To the Graduate Council:

I am submitting herewith a thesis written by Lori A. Schmitt entitled "Optimization of DNA Entrapment in pH-Sensitive Liposomes." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Leaf Huang

Leaf Huang, Major Professor

We have read this thesis
and recommend its acceptance:

Robert N Moore

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

OPTIMIZATION OF DNA ENTRAPMENT IN pH-SENSITIVE LIPOSOMES

A Thesis

Presented for the

Master of Science

Degree

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Lori A. Schmitt

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Dedicated to mom and dad.

Without you, this never
would have happened.

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ABSTRACT

The pH-sensitive liposomes commonly used for DNA delivery, DOPE:CHOL:OA (4:4:2), upon further investigation, were found to be very inefficient in their ability to entrap plasmid DNA. This was a result of both the liposome composition and the size of the plasmid.

We have developed another liposome which maintains the pH-sensitive characteristic of the previous composition but greatly enhances the DNA entrapment. These liposomes, composed of DOPE:DOSG (7:3), show 50% content release at pH 5.3 and have been able to entrap up to 31% of the added plasmid DNA after the plasmid had been reduced in size by 600 base pairs. It is expected that further reductions in the plasmid size will enhance entrapment to an even greater extent.

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LIST OF ABBREVIATIONS

CHOL:	cholesterol
DOC:	deoxycholic acid
DOPC:	dioleoylphosphatidylcholine
DOPE:	dioleoylphosphatidylethanolamine
DOSG:	dioleoylsuccinylglycerol
DOTMA:	(N-(1-(2,3,-Dioleyloxy)propyl)-N,N,N-trimethylammonium chloride)
OA:	oleic acid
SUV:	small unilamellar vesicle

CHAPTER I

INTRODUCTION

In the past several years, understanding the ways in which living cells express the information encoded by their genes has been of major importance. The field of recombinant DNA technology is rapidly becoming a primary tool in developing this understanding.

Of major interest to pioneers of this field are genetic disorders (1,2). Why are cells sometimes deficient in major genes? Is it that the genes are not present or perhaps that the enzymes necessary to copy these genes are not present? The central theme of gene therapy is to insert normal exogenous genes into target cells where their products will replace or work with defective or absent gene products, hence aiding the cell in normal function.

However, before gene therapy can be accepted as a successful means of treatment, several requirements must be met. First, a method must be developed which provides specific and efficient delivery of genes to the target cells. Second, there must be a minimal immune response toward the cells expressing the foreign gene. Thirdly, stable expression of the integrated gene must occur.

In the search of DNA vectors for gene therapy, several possibilities have arisen, but so far there have been few that meet the aformentioned requirements. For example, autologous bone marrow cells have been transfected by normal genes in vitro and implanted into patients (3,4).

This procedure allows for a minimal immune response, however, the number of transformants are so few that the disorder is still difficult to correct.

Retroviruses also appeared as potential vehicles for gene transfer. They possess a unique structure, mode of replication, and have the ability to infect a wide variety of cells (5). However, drawbacks such as loss of expression of transferred genes (6), random integration of viral genes into the host genome (7), and lack of control of cell specificity (8) frequently occur with this method.

Electroporation, another alternative, works by breaking down the cell membranes with an electrical current which ultimately allows DNA to enter the cells through pores. This method has been successful in the transfection of yeast spheroplasts (9), plant protoplasts (10,11), and mammalian cells (12,13), and is appealing due to its simplicity compared to other techniques, greater degree of control and reproducibility, avoidance of potentially lethal chemical reagents, and comparable transfection frequency. The major drawback of this technique, however, is its inability to be carried out in vivo which is a primary goal for successful gene therapy.

A method of gene therapy which does allow for in vivo transfection is one developed by Wu and Wu. Recently, they made a gene delivery system which utilizes the asialoglycoprotein receptors of hepatocytes(14,15,16). Here the DNA carrier consists of : 1) a galactose-terminal (asialo-) glycoprotein, and 2) a polycation that can bind DNA strongly and without damage.

The advantage of this system is the ability to target to unique receptors of the liver cells which will then internalize the DNA by receptor mediated endocytosis. In vivo expression of exogenous DNA has been achieved by this method, which is a major advantage to the technique. In these in vivo experiments, the conjugate was injected i.v. and so went directly to the liver where the receptor was. Liver cells are unique in their expression of this receptor, so other types of cells are non-targetable by this technique unless other specific receptors are utilized whose ligand can similarly be conjugated to DNA.

A method of DNA delivery which has become of great interest in the past decade is that of liposomal delivery. Liposomes first became of interest two decades ago when their ability to carry antitumor agents, antibiotics, hormones, and immunomodulators was seen. Their biodegradability and lack of toxicity was also a plus to the technique (17). It was eventually found that the specificity of delivery of these molecules could be enhanced by coupling of monoclonal antibodies (18) or antigens (19) to the liposome.

More recently, liposomes have been used as vehicles for DNA delivery. Successful transfection has been achieved using lipid compositions which deliver by fusion or endocytosis, however, the transfection efficiency is not very high (1). Liposomes consisting of cationic lipids (20) or quaternary ammonium detergents (21,22) have surfaced recently and have had transfection efficiencies comparable to other methods in vitro. However, these liposomes do not entrap DNA inside. Rather, they adsorb the negatively charged molecule on

their surface. This idea would not seem beneficial in an in vivo situation where nucleases come into play.

More recently, liposomes which release their contents as a result of drops in pH, pH-sensitive lipsomes, have become popular for delivery of DNA. These liposomes, consisting of phosphatidylethanolamine (PE) and oleic acid (OA) or palmitoyl homocysteine (PHC), have been used for the transfection of plant protoplasts (23), L-cells (24), and RDM4 lymphoma cells (25).

Liposomes are termed pH-sensitive with regards to their inability to form stable bilayers at low pH. This is mainly due to the presence of unsaturated PE molecules. Unsaturated PE is a zwitterionic lipid consisting of a small head group and large acyl chains which favors a hexagonal phase rather than a bilayer conformation at physiological conditions. In the presence of a second amphipathic component such as OA or PHC, however, PE containing liposomes can be made to take on the otherwise unfavorable bilayer form. This is due to the negatively charged head groups of the second component, which causes repulsions between the adjacent PE bilayers. When the pH is lowered, the negative head groups become neutralized and so the liposome reverts back to the preferred hexagonal phase. These PE containing liposomes are also known to be capable of fusion with other membranes (26). By following this logic, one can form stable liposomes at neutral pH which contain the DNA to be delivered. Once these liposomes are endocytosed they may, as a result of the acidic environment, fuse with the endosomal membrane and release their contents into the cytoplasm (27,28,29). Subsequent entry into the nucleus and

integration of the released DNA into the host chromosome has the potential to allow for long term transformation. This last step is not absolutely necessaryfor gene expression; it can occur even if the gene is not integrated into the host chromosome, however, its chances to be continually expressed are greatly increased.

Integration of the delivered DNA has been shown, by microinjection studies, to be dependent on the physical form of the plasmid DNA (30). When only a few plasmid DNA molecules were injected, the transformation frequency was 40-fold higher after injection of linearized molecules. This is expected to be due to a lack of preferred sites of integration within the plasmid sequence, whereas linear fragments are inserted through their ends.

The purpose of this thesis project is to first evaluate the pH-sensitive liposome composition previously used in the lab, i.e. the DOPE:CHOL:OA liposomes. We then designed experiments to further improve the stability and the delivery properties of the pH-sensitive liposomes. We have established a reliable method to measure the DNA entrapment efficiency of the liposomes. A new pH-sensitive liposome composition has been developed for DNA delivery. The liposomes are characterized in terms of DNA entrapment efficiency, pH-sensitivity and serum stability.

CHAPTER II

EVALUATION OF DNA ENTRAPMENT OF pH-SENSITIVE LIPOSOMES COMPOSED OF DOPE:CHOL:OA (4:4:2)

A. INTRODUCTION

The pH-sensitive liposomes described previously were composed of DOPE:CHOL:OA (molar ratio of 4:4:2). They were coupled to anti H2Kk antibodies specific for the H2Kk antigen on the surface of RDM4 cells (24,25) and were prepared by the detergent dialysis method (31). By entrapping a plasmid (pBBO.6) which contains the E. Coli chloramphenicol acetyl transferase (CAT) gene, it was easy to assay for transfection because this gene is absent in eucaryotic cells. To further control the expression of the exogenous CAT gene, a cAMP regulatory element, upstream from the phosphoenolpyruvate carboxykinase (PEPCK) gene, was used as a promoter of the plasmid (32). It has been shown that in order for the cAMP regulatory element to function in gene expression, transactivating factors, which are produced by specific tissue types, must be present (33).

In the experiments done with these liposomes, the target cells (RDM4) were placed in the peritoneal cavity of Balb C nude mice. After i.p. injection of the liposomes, there was uptake and expression of the CAT gene by the RDM4 cells, but, there was also uptake of the liposomes by organs rich in cells of the reticuloendothelial system (e.g. Kupffer cells in liver). These cells, however, were not able to express the exogenous gene (34).

Unpublished data from C. Lee suggests that these cells do not contain measurable amounts of these transactivating factors, hence even in the presence of cAMP, the CAT gene could not be transcribed.

The original purpose of the project was to deliver exogenous genes to macrophage cells. The lack of transactiving factors produced by macrophage cells has led us to the use of plasmid pSV2 CAT which is under the control of the SV40 promoter, and so lacks the requirement of cAMP and transactivating factors.

When this plasmid was encapsulated inside of DOPE:CHOL:OA liposomes, no detectable CAT activity was obtained in cultures of macrophage or L929 cells. This plasmid did, however, allow for expression in L929 cells when used in conjunction with DOTMA:DOPE liposomes (i.e. Lipofectin) which are positively charged and act by adsorbing the negatively charged DNA to their surface. This has led to the physical studies of the DOPE:CHOL:OA liposomes presented in this chapter.

B. MATERIALS AND METHODS

B.1. Materials:

Dioleoylethanolamine (DOPE) was purchased from Avanti (Birmingham, Al.). Oleic acid (OA), cholesterol, and octylglucoside were purchased from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories. Plasmid pSV2CAT was a gift from Dr. Wesley D. Wicks. The plasmid DNA was isolated using the pz523 column procedure (35).

B.2. Nick Translation:

DNA was radiolabeled by nick translation with γ-[32P]-dCTP (36). In a typical experiment, 10 ug of plasmid DNA was mixed with 5 ul dGTP,TTP,ATP, 5 ul γ-[32P]-dCTP, 31 ul distilled water, and 1 ul DNA polymerase. After mixing, the sample was incubated for no more than 15 minutes at 18°C. After the incubation, the reaction was stopped with 5 ul stop buffer (EDTA) and the volume was brought to 100 ul with STE (10 mM Tris-HCl, 100 mM NaCl, 1mM EDTA pH 8.0). To remove unincorporated dCTP, the sample was applied to a Biogel P-6 column equilibrated with STE buffer and centrifuged for 5 min. at 1500 rpm. The radiolabeled DNA eluted in the spun out fraction and was stored at -20°C.

B.3. Liposome Preparation:

Liposomes were prepared by methods decribed previously (31,37). Briefly, lipid films composed of DOPE:CHOL:OA (4:4:2 molar ratio) were dried under nitrogen and vacuum dessicated to remove residual traces of chloroform. The lipid films were hydrated in Buffer L (Hepes 10mM, EGTA 1mM, NaCl 150mM) and sonicated to form SUVs. pH was maintained at 8.0 throughout the preparation. In order to entrap DNA, octylglucoside was added at a 10-fold molar excess to the lipid. 200 ug of plasmid DNA was added and the mixture was dialyzed against Tris-HCl buffer (Tris-HCl 10mM, EDTA 1mM, NaCl 150mM, pH 8.0) for 12 hours without stirring, followed by 36 hours with stirring. The dialysis buffer, containing SM-2 beads

(generally 1gram/100 ml buffer) was changed after the first 24 hours. All liposome preparations were labeled with [3H] hexadecyl cholestanyl ether (CE) at 5 x10^4 cpm/umole lipid.

B. 4. Entrapment Analysis:

Liposome preparations were analysed for DNA entrapment by Ficoll gradient centrifugation (38). Briefly, liposomes were mixed (1:1 v/v) with 25% ficoll in Tris-HCl buffer. Three mls. of 10% ficoll were carefully added on top of this mixture followed by 1 ml of Tris-HCl buffer. The samples were centrifuged for 30 minutes at 3000 rpm and 4°C. Following centrifugation, 500 ul aliquots were taken (starting from the top and continuing through to the bottom) and counted for 32P and 3H distribution.

C. RESULTS

In pursuing this physical study, one of the first aspects investigated was the amount of entrapment of plasmid DNA inside the liposome. Since this was determined by using 32P-labeled DNA, we have first analyzed the DNA.

C.1. 32P-labeled DNA:

Nick translation of plasmid DNA, when done by common protocols (8), results in a majority of the plasmid DNA becoming nicked and so of lower molecular weight. This is shown by agarose

gel electrophoresis of the nicked DNA followed by autoradiography (Fig. 1, Appendix). Being smaller in size, these fragments are more likely to be encapsulated inside the liposomes than the otherwise large intact plasmid. Furthermore, these fragments are pieces of the intact gene rather than the whole gene, and are not likely to be expressed into active protein. These fragments do, however, add to the OD 260 reading and lead to an overestimation of the intact DNA entrapped.

In order to overcome this problem, greater quantities of DNA (10 ug) were used in the nick translation, and a shorter period of time (10 - 15 min.) was used in the reaction. This allowed for more labelling of the plasmid with very minimal small molecular weight products, as shown on agarose gels followed by autoradiography (Figs. 2 and 3). Thus, the DNA being used for the quantitation of entrapment was more representative of the DNA actually being used for delivery. In other words, the majority of the nicked DNA remained in the supercoiled configuration while a very small amount is actually nicked.

C.2. Entrapment Analysis:

Previously, nicked plasmid DNA was analyzed for entrapment by running the liposome preparation over a CL-2B column. This column supposedly separated liposome bound/entrapped DNA from free DNA. The liposome peak was then analyzed by an OD260 reading to determine the DNA content. Using this method, the liposomes were determined to entrap 16.5% of the input DNA (39).

Using this DNA to assay for encapsulation, it was seen that the CL-2B column was not efficient in separating free DNA from lipid associated DNA. As shown in Figure 4, DNA, whether in the absence or presence of lipid, elutes from the column in the same void volume fractions. This is probably due to the fact that both the liposomes and the plasmid are relativiely large in size, a factor that is reconsidered later in this thesis. At any rate, the column method of separation is very inefficient, leading to an overestimation of the amount of DNA entrapment in liposomes.

To achieve a more accurate method of separation, ficoll gradient centrifugation was used. Here liposomes were floated through a density gradient. Although the liposomes and the plasmid are large in size, they differ greatly in their buoyant densities, and therefore can be separated easily.

In these experiments the migration of the plasmid and liposome alone was first examined. As shown in Figure 5, this method provides a definite mechanism of separation. The plasmid DNA which is of greater density remains at the bottom of the gradient while the less dense liposome floats to the top.

When liposomes were prepared in the presence of DNA, or DNA was added to preformed liposomes, this method of separation shows very little association between the molecules. Figure 6 represents the gradient profile of liposomes made in the presence of DNA. Here only 5.9% of the recovered DNA is liposome associated. This does not tell us if the DNA is inside or adsorbed to the surface. In Figure 7, DNA was added to preformed liposomes and then floated through the gradient.

In this case approximately, 7.0% of the DNA was liposome associated, all of which is assumed to be by adsorption.

Judging from these results , it appears that this liposome preparation entraps very little DNA. This could be due to charge repulsions between the negatively charged DNA and the negatively charged lipid or the inability of the DNA to be entrapped because of its large size.

C.3. Lipid Composition and Size of Solute for Entrapment:

To examine further the lack of entrapment by the DOPE:CHOL:OA liposomes, two major changes were made. First, to test the possibility of a charge repulsion problem and/or a size of the solute problem, 3H-inulin was used. Second, the lipid composition was changed to see if differences in entrapment could occur.

The inulin molecule is a polysaccharide which is several times smaller than the pSV2CAT plasmid, having a m.w. of 5200. It is also uncharged, and so may elimininate the charge repulsion possibly seen with the DNA. Using 3H-inulin in conjuction with the 32P DNA, floating assays were done on liposomes made in the presence of both molecules or on preformed liposomes with both molecules adsorbed to the surface. In these experiments the lipid was not labeled with any radioactive marker.

In Figure 8, it seems that although there was very little entrapment of either molecule, there was slightly more inulin entrapped (3.2% compared to 2.1% of the DNA). To see if this was due to size or charge, both molecules were added to preformed

liposomes and then floated through the gradient. As Figure 9 shows, both molecules were adsorbed to the same extent (inulin 2.3% and DNA 2.1%). This rules out the charge question and suggests that the size of the molecule being entrapped may be the limiting factor in these experiments.

The second change made to study the entrapment problem was to modify the composition of the liposome itself. The new liposomes were composed of dioleoylphoshatidylcholine (DOPC):CHOL:OA (4:4:2) and , like the DOPE:CHOL:OA liposomes, bore a net negative charge. The PC molecule itself consists of the same long fatty acid chains that the DOPE molecule has, but it has a considerably larger and more hydratable headgroup. As a result, PC can form a stable bilayer by itself. To see if the entrapment could be improved with a more stable liposome the 3H-inulin and 32P-DNA experiments were repeated.

In Figure 10, the molecules were adsorbed to preformed liposomes. Here we see a minor increase in adsorption compared to the DOPE liposomes. However, both molecules were adsorbed to the same extent, (3.8% for both inulin and DNA), again ruling out a charge problem. Both molecules were then included in the buffer for the preparation of DOPC liposomes. As seen in Figure 11, there is again more trapping than in the DOPE liposomes, and also there is more trapping of the smaller inulin molecule (4.5%) than of the DNA (2.4%).

D. DISCUSSION

These experiments suggest that both the size of the solute molecule being entrapped and the liposome composition itself are limiting factors in the entrapment of DNA in liposomes ,which may in turn be the limiting factor determining the delivery efficiency to cells.

In order to be successful in transfecting cells with foreign genes, the efficiency of delivery must be great enough to allow for maximal delivery of the DNA available in the system. The current system is obviously not meeting these requirements.

First of all, by not entrapping much DNA inside the liposomes, we are not providing enough DNA for delivery, hence resulting in low levels of transfection. In spite of the increased amounts of DNA adsorbed to preformed liposomes, this is not likely to be useful in the in vivo experiments as there are abundant amounts of nuclease activity in the tissue fluid which would degrade this unprotected DNA.

The liposome composition being used is obviously very important for efficient delivery. Both compositions studied showed low levels of entrapment. DOPE, as mentioned earlier, is not able to form stable bilaylers by itself. However, this is the aspect of the lipid that makes it an excellent molecule for delivery. DOPE bilayers can be stabilized to entrap solute molecules and then destabilized, following fusion with the endosome membrane, to release its contents into the cytoplasm and avoid lysosomal degradation.

Obviously, changes in the system must be made that maintain the pH-sensitivity characteristic, yet entrap more DNA.

Although DOPC liposomes showed slightly better entrapment, they are not suitable to be used as a delivery system. As noted earlier, DOPC is able to form stable bilayers by itself. This aspect, even in the presence of the negatively charged OA molecule, makes it lack pH-sensitivity. It has been shown that lipsomes composed of mainly PC are generally much less effective in drug delivery than the corresponding PE-containing liposomes (39,40).

The data presented in this chapter also indicate the difficulty of entrapping a large molecule such as the plasmid DNA inside of liposomes. pSV2 CAT is a 5 kb plasmid. Even in the supercoiled rod configuration, it is approximately 0.1 um in length. The DOPE:CHOL:OA liposomes prepared by the detergent dialysis method have an average diameter of 0.5 um (41). It is evident that the size of the solute is not much smaller than the liposome itself. Steric hinderance would be a major problem. It would be desirable to prepare larger liposomes and/or to use smaller plasmid DNA or even linear DNA fragments.

OA is an amphiphile which possesses only a weak activity to stabilize the DOPE bilayer phase (42). It is also necessary to include a high concentration of CHOL to increase the liposome stability in buffer and in blood (41). However, this composition is obviously not ideal for DNA entrapment. We have thus turned to other amphiphiles with stronger DOPE bilayer stabilization activity and avoided the use of CHOL.

CHAPTER III

EVALUATION OF DNA ENTRAPMENT OF pH-SENSITIVE LIPOSOMES COMPOSED OF DOPE:DOSG (7:3)

A. INTRODUCTION

In looking for other suitable amphiphiles to stabilize the DOPE bilayer, we turned away from the single chain fatty acids and looked at the possibility of double chain amphiphiles.

It has been shown by several groups, that single chain amphiphiles can readily dissociate from the membranes in which they are incorporated (43-47). It must be considered then if these liposomes would really maintain their stability when in the presence of those agents which could possibly extract the amphiphiles. Those agents include biological membranes or serum components, and so should be considered when heading towards in vivo work.

In the past couple of years, a series of double chain amphiphiles have been prepared (48). These amphiphiles were designed to have characteristics similar to the single chain amphiphiles, i.e. they have a net negative charge at pH 7.0, but become protonated at a mildly acidic pH. It was our thought to incorporate one of these amphiphiles into the DOPE bilayer and examine its DNA entrapment efficiency and pH-sensitivity.

The amphiphile chosen for these studies was dioleoylsuccinylglycerol (DOSG), a nonphospholipid consisting of two

oleic acid chains and a protonatable hemisuccinate head group. In work done in our lab (42), it was seen that a derivative of this lipid, DOSG, is able to stabilize DOPE bilayers and the resulting liposomes exhibit a lower acid sensitivity than the liposomes composed of DOPE and OA. The work in this chapter reflects studies done to elucidate the DNA entrapment efficiency of DOPE:DOSG liposomes.

B. MATERIALS AND METHODS

B. 1. Materials:

Dioleoylethanolamine (DOPE) and Dioleoylsuccinylglycerol (DOSG) were purchased from Avanti (Birmingham, Al.). Cholesterol, calcein and octylglucoside were purchased from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories. Plasmid pSV2CAT was a gift from Dr. Wesley D. Wicks. Plasmid pSV2CAT-intron, similar to the pSV2CAT plasmid except 600 bp of the noncoding intron is removed, was a gift from Genentech, Inc. Both plasmids were isolated using the pz523 column procedure (35).

B. 2. Nick Translation:

Nick translation of both plasmids followed the same protocol as in Chapter II, sec. B. 2.

B.3. Liposome Preparation:

Liposomes containing DOPE:CHOL:DOSG (4:4:2) or DOPE:DOSG (8:2 and 7:3) were prepared following the procedure outlined in Chapter II, sec.B.3. For pH-sensitivity experiments, calcein was added to the liposome suspension, following the addition of octylglucoside, to a final concentration of 50 mM. DNA was not included in these experiments. The liposomes were dialyzed against Tris:HCl buffer containing 50 mM calcein.

B. 4. Entrapment Analysis:

Analysis of DNA entrapment by Ficoll density gradient centrifugation was identical to the procedure used in Chapter II, sec. B. 4.

B. 5. pH-Sensitivity:

Liposomes made in the presence of 50 mM calcein were passed over a Sephadex G-75 column to remove any unencapsulated calcein. Using a Perkin Elmer LS5 spectrofluorometer with ex=490nm and em=520nm, the fluorescence of the liposomes was measured. In these experiments, 10 ul of calcein entrapped liposomes were added to 1.99 ml of Tris:HCl buffer at various pHs and incubated at room temperature with continuous stirring for 5 minutes. The appropriate amount of NaOH was added to the sample to return the pH to 8.0 and a fluorescence reading was taken.

Forty ul of 5% DOC was then added to allow for complete release of the calcein and another reading was taken.

Using the dequenching of calcein fluoresence, the percent release by the liposomes was measured using the following equation:

$$\% \text{ Release} = \frac{F - Fo}{Ft - Fo} \times 100$$

where Fo is the fluorescence of the liposomes at pH 8.0, F is the reading after treatment at the lower pH followed by the titration back to pH 8.0, and Ft is the reading after complete release by DOC. Since the liposomes were added directly to buffers of various pHs, the Fo reading of the liposomes was taken every 15 minutes to ensure that they were not leaky over time at pH 8.0. The percent quenching was calculated by using the fluorescence before and after addition of DOC and using the equation:

$$\% \text{ Quenching} = 1 - \frac{Fo}{Ft} \times 100$$

These liposomes remained quenched in the upper 80% range throughout the experiment, indicating their stability over time at pH 8.0.

B.6. Serum Stability:

Liposomes composed of DOPE:DOSG (7:3) were prepared by dialysis or by sonication in the presence of 50 mM calcein. The unentrapped calcein was removed by using a Sephadex G-75 column. In these experiments, 250 ul of each sample was mixed with 250 ul

of either normal human serum or column buffer, both of which had been warmed at 37°C for 15 minutes, and the mixtures were placed back at 37°C. At time zero and designated time points later, 20 ul of the mixture was mixed with 1.98 ml of column buffer. The fluorescence was measured and the % release was calculated identically to the pH sensitivity experiments. However, Fo is the fluorescence of the sample at time zero, F is the fluorescence at the designated time points, and Ft is the total fluorescence after DOC addition.

C. RESULTS

The purpose of changing the composition of the liposomes was to obtain a greater degree of DNA entrapment. pH-sensitivity, however, was a credential that had to be maintained. Therefore, both properties are examined with the new lipid compositions.

C. 1. Entrapment Analysis:

As in the previous chapter, DNA entrapment was analyzed by using the Ficoll floatation assay. In initial experiments, the OA component was replaced with the double chain amphiphile DOSG. Here, entrapment was analyzed with or without cholesterol. In these studies, the compositions were at molar ratios of 4:4:2 (DOPE:CHOL:DOSG) or 8:2 (DOPE:DOSG), and entrapment was examined for DNA and inulin added to preformed liposomes or added in the buffer of the liposomes to be dialyzed.

Figures 12 and 13 are profiles of the DOPE:CHOL:DOSG liposomes after floatation through the gradient. In figure 12, DNA and inulin were added to the preformed liposomes before floatation. As is seen, approximately 7.4% of DNA and 8.3% of inulin are liposome associated. When added to the liposomal buffer and dialyzed to be entrapped, 8.1% of the DNA and 12.6% of the inulin are liposome associated, as shown in figure 13. These results indicate that most of the DNA associated with the liposome was due to adsorption. However, some inulin entrapment was seen with this composition.

In the next experiments, the cholesterol component was removed and the floatation was repeated. Here we see a great increase in liposome association, with 9.8% of the DNA and 12% of the inulin being associated in the preformed samples, and 16% of the DNA and 21.8% of the inulin associated with those samples dialyzed to entrap (Figures 14 and 15 respectively).

These results may be explained by the great differences in the size of the different liposomes. The liposomes made in the presence of cholesterol were approximately 0.3 um in diameter after dialysis and extrusion. Those made without cholesterol were 0.5 um in diameter, allowing for more entrapment of the large DNA plasmid. However, entrapment of inulin is still better than that of DNA, indicating that size of the solute is still a major determining factor for entrapment. The amount of entrapment with the DOPE:DOSG liposomes is better than the previous composition being used, however, there is room for improvement. This will be considered later.

C. 2. pH-Sensitivity:

In trying to assess the validity of the new liposome composition, we also had to test whether the required pH-sensitivity characteristic was present. It has previously been shown (42) that liposomes composed of DOPE:DOSG (8:2) are pH-sensitive, showing 50% release at pH 6.5. However, these liposomes were made by sonication and the average diameter of the liposome was less than 0.1 um. Although the composition is the same, preparations of liposomes made by different methods may differ in their characteristics. The pH-sensitivity of the DOPE:DOSG liposomes made by the dialysis method must be examined.

It was found that these liposomes were in fact, quite different from the soniciated liposomes. The entrapped calcein leaked out at a rate of 1.5%/min at pH 8 and room temperature, such that the measurement of pH-sensitivity of the liposome could not be performed. Although the DOSG offers one extra stabilizing fatty acyl chain compared to the previously used OA, the presence of twice as much DOPE in the system most likely requires more than twice as much of the stabilizers.

Therefore, we concluded that although the liposomes composed of DOPE:DOSG (8:2) are stable enough to entrap large molecules such as DNA and inulin, they are leaky to small molecules such as calcein. These results indicate that liposomes of this composition have a borderline stability, and further improvement in composition is needed.

To improve the liposome composition, we decided to increase the DOSG concentration . Changing the composition to include more DOSG and thus less DOPE did allow for the preparation of stable and pH-sensitive liposomes by the detergent dialysis method. It was seen that the liposome preparations could remain up to 80% quenched for at least two weeks when kept under nitrogen and stored at 4 C.

Experiments done to examine the pH-sensitivity of the DOPE:DOSG (7:3) liposome composition showed that they release 50% of their contents at a lower pH than liposomes made of the same composition but by a different method. As Figures 16 and 17 show, sonicated liposomes composed of DOPE:DOSG (7:3) release 50% of their contents at pH 7.0. Those made by dialysis release the same amount at approximately pH 5.3, indicating that they are more stable than the sonicated type and so less pH sensitive. This aspect, however, essentially guarantees that the contents of the liposome will not be released until they reach the late endosomes, because the pH of the late endosomes is 5-5.5 (49,50).

The differences in pH-sensitivity of the two types of liposomes can be explained by their size differences. As was mentioned earlier, DOPE is not able to form a stable bilayer under physiological conditions. This is due to its inverted cone shape; a result of the small, poorly hydratable, head group and bulky acyl chains. In order for DOPE to be stabilized, a second component of the opposite shape is necessary. DOPE packs well in the inner monolayer of a small liposome with high curvature, because the lipid packing resembles that of HII phase. Packing of DOPE, together with the

stabilizer, in the outer monolayer is not energetically favorable due to the concave curvature. Thus, the small sonicated liposomes are inherently unstable. Liposome destabilization occurs when only a small fraction of the stabilizer is protonated. On the other hand, larger liposomes of smaller curvature are more stable due to the relief of the packing constrain in the outer monolayer. These liposomes destabilize only when a larger fraction of the stabilizer is protonated.

C. 3. Serum Stability:

If one is going to eventually use this liposome composition in vivo, its stability in serum must be analysed. It has previously been shown (42) that liposomes composed of DOPE:DOSG (8:2) are serum stable when prepared by sonication.

In the experiments done here, the composition is DOPE:DOSG (7:3). The liposomes were prepared by sonication as a comparison to the 8:2 composition, and also by dialysis. As shown in Figure 18, those liposomes prepared by sonication, in the presence of serum or buffer , are very stable and show little release over a 2 hour period. The liposomes prepared by dialysis are stable in the presence of buffer , but show 60% release within the first 5 minutes after exposure to serum, with a continuous but slower leakage thereafter. Thus the liposomes prepared for DNA entrapment are not stable in serum.

The lack of serum or plasma stability of liposomes prepared by dialysis is likely due to its large size (average diameter = 239 nm).

Work done in this laboratory (51) has shown that the serum stability of liposomes composed of DOPE as the major phospholipid ingredient depends on the diameter of the liposomes. Liposomes smaller than 200 nm are stable; larger liposomes are progressively less stable in serum. The sonicated liposomes used in this experiment had an average diameter of 170 nm, as measured by dynamic laser light scattering, and these liposomes are quite stable in serum (Fig. 18). It has been proposed that serum apolipoprotein(s) inserts into the outer monolayer of a small liposome and provides stability against the lytic activity of other serum components (51). Insertion of apolipoprotein(s) to the larger liposome membranes is probably nonexisting or taking place only at a slow rate.

C. 4. Entrapment Analysis of DOPE:DOSG (7:3):

The presence of DOSG allowed for greater trapping in otherwise leaky DOPE:DOSG (8:2) liposomes, and increasing the amount of DOSG made the liposomes more stable. Therefore, in the next part of the experiment we examined the trapping efficiency of a smaller plasmid in this new, more stable, DOPE:DOSG (7:3) liposome.

The new plasmid, pSV2CAT-intron, was identical to the previous DNA that was used in terms of the gene it coded for; however, a part of the non-coding intron (approximately 600 base pairs) was removed. It was thought that this somewhat smaller plasmid would be entrapped more efficiently than the larger plasmid.

Entrapment of this smaller plasmid in the more stable liposome composition was analyzed by Ficoll gradient centrifugation. Again, the DNA was added to preformed liposomes or to the liposomal buffer before dialysis. In Figure 19, the DNA and inulin were added to preformed liposomes. Here, there was approximately 19% of DNA and 20% of inulin associated with the liposome. When the liposomes were made in the presence of the DNA and inulin, there was a 17% and 51% association respectively as seen in Figure 20. Clearly, the entrapment of inulin of the more stable composition is very high. However, the entrapment of DNA seems not enhanced. The great increase in the DNA association, however, was a definite improvement over the previous compositions . At this point another factor was considered.

In the previous experiments to analyze DNA entrapment, 10 ul of 32P-DNA was used. In an actual nick translation, 10 ug of plasmid was nicked in a total volume of 100 ul, hence the 10 ul being analyzed only contained 1 ug of DNA. This DNA is an accurate representation of the DNA that would later be entrapped for delivery, so it is sufficient for the entrapment analysis. However, we wondered if this entrapment would change if the actual amount of DNA used in a real liposome preparation was floated through the gradient. To study this we simply mixed 20 ul of the labeled DNA with 180 ug of unlabeled plasmid DNA and repeated the experiments. Much to our surprise, the amount of DNA entrapment did increase, but only in those experiments where the liposomes were made in the presence of the DNA. As is seen in Figure 21, when 180 ug of plasmid DNA was placed in the liposomal buffer, approximately 31%

became liposome associated. The same amount of DNA added to preformed liposomes became associated to roughly the same extent as when only 1 ug of labeled DNA was used (15.5%).

The increase in association seen with the liposomes made in the presence of DNA may simply be due to the fact that the high concentration of DNA has caused saturation of DNA adsorption on the liposomes. At low concentration (1ug), only adsorption of DNA to the liposome membrane is seen.

D. DISCUSSION

These experiments do indeed prove that entrapment of plasmid DNA in liposomes is very dependent on the liposome composition and possibly the size of the DNA itself. With various alterations in the two components perhaps a combination can eventually be acheived whereby the liposome is very stable, pH-sensitive and not subject to component extraction, and the DNA is small enough to increase entrapment to the extent of being over 50%.

We have shown here that by removing the cholesterol from the liposome composition, both DNA and inulin entrapment have increased. Cholesterol is included in the previous lipid compositions to increase the serum stability of the liposome (40). As we have seen, the current liposome composition is not serum stable. Therefore, the appropriate amount of cholesterol may be added back to the liposome composition to increase the serum stability without sacrificing the extent of DNA entrapment. Alternatively, other

amphiphiles such as dipalmitoylsuccinylglycerol (DPSG) may be tested as a substitute for DOSG. DPSG is a stronger bilayer stabilizer than DOSG (41), and therefore may provide a higher level of stability of the liposome against the serum assult. The stability of the DOPE:DOSG (7:3) liposomes in buffer should allow it to be . useful for in vitro studies.

We have shown that by removing the OA, a component which can be extracted by biological membranes or serum components and so cause liposome instability, we can add DOSG, a component that not only has the same stabilizing activity and pH-dependence effect as OA, but is not as extractable and less pH-sensitive (i.e. releases at lower pH, most likely in the late endosomes). This alteration does indeed allow for the liposomes to have the required characteristics mentioned earlier.

The high level of DNA entrapment was seen with a plasmid which is 600 bases shorter than the previous plasmid used. It is possible that by decreasing the size of the plasmid, the amount of entrapment increases. If this is the case, one can only assume that decreasing the size of DNA further (i.e. to the extent of fragments consisting of the promoter and gene of interest only), will result in the entrapment efficiency being increased even more.

The future of this project lies in the area of using the new liposome composition of DOPE:DOSG (7:3) for in vitro transfections with the small plasmid DNA. Hopefully, this composition will help to overcome the lack of transfection previously seen with the DOPE:CHOL:OA liposomes and will successfully transfect L-929 cells.

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APPENDIX

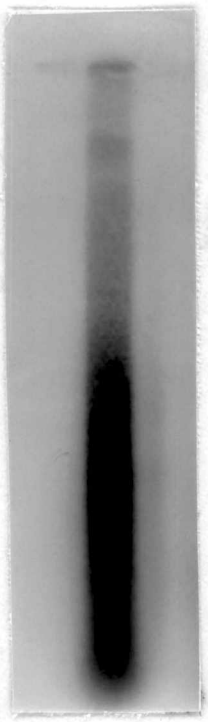


FIGURE 1. An autoradiogram of nick translated pSV2CAT plasmid DNA. 1 ug of plasmid DNA was nicked under the conditions described in common protocols (1 ug of DNA, reaction time 1/2 hour). As can be seen, under these conditions, a majority of the plasmid DNA becomes small molecular weight fragments and so is an inaccurate representation of the actual plasmid being entrapped.

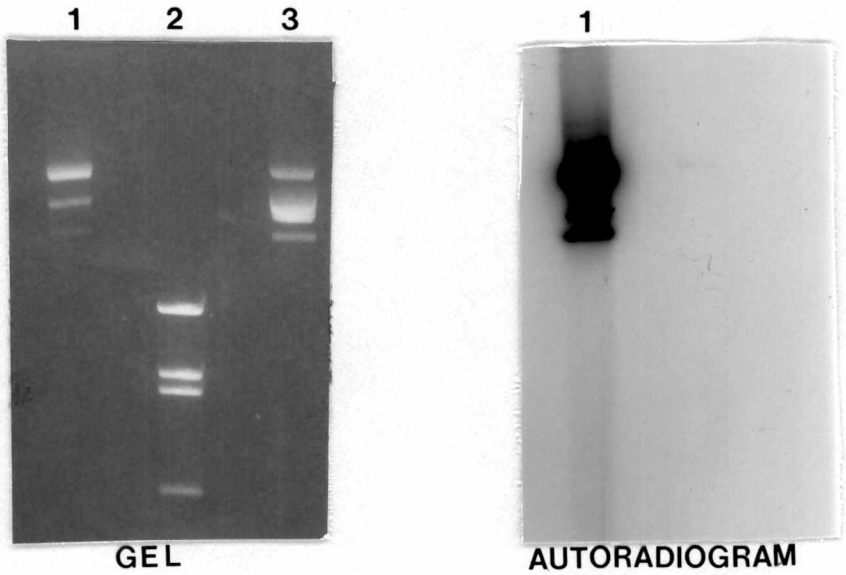


FIGURE 2. A 1% agarose gel of 1 ug each of nicked pSV2CAT, standard DNA, and cold plasmid DNA (lanes 1-3 respectively). After nick translation (see figure 3 legend for method), 1 ug of the DNA was run in conjunction with the cold plasmid DNA. This justifies the presence of intact plasmid DNA following nick translation.

FIGURE 3. An autoradiogram of pSV2CAT DNA nick translated under modified conditions. Here 10 ug of plasmid DNA were reacted for no more than 15 minutes. After the DNA was run on the gel (Fig. 2), the gel was dried and autoradiographed for 24 hours. As is shown in lane 1 there is a great decrease in the small molecular weight products made by the previous method. Lanes 2 and 3 are the standard DNA and the cold plasmid DNA respectively. They do not appear because they were not labeled in the above gel.

FIGURE 4. Elution profile of free DNA (⊡) or lipid associated DNA (◆). 10 ul of labelled DNA was either mixed with 90 ul of liposomes (.9 umole lipid) or Tris:HCl Buffer (pH 8.0). The samples were run over a 20 ml sepharose CL-2B colum equilibrated with Tris:HCl and eluted into .5 ml fractions. The fractions were counted for 32P. The DNA elutes from the column at the void volume fractions whether in the presence or absence of lipid.

FIGURE 5. Floatation assay of free liposomes and free 32P-DNA by ficoll density gradients. 500 ul of liposome (5 umole lipid) or DNA (1 ug; 10 ul in 490 ul Tris:HCl buffer) were mixed with 500 ul of 25% ficoll. 3 ml of 10% filoll were layered on top followed by 1 ml of Tris:HCl buffer. The gradients were spun for 30 min at 3000 rpm at 4 C. 500 ul aliquots were taken from the gradient, starting from the top, and total radioactivity was counted. DNA labelled with 32P and lipid was labelled with 3H. Free DNA (◆) remains at the bottom of the gradient while free liposomes (⊡) float to the top.

FIGURE 4.

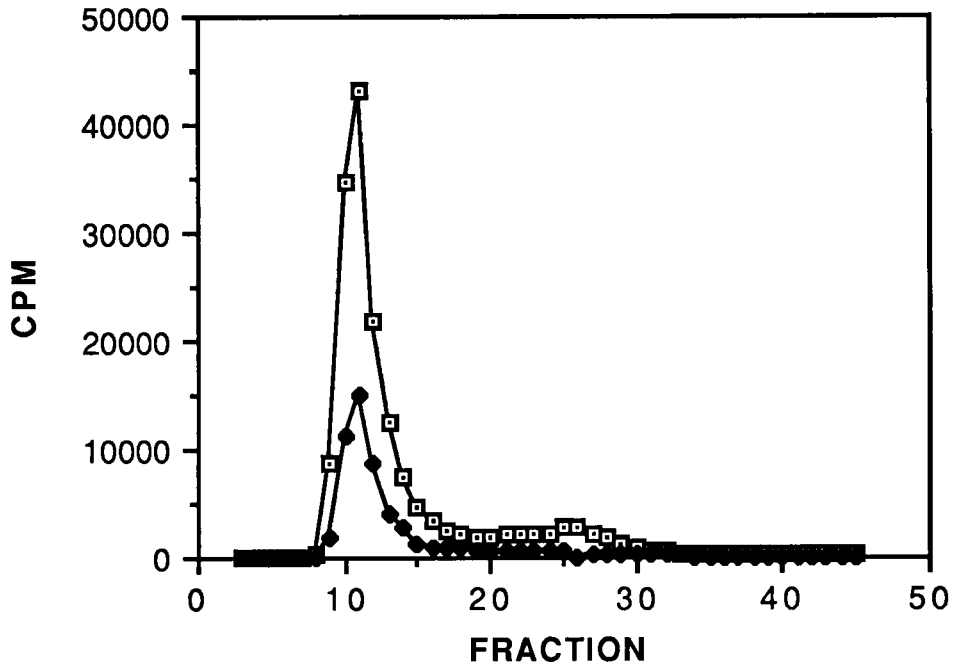


FIGURE 5.

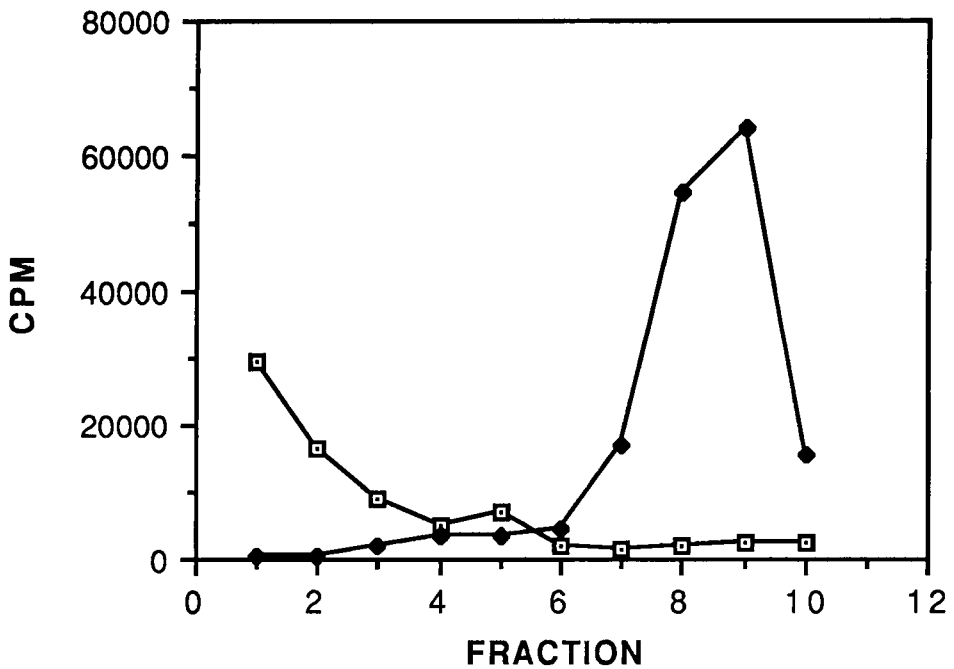


FIGURE 6. Floatation assay of liposomes (5 umole lipid) prepared in the presence of DNA (1 ug). Procedure is the same as in Figure 5. Floatation allows for clear separation of the two molecules with the 3H lipid (⊡) at the top and the 32P-DNA (◆) at the bottom. Approximately 6.0% of the total DNA is liposome associated.

FIGURE 7. Floatation assay of preformed liposomes (5 umole lipid) with DNA (1 ug) added. The procedure is the same as in Figure 5. 3H lipid (⊡) floats to the top while 32P-DNA (◆) remains at the bottom. Only 7.0% of the DNA is liposome associated, and assumed to be through adsorption.

FIGURE 6.

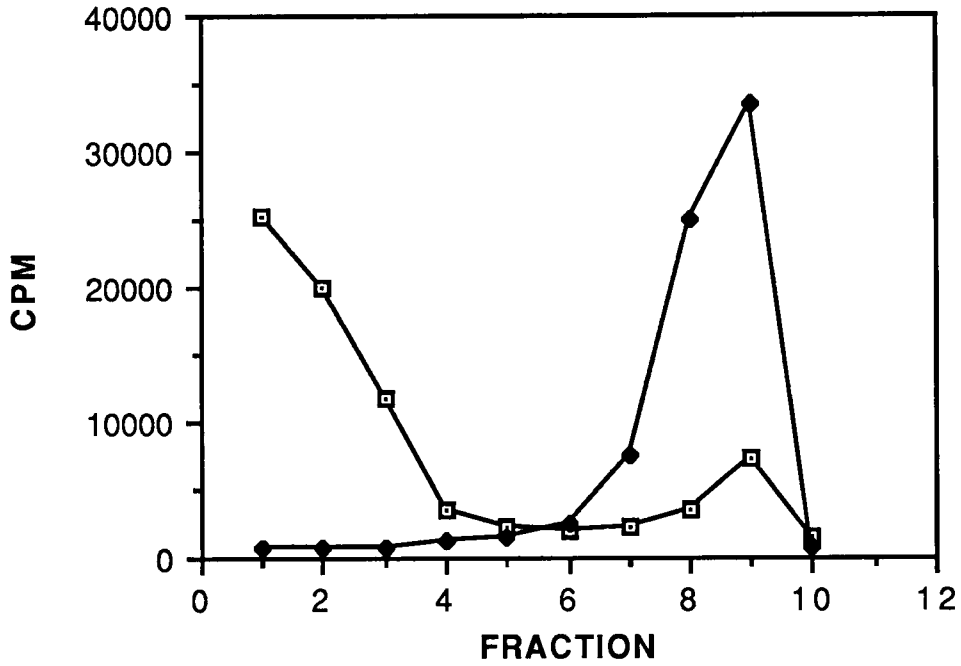


FIGURE 7.

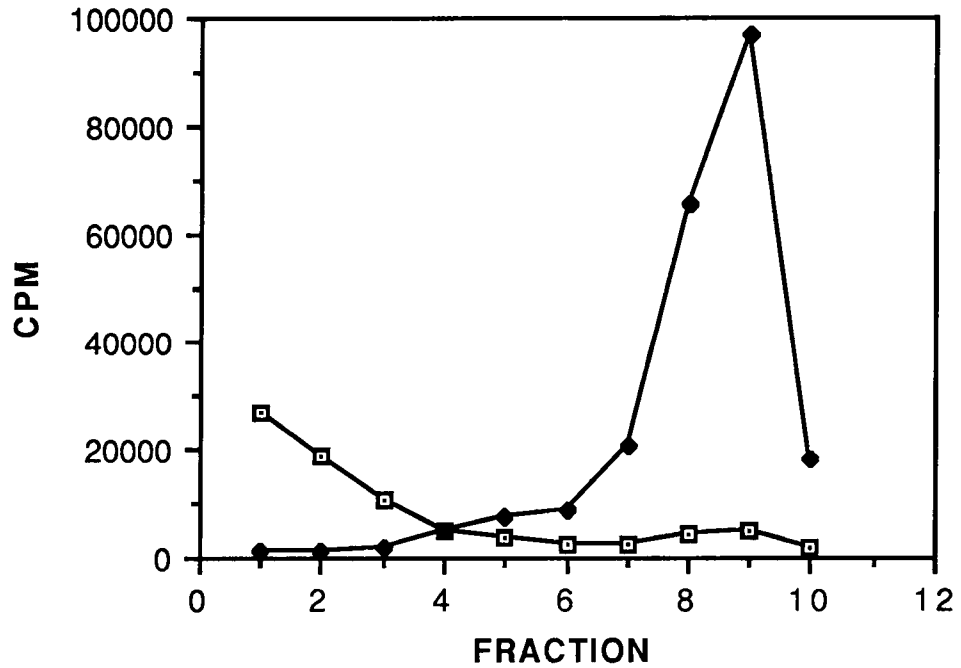


FIGURE 8. Floatation assay of 3H-inulin (⧈) and 32P-DNA (◆) entrapped in DOPE:CHOL:OA liposomes. 10 ul (2.75 dpm/ul, 3200 mCi/mmole) 3H-inulin and 1 ug 32P-DNA were floated as previously described. Approximately 3.2% of the inulin was entrapped whereas 2.1% of the DNA was entrapped.

FIGURE 9. Floatation of 3H-inulin (⧈) and 32P-DNA (◆) added to preformed DOPE:CHOL:OA liposomes. Both molecules are lipid associated to the same extent (2.3% for inulin and 2.1% for DNA).

FIGURE 8.

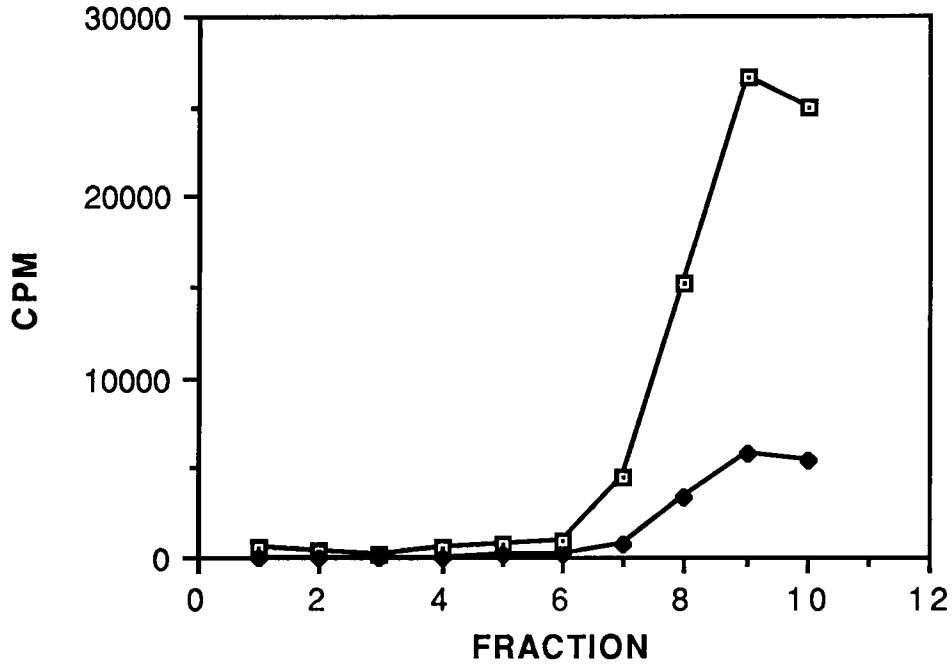


FIGURE 9.

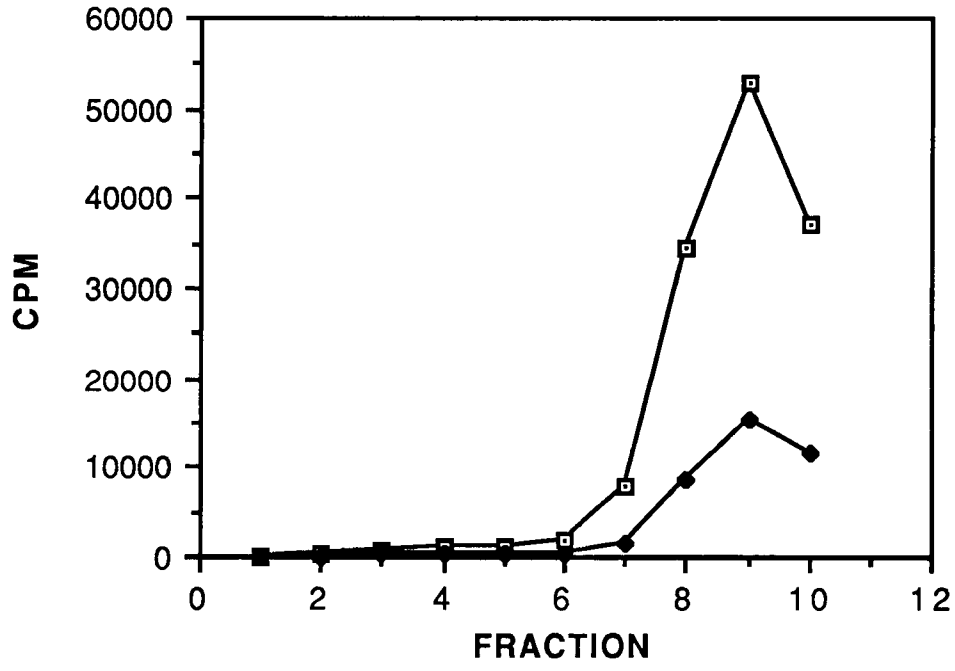


FIGURE 10. Floatation assay of 3H-inulin (⧈) and 32P-DNA (◆) adsorbed to DOPC:CHOL:OA liposomes. Adsorption was greater than with the previous composition tested, however, it was again to the same extent for both molecules, i.e. 3.8%.

FIGURE 11. Floatation assay of 3H-inulin (⧈) and 32P-DNA (◆) entrapped in DOPC:CHOL:OA liposomes. An increase in trapping is seen by this composition and also in terms of the smaller inulin molecule (4.5% for inulin and 2.4% for DNA).

FIGURE 10.

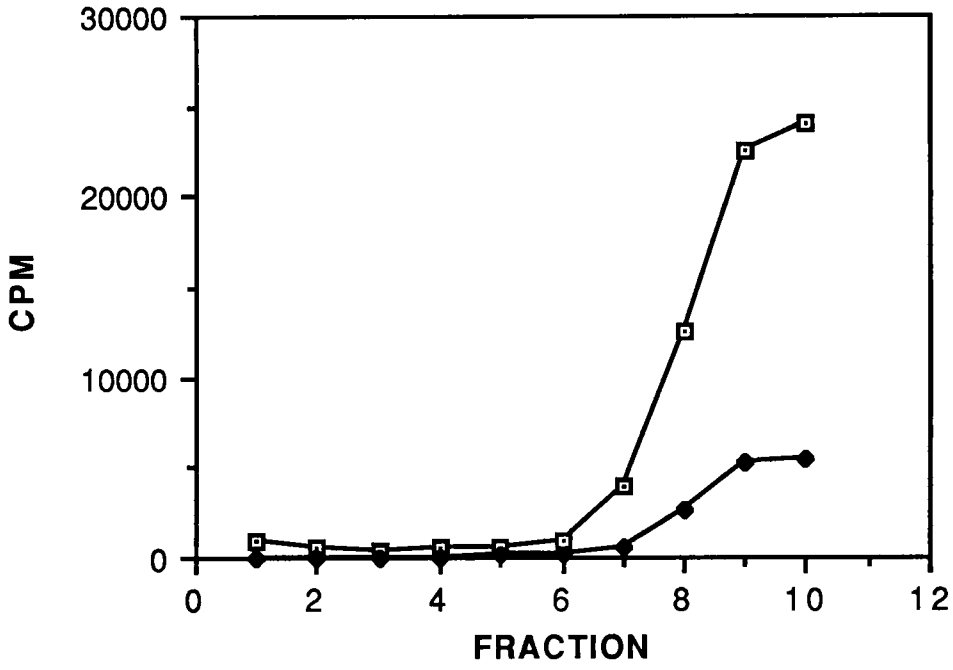


FIGURE 11.

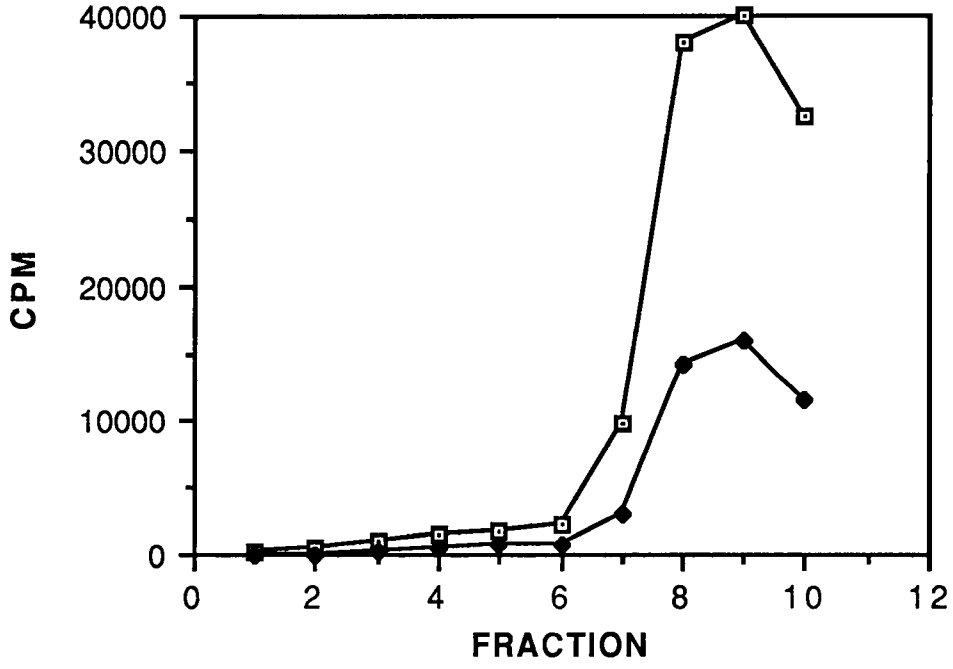


FIGURE 12. Floatation assay of 1 ug 32P-DNA and 3H-inulin added to preformed DOPE:CHOL:DOSG liposomes (5 umole lipid). Both molecules remain at the bottom on the gradient except for 7.4% of the DNA (◆) and 8.3% of the inulin (⊡).

FIGURE 13. Floatation assay of 32P-DNA and 3H-inulin added to the DOPE:CHOL:DOSG liposomal suspension before dialysis. Here 8.1% of the DNA (◆) and 12.6% of the inulin (⊡) is liposome associated.

FIGURE 12.

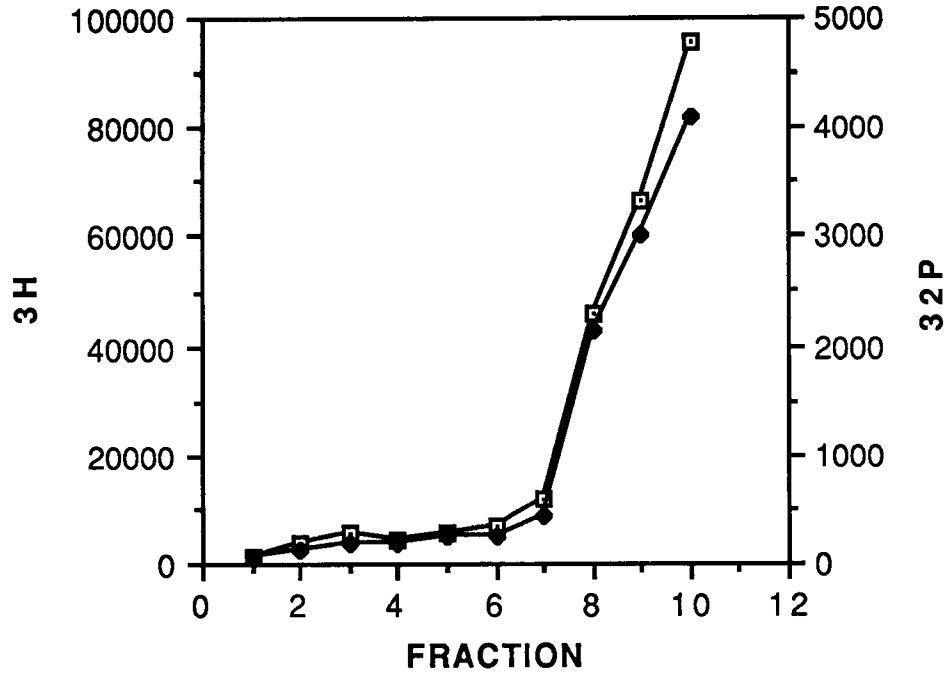


FIGURE 13.

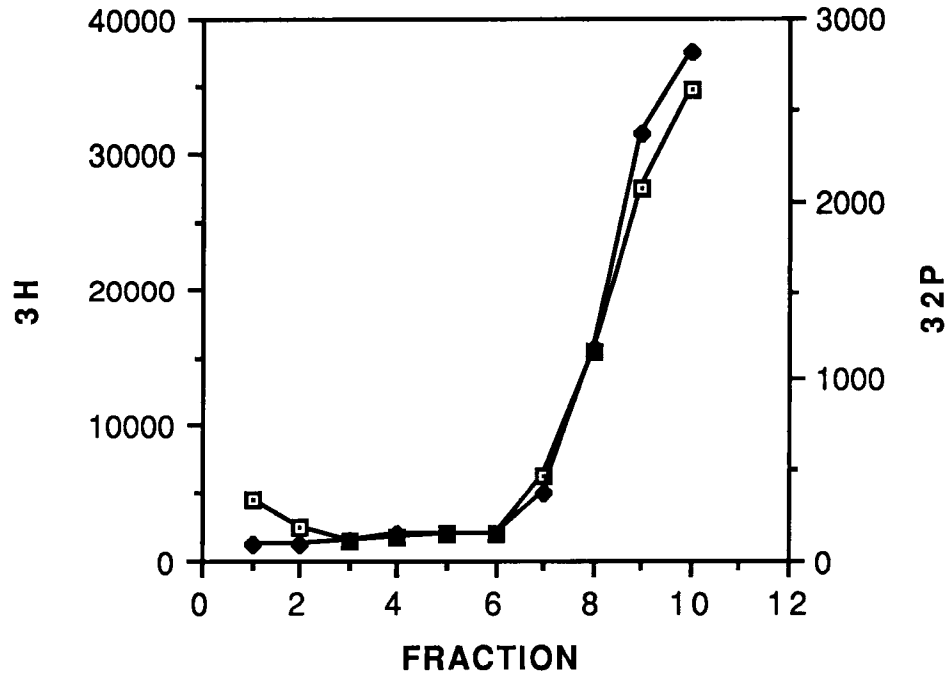


FIGURE 14. Floatation assay of 32P-DNA and 3H-inulin added to preformed DOPE:DOSG (8:2) liposomes. 12% of the inulin (⊡) and 9.8% of the DNA (◆) are liposome associated.

FIGURE 15. Floatation assay of DOPE:DOSG (8:2) liposomes dialyzed in the presence of 32P-DNA and 3H-inulin. 22% of the inulin (⊡) and 16% of the DNA (◆) are liposome associated.

FIGURE 14.

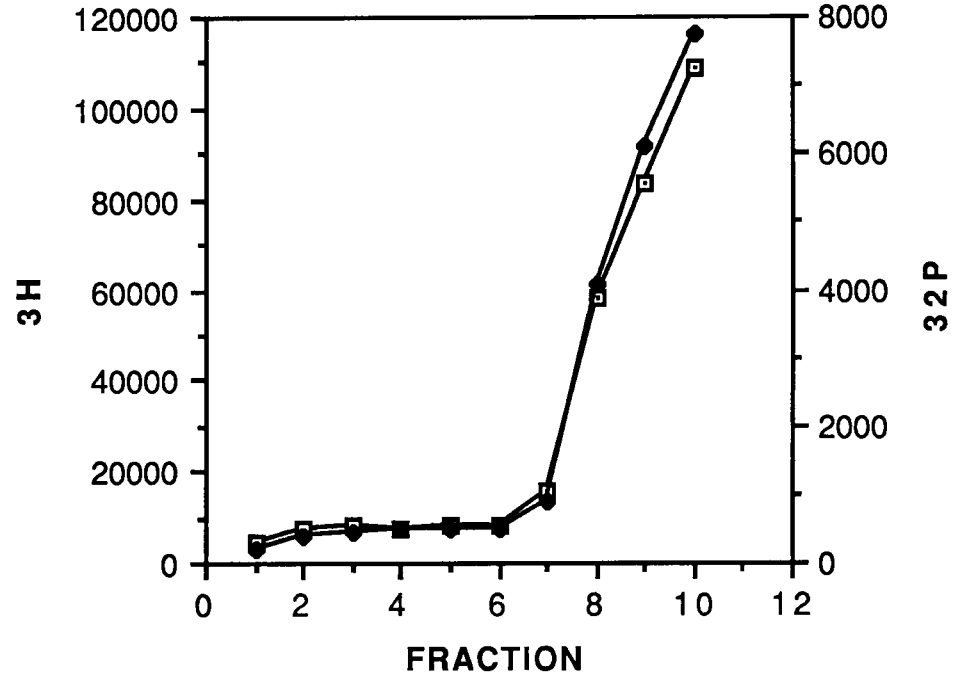


FIGURE 15.

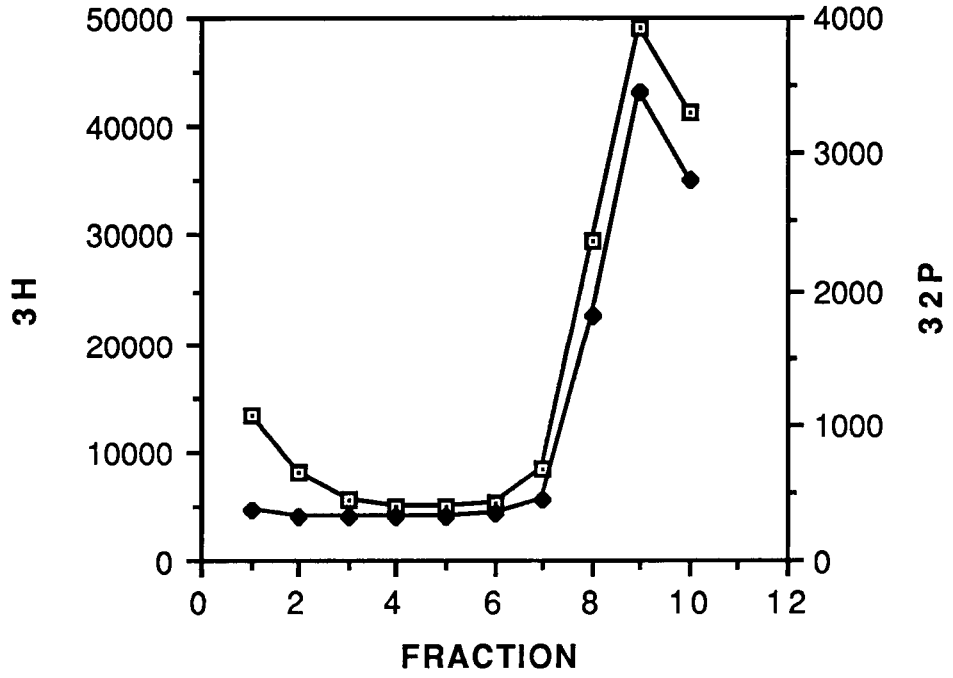


FIGURE 16. pH-sensitivity curve of sonicated DOPE:DOSG (7:3) liposomes. Sonicated liposomes release 50% of their contents at pH 7.0.

FIGURE 17. pH-sensitivity curve of DOPE:DOSG (7:3) liposomes prepared by dialysis. These liposomes are much less pH-sensitive, releasing 50% of their contents at pH 5.3.

FIGURE 16.

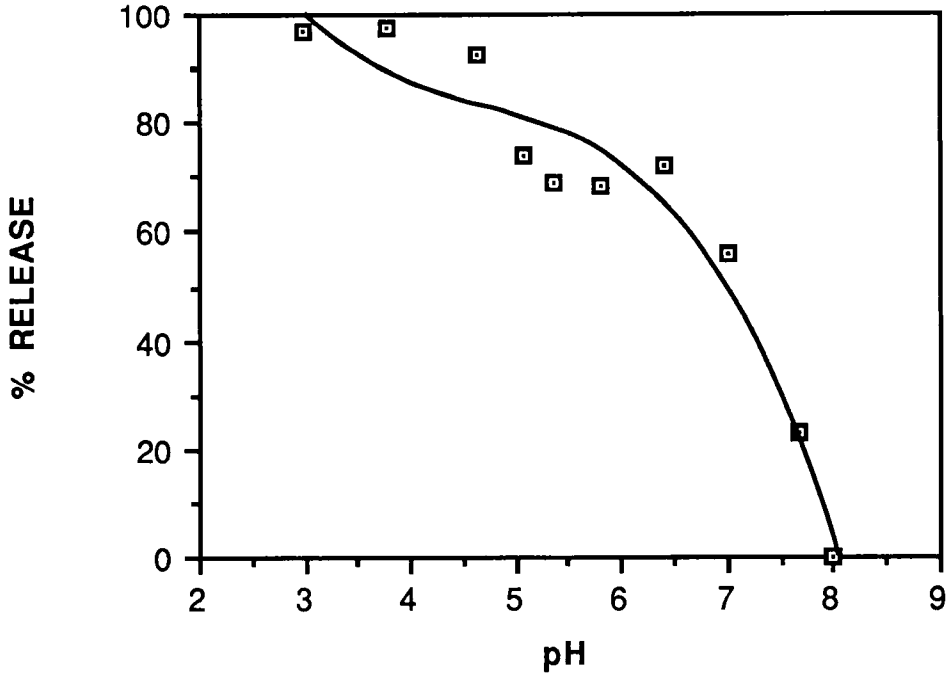
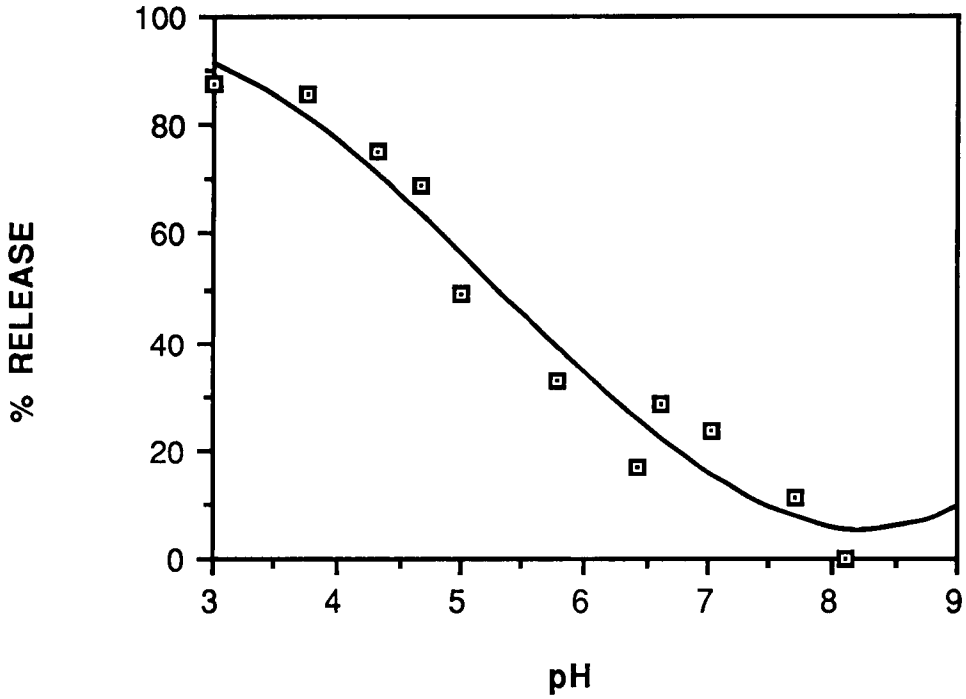


FIGURE 17.



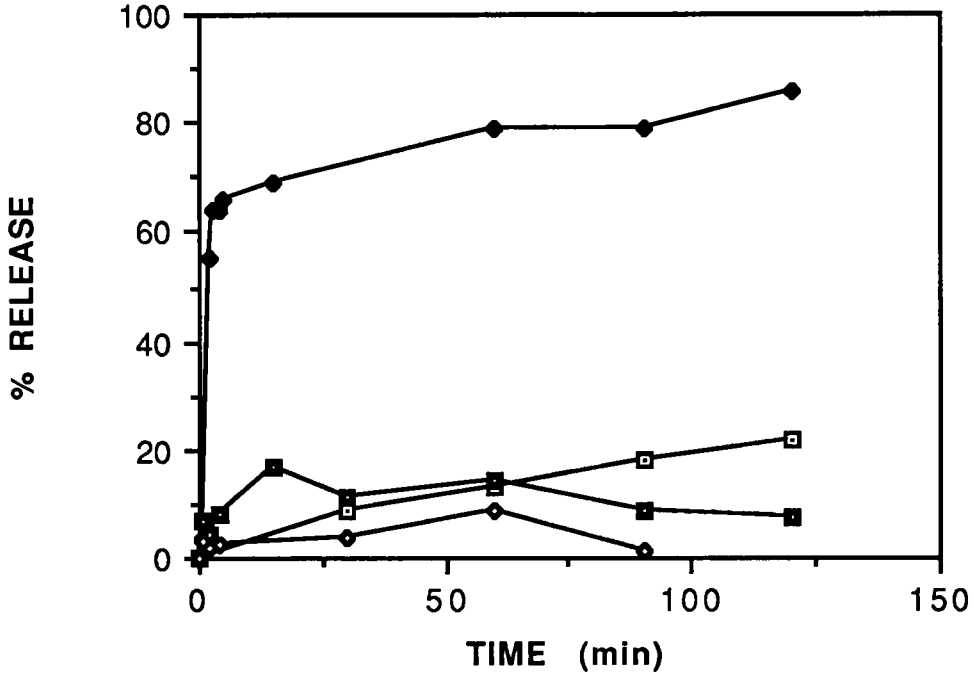


FIGURE 18. Serum stability of DOPE:DOSG (7:3) liposomes. Liposomes were prepared by dialysis or sonication and tested for their stability in 50% buffer or serum over a 2 hour period. Those liposomes prepared by sonication are very stable in both serum (□) and buffer (◇). Liposomes prepared by dialysis are stable in buffer (⊡), but are quite leaky in the presence of serum (◆).

FIGURE 19. Floatation assay of 3H-inulin and 32P-DNA added to preformed DOPE:DOSG (7:3) liposomes. 19% of the smaller pSV2CAT-intron DNA is liposome associated (◆) while 20% of the inulin (⊡) is associated.

FIGURE 20. Floatation assay of 3H-inulin and 32P-DNA added to the DOPE:DOSG (7:3) liposomal suspension prior to dialysis. 17% of the DNA (◆) and 51% of the inulin (⊡) is liposome associated.

FIGURE 19.

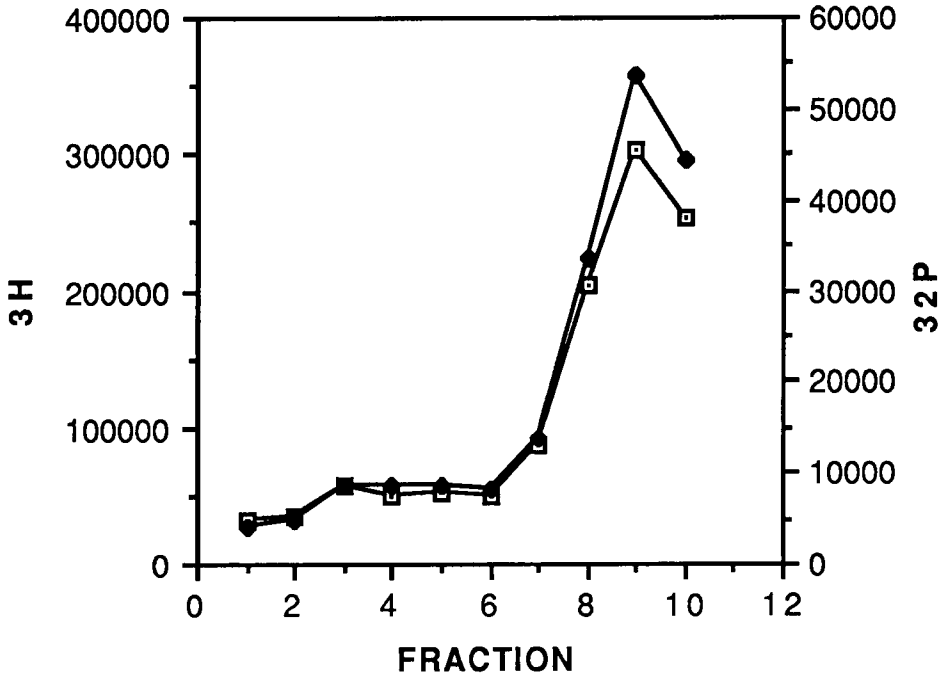
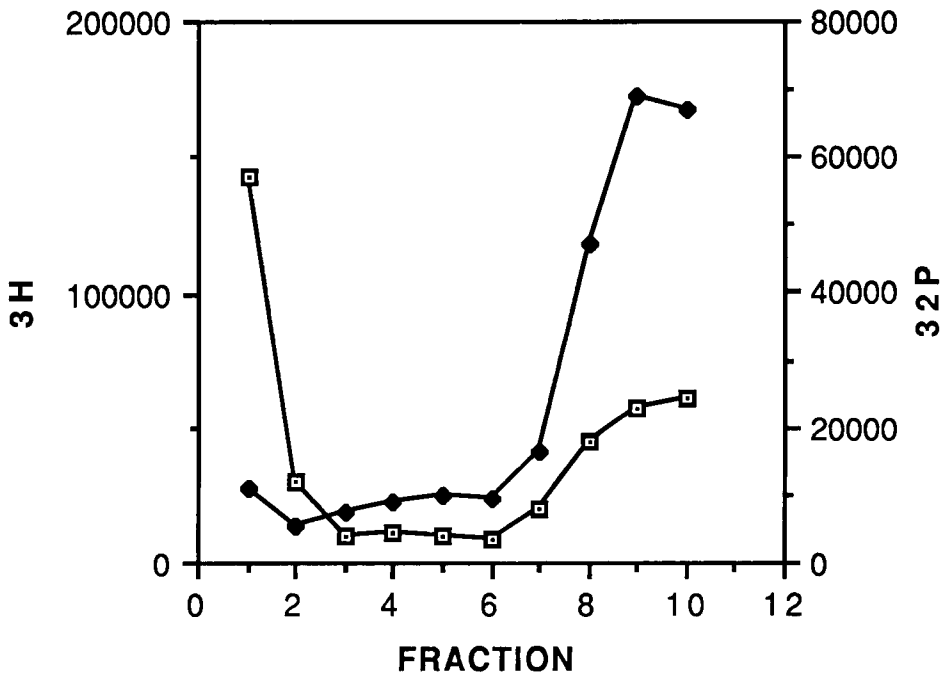


FIGURE 20.



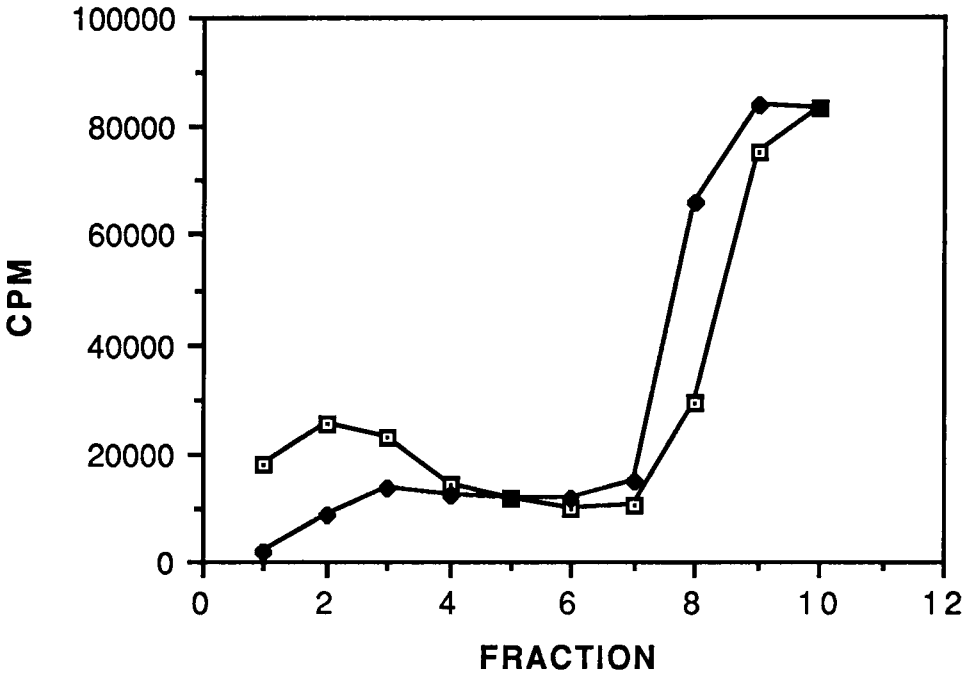


FIGURE 21. Floatation assay of 200 ug 32P-DNA added to preformed liposomes (◆) or placed in the liposomal buffer and dialyzed (⊡). 15.5% of the DNA is liposome associated in the preformed samples while 31% of the DNA is associated in the dialyzed samples. No 3H-inulin was used in this experiment.

VITA

Lori Ann Schmitt was born in Pittsburgh, Pennsylvania on December 10, 1964. After graduating from North Hills High School in 1982, she entered Allegheny College in Meadville, Pennsylvania. She graduated in June of 1986 with a Bachelor of Science degree in Biology. In the fall of 1986, she accepted a teaching assistantship at the University of Tennessee, Knoxville and began working towards a Master's degree in Biochemistry. This degree was awarded in April of 1989.