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

Theodore M. Wynnychenko

ENTITLED Synthesis and Purification of Dehydroergosterol

and Preliminary Studies with Model Biological Membranes

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Degree of Bachelor of Science in Biochemistry

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Instructor in Charge

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HEAD OF DEPARTMENT OF Biochemistry

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Synthesis and Purification of Dehydroergosterol and Preliminary Studies with Model Biological Membranes

by

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ABBREVIATIONS

DUA	N-(5-dimethylaminonaphthalene-1-sulfonyl)amino-undecanoic acid
NBD	1-acyl-2-[N-(nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl]
PA	Phosphatidic acid
PC	Phosphatidylcholine
DOPA	Dioleoylphosphatidic acid
DOPC	Dioleoylphosphatidylcholine
NBD-PA	NBD-phosphatidic acid
NBD-PC	NBD-phosphatidylcholine
DUA-PA	DUA-phosphatidic acid
DUA-PC	DUA-phosphatidylcholine
Ergosterol	$\Delta^{5,7,22}$-Ergostatrien-3ß-ol
Dehydroergosterol	$\Delta^{5,7,9(11),22}$-Ergostatetraen-3ß-ol

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SYNTHESIS AND PURIFICATION OF DEHYDROERGOSTEROL

Introduction

Fluorescence microscopy with digital image processing is currently being used to investigate membrane structure (8). When a fluorescent probe is attached to a membrane component, such as phosphatidylcholine, phosphatidic acid, or a membrane protein, and the component is then incorporated in a lipid bilayer, monitoring the relative fluorescence intensity of the probe throughout the bilayer can provide one with detailed information as to the structure of the lipid bilayer, or membrane (8).

Previous work carried out in the laboratory has been almost exclusively involved with labeling a phospholipid with a fluorophore and then monitoring the fluorescence with fluorescence microscopy as a means of determining the distribution the phospholipid. However, the biological membrane contains many other components, for example, cholesterol and proteins, all of which are involved in determining its structure, and thus, its function. Proteins, just as phospholipids, can be labeled with fluorescent probes, without seriously degrading their natural behavior. This is the case because proteins and phospholipids are large enough that a small fluorescent probe does not seriously perturb their structure. However, this would not be case with cholesterol, where the probe and cholesterol would be similar in size. Thus, labeling cholesterol with an exogenous fluorescent probe would seriously hamper a molecule like cholesterol in behaving naturally, and would, therefore, be useless for studying cholesterol naturally present in biological membranes.

A way to avoid this problem is to use a compound that is naturally fluorescent and behaves similarly to cholesterol. Dehydroergosterol, $\Delta^{5,7,9(11),22}$-Ergostatetraen-3ß-ol (Figure 1), is such a compound. It is not a naturally occurring steroid, but has been found to behave comparably to cholesterol in biological membranes (11). Structurally, dehydroergosterol is very similar to

cholesterol (Figure 2), and the fact that it contains conjugated π-bonds which allow it to fluoresce naturally make it an ideal choice for use in studying the distribution of cholesterol in model biological membranes with fluorescence microscopy.

FIGURE 1: Dehydroergosterol

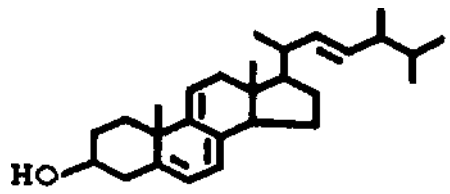
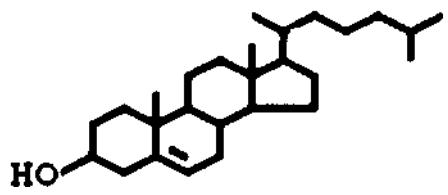
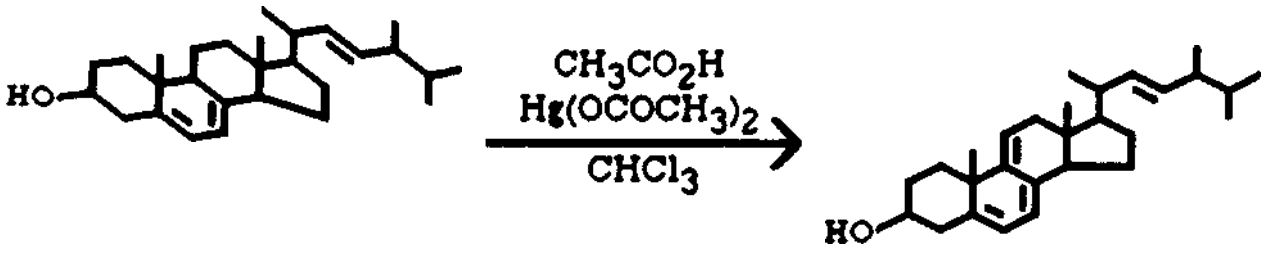


FIGURE 2: Cholesterol



Dehydroergosterol can be synthesized from ergosterol, a naturally occurring sterol in plants. by dehydration (Figure 3) (1). The crude product is then purified using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). This compound has not been previously synthesized in the laboratory.

FIGURE 3: Synthesis of dehydroergosterol from ergosterol.



Materials and Methods

Ergosterol was purchased from Sigma Chemical Company (St. Louis, MO). Dehydroergosterol was synthesized directly by the procedure given by Smutzer (1), a modification of the procedure originally presented by Windhaus (2).

2 grams of ergosterol were placed in a 50 ml reaction vessel. This was then dissolved in 17 ml of glacial acetic acid and 20 ml of chloroform. To this solution was added 3 grams of mercuric acetate. The reaction was allowed to proceed in the dark for 24 hours under a nitrogen atmosphere. The mixture was kept at 37-40°C with constant stirring for the duration of the reaction. After completion, mercurous acetate was removed by filtering the mixture twice. The mixture was then concentrated by rotary evaporation to 5 ml. This final volume was then recrystalized in ethanol, filtered, and washed with ice-cold ethanol. The product was then dissolved in ether and filtered thr ugh Whatman No. 1 filter paper. The filtrate was placed under a stream of nitrogen to remove the ether. When the product was dry, it was again recrystalized in ethanol, this time allowing the recrystallization to proceed overnight at -5°C. The product was then dried overnight under house vacuum. This crude product was then resuspended in chloroform and stored in the dark under nitrogen at -5°C.

The crude product was then purified by high performance liquid chromatography (HPLC), by a procedure similar to that given by Fischer et al. (3). A 10 µl sample of the crude product in chloroform was injected onto a reverse phase C-18 preparatory HPLC column (10.0 mm ID x 250 mm). The column was equilibrated with methanol/acetonitrile (75:25 v/v). A flow rate of 5.0 ml/min was used. The elution from the column was followed by monitoring absorbance at 328 nm, and the single peak at 12 minutes was collected, and stored in the dark under nitrogen. A number of HPLC runs were performed at one time (approximately 10), to obtain a sufficient quantity of dehydroergosterol. When the HPLC runs were completed, the fractions obtained were combined.

The dehydroergosterol was then extracted into chloroform by using a slightly modified version of the procedure given by Bligh and Dyer (4). To 5 ml volume of the methanol/acetonitrile (3/1 v/v) solution was added 3 ml of water. The solution was shaken. Then 3 ml of chloroform were added, and the solution was again shaken. The solution was then spun in a desktop centrifuge for approximately 10 minutes. The top, polar, phase was drawn off. 6 ml more water was then added to lower, non-polar, phase, mixed, and spun for 10 minutes in a desktop centrifuge. The top phase was again drawn off, and the chloroform phase was then dried down under nitrogen to approximately one third original volume (approximately 1 ml).

This solution was then checked for purity by obtaining a fluorescence emission spectra. The sample was excited at 327.8 nm at emission was monitored from 340 nm to 540 nm. The presence of a intense peak at approximately 400 nm was indicative of remaining contamination.

Therefore, the sample was further purified by thin layer chromatography (TLC). The product in chloroform was applied to a 250 µm Silica Gel G TLC plate which had been pre-run in acetone. The plate was run with hexane/diethylether/glacial acetic acid (60/20/1 v/v/v). Two fluorescent bands were observed. Staining the plate with iodine, in order to detect the presence of other bands with double bonds, revealed no additional bands. The bands where scraped and collected. The silica was then washed with at least 30 ml of chloroform/methanol/water (50/100/5 v/v/v). The solution was added to the silica, thoroughly mixed, and then spun down for 5 to 10 minutes in a desktop centrifuge. The washes were collected and the product was drawn into a chloroform phase by the procedure given by Bligh and Dyer (4).

This final product was then checked for purity by fluorescent excitation and emission spectra, and absorption spectra. Furthermore, a sample was reinjected onto a 4.6 mm ID by 250 mm reverse phase C-18 HPLC column, equilibrated with acetonitrile/methanol (1/3 v/v), and followed by monitoring absorbance and fluorescent emission.

Results and Discussion

The sample was checked for purity by obtaining fluorescence emission and excitation spectra. For excitation spectra, emission at 380 nm was monitored, while the sample was excited from 200 nm to 360 nm, while for emission spectra the sample was excited at 320 nm, and emission was monitored from 340 nm to 550 nm. Spectra were corrected with rhodamen, to correct for variations in lamp output. These spectra (Figure 4 and Figure 5) were identical to those previously published (1,5). The absorbtion spectra of the product was also taken, and it was identical to previously published results (Figure 6) (3). The absorption maximum was seen at 326 nm, while maximum excitation was seen at 324.5 nm. These maxima are almost identical, and their difference is probably due to the instrumentation used to obtain the spectra.

Samples were also injected onto a C-18 reverse phase HPLC with an inner diameter of 4.6 mm. A flow rate of 1.0 ml/min of acetonitrile/methanol (1/3 v/v) solvent was used (3). Monitoring the sample by fluorescent emission at 378 nm after excitation at 327 nm, resulted in a single large peak at approximately 9 minutes. Furthermore, monitoring by absorbance at 280 nm and 324 nm, also resulted in single peaks at approximately 9 minutes (Figures 7, 8, and 9). These wavelenghts were chosen because they are they represent the maximum absorption of ergosterol and dehydroergosterol, respectively. Thus, monitoring the sample at 280 nm, the absorption maximum for ergosterol, not dehydroergosterol, and only seeing one peak for dehydroergosterol, indicates the absence of ergosterol.

Finally, by taking the ratio of the solution's absorbance at 282 nm to its absorbance at 324 nm, and consulting a standard curve provide by Fischer, et al., it is possible to determine the amount of $\Delta^{5,7}$-sterol contamination present in the final product (3). This ratio for the product after the HPLC separation was 0.55, or approximately 85 mole % dehydroergosterol; while it was 0.22 for the product after separation by TLC, or approximately 100 mole % dehydroergosterol.

After purification the concentration of dehydroergosterol was determined by reading the

absorbance of the product in chloroform at 327.8 nm. Using this and the published value of the extinction coefficient, 11200 $M^{-1}cm^{-1}$, the concentration of dehydroergosterol in chloroform was easily obtained (1).

The actual synthesis of dehydroergosterol was relatively easy to accomplish. However, the purification of the reaction mixture was not. This was primarily due to the fact that the product, dehydroergosterol, differed from the reactant, ergosterol, by only one double bond in the 9 position on the sterol ring. After several attempts with varying methods, it was determined that the only method of effectively separating product from reactant was by HPLC, as the structural difference that was being exploited to achieve this separation was not a major one. The HPLC method used was similar to that presented by Fischer (3). This method is very time consuming and relatively expensive, but is needed in order to assure complete separation of the dehydroergosterol from the ergosterol.

However, use of the time consuming HPLC procedure brought on another problem. As the product was being separated, it was exposed to an oxygen atmosphere, which degraded the dehydroergosterol. The change here was more pronounced, and it was found that using thin layer chromatography with a hexane/diethylether/glacial acetic acid (60/20/1 v/v/v) separated the product into two bands, with R_f values of 0.21 and 0.12. The band at the R_f of 0.12 fluoresced with a bluish tint, while the one at $R_f = 0.21$ fluoresced with a greenish tint. Furthermore, iodine staining was more intense in the band at $R_f = 0.12$. Fluorescence emission spectra of the two bands confirmed the band at $R_f = 0.12$ to be the pure dehydroergosterol. The band at $R_f = 0.21$ is believed to be oxidized dehydroergosterol, but this was never confirmed.

With the highly pure dehydroergosterol of known concentration, it is now possible to examine the role of cholesterol in a biological membrane, a previously unstudied area in our laboratory. The following section of this paper details preliminary investigations with this fluorescent probe.

PRELIMINARY STUDIES WITH DEHYDROERGOSTEROL IN MODEL BIOLOGICAL MEMBRANES

Introduction

The fluid mosaic model of a biological membrane proposed by Singer describes the membrane as a random sea of phospholipids which are free to move laterally (6, 7). Inserted in this sea are proteins which are also free to diffuse laterally. However, recent studies have shown that the membrane is not completely homogeneous, and that phospholipids and proteins are sequestered into different domains within the membrane.

Recently, fluorescent microscopy has been used to visualize the formation of phospholipid domains in vesicles (8). It was found that in vesicles made with 5% fluorescently labeled phosphatidic acid (NBD-PA) in 95% phosphatidylcholine (PC), a domain formed in the membrane upon the addition of 2 mM Ca^{2+}. This was visualized by the creation of an area in the membrane of increased fluorescence intensity. It is believed that the positively charged cation is bridging the negatively charged acidic head groups of the phospholipid. This imposes order onto the previously random membrane, creating two distinct domains in the vesicles, one rich in phosphatidic acid and one rich in phosphatidylcholine. Furthermore, it was determined, through polarization studies, that the area which was high in phosphatidic acid was more rigid than the phosphatidic acid poor area. This conclusion was reached by observing an increase in the polarization of the fluorophore in the region of the patch, which indicates that the fluorophore was in an environment that restricted random movement of the fluorophore, which, in turn, suggested a less fluid area of the membrane (9).

Studies were also carried out in vesicles that contained 65% PC, 5% NBD-PA, and 30% cholesterol (8). In these vesicles, the formation of areas of increased fluorescence intensity was

also observed, indicating patch formation. However, there was no information concerning the role of cholesterol in these vesicles. Through the use dehydroergosterol, one would be able to directly observe the role of cholesterol in patch formation in ideal biological membranes (i.e., whether the cholesterol was sequestered into the phosphatidic acid domain, excluded from the domain, or unaffected by the domain).

Further studies with dehydroergosterol can be carried out in vesicles with different chain lengths, and with non-homogeneous chain lengths (vesicles made with phospholipids extracted from egg yolk, for example). These would all be valuable in determining the behavior of cholesterol in biological membranes.

The results presented here indicate a beginning into the studies that can be carried out with dehydroergosterol. When speaking of membrane structure, it is clearly necessary to include all membrane components, in order to understand the behavior of the membrane under physiological conditions. Dehydroergosterol presents a relatively easy method of studying the behavior of cholesterol in the biological membranes, allowing one to obtain a fuller understanding of domain formation in membranes and overall membrane structure.

Materials and Methods

Dioleoylphosphatidic acid (DOPA), dioleoylphosphatidylcholine (DOPC), and their fluorescently labeled derivatives, NBD-PA, NBD-PC, DUA-PA, and DUA-PC, were synthesized by previously developed procedures from our laboratory.

Large unilamellar vesicle were made by taking a total of 250 nmoles of lipid and dehydroergosterol in 0.5 ml water, as per the procedure given by Darszon et al. (8).

Images of the vesicles were taken by an epifluorescence microscope, attached to a charged coupled device camera and digital image processor, as described by Haverstick and Glaser (8). Images were captured by averaging 75 frames of a vesicle, taken at a rate of 30 frames per second. The digital processor then applied a pseudo color operation onto the images, which applies color to the black and white image as a function of fluorescent intensity. Very low intensity areas of the image are given a blue color, and as the intensity increases, the color changes to green then yellow, with increasing intensity, and, the most intense areas of the image, are given a bright red color. All figures of vesicles presented here are photographs taken directly from the video monitor with a camera equipped with a 90 mm lens.

Results and Discussion

Preliminary studies were undertaken with dehydroergosterol. These studies were simply attempts to visualize the dehydroergosterol with our fluorescent microscope. While this seems rather basic, it was very necessary. First, the emission maximum of dehydroergosterol is at approximately 376 nm, a wavelength of light that is very far into the blue region of the visible spectra, if not into the ultraviolet. Secondly, the microscope did not have built in filter settings that would allow the dehydroergosterol to be excited at its maximum and pass its maximum emission. Finally, energy in this range is readily absorbed by glass. Thus, the glass cover slips that were used to cover the vesicles could be responsible for absorbing a significant amount of the already faint fluorescent emission.

Many different filter configurations were tried to bring about the brightest possible emission from the dehydroergosterol. In the end, it was determined that the best solution available was to use filter block 2 of the microscope, which would permit exciting light down to 345 nm to strike the vesicles, and then allow emitted light over 430 nm to pass to the camera, even though the excitation and emission maxima of dehydroergosterol are 327 at 376 nm, respectively. It was also found that the use of quartz cover slips also improve the signal obtained, and that the lens should emersed in glycerol, and not in the oil that was previously used, when it was found that the oil absorbed light below 400 to 450 nm very effectively. Both of these changes increased the fluorescence seen in the dehydroergosterol vesicles; however, these vesicles were still dim to the naked eye, and difficult to observe. This dimness is due to, in a large part, the fact that the excitation of, and emission from, the dehydroergosterol are not at their maxima, and substantial increases in fluorescence intensity could still be seen by obtaining filters which allowed the passage of light closer to the excitation and emission maxima of the fluorophore.

Once the best configuration of the microscope was obtained, studies were begun to observe the role of dehydroergosterol during phosphatidic acid domain formation upon the addition of Ca^{2+}

(2). Originally, it was hoped that the vesicle could be labeled with both the fluorescent dehydroergosterol and a fluorescently tagged phospholipid. The advantage of this is readily apparent. With double labeled vesicles, one could directly observe the formation of the lipid domain, and then compare this to any changes in dehydroergosterol distribution. In our laboratory, there are two common fluorescent labels employed for phospholipid, NBD and DUA. The emission spectra of DUA is very similar to the dehydroergosterol, and, thus, would not be very effective for use with dehydroergosterol, as the emissions from these two compounds could not be distinguished. However, NBD labeled phospholipids, with a maximal excitation at approximately 460 nm and maximal emission at approximately 520 nm, were hoped to be used with the dehydroergosterol.

Toward this end, the first determination made was to confirm that the emissions from the two fluorophores were distinguishable. Images were obtained of vesicles containing 5 mole % dehydroergosterol in DOPC; 5 mole % NBD-PA in DOPC; and 5 mole % dehydroergosterol with 5 mole % NBD-PA in DOPC (Figure 10). These images were obtained under filter blocks 2 and 4 of the fluorescent microscope. As has been previously discussed, filter block 2 gave the best results for dehydroergosterol, while filter block 4, which permits exciting light of 350-460 nm to reach the stage, and passes excited light above 515 nm, has been used for the monitoring of NBD labeled phospholipids. As can be seen in figure 10, the vesicle containing only NBD-PA is clearly visible with filter block 4 set, and not at all visible with filter block 2. The reverse is true for vesicle labeled with dehydroergosterol; they are clearly visible under filter block 2, but not visible under filter block 4. Finally, as would be expected, a vesicle labeled with both dehydroergosterol and NBD can be seen with both filter blocks 2 and 4. This shows that the microscope is able, through the use of the different filter blocks, to distinguish between dehydroergosterol emission and NBD emission. From this point, fluorescence obtained through filter block 2 will be referred to as fluorescence from the dehydroergosterol position, and the fluorescence obtained through filter

block 4 as fluorescence from the NBD position.

These preliminary investigations showed that dehydroergosterol was bleached relatively quickly, and that care would have to be taken to make sure that, later, when a vesicle was found to study, it was exposed to the high intensity light of the microscope for as short a time as possible.

However, while carrying out these experiments, it was observed that the vesicles that contained both dehydroergosterol and NBD appeared brighter under the dehydroergosterol position, than the vesicles which contained only dehydroergosterol. There was no noticeable difference in the intensities of vesicles, whether or not they contained dehydroergosterol, under the NBD position. This seemed to suggest that there might be something other than simple emission happening under the dehydroergosterol position when both fluorophores were present in the vesicle. Fluorescence emission spectra of vesicles containing only 4 mole % dehydroergosterol in DOPC, and vesicles containing both 4 mole % dehydroergosterol and 4 mole % NBD-PA in DOPC, at various exciting wavelengths were taken (Figures 11, 12, 13, 14, 15, 16, 17, and 18). The emission spectra were taken with the fluorescence microscope in mind. Thus, vesicles were excited at 340 nm and 380 nm, while watching emission above 430 nm, duplicating the excitation and emission characteristics of the dehydroergosterol under the dehydroergosterol position (filter block 2). Similarly, excitation at 405 nm and 460 nm, with emission above 510 nm, duplicated the conditions under filter block 4. Figures 19 and 20, the excitation and emission spectra of NBD in chloroform are provided as a reference.

From these spectra, it is apparent that in the vesicles that contain both dehydroergosterol and NBD labeled phospholipid there is significant, if not complete, energy transfer from the excited dehydroergosterol to the NBD label on the phospholipid. This energy transfer is not particularly suprising after considering the fact that the emission spectra of dehydroergosterol overlaps with the excitation spectra of NBD and that the two fluorophores are very close to one another in a relatively restricted environment. The energy transfer also explains why the NBD labeled vesicles

were brighter under the dehydroergosterol position, than the vesicles which only contained dehydroergosterol. The double labeled vesicles are emitting a significant percentage of their light at higher wavelengths, which are more easily seen, and thus appear brighter, than the vesicles that emit all of their light near the ultraviolet end of the visible spectrum.

At first, such significant energy transfer from the dehydroergosterol to the NBD would appear to indicate that both probes cannot be used if data on the distribution of dehydroergosterol is wanted, because even while using filter block 2 (the dehydroergosterol position), one would actually be visualizing the emission from NBD. However, after further consideration, the energy transfer from dehydroergosterol to NBD does not completely destroy the possibility of using both probes together to obtain data on the distribution of dehydroergosterol in the biological membrane. Even though both the dehydroergosterol position and the NBD position provide NBD emission, the origin of this emission is from different sources. While using the NBD position, NBD is being directly excited, and is then emitting light, allowing one to determine its distribution through the vesicle. But, if the vesicle is visualized under the dehydroergosterol position, the dehydroergosterol is being excited, transferring its energy to the NBD, which is then emitting; a completely different process from the one obtained under the NBD position.

This provides a way of monitoring both NBD and dehydroergosterol, in a limited case. Using energy transfer as a way of monitoring dehydroergosterol would only be valid if the NBD is leaving the area the dehydroergosterol is entering. For example, consider a vesicle with dehydroergosterol and NBD-PA. If, upon the addition of calcium, dehydroergosterol leaves the area into which the NBD-PA is being sequestered, one could visualize the formation of the PA region by directly monitoring NBD fluorescence. Then, if there is enough of the NBD-PA left outside the patch, one could visualize, under the dehydroergosterol position, an area in the vesicle's membrane where the fluorescent intensity is low. This area of lower intensity under the dehydroergosterol position would correspond to the area of increased intensity under the NBD

position. This would occur due to the method that NBD is being excited before it emits in each case. Under the NBD position, NBD is directly excited, so that NBD-PA's distribution in the vesicle can be determined form the image obtained. However, under the dehydroergosterol position, only NBD that is present in the area that contains dehydroergosterol would emit light. Thus, even though there is an area that has a high concentration of NBD label, if the excited dehydroergosterol is outside this area, then the emission directly from the dehydroergosterol and the emission from the NBD that received its energy from energy transfer from dehydroergosterol outside the area of the NBD-PA patch, would be more intense than emission from the high concentration of NBD in the patch, causing the NBD-PA rich region to appear less intense.

This is very dependent on the relative concentrations of NBD and dehydroergosterol. If the patch appeared in the same place under both the dehydroergosterol and NBD positions, one could not be sure of dehydroergosterol's distribution in the membrane. An increase in fluorescence of both labels appears in the same area, could be caused by actual sequestering of dehydroergosterol into the same region as the NBD, or by the incomplete exclusion of dehydroergosterol from the NBD area of the vesicle (i.e., even though the dehydroergosterol is leaving the NBD enriched area, this exclusion is not complete, and enough is remaining in the NBD region to make that region appear more intense than the rest of the vesicle).

Even with this ambiguity if the patch appears in the same area with both positions, the patch appearing in different areas under the two positions would clearly indicate that the dehydroergosterol is leaving the area with high NBD concentration. Thus, if the dehydroergosterol appears to be in the same area as NBD-PA, and it is believed that dehydroergosterol is being sequestered into this region, making vesicles with NBD-PC should show distinct regions for NBD and dehydroergosterol.

Vesicles were then made with 4 mole % dehydroergosterol, 4 mole % NBD-PA, and 92 mole % DOPC, to examine whether the dehydroergosterol was being excluded from the area of the

phosphatidic acid patch. Images were obtained at time 0 to 2 min, 15 min, and 25 min, under the dehydroergosterol and NBD positions for a number of vesicles (Figures 21 and 22). In figures 21, and 22, one can clearly see that after 15 minutes in the presence of Ca^{2+}, two distinct regions in the membrane form. By watching NBD emission, one sees the actual phosphatidic acid domain forming in the vesicles membrane. Then, by examining the vesicle under the dehydroergosterol position, one sees a region of increased fluorescence intensity that does not correspond to the area of the phosphatidic acid domain. This appears to indicate that the dehydroergosterol is leaving the area of the more highly rigid phosphatidic acid domain. Figure 23, is included to show the ambiguity that can arise with these double labeled vesicles. After 15 minutes, it appears as if the dehydroergosterol is being drawn into the area of the patch. However, this result is probably due to the fact that the vesicle was made with 7 mole % dehydroergosterol, and that, even though the majority of the dehydroergosterol was excluded from the phosphatidic acid domain, there was enough left there to cause significant excitation of the NBD-PA.

Vesicles were also made with 5 mole % dehydroergosterol, 5 mole % DOPA, and 90 mole % DOPC. These vesicles, as has been previously stated, are much harder to observe, but they can be found. Figure 24 is one such vesicle after addition of Ca^{2+}. One can see the formation of a domain in the vesicle that is excluding dehydroergosterol. This domain is comparable in size to the PA domains in other vesicles with the same percentage of PA. At this time, this event has not been observed in a large number of vesicles, but it provides further evidence that the dehydroergosterol is being excluded form the highly rigid PA domain.

It was also observed, that when very high concentrations of dehydroergosterol were used (30 mole %), vesicles were still difficult to detect by dehydroergosterol's emission. This indicated that dehydroergosterol might be self-quenching, and when present in these higher concentrations, the dim fluorescence that is seen at lower concentrations is lost. To test this, a series of experiments was done with the fluorometer.

First, quantitative data the emission of dehydroergosterol in chloroform at 378 nm after excitation at 327.8 nm was obtained. This data is presented in table 1, and is graphed in figures 25A and 25B. The data shows that dehydroergosterol is indeed a self quenching molecule although the effect is quite small. This is determined by the fact that as the concentration of dehydroergosterol is increased, there is not a corresponding linear increase in the intensity of emission. Instead, the increases in intensity become smaller with constant increases in concentration. The absorbance of the solution at the exciting wavelength (327.8 nm) was also checked and kept below 0.10. This was done to insure that fluorescence in the more concentrated samples was not being lost due to inner filter effects.

Furthermore, vesicles were made with 5 mole % DOPA, with increasing amounts of dehydroergosterol and decreasing amounts of DOPC. These vesicles were made by drying down the components, adding distilled water, vortexing the sample, and then sonicating it for 1 minute. The solution was then left undisturbed for 2 hours. The data obtained is presented in table 2, and graphed in figure 26. The fluorescence of the vesicles was obtained before and after addition of Triton X-100. Triton X-100 is able to disrupt the phospholipid bilayer, thus freeing its components. Therefore, the fluorescence observed before addition of Triton X-100 is from dehydroergosterol that is in the bilayer, while the fluorescence after is from dehydroergosterol in Triton X-100 micelles When dehydroergosterol is confined to the bilayer it has less space available to it and is more concentrated. Thus, when it is solubilized by Triton X-100, it becomes less concentrated, and less likely to self-quench. In Figure 26 an increase in fluorescence is seen after addition of Triton X-100, indicating that dehydroergosterol is indeed self-quenching. This increase is not an artifact produced by light scattering in solution, as the absorbances before and after the addition of Triton X-100 were the same (Table 2). Finally, there does not appear to be an increased quantum yield of dehydroergosterol in the Triton X-100 micelles. However, there may be a decreased quantum yield in the micelles, which would increase the change in flourescence observed

upon addition of Triton X-100, but more work is needed to confirm this.

After it was determined that the dehydroergosterol does quench, vesicles were made with 5 mole % dehydroergosterol, 5 mole % DOPA, and 90 mole % DOPC. These vesicles then had either 3 mM Ca^{2+} or distilled water added to them and they were watched over time in the fluorometer (Table 3 and Figure 27). The vesicles that had water added to them retained a constant, or nearly constant, fluorescence intensity over time, or a slightly diminished intensity due to bleaching of the fluorophore. Vesicles that had calcium added lost a significant amount of fluorescence, over the water controls. Again absorbances were determined to make sure they were below 0.10, and the absorbance change was determined to be below 0.030, to rule out light scattering in the solution as the cause of the decrease in fluorescence. The decrease in fluorescence in the vesicles to which calcium had been added, coupled with the knowledge that dehydroergosterol self quenches, provides further evidence that the dehydroergosterol is undergoing a change in the membrane, but does not provide information about whether it is being included or excluded from the phosphatidic acid domain.

This conclusion is reached by realizing that when the intensity of dehydroergosterol emission goes down, the dehydroergosterol must be quenching itself, which requires a higher concentration of dehydroergosterol. Thus, when the membrane is reorganized by calcium, the concentration of dehydroergosterol in the membrane must be going up. Since there has been no change in the amount of dehydroergosterol present, the only way to have an increase in concentration is by forcing what is already present to occupy less area. Therefore, the dehydroergosterol is being confined to a particular area, or domain, in the membrane.

Taken together, this data provides rather conclusive evidence concerning the distribution of dehydroergosterol after a phosphatidic acid domain has been formed with calcium. However, more work must be done to confirm these results and ensure that they are repeatable.

CONCLUSION

The results presented here are a beginning in the kinds of studies that can be done with dehydroergosterol, a very useful fluorescent probe for cholesterol. In fact, many studies have to been done before any final conclusions have can be drawn about cholesterol's role in the membrane. Some experiments that could be easily accomplished currently include changing the amounts and types of phospholipids in the membrane, as well as the concentration of cholesterol present in the membrane.

Knowledge of the structure and distribution of phospholipids and other components in the biological membrane could provide insight into many cellular processes that deal with the membrane, such as fusion of sperm and egg during fertilization, endocytosis in microbes, and exocytosis causing neurotransmitter release in axons. Dehydroergosterol provides a very efficient and direct means of studying one of these membrane components, and may provide great insight into the molecular mechanisms of membrane mediated cellular events.

Table 1: Quenching data

DATA SET 1

Concentration (mM)	Fluorescence (excitation 327.8 nm/ emission 378.0 nm)
0.00129	203.724
0.00193	352.853
0.00257	445.852
0.00321	541.549
0.00386	641.848
0.00450	719.352
0.00514	759.897

DATA SET 2:

Concentration (mM)	Fluorescence (excitation 327.8 nm/ emission 378.0 nm)
0.00257	508.735
0.00514	824.620
0.00771	1311.327
0.00154	1703.856

Table 2: Quenching in Vesicles Upon Addition of Triton X-100

Mole % Dehydroergosterol	Fluorescence Before Triton (327.8 nm/378.0 nm)	Fluorescence After Triton (327.8 nm/378.0 nm)
0.25	65.427	129.891
1.0	262.166	378.953
3.0	348.357	1008.731
5.0	937.706	1479.729
10.0	1490.437	2541.254

Mole % Dehydroergosterol	Absorbance 327.8 nm Before Triton	Absorbance 327.8 nm After Triton
0.25	0.022	0.014
1.0	0.023	0.016
3.0	0.103	0.084
5.0	0.100	0.111
10.0	0.259	0.262

Table 3: Quenching Upon Addition of Calcium

Time (minutes)	Vesicles with 0 mM Calcium Added (327.8 nm/378.0 nm)	Vesicles with 3 mM Calcium Added (327.8 nm/378.0 nm)
0	1741.426	1583.717
15	761.931	1329.998
30	632.074	1136.321

Figure 4:

Fluorescence excitation spectra of dehydroergosterol in ethanol. Emission was monitored at 380 nm. The maximum excitation is seen at 325.0 nm.

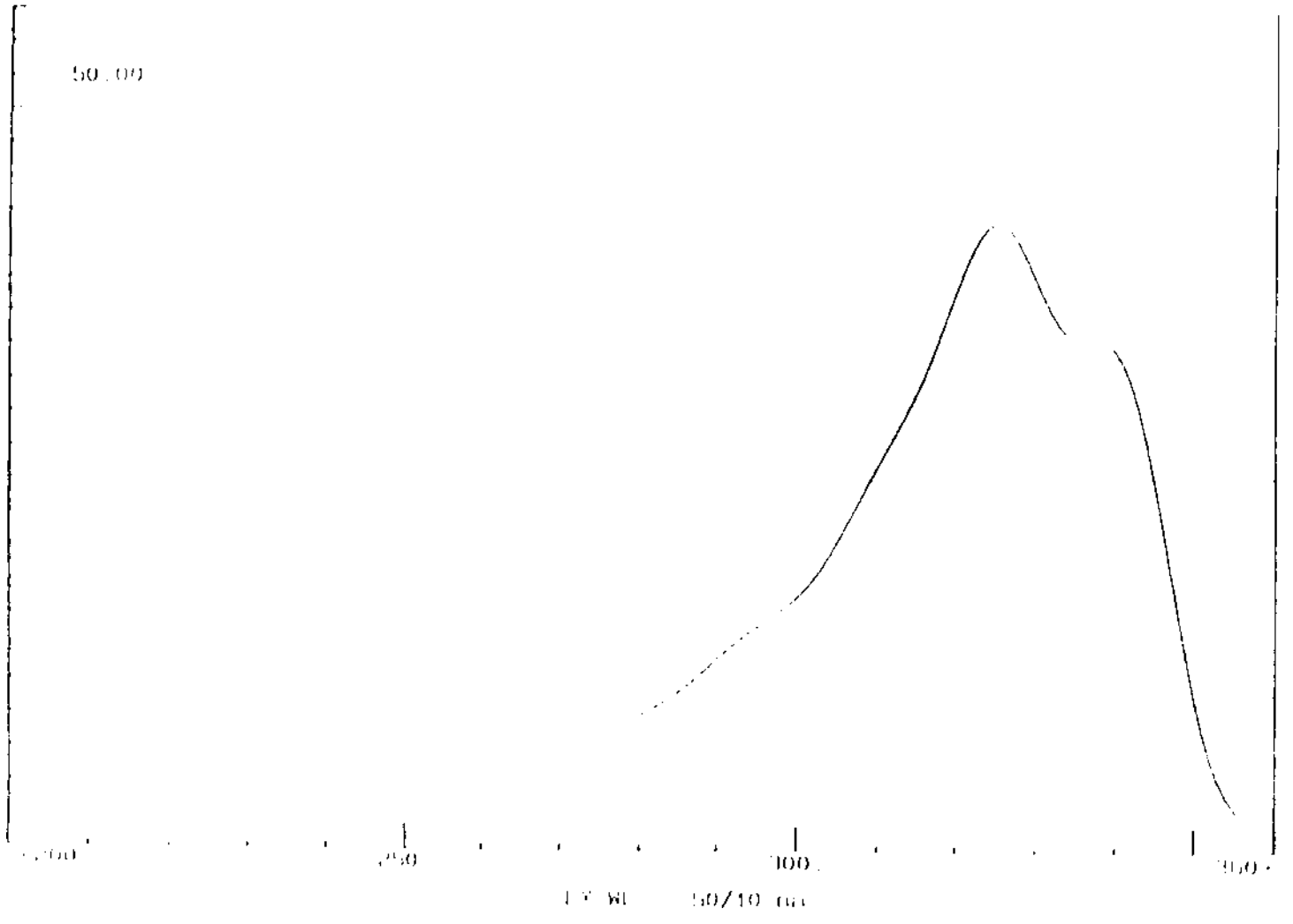


Figure 5:

Fluorescence emission spectra of dehydroergosterol in ethanol. Sample was excited at 320 nm. Maximum emission at 371 nm.

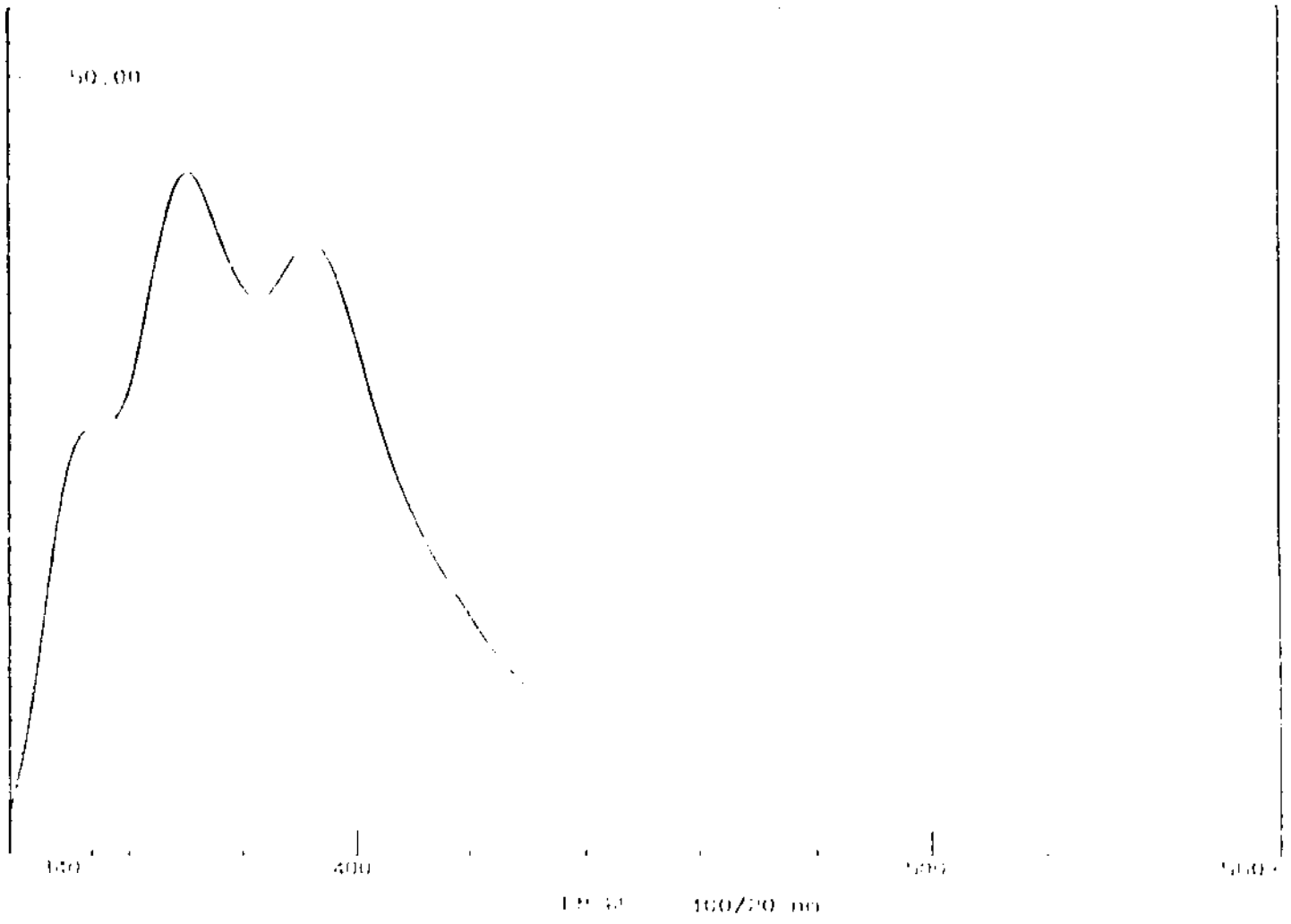


Figure 6:

Absorption spectra of dehydroergosterol in ethanol. The maximum absorbance is seen at 326 nm.

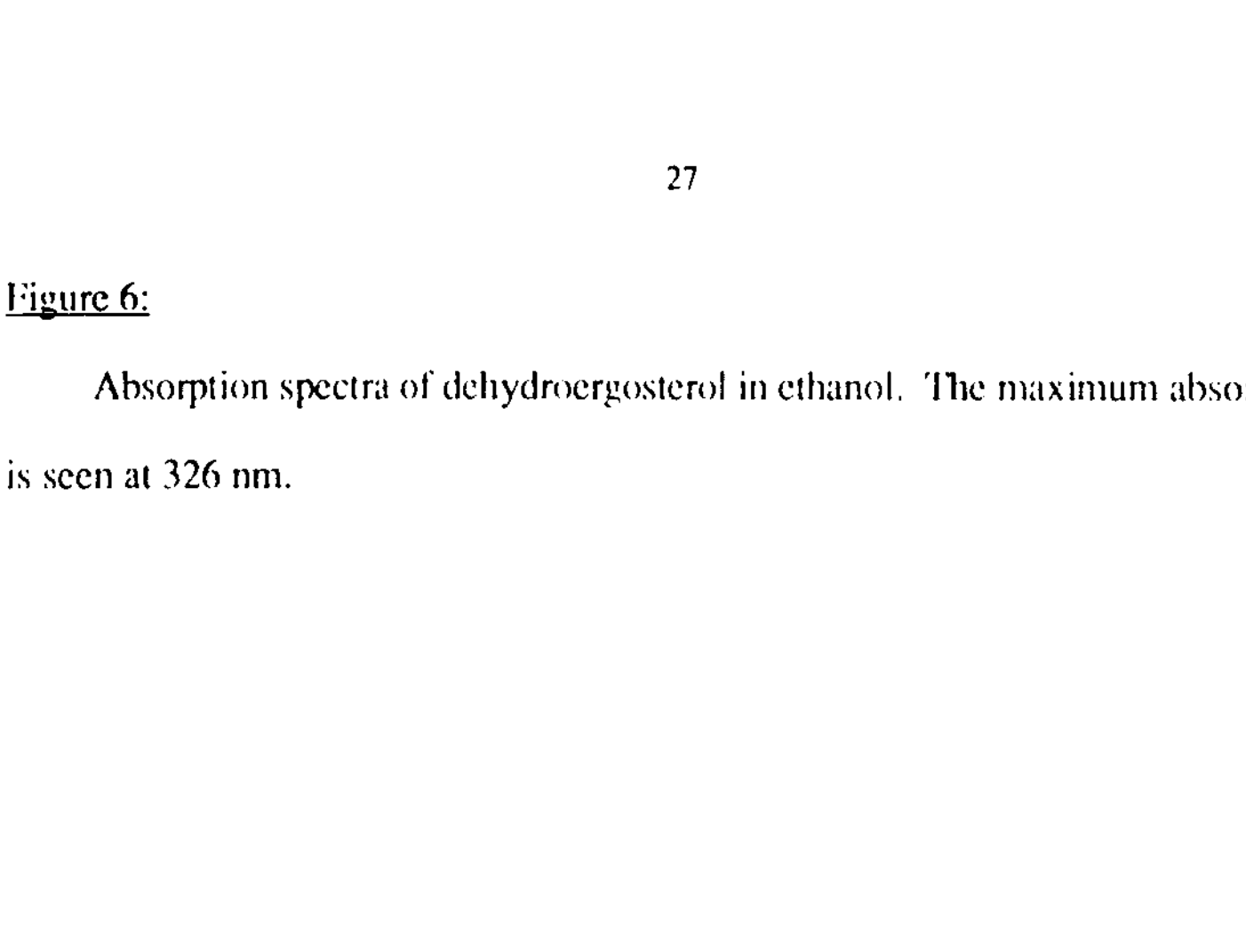


Figure 7:

Fluorescently monitored HPLC run of pure dehydroergosterol (4.6 mm inner diameter by 250 mm C-18 HPLC column, 10 µl injection, methanol/acetonitrile (3/1 v/v) solvent system, flow of 1.0 ml/min). Monitored fluorescently with excitation at 324 nm, and emission at 376 nm. The small peak at 14.11 min. is due to contamination in the chloroform solvent.

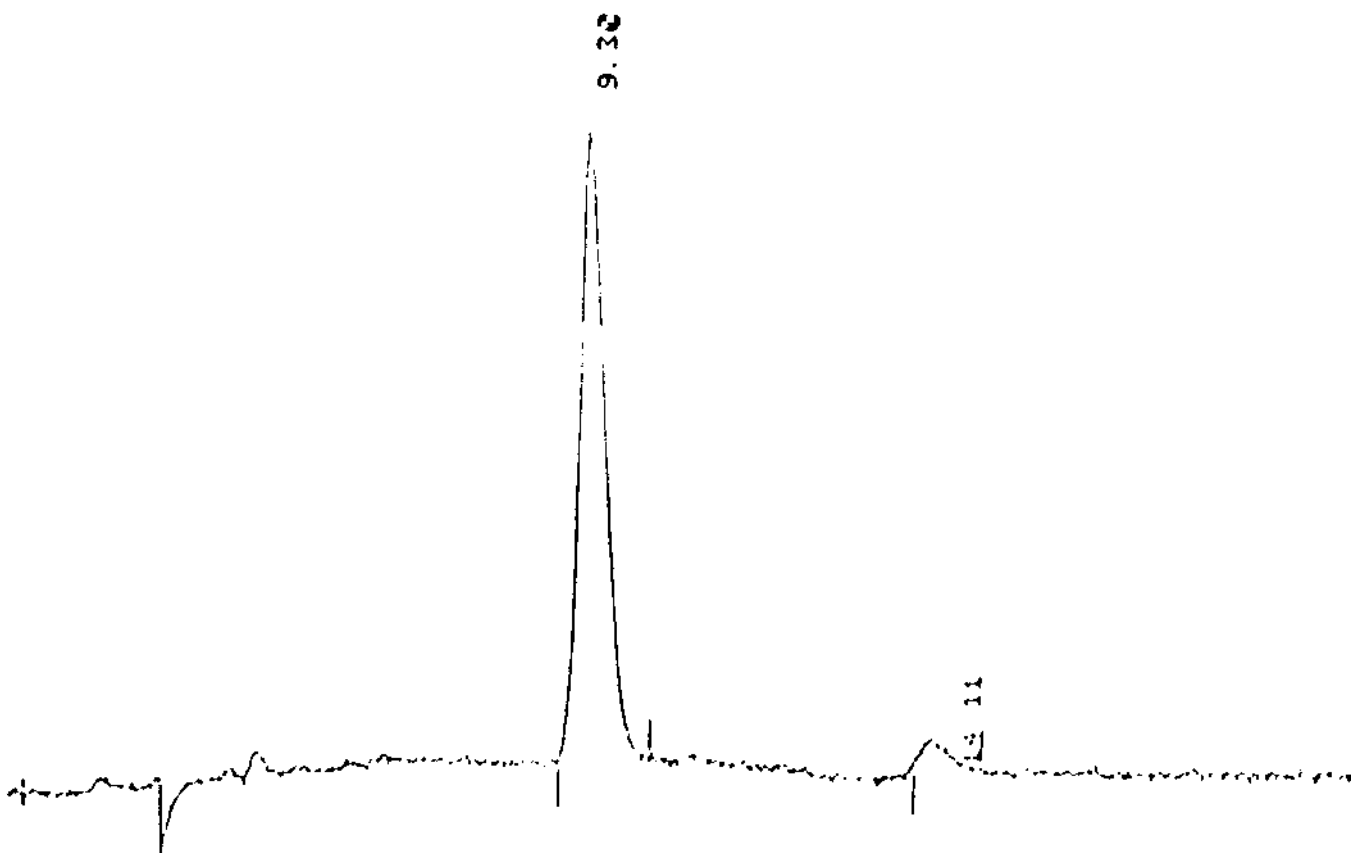


Figure 8:

HPLC run of pure dehydroergosterol (4.6 mm inner diameter by 250 mm C-18 HPLC column, 10 µl injection, methanol/acetonitrile (3/1 v/v) solvent system, flow of 1.0 ml/min). Monitored by absorbance at 280 nm. The small peaks early in the run are due to chloroform.

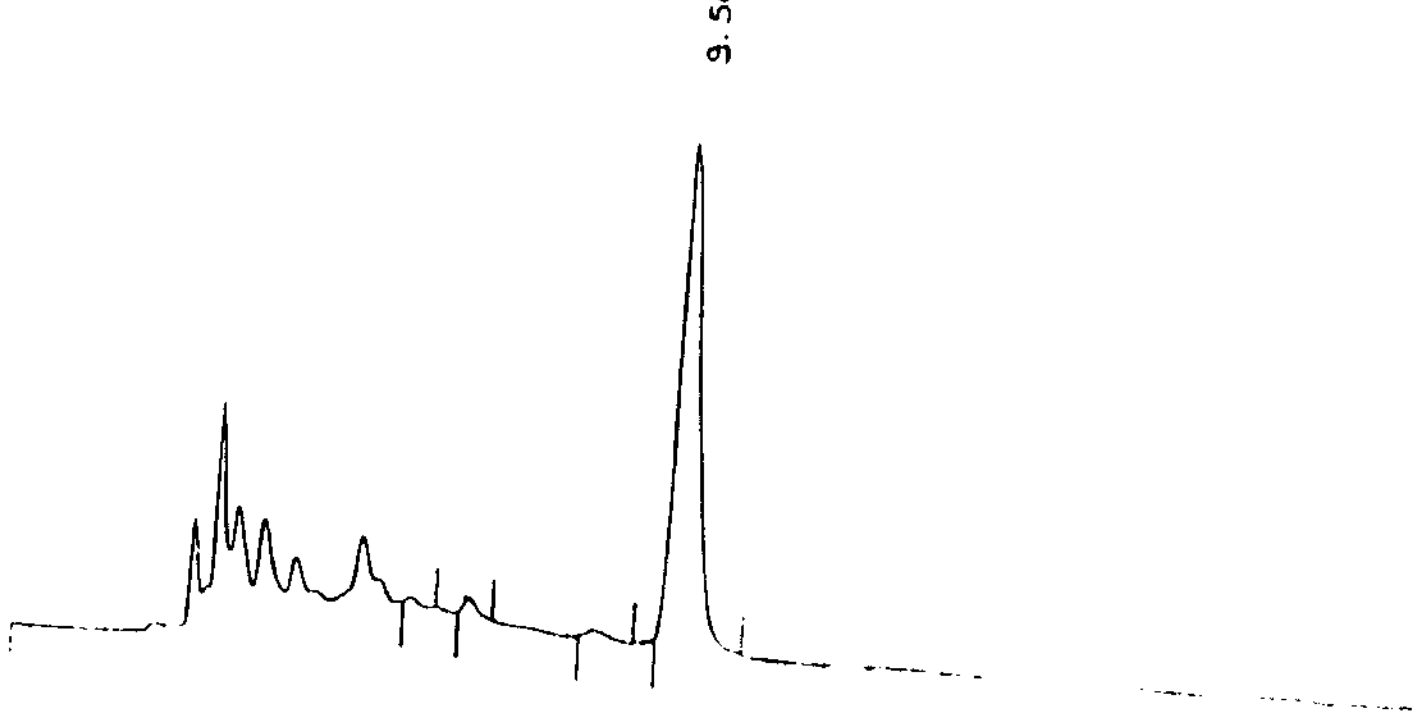


Figure 9:

HPLC run of pure dehydroergosterol (4.6 mm inner diameter by 250 mm C-18 HPLC column, 10 µl injection, methanol/acetonitrile (3/1 v/v) solvent system, flow of 1.0 ml/min). Monitored by absorbance at 324 nm. The small peaks early in the run are due to chloroform.

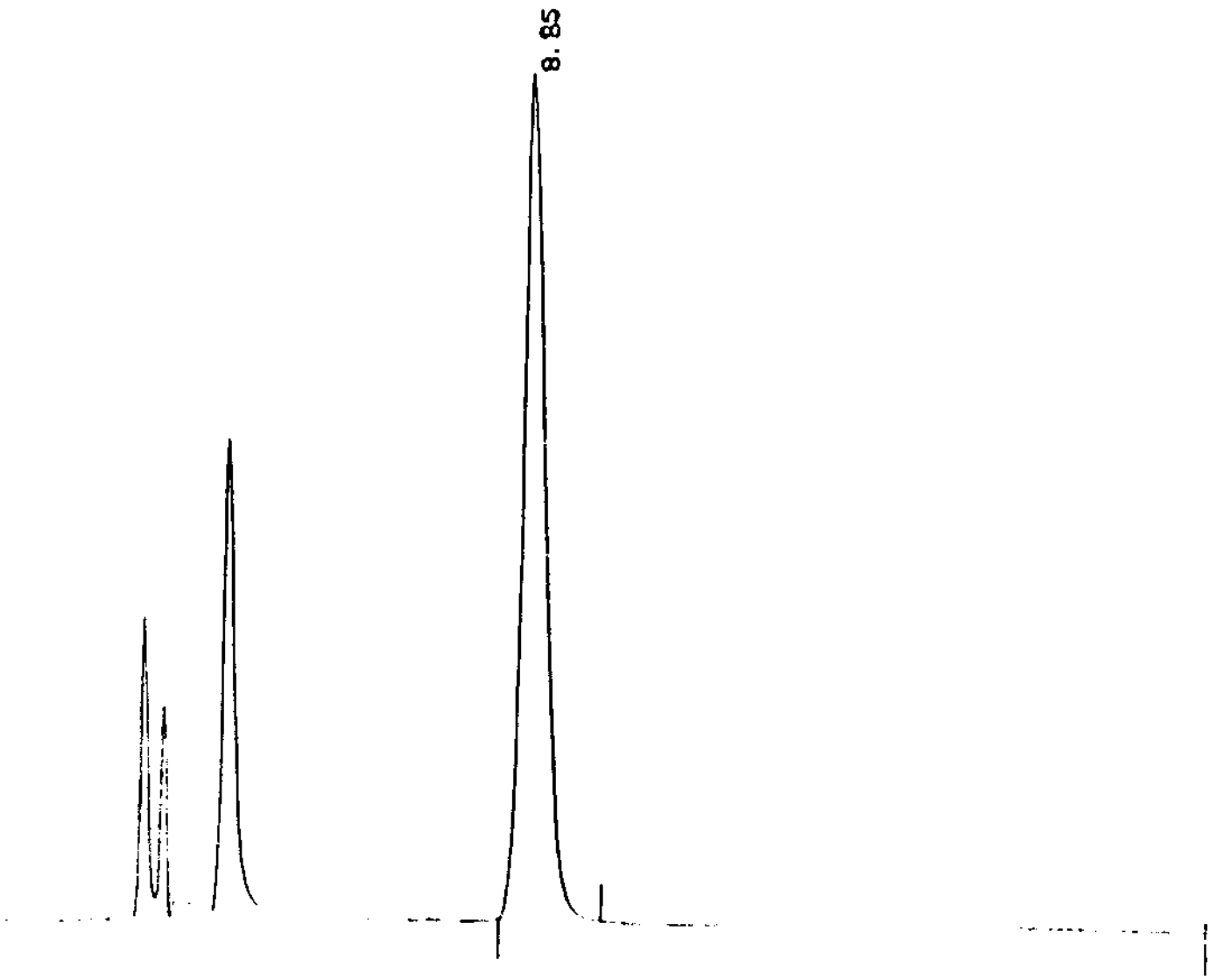


Figure 10:

Visualization of dehydroergosterol. Vesicles are composed of top panel, top row: 5 mole % NBD PA in 95 mole % DOPC; top panel, bottom row: 5 mole % dehydroergosterol and 5 mole % NBD PA in 90 mole % DOPC (the fluorescent position 3 has a very high background); and bottom panel, 5 mole % dehydroergosterol in 95 mole % DOPC. Left hand images are with position 3 (dehydroergosterol position), and right hand images are with position 4 (NBD position).

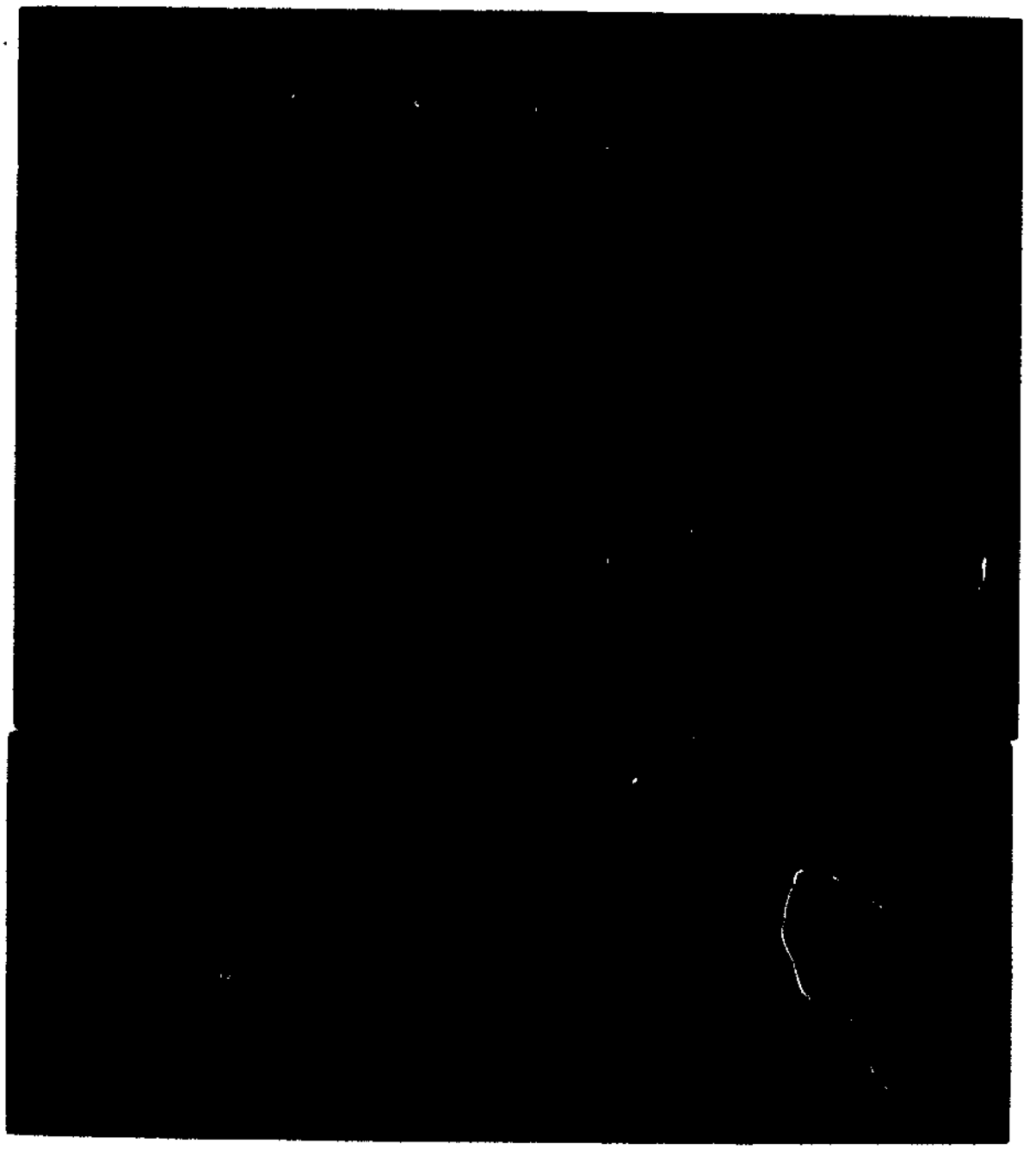


Figure 11:

Emission spectra of vesicles containing NBD-PA and dehydroergosterol. Excitation at 340 nm. Note the absence of dehydroergosterol fluorescence.

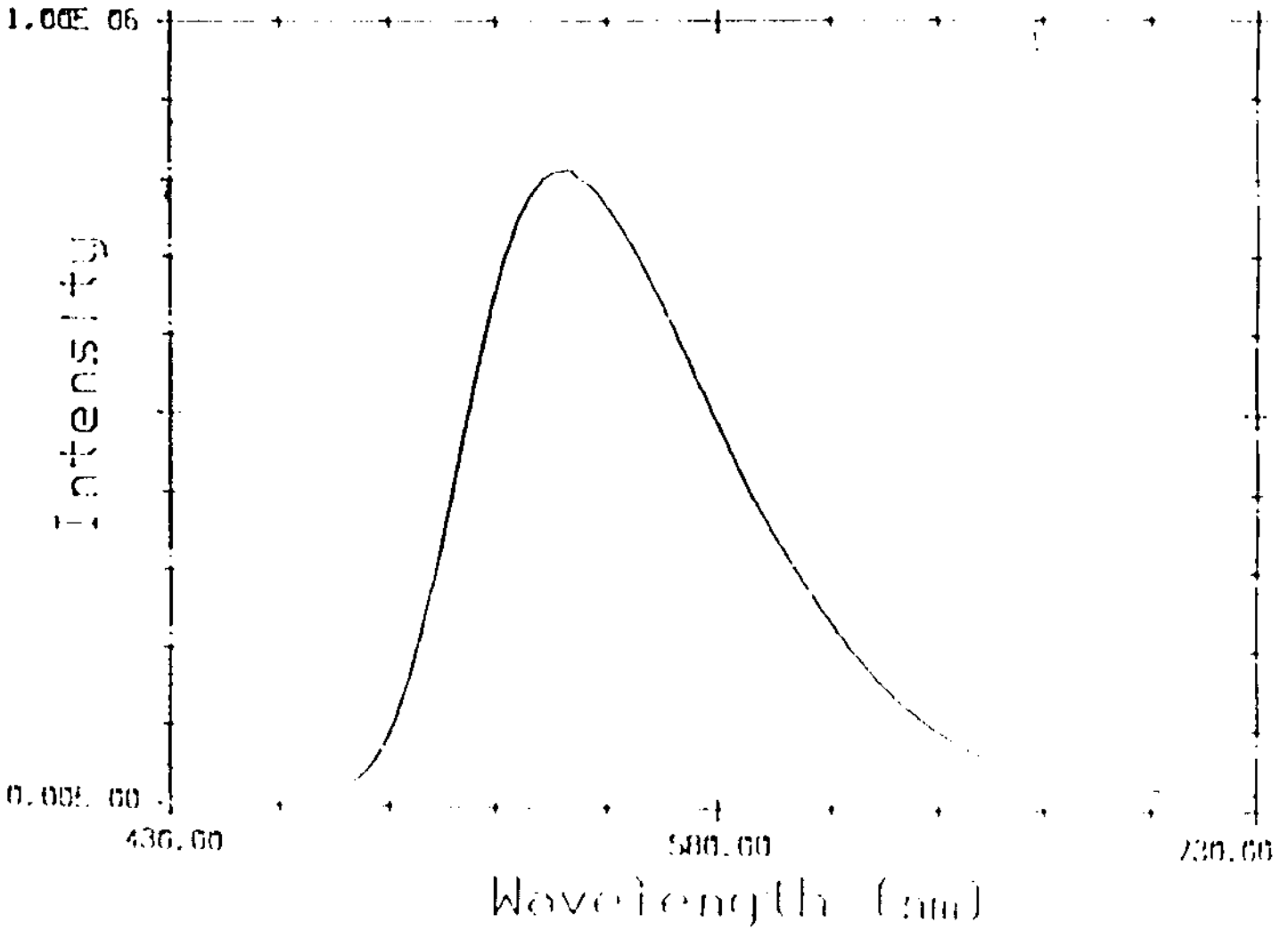


Figure 12:

Emission spectra of vesicles containing NBD-PA and dehydroergosterol. Excitation at 380 nm. Note the absence of dehydroergosterol fluorescence.

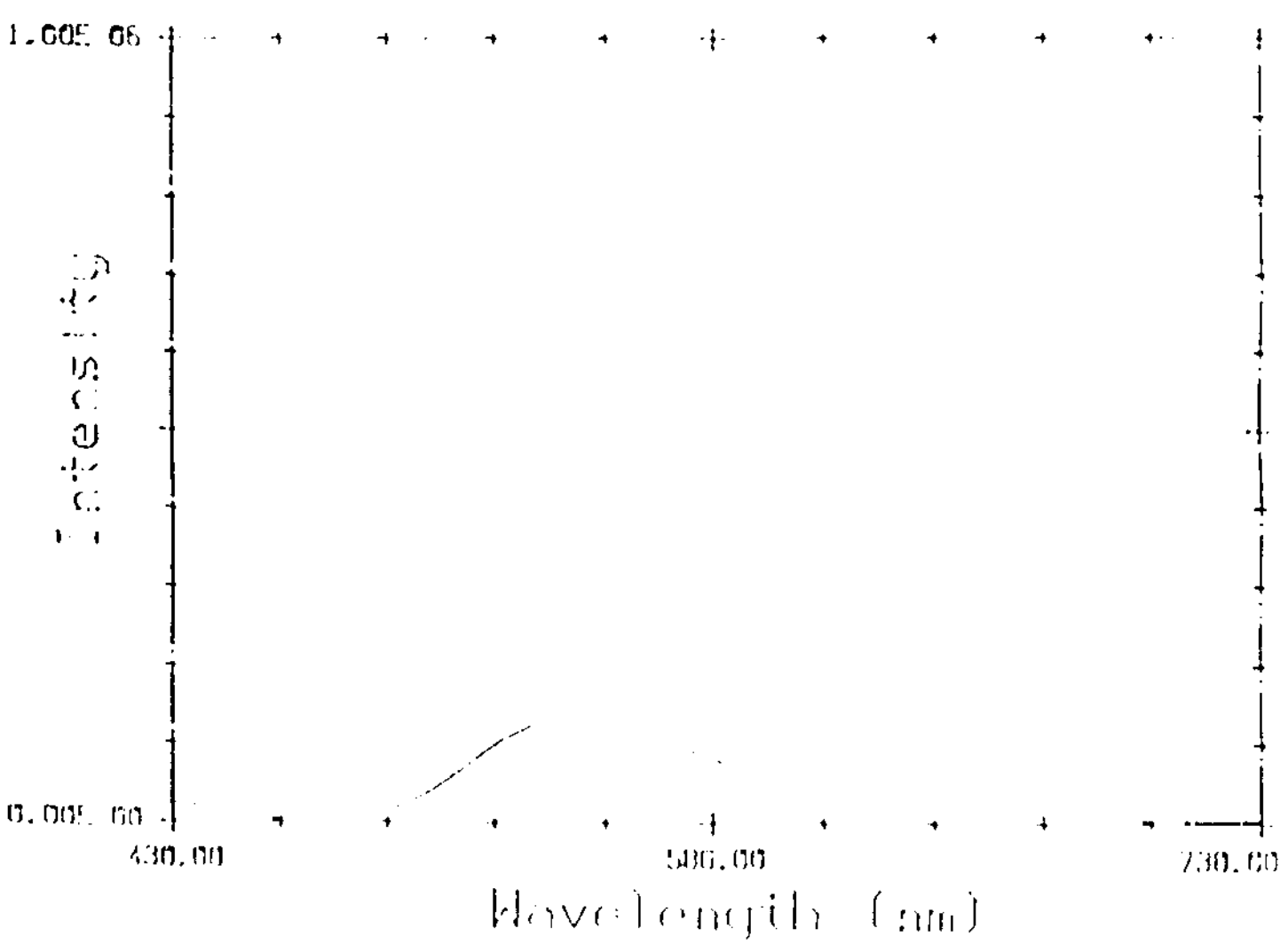


Figure 13:

Emission spectra of vesicles containing NBD-PA and dehydroergosterol. Excitation at 405 nm.

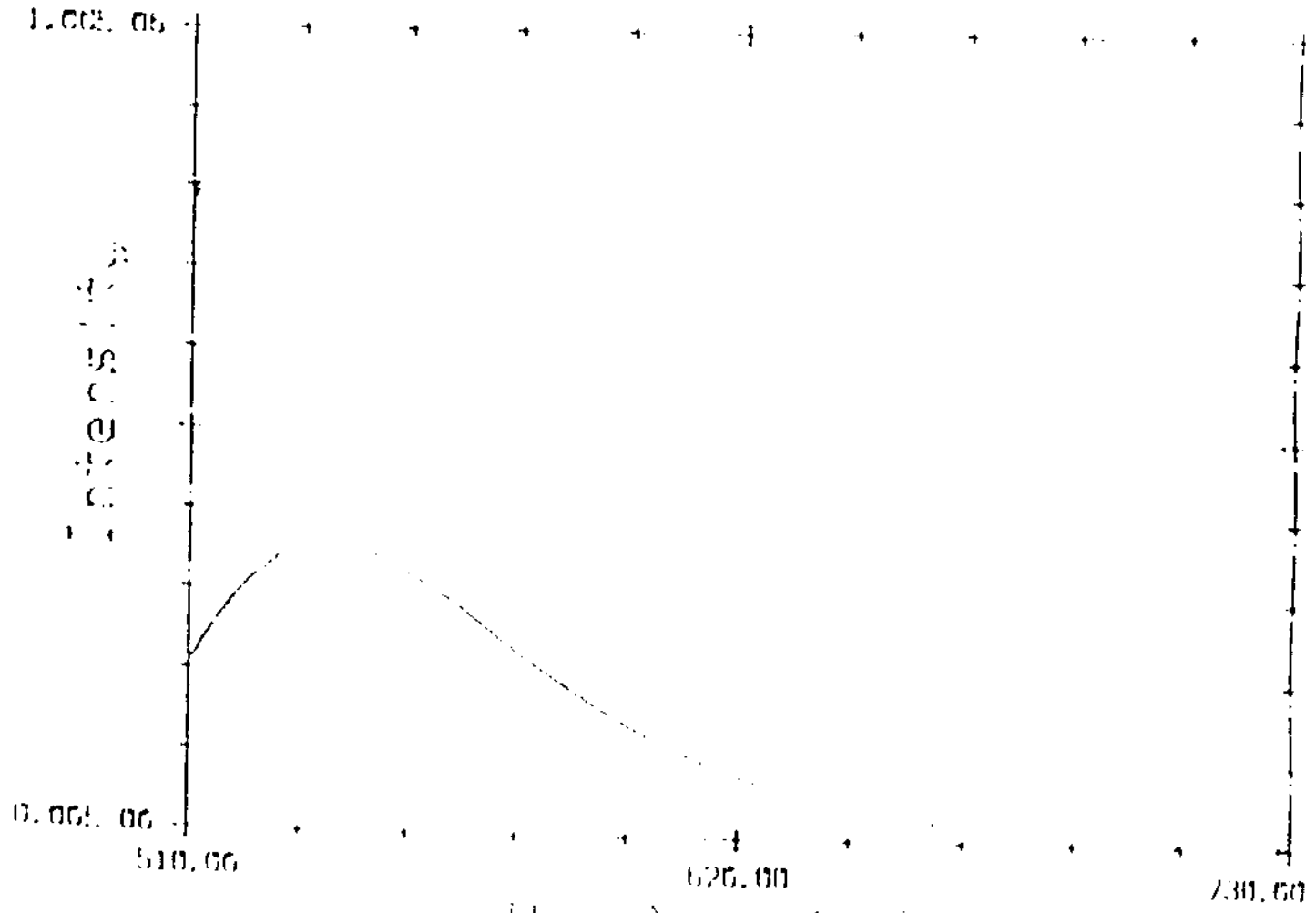


Figure 14:

Emission spectra of vesicles containing NBD-PA and dehydroergosterol. Excitation at 460 nm.

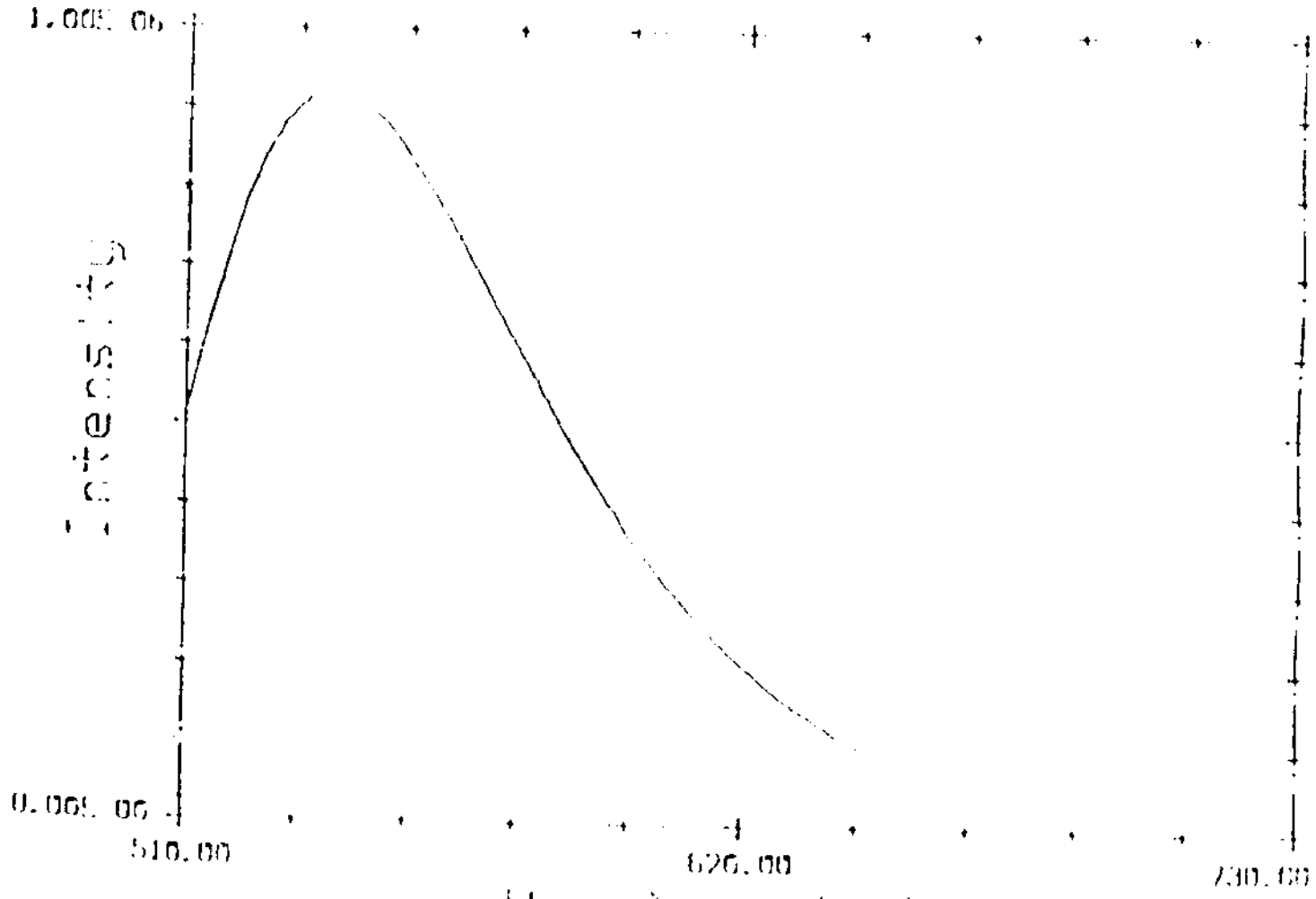


Figure 15:

Emission spectra of vesicles containing dehydroergosterol. Excitation at 340 nm. Note the presence of dehydroergosterol fluorescence.

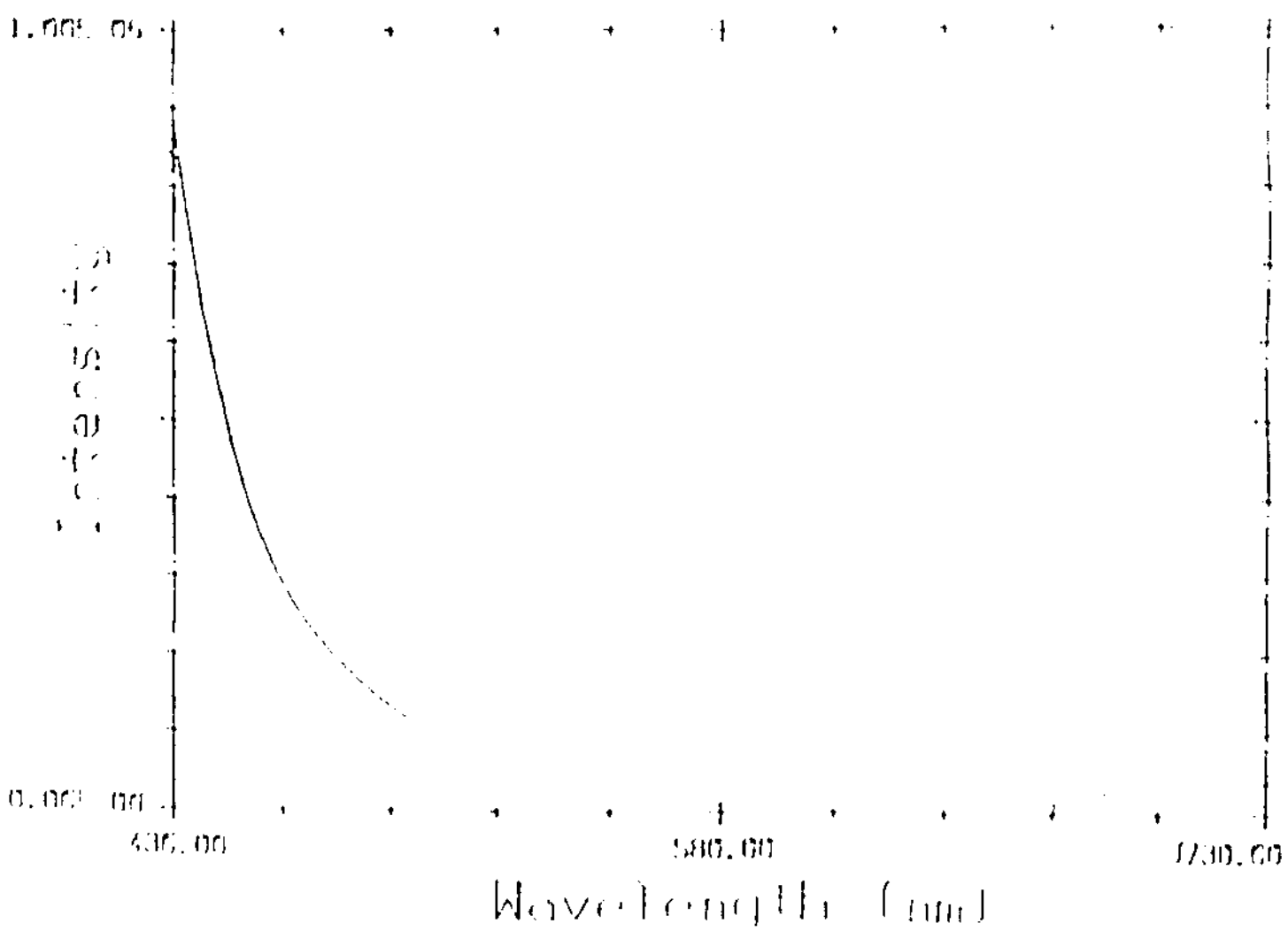


Figure 16:

Emission spectra of vesicles containing dehydroergosterol. Excitation at 380 nm.

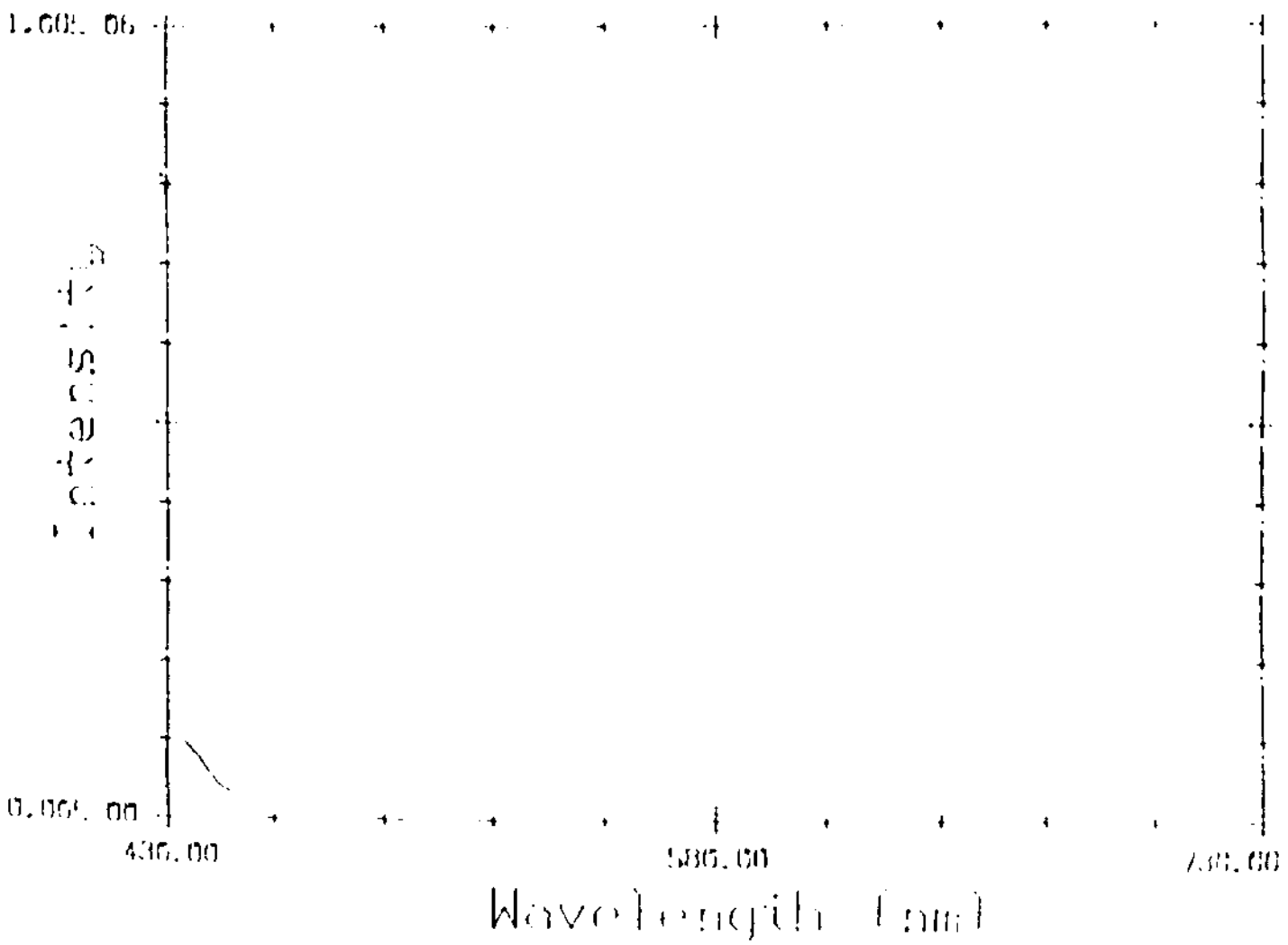


Figure 17:

Emission spectra of vesicles containing dehydroergosterol. Excitation at 405 nm.

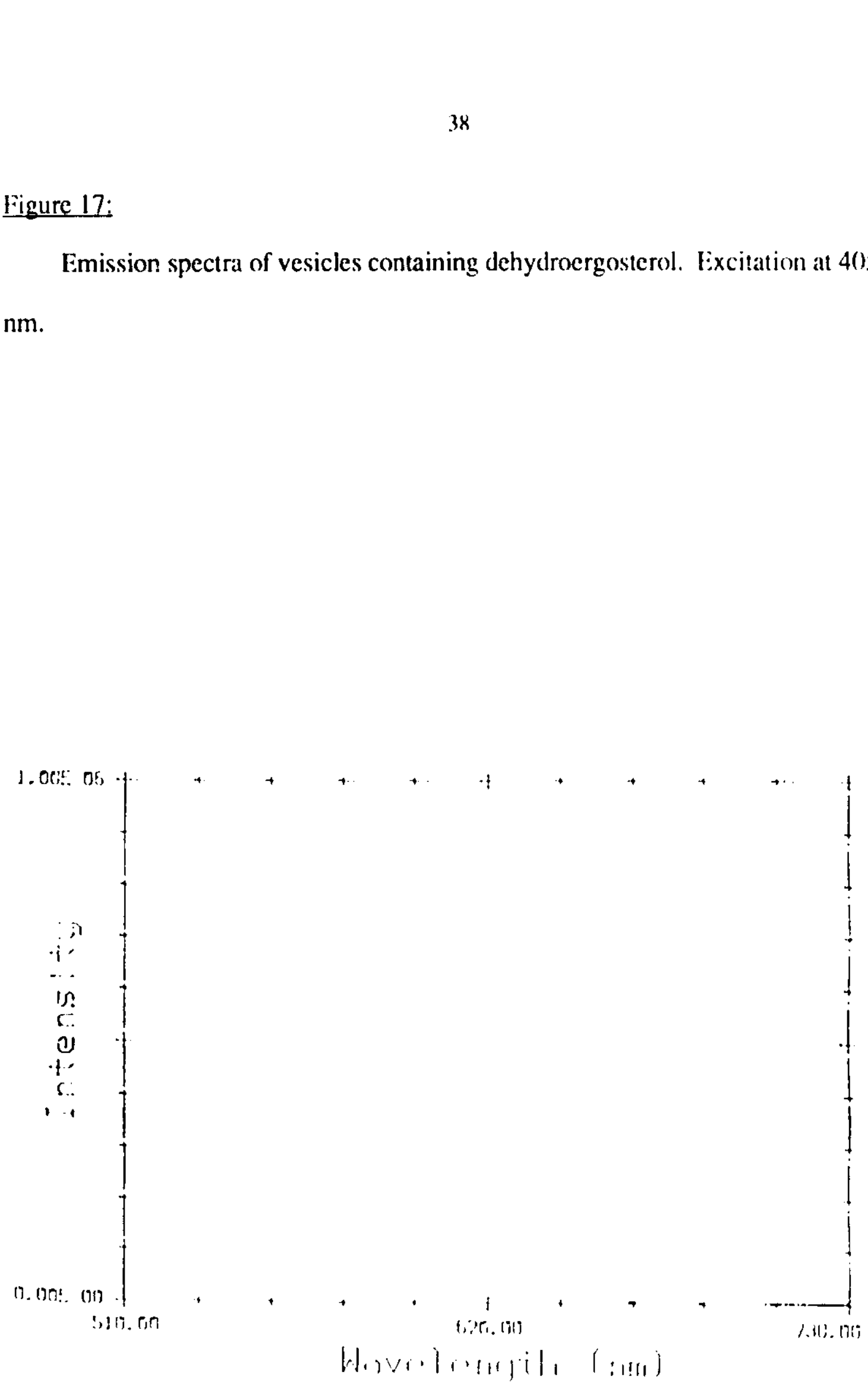


Figure 18:

Emission spectra of vesicles containing dehydroergosterol. Excitation at 460 nm.

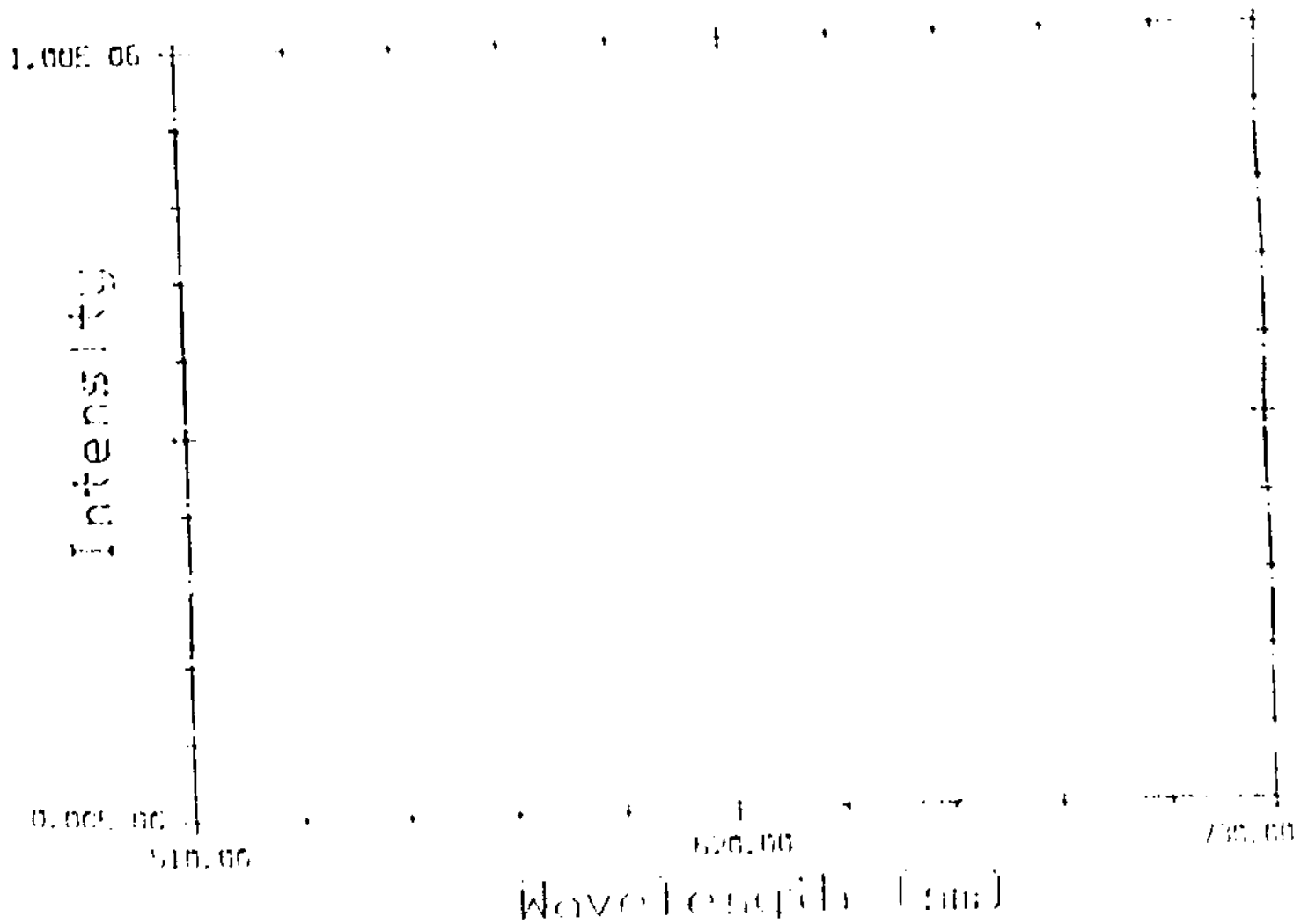


Figure 19:

Fluorescence excitation spectra of NBD in chloroform. Emission was monitored at 520 nm.

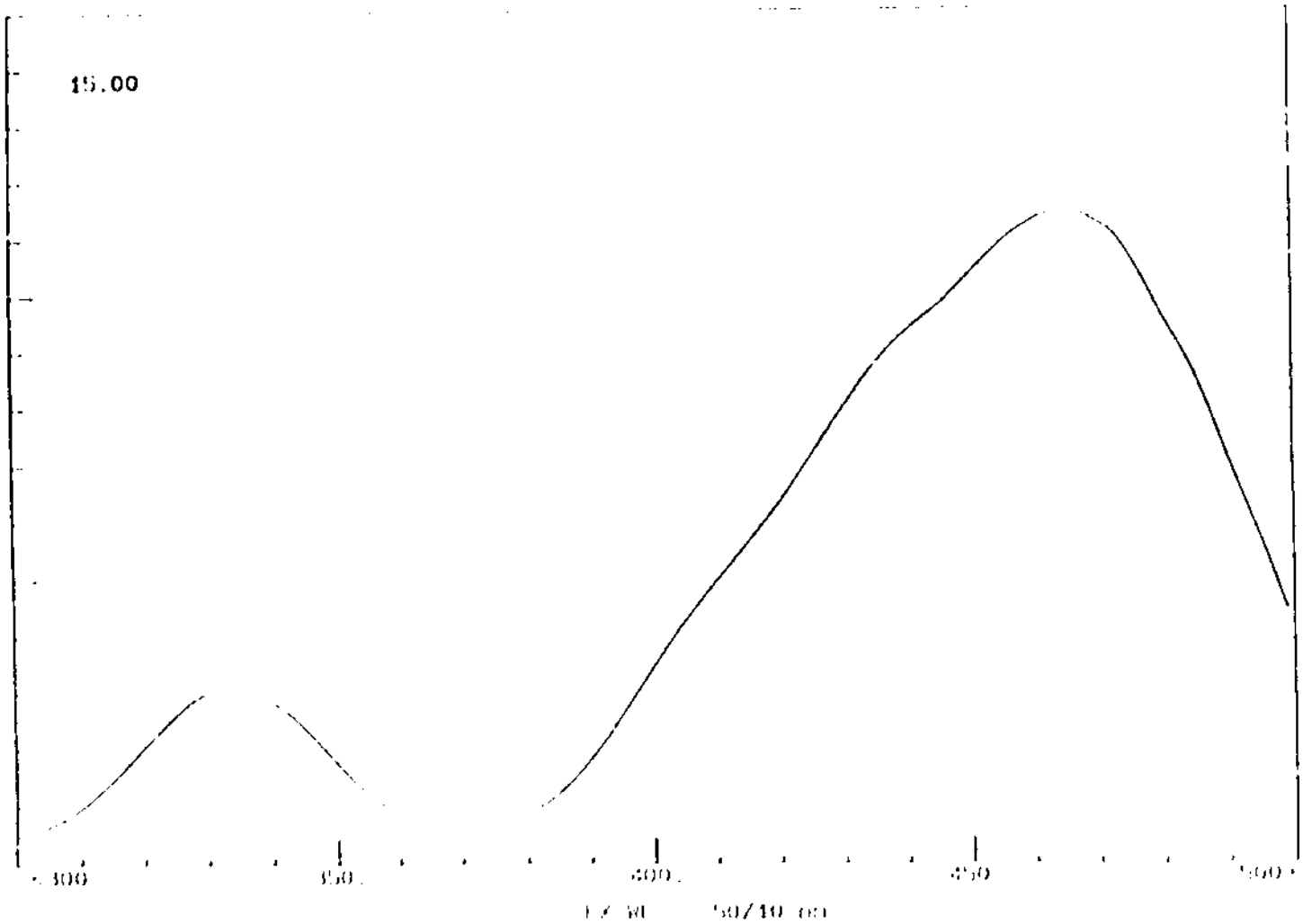


Figure 20:

Fluorescence emission spectra of NBD in chloroform. Sample was excited at 460 nm.

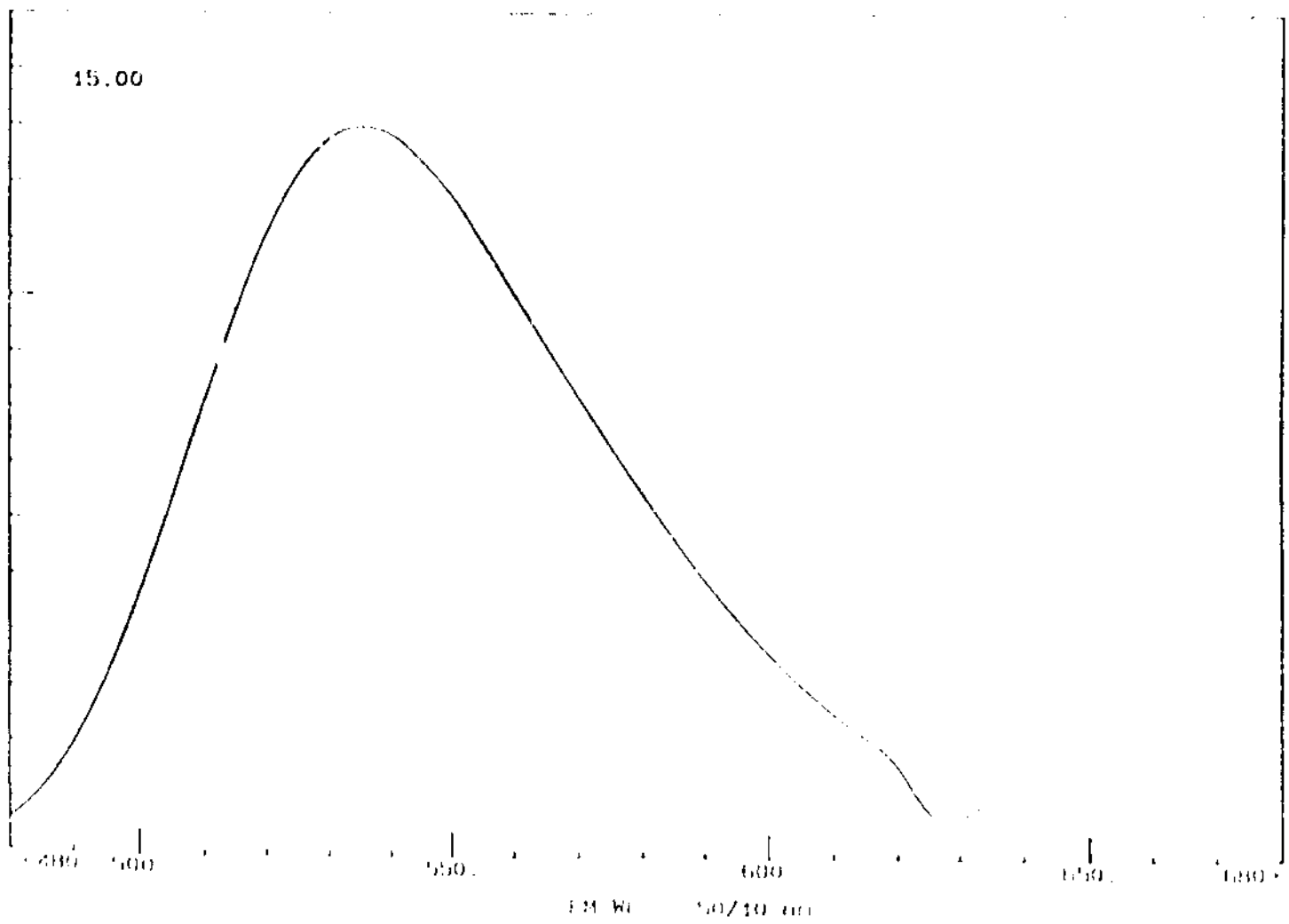


Figure 21:

Vesicle containing 4 mole % dehydroergosterol, 4 mole % NBD-PA, and 92 mole % DOPC. Top row is at 0 to 2 minutes after addition of Ca^{2+}, bottom row is 15 to 20 minutes after addition. Left hand images are with dehydroergosterol position, and right hand images are with NBD position. Note the exclusion of dehydroergosterol from the PA domain. This is difficult to see, as the vesicle became very dim over time.

Figure 22:

Second set of images. Vesicle containing 4 mole % dehydroergosterol, 4 mole % NBD-PA, and 92 mole % DOPC. Top row is at 0 to 2 minutes after addition of Ca^{2+}, bottom row is 15 to 20 minutes after addition. Left hand images are with dehydroergosterol position, and right hand images are with NBD position. Note the exclusion of dehydroergosterol from the PA domain. The NBD images are dim due to the use of a neutral density filter.

Figure 23:

Vesicle containing 7 mole % dehydroergosterol, 5 mole % NBD-PA, and 88 mole % DOPC. Top row is at 0 to 5 minutes after addition of Ca^{2+}, bottom row is 15 to 20 minutes after addition. Left hand images are with dehydroergosterol position, and right hand images are with NBD position. Notice that with the added dehydroergosterol, the patches appear to coincide.

Figure 24:

Vesicle containing 5 mole % dehydroergosterol and 5 mole % DOPA in 90 mole % DOPC. Top left-hand image is at 0 to 5 minutes after addition of Ca^{2+}, bottom left-hand image is 15 to 20 minutes after addition. Images are with dehydroergosterol position. Note the exclusion of dehydroergosterol from the upper-right area of the vesicle. The image on the right is taken with phase contrast microscopy, and is presented to show that the vessicle is not multilamellar.

Figure 25A:

Self quenching of dehydroergosterol in chloroform. This is indicated by the fac that there is not a linear relation between fluorescence and concentration, as the concentration is increased. Both data sets are plotted.

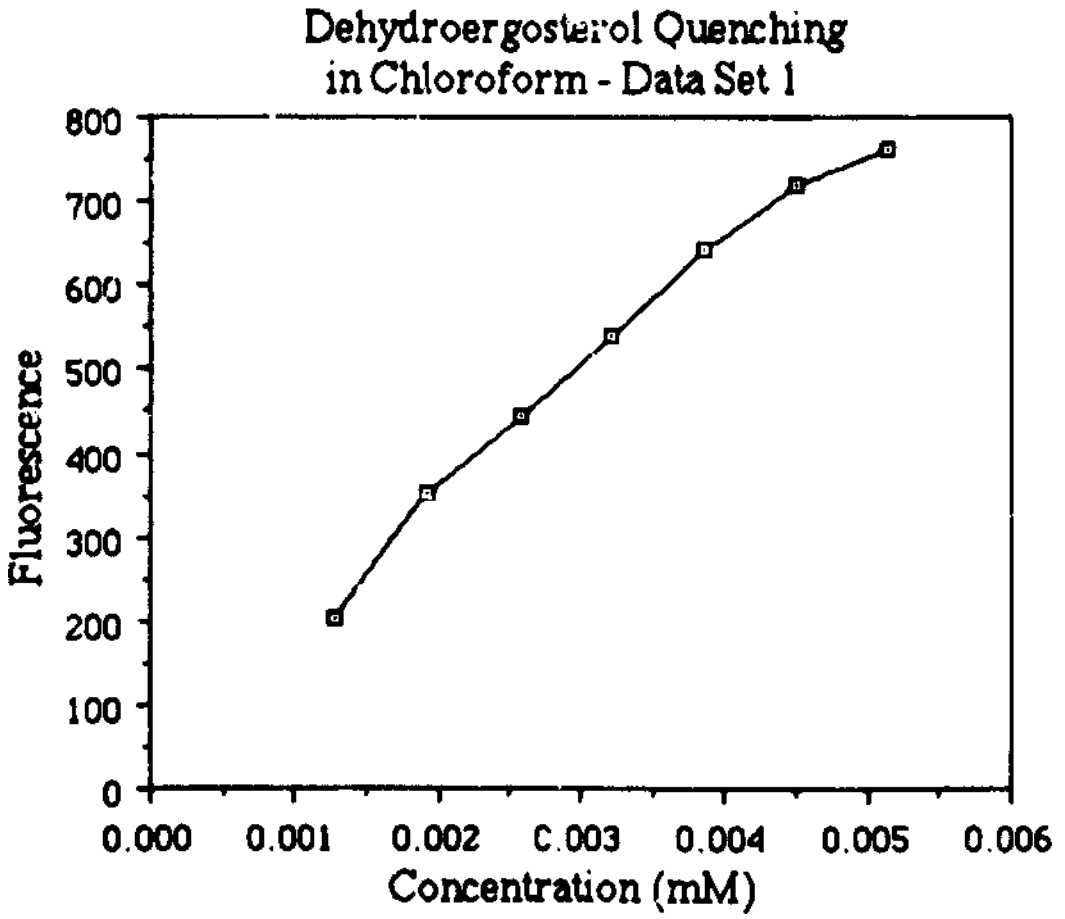


Figure 25B:

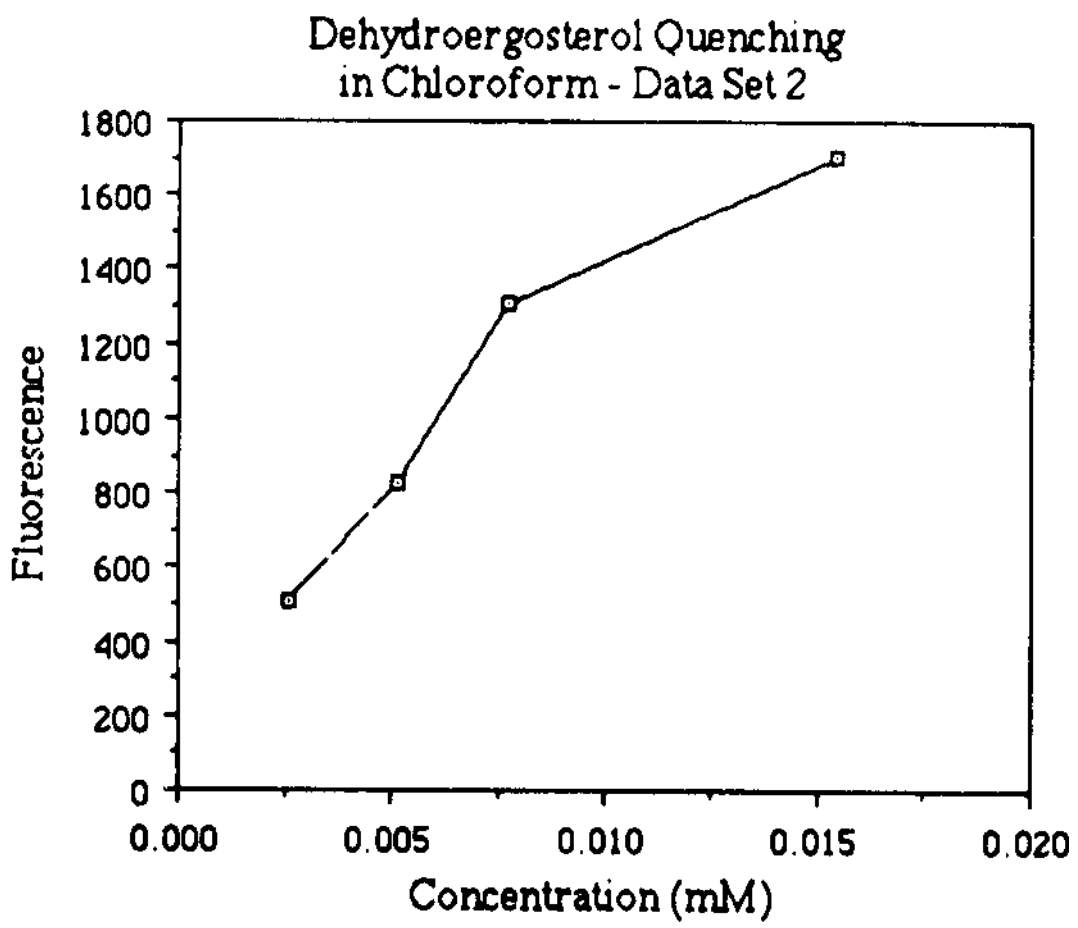

Dehydroergosterol Quenching
in Chloroform - Data Set 2
Fluorescence
1800
1600
1400
1200
1000
800
600
400
200
0
0.000
0.005
0.010
0.015
0.020
Concentration (mM)

Figure 26:

Fluorescence increase of dehydroergosterol after disruption of vesicles with Triton X-100. Final Trition concentration was 1 %. Vesicles were prepared with 5 mole % DOPA, the indicated precentage of dehydroergosterol, and the remaining precentage DOPC. At 1 % dehydroergosterol there is a 44.5 % increase in fluorescence intensity upon addition of Triton X-100; at 5 % dehydroergosterol the increase is 57.8 %; and at 10 % dehydroergosterol the increase is 70.5 %.

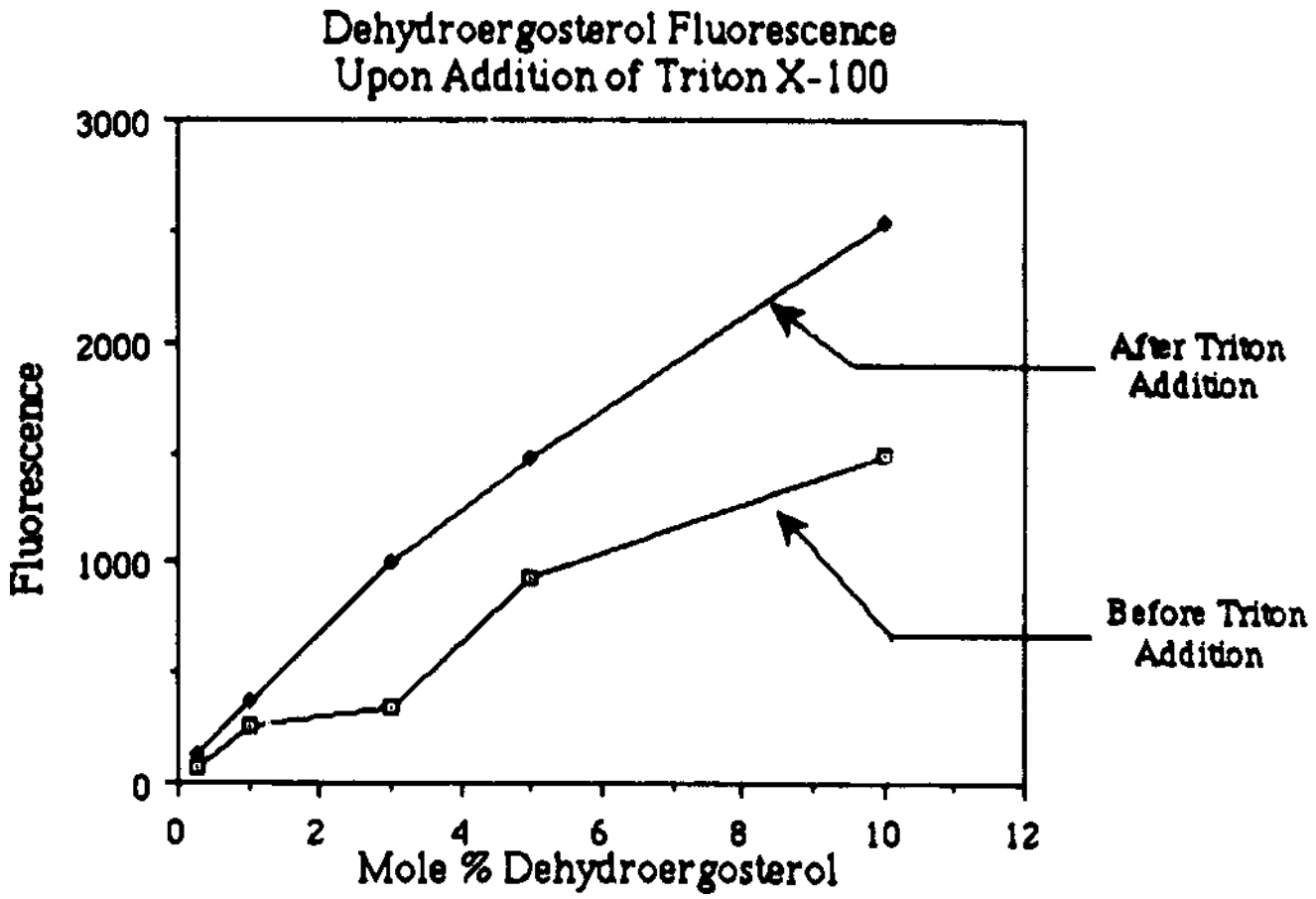
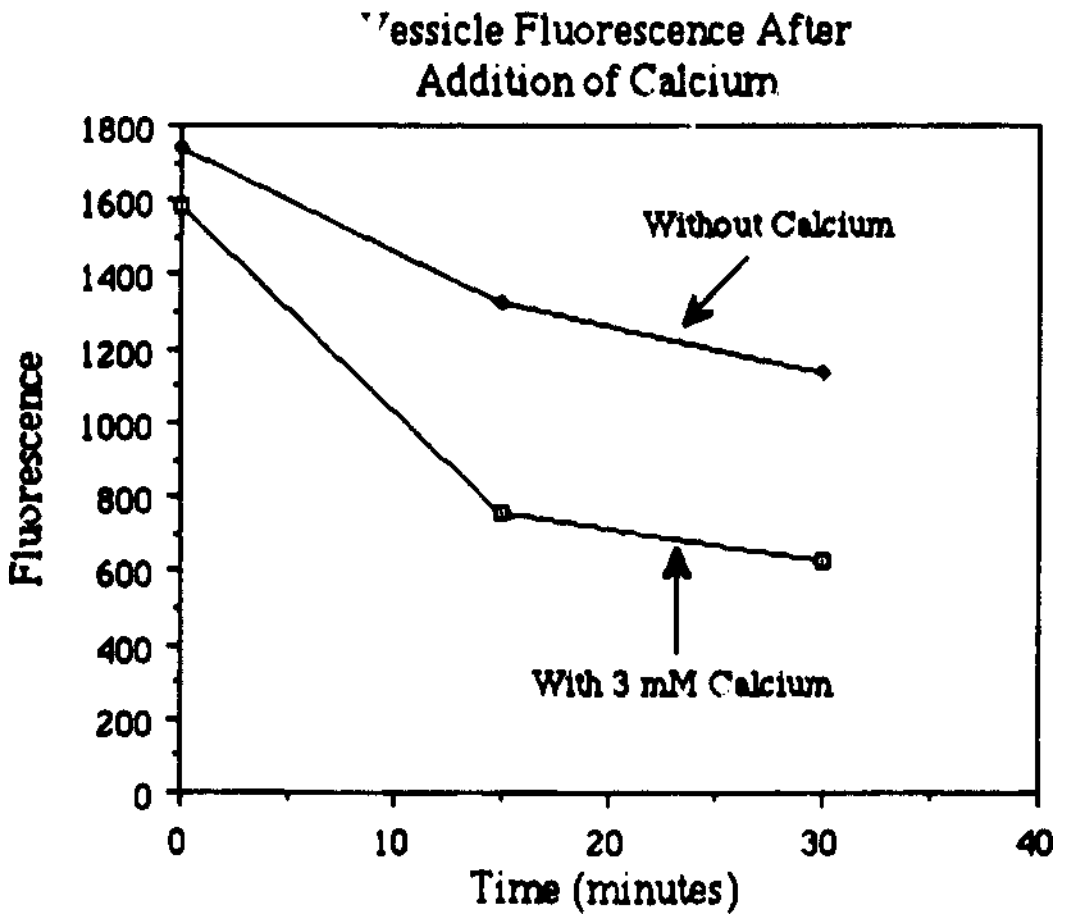


Figure 27:

Decrease in fluorescence of vesicles upon addition of calcium. Vesicles were made with 5 mole % DOPA, 5 mole % dehydroergosterol, and 90 mole % DOPC. Vesicle solutions had either distilled water or calcium solution added. Notice that after 15 and 25 minutes, the vesicle solution that had Ca^{2+} added has a significantly greater drop in fluorescence than the vesicle solution to which water was added. The decrease in the fluorescence of the control is due to bleaching of the fluorophore.



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