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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED SYNTHESIS OF FLUORESCENTLY LABELLED PHOSPHOLIPIDS and PRELIMINARY STUDIES INTO THE FEASIBILITY OF POLARIZATION MICROSCOPY

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SYNTHESIS OF FLUORESCENTLY LABELLED PHOSPHOLIPIDS

and

PRELIMINARY STUDIES INTO THE FEASIBILITY OF "POLARIZATION MICROSCOPY"

BY

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ABBREVIATIONS

C_4-dansyl	N-(5-dimethylaminonaphthalene-1-sulfonyl)amino-butyric acid
DUA	N-(5-dimethylaminonaphthalene-1-sulfonyl)amino-undecanoic acid
DCC	N,N'-dicyclohexylcarbo-diimide
DOPA	Dioleoylphosphatidic acid
DOPC	Dioleoylphosphatidylcholine
DUA-PC	DUA-phosphatidylcholine
DUA-PA	DUA-phosphatidic acid
PC	Phosphatidylcholine
PA	Phosphatidic acid
PS	Phosphatidylserine
EDTA	Ethylenediamine tetra-acetic acid

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PART I: SYNTHESIS OF C_4-DANSYL-PHOSPHATIDYLETHANOLAMINE, C_4-DANSYL-PHOSPHATIDIC ACID, AND DUA-SPHINGOMYELIN

INTRODUCTION

One method of studying biological membranes is by the use of fluorescence. If a fluorescent probe is attached to a phospholipid or a protein in the membrane, its movement and behavior in the membrane can be studied. These types of studies are carried out in the laboratory of Dr. M. Glaser in the form of polarization studies and fluorescence microscopy. By the use of these fluorescence techniques, the response of phospholipids in the membrane to various conditions can be studied in great detail.

Dr. Glaser's laboratory uses a variety of fluorescently labelled phospholipids in its studies. For the studies presented here, the fluorescent probe is attached to the acyl chain of the phospholipid. This allows for the study of the effects of the head group. The synthesis of these probes is often performed by modification of another fluorescent phospholipid. In the case of labelled phosphatidylethanolamine (PE) and phosphatidic acid (PA), the synthesis occurs via a transphosphatidylation reaction by cabbage phospholipase D with a starting material of fluorescently labelled phosphatidylcholine (PC). The product is then purified by thin layer chromatography. The work presented here involves the synthesis of C_4-dansyl (N-(5-dimethylamino-naphthalene-1-sulfonyl)amino butyric acid) phosphatidylethanolamine (C_4-dansyl-PE) and phosphatidic acid (C_4-dansyl-PA) from C_4-dansyl-PC. C_4-dansyl phospholipids have not been previously synthesized in our laboratory but they were needed for use because of the short acyl chain. The short acyl chain will allow for spontaneous transfer of phospholipids between two populations. Therefore C_4-dansyl phospholipids can be used in the same manner as C_8-labelled

phospholipids were used by Pagano (1).

Another constituent of membranes which can be used as a tool in membrane studies is sphingomyelin. This can also by used in fluorescence studies if a fluorescent probe is attached to it. DUA (N-(5-dimethyl-aminonaphthalene-1-sulfonyl)amino undecanoic acid) labelled sphingomeylin was synthesized by condensation of the free DUA with lyso-sphingomyelin (sphingosylphosphorylcholine) in chloroform. The lyso-sphingomyelin was obtained by hydrolysis of sphingomyelin with an acidic reagent. This compound had also not been previously synthesized in the laboratory.

MATERIALS

C_4-dansyl was purchased from Chemical Dynamics Corp. (South Plainfield, NJ) and was attached to phosphatidylcholine by the procedure of DUA-PC synthesis of Haverstick and Glaser (2). The only modification was that C_4-dansyl was used as the starting material instead of DUA.

Cabbage phospholipase D and sphingomyelin were purchased from the Sigma Chemical Co. (St. Louis, MO). N,N'-dicyclohexylcarbo-diimide (DCC) used in the synthesis of C_4-dansyl phosphatidylcholine and DUA-sphingomyelin was purchased from the Aldrich Chemical Co., Inc. (Milwaukee, WI). DUA was purchased from Molecular Probes (Junction City, OR).

METHODS AND RESULTS

Miscellaneous methods

The technique used to extract products from either the reaction mixture or the elutant solution was that of Bligh and Dyer (3). Yields of the syntheses were determined by phosphate content, following perchloric acid digestion (4).

Synthesis of C_4-dansyl-phosphatidylethanolamine

Phosphatidylethanolamine labelled with the fluorescent probe C_4-dansyl was made by transphosphatidylation with cabbage phospholipase D. This synthesis was based on a procedure by Yang, et al. (5) as modified by Comfurius and Zwaal (6).

One micromole of C_4-dansyl phosphatidylcholine was dried completely under nitrogen and resuspended in 2 ml of diethyl-ether. To this, 7.2 ml of a solution which was 100 mM sodium acetate, 100 mM $CaCl_2$, pH 5.6, was added. The enzyme was brought up to 2 mg per ml in a solution of 2 mM TES, pH 7.0. One mg (0.5 ml of the above solution) was added to the reaction tube, along with 4.0 ml of ethanolamine, pH 5.6. The pH of the reaction mixture was critical to the success of the synthesis since the enzyme has a very narrow pH optimum for full activity (5).

The reaction was run in a shaking water bath at 30°C for 15 to 18 hours and stopped by the addition of 14 ml of 100 mM EDTA, pH 7.0. The ether was then blown off under nitrogen and the product was extracted by adding 16 ml of chloroform to the solution followed by 8 ml methanol. The mixture was shaken and centrifuged in a bench-top centrifuge for 5 minutes. The upper,

aqueous phase was discarded and 8 ml of methanol were added to the lower phase followed by 8 ml of water and another 8 ml of methanol. The mixture was again shaken and centrifuged. The aqueous layer was discarded.

The lower phase was dried completely under nitrogen and resuspended in 250 to 500 microliters of chloroform. The product was then isolated by thin layer chromatography on Silica Gel G plates (Analtech, Newark, DE) using a solvent system of 65:25:4, v/v chloroform:methanol:H_2O. In this system, PE moves to an R_f value of 0.55 while unreacted PC has an R_f of 0.4 and by-product PA stays at the origin.

The product was scraped from the plate into a tube and eluted with 50:100:5, v/v chloroform:methanol:H_2O. The elutant was dried down and extracted by the Bligh-Dyer technique (3). Yield of the product was tested by phosphate determination (4) and was determined to be 16%. This was very low as compared to the yields obtained when DUA-PE was synthesized using the same method. The low yield may be due to the fact that the C_4-dansyl-PC is not as readily recognized by the phospholipase D as a substrate because of its extremely short acyl chain.

Synthesis of C_4-dansyl-phosphatidic acid

Phosphatidic acid labelled with C_4-dansyl was made by enzymatic digestion of C_4-dansyl-PC with cabbage phospholipase D in a procedure similar to that used for the formation of C_4-dansyl-PE. This was also based on the procedure of Yang, et al. (5) and Comfurius and Zwaal (6).

One micromole of C_4-dansyl-phosphatidylcholine was dried completely and resuspended in 2.0 ml diethyl-ether. Eleven ml of a solution which was 100 mM sodium acetate, 100 mM $CaCl_2$, pH 5.6 were added, along with one ml of a

cabbage phosphlipase D solution which was 2 mg/ml in 2 mM TES, pH 7.0.

The reaction was run at room temperature in a shaking water bath for 15 to 18 hours and stopped by the addition of 14 ml of 100 mM EDTA, pH 7.0. The ether was blown off and 16 ml of chloroform followed by 8 ml methanol were added. The mixture was shaken briefly and centrifuged in a bench-top centrifuge for 5 minutes. The aqueous phase was discarded while 8 ml of methanol, 8 ml water, and 8 ml methanol were added to the lower phase. The mixture was again shaken and centrifuged and the aqueous phase discarded. The lower phase was dried completely and resuspended in 250 to 500 microliters of chloroform for purification.

The product was isolated by thin layer chromatography on the same plates as used for the C_4-dansyl-PE synthesis with the same solvent system. PA remained at the origin in a broad band while unreacted PC had an R_f value of 0.4. Product was scraped into a tube and eluted from the silica with 50:100:5:0.5, v/v chloroform:methanol:H_2O:HCl. The elutant was then dried down and extracted by the technique of Bligh and Dyer (3). Yield of the reaction was determined by phosphate assay (4) to be 35%. This yield was again low compared to DUA-PA synthesis. This concurs with the explaination given above, i.e. that C_4-dansyl-PC may not be a good substrate for the phospholipase D.

Synthesis of DUA-sphingomyelin

Sphingomyelin was prepared with DUA attached in the fatty acid position. Sphingomyelin was first hydrolyzed to lyso-sphingomyelin and then the DUA was covalently attached. The procedure used was a modification of that of Cohen et al. (7).

The first step in the synthesis was the hydrolysis of sphingomyelin to lyso-sphingomyelin. Sphingomyelin was brought up in a solution which was 2 mg of sphingomyelin per ml in aqueous methanolic reagent (8). The reagent consisted of 8.6 ml HCl, 9.4 ml H_2O, and 82 ml methanol.

The reaction was run for 18 hours at 65 to 75°C and stopped by adding chloroform, methanol and water to a final ratio of 8:4:3, v/v. The solution was mixed and allowed to separate into phases. The upper phase contained most of the lyso-sphingomyelin (95%). The lower phase was extracted with pure solvent upper phase (chloroform:methanol:water in a ratio of 3:48:47, v/v) (9). The upper phases were combined and extracted with pure solvent lower phase (86:14:1, v/v chloroform:methanol:water) (9). The pH of the upper phase was adjusted to 12 to 13 with NaOH to bring the lyso-sphingomyelin to the lower phase. This step was repeated and the lower phases were combined and dried down.

Throughout the course of the extraction procedure, fractions were saved for analysis by thin layer chromatography. Samples from each step of the extraction were run on Silica Gel G plates in a solvent system of 65:35:2.5:2.5, v/v chloroform:methanol:ammonium hydroxide:water. In this system, the sphingomyelin has an R_f value of 0.2, while lyso-sphingomyelin moves to 0.09.

The lyso-sphingomyelin product was identified with iodine vapor staining for carbon double bonds, molybdate stain for phosphate (10), ninhyrin reagent for primary amino groups, and Dragendorff reagent for choline (11).

The lyso-sphingomyelin was purified from the final lower phase of the extraction by thin layer chromatography in the same solvent system as above. The product was scraped from the plate into a tube and eluted from

silica with 50:100:5, v/v chloroform:methanol:water. The elution required a large volume of solution (50 ml) to remove the product from the silica. The elutant was then extracted by the method of Bligh and Dyer (3), and the upper phase was made basic (pH 12) to again bring the lyso-sphingomyelin to the lower phase. The phases were separated and the upper phase was extracted again with chloroform. The lower phases were combined and dried down after repeating the final chloroform extraction at least twice. The yield was then determined by phosphate analysis (4) to be approximately 10%. The low yield obtained indicated that more extraction of the final upper phase was needed or that the product was still attached to the silica. Therefore the silica was eluted several more times with the elution solvent and extracted. The final upper phase was again extracted with chloroform several times. In this way the yield was increased to 30%.

The next step in the synthesis was the attachment of DUA. Initially, the attachment was tried in toluene because of a similar procedure used for DUA-PC (2). However, the combination of an extremely low yield, unusual emission spectra, and a residue found at the bottom of the reaction vessel led to the conclusion that the lyso-sphingomyelin was not dissolved in the toluene. Thus the reaction was tried again in chloroform. First, an acid anhydride was formed by mixing 20 mg of DUA with 10 mg DCC in toluene for 1.5 hours at room temperature in the dark. The solution was then centrifuged in a desk-top centrifuge and the supernatent dried down under nitrogen and resuspended in 5 ml of chloroform.

The lyso-sphingomyelin was prepared by drying down 10 ml of a 1 mg per ml solution in a round bottomed flask of a Buchler Rotovap to complete dryness and then resuspending in 10 ml chloroform.

The acid anhydride solution was then added to the lyso-sphingomyelin solution and sodium oxide beads were added. The reaction was run by slowly evaporating the chloroform while holding the reaction vessel in a 70°C oil bath. Another 10 ml of chloroform was added and the evaporation repeated. This procedure was repeated up to 10 times. The progress of the reaction was followed by thin layer chromatography in a solvent system of 65:35:2.5:2.5, v/v chloroform:methanol:ammonium hydroxide:water. The appearance of DUA-sphingomyelin was visualized by its fluorescence at an R_f of 0.09.

When the reaction was complete, the sample was dried completely under nitrogen. The product was purified by thin layer chromatography in the above solvent system after resuspending in 1 ml chloroform. The product was eluted from the silica with 50:100:5, v/v chloroform:methanol:ammonium hydroxide, extracted by the Bligh-Dyer technique (5), and yield was determined by the phosphate analysis (4) to be 70%. The DUA-sphingomyelin was identified by emission spectra. Figure 1A shows an excitation scan of the DUA-sphingomyelin compared to free DUA dissolved in the same solvent (chloroform). As can be seen, the spectra are almost identical. To further characterize the DUA-sphingomyelin, emission spectra on both samples were also run (Figure 1B). Again, the scans were almost identical.

Upon completion of the above three syntheses, it was possible to use the fluorescent phospholipids synthesized in the studies of phospholipids and membranes. The lipids synthesized were ones which had previously not been uced in our laboratory, thus new properties and behaviors could be studied. In the next section of this paper, behaviors of acidic phospholipids in the

presence of cations are studied. Although the studies did not include the compounds synthesized, they are examples of the type of work in which these compounds could be used.

PART II: PRELIMINARY STUDIES ON THE FEASIBILITY OF "POLARIZATION MICROSCOPY"

INTRODUCTION

The fluid mosaic model for biological membranes proposed by Singer views the plasma membrane as a fluid, random sea of phospholipids in which membrane proteins are inserted (12). This model suggests that there is free lateral movement of the phospholipds in the membrane (13). However, recent studies have shown that there may be domain formation in membranes. An example of this is the formation of cation-acidic phospholipid complexes in lipid vesicles. These complexes have been visualized by the use of fluorescent microscopy by Haverstick and Glaser (2). In this work, calcium and cadmium were shown to cause fluorescent acidic phospholipids to form patches in vesicles. These patches were viewed with a fluorescence microscope which was connected to a digital analysis imaging system. Thus, Ca^{2+} complexes could be viewed very clearly in the vesicles. If a patch (or domain) forms in a membrane system, there is a loss of randomness in the system. The acidic phospholipids have now been sequestered into a small area of the vesicle. In this area of the vesicle there may be a large number of phospholipids with the same head group packed together and held in place by the ionic interactions of Ca^{2+} with the negatively charged phospholipid. Thus there is probably a loss of free, random movement of these phospholipids in the vesicle. The area of the patch thus becomes more rigid, almost comparing to a "gel" phase or state which a phospholipid bilayer might exist at low temperature. One way to measure this change in fluidity is by polarization measurements. Increased polarization of a fluorophore would mean that the probe (in this case, attached to the phospholipid) is restricted in its movement. Thus, if a domain or patch is

visualized by fluorescence microscopy and the image analyzed by the computer shows an increase in polarization, this would indicate that a region exists where in fact the bilayer is not a fluid, random structure, but rather a rigid, well defined system.

The techniques used in this work made it possible to study this phenomenon because of the following factors: 1) a vesicle system in which "patches" of fluorescent phospholipids could be seen, 2) a computer system which digitized the images captured from the microscope, 3) polarizers on the excitation and emission light paths of the microscope which allow for viewing the vesicles at perpendicular and parallel light, and 4) a computer system with which image ratioing could be done.

The polypeptide ionophore gramicidin was used in these studies because of previous work done in our laboratory by Wang et al (14). Gramicidin is known to insert into the bilayer and it is easily labelled with a fluorophore. The work of Wang et al. (14) showed that gramicidin has no specificity for phospholipids, and preliminary experiments had shown that gramicidin is excluded from cation-acidic phospholipid patches. Thus gramicidin was used in these experiments to study the effect on the rest of the vesicle when a patch forms.

The results given here show a correlation between patch formation and increased polarization in vesicle systems which contain acidic phospholipids. No patch formation was seen for vesicles containing a neutral phospholipid (phosphatidylcholine). This work has been only an introductory study, and will be repeated in greater detail in future work. However, preliminary results indicate that a) the techniques of polarization microscopy and digital image ratioing will work in the studies

of these vesicles and b) regions are forming in the vesicle system that are rigid and defined as compared to the rest of the vesicle.

MATERIALS AND METHODS

Dioleoylphosphatidic acid (DOPA) was synthesized from dioleoylphosphatidylcholine (DOPC, from Avanti Polar Lipids, Inc., Birmingham, AL) as outlined for C_4-dansyl-PA in Part I. Similarly, DUA-phosphatidic acid (DUA-PA) was synthesized from DUA-phosphatidylcholine (DUA-PC).

Dioleoylphosphatidylserine (DOPS) and DUA-phosphatidylserine (DUA-PS) were synthesized from DOPC and DUA-PC respectively by the procedure for C_4-dansyl-PE synthesis outlined in Part I except that 40% (w/v) L-serine at pH 5.6 was used. In addition, the reaction was run at 45°C for 90 minutes (5). The product was purified as before except with a solvent system of 65:40:5, v/v chloroform:methanol:ammonium hydroxide and it was eluted with 50:100:5:0.5, v/v chloroform:methanol:water:HCl.

Gramicidin A was purchased from Sigma Chemical Co. (St. Louis, MO). Dansyl was attached to the gramicidin by a procedure similar to that of Ventch and Blout (15). The gramicidin was mixed with dansyl chloride in acetone. Sodium carbonate was added and the reaction was stirred for three hours in the dark. Gramicidin and dansyl-gramicidin were separated from free dansyl by passing the reaction mixture over a Sephadex LH-20 column (1cm x 37cm) equilibrated with methanol. Product was identified by emission spectra. Figure 2A shows the characteristic dansyl emission spectra when the molecule is excited at 350 nm (see also Figure 1B). Figure 2B shows the emission of the tryptophan in the poly-peptide when the molecule is excited at 280 nm. The second peak in Figure 2B is due to dansyl emission.

Large vesicles for use with the fluorescence microscope were prepared by

the procedure of Darzon et al. (16). Vesicles were prepared with 250 nmoles lipid per 500 microliters water.

Polarization values for the vesicles were obtained using an SLM 8000 fluorometer. Fluorescent values were obtained and polarization calculated using the formula P equals (intensity of parallel fluorescence minus that of perpendicular fluorescence) over the sum of the two.

Vesicles were viewed with a fluorescence microscope attached to a low-light intensity charge coupled device camera and digital image processor. The microscope instrumentation is described in full by Haverstick and Glaser (2). Images were captured as averages of 150 frames captured at a rate of 30 frames per second and a pseudo color program was applied to more clearly view the distribution of fluorescence.

Images used for direct correlation with polarization values were obtained by first capturing an image of the vesicle taken with parallel polarized light. This image was stored by the computer and another image was captured using perpendicular polarized light. The computer then divided these images (parallel over perpendicular) using the equation $A/B = \text{antilog}(\log A - \log B)$. The image so generated showed actual areas in the vesicle where there was a significant difference between the images captured using parallel and perpendicularly polarized light. In order to maximize the differences in the ratioed images, the output from the computer to the monitor was expanded such that values from 100 to 150 were assigned values from 1 to 256 (see reference 2). All figures of vesicles are photographs taken directly of the computer monitor. In all cases an image of the vesicle under fluorescent illumination (parallel light) is shown for reference to the ratioed image.

RESULTS

The experiment was conducted with nine different sets of vesicles, each containing a different combination of lipids. Each set consisted of four replicates along with a blank which consisted of 100% DOPC. After formation of the vesicles, absorbance readings were taken at 340 nm. Absorbance readings served as an indication as to the presence and quality of the vesicles. Absorbance readings for normal vesicles were centered around 0.1. A much higher reading than this would indicate that the vesicles were clumped, or somehow enlarged, and that light was scattered. Any vesicle preparations which gave high absorbance readings were not used in the experiment. Polarization measurements were then obtained from the fluorometer and the vesicles were viewed with the microscope and images stored. These values and images served as the initial time frame of the experiment. They were also used to verify the presence and quality of the vesicles.

After the initial measurements were taken, cations were added to the vesicles. Additions to the four replicates were as follows: 1) 2 mM $CaCl_2$, 2) 4 mM NaCl, 3) 0.5 mM $PrCl_3$, 4) no additions. Calcium was used based on the work of Haverstick and Glaser (2). Sodium and no additions were used as controls, and praseodymium has been used previously in nuclear magnetic resonance studies (17). It was used here because of interest as to whether or not the acidic phospholipids would interact differently with the molecule than they do with calcium and perhaps form slightly different patterns of patching. Immediately after addition of cations, absorbance at 340 nm was measured, along with polarization. These values served as the

zero time point of the experiment.

Measurements were again taken after 20 to 24 hours in the presence of the cations. Absorbance and polarization measurements were taken and the vesicles were viewed in the microscope. It was at this time that parallel and perpendicular images were captured, and the divided images obtained.

The vesicles composed of 100% DOPC served as a blank in obtaining polarization values throughout the 24 hour experiment. No cations were added to these vesicles. Since these vesicles contained no fluorescent compounds, they could not be viewed under the microscope.

The first set of vesicles in the experiment consisted of a 99:1 ratio of DOPC to DUA-PC. The results with calcium addition served as positive controls based on the work of Haverstick and Glaser (2). Therefore, other than for comparisons sake, these results will not be discussed further. Polarization over time is shown in Table 1A. All values of polarization given in Tables 1 and 2 are very low for vesicles containing dansyl. There are two possible explainations for these low values. One is that there was not enough signal, i.e. that the dansyl concentration was not high enough in the cuvette. The second possibility is that the extremely heterogeneous sizes of the vesicles in some way interfered with the measurements being taken. For those values listed as 0 in the tables, the actual calculations were less than 0.005. The vesicles to which NaCl was added serve as controls in these experiments. The polarization values for vesicles with Pr^{3+} added show no change as compared to the controls. Figure 3A is an example of vesicles after 24 hour treatment with Pr^{3+}. Figure 3B is the ratioed image of this same vesicle viewed under parallel and perpendicular light. The lack of change in polarization along with the lack of change in

intensity and distribution of fluorescence in these vesicles seems to indicate that there is no patching of the phospholipids in these vesicles along with no change in rigidity of the bilayer. These results are consistent with those found by Haverstick and Glaser (2).

As outlined in the introduction, there was interest in the effect of gramicidin on this system. Therefore, the ionophore was included in the vesicles when they were made and the experiment was repeated. Polarization values for this vesicle system are shown in Table 1B. Again no significant change in polarization over time in the vesicles containing Pr^{3+} as compared to the vesicles used as controls was seen, although the initial reading was high. These results were as expected, since gramicidin has no specific interations with phospholipids (14).

Computer images of the vesicle after Pr^{3+} addition are shown in Figure 4. Figure 4A is the fluorescent image and Figure 4B is the ratioed image. These images show a very uniform distribution of intensity of fluorescence in the vesicles. Thus the presence of gramicidin in these vesicles does not seem to cause any type of patching of fluorescent phospholipids by itself, nor does it interact with the Pr^{3+}.

In order to study more closely if in fact the gramicidin interacts with Pr^{3+}, the dansyl group was attached to the gramicidin, and vesicles consisting of DOPC and dansyl-gramicidin were studied. Polarization over time is shown in Table 1C. No change in polarization is seen as compared to the controls. The computer image shows a uniform distribution of intensity (Figure 5). Figure 5A is a fluorescent image of a respresentative vesicle and Figure 5B is the ratioed image of the same vesicle. From these data, it appears that dansyl-gramicidin does not

interact with Pr^{3+}.

As a result of the work of Haverstick and Glaser, it was known that PA formed patches in the presence of calcium. Thus, the next group of vesicles were used to study the effects of Pr^{3+} on PA. Polarization over time is given in Table 2A. The vesicles to which Pr^{3+} has been added show a significant change in polarization after 24 hours. This is complimented by the image obtained (Figure 6), which shows three patches in the vesicle where the ratio of parallel to perpendicular images shows a significant difference between the two. One can clearly see from these pictures the patches which have formed. These data show that the DUA-PA has patched in the presence of Pr^{3+} (Figures 6A and 6B) and this patching has caused restricted movement of the DUA-PA (increased rigidity) in that area, as indicated by both the increase in polarization over time (Table 2A) and the ratioed image (Figure 6C).

Again, the effects of gramicidin on patching behavior was studied by adding gramicidin to the vesicle system. Polarization over time is given in Table 2B. In order to more directly compare this study with that of Haverstick and Glaser (2), the results for calcium addition are presented here. Significant changes can be seen with Ca^{2+} addition as compared to the controls. Changes are also seen in images of these vesicles. Figure 7A shows the fluorescence image, while Figure 7B shows the ratio of parallel and perpendicular images. These data show that the presence of gramicidin doesn't affect the patching of the DUA-PA with Ca^{2+}. Similar results were seen with Pr^{3+}.

The effect of gramicidin on the interaction of PA with calcium and praseodymium was studied by making up vesicles with unlabelled PA (DOPA)

and using gramicidin with dansyl attached to it. It was expected based on preliminary experiments that the dansyl-gramicidin would be excluded from the PA patch induced by treatment with the cations. Polarization over time for vesicles containing DOPC:DOPA:dansyl-gramicidin is given in Table 2C. These vesicles treated with Pr^{3+} do not show a large change as compared to the controls, but the images (Figures 8A and 8B) do show areas of some differences between parallel and perpendicular light. This may be explained by the following: the fluorescent label is now attached to the gramicidin and not to the phospholipid. When the unlabelled PA patches in the presence of Pr^{3+}, the dansyl-gramicidin moves into the rest of the vesicle. Thus we see a more diffuse area of increased intensity in the ratioed image and polarization values which indicate less restriction of movement than shown above for vesicles with PA and gramicidin (Table 2B and Figure 8).

The above experiments were also performed on vesicles containing phosphatidylserine. However, there was some difficulty in locating these vesicles under the microscope after 24 hours. Initial experiments had indicated that vesicles with PS tended to fuse together, and this was seen in the experiment by an increased absorbance at 340 nm. However, the experiments did yield preliminary results which indicated that PS also patches in the presence of Pr^{3+} as predicted by Haverstick and Glaser (2). The presence of gramicidin and dansyl-gramicidin gave similar results as were presented for vesicles containing PA.

DISCUSSION

The technique of polarization microscopy has been used in this study to show that when acidic phospholipids are sequestered into a patch in the presence of Pr^{3+}, these phospholipids are restriced in their movement and a region of increased rigidity now exsists in the vesicle bilayer. The results are an introductory attempt to correlate visualization of patching of acidic phospholipids in the presence of Ca^{2+} and Pr^{3+} with changes in polarization in vesicles over a 24 hour time period. The ability to use polarized exciting light combined with the capacity for digitalization of the images and subsequent arithmatic manipulation of the images has made this study possible. Vesicles with various lipid compositions were studied to determine the effect of different phospholipids, the effect of the presence of a protein in the vesicle and the effect of labelling the protein instead of the phospholipid.

The vesicles in this study can be divided into three groups based on presence of gramicidin. The three groups are: those which contain DUA-phospholipid and no gramicidin; those which contain DUA-phospholipid and unlabelled gramicidin; and finally those which contain dansyl-gramicidin and unlabelled phospholipids. By comparing results obtained for PC and PA within each of these three groups, the effects of gramicidin and dansyl-gramicidin in each system can be studied.

The first group consists of the vesicles presented in Tables 1A and 2A. Polarization values of these vesicles over time are compared in Figure 9.

The graph shows that vesicles containing DUA-PA show a significant increase in polarization after the addition of Pr^{3+}, however, vesicles

containing DUA-PC show no increase in polarization. These observations correlate with Figures 3 and 6, which are images of the vesicles containing DUA-PC and DUA-PA respectively. This shows that vesicles containing PC do not patch in the presence of Pr^{3+} and there is no change in bilayer rigidity, while vesicles that contain DUA-PA do form patches with Pr^{3+} and when this occurs there is an increased rigidity and restriction of movement of the fluorescent phospholipids in the vesicle.

The second group of vesicles in this comparison are those containing DUA-phospholipid and gramicidin. This includes vesicles seen in Tables 1B and 2B.

Polarization values for these vesicles are compared in Figure 10. For the vesicles which contain DUA-PC and gramicidin, no change is seen over time. This is also shown in Figure 4. The vesicles containing DUA-PA and gramicidin show an increase in polarization after 24 hours and this also can be visualized in patching of DUA-PA as seen in Figure 7.

Overall, this group of vesicles shows again the patching phenomenon in vesicles containing PA but not PC as might be expected based on earlier results from the laboratory (2). The presence of unlabelled gramicidin in the vesicles seems to have no effect on patching behavior.

The final group of vesicles to be considered are those which contain dansyl-gramicidin. These vesicles are those presented in Tables 1C and 2C. Comparison of polarization values for these vesicles are found in Figure 11. Little change is seen in vesicles containing DOPC and dansyl-gramicidin. This is also shown by lack of patching in Figure 5. Thus, dansyl-gramicidin does not patch in PC vesicles that have been treated with Pr^{3+}. Figure 9 shows diffuse patching of dansyl-gramicidin in vesicles

with PA. This corresponds to the polarization data for these vesicles which does not change over time. This can be explained as before in that the dansyl-gramicidin is occupying the area of the vesicle enriched in PC, which is still fluid.

The overall conclusion from this comparison is that no patching seems to be occuring in PC vesicles, while patching of dansyl-gramicidin is seen in vesicles containing PA. The dansyl-gramicidin responds to patching of unlabelled PA by moving to other areas of the vesicles and thus there isn't as much restriction of movement and changes in bilayer rigidity seen.

As indicated in the previous section, vesicles containing PS were also examined for patching and changes in polarization over time. Although preliminary results were suggestive that PS behaves similarly to PA, problems with fusion of the vesicles prevented good polarization measurements and microscope images.

From the data presented here, it can be seen that phosphatidic acid forms patches in vesicles in the presence of the cation praseodymium. The formation of these patches corresponds to increases in polarization over a 24 hour period. No patch formation or subsequent increase in polarization was seen in vesicles containing PC. The patch formation corresponding to increases in polarization show a restriction of movement of the phospholipids in that area of the patch.

The techniques presented in this work outline the procedures used to view patch formation in vesicles and digitization of the images obtained so that actual changes in polarization can be visualized. This new technique which we have referred to as polarization microscopy has been used to show regions in bilayer systems which exhibit changes in rigidity and may be

used in the future to show actual domain formation in the plasma membranes of cells.

TABLE 1

Polarization values for synthetic phospholipid vesicles containing phosphatidylcholine as thu only phospholipid

A: Vesicles containing 99 mole % DOPC and 1 mole % DUA-PC

Time	2.0 mM NaCl	0.5 mM $PrCl_3$
Pre-cation	0.06	0.02
Zero time	0.08	0.01
20 hours	0.04	0.01

B: Vesicles containing 94 mole % DOPC, 1 mole % DUA-PC and 5 mole % gramicidin

Time	2.0 mM NaCl	0.5 mM $PrCl_3$
Pre-cation	0	0.12
Zero time	0.04	0.13
20 hours	0	0.13

C: Vesicles containing 95 mole % DOPC and 5 mole % dansyl-gramicidin

Time	2.0 mM NaCl	0.5 mM $PrCl_3$
Pre-cation	0	0.01
Zero time	0	0.03
20 hours	0	0.02

TABLE 2

Polarization values for synthetic phospholipid vesicles containing phosphatidylcholine and phosphatidic acid

A: Vesicles containing 90 mole % DOPC, 9 mole % DOPA and 1 mole % DUA-PA

Time	2.0 mM NaCl	0.5 mM $PrCl_3$
Pre-cation	0.10	0.01
Zero time	0.10	0.06
20 hours	0.02	0.13

B: Vesicles containing 85 mole % DOPC, 9 mole % DOPA, 1 mole % DUA-PA and 5 mole % gramicidin

Time	2.0 mM NaCl	2.0 mM $CaCl_2$
Pre-cation	0.01	0.10
Zero time	0	0.10
20 hours	0.01	0.16

C: Vesicles containing 85 mole % DOPC, 10 mole % DOPA, and 5 mole % dansyl-gramicidin

Time	2.0 mM NaCl	0.5 mM $PrCl_3$
Pre-cation	0	0
Zero time	0	0
20 hours	0.01	0.01

FIGURE 1:

Excitation (A) and emission (B) scans of DUA-sphingomyelin (——) and free DUA (---). Both samples were dissolved in chloroform. Free DUA was used for comparison.

(A) Fluorescence emission at 505 nm was monitored while exciting the dansyl moiety from 300 to 450 nm.

(B) Fluorescence emission was monitored from 375 to 625 nm while exciting the samples at 350 nm.

FIGURE 1

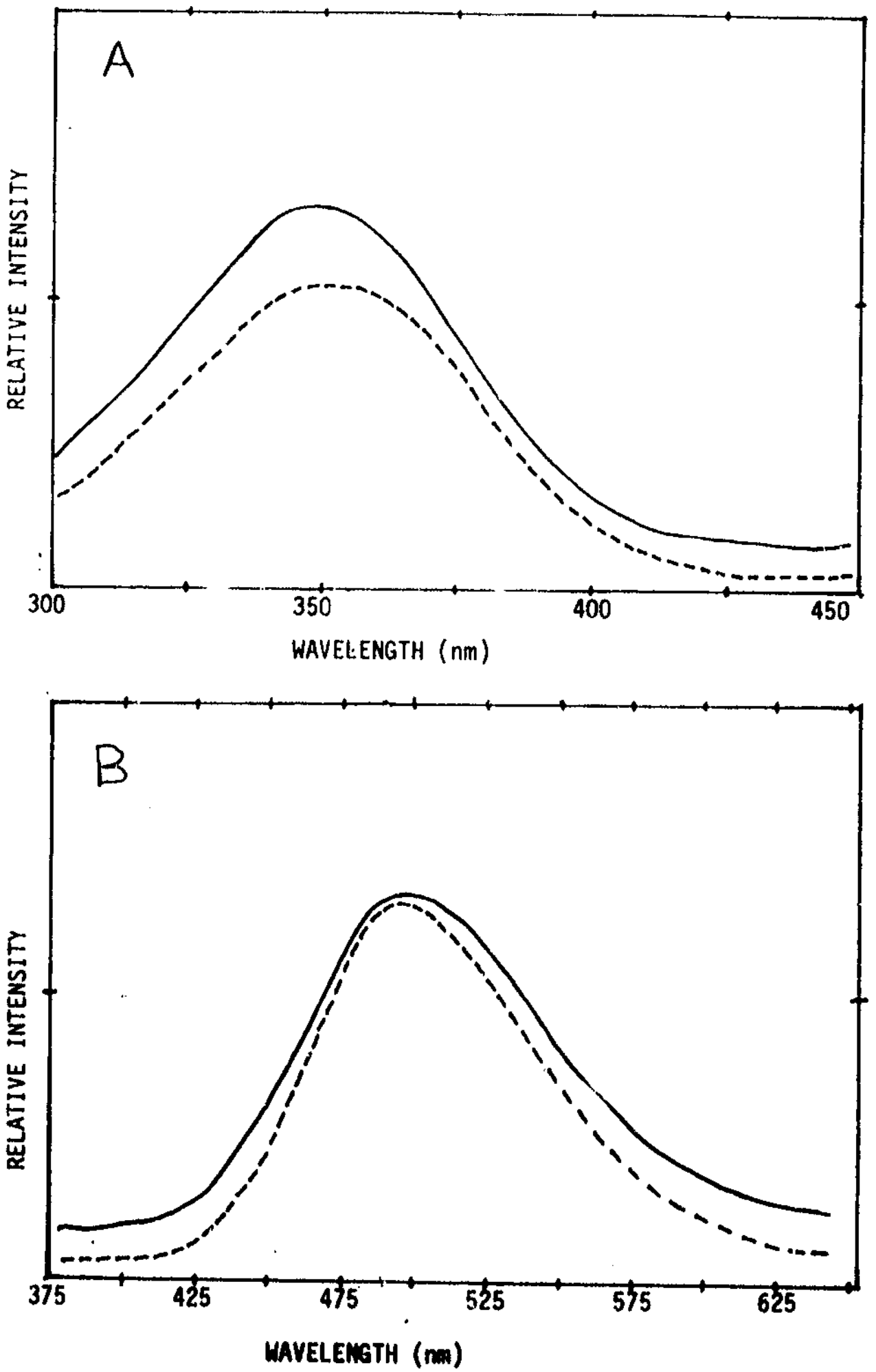

A
RELATIVE INTENSITY
300
350
400
450
WAVELENGTH (nm)
B
RELATIVE INTENSITY
375
425
475
525
575
625
WAVELENGTH (nm)

FIGURE 2:

Emission spectra of dansyl-gramicidin. Gramicidin A, labelled with dansyl and separated from unreacted dansyl as outlined in the methods section, was scanned for emission while exciting the molecule at (A) 350 nm for dansyl fluorescence and (B) 280 nm for tryptophan fluorescence.

FIGURE 2

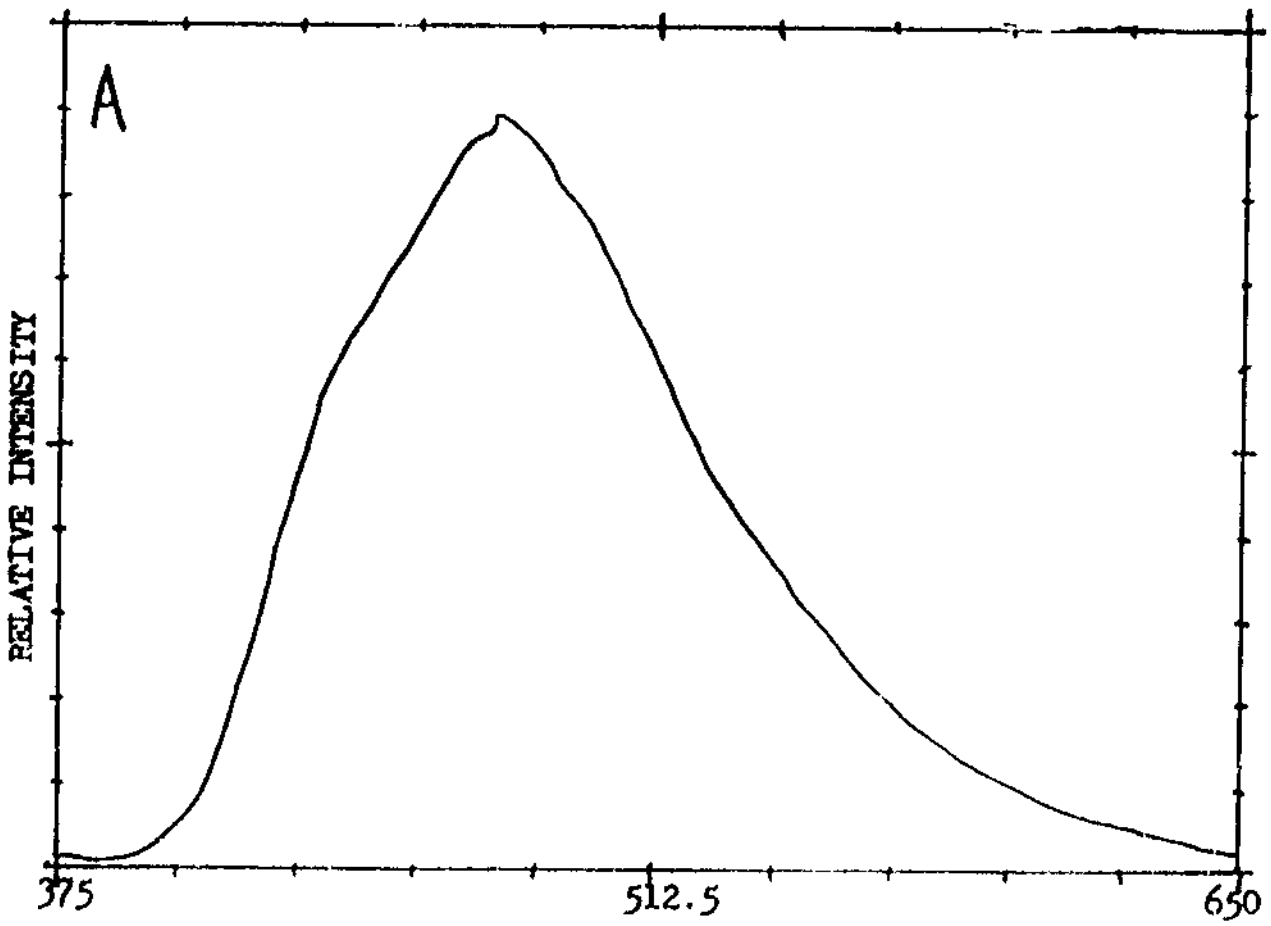

A
RELATIVE INTENSITY
375
512.5
650
WAVELENGTH (nm)
B
RELATIVE INTENSITY
300
375
450
WAVELENGTH (nm)

FIGURE 3:

Fluorescence image taken under parallel light (A) and ratioed image (B) of a vesicle composed of DOPC and DUA-PC following 24 hour exposure to 0.5 mM $PrCl_3$. The vesicle was hydrated from a phospholipid film containing 99 mole % DOPC and 1 mole % DUA-PC. Image capture and image ratioing are outlined in detail in the methods section of Part II.

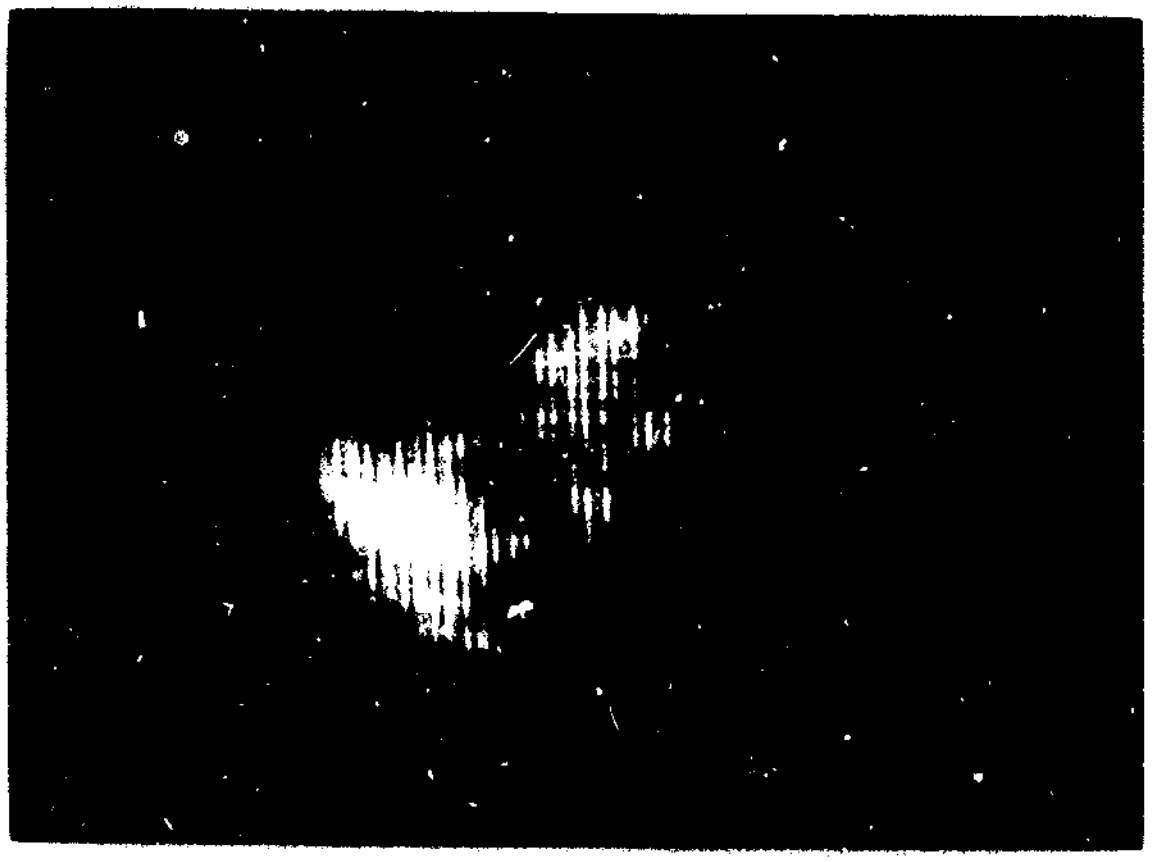


FIGURE 4:

Fluorescence image taken under parallel light (A) and ratioed image (B) of a vesicle composed of DOPC, DUA-PC and gramicidin following 24 hour exposure to 0.5 mM $PrCl_3$. The vesicle was hydrated from a phospholipid film containing 94 mole % DOPC, 1 mole % DUA-PC and 5 mole % gramicidin.

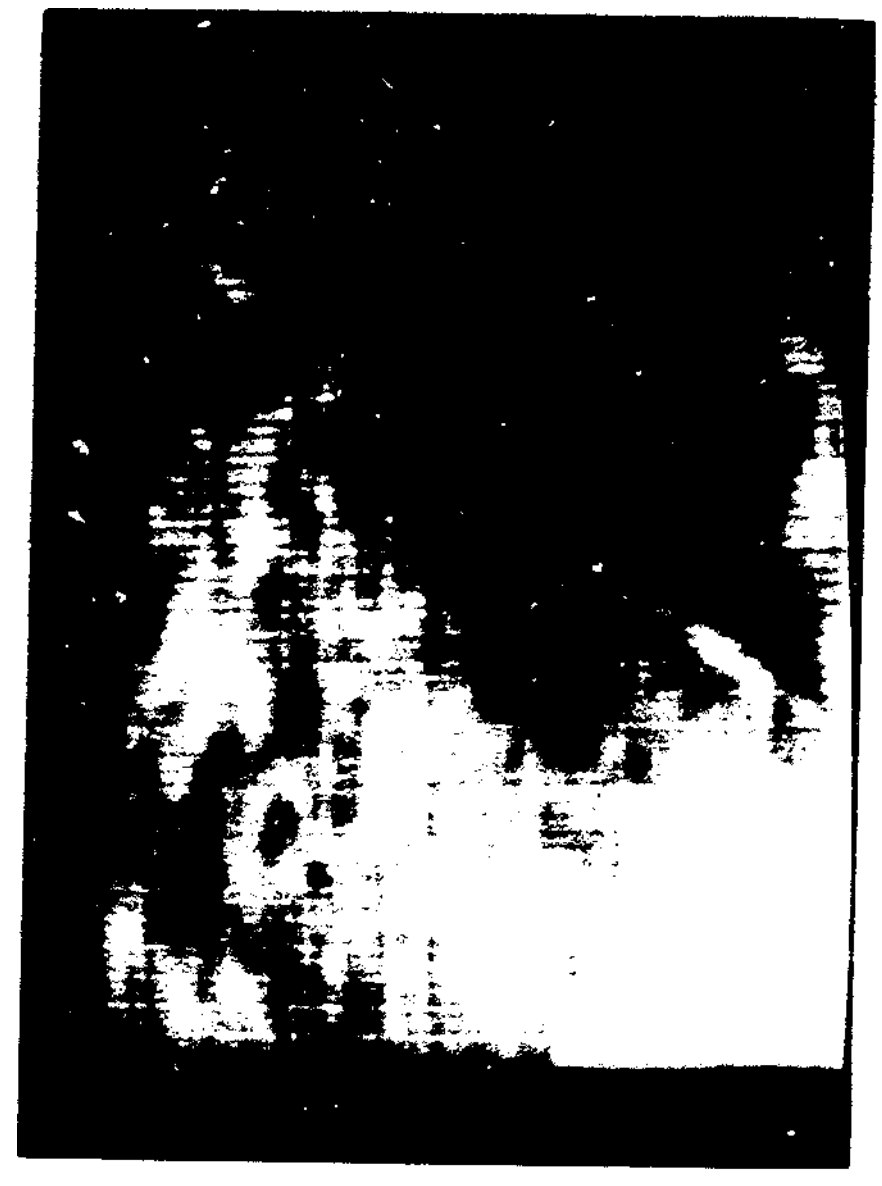


FIGURE 5:

Fluorescence image taken under parallel light (A) and ratioed image (B) of a vesicle composed of DOPC and dansyl-gramicidin following 24 hour exposure to 0.5 mM $PrCl_3$. The vesicle was hydrated from a phospholipid film containing 95 mole % DOPC and 5 mole % dansyl-gramicidin.

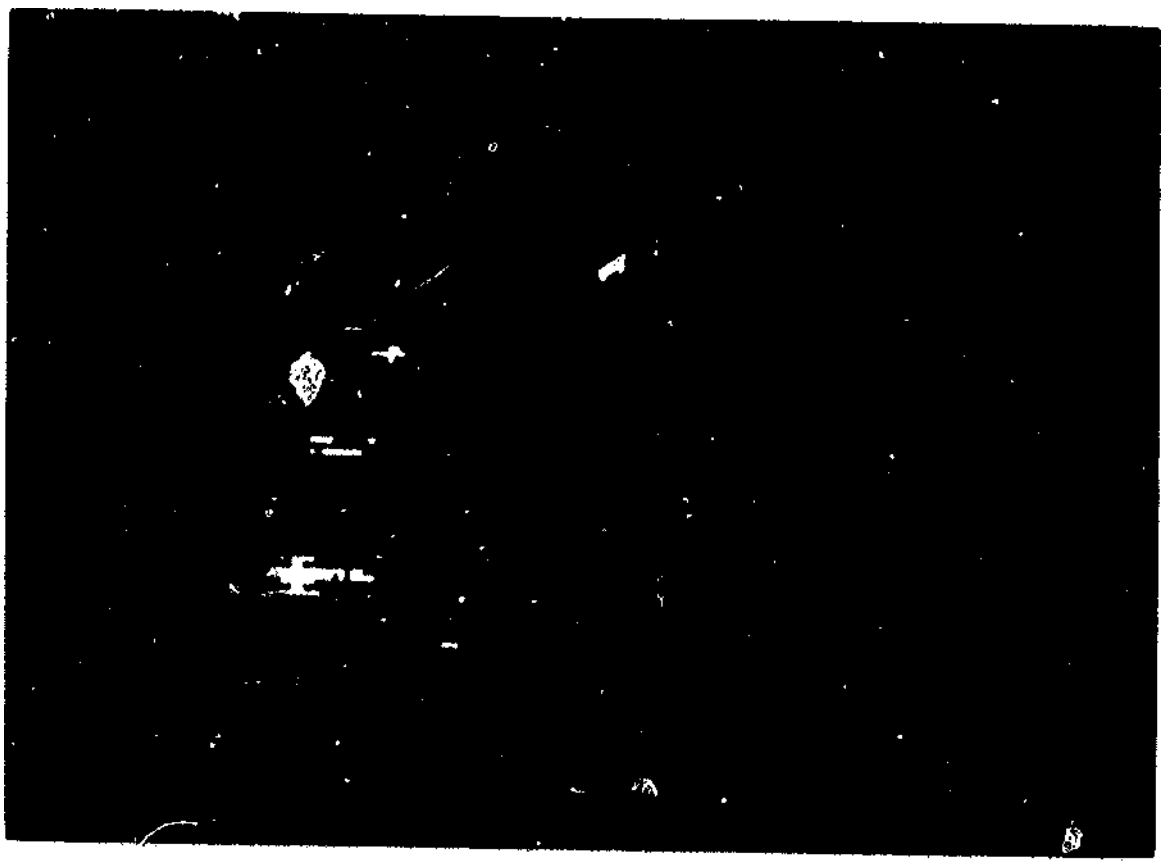


FIGURE 6:

Fluorescence image of a vesicle taken under parallel (A) and perpendicular (B) light and the computer generated ratio of the two images (C) following 24 hour exposure of the vesicle preparation to 0.5 mM $PrCl_3$. The vesicle was hydrated from a phospholipid film containing 90 mole % DOPC, 9 mole % DOPA, and 1 mole % DUA-PA.

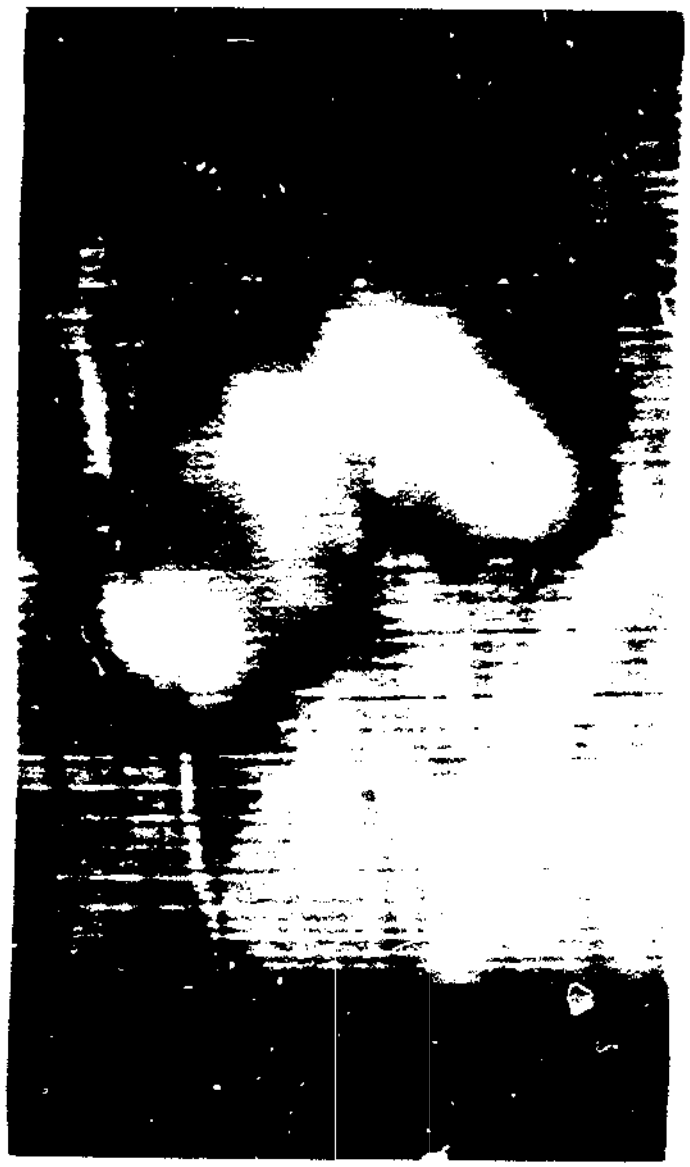


FIGURE 7:

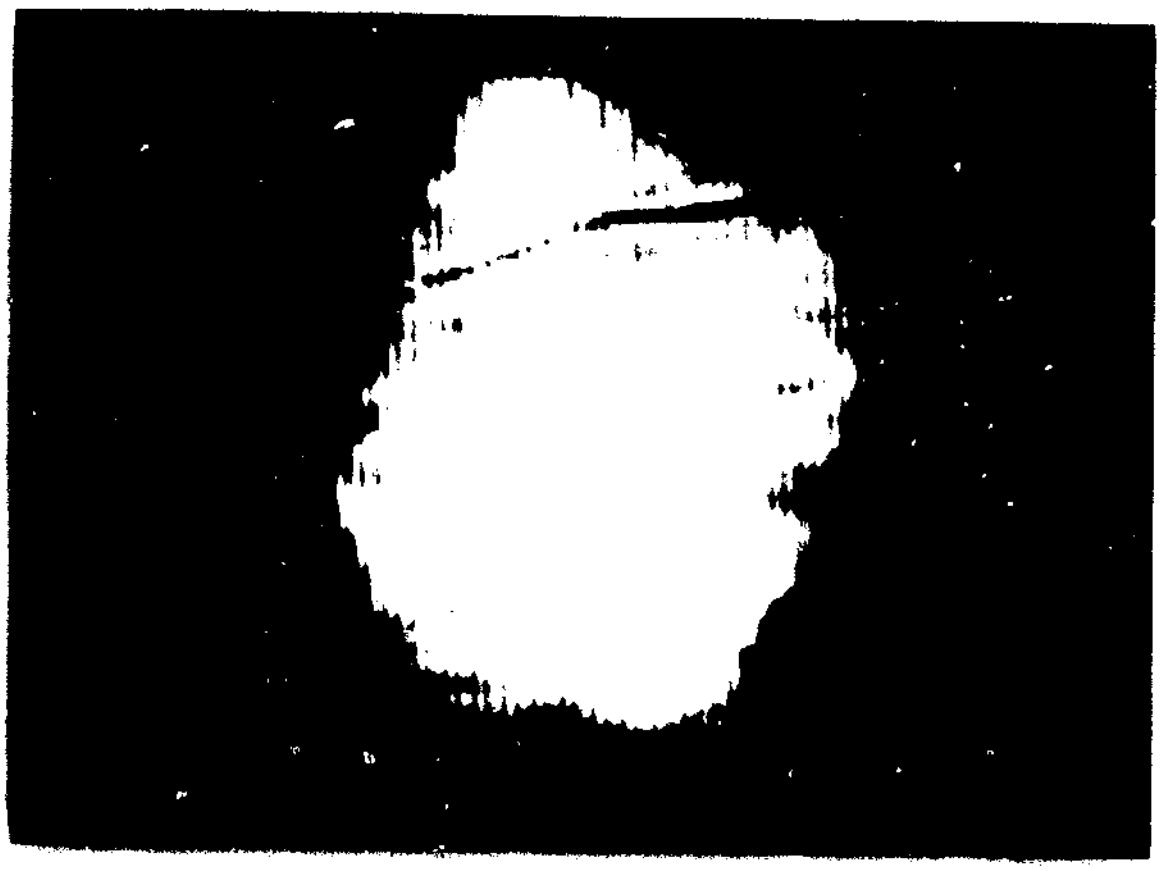
Fluorescence image taken under parallel light (A) and ratioed image (B) of a vesicle composed of DOPC, DOPA, DUA-PA, and gramicidin folllowing 24 hour exposure to 2.0 mM $CaCl_2$. The vesicle was hydrated from a phospholipid film composed of 85 mole % DOPC, 9 mole % DOPA, 1 mole % DUA-PA and 5 mole % gramicidin.

FIGURE 8:

Fluorescence image taken under parallel light (A) and ratioed image (B) of a vesicle composed of DOPC, DOPA, and dansyl gramicidin following 24 hour exposure to 0.5 mM $PrCl_3$. The vesicle was hydrated from a phospholipid film composed of 85 mole % DOPC, 10 mole % DOPA and 5 mole % dansyl-gramicidin.

FIGURE 8

A



B

FIGURE 9:

Comparison of polarization values over time. (——) vesicles composed of 99 mole % DOPC and 1 mole % DUA-PC. (---) vesicles composed of 90 mole % DOPC, 9 mole % DOPA, and 1 mole % DUA-PA. Polarization values were determined using an SLM 8000 spectrofluorometer as outlined in the methods section. PRE indicates vesicles before any additions, ZERO indicates immediately after the addition of 0.5 mM $PrCl_3$ to the cuvette and 20 indicates 20 hours after the addition of the cation.

FIGURE 9

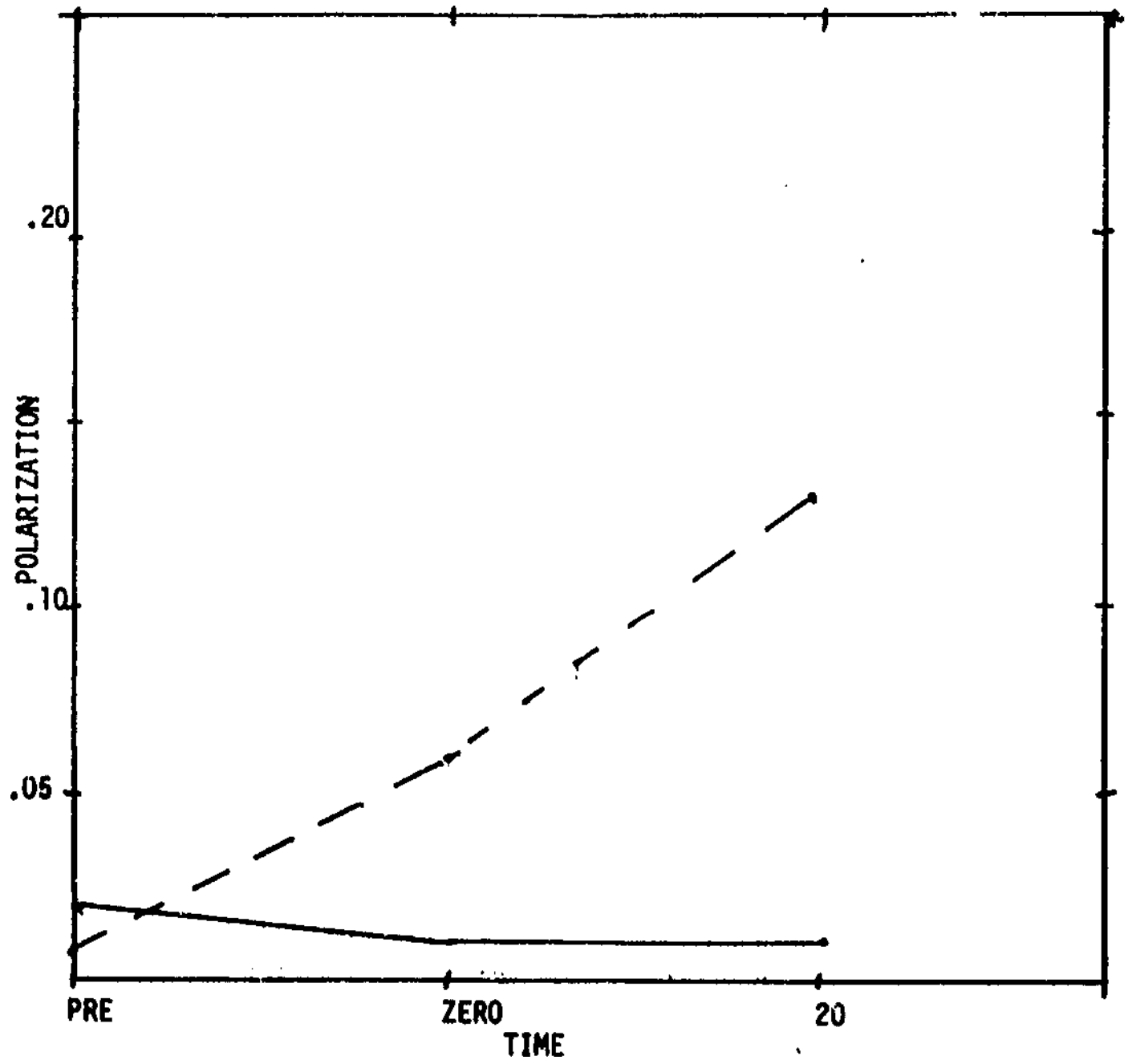


FIGURE 10:

Comparison of polarization values over time. (——) vesicles composed of 94 mole % DOPC, 1 mole % DUA-PC and 5 mole % gramicidin. (---) vesicles composed of 85 mole % DOPC, 9 mole % DOPA, 1 mole % DUA-PA and 5 mole % gramicidin. PRE indicates vesicles before any additions, ZERO indicates immediately after the addition of 0.5 mM $PrCl_3$ to the cuvette and 20 indicates 20 hours after the addition of the cation.

FIGURE 10

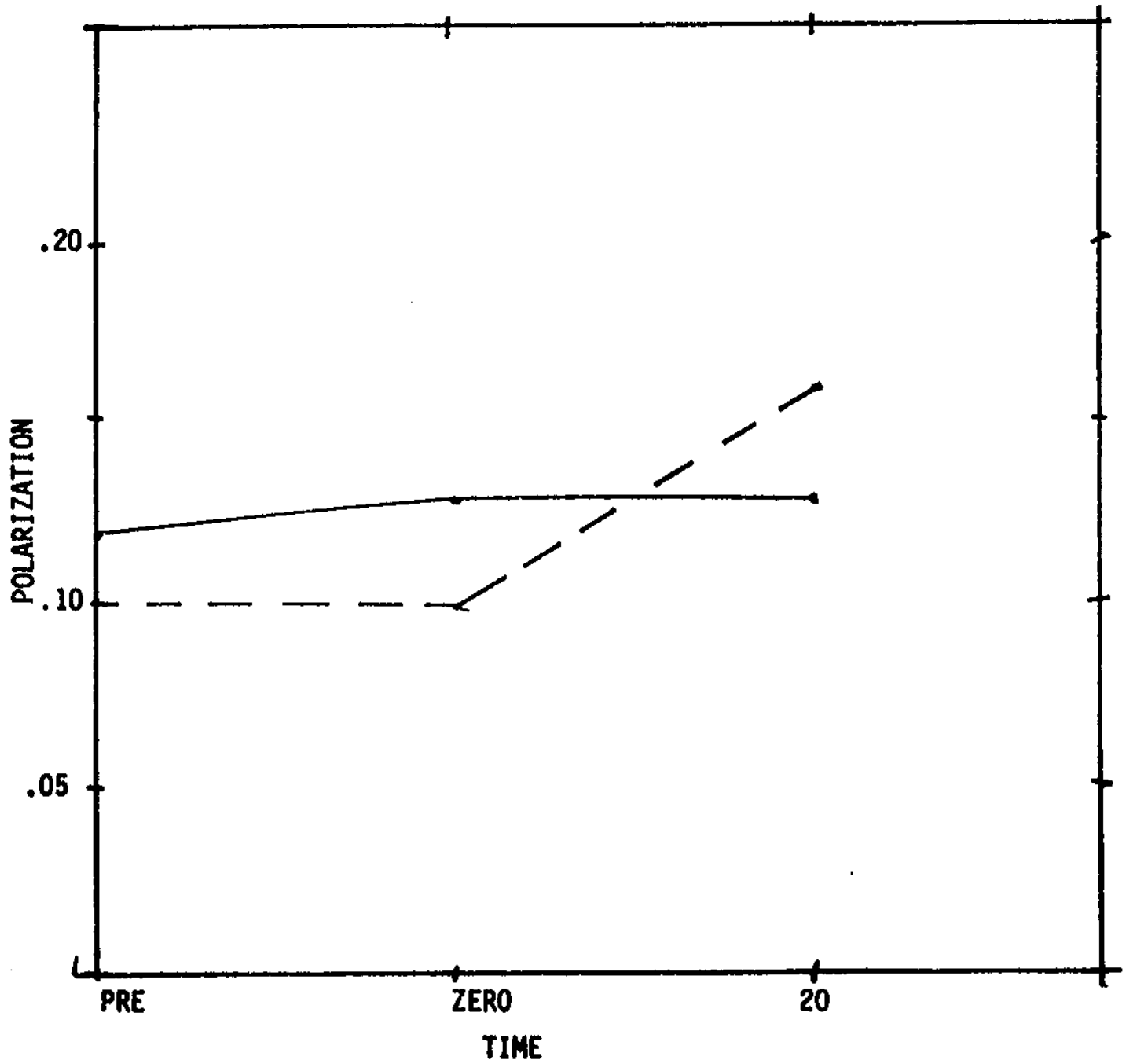
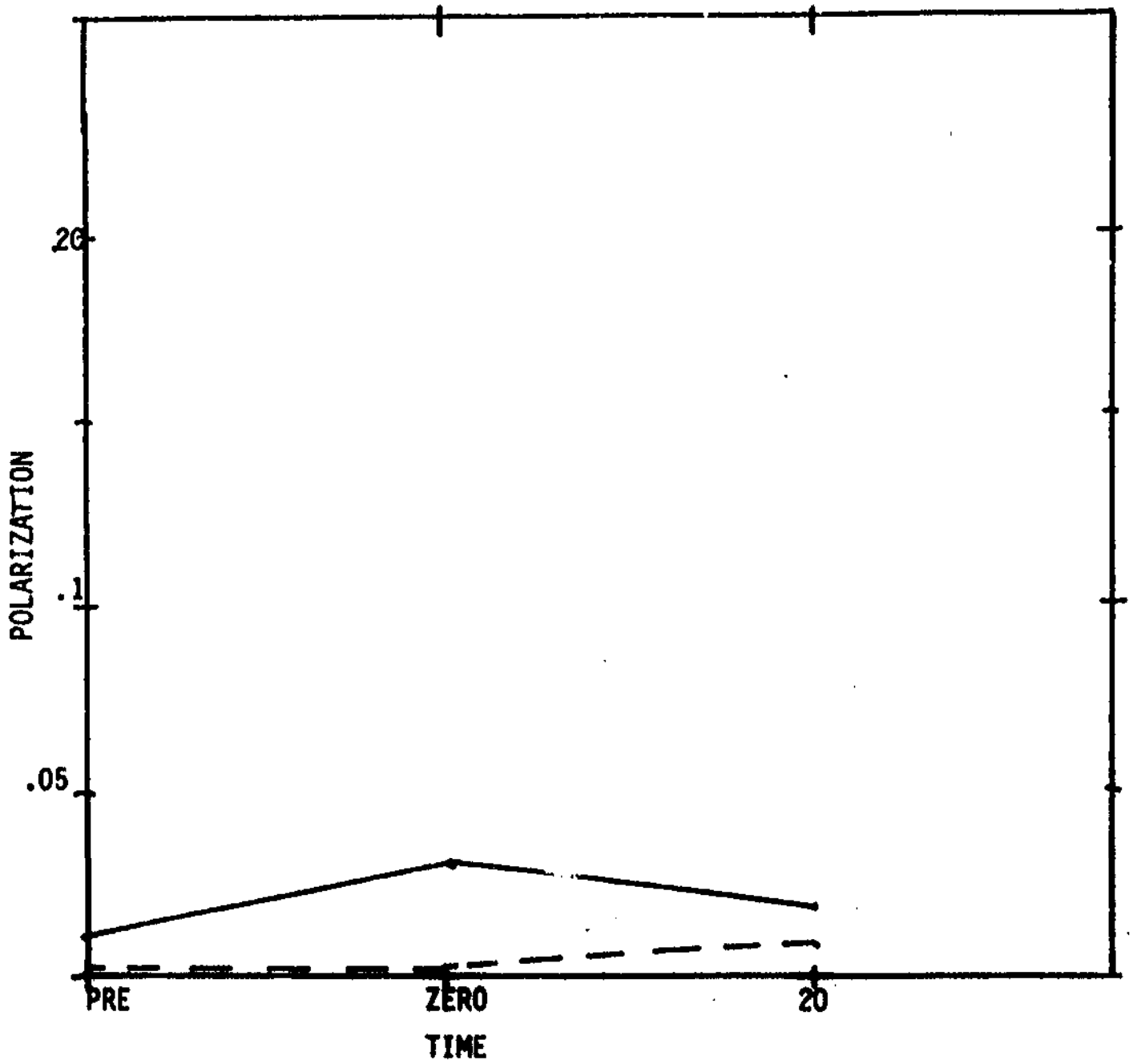


FIGURE 11:

Comparison of polarization values over time. (——) vesicles composed of 95 mole % DOPC and 5 mole % dansyl-gramicidin. (---) vesicles composed of 85 mole % DOPC, 10 mole % DOPA, and 5 mole % dansyl-gramicidin. PRE indicates vesicles before any additions, ZERO indicates immediately after the addition of 0.5 mM $PrCl_3$ to the cuvette and 20 indicates 20 hours after the addition of the cation.

FIGURE 11



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