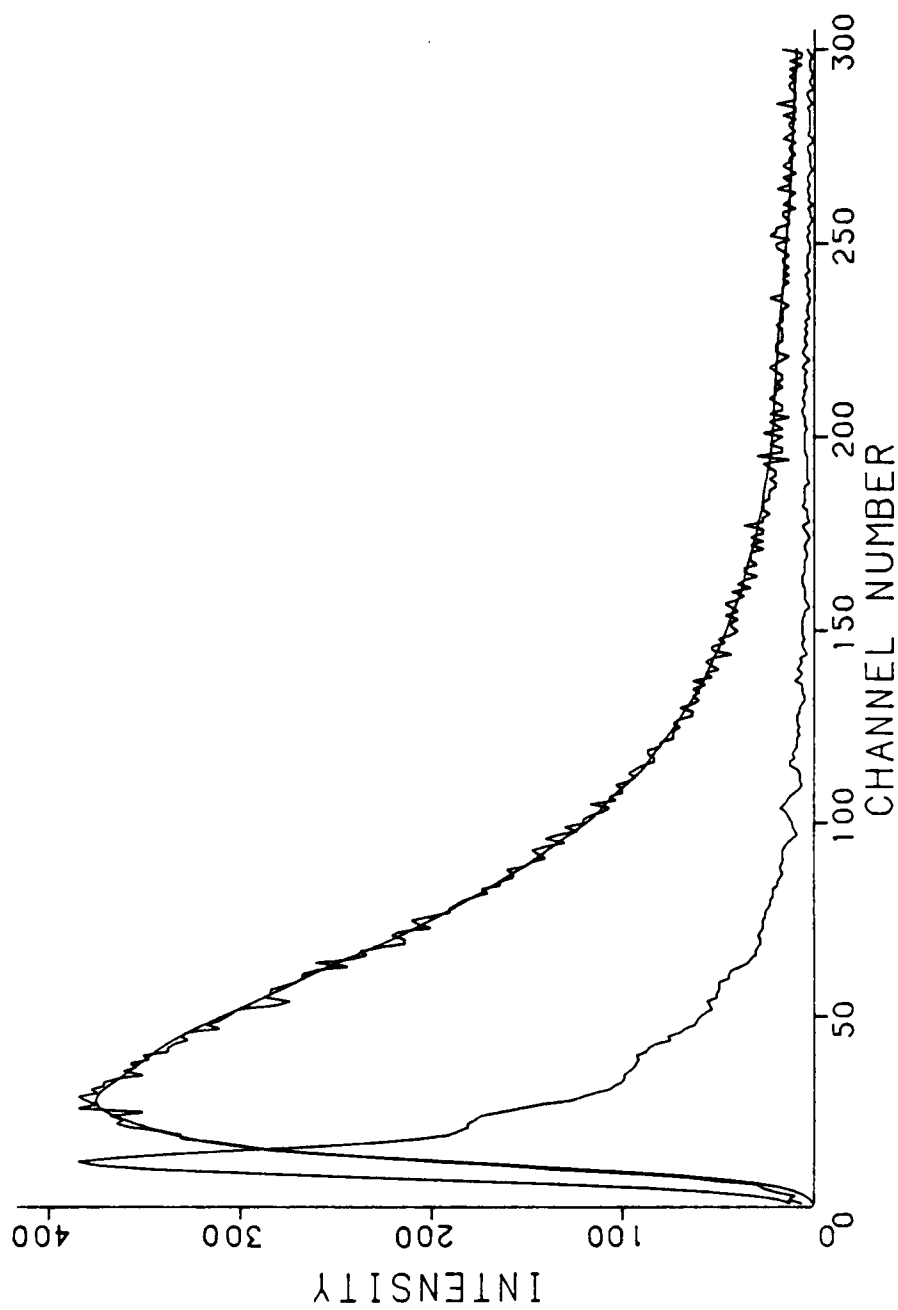


Figure 19. Plot of lamp and I_V-I_H intensities for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

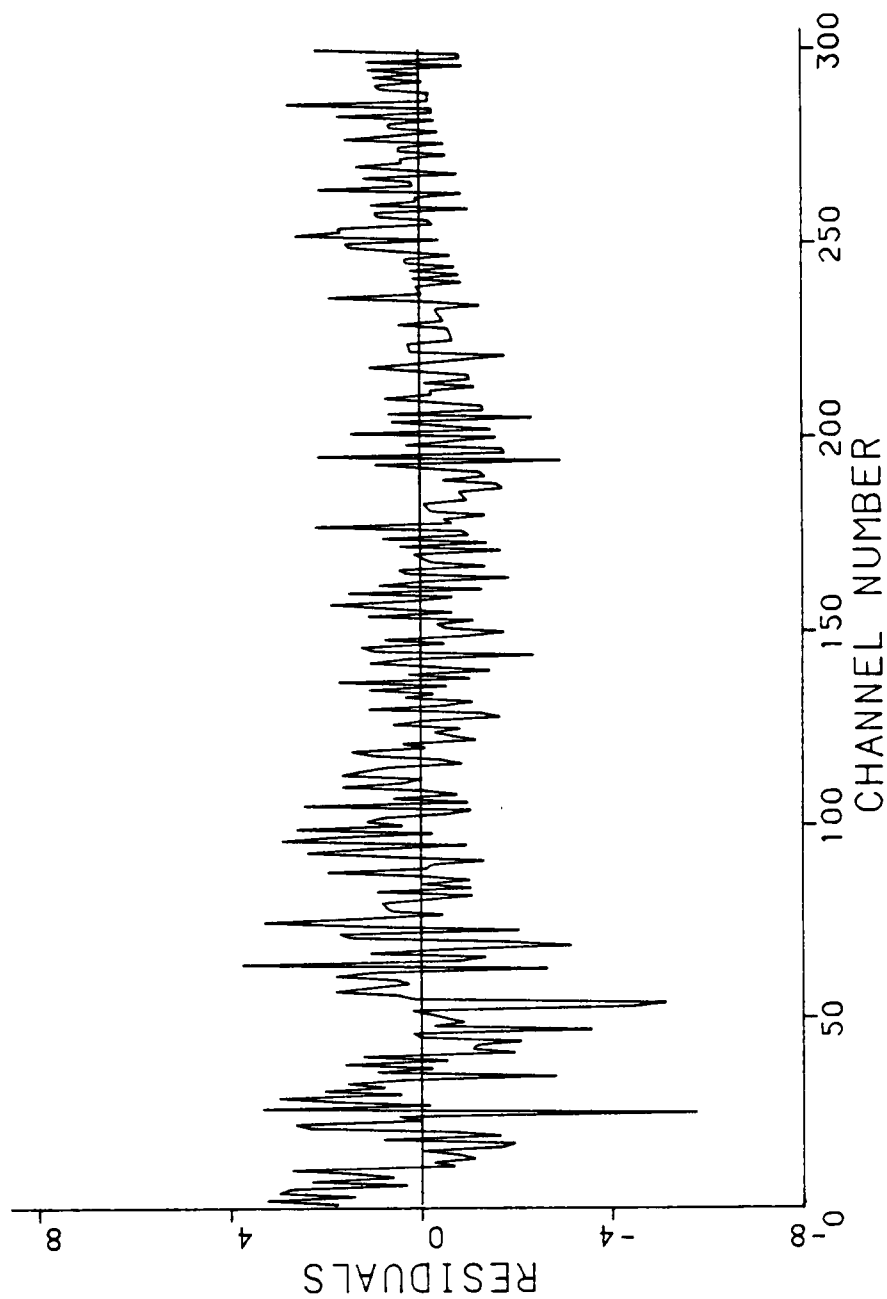


Figure 20. Plot of residuals for fit to $I_V - I_H$ for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

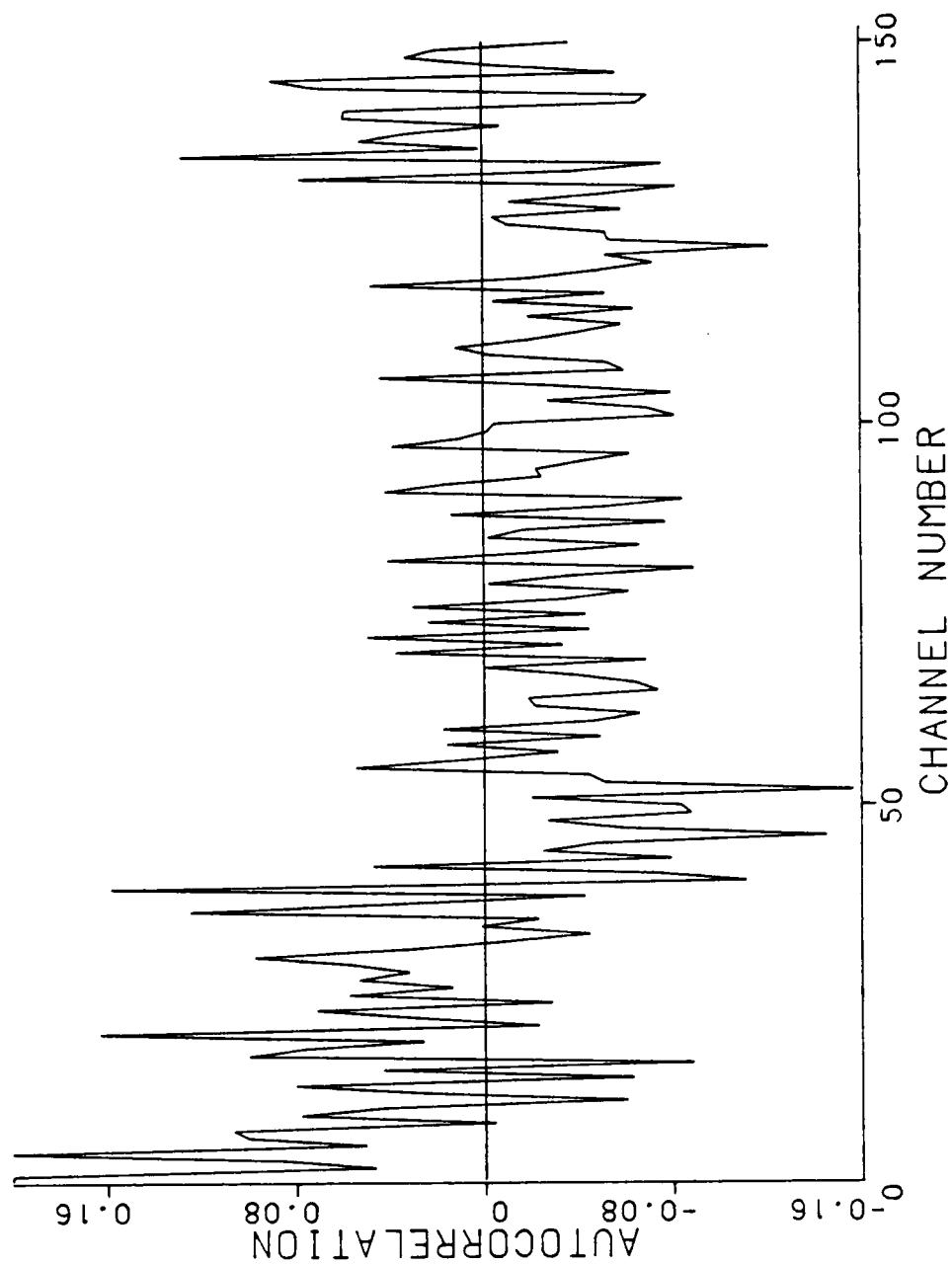


Figure 21. Plot of autocorrelation function for fit to I_y-I_y for DPH in sonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

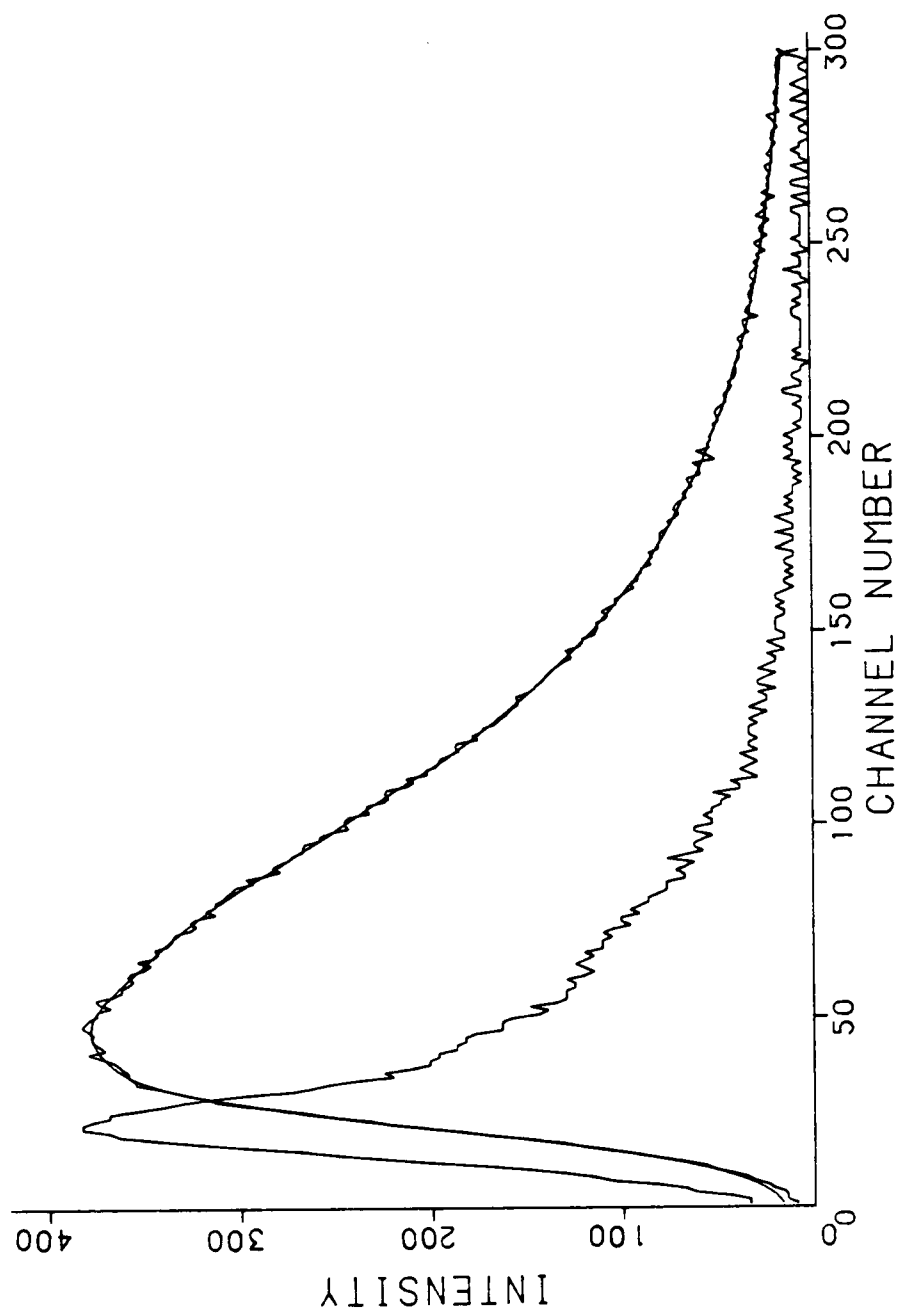


Figure 22. Plot of lamp and total fluorescence intensities for DPH in sonicated DMPC at 33°C in the absence of melittin. Channel width = 0.209 ns.

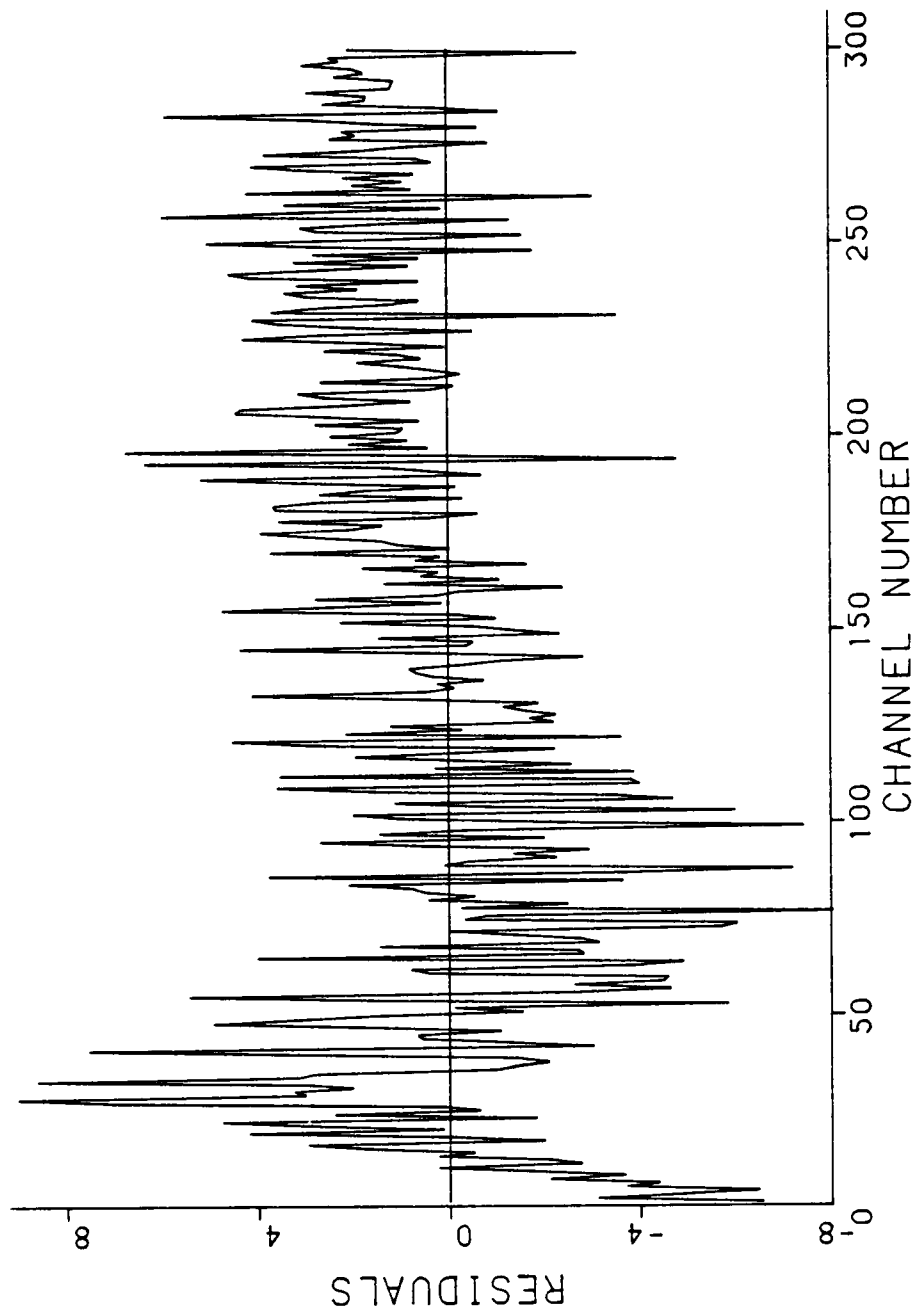


Figure 23. Plot of residuals for single exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C in the absence of melittin.

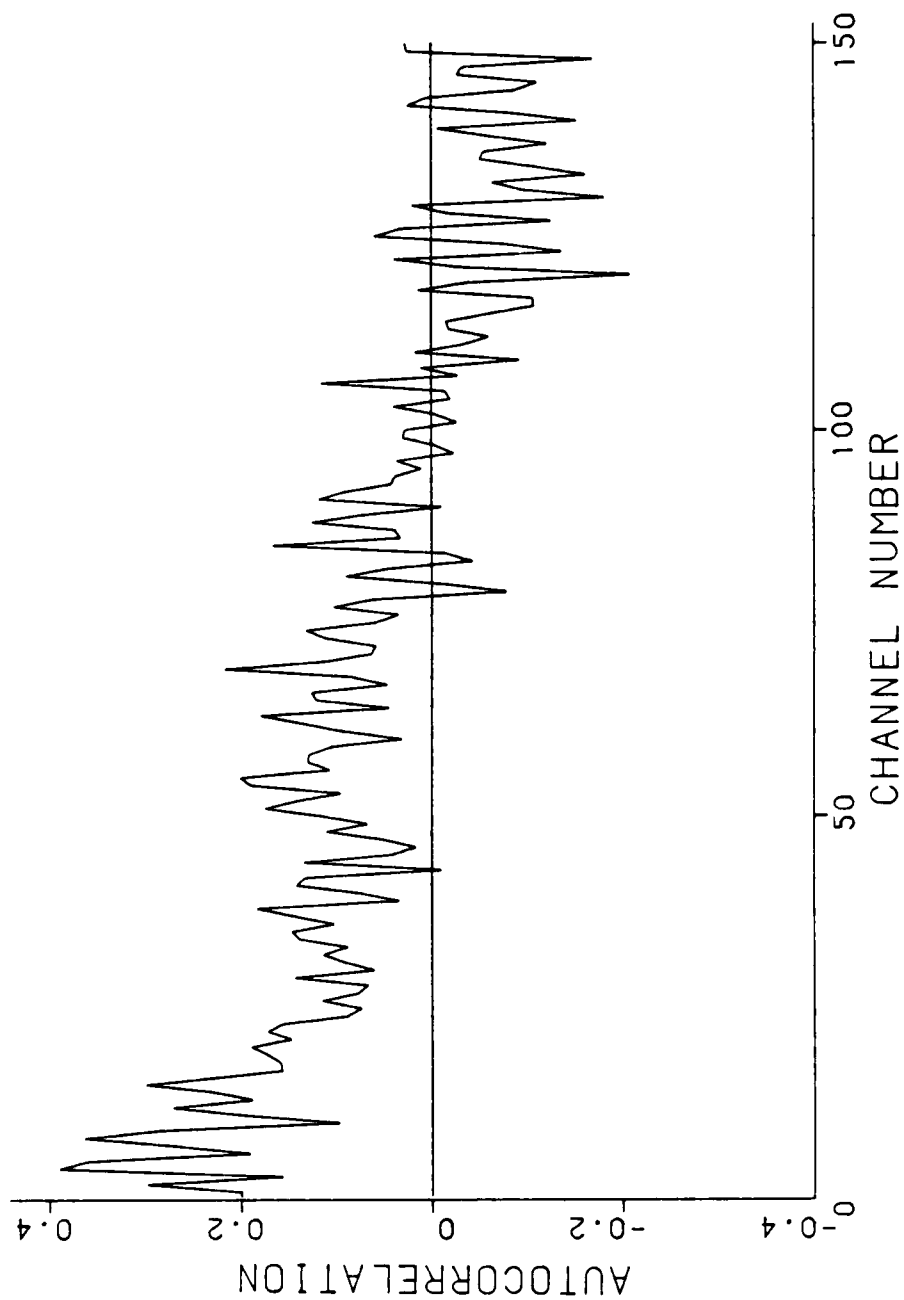


Figure 24. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C in the absence of melittin.

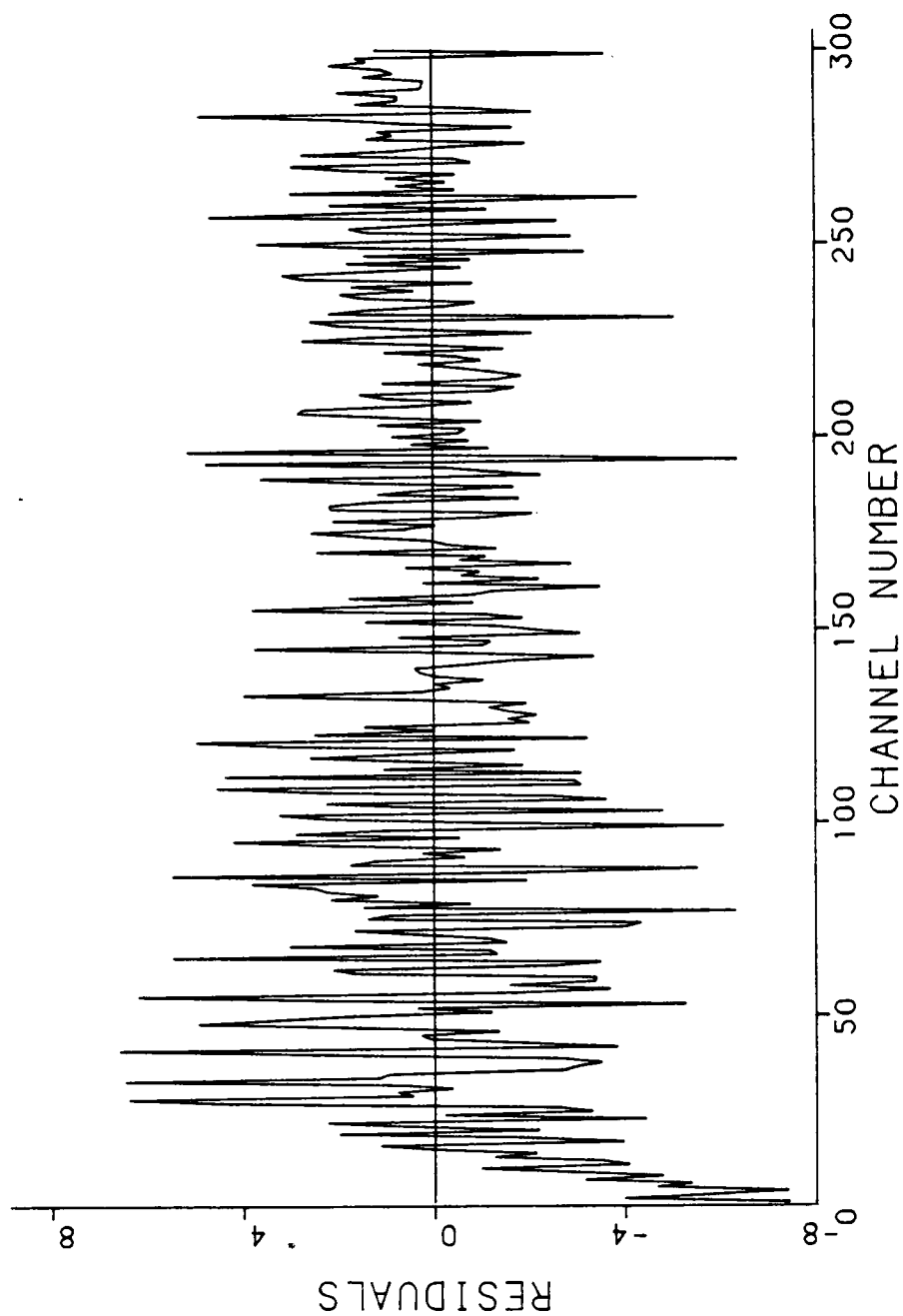


Figure 25. Plot of residuals for double exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C in the absence of melittin.

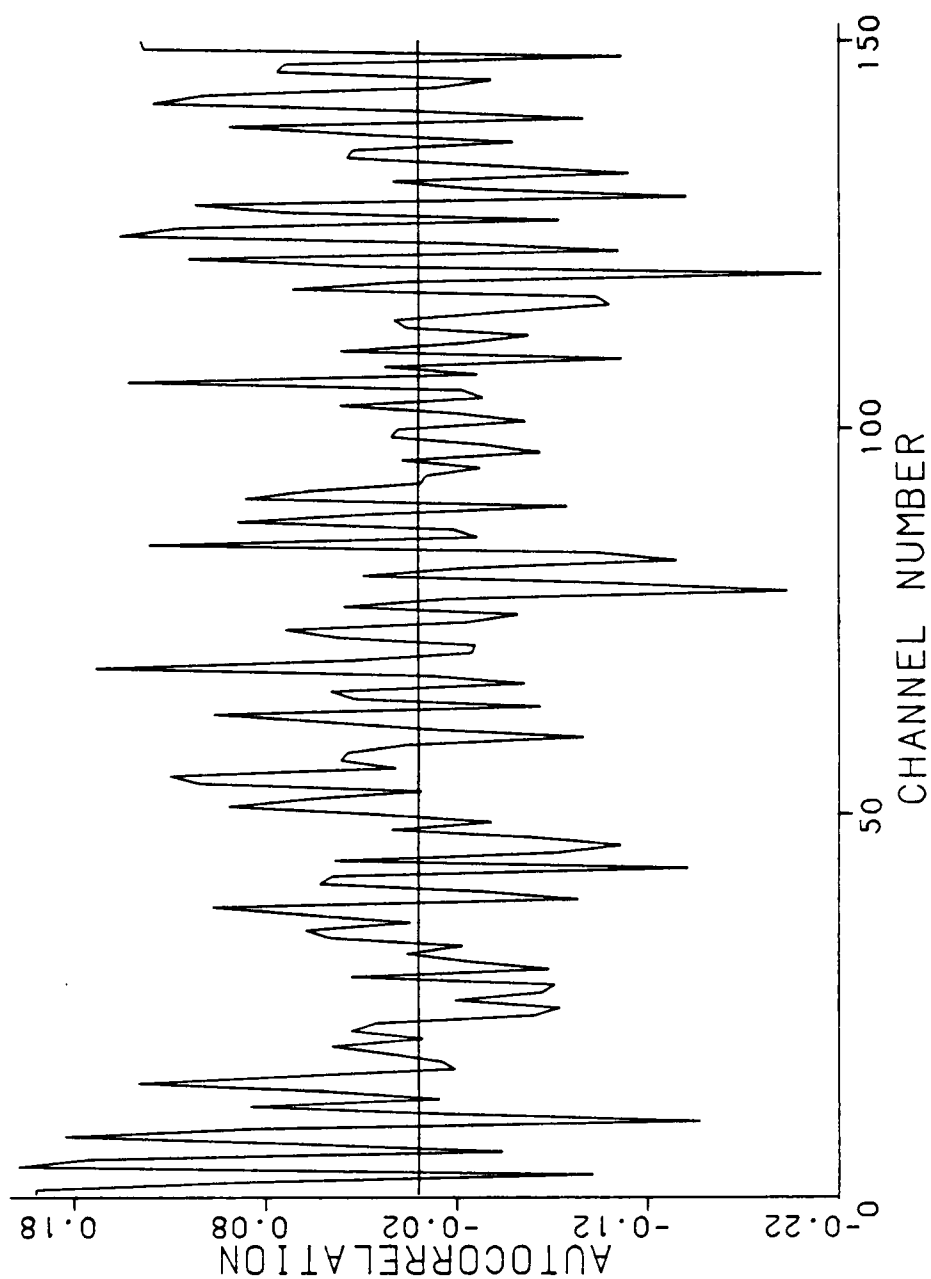


Figure 26. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C in the absence of melittin.

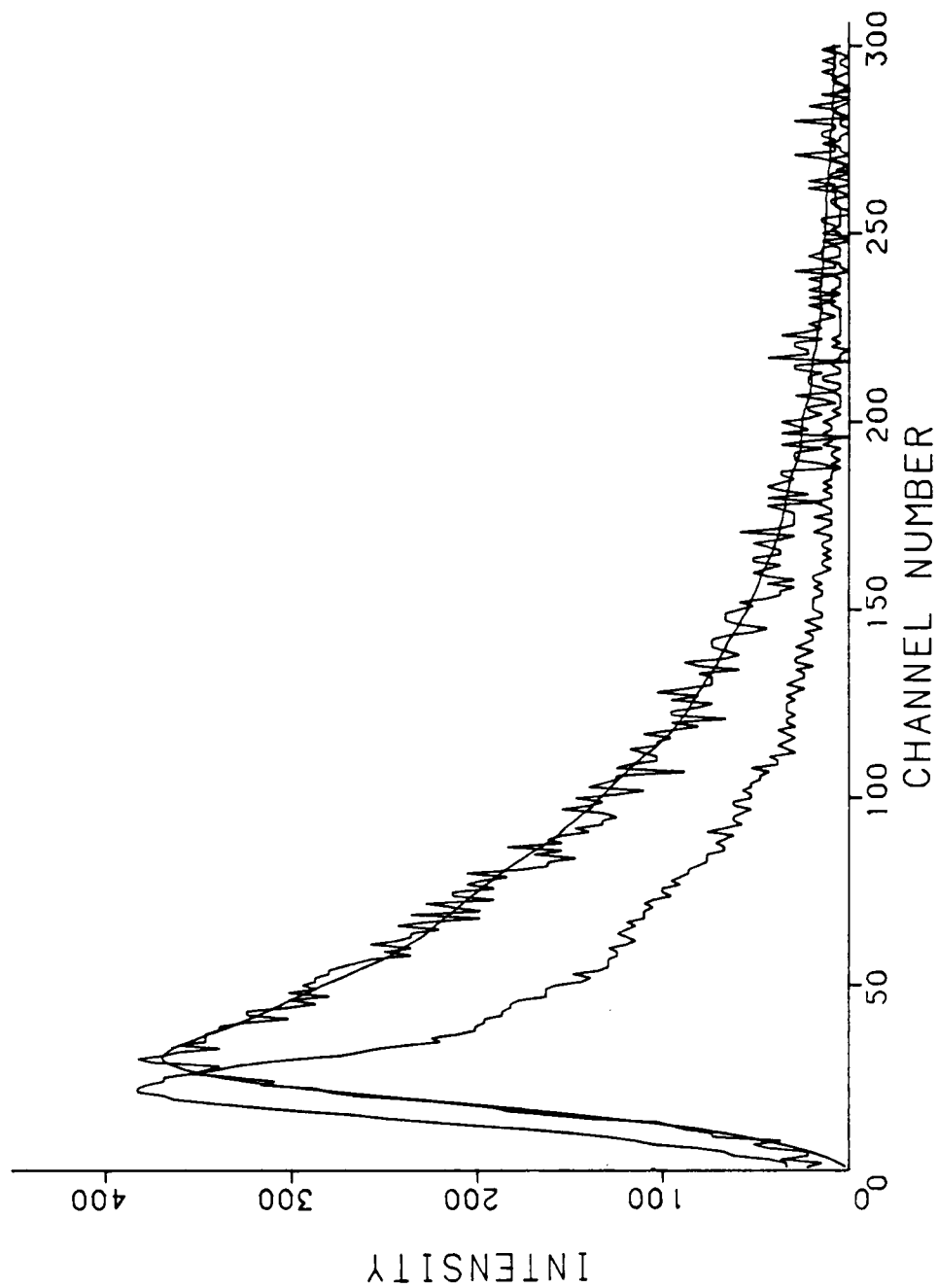


Figure 27. Plot of lamp and $I_{\gamma} - I_H$ intensities for DPH in sonicated DMPC at 33°C in the absence of melittin.

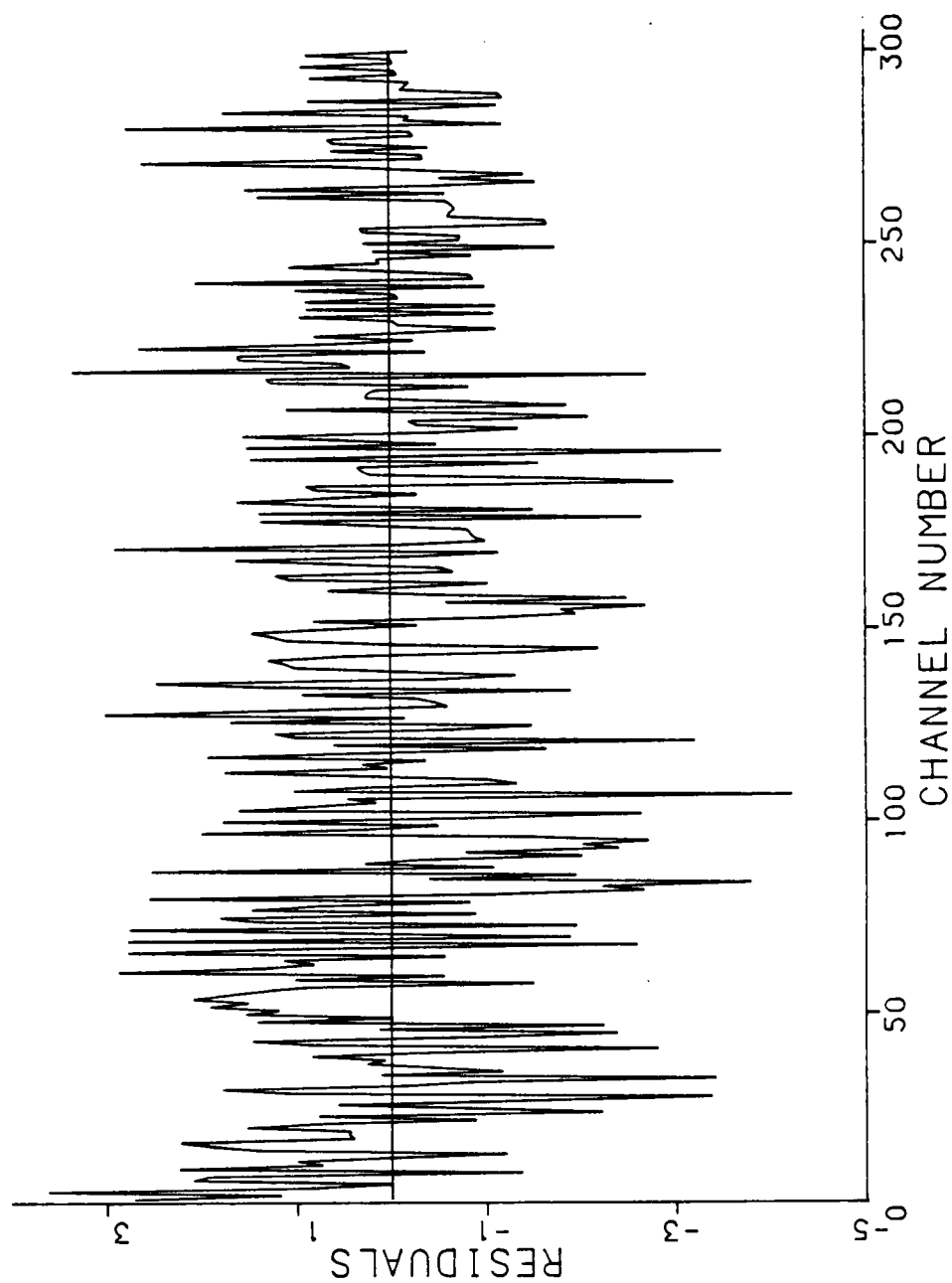


Figure 28. Plot of residuals for fit to $I_V - I_H$ for DPH in sonicated DMPC at 33°C in the absence of melittin.

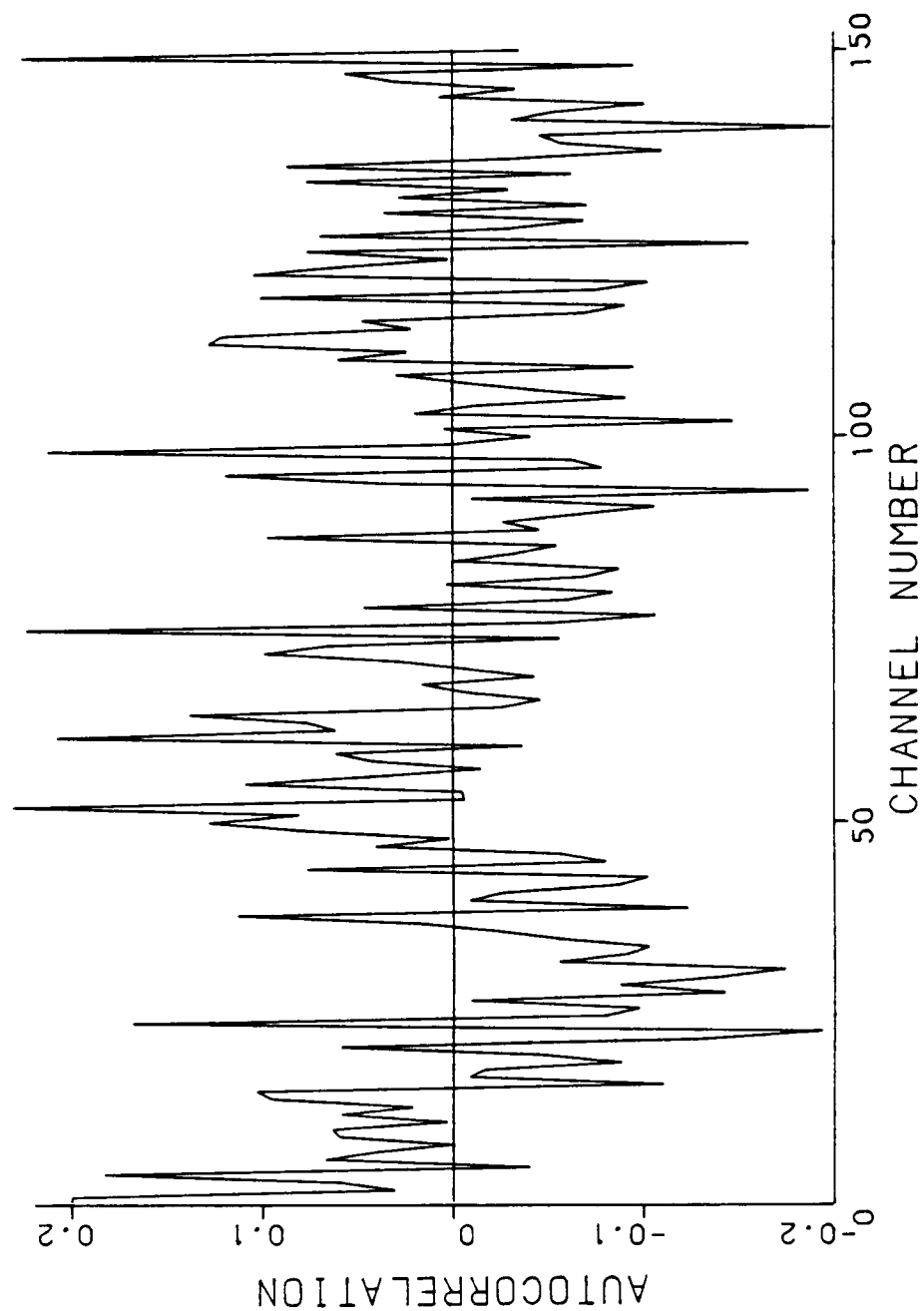


Figure 29. Plot of autocorrelation function for fit to $I_V - I_H$ for DPH in sonicated DMPC at 33°C in the absence of melittin.

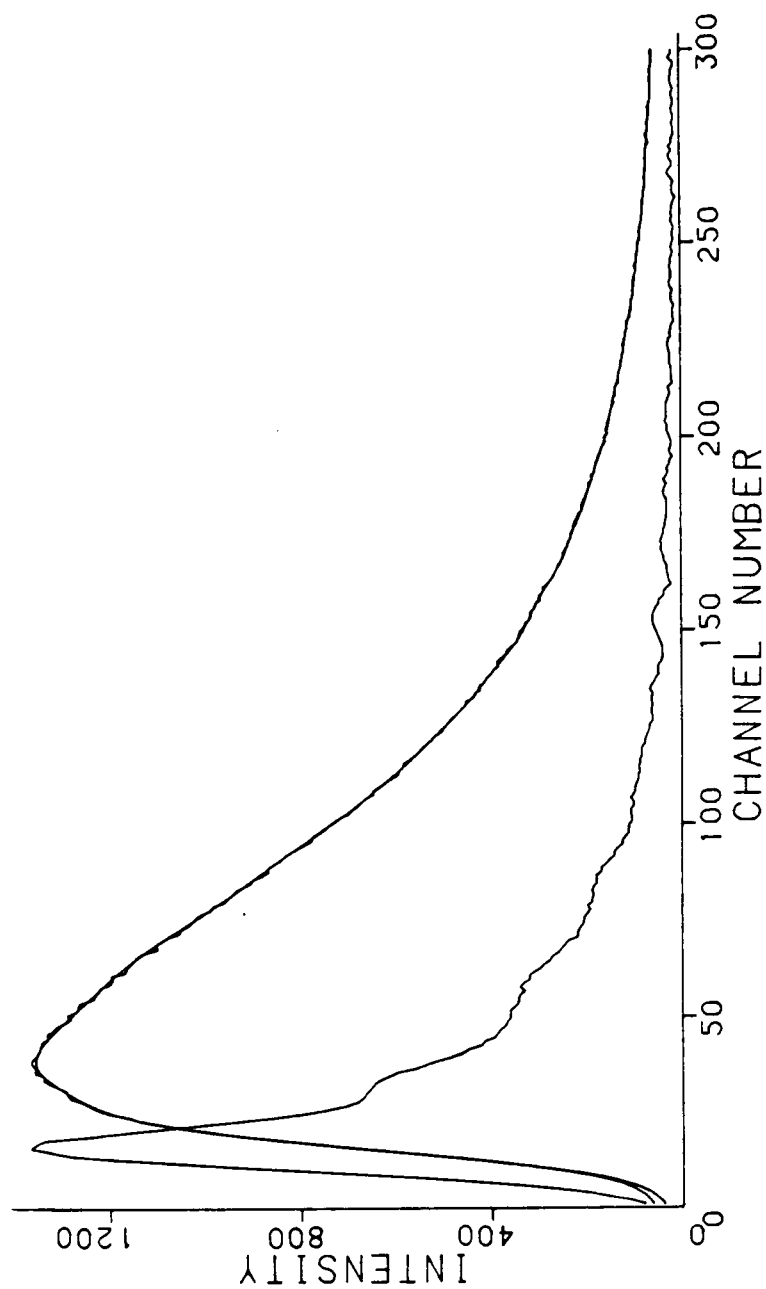


Figure 30. Plot of lamp and total fluorescence intensities for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$. Channel width = 0.237 ns.

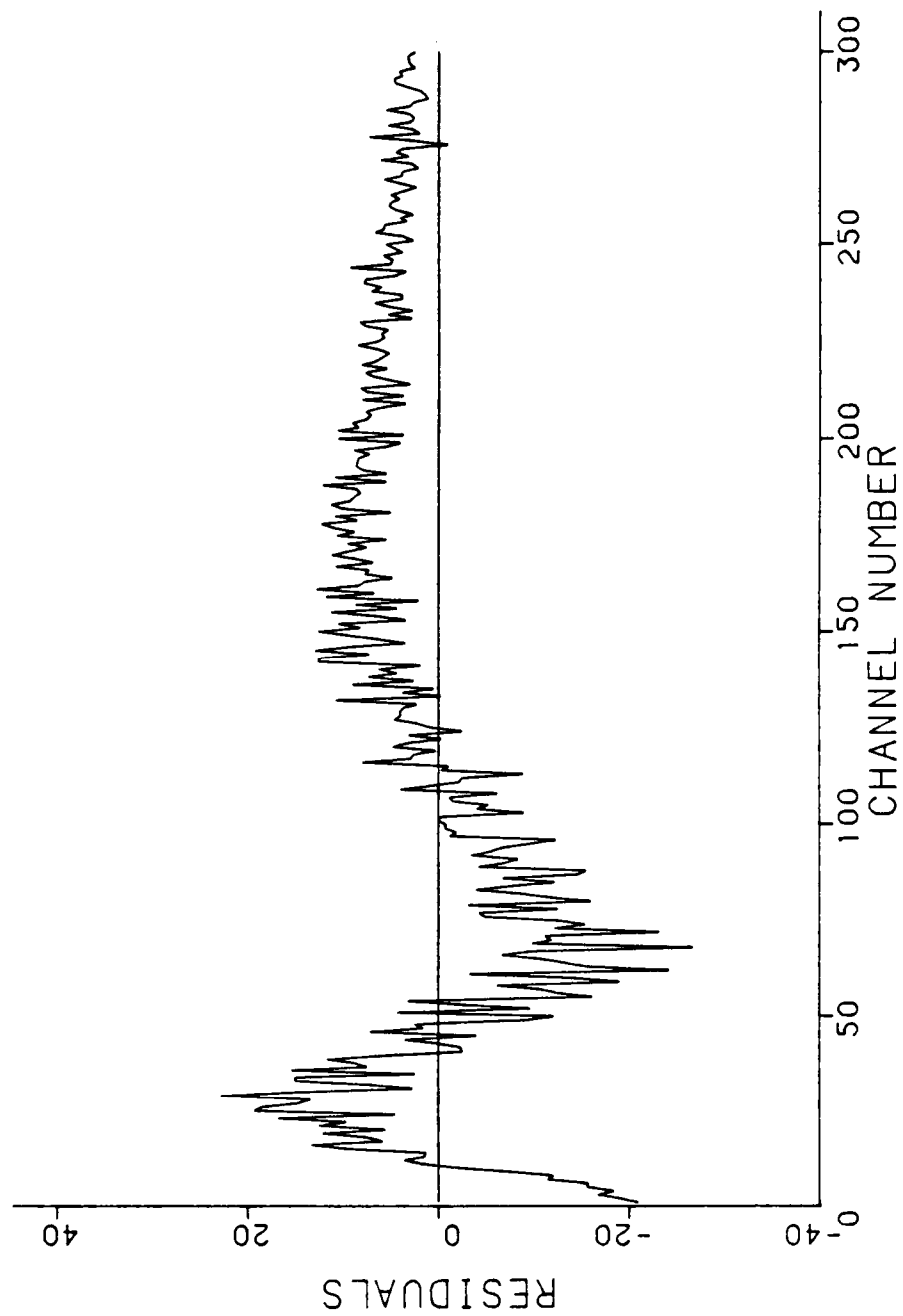


Figure 31. Plot of residuals for single exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

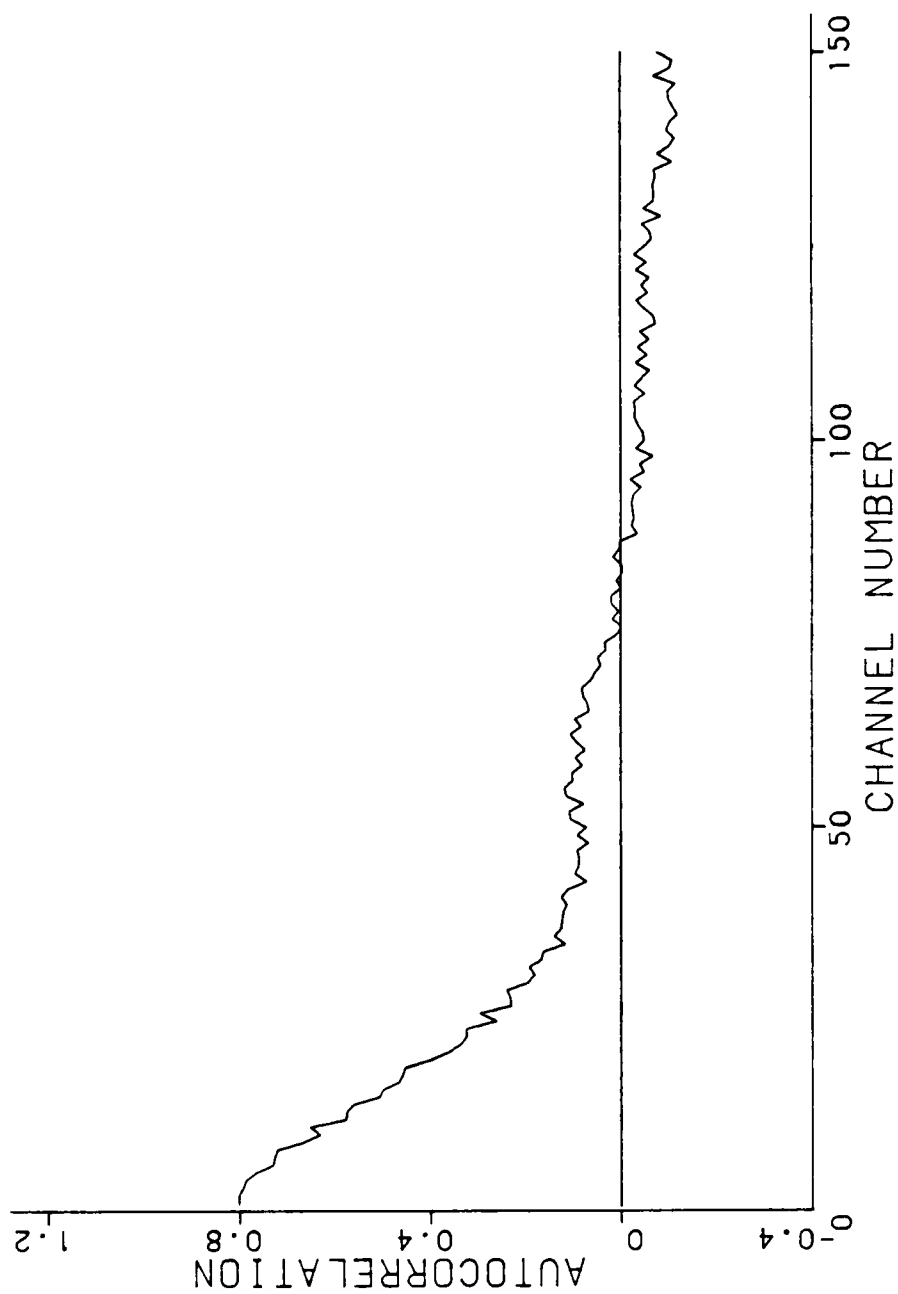


Figure 32. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

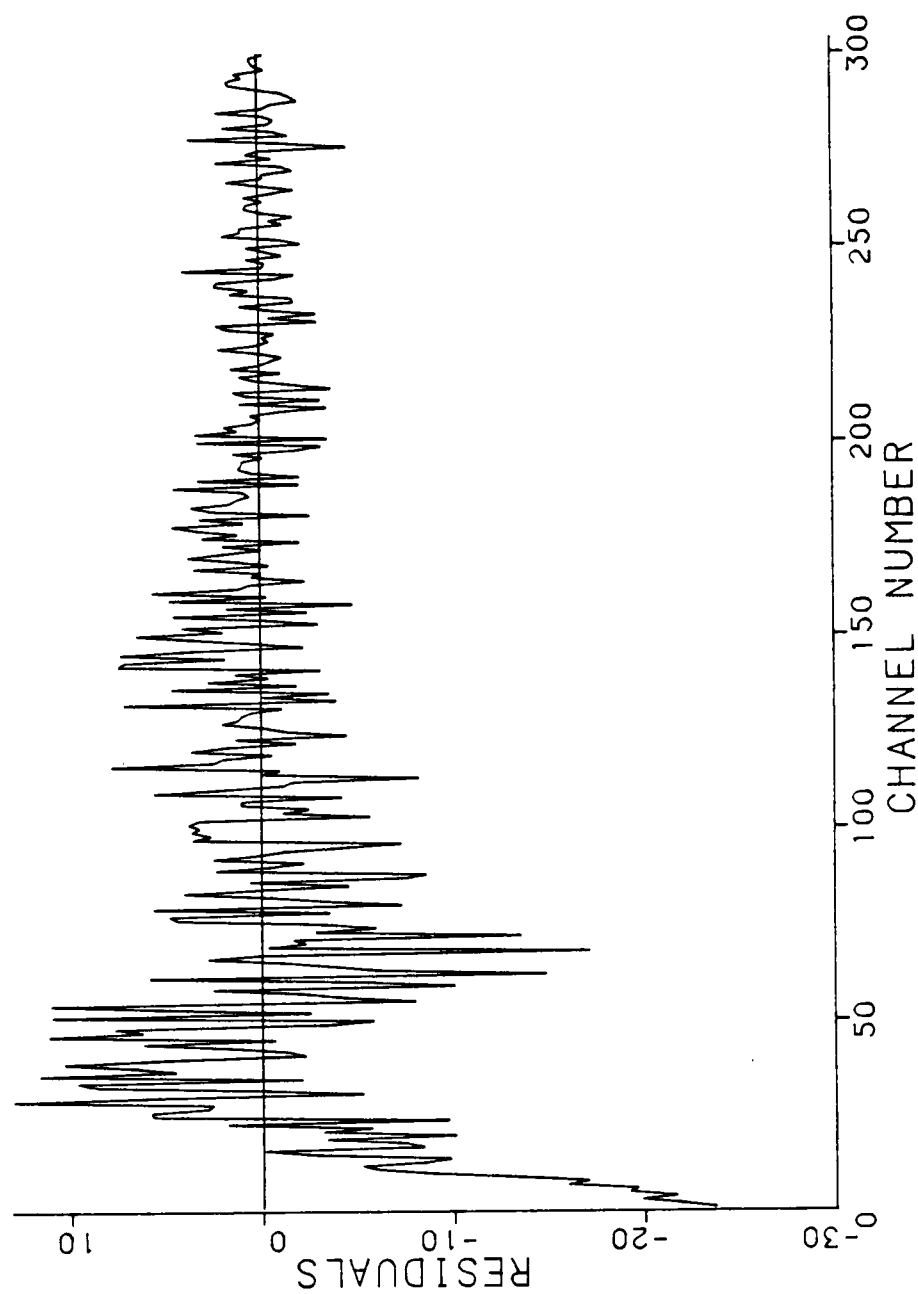


Figure 33. Plot of residuals for double exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

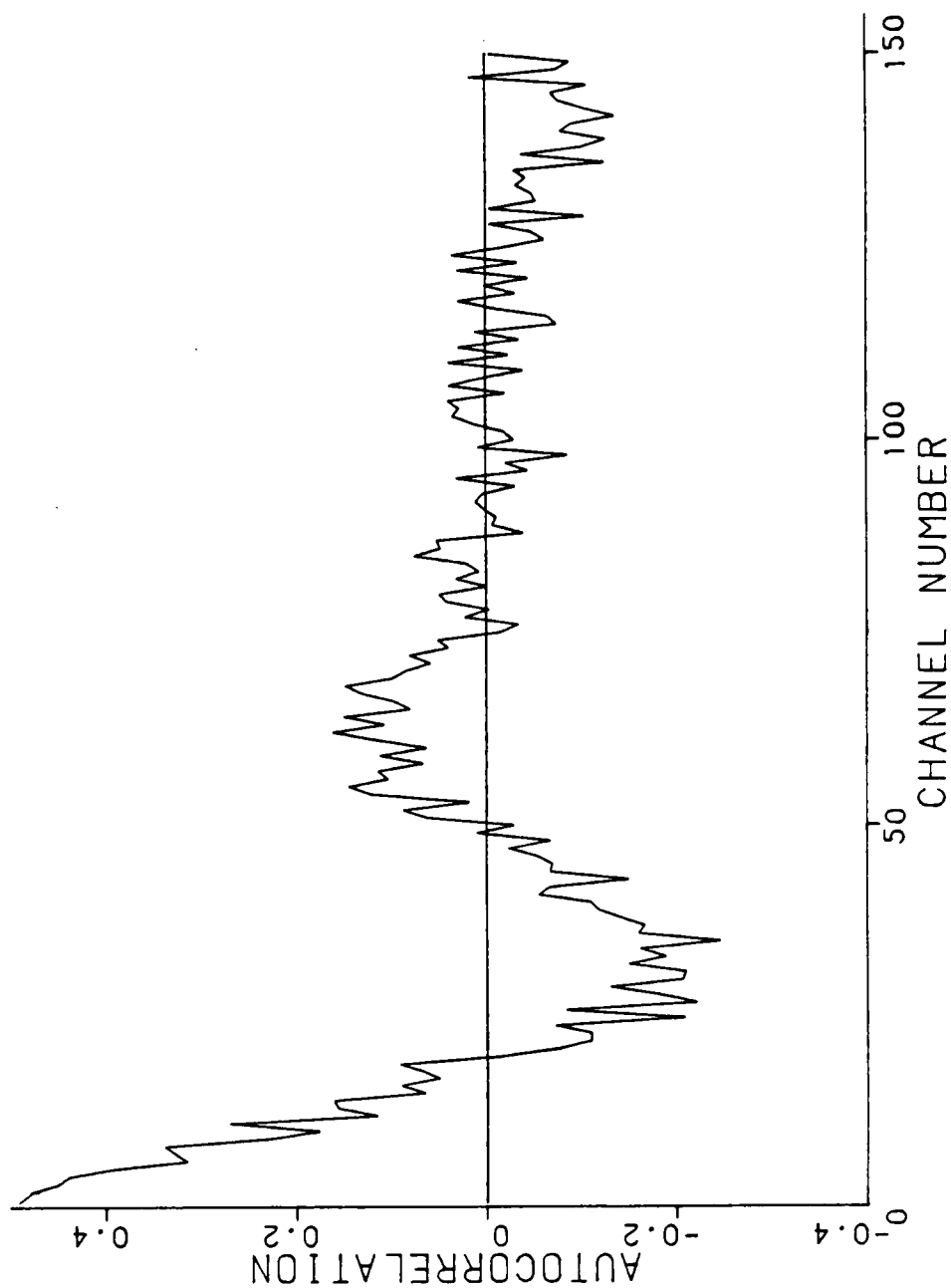


Figure 34. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

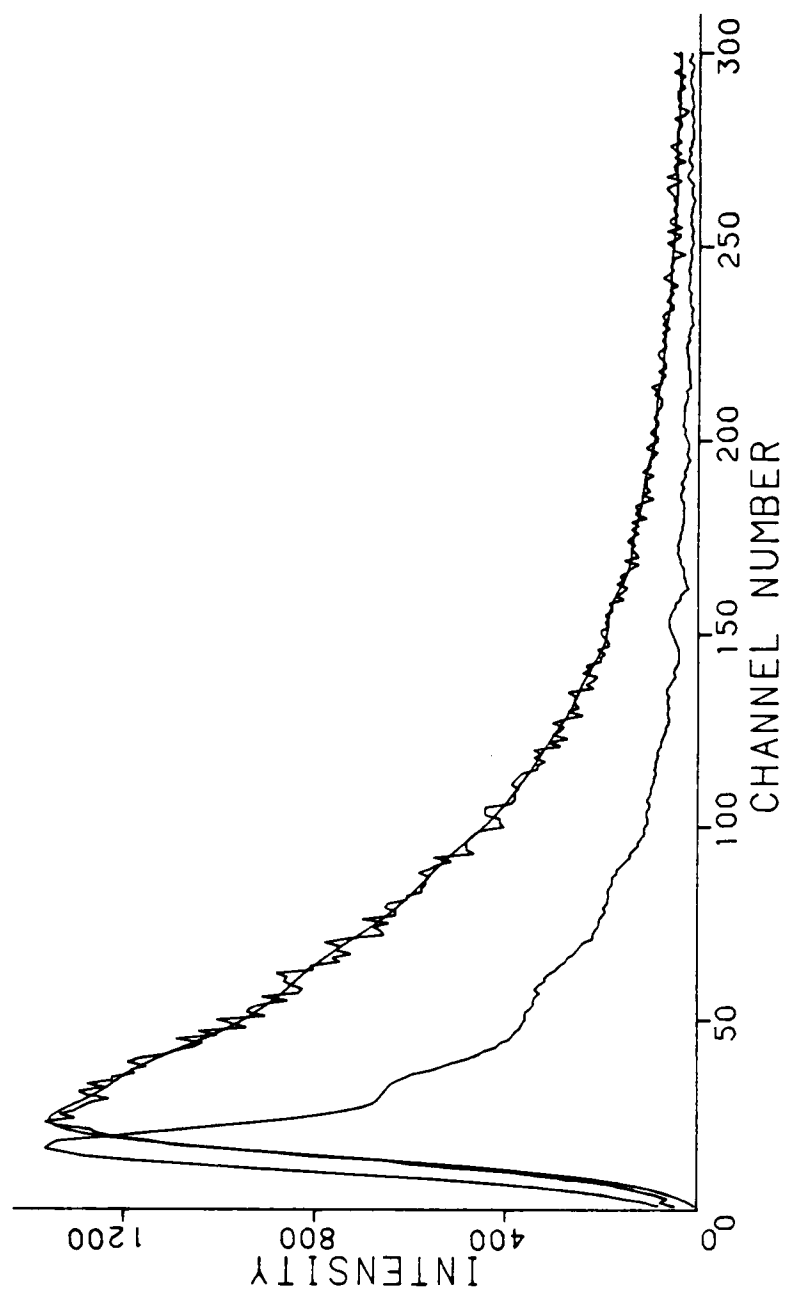


Figure 35. Plot of lamp and $I_V - I_H$ intensities for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

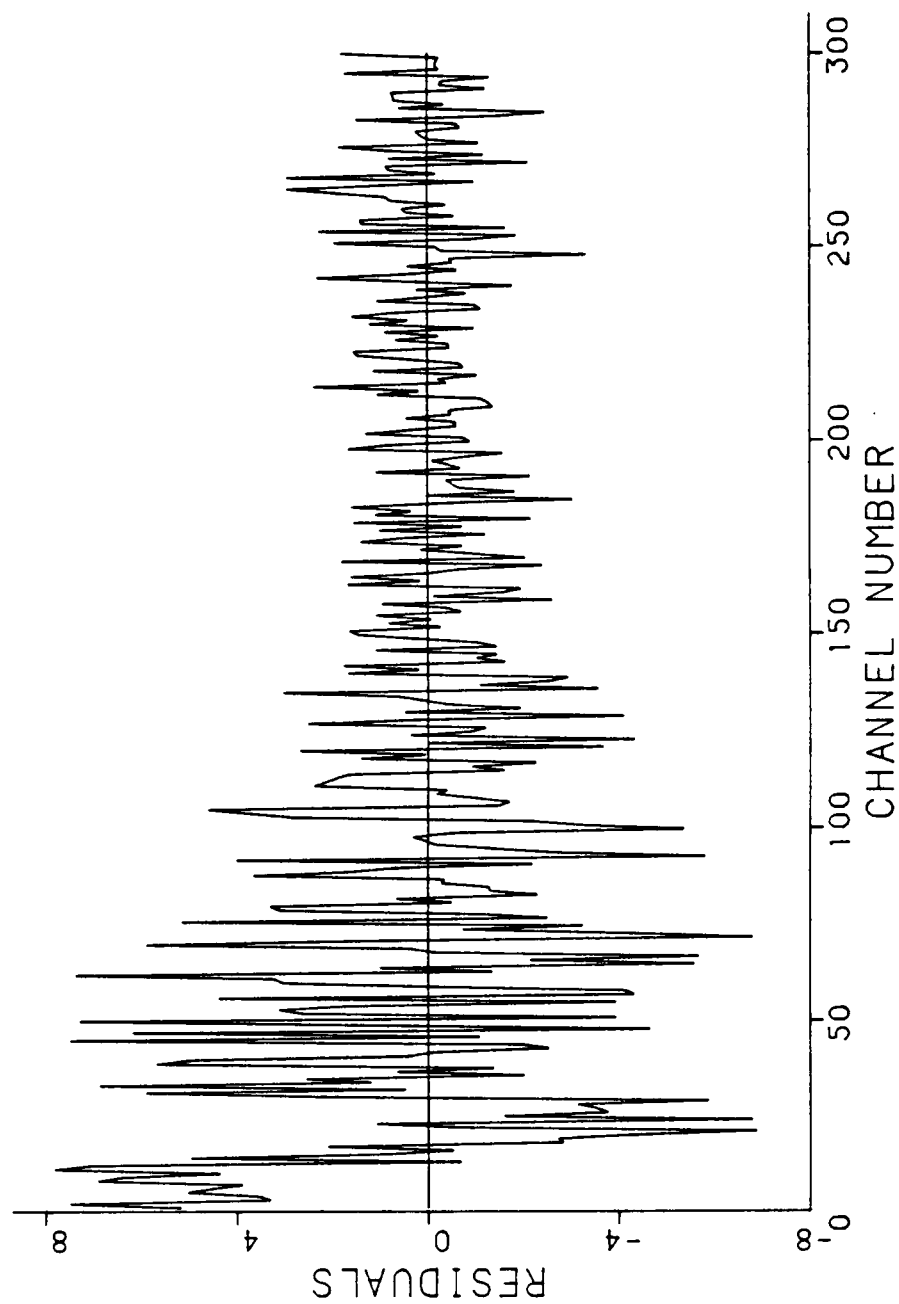


Figure 36. Plot of residuals for fit to I_V-I_H for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

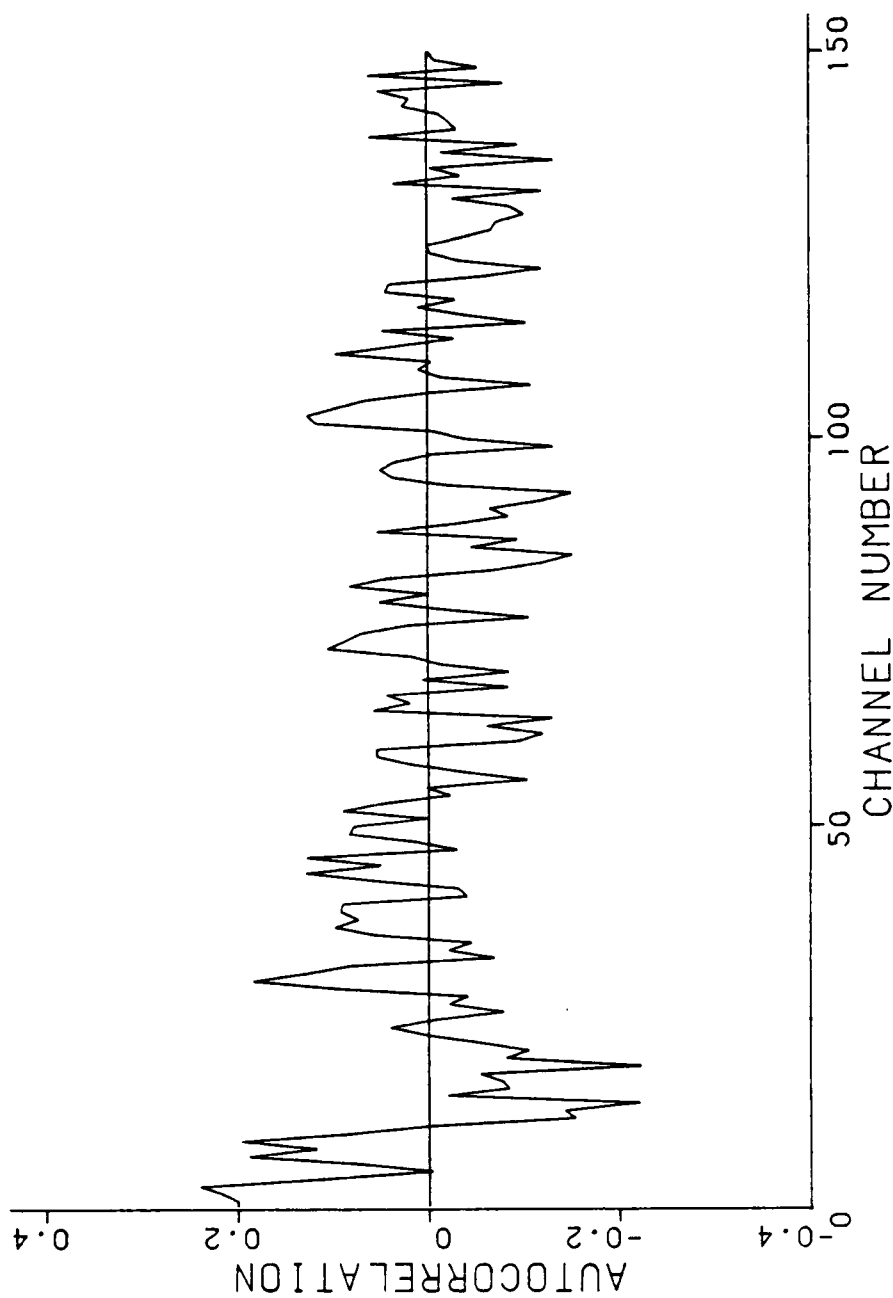


Figure 37. Plot of autocorrelation function for fit to $I_V - I_H$ for DPH in sonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

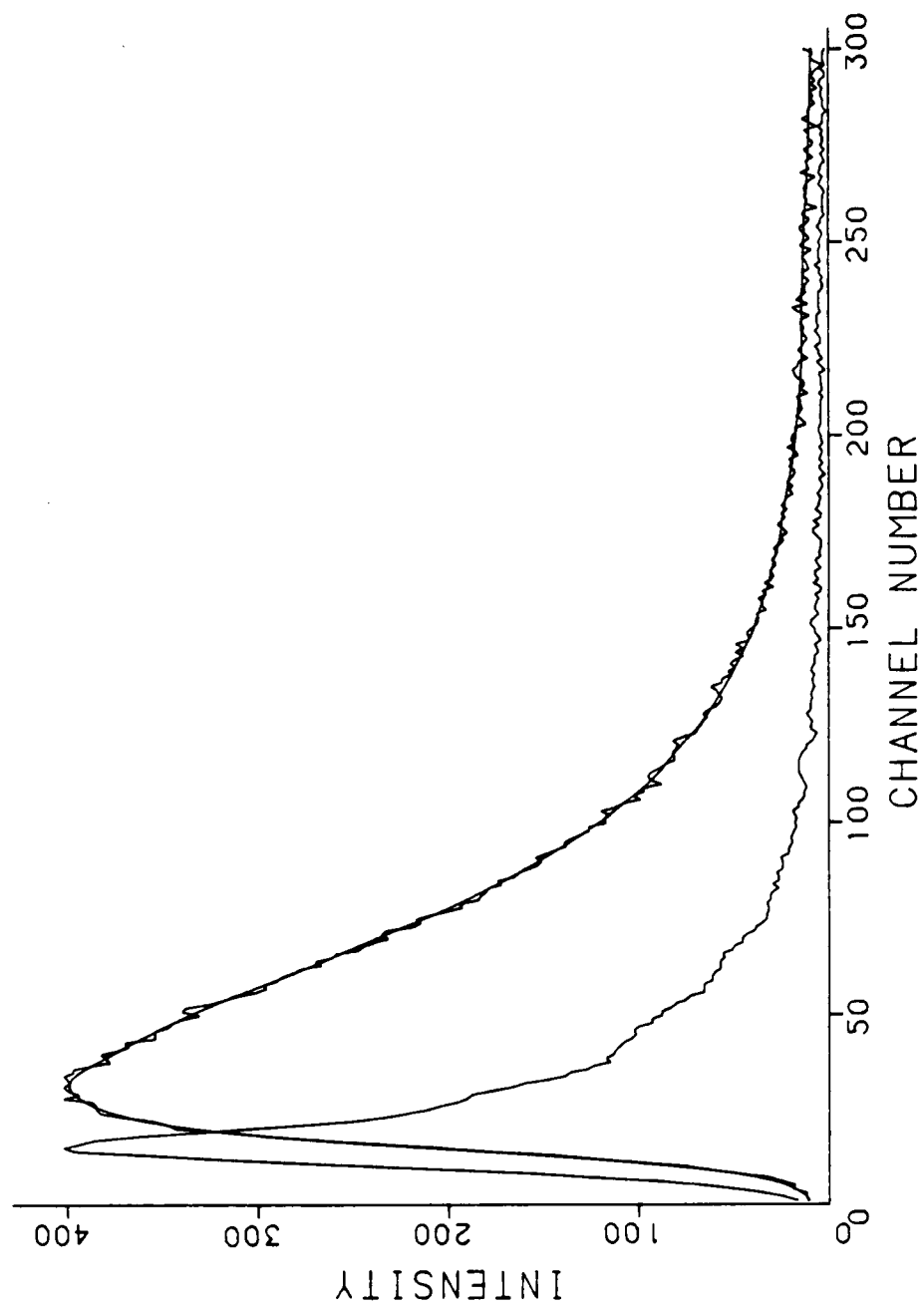


Figure 38. Plot of lamp and total fluorescence intensities for DPH in nonsolubilized DMPC at 16°C in the absence of melittin. Channel width = 0.324 ns.

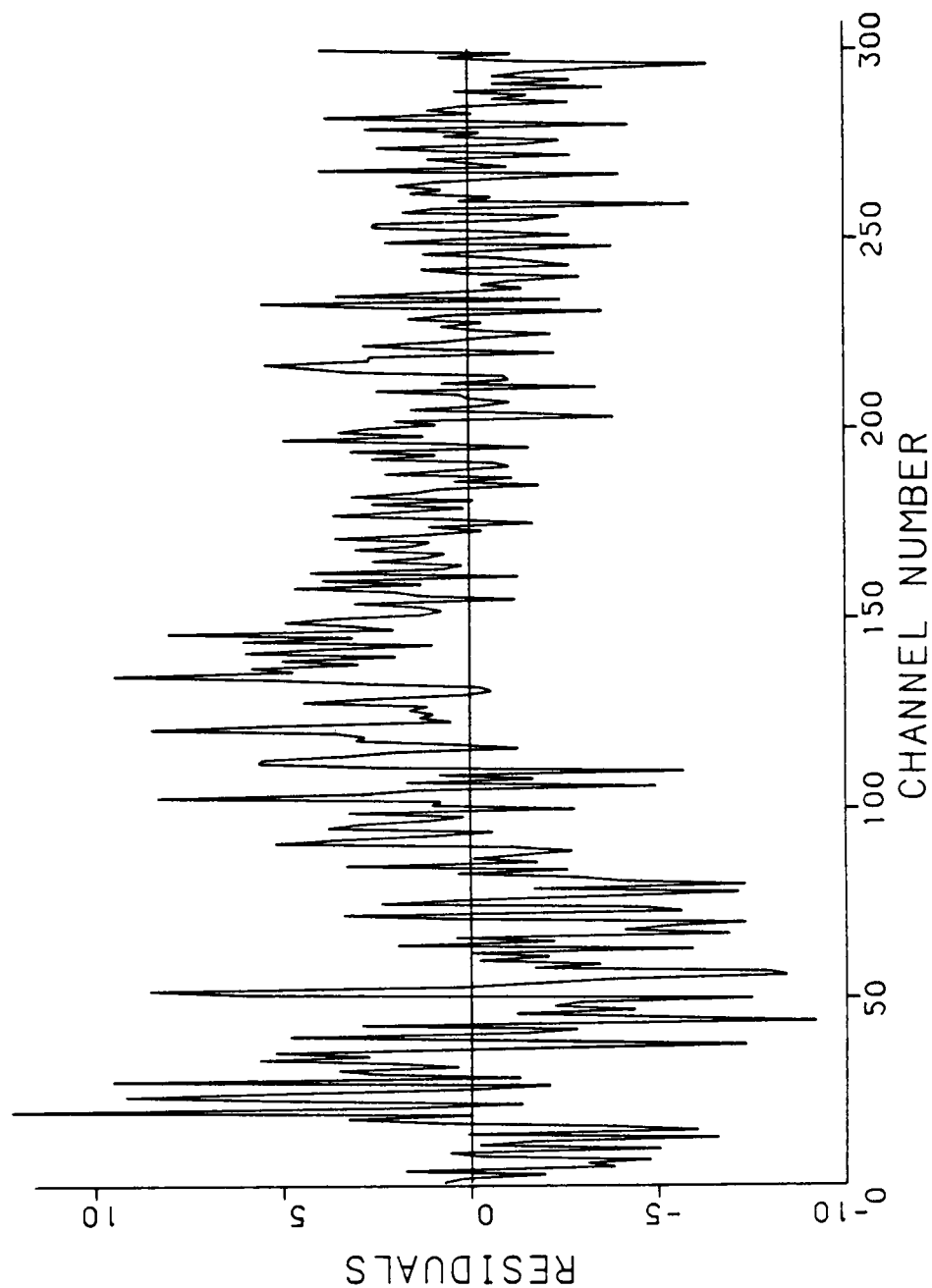


Figure 39. Plot of residuals for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

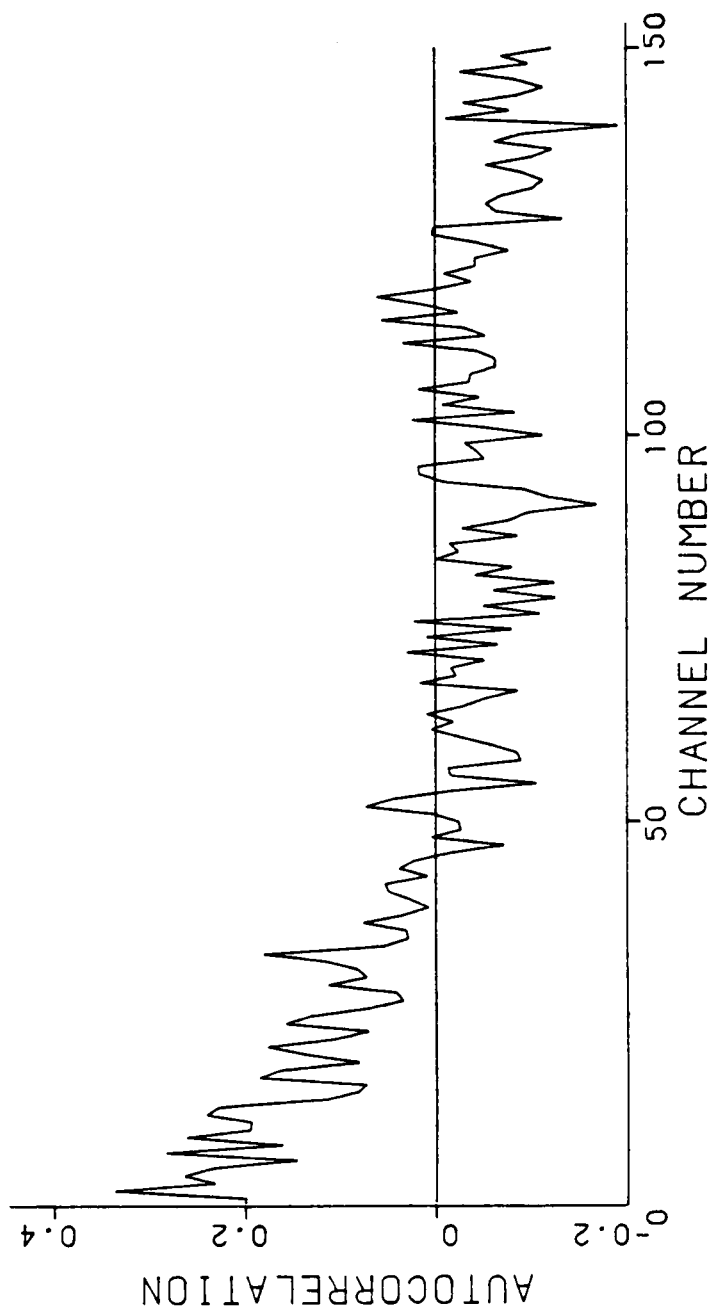


Figure 40. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

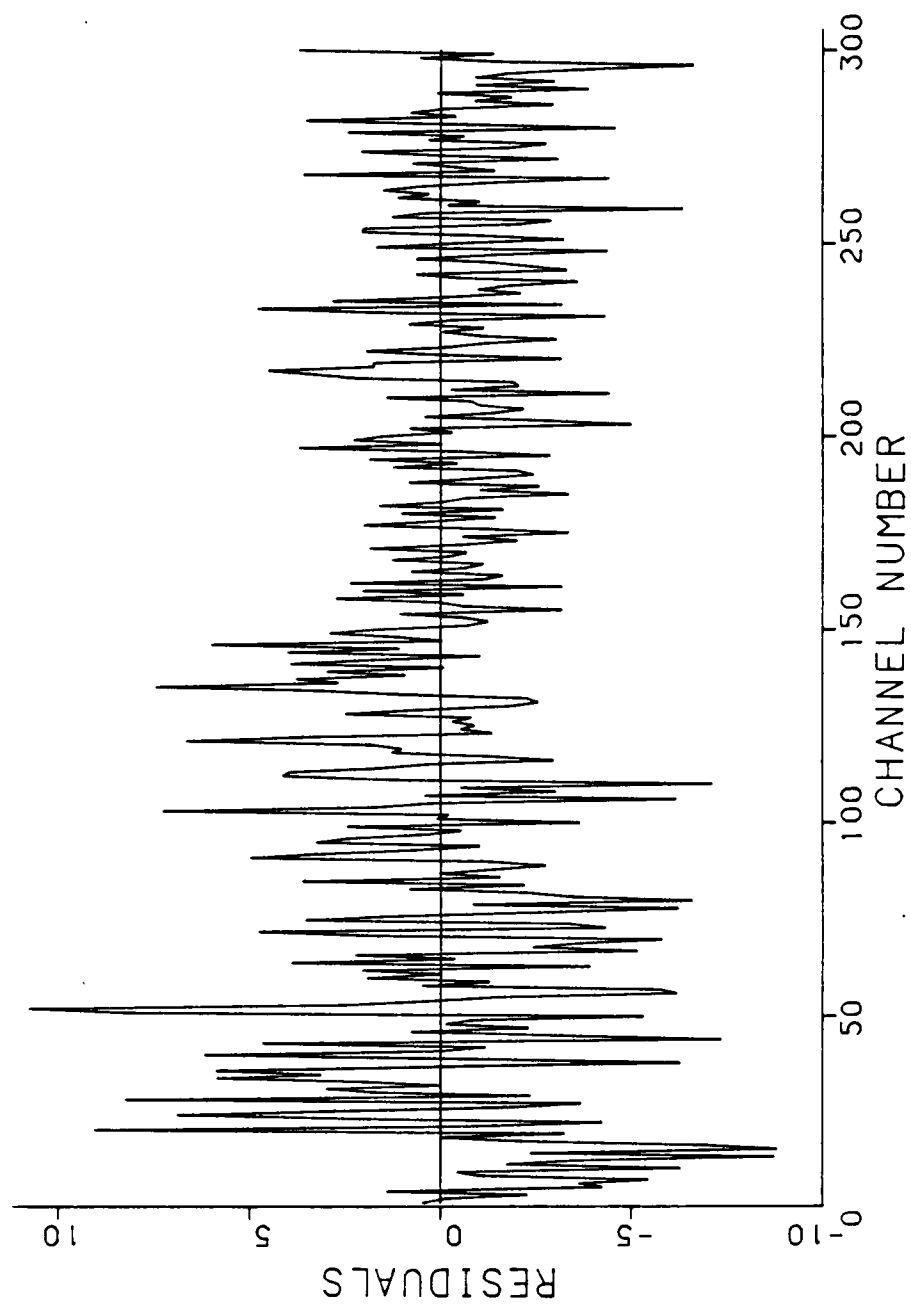


Figure 41. Plot of residuals for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

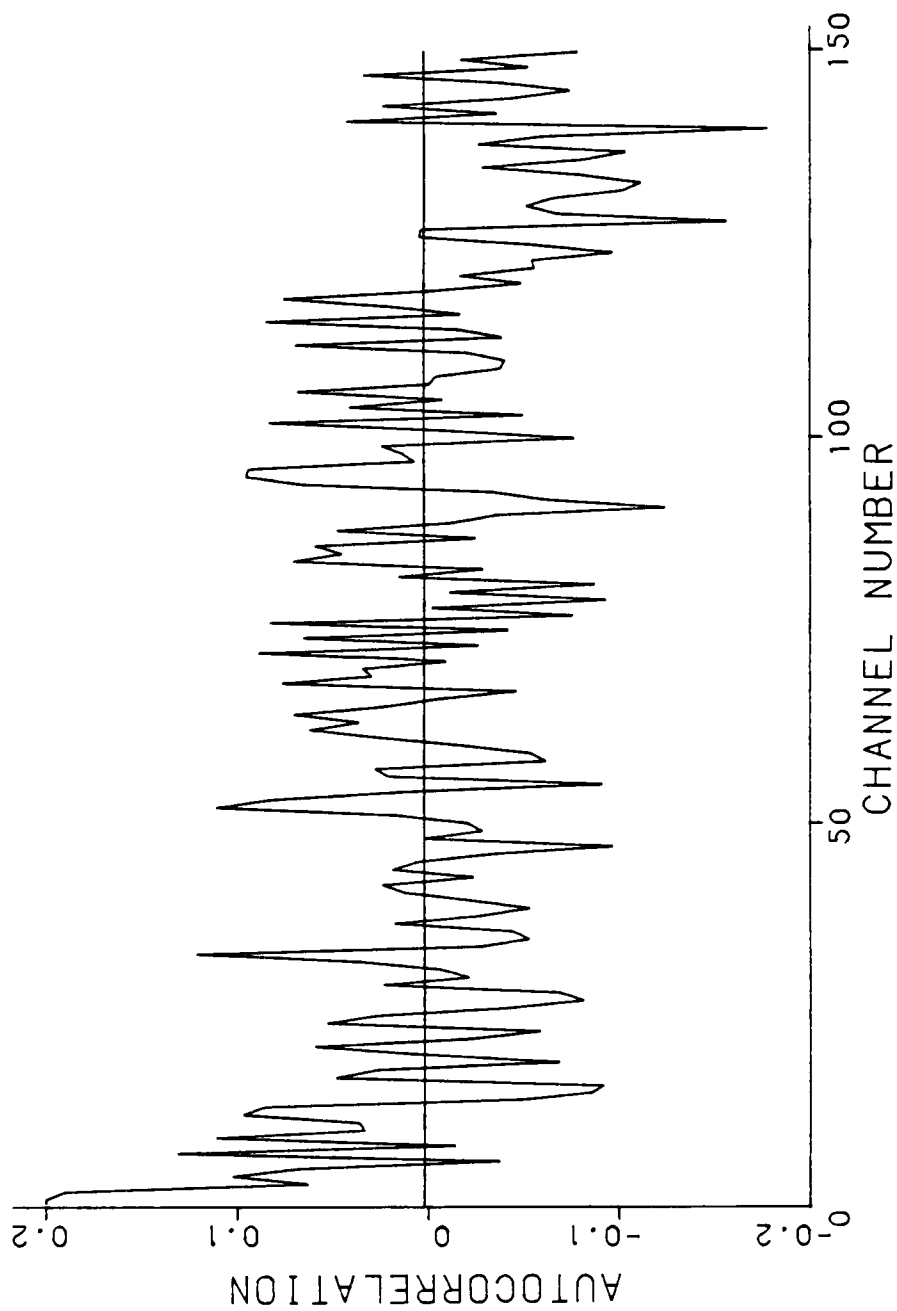


Figure 42. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

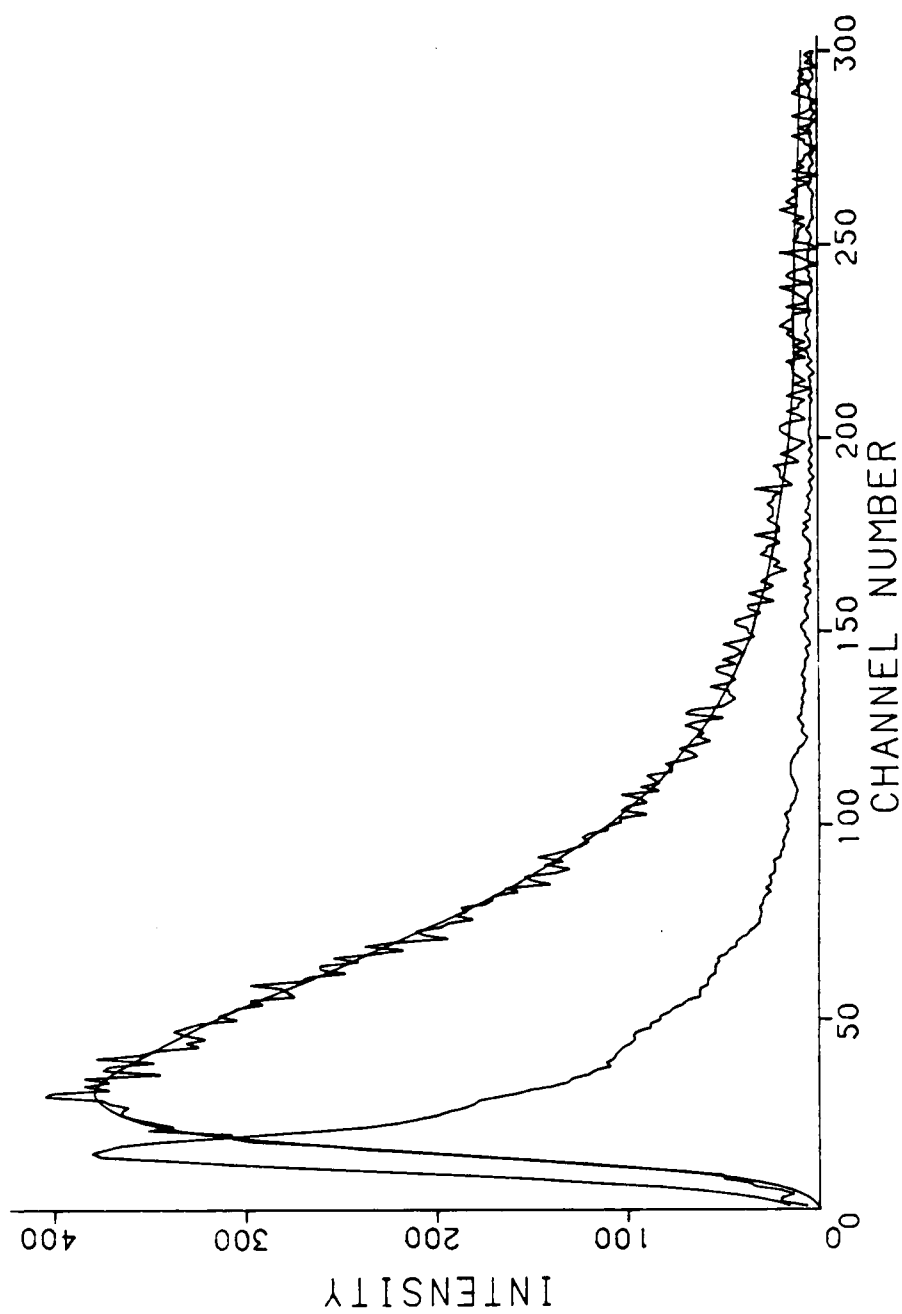


Figure 43. Plot of lamp and $I_V - I_H$ intensities for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

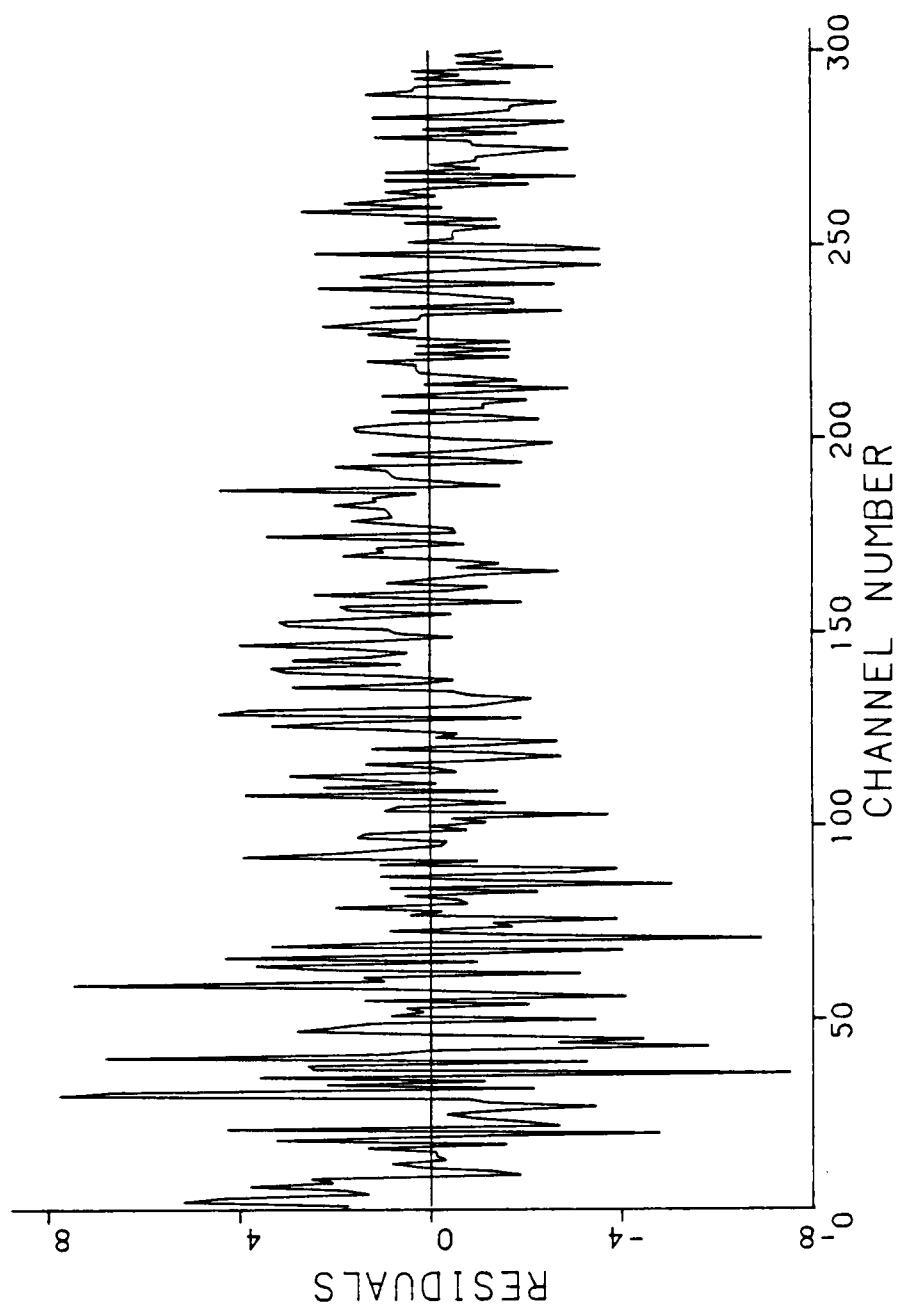


Figure 44. Plot of residuals for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

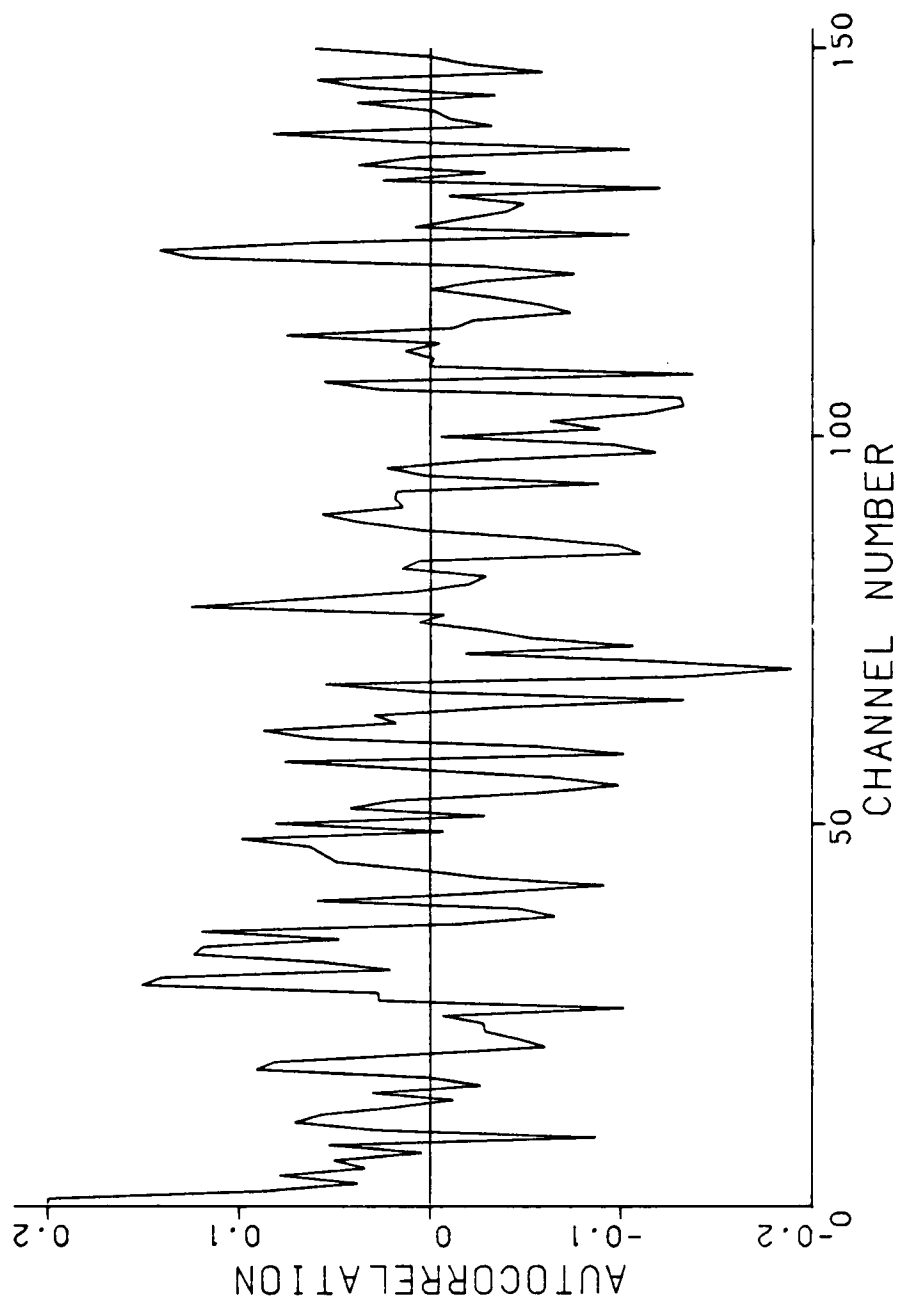


Figure 45. Plot of autocorrelation function for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 16°C in the absence of melittin.

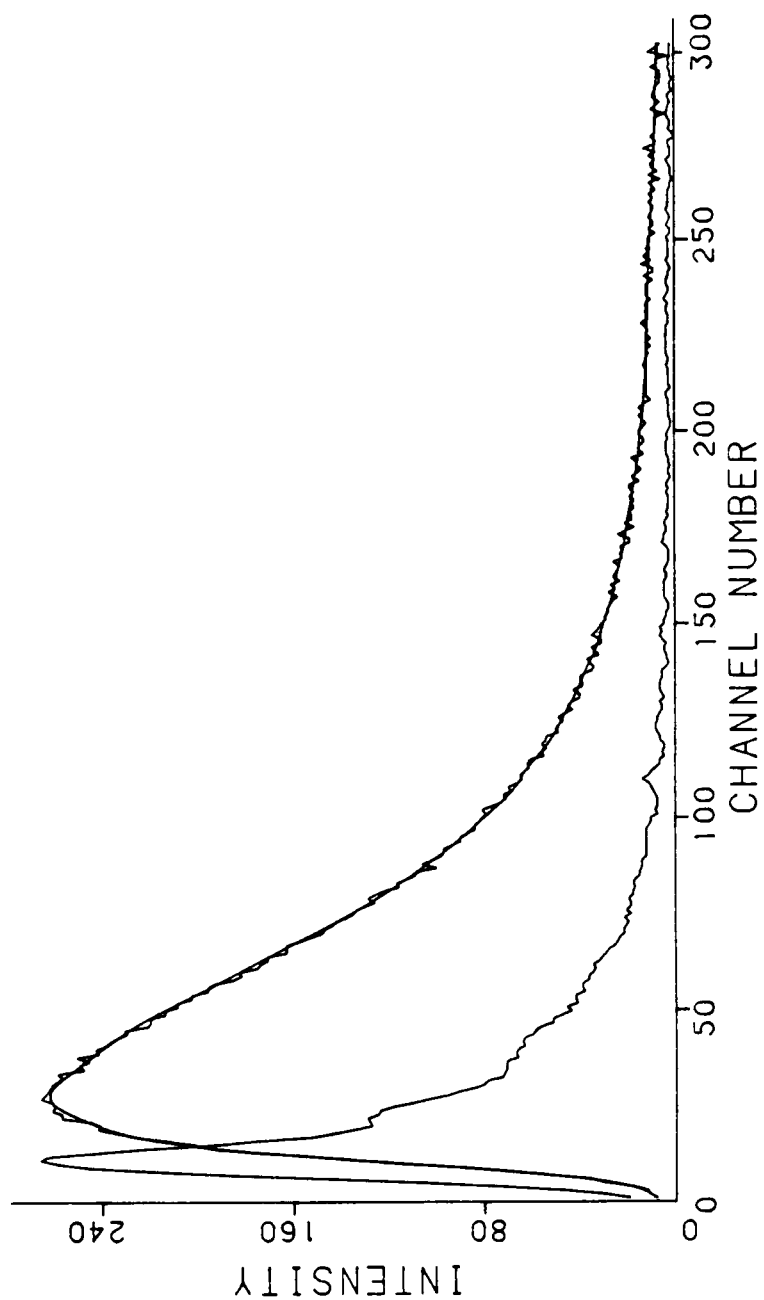


Figure 46. Plot of lamp and total fluorescence intensities for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$. Channel width = 0.327 ns.

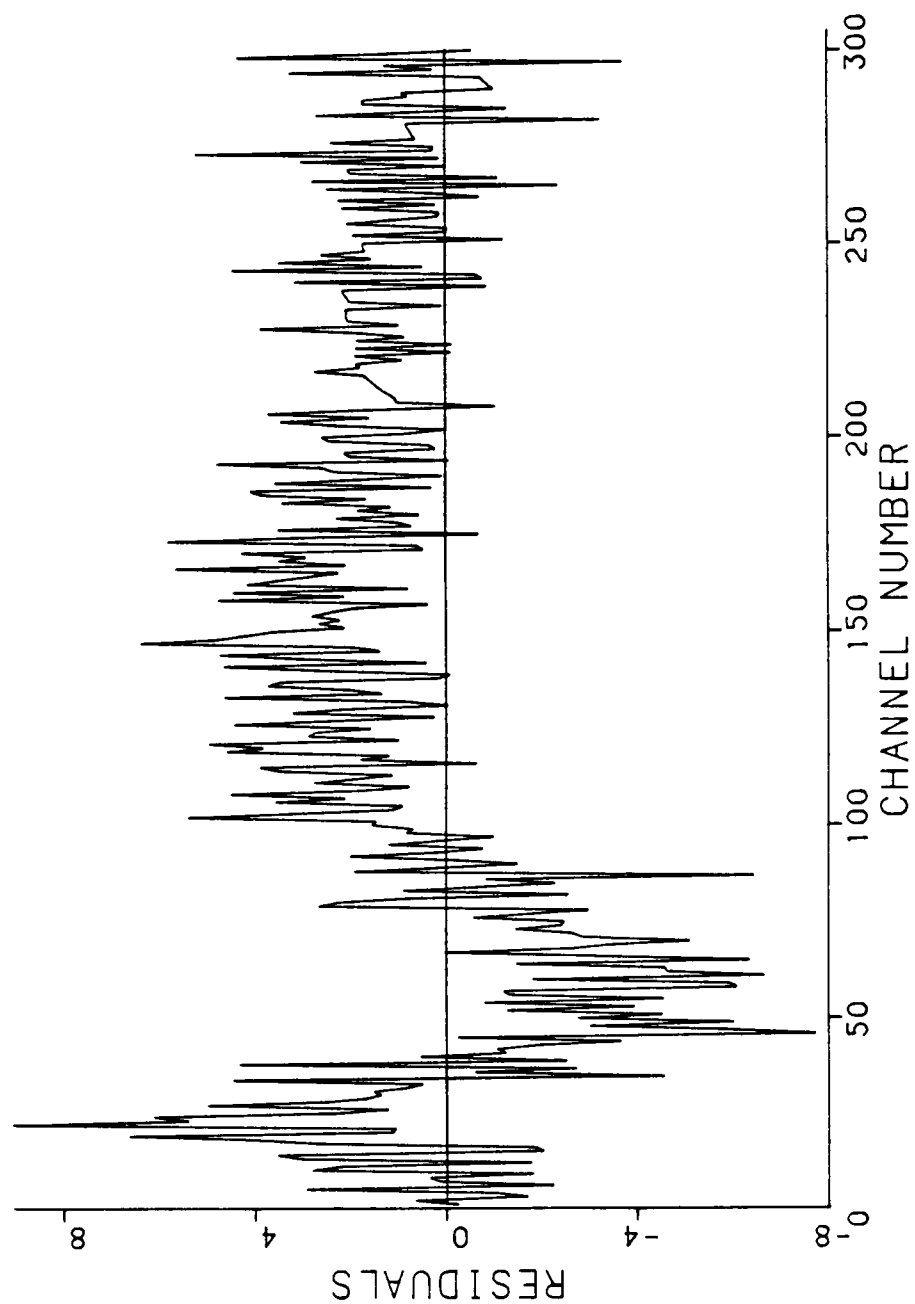


Figure 47. Plot of residuals for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

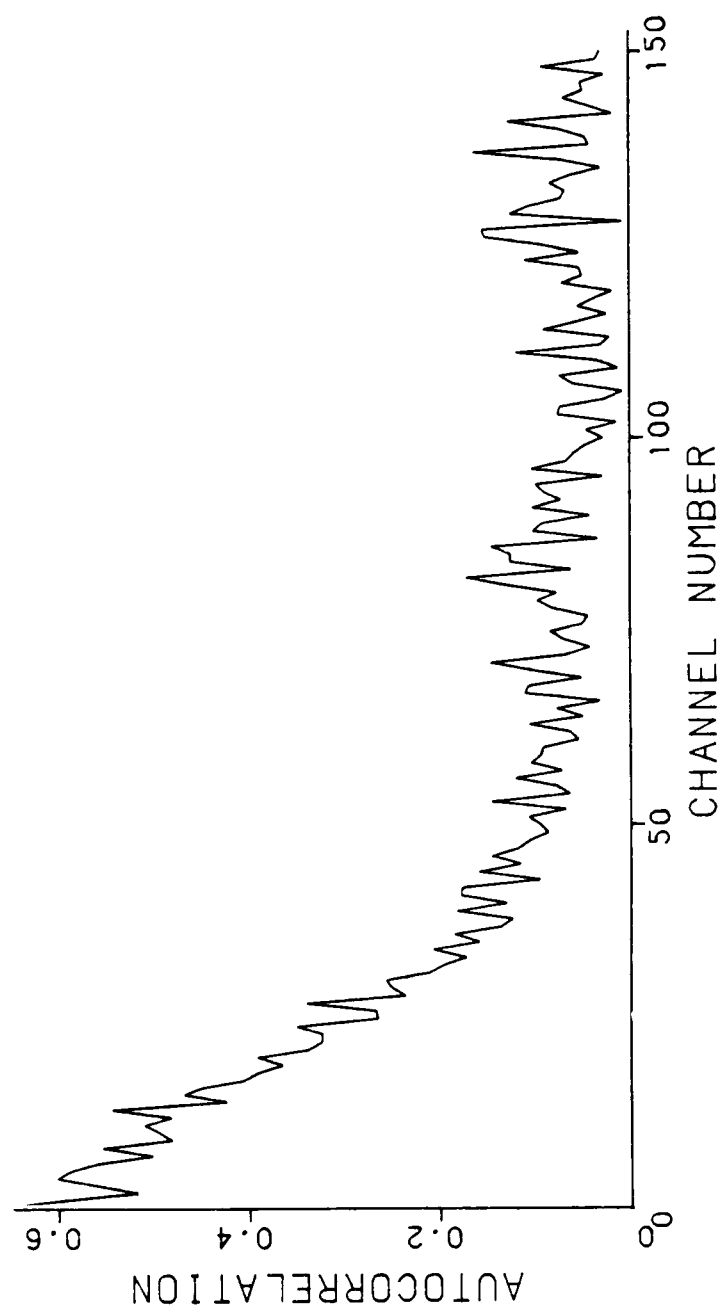


Figure 48. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in nonsolubilized DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

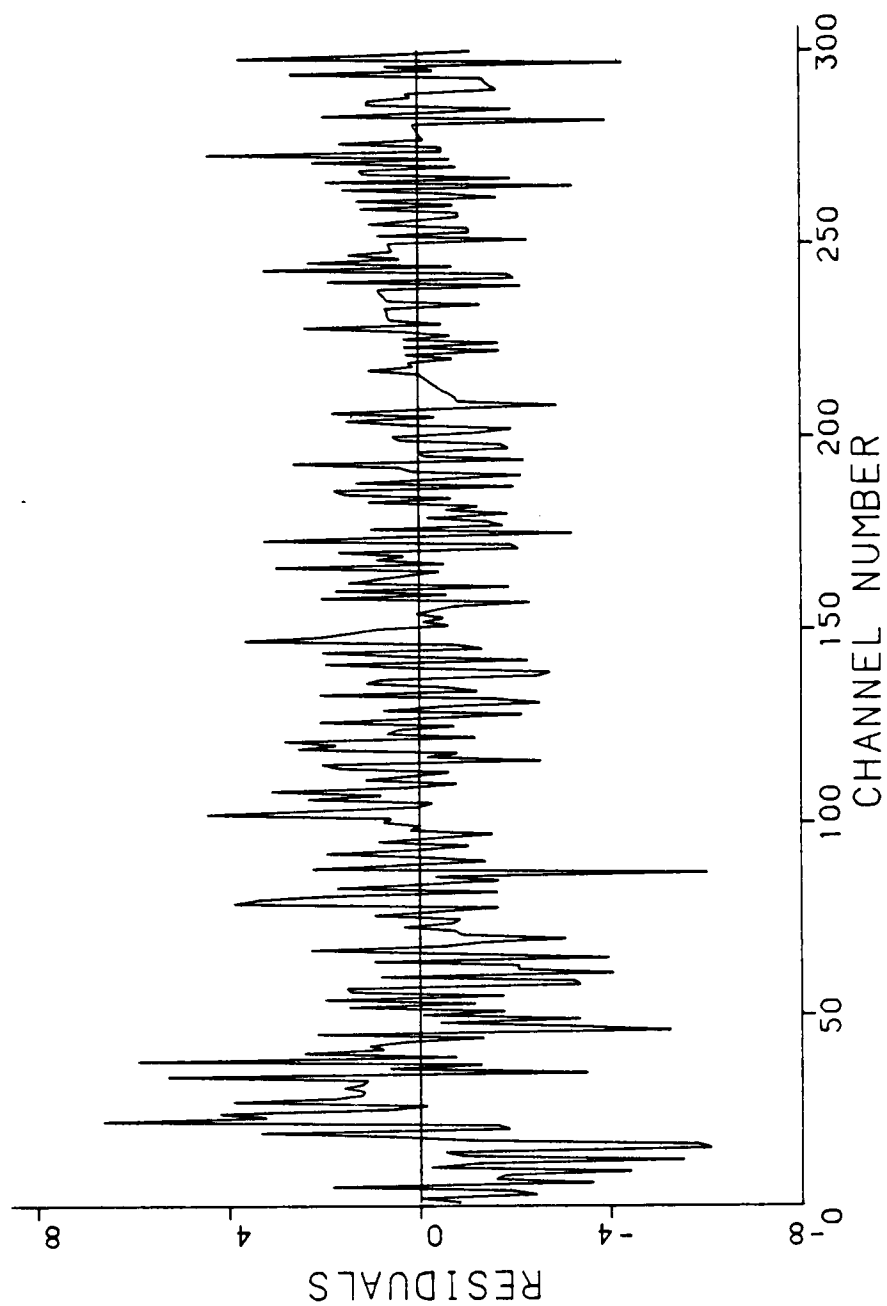


Figure 49. Plot of residuals for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

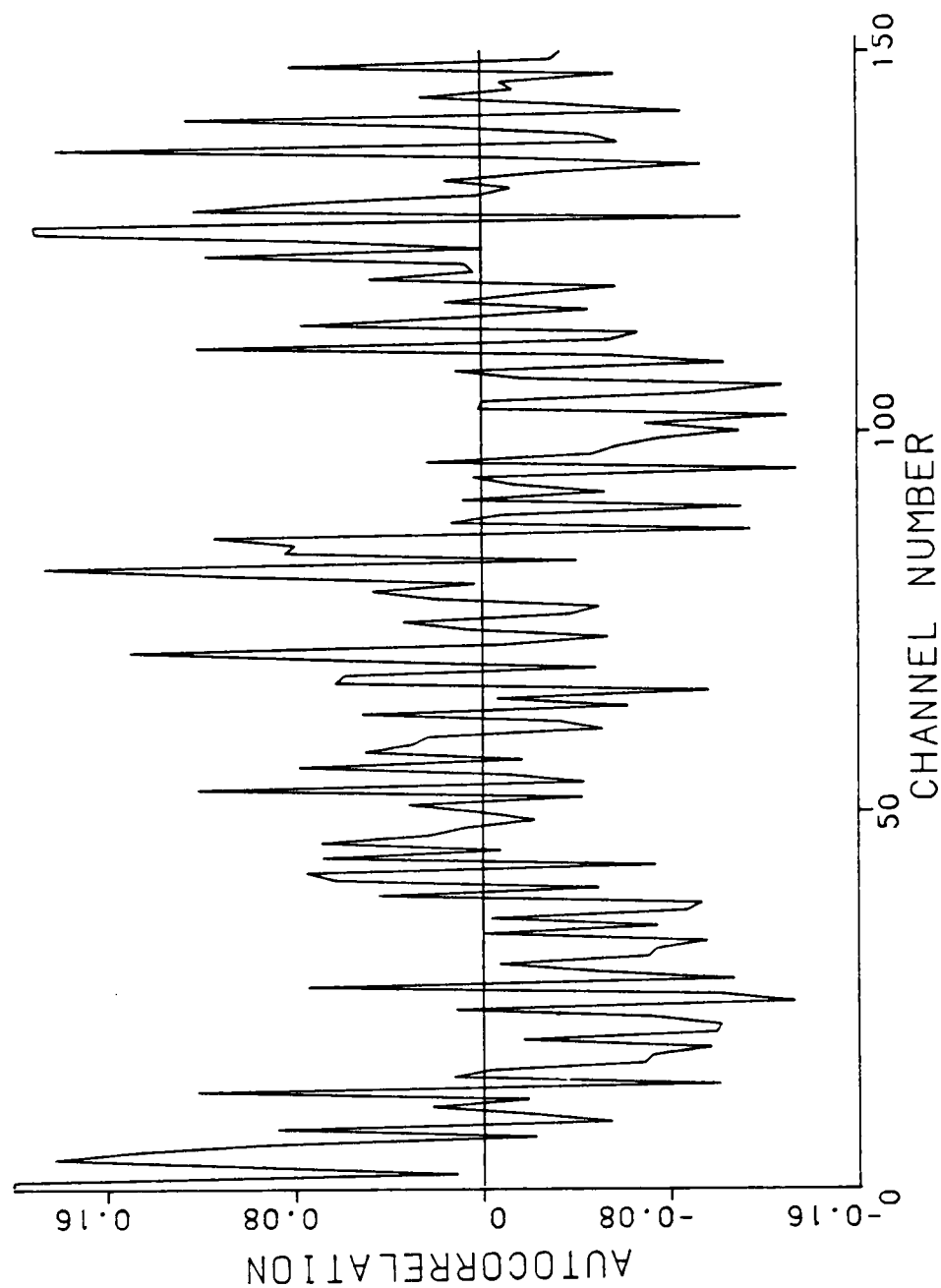


Figure 50. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

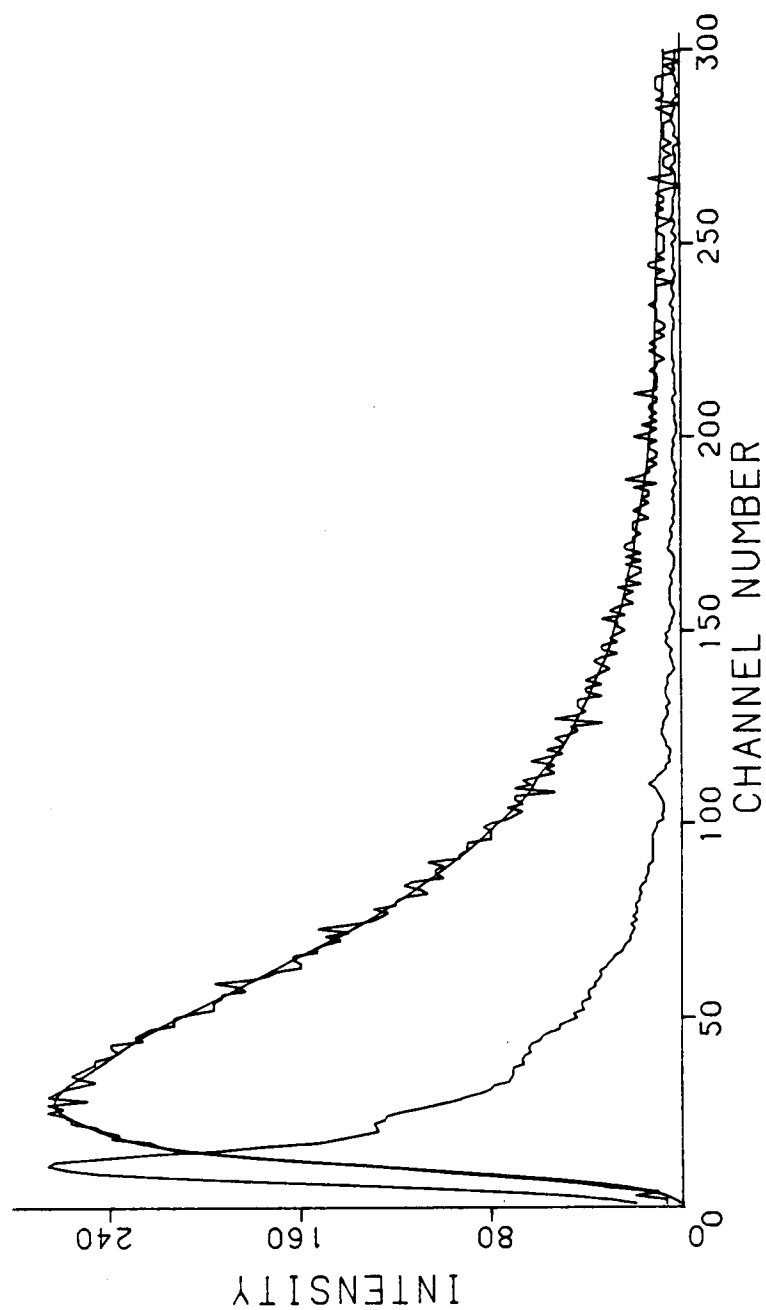


Figure 51. Plot of lamp and I_V-I_H intensities for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of ~1/60.

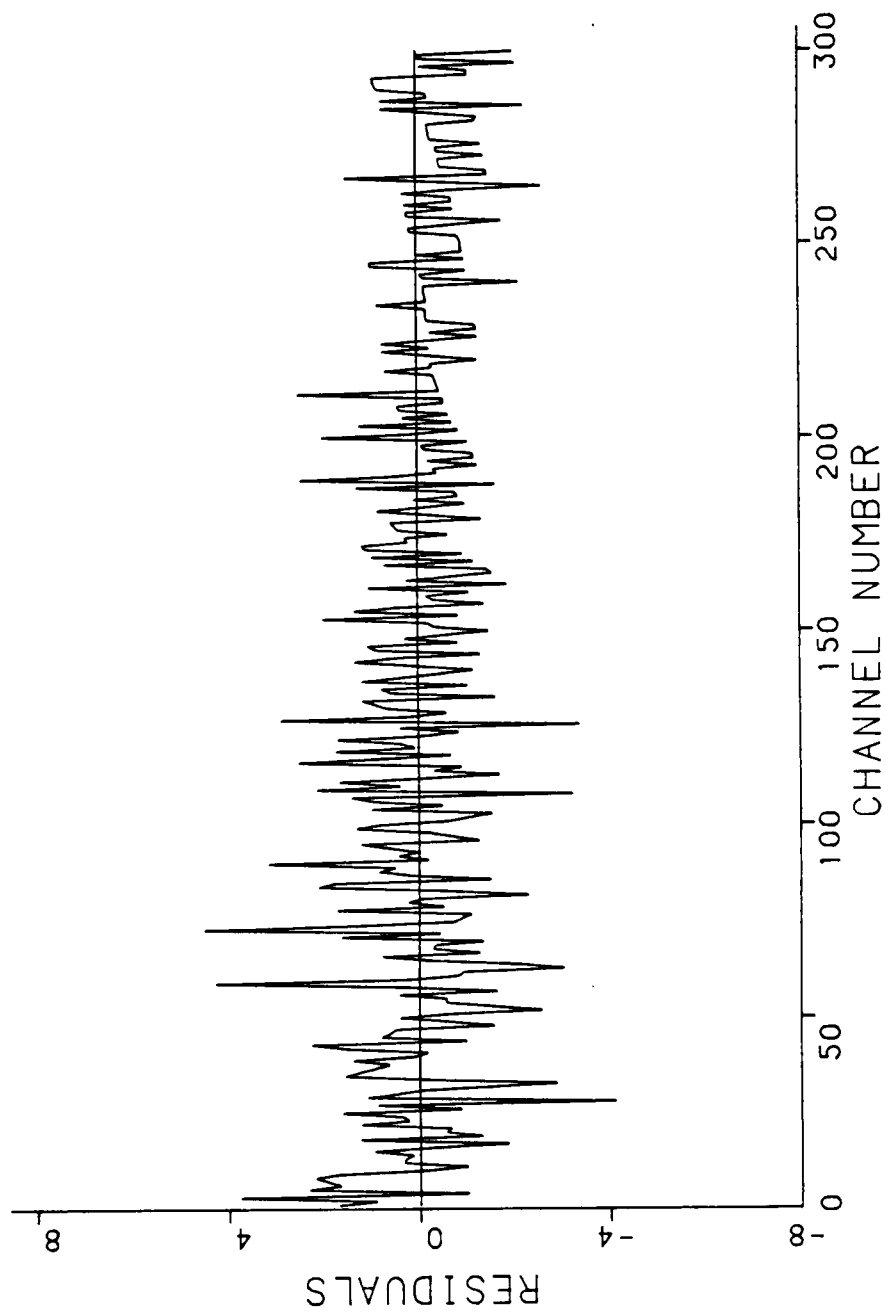


Figure 52. Plot of residuals for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

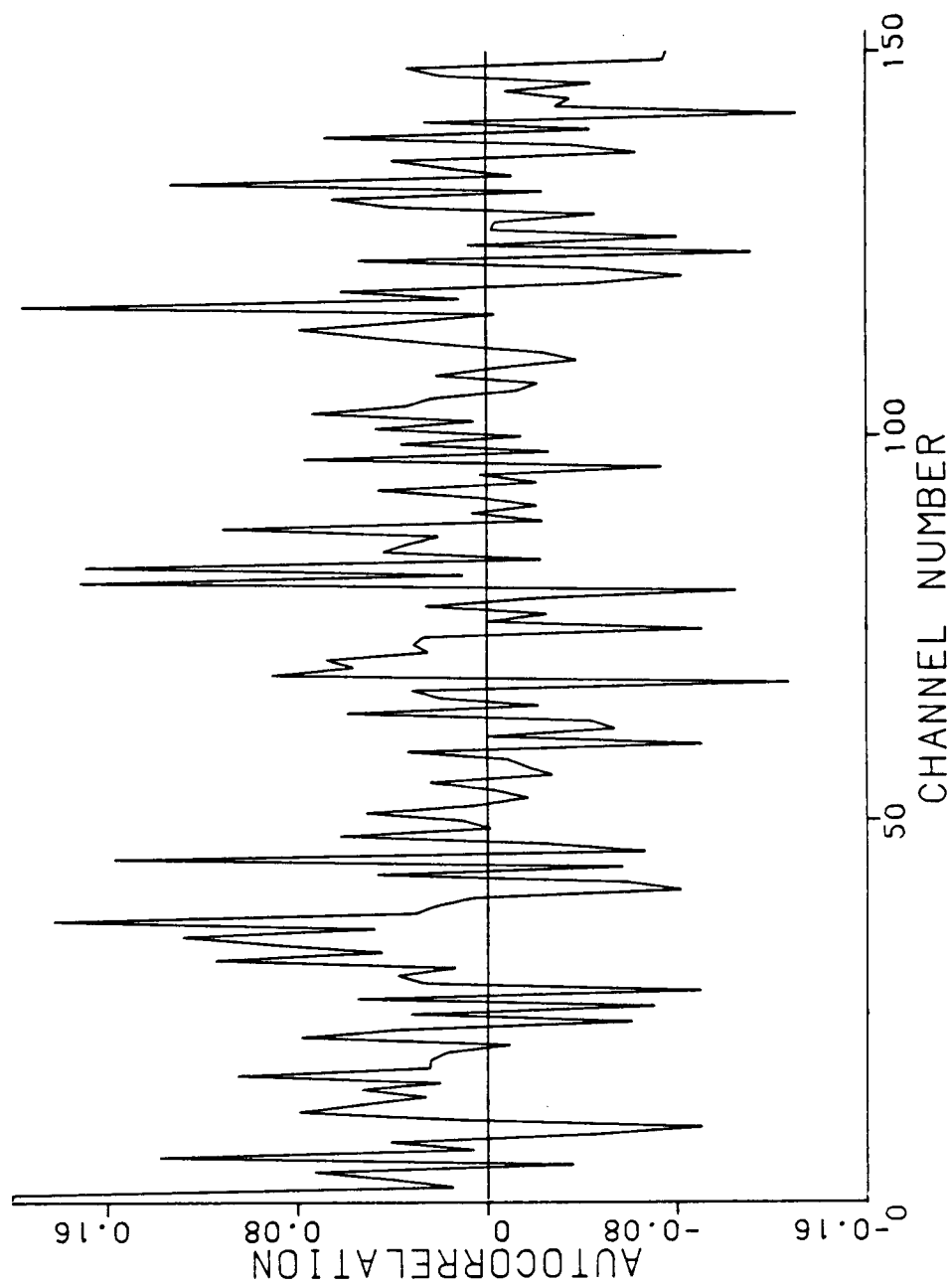


Figure 53. Plot of autocorrelation function for fit to I_V-I_H for DPH in nonsonicated DMPC at 16°C with a melittin to lipid molar ratio of $\sim 1/60$.

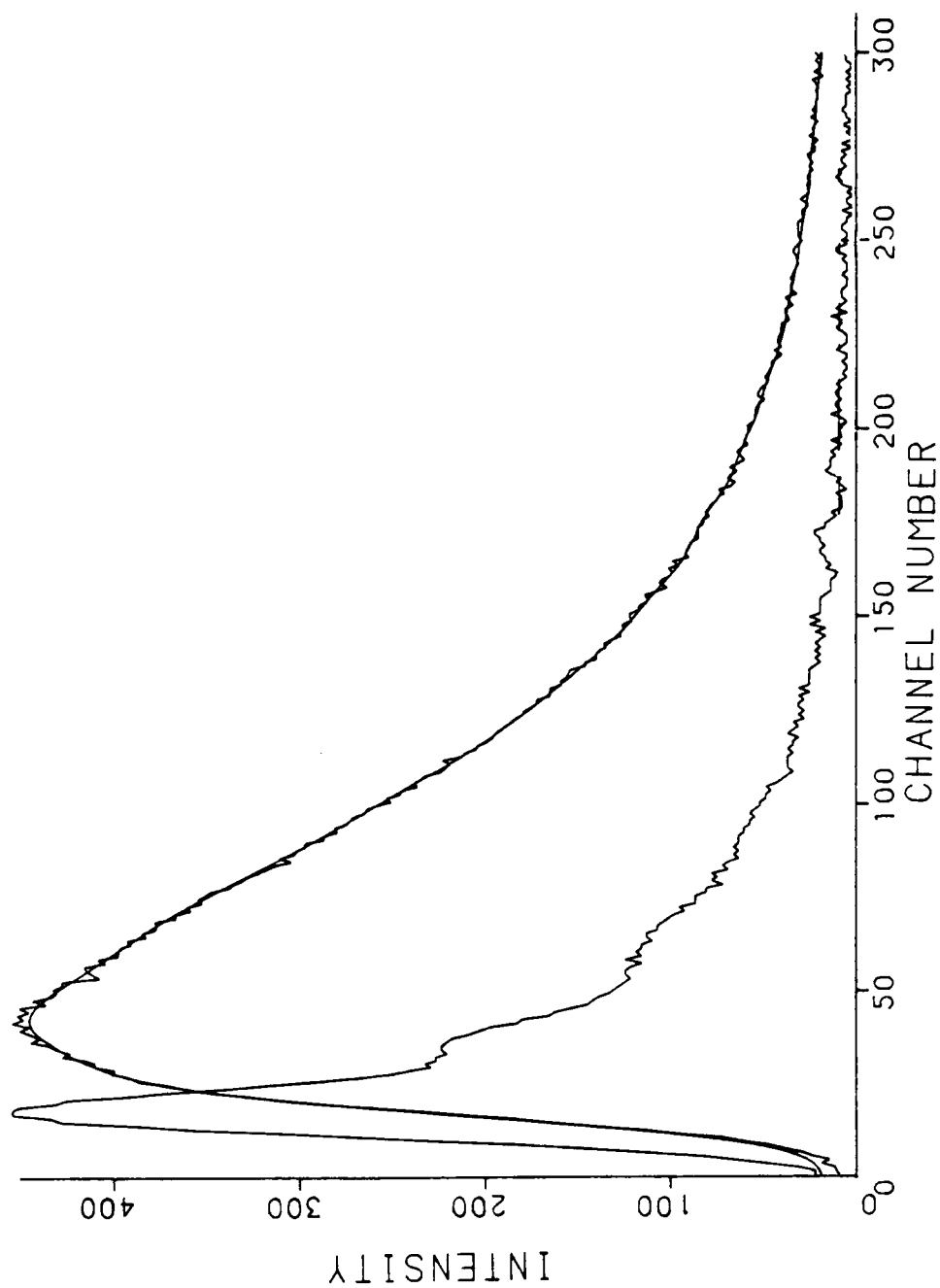


Figure 54. Plot of lamp and total fluorescence intensities for DPH in nonsonicated DMPC at 33°C in the absence of melittin. Channel width = 0.212 ns.

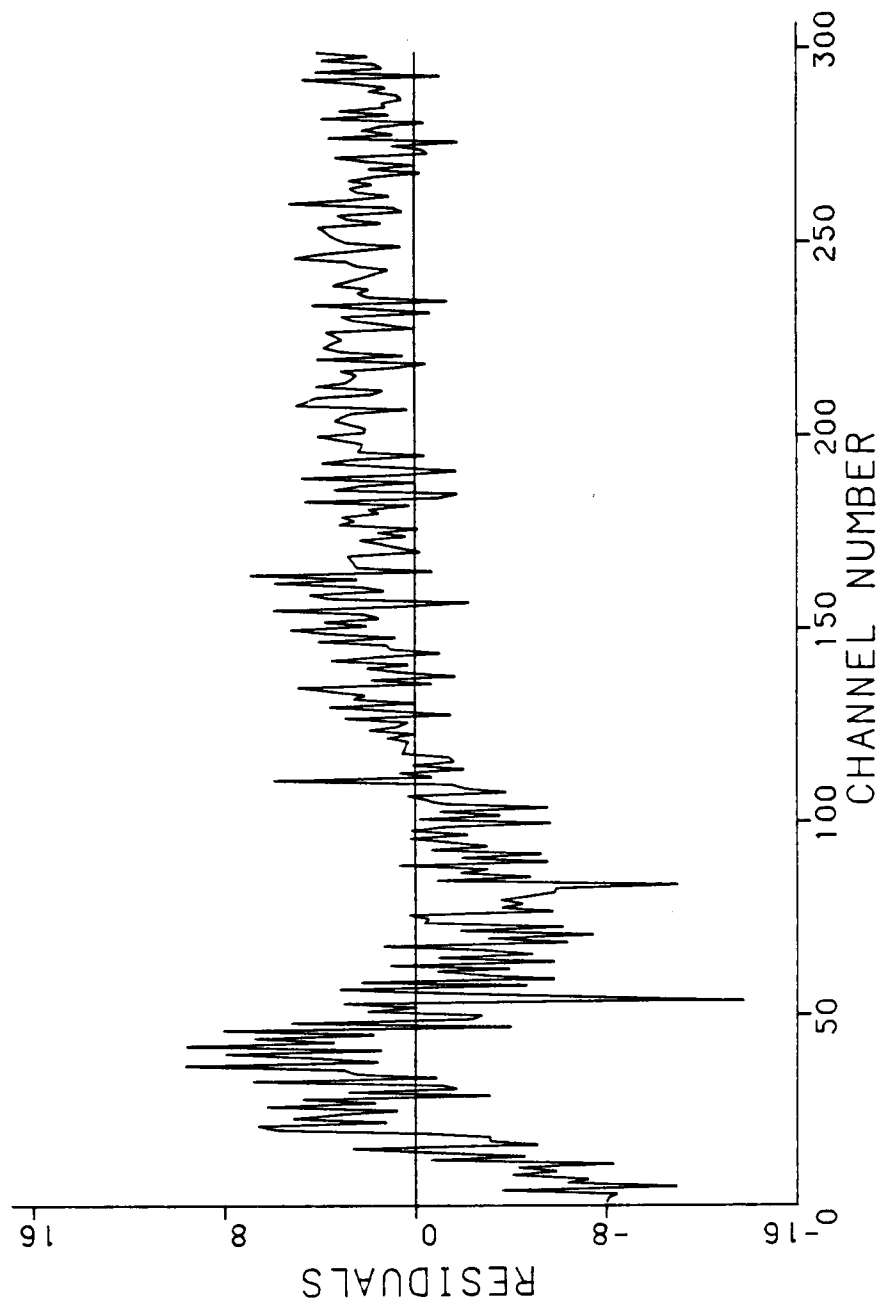


Figure 55. Plot of residuals for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

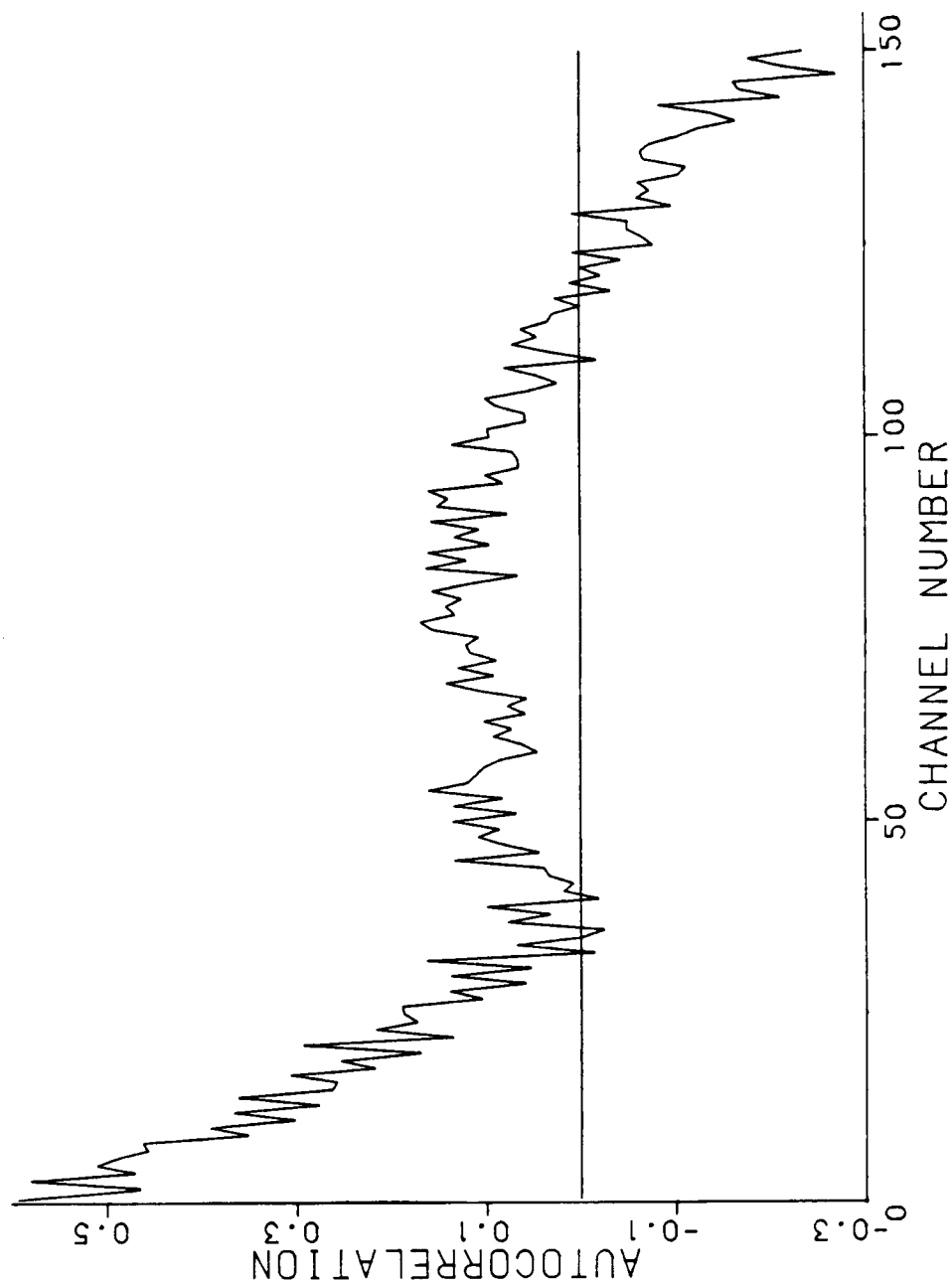


Figure 56. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

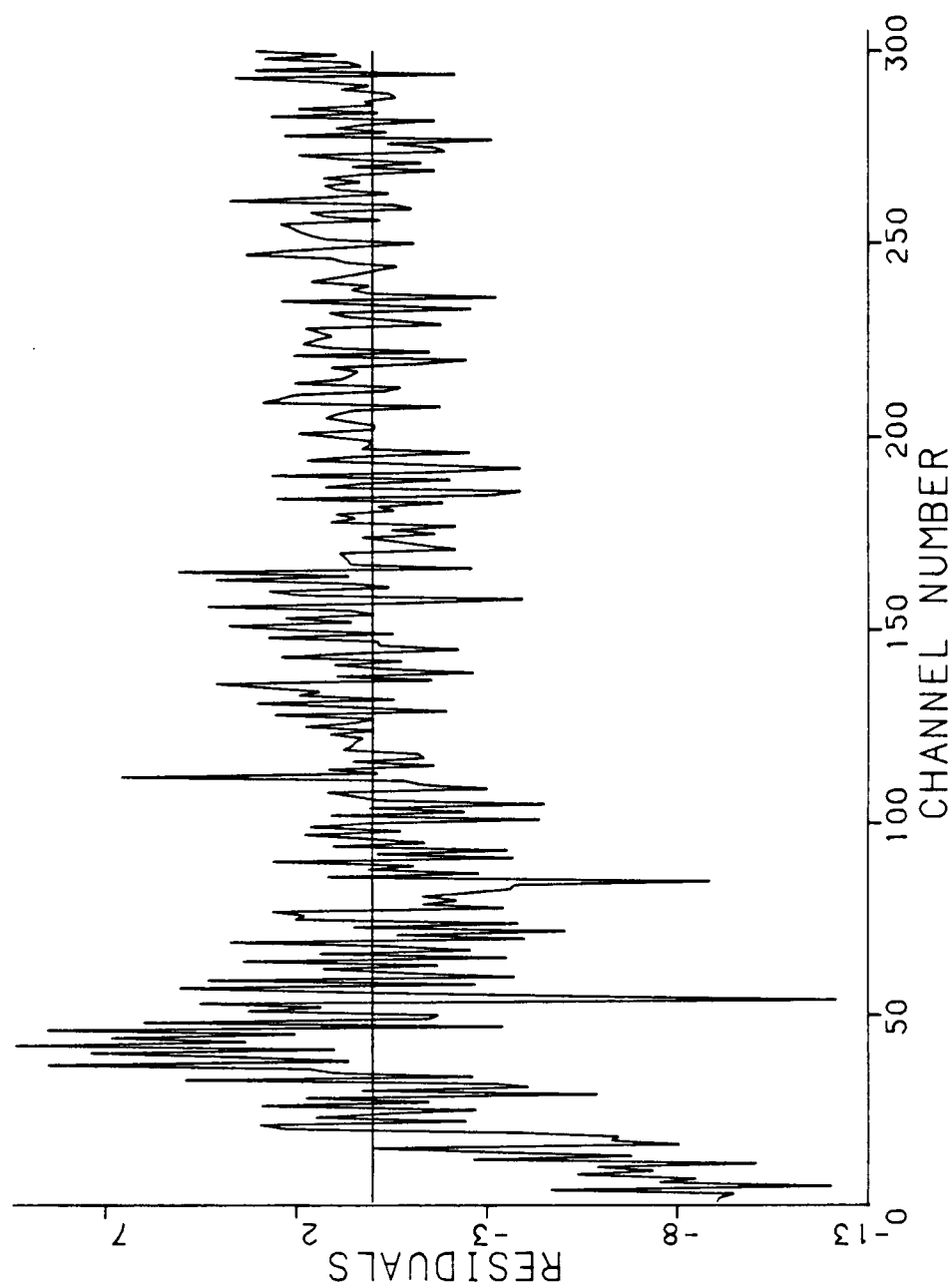


Figure 57. Plot of residuals for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

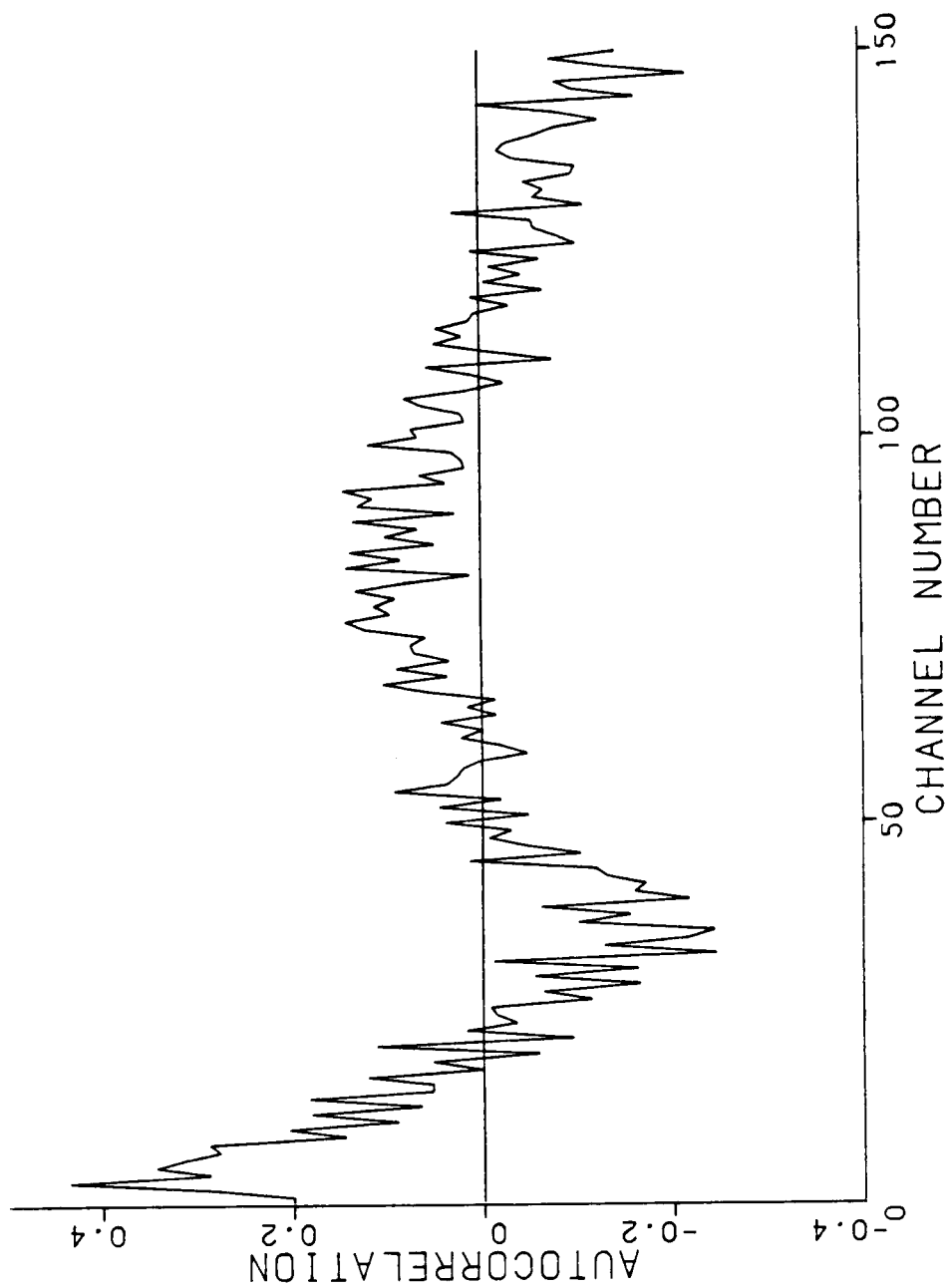


Figure 58. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

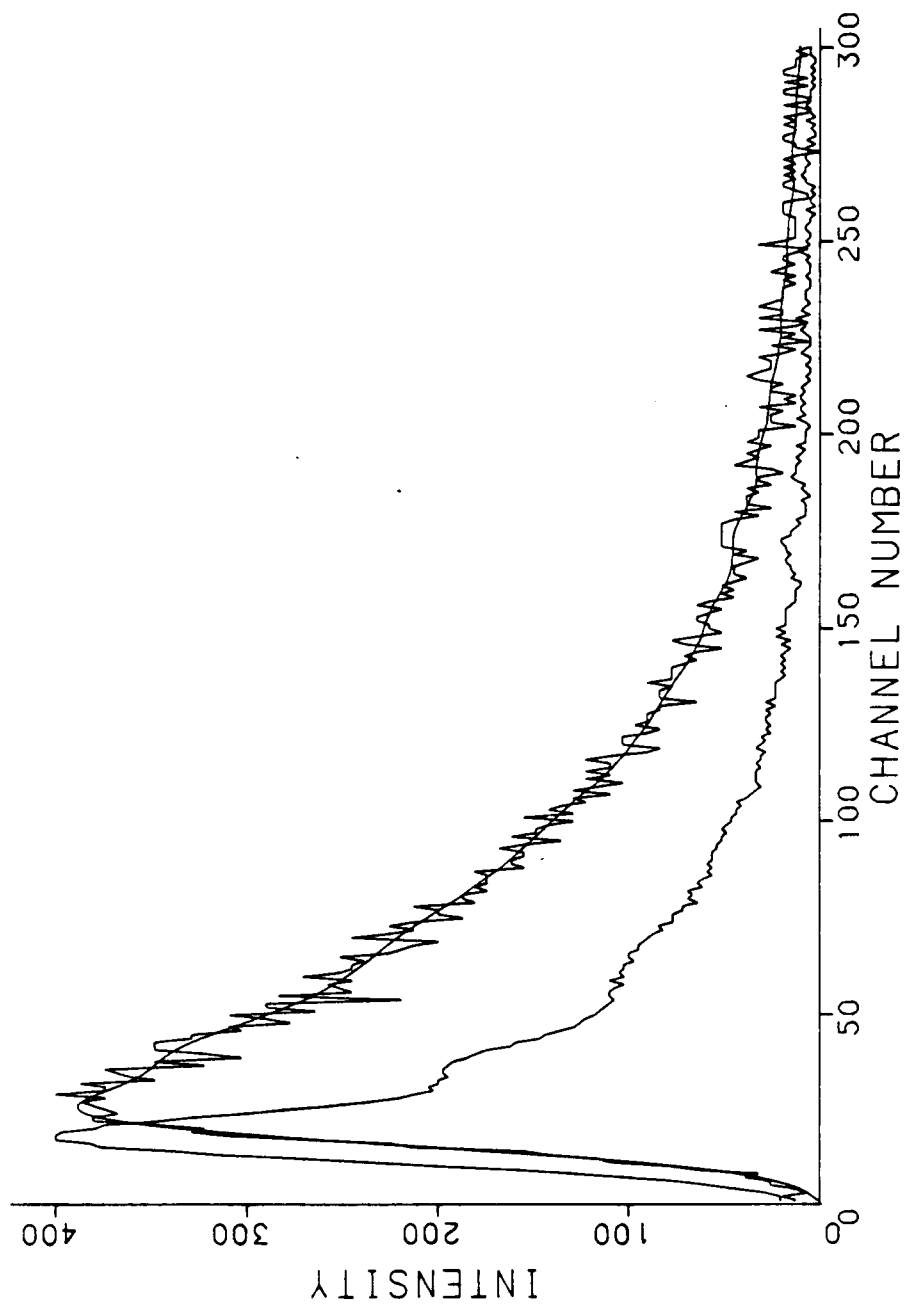


Figure 59. Plot of lamp and $I_V - I_H$ intensities for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

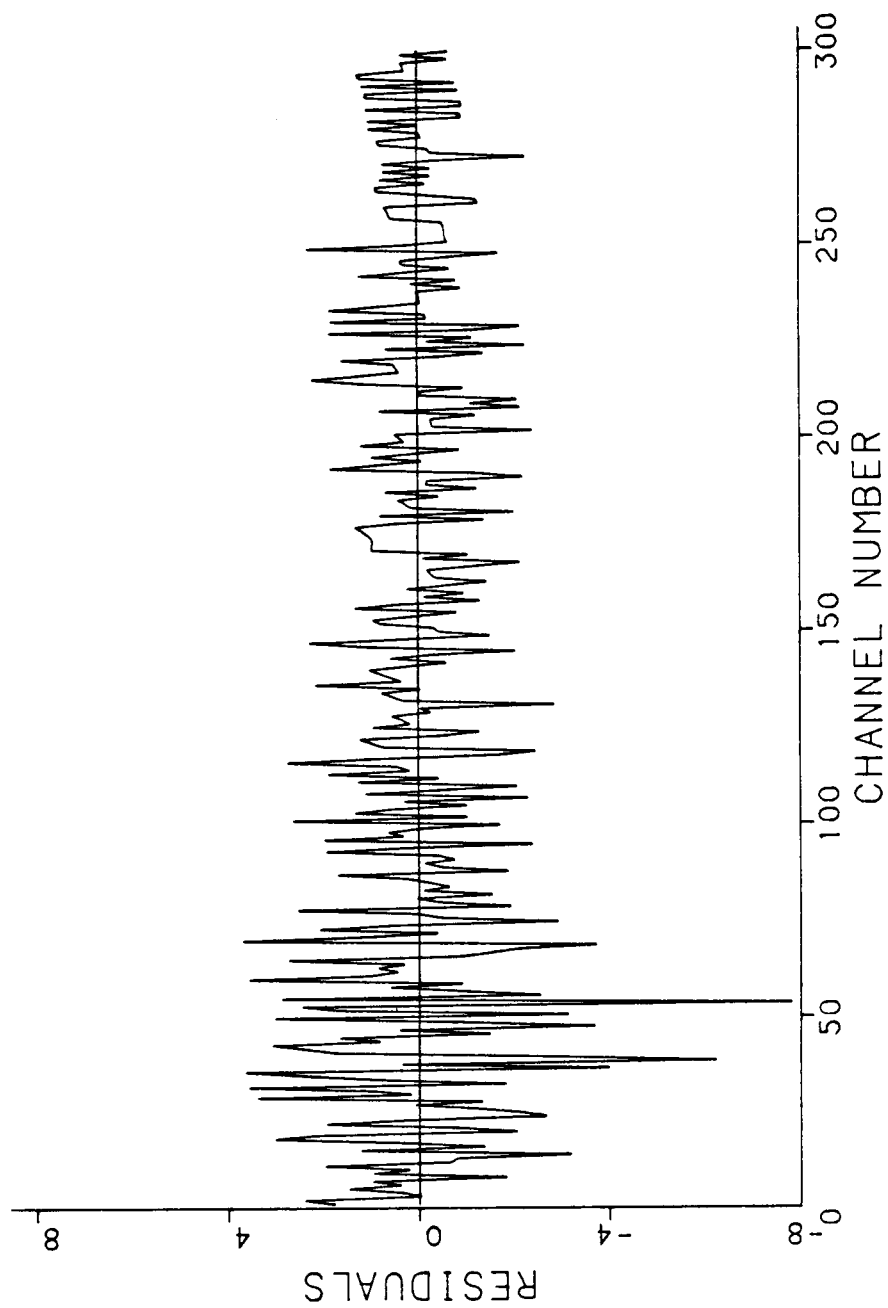


Figure 60. Plot of residuals for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

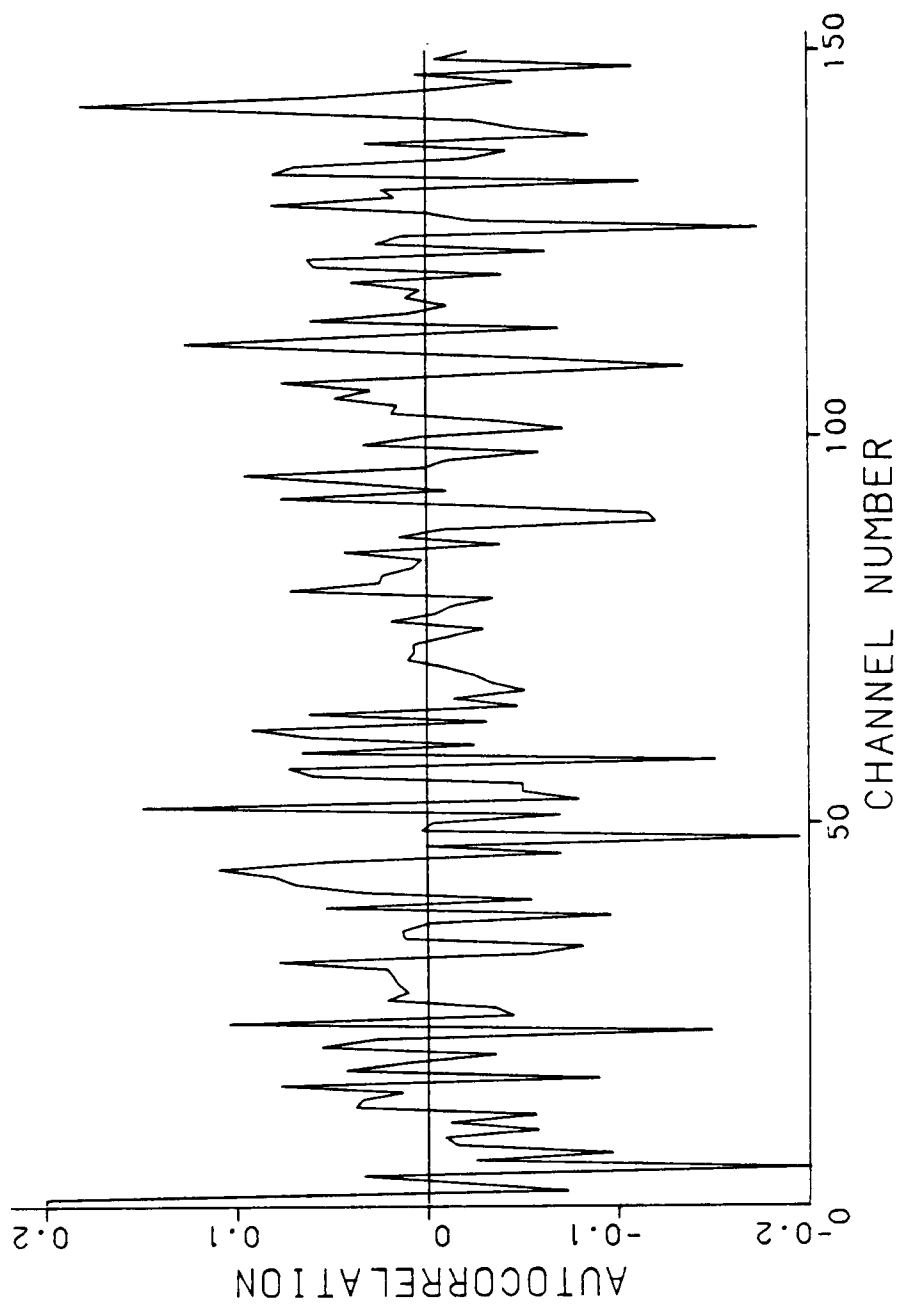


Figure 61. Plot of autocorrelation function for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 33°C in the absence of melittin.

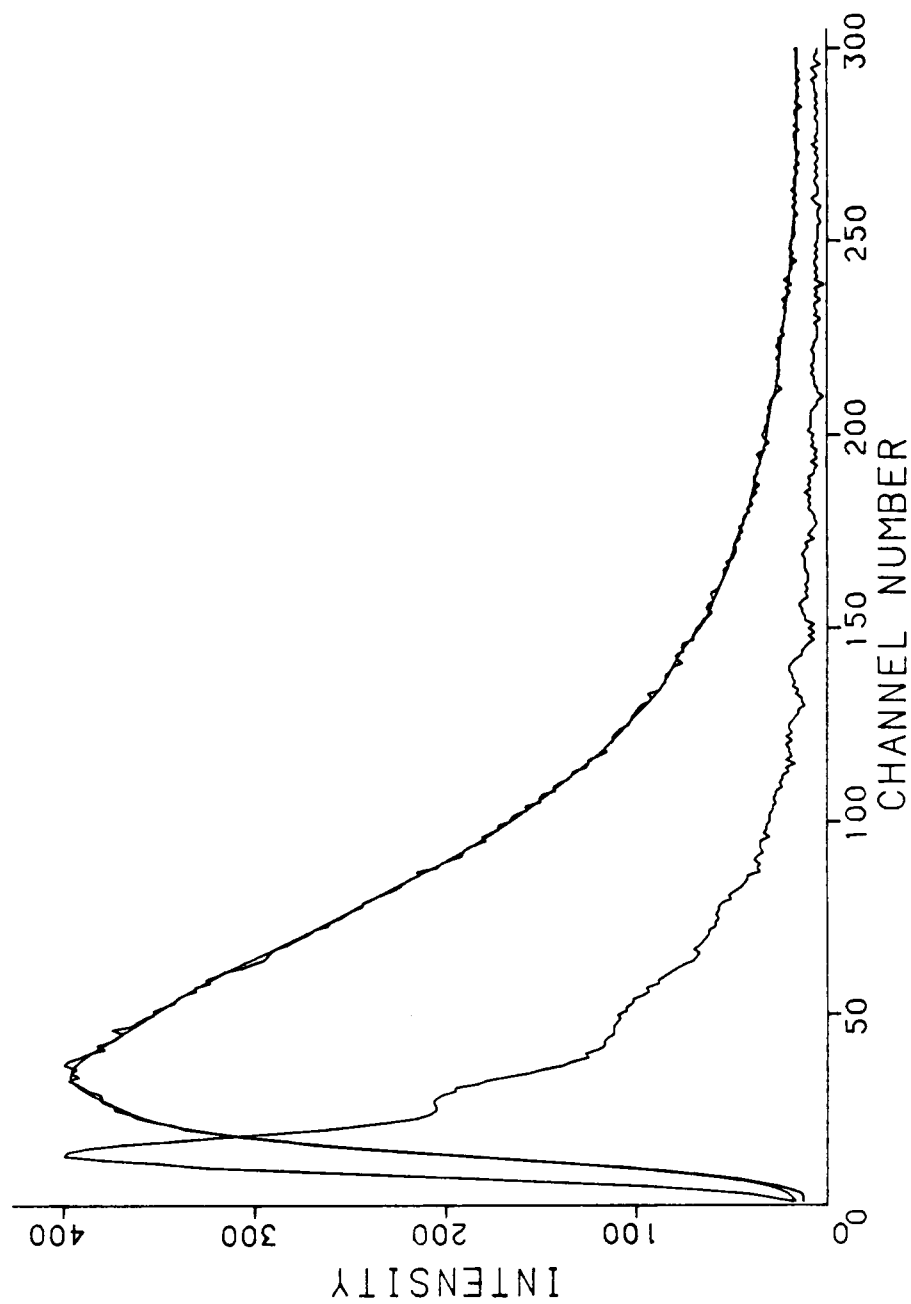


Figure 62. Plot of lamp and total fluorescence intensities for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$. Channel width = 0.259 ns.

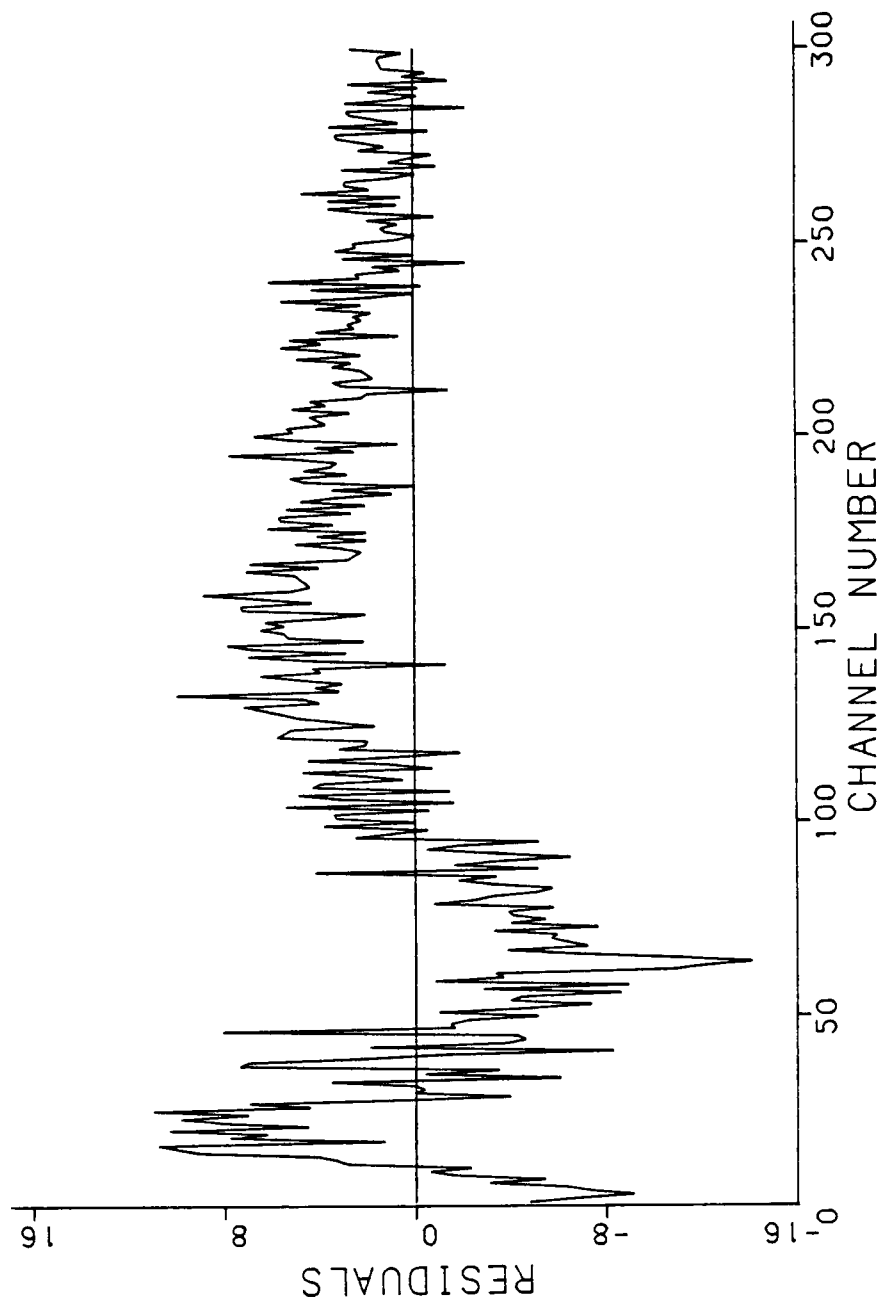


Figure 63. Plot of residuals for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

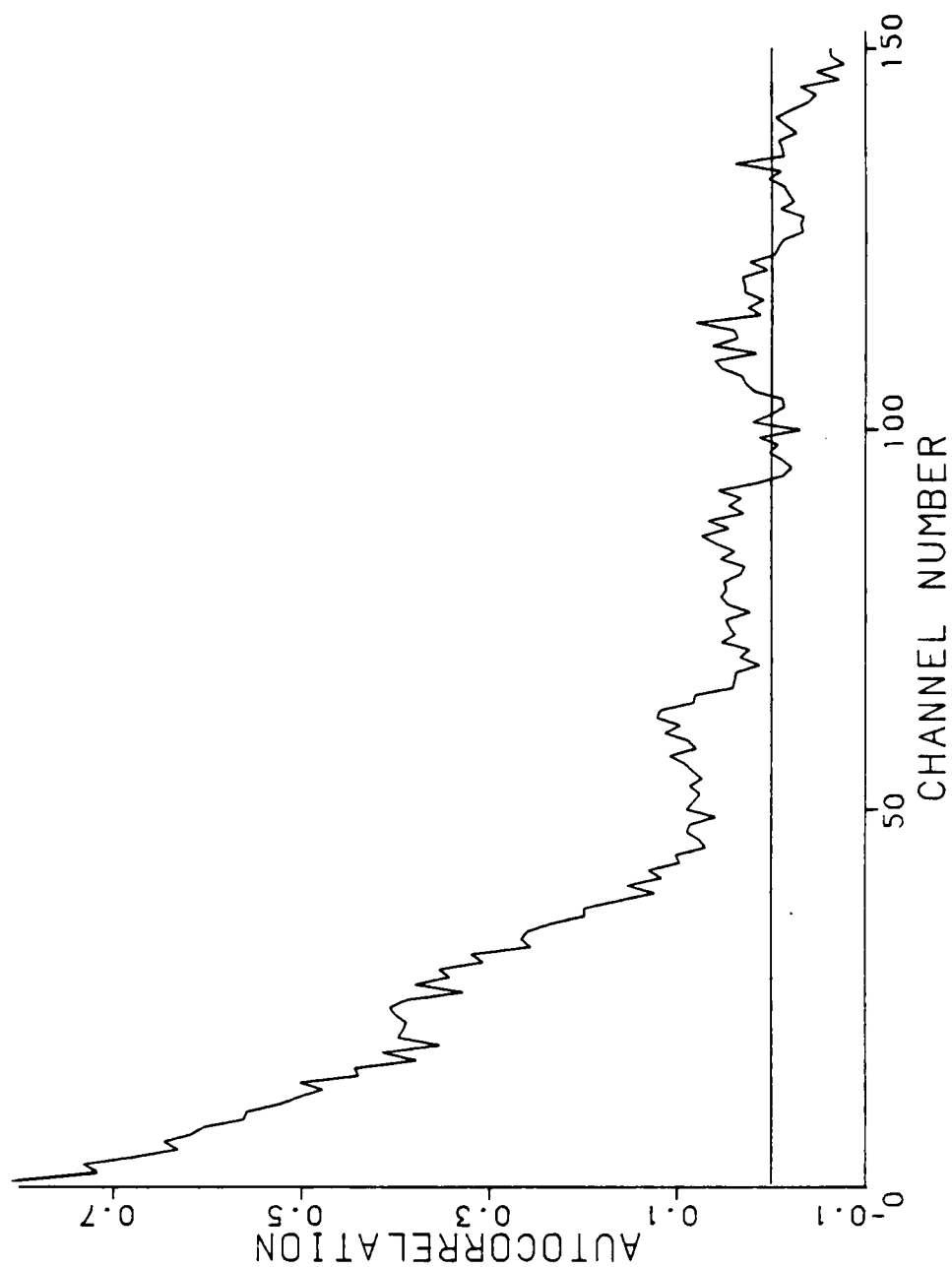


Figure 64. Plot of autocorrelation function for single exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

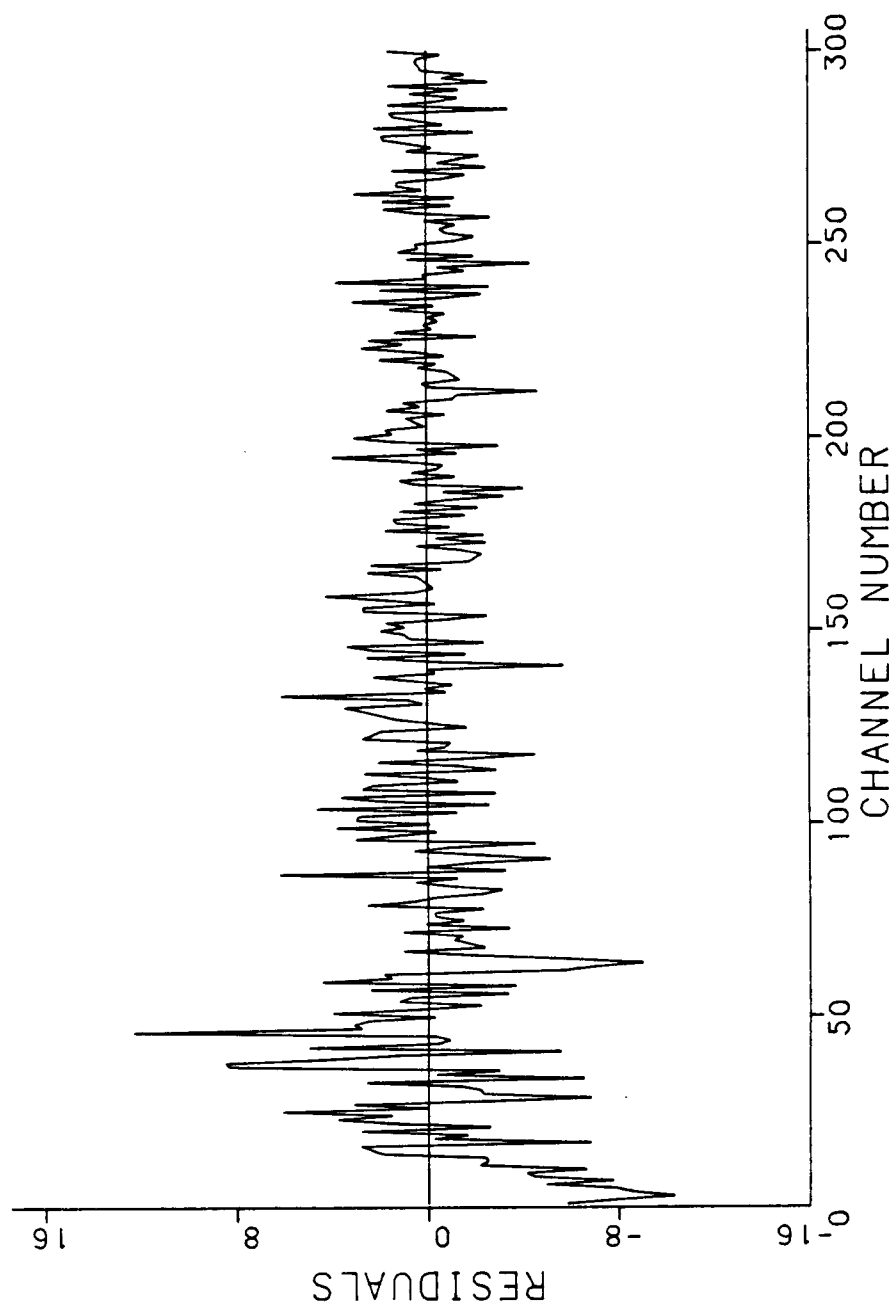


Figure 65. Plot of residuals for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

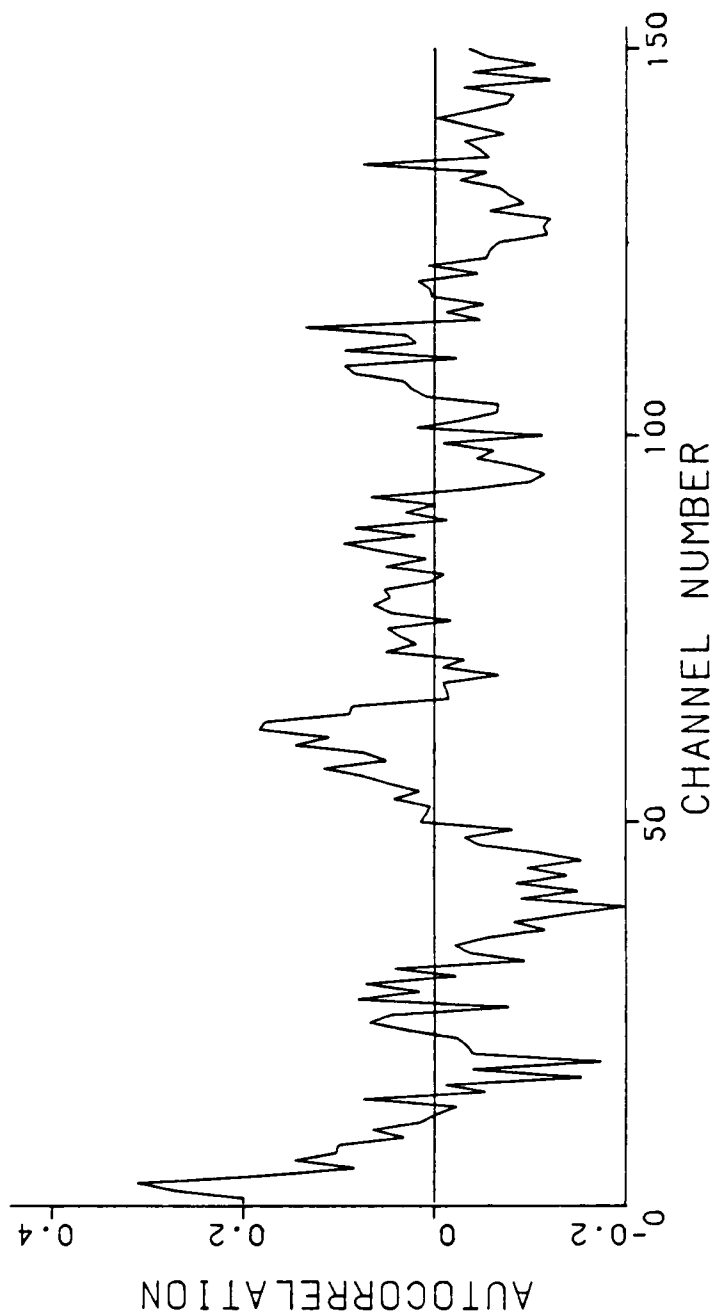


Figure 66. Plot of autocorrelation function for double exponential fit to total fluorescence for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

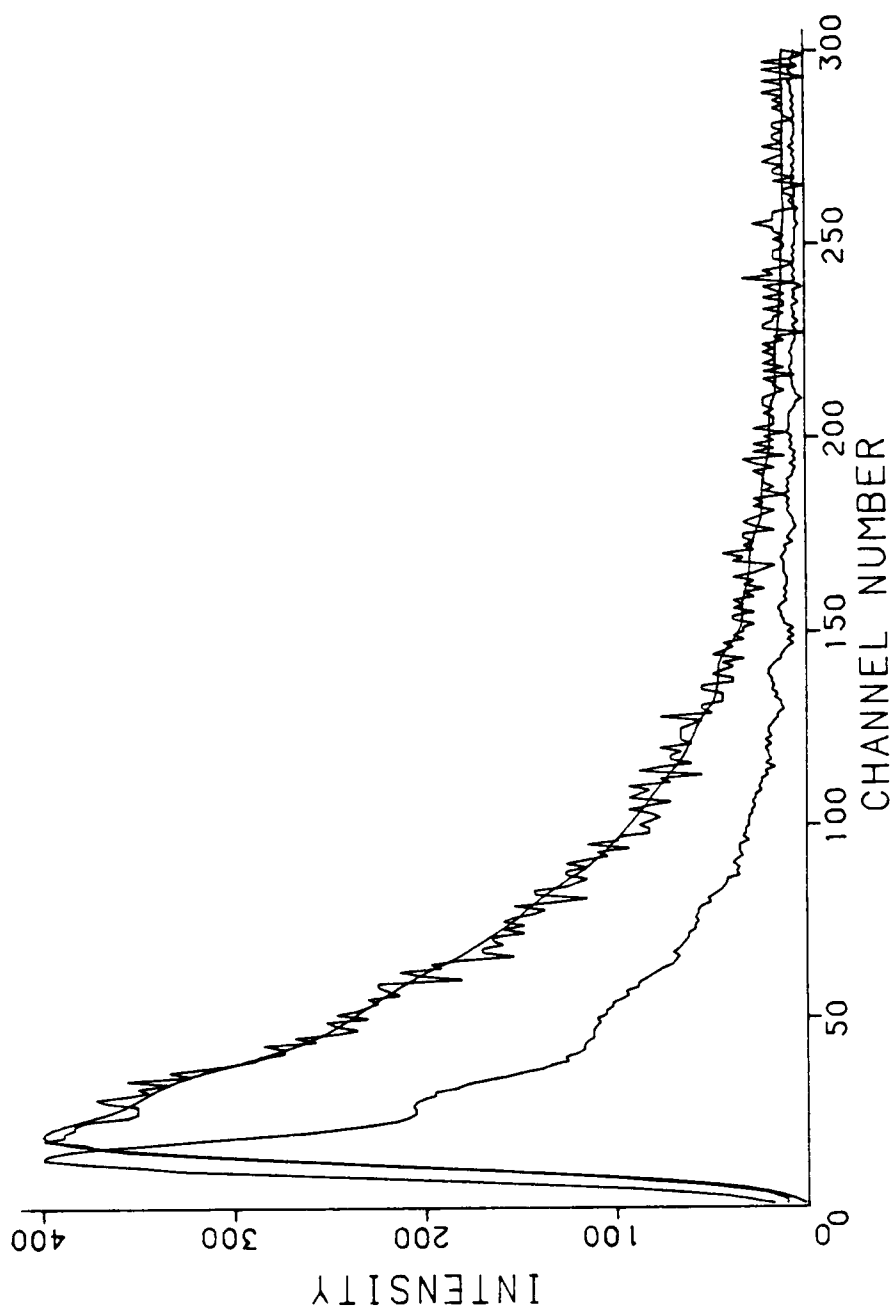


Figure 67. Plot of lamp and I_V-I_H intensities for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

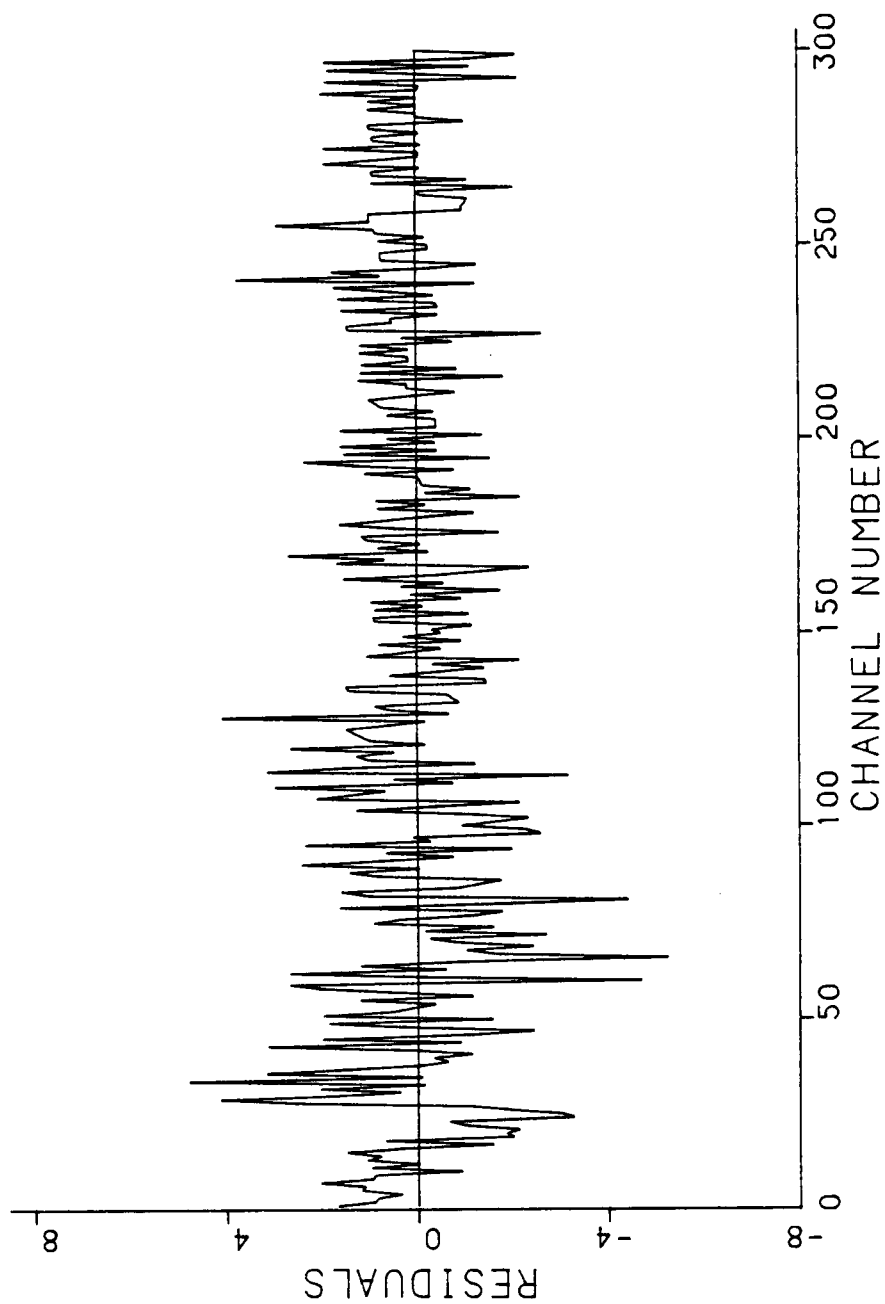


Figure 68. Plot of residuals for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

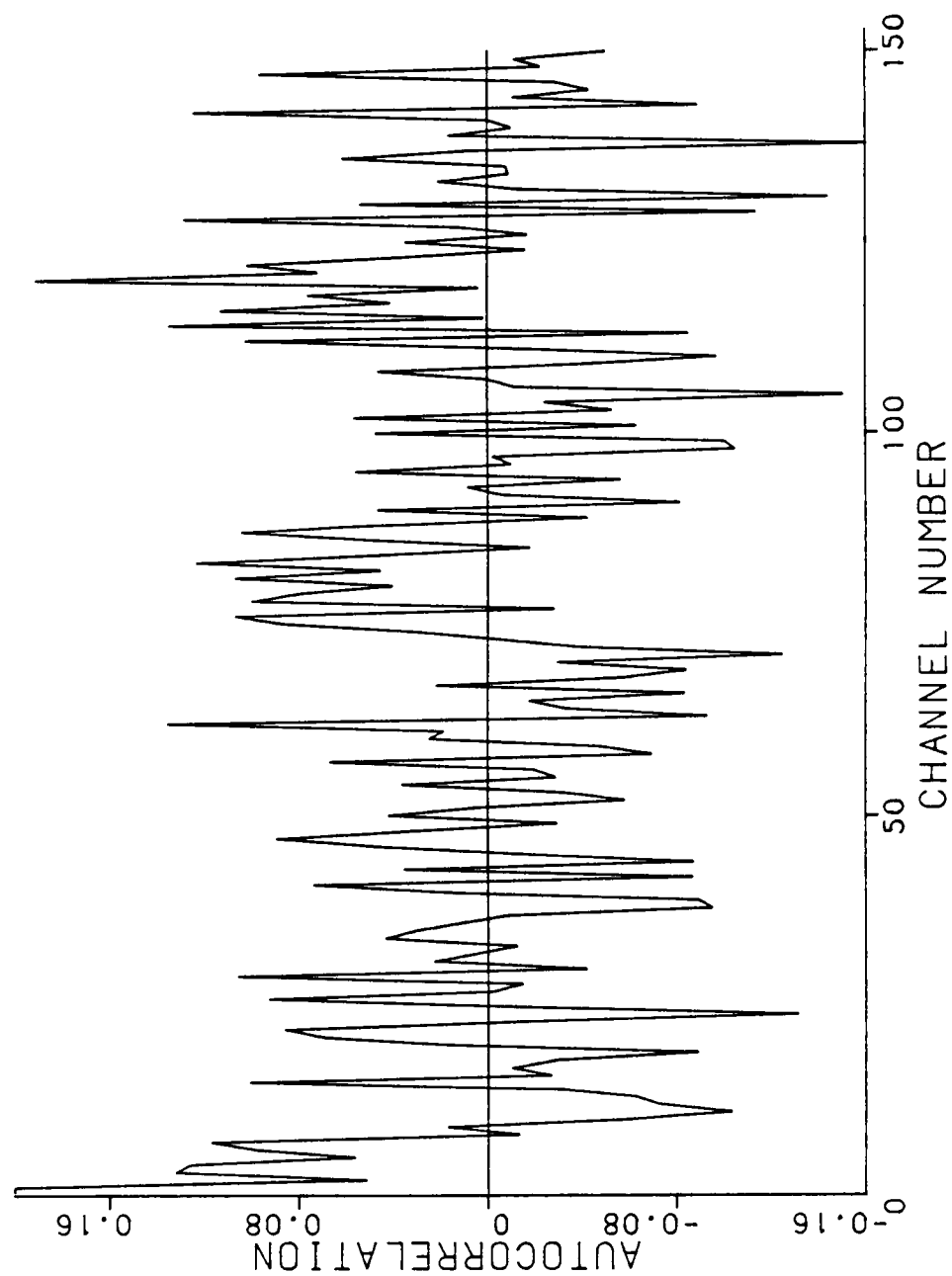


Figure 69. Plot of autocorrelation function for fit to $I_V - I_H$ for DPH in nonsonicated DMPC at 33°C with a melittin to lipid molar ratio of $\sim 1/60$.

fluorescence, lamp profile and fluorescence decay for I_V-I_H , and residuals and autocorrelation for the fit to I_V-I_H . Finally, Figures 70 and 71 show the time-decay of the emission anisotropy, $r(t)$, as given by the best-fit parameters r_0 , r_∞ , and ϕ , for the above listed set of experimental conditions.

It should be noted that for some of the fluorescence intensity vs. channel number graphs the lamp and fluorescence intensities were rescaled in order to obtain computer-generated graphs of the desired size. Also, by definition the first value of the autocorrelation function is unity. This initial value was instead varied from graph to graph, again in order to obtain the desired scaling on the computer-generated graphs. These were the only changes made in graphing the data, however.

The enhancement of the weakly allowed 0-0 vibronic transition in the fluorescence spectrum of pyrene was also used to study the effect of melittin on phospholipid membranes. The enhancement ratio R , defined to be the ratio of the 0-0 vibronic band intensity at ~ 376 nm to that of the band at ~ 387 nm, was measured in DMPC unilamellar and multilamellar liposomes, in the absence of melittin and in the presence of melittin at a protein to lipid molar ratio of $\sim 1/60$. Figures 72 through 75 show representative pyrene spectra in liposomes under various experimental conditions.

Previous work from this laboratory (Lianos and Georghiou, 1979a and Lianos and Georghiou, 1979b) showed that the enhancement of the 0-0 vibronic transition in the fluorescence spectrum of pyrene in polar

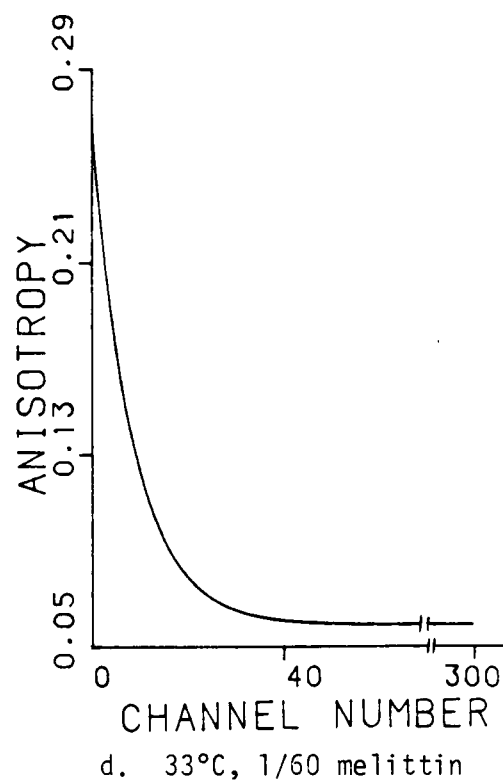
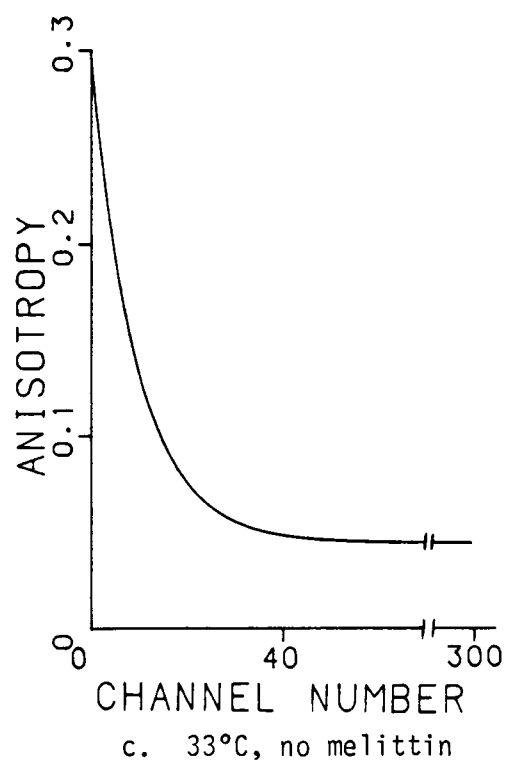
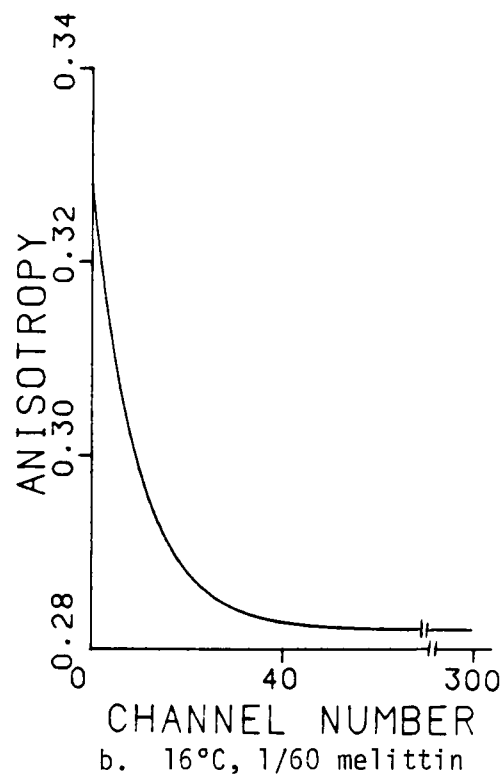
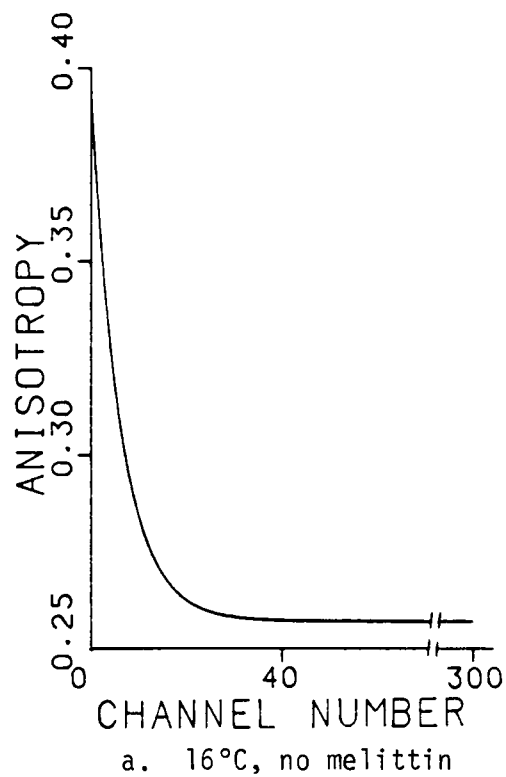


Figure 70. Plots of anisotropy decays for sonicated samples.

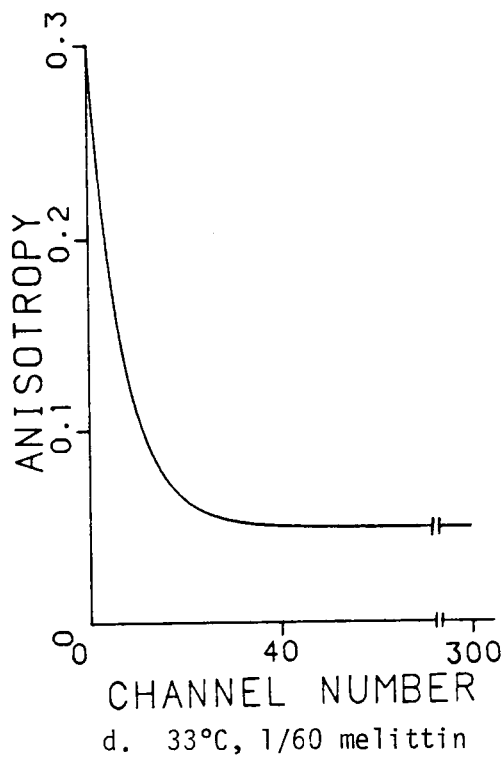
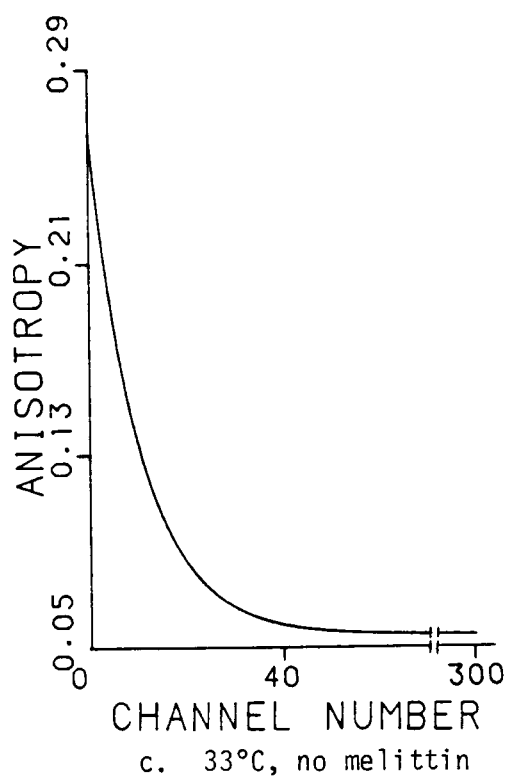
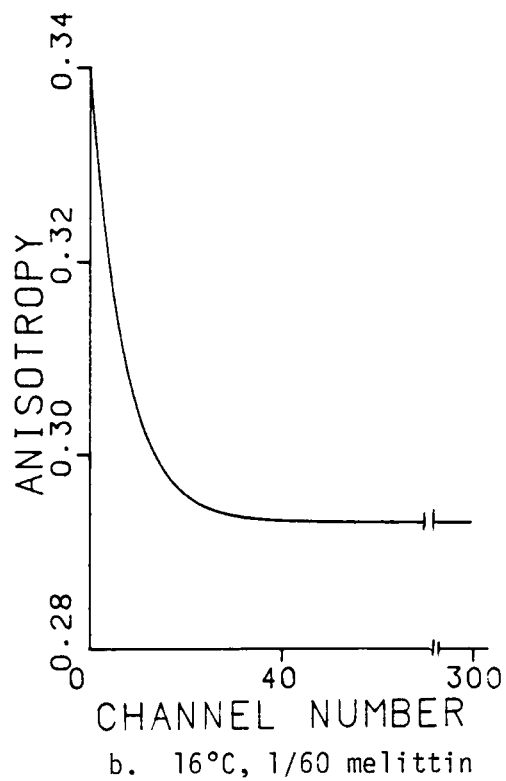
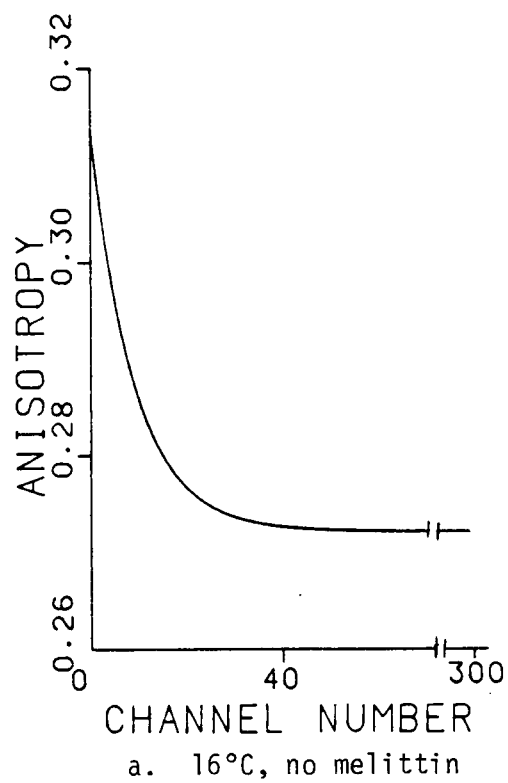


Figure 71. Plots of anisotropy decays for nonsonicated samples.

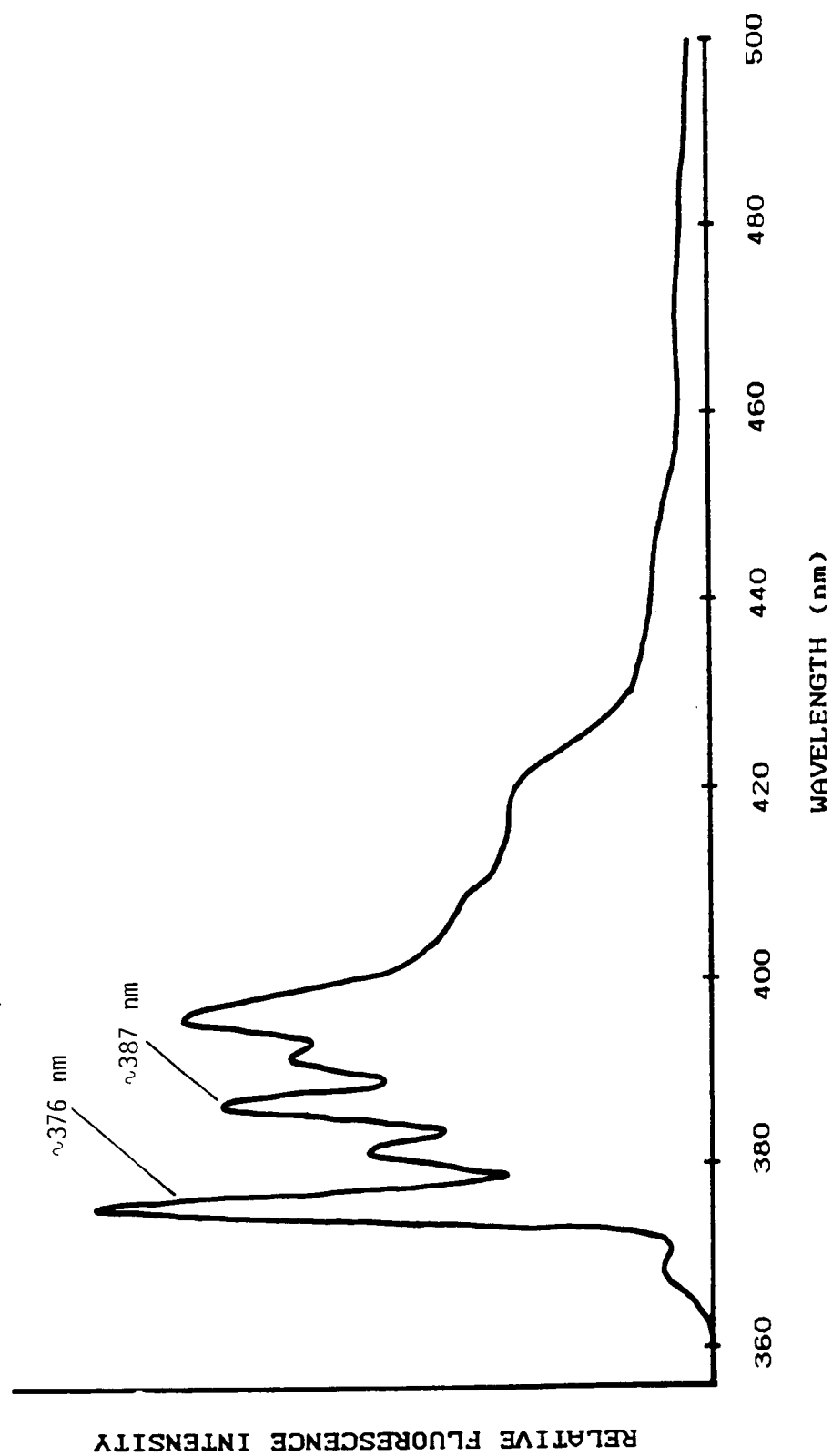


Figure 72. Fluorescence spectrum of pyrene embedded in sonicated DMPC liposomes at 16°C in the absence of melittin.

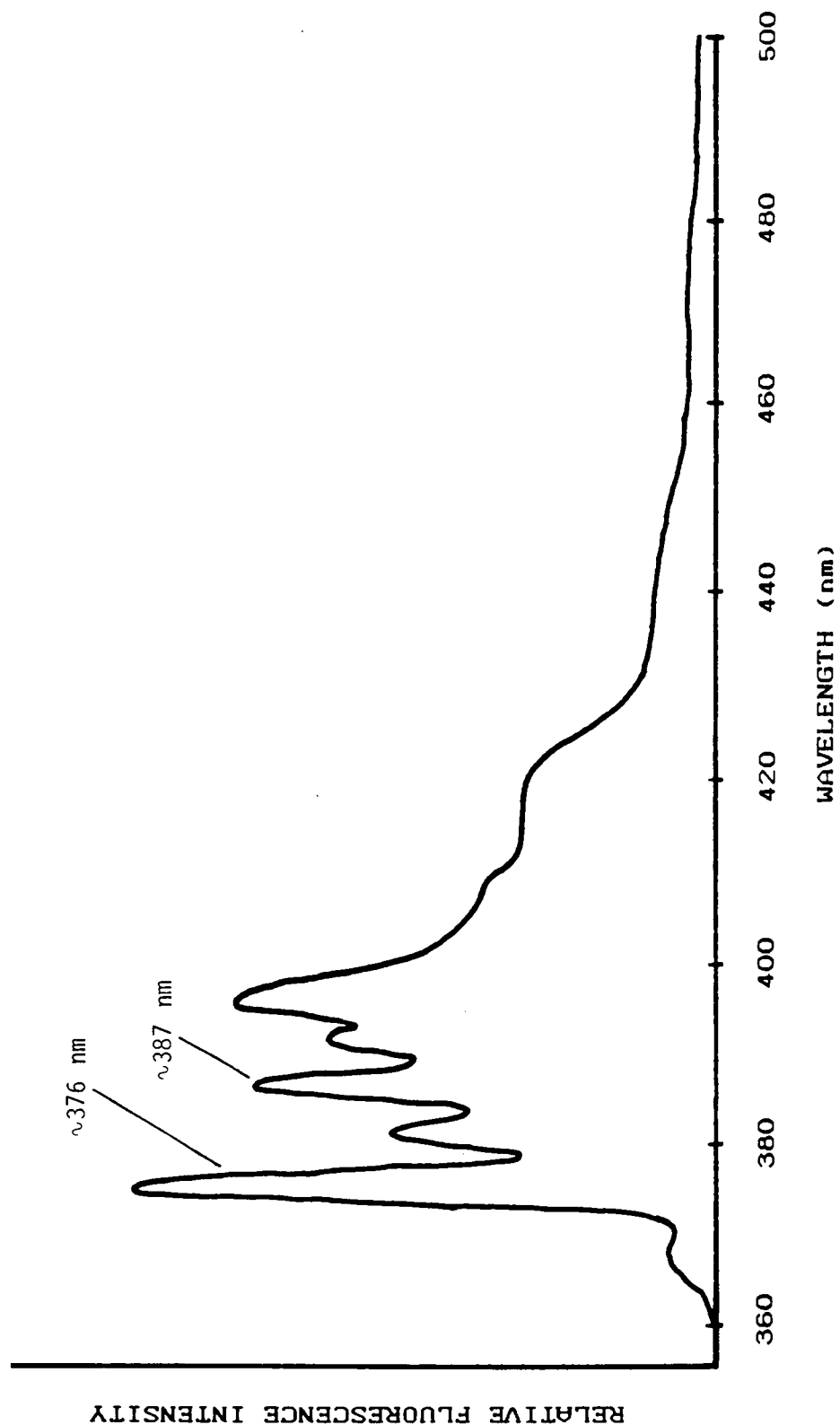


Figure 73. Fluorescence spectrum of pyrene embedded in sonicated DMPC liposomes at 16°C with melittin in a protein to lipid molar ratio of ~1/60.

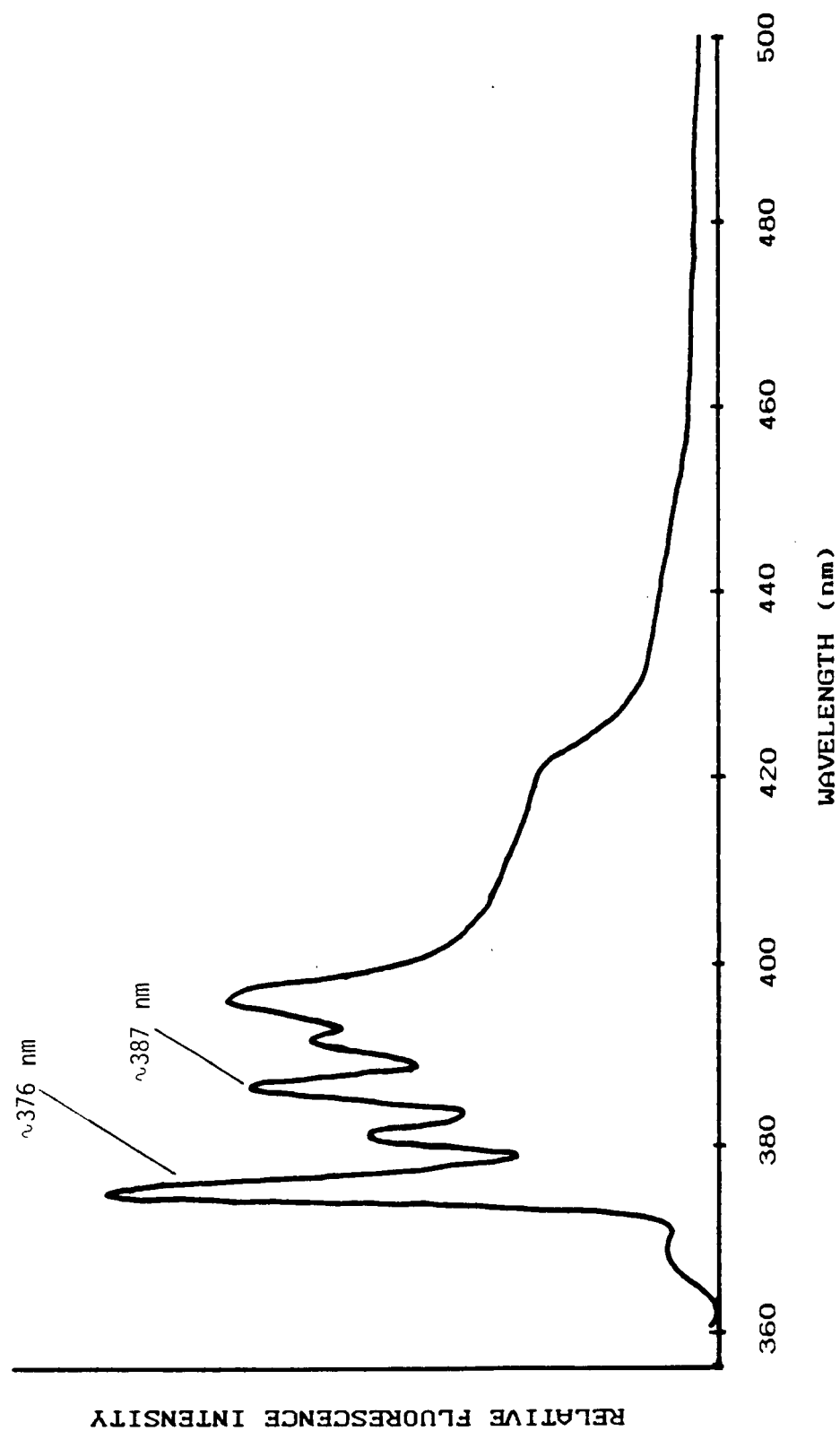


Figure 74. Fluorescence spectrum of pyrene embedded in nonsonicated DMPC liposomes at 16°C in the absence of melittin.

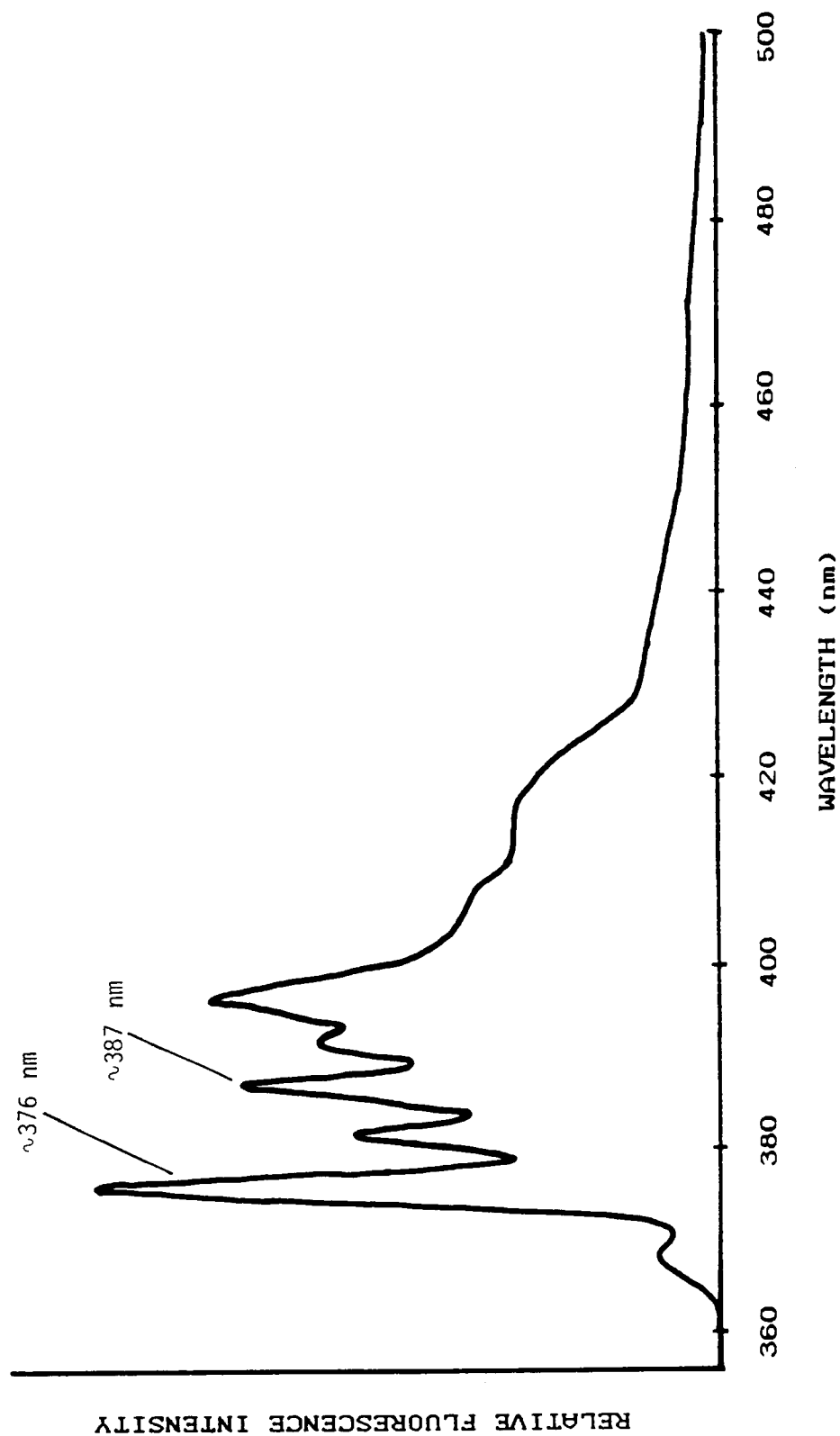


Figure 75. Fluorescence spectrum of pyrene embedded in nonsonicated DMPC liposomes at 16°C with melittin in a protein to lipid molar ratio of $\sim 1/60$.

solvents (Nakajima, 1971) is the result of ground-state molecular complex formation between pyrene and polar groups which reduces the symmetry of the molecule and renders the transition more allowed. The enhancement ratio R is then a measure of the effective polarity of the microenvironment of the fluorophore. Lianos et al. (1980) attributed the high values of R , when using pyrene as a fluorescent probe of egg phosphatidylcholine and dipalmitoylphosphatidylcholine vesicles (matching those in butanol and ethanol, respectively, at room temperature), to interaction with polar groups of the glycerol backbone. Above the transition temperature of the latter lipid the R value decreased, matching that in ether, implying that pyrene was penetrating to some extent further into the bilayer. They considered and then rejected the possibility of attributing the observed high values of R to small amounts of water that may be penetrating into the bilayer as it was found that in butanol-heptane mixtures it takes at least 1 M of butanol to induce a barely discernible enhancement of the 0-0 band. Also, it takes ~ 8 M of butanol (Georghiou and Mukhopadhyay, 1981) to increase R from about 0.6 in heptane to 1. (The lowest value of R in DPPC vesicles was about 1.1.) That the fluorescent probe is located in the vicinity of the glycerol backbone is also supported by its reported (Charlton et al., 1976) fast transfer between lipids (in ~ 3 ns). The enhancement phenomenon was employed (Georghiou and Mukhopadhyay, 1981) for studying the effects of cholesterol on the structure and on the fluidity of model membranes and in determining their phase transition temperature. In the present study the

enhancement ratio R was measured as a function of temperature from 2°C to 36°C for sonicated samples and from 5°C to 32°C for nonsonicated samples. The experiments were repeated once for the case of SUVs and twice for the case of MLVs. The results of the measurements are shown in Figures 76 and 77. Figure 76 shows R as a function of temperature for sonicated samples in the absence (dashed line) and in the presence (solid line) of the protein. Figure 77 shows the same information for nonsonicated samples. Again, the dashed line represents the case of pure lipid while the solid line represents the case of lipid plus protein.

The previous two fluorescence techniques (nanosecond emission anisotropy of DPH and the enhancement of the forbidden 0-0 vibronic transition in pyrene) were used to examine the effects of melittin on the order and fluidity of DMPC liposomes at various temperatures. It has also been reported that melittin induces the fusion of acidic liposomes (Eytan and Almary, 1983) and the large unilamellar vesicles of certain zwitterionic phospholipids (Morgan et al., 1983). Aggregation and/or fusion was also observed in our laboratory for DMPC SUVs through light scattering measurements made by monitoring the apparent absorbance of samples at 450 nm as a function of temperature, using a Cary 14 spectrophotometer (data not included). That such protein-induced fusion would also contribute, along with the binding of the protein to the membrane, to the observed changes in membrane order and fluidity made it desirable to verify that fusion was actually taking place, and if so to what extent. To this end a fusion

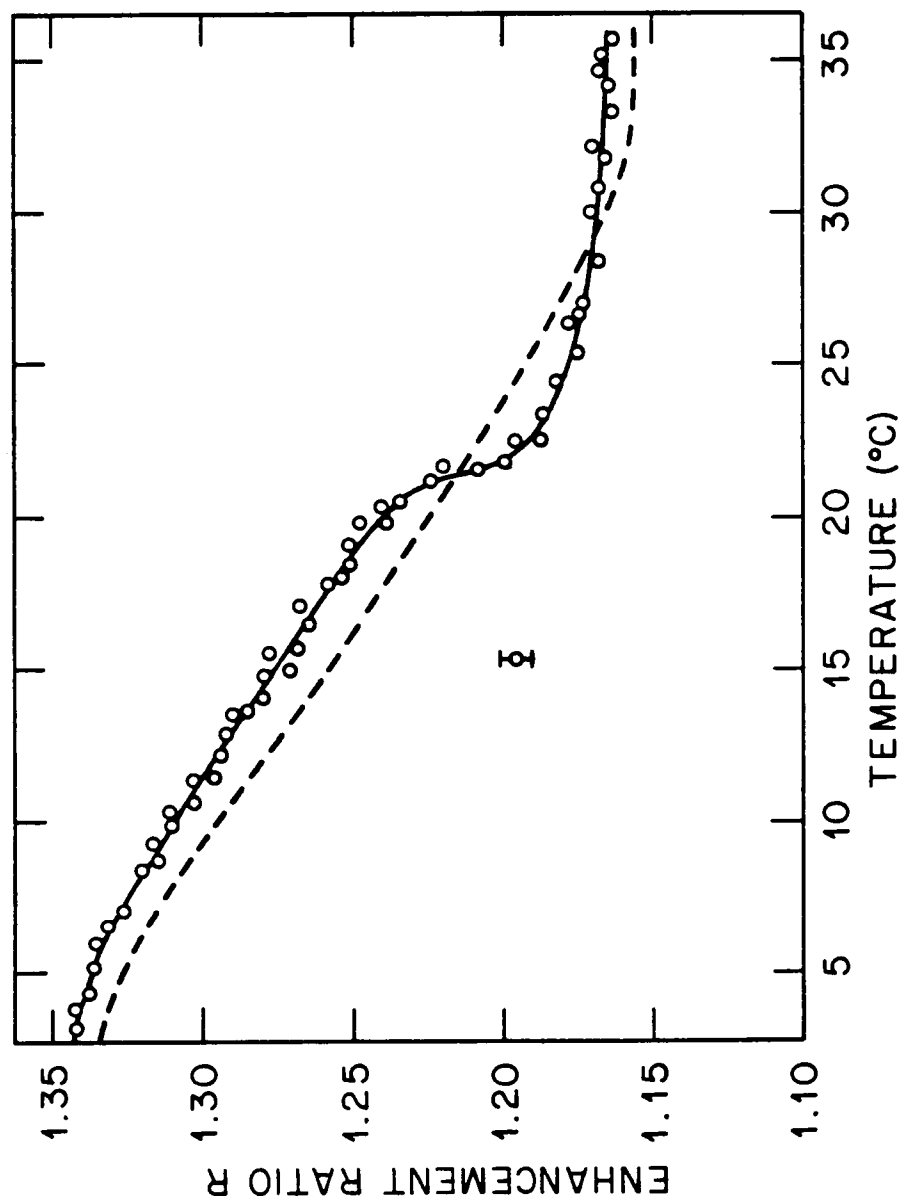


Figure 76. Plot of the enhancement ratio of pyrene vs. temperature for sonicated DMPC liposomes, in the absence of melittin (dashed line) and in the presence of melittin at a protein to lipid molar ratio of 1/60 (solid line).

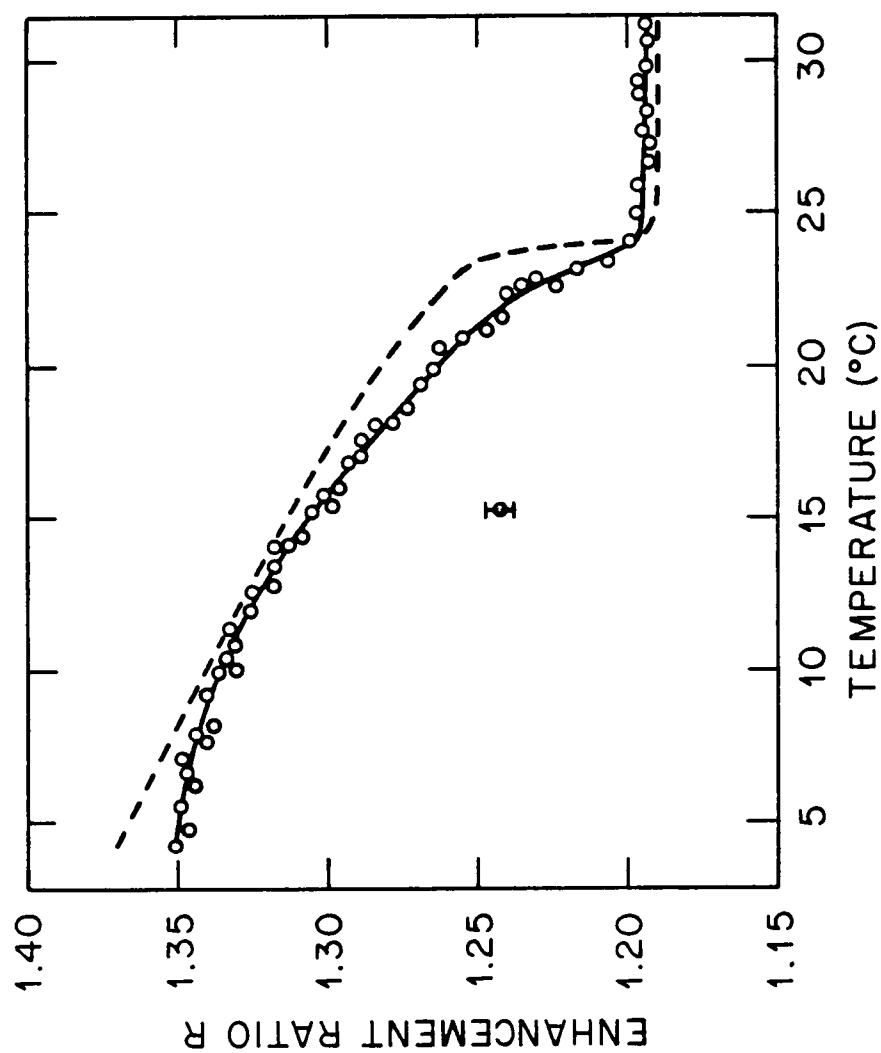


Figure 77. Plot of the enhancement ratio of pyrene vs. temperature for nonsonicated DMPC liposomes, in the absence of melittin (dashed line) and in the presence of melittin at a protein to lipid molar ratio of $\sim 1/60$ (solid line).

assay was employed which measures the extent of the fusion-induced mixing of membrane contents (Gad and Eytan, 1983). The assay works by measuring the change in the efficiency of energy transfer from chlorophyll b molecules to chlorophyll a molecules embedded in the membrane when such a fluorescently-labeled liposome fuses with non-labeled liposomes and the chlorophyll concentration is diluted. Such a dilution will reduce the efficiency of energy transfer. This can be measured by observing the fluorescence intensity at the appropriate wavelengths. Through comparison of the efficiency with the energy transfer efficiencies of known chlorophyll-in-lipid concentrations the amount of chlorophyll dilution can be determined and the number of liposomes fusing together can be calculated. In performing the assay one part chlorophyll labeled liposomes is mixed with ten parts nonlabeled liposomes. This ensures that if fusion takes place a labeled liposome will almost certainly fuse with a nonlabeled one. (The fusion of two labeled liposomes could not be detected as such an event would not result in a dilution of the chlorophylls, and would be without the desired reduction in energy transfer efficiency.) As previously stated, the chlorophyll-in-membrane concentration, both before and after fusion, is determined through comparison of the respective energy transfer efficiencies with those of known concentrations.

The samples were excited at 468 nm and the fluorescence intensity was measured at 658 nm, 677 nm and 700 nm, at a resolution of 3.3 nm. The efficiency of energy transfer was calculated as

$$\text{Energy transfer efficiency} = \frac{I_{677} - I_{700}}{I_{658} - I_{700}} .$$

Figure 78 shows a plot of the energy transfer efficiencies for the four calibration concentrations used (2.4%, 1.9%, 1.2% and 0.48% chlorophyll in lipid by weight).

Labeled and nonlabeled liposomes were mixed, as described above, and the efficiency of energy transfer was measured both before and after incubation of the sample with melittin at 33°C. (The protein to lipid molar ratio was ~1/60.) The chlorophyll concentrations, before and after fusion, were determined using the calibration curve and the extent of fusion was calculated as (Gad and Eytan, 1983)

$$\% \text{ fusion} = \left(\frac{C_0}{C_t} - 1 \right) \cdot \frac{100}{D-1}$$

where C_0 is the initial chlorophyll concentration (before fusion), C_t is the chlorophyll concentration in the fused liposomes and D is the maximum possible dilution, which depends on the ratio of nonpigmented to pigmented liposomes. (D was always eleven in the experiments reported here.)

The calculated percentage gives the amount of fusion as a fraction of the maximum possible which could take place. For example, if no fusion occurred, $C_t = C_0$ and the % fusion = 0. If one pigmented liposome fused with ten nonpigmented liposomes (the maximum possible), $C_t = \frac{1}{11} C_0$ and the % fusion = 100%. Fusion of one pigmented with five

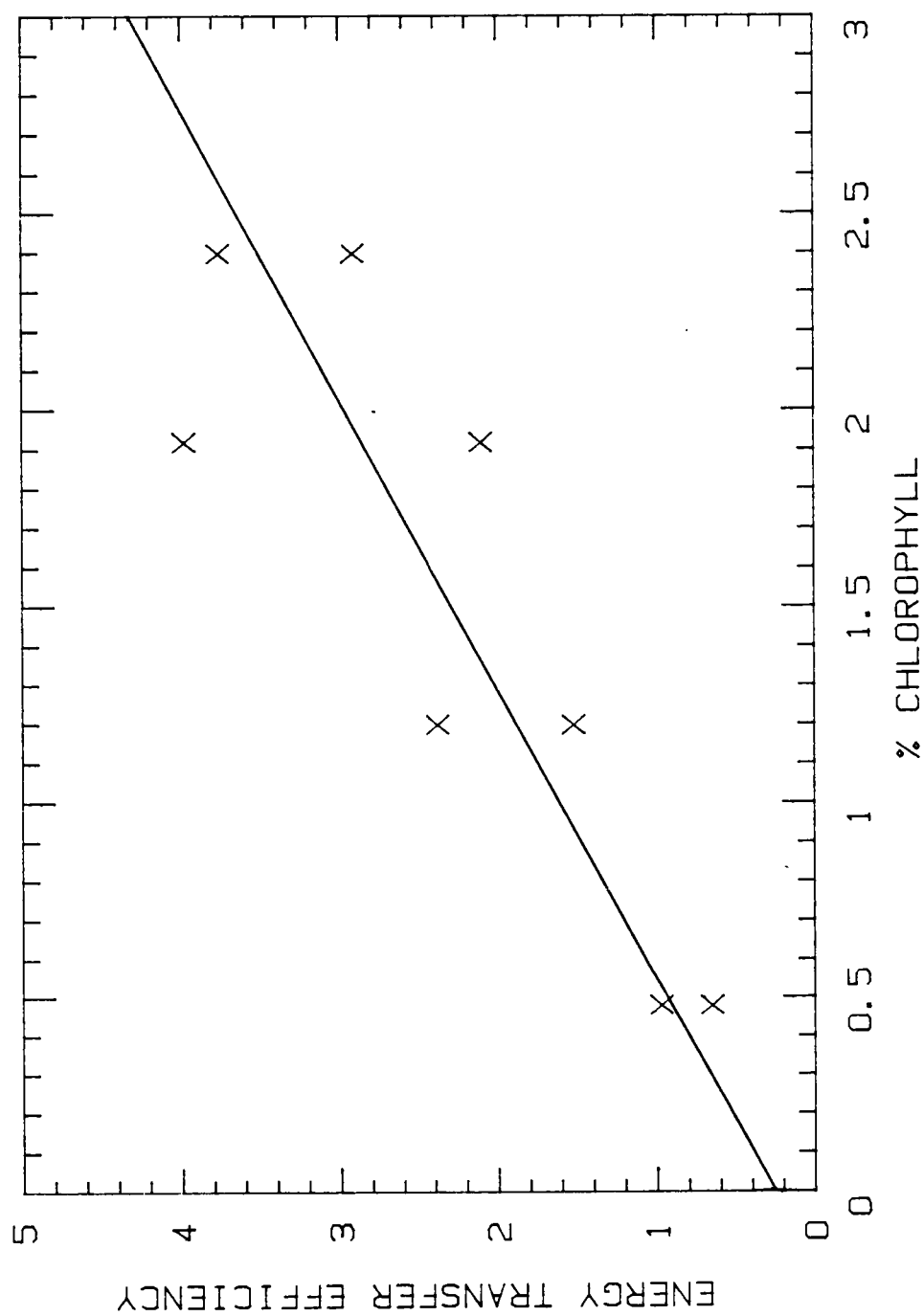


Figure 78. Calibration curve for the fusion assay.

nonpigmented liposomes ($C_t = \frac{1}{6} C_0$) would yield 50% fusion. (These examples assume ten parts nonlabeled and one part labeled liposomes mixed together, for which D is eleven.)

In the case of our own system, it was found that on the average melittin caused the fusion of approximately three liposomes in the case of unilamellar vesicles. Within the limits of detection for these less concentrated samples, no melittin induced fusion was observed for the multilamellar vesicles. The same measurements, made using a different fusion assay being developed in this laboratory (Bradrick and Georghiou, to be published) gave the same result in the case of DMPC unilamellar vesicles.

CHAPTER IV

DISCUSSION

The purpose of this study is to further elucidate the nature of the interaction of melittin with phospholipid membranes. It is known that at high concentrations melittin lyses model and natural biological membranes (Weissmann et al., 1969; Sessa et al., 1969; Olson et al., 1974), while at lower concentrations it causes structural changes in the liposomes and prepares the membrane for attack by the enzyme phospholipase A_2 (Habermann, 1972; Vogt et al., 1970; Mollay and Kreil, 1974; Mollay et al., 1976; Yunes et al., 1977). Previous work from this laboratory which examined the fluorescence of the protein's single tryptophan residue, implied that melittin aggregates when it binds to membranes and that the tryptophan residue is not buried very deeply in the bilayer (Georghiou et al., 1982a and Georghiou et al., 1982b). Questions remain, however, concerning the form and manner in which melittin binds to membranes, as well as the nature of the effect it has on membrane structure and dynamics.

For this study multilamellar vesicles were included because we wished to correlate our nanosecond emission anisotropy and pyrene 0-0 vibronic enhancement results with the results of Raman spectroscopic studies being carried out in our laboratory.

Before discussing the results obtained from the various experimental techniques used in this particular study, it should be

pointed out that some care must be exercised in interpreting the results presented here as they pertain to unilamellar vesicles.

As was previously stated, it has been shown that melittin induces fusion in sonicated acidic vesicles (Eytan and Almary, 1983) and in large unilamellar vesicles of certain zwitterionic phospholipids (Morgan et al., 1983). Similarly, DMPC SUVs will fuse when their temperature is taken below about 12°C. This type of behavior has been seen in DPPC SUVs (Suurkuusk et al., 1976), and the results of a differential scanning calorimetric study (carried out by us in collaboration with Dr. Ernesto Freire of the Department of Biochemistry) verified it in our system as well. The experiments, done to examine the binding of melittin to phospholipid membranes, were initially performed on DMPC SUVs prepared as described above. The results showed that for scans begun, of necessity, far below T_m (i.e., at 7°C) two separate peaks, one quite broad and centered at ~18°C, the other quite narrow and centered at 24°C (the transition temperature for DMPC MLVs), were found to exist. Repeated scans made on the same sample showed that with each heating and cooling cycle the low temperature peak decreased in height and the high temperature peak increased, indicating that an increasing amount of temperature-induced fusion was taking place with each new scan. Such behavior is judged not to affect the nanosecond emission anisotropy measurements, as there the lowest temperature examined was 16°C. For the pyrene 0-0 vibronic enhancement measurements, the sample temperature was taken down to

$\sim 4^{\circ}\text{C}$, however, so that temperature-induced vesicle fusion has taken place over the course of that type of measurement.

Finally, as has been pointed out (Dasseux et al., 1984) care must be taken in comparing the results of studies of lipid-protein interactions carried out using different experimental techniques (e.g., fluorescence depolarization and anisotropy, Raman spectroscopy, ESR, etc.). As was stated above, melittin has the ability to self-associate and form tetramers in solutions. As would be expected, the equilibrium rates of association and dissociation for this process depend on the absolute concentration of the melittin in solution. Also, the rates of association and dissociation for the binding of melittin to phospholipid membranes depend on the melittin concentration (Faucon et al., 1979a and Faucon et al., 1979b), so that the effect which the protein has on a membrane will depend on the form the protein (and the liposome) has when it binds and the degree to which it binds, both of which are concentration dependent. Thus, for experimental techniques which require quite different concentrations of lipid (e.g., Raman spectroscopy and fluorescence spectroscopy, for which sample concentrations can differ by a factor of 10^2 to 10^3) use of the same protein to lipid molar ratio will not necessarily allow one to compare the results obtained using the different techniques.

In examining the results of the experiments described herein it is more convenient to order the discussion in terms of the different melittin-lipid systems (unilamellar and multilamellar vesicles) than to arrange the discussion in order of the different experimental

techniques employed (nanosecond emission anisotropy, the enhancement of the 0-0 vibronic transition in pyrene, and the chlorophyll-based fusion assay.

In general, the effect which melittin has on lipid vesicles depends on the state of the liposomes, i.e., whether they are above or below their transition temperature, on the type of lipid molecule (acyl chain length and net charge), and on whether the liposomes have unilamellar or multilamellar structures (Dasseux et al., 1984). From the work presented here it is evident that for DMPC unilamellar liposomes above T_m , melittin induces vesicle fusion. From the data in Table II (p. 27) it can be seen that the resulting structures have more order than DMPC SUVs at the same temperature in the absence of the protein. The order is even greater than that of multilamellar vesicles under the same conditions, although the rates of diffusion are the same for all the systems at 33°C. This would suggest that the structures formed at 33°C under melittin-induced fusion are different from those of MLVs. The presence of melittin in SUVs below T_m still causes an increase in the order parameter and a decrease in the rate of the probe's diffusion. In the absence of the protein the order parameter is somewhat lower than that for MLVs under the same conditions (Table II). Comparison of the order parameters for SUVs at 33°C and 16°C in the absence of melittin with the order parameters for MLVs at those temperatures (cf. Table II) suggests that the sample is composed of liposomes having acyl chain structure similar to that of MLVs. Results of the pyrene 0-0 vibronic enhancement studies are at

odds with this interpretation, however. In both cases (with and without protein) it was found that at all temperatures studied, pyrene in "unilamellar" vesicles gives a lower enhancement ratio R than when it is in multilamellar vesicles (cf. Figures 76 and 77, pp. 101 and 102). This means that pyrene is allowed to penetrate further into the nonpolar region of the bilayer in SUVs than in MLVs, and that the order and fluidity of the former are still different from those of the latter, even below T_m . The observed increase in order at 16°C, 33°C and T_m with the addition of melittin is consistent, however, with the increase in the enhancement ratio seen at the same temperatures. Clearly, data from freeze fracture electron microscopy are needed to establish the morphology of the structures.

The nanosecond emission anisotropy of DPH in DMPC multilamellar vesicles indicates that the order of the vesicles increases below the transition temperature, as would be expected. Only in the vicinity of the transition temperature does the presence of the protein have any effect on the order of the membrane, and there it decreases it. The rate of diffusion of the probe molecule (and thus the probe's microviscosity) is not changed from that for the pure lipid at any of the temperatures examined. Also, no fusion took place with the addition of melittin to MLVs at 33°C. Recent freeze fracture electron microscopy studies (Dufourcq et al., in press) have shown that, over a range of protein to lipid molar ratios which includes 1/60, the addition of melittin to DPPC dispersions yields a mixture in which some but not all of the MLVs are solubilized into large unilamellar

vesicles. These experiments were carried out at a lipid concentration of 15 mM (one hundred times the concentration used here) so that it is again necessary to exercise caution in extending such results to the system as studied here. Also at this point it cannot be said just how much solubilization has taken place for a protein/lipid ratio of 1/60. We did find, however, that for our concentrations melittin does reduce the amount of light scattering (measured as apparent absorbance at 450 nm) from DMPC MLVs, implying that some such solubilization was in fact taking place. From the nanosecond emission anisotropy and pyrene 0-0 vibronic enhancement studies it is clear that the resulting structures still retain much of the order which the MLVs had before solubilization.

CHAPTER V

CONCLUSION

The above analysis indicates that the binding of melittin to DMPC unilamellar vesicles brings about an increase in the enhancement of the 0-0 vibronic band of pyrene when the lipid sample is below the transition temperature and above a temperature of 30°C. Such an increase in the enhancement is interpreted as meaning that the position of the probe molecule has been shifted toward the lipid polar head groups, relative to its position in the membrane in the absence of melittin. This dislocation is the result of melittin-induced structural changes in the liposome which bring about an ordering of the lipid acyl chains and a reduction in their fluidity. Such an interpretation is consistent with the results obtained through the measurement of the fluorescence anisotropy of DPH molecules embedded in DMPC unilamellar liposomes. At 16°C, 33°C and at the transition temperature of the liposomes it was found that the binding of melittin brought about an increase in the order parameter and a decrease in the probe molecule's mobility. Such an increase in lipid order and rigidity appears to be due, at least in part, to melittin-induced fusion of the vesicles, in which structures possessing a larger radius of curvature and consequently more order are presumably formed.

Measurements of DPH fluorescence anisotropy made on multilamellar vesicles under similar conditions show that melittin has

little effect on the order of the acyl chains for samples above and below the transition temperature. Studies employing the enhancement of pyrene's 0-0 vibronic transition gave results in agreement with this. There it was found that the binding of melittin caused virtually no change in the enhancement ratio, implying that acyl chain order and fluidity are left largely unaffected. Melittin does cause some solubilization of the MLVs, however. Either the order and fluidity of the resulting structures remains largely unchanged or else the degree of solubilization at 1/60 melittin is small enough so that the solubilized membranes contribute little to the overall behavior of the sample. Comparison of the results for DMPC unilamellar and multilamellar vesicles leads us to conclude that the effect on such liposomes is largely confined to unilamellar vesicles. For these the principal effect of melittin binding is the fusing of the vesicles into larger, more ordered structures.

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VITA

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