To the Graduate Council:

I am submitting herewith a thesis written by Chen-Yen Wang entitled "Polyhistidine Mediates an Acid Dependent Fusion of Negatively Charged 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:

Ernst Freer

Robert H. Feinberg

Accepted for the Council:

Lew Minkel

The Graduate School

4

POLYHISTIDINE MEDIATES AN AICD DEPENDENT FUSION OF NEGATIVELY CHARGED LIPOSOMES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Chen-Yen Wang

March 1984

ACKNOWLEDGMENTS

I would like to express my deep appreciation and thanks to Dr. Leaf Huang for his expert guidance, assistance and patience during my studies in his laboratory. I would also like to express thanks to my lab mates and peers for the answers to my questions, and a major thanks to the office secretaries for their friendship and assistance.

I would also like to express my deepest appreciation to my father for his support, understanding and encouragement.

ABSTRACT

Polyhistidine facilitates the fusion of negatively charged liposomes prepared by sonication. Liposome fusion was demonstrated by (a) negative stain electron microscopy, (b) gel filtration and (c) resonance energy transfer of the fluorescent phospholipids. Liposome fusion required the presence of polyhistidine, whereas histidine at equivalent concentrations had no effect. Little or no liposome fusion was detectable at pH 7.4, but it was greatly enhanced when the pH of medium was reduced below 6.5. Although acidic phospholipid is necessary for fusion, liposomes made of acidic lipids alone showed only low levels of fusion activity. Liposomes composed of mixtures (1:1) of a negatively charged phospholipid and a neutral phospholipid such as phosphatidylcholine, phosphatidylethanolamine, but not sphingomyelin, showed high levels (about 80%) of fusion competency. For liposomes made of PE/PS (1:1), fusion at pH 5.2 and 2.5 g/ml of polyhistidine resulted in an increase in the average liposome diameter from 300 to 2,400 A, indicating multiple rounds of fusion had occurred. Liposome fusion was not very leaky as revealed by the lack of release of encapsulated calcein. For PE/PS (1:1) liposomes, about 10% of dye leakage was observed for up to about 30% liposome fusion and about 45% leakage at 80% liposome fusion. Since polyhistidine becomes a strong polycation at acidic pH, liposome fusion may be a direct result of the bilayer phase separation induced by the binding of polyhistidine with the negatively charged phospholipids. Therefore, this phenomenon is similar to the liposome fusion induced by other polycations such as polylysine at neutral pH. This system may serve as a model for studies on the acid dependent fusion

of viral envelopes with target cell membranes, such as in the case of the Influenza, Vesicular Stomatitis and Semliki Forest Viruses.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	4
	Materials	4
	Liposome Preparation	4
	Liposome Fusion	5
	Quantitation of Liposome Fusion	5
	Gel Filtration	6
	Electron Microscopy	6
	Liposome Leakage	7
III.	RESULTS	8
	Fusion Measured by Resonance Energy Transfer	8
	Gel Filtration of Liposomes	9
	Effect of Lipid Composition	11
	Fusion Revealed by Electron Microscopy	12
	Effect of pH of Medium	13
	Effect of Polyhistidine Concentration	13
	Liposome Leakage during Fusion	14
IV.	DISCUSSION	15
LIST OF REFERENCES		21
APPENDIX		25
VITA		38

LIST OF TABLES

TABLE		PAGE
1.	Effect of order of addition on liposome fusion	26
2.	Dependence of liposome fusion on lipid composition	27
3.	Change of liposome diameter with polyhistidine and acid treatments as revealed by negative stain electron microscopy	28
4.	Calculations of protonated histidine residues at polyhistidine concentrations which caused 50% maximal liposome fusion	29

LIST OF FIGURES

FIGURE		PAGE
1.	Test of the validity of eq. (1)	30
2.	Time course of fluorescence enhancement during polyhistidine induced liposome fusion	31
3.	Gel filtration of liposomes	32
4.	Negative stain electron micrographs of PE/PS (1:1) liposomes	33
5.	Size distribution of PE/PS (1:1) liposomes measured by negative stain electron microscopy	34
6.	Effect of medium pH on liposome fusion	35
7.	Effect of polyhistidine concentration on liposome fusion	36
8.	Liposome leakage during fusion	37

LIST OF ABBREVIATIONS

PS = phosphatidylserine

PG = phosphatidylglycerol

PE = phosphatidylethanolamine

PA = phosphatidic acid

PC = phosphatidylcholine

CL = cardiolipin

Sph = Sphingomyelin

N-NBG-PE = N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine

N-Rh-PE = N-(Lissamine Rhodamine B sulfonyl) phosphatidylethanolamine

PBS = phosphate buffered saline (547 mM NaCl, 11 mM KCl, 6 mM KH_2PO_4 and 4.5 mM Na_2HPO_4)

CHAPTER I

INTRODUCTION

Membrane fusion has been extensively studied with liposome model membranes (for reviews see Papahadjopoulos et al., 1979 and Nir et al., 1983). In most of the studies to date divalent cations such as Ca^{+2} have been used to induce fusion of negatively charged liposomes e.g. those composed of phosphatidylserine (PS)[1]. The evidence points to a mechanism in which Ca^{+2} plays a dual role: it promotes liposome aggregation (Nir et al., 1980), and induces a lipid phase separation which results in fusion (Newton et al., 1978). The concentrations of Ca^{+2} required to induce liposome fusion are generally much higher than the intracellular Ca^{+2} concentrations which trigger the fusion of intracellular membranes, raising some doubt about the physiological relevance of the model studies. Recent studies, however, have shown that in the presence of proteins such as synexin (Hong et al., 1981) and polyamines (Schuber et al., 1983) much lower Ca^{+2} concentrations are necessary to induce fusion.

Effects similar to those observed with divalent cations have been observed with cationic proteins and polypeptides such as polylysine (Hartmann & Galla, 1978). These molecules have the capacity to promote fusion of small unilamellar vesicles containing acidic phospholipids such as PS (Gad, 1983). However, the binding of the polypeptides to the liposomes, in contrast to divalent cations, is practically irreversible (Gad, 1983). Other polypeptides such as polymyxin are also able to induce bilayer membrane fusion. In this case, a lipid-peptide domain is established between the positively charged polypeptide and the negative membrane surface; neutral lipid bilayer membranes are unable to interact with these polypeptides (Sixl & Galla, 1981). Myelin basic protein

also has the capacity to induce PG/PC liposome fusion (Lampe et al., 1983). In all of these cases the involvement of lipid phase separation presumably plays a similarly important role for fusion as in the case of divalent cations.

All except a few (Schenkman et al., 1981; Blumenthal et al., 1983; Connor et al., 1984) of the published model studies have described liposome fusion at neutral pH. Recently a new class of membrane fusion which takes place at a mildly acidic condition has been demonstrated for the fusion of viral envelopes with target membranes (White et al., 1981; Marsh et al., 1982). Although the mechanism of membrane fusion is still obscure, it is clear that the viral glycoproteins are required (White & Helenius, 1980; Marsh et al., 1983; Gething & Sambrook, 1982). It has been proposed (White & Helenius, 1980; Maeda et al., 1981) that the viral envelopes fuse with the endosome/lysosome membranes once the virions are internalized by the receptor-mediated endocytosis and exposed to the acidic environment inside the endosomes/lysosomes (Tycko & Maxfield, 1980). The fusion event results in the release of the viral genome into the cytoplasm which is part of the normal infection process for the class of viruses including Influenza, Semliki Forest, Vesicular Stomatitis Viruses and perhaps others.

In view of the complexity of the natural membranes and glycoproteins, we have studied the acid dependent membrane fusion with the liposome model membranes and have used a well-defined polypeptide, i.e. polyhistidine, to induce the fusion. Polyhisitidine is not a strong polycation at the neutral pH. However, due to the weakly acidic imidazole side chains, it can be converted into a relatively strong polycation at mildly acidic conditions. In this communication, we report the effect of polyhistidine on the fusion events and

physical changes of the negatively charged liposomes as studied by gel filtration, electron microscopy and resonance energy transfer of the fluorescent lipids.

CHAPTER II

MATERIALS AND METHODS

Materials

N-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (N-NBD-PE) and N-(Lissamine Rhodamine B sulfonyl) phosphatidylethanolamine (N-Rh-PE) were obtained from Avanti Polar-lipids, Inc. (Birmingham, AL). Egg phosphatidylethanolamine (PE), brain phosphatidylserine (PS), phosphatidyl-L-glycerol (PG), phosphatidic acid (PA), egg phosphatidylcholine (PC), bovine heart cardiolipin (CL), brain sphingomyelin (Sph), poly-L-histidine (average MW 9,000), poly-L-glutamic acid (average MW 17,000), L-histidine and calcein were purchased from Sigma. All other chemicals were reagent grade.

Liposome Preparation

The preparation of liposomes was done following the method of Szoka & Papahadjopoulos, 1980. Briefly, ten μ moles of the appropriate lipid mixture was dried to a film under N_2 and vacuum dessicated for at least 30 minutes. The dry lipid was resuspended in 1 ml phosphate buffered saline (PBS), pH 7.4 and sonicated for 20 minutes in a bath sonicator (Laboratory Supplies, Hicksville, NY). To label the liposomes, 0.5 mol% of N-NBD-PE and 2 mol% N-Rh-PE were included in the lipid mixtures and the liposomes were prepared identically as the unlabelled ones. Calcein-encapsulated liposomes were prepared by sonicating in 1 ml of PBS containing 40 mM calcein. Untrapped calcein was removed by passage through a Sephadex G-50 column. The sonication procedure produced no degradation of the lipids as detected by thin layer chromatography.

Liposome Fusion

Concentrated polyhistidine in PBS pH 7.4 was added at room temperature to a 2 ml sonicated liposome suspension in PBS (0.15 mM lipids). The pH of the mixture was then reduced to the desired value by adding 10 μl HCl of appropriate concentration. The suspension immediately became turbid. After about 5 min at room temperature, equivalent amount of NaOH was added to bring the pH back to 7.4. Although the suspension became less turbid after the addition of NaOH, it was still more turbid than the original liposome suspension.

Quantitation of Liposome Fusion

The method of resonance energy transfer described by Struck (Struck et al., 1981) was used to quantitate liposome fusion. In this assay, two fluorescent lipids with favorable spectral overlap are included in the same liposome membranes at a relatively high concentration resulting in an efficient resonance energy transfer. When these liposomes fuse with liposomes without fluorescent lipids, dilution of the fluorescent lipids occurs which results in a decrease in the resonance energy transfer (Stryer & Hoagland, 1967) and a consequent "dequenching" of the donor emission. We have used N-NBD-PE as a donor and N-Rh-PE as an acceptor fluorescent lipid. The efficiency of energy transfer was measured by the emission at 530nm of N-NBD-PE when the samples were excited at 470nm. As a result of fusion, fluorescence of N-NBD-PE was enhanced. A complete mixing of liposomal lipids during fusion will result in a maximal fluorescence enhancement which is dependent on the ratio of unlabeled to labelled liposomes in the fusion mixture. The percent liposome fusion used in this paper is defined as

$$\% \text{ fusion} = \frac{F_f/F_i - 1}{n} \times 100 \qquad (1)$$

Where F_i and F_f are the fluorescence intensities of N-NBD-PE before and after the fusion reaction, respectively. n is the concentration ratio of unlabelled to labelled liposomes in the mixture. In order to check the validity of equation (1), labelled and unlabelled liposomes were mixed at different ratios and the initial fluorescence, F_i was measured. The mixtures were then sonicated thoroughly to mix all lipids and the final fluorescence, F_f was obtained. As can be seen in Fig. 1*, a plot of $F_f/F_i - 1$ vs. n is a straight line with a slope equal to 1 up to $n = 2$, indicating that under these conditions equation (1) is valid. For $n > 2$ the fluorescence enhancement was less than the theoretical value. All experiments were done with $n = 2$.

Gel Filtration

One ml liposome suspension in PBS was applied to a Biogel A-50M column (30 x 1.5 cm) and fractions of 0.5 ml were collected. Elution was carried out with PBS (pH 7.4). To eliminate absorptive effects, the gel was first saturated with lipid dispersion and then washed and equilibrated with PBS. The recovery yield of the eluted liposomes of the lipid-treated column was about 90%. Elution profiles were examined by measuring fluorescence of N-NBD-PE of each fraction in the presence and absence of 0.2% triton X-100.

Electron Microscopy

Sonicated liposomes which had undergone different fusion treatments were negatively stained with 0.5% uranyl acetate or potassium phosphotungstate and

*All Figures appear in Appendix.

were viewed with a Zeiss or JEM6C electron microscope. Photographs were taken at 40,000 to 50,000 x magnification and further enlarged photographically.

Liposome Leakage

Liposomes were prepared as described above in a buffer containing 40 mM calcein. The calcein-entrapped liposomes were freed of untrapped calcein by passing them through a column of Sephadex G-50 (Gad, 1983). Fluorescence intensity of calcein-entrapped liposomes was measured in 2ml PBS. At high concentration, fluorescence of calcein is efficiently self-quenched. Enhancement of fluorescence occurs when the dye leaks from the liposomes (Allen and Cleland, 1980). Calcein fluorescence was measured at λ_{ex} = 490 nm and λ_{em} = 530 nm. Liposome leakage is defined as

$$\% \text{ leakage} = \frac{I_f - I_i}{I_t} \times 100$$

Where I_i, I_f and I_t are the fluorescence intensities of calcein in the original liposomes, in liposomes after acid treatment, and in the presence of 0.2% triton X-100, respectively. The amount of fluorescence due to free calcein in the original liposome preparation was less than 10% and could be detected by the quenching of calcein fluorescence after addition of 5 mM $CoCl_3$ (Oku et al., 1982). This was subtracted from I_t.

CHAPTER III

RESULTS

Fusion Measured by Resonance Energy Transfer

The time course of fusion for PE/PS (1:1) liposomes is shown in Fig. 2. As can be seen, the fluorescence intensity slightly increased when polyhistidine was added to the liposome-containing medium. However, the increase caused by polyhistidine was small compared to the increase observed following the addition of acid. Furthermore, the fluorescence increase after acid addition was very rapid; greater than 75% of the increase took place in the first minute. This large increase in fluorescence was not due to increased quantum yield of N-NBD-PE at acidic pH, since addition of NaOH to return the pH to 7.4 only slightly reduced the fluorescence intensity. Furthermore, addition of excess polyglutamic acid did not affect the increased fluorescence, although the mixture was visually less turbid. Also shown in Fig. 2 is the change of light scattering during fusion which generally paralleled the change in fluorescence. The magnitude of light scattering was, however, much smaller than that of the fluorescence (5-18%) so that it did not contribute significantly to the fluorescence signal. Addition of polyglutamic acid after the acid treatment showed a slight decrease in turbidity, indicating that the fused liposomes were disaggragated by the addition of polyanion.

In order to ascertain that observed fluorescence increase is truly induced by polyhistidine at acidic condition, an experiment was performed in which the order of addition of various reagents was altered. As can be seen in Table 1*, large fluorescence increase expressed as percent fusion according to

*All Tables appear in Appendix.

eq. (1) was observed only when polyhistidine was exposed to an acidic medium. Addition of base before acid, i.e. an alkaline treatment of the liposomes, could not generate large fluorescence increase. Acid treatment before the addition of polyhistidine also had no effect. This is true for both types of the fusion-competent liposomes tested, i.e. PE/PS (1:1) and PC/PS (1:1).

Gel Filtration of Liposomes

To determine the reliability of fluorimetric measurement, and to demonstrate liposome fusion using a different technique, we fractionated various pre-treated liposomes on the basis of particle size by gel filtration. Mixtures of 2 parts of unlabelled and 1 part of labelled liposomes (n=2) were treated sequentially with polyhistidine and then with acid (pH 5.2) and base (pH returned to 7.4), with polyhistidine alone or with acid-base alone. These samples were then chromatographed on an A50M column and the fractions were assayed for N-NBD-PE fluorescence in the presence (F_t) and absence (F) of triton-X100. Since F_t represents the total, unquenched fluorescence of N-NBD-PE, the efficiency of resonance energy transfer (E) of each fraction is calculated as

$$E = 1 - \frac{F}{F_t} \qquad (3)$$

according to Struck et al. (1981). As labelled liposomes fuse with unlabelled ones, quenching of N-NBD-PE fluorescence decreases which results in greater F and hence smaller E values. Chromatograms of the untreated liposomes (Fig. 3A) showed mainly a broad included peak with high E values across the chromatogram, indicating some size heterogeneity of the unfused liposome population. After treatment with polyhistidine (Fig. 3B), large amounts of liposome appeared in the void volume fractions, with the remaining liposomes eluted in the included volume fractions. The efficiencies of energy transfer

of the liposomes were slightly lower, particularly in the void volume fractions. This result indicated that the treatment of polyhistidine alone caused liposome aggregation accompanied by a low level of liposome fusion. After the addition of polyhistidine, subsequent acid-base treatment of liposomes produced a large increase in NBD-PE fluorescence and a total shift of liposome elution to the void-volume fractions (Fig. 3C). Furthermore, the efficiencies of energy transfer in these fractions were much lower than the untreated liposomes. Therefore, the combined treatment of polyhistidine and acid-base had resulted in a high level of liposome fusion with accompanied size increase. Treatment of liposomes by acid alone also brought some degree of liposome aggregation as indicated by the enlarged peak at the void volume fractions (Fig. 3D). There was no liposome fusion detected since the efficiencies of energy transfer across the chromatogram was essentially unchanged from those of the untreated liposomes. Furthermore, liposome aggregation in this case did not seem to be stable, since subsequent examination of liposomes in the void volume fractions by the negative-stain electron microscopy failed to reveal the aggregated liposomes (data not shown).

It is worth noting that liposomes eluted in fractions 30 to 38, shown in Fig. 3B, after polyhistidine treatment could be induced to fuse with an acid treatment alone without additional polyhistidine addition. The fused liposomes eluted in the void volume fractions with low E values (about 0.7) after being applied to the same A50M column (profile not shown). Since the polyhistidine used was quite small (average M.W. 9,000), it should be eluted near the end of the column volume fractions and well separated from the liposomes. These results suggested that enough polyhistidine binds with liposomes at neutral pH such that fusion can take place upon subsequent acid-base treatment. This interesting observation was not further explored in the present study.

Effect of Lipid Composition

We have examined the dependence of fusion on the lipid composition of the liposomes (Table 2). It is clear that fusion requires the presence of acidic lipid in the liposomes; no fusion was observed with neutral liposomes. Although liposomes made of neutral lipids had no fusion activity, neutral lipids could be used together with acidic lipids to form fusion competent liposomes. In fact, liposomes made of a mixture of neutral and acidic lipids showed highest fusion activities. Liposomes composed of acidic lipids alone (e.g. PS, PG or CL) only had low activities. This observation can not be explained by the low polyhistidine to acidic lipid ratios used for liposomes containing only acidic lipids, since the fusion only increased to moderate levels when the polyhistidine concentration was doubled. Of the acidic lipids which supported fusion, the activities of PS, PA and PG were about the same. The activity of CL was considerably lower. Since CL carries two negative charges per molecule, we have also tested a 2:1 combination of PE or PC with this lipid. The fusion activity was still lower in these cases. Of the neutral lipids tested, PE and PC showed about equal ability to form fusion competent liposomes with acidic lipids. Sph was much less active. This aspect was further explored in an experiment in which the acidic lipid (PS) content was kept at 30% and the remaining 70% was made of mixtures of PE and Sph at different ratios. The data (not shown) showed that replacement of PE with Sph resulted in a drastic decrease in liposome fusion. No fusion could be observed when more than half of the PE was replaced. These results clearly demonstrated that Sph, although having the same head group as PC, inhibits the fusion competency of the liposomes.

Fusion Revealed by Electron Microscopy

The morphology of the liposomes was examined by negative stain electron microscopy and the average diameters of the liposomes were measured (Table 3). Before the addition of polyhistidine, sonicated liposomes were unilamellar and rather homogeneous in size with an average diameter ranging from 170 to 400Å, depending on the lipid composition. Addition of polyhistidine at pH 7.4 generally caused a small increase in liposome diameter; the magnitude of increase ranged from 5 to 25%, again depending on the lipid composition. Subsequent treatment of liposomes with acid-base resulted in a large increase in liposome size. Particularly, the size of PE/PS (1:1) liposomes increased about 7 fold.

The electron micrographs of the PE/PS (1:1) liposomes are shown in Fig. 4. Aggregation occurred on polyhistidine treated liposomes (Fig. 4B and C). This observation is consistent with the notion that polyhistidine binds with liposomes even at pH 7.4 which results in liposome aggregation.

A size distribution curve for PE/PS (1:1) liposomes (Fig. 5A), showed the size range of the original liposomes was about 170-520 A with a large proportion being about 260-270 Å in diameter. After treatment with 2.5 μg/ml of polyhistidine, the size range was about 260-650 A with a mean diameter of 420 A. In comparing the elution profile of PE/PS (1:1) liposomes in Fig. 3B with the size distribution in Fig. 5B, the results are consistent. It indicated that some of polyhistidine-treated liposomes fused having lower efficiency of energy transfer and some of liposomes aggregated whose efficiency was close to those of the original liposomes. Size distribution of the fused liposomes shown in Fig. 5C presented a large shift. Most of liposomes were in the range of 1,800-3,000 Å diameter. The mean diameter of fused liposomes was 2,080

A which was 7 times the size of the original ones, indicating massive fusion had occurred. The broad distribution also indicated that the extent of liposome fusion varied. The size distribution of acid treated liposomes (Fig. 5D), showed that they appeared in the range of 170-570 Å with mean diameter of 340 Å. These results clearly showed that the acid treatment alone could not induce fusion of the PE/PS liposome.

Effect of pH of Medium

The extent of liposome fusion was critically dependent on the pH of the acid treatment. Fig. 6 shows the effect of the medium pH on fusion of liposomes of four different lipid compositions. As can be seen, at pH 7.4 there was little or no fusion observed by the resonance energy transfer method, except for liposomes made of PE/PS (1:1) which showed a low level (about 16%) of fusion at neutral pH. Fusion was greatly enhanced when the medium pH was lowered below 7. At pH 5, approximately 80% fusion was observed for PE/PS (1:1), PC/PS (1:1) and PC/PG (1:1) liposomes. The PC/CL (2:1) liposomes showed lower level of fusion activity at all pH values examined. In the absence of polyhistidine, little or no fusion of liposomes upon acid treatment was observed. This was the case for all liposomes tested.

Effect of Polyhistidine Concentration

The effect of polyhistidine concentration on the extent of liposome fusion is shown in Fig. 7. Liposomes of four different lipid compositions were examined at pH 7.4, 6.5 and 5.2. It was clear that fusion of liposomes required the presence of polyhistidine in a concentration dependent manner. In general, maximal fusion activity was observed at polyhistidine concentration of 5-10 μg/ml at pH 5.2, regardless of the lipid composition. At less acidic conditions

(pH 6.5) considerably more polyhistidine was required to achieve the comparable level of fusion. In some cases such as the PC/PS (1:1) and PC/PG (1:1) liposomes, maximal level of fusion was about 20% lower than that at pH 5.2. At pH 7.4, only PE/PS (1:1) liposomes showed significant fusion at high polyhistidine concentrations ($\geq 20 \mu$g/ml), other types of liposome showed only a small degree of fusion even at high concentrations. No fusion was observed when polyhistidine was replaced by histidine, polyglutamic acid or a random polymer of Glu, Ala and Tyr, even at 20 μ g/ml.

Liposome Leakage during Fusion:

To investigate whether leakage of liposome contents accompanied fusion, calcein-entrapped liposomes were prepared. Liposomes can be loaded with calcein to a concentration of about 10^{-3}M without concern for quenching due to excimer formation (Oku et al., 1982). To use it as a marker for leakage, 40 mM calcein was trapped in PE/PS (1:1) liposomes without fluorescent lipids. As a parallel experiment, liposomes containing fluorescent lipids were also prepared and liposome fusion was measured by the resonance energy transfer method. Curves in Fig. 8 show that the tendency of liposome leakage was similar to that of liposome fusion, i.e. both fusion and leakage only took place at acidic pH. However, there was no proportional relationship between these two events; the extent of leakage was generally lower than that of fusion. This was particularly clear at pH between 4 and 5, under which conditions the extent of fusion was greater than 80% whereas the leakage was saturated at about 45%. Thus, it was possible to maximally fuse liposomes with substantial retention of their trapped contents.

CHAPTER IV

DISCUSSION

In the present study, we have shown that polyhistidine mediates an acid dependent fusion of negatively charged liposomes. Our results indicate that fusion requires the exposure of liposomes to polyhistidine at a mildly acidic condition. At reduced pH, polyhistidine is partially protonated and becomes a polycation which can readily aggregate the negatively charged liposomes. The fraction of the histidine residues protonated in the polyhistidine molecules at a given pH can be calculated according to (Patchornik et al., 1957). As can be seen in Table 4, at concentrations of polyhistidine which caused 50% of maximal fusion of liposomes of a given lipid composition, approximately same amount of the protonated histidine residues were present in the polyhistidine molecules, regardless of the pH of the acid treatment used. These calculations provide quantitative evidence that it is the cationic polypeptide which serves as a driving force for the fusion event.

The binding of the protonated polyhistidine with the negatively charged phospholipids in the liposomes is likely to bring about a phase separation in the liposome bilayers, due to the lateral mobility of the phospholipids. Such phase separation has been demonstrated in the case of Ca^{+2} (Papahadjopoulos et al., 1977; Ohnishi & Ito, 1974) and polylysine (Galla & Sackmann, 1975; Gad et al., 1979) induced fusion. The packing of the acyl chains of the negatively charged phospholipids bound to bivalent or polyvalent cations is in a more ordered state than those of the unbound phospholipids (Papahadjopoulos et al., 1977; Hammes & Schullery, 1970). It is reasonable to assume that a similar situation occurs with polyhistidine binding to the negatively charged

phospholipids so that both "solid" and "fluid" domains coexist in the liposome bilayers. Papahadjopoulos et al. (1979) have proposed that phase separated phospholipid bilayers in close apposition are prone to fuse at the domain boundaries due to high degree of local destabilization. In the case of PE/PS (1:1) liposomes, domains enriched with PE may undergo bilayer to hexagonal (H_{II}) phase transition or the formation of the inverted micelles or lipidic particles (for review, see Cullis & de Kruijff, 1979). Such non-lamellar structures of the phospholipids have been postulated to be a strong driving force for membrane fusion (Verkleij et al., 1979; Verkleij et al., 1980). Indeed, although the resonance energy transfer assay showed that liposomes containing PE/PS and PC/PS had approximately equal degree of fusion at same conditions (Table 2), electron microscopic observations revealed that PE/PS liposomes fused to much larger liposomes than the PC/PS liposomes (Table 3). This difference may be related to the ability of PE to undergo a bilayer to H_{II} phase transition under the conditions used in this study.

The ratios of the protonated histidine residues to the negative charges of the liposomes for 50% of maximal fusion ranged between 0.06 to 0.2, depending on the lipid composition of the liposomes (Table 4). Binding of polyhistidine to the negatively charged liposomes is unlikely to bring about a total charge neutralization of the liposomes even at saturation concentrations of polyhistidine. This is due to the steric hindrance for the binding of polymeric molecules to the liposome surface. Thus the equilibrium state of liposome aggregation, which determines the extent of liposome fusion, must be the result of a balance between the attractive forces facilitated by the protonated polyhistidine and the repulsive forces among the residual negative charges on the liposomes. This might explain why the liposomes made of negatively

charged phospholipids alone had lower fusion activities than those composed of equimolar mixtures of neutral and negatively charged phospholipids. The former liposomes would contain more residual negative charges, and hence less liposome aggregation and fusion, than the latter ones when the binding of polyhistidine is saturated. It is predicted that the difference in fusion competency of the two liposome types will diminish if polyhistidine of smaller molecular weight is used as a fusogen, since less steric hindrance of binding is expected. However, larger size polyhistidine molecules should be more effective in bringing about the phase separation in the liposome bilayer. The determination of the optimal polymer size for liposome fusion must awaits further experimentation.

Polyhistidine mediated liposome fusion at low pH is a much more effective process than the Ca^{+2} induced fusion of similar liposomes. For example, concentrations of Ca^{+2} at mM range are required for the fusion of small unilamellar liposomes of PS (Papahadjopoulos et al., 1976). In the present study, polyhistidine at subμM to μM range can bring about efficient fusion of PS liposomes at pH 5.2 (Table 2). Ca^{+2} does not induce fusion of liposomes until the ratio of the positive charge of Ca^{+2} to the negative charge of the liposomes exceeds 0.7 (Nir et al., 1980). Polyhistidine can effectively fuse liposomes at a charge ratio of 0.2 or less (Table 4). Polylysine is even more effective in inducing liposome fusion in that only nM concentrations of polylysine are required (Gad et al., 1982). This is probably due to the polymeric nature of the polyhistidine and polylysine which should be more effective to induce the bilayer phase separation. Ca^{+2} induced liposome fusion also exhibits a threshold Ca^{+2} concentration only above which effective fusion is observed. In the present study, no clear threshold phenomena are noted, again indicating

more efficient fusogen activity of polyhistidine than Ca^{+2}. Dilution of the negative charge density of the liposomes by mixing PS with PC generally results in reduced fusion competency and/or increased threshold concentration for the Ca^{+2} induced fusion (Hoekstra et al., 1979; Duzgunes et al., 1981). For the polyhistidine mediated fusion, the opposite is true. This is probably because there is no steric hindrance in Ca^{+2} binding to PS and a nearly total charge neutralization of the PS liposomes can be achieved at saturation Ca^{+2} concentration (Newton et al., 1978).

Polyhistidine has a random coil conformation at acidic pH due to repulsive forces between the protonated histidine residues (Lindemann & Zundel, 1978). It is possible that upon binding with the negatively charged phospholipids, ∂-helical conformation is stabilized due to charge neutralization. This is the case for polylysine (Hammes & Schullery, 1970). In the case of polylysine, it has been shown that in addition to the electrostatic charge interactions, polylysine can interact hydrophobically with the acyl chains of the phospholipids (Hammes & Schullery, 1970; Pawagi & Campbell, 1981), providing additional destabilization effect to the lipid bilayer. It is doubtful that polyhistidine could do the same thing, because of the relatively bulky imidazole group of the side chain. This may be why polylysine is a more potent fusogen than polyhistidine.

Our results also showed that the polyhistidine mediated liposome fusion is accompanied by the leakage of the liposome content, at least for the PE/PS (1:1) liposomes. Although the liposome leakage qualitatively correlates with the liposome fusion, there are quantitative differences. For example, at pH 6.5 and [polyhistidine] = 5 μg/ml, PE/PS (1:1) liposomes on the average had gone through one round fusion (4 out of 9 collision events at n = 2, or about

44% of maximum dilution of the fluorescent lipids). Under the identical conditions the extent of liposome leakge was only 16% (Fig. 8). Liposome leakage saturated at about 45% when fusion reached maximal level at about 80%. Similar phenomenon has been observed for the Ca^{+2} induced liposome fusion (Wilschut et al., 1982; Vanderwerf & Ullman, 1980). Detailed measurements of the initial velocities for fusion and leakage have led to the conclusion that only the first few rounds of fusion of the small liposomes are leaky, hence preserving the contents as further fusion proceeds (Wilschut et al., 1980; Nir et al., 1983). Similar mechanisms may be operating in the present fusion system, which would explain the observation that the extent of liposome leakage is always lower than fusion. Future kinetic measurements may provide verifications of this hypothesis.

The present study may be related to the acid dependent fusion of the viral envelopes with the target membranes as in the cases of the Influenza (Matlin et al., 1981), Semliki Forest (White & Helenius, 1980; White et al., 1980) and Vesicular Stomatitis Viruses (White et al., 1981). It is believed that the fusion event takes place in the acidic endosomes or lysosomes, resulting in the release of viral genomes into the cytoplasm of the infected cells (Matlin et al., 1982; Kennedy, 1976). The amino acid sequences of the viral glycoproteins responsible for fusion have been studied with the cloned DNA (Porter et al., 1979; Garoff et al., 1980; Gething et al., 1980). Although there exists no significant stretch of the polypeptide chains enriched with histidine residues, it is possible that the histidine residues may be brought together as a result of polypeptide chain folding. The protonation of these residues at the acidic pH inside the endosomes/lysosomes and the subsequent binding of these residues to the negatively charged lipids in the target membrane may

trigger a phase separation and eventually lead to the fusion of the envelope with the target membrane. Recent studies using liposomes containing reconstituted Vesicular Stomatitis Virus G protein showed that the acid dependent fusion of these liposomes with model target membranes requires the presence of the negatively charged lipids in the target membranes (Eidelman et al., 1984). This observation is in apparent agreement with our hypothesis.

LIST OF REFERENCES

LIST OF REFERENCES

Allen, T.M. and Cleland, L.G. (1980). Biochim. Biophys. Acta 597, 418-426.

Blumenthal, R., Henkart, M. and Steer, C.J. (1983). J. Biol. Chem. 258, 3409-3415.

Connor, J., Yatvin, M.B. and Huang, L. (1984). Proc. Natl. Acad. Sci. USA in press.

Cullis, P.R. and De Kruijff, B. (1979). Biochim. Biophys. Acta 559, 399-412.

Duzgunes, N., Wilschut, J., Fraley, R. and Papahadjopoulos, D. (1981). Biochim. Biophys. Acta 642, 182-195.

Eidelman, O., Schlegal, R., Tralka, T.S. and Blumenthal, R. (1984). Biophys. J. 45, 72a.

Gad, A.D. (1983). Biochim. Biophys. Acta 728, 377-382.

Gad, A.E., Broza, R. and Eyton, G.D. (1979). Biochim. Biophys. Acta 556, 181-195.

Gad, A.E., Silver, B.L. and Eytan, G.D. (1982). Biochim. Biophys. Acta 690, 124-132.

Galla, H.J. and Sackmann, E. (1975). Biochim. Biophys. Acta 401, 509-529.

Garoff, H., Frischauf, A.M., Simons, K., Lehrach, H. and Delius, H. (1980). Nature 288, 236-241.

Gething, M.J. and Sambrook, J. (1982). Nature 300, 598-603.

Gething, M.J., Bye, J. Skehel, J. and Waterfield, M. (1980). Nature 287, 301-306.

Hammes, G.G. and Schullery, S.E. (1970). Biochem. 9, 2555-2559.

Hartmann, W. and Galla, H.J. (1978). Biochim. Biophys. Acta 509, 474-490.

Hoekstra, D., Yaron, A., Carmel, A. and Scherphof, G. (1979). FEBS Lett. 106, 176-180.

Hong, K., Duzgunes, N. and Papahadjopoulos, D. (1981). J. Biol. Chem. 256, 3641-3644.

Kennedy, S.I.T. (1976). J. Mol. Biol. 108, 491-511.

Lampe, P.D., Wei, J. and Nelsestuen, G.L. (1983). Biochem. 22, 1594-1599.

Lindemann, R. and Zundel, B. (1978). Biopolymers 17, 1285-1304.

Maeda, T., Kawasaki, K. and Ohnishi, S.I. (1981). Proc. Natl. Acad. Sci. USA 78, 4133-4137.

Marsh, M., Wellsteed, J., Bolzau, E. and Helenius, A. (1982). J. Cell Biol. 95, 418a.

Marsh, M., Bolzau, E. and Helenius, A. (1983). Cell 32, 931-940.

Matlin, K., Reggio, H., Helenius, A. and Simons, K. (1981). J. Cell Biol. 91, 601-613.

Matlin, K., Reggio, H., Helenius, A. and Simons, K. (1982). J. Mol. Biol. 156, 609-631.

Newton, C., Pangborn, W., Nir, S. and Papahadjopoulos, D. (1978). Biochim. Biophys. Acta 506, 281-287.

Nir, S., Bentz, J. and Wilschut, J. (1980). Biochem. 19, 6030-6036.

Nir, S., Bentz, J., Wilschut, J. and Duzgunes, N. (1983). Progress in Surface Science 13, 1-124.

Ohnishi, S.I. and Ito, T. (1974). Biochem. 13, 881-887.

Oku, N., Kendall, D.A. and Macdonald R.C. (1982). Biochim. Biophys. Acta 691, 332-340.

Papahadjopoulos, D., Poste, G., and Vail, W.J. (1979). Meth. Memb. Biol. 10, 1-121.

Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977). Biochim. Biophys. Acta 465, 579-598.

Papahadjopoulos, D., Vail, W.J., Pangborn, W.A. and Poste, G. (1976). Biochim. Biophys. Acta 448, 265-283.

Patchornik, A., Berger, A. and Katchalski, E. (1957). J. Am. Chem. Soc. 79, 5227-5230.

Pawagi, A.B. and Campbell, I.M. (1981). Can. J. Biochem. 59, 404-411.

Porter, A.G., Barber, C., Carey, N.H., Hallewell, R.A., Threlfall, G. and Emtage, T.S. (1979). Nature 282, 471-477.

Schenkman, S., Anaujo, P.S., Sesso, A., Quina, F.H. and Chaimovich, H. (1981). Chem. Phys. Lipids 28, 165-180.

Schuber, F., Hong, K., Duzgunes, N. and Papahadjopoulos, D. (1983). Biochem. 22, 6134-6140.

Sixl, F. and Galla, H.J. (1981). Biochim. Biophys. Acta 643, 626-635.

Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981). Biochem. 20, 4093-4099.

Stryer, L. and Haugland, R.P. (1967). Proc. Natl. Acad. Sci. USA 58, 719-726.

Szoka, F., Jr. and Papahadjopoulos, D. (1980). Annu. Rev. Biophys. Bioeng. 9, 467-508.

Tycko, B. and Maxfield, F.R. (1980). Cell 28, 643-652.

Vanderwerf, P. and Ullman, E.F. (1980). Biochim. Biophys. Acta 596, 302-314.

Verkleij, A.J., Mombers, C. Leunissen-Bijvelt, J. and Ververgaert, P.H.J.T. (1979). Nature 279, 162-163.

Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Cullis, P.R. and DeKruijff, B. (1980). Biochim. Biophys. Acta 600, 620-624.

White, J. and Helenius, A. (1980). Proc. Natl. Acad. Sci. USA 77, 3273-3277.

White, J., Kartenbeck, J. and Helenius, A. (1980). J. Cell Biol. 87, 264-272.

White, J., Matlin, K. and Helenius, A. (1981). J. Cell Biol. 89, 674-679.

Wilschut, J., Duzgunes, N., Fraley, R. and Papahadjopoulos, D. (1980). Biochem. 19, 6011-6021.

Wilschut, J., Holsappel, M. and Jansen, R. (1982). Biochim. Biophys. Acta 690, 297-301.

APPENDIX

Table 1. Effect of Order of Addition on Liposome Fusion.

Order of addition	Fusion (%) PE/PS (1:1)	PC/PS (1:1)
liposome[a] → polyhistidine[b] → acid[c] → base	74.8	72.0
liposome → acid → polyhistidine → base	70.3	70.0
liposome → polyhistidine → base[d] → acid	18.7	28.4
liposome → base → polyhistidine → acid	16.1	23.6
liposome → base → acid → polyhistidine	10.4	12.3
liposome → acid → base → polyhistidine	10.6	12.1

a. [lipid] = 0.15 mM

b. [polyhistidine] = 5 μg/ml

c. acid = pH 5.2

d. base = pH 10

Table 2. Dependence of liposome fusion on lipid composition[a]

Lipid composition	Fusion (%)
PE:PS (1:1)	75
PE:PA (1:1)	82
PE:PG (1:1)	80
PE:CL (1:1)	35
PE:CL (2:1)	50
PC:PS (1:1)	72
PC:PG (1:1)	83
PC:CL (1:1)	48
PC:CL (2:1)	57
PC	0
Sph:PS (1:1)	23
Sph	0
PE:PC (1:1)	0
PE:Sph (1:1)	0
PS	27
PG	9
CL	0
PS[b]	44
PG[b]	37
CL[b]	13

a. [polyhistidine] = 5 μg/ml, [lipid]= 0.15 mM, pH of acid treatment = 5.2.

b. [polyhistidine] = 10 μg/ml, other conditions unchanged.

Table 3. Change of liposome diameter with polyhistidine and acid treatments as revealed by negative stain electron microscopy[a]

Lipid composition	Liposomes, A	Polyhistidine treated liposomes[b], A	Polyhistidine & acid treated liposomes[c], A
PE:PS (1:1)	296 ± 59 (107)	424 ± 85 (88)	2084 ± 710 (50)
PE:CL (2:1)	354 ± 93 (58)	528 ± 80 (109)	1125 ± 375 (100)
PE:PA (1:1)	405 ± 101 (140)	429 ± 108 (117)	515 ± 106 (98)
PE:PG (1:1)	365 ± 81 (117)	366 ± 95 (81)	565 ± 41 (87)
Sph:PS (1:1)	208 ± 46 (100)	220 ± 44 (100)	300 ± 58 (94)
PC:PS (1:1)	192 ± 37 (150)	248 ± 48 (141)	568 ± 142 (102)
PC:PG (1:1)	180 ± 40 (115)	270 ± 45 (107)	331 ± 87 (96)
PC:CL (2:1)	252 ± 50 (97)	370 ± 70 (95)	750 ± 187 (74)

a. all data are expressed as mean ± standard deviation (number of measurements).

b. [polyhistidine] = 5 μg/ml

c. pH of acid treatment = 5.2, pH returned to 7.4 before examined by electron microscopy.

Table 4. Calculations of Protonated Histidine Residues at Polyhistidine Concentrations Which Caused 50% Maximal Liposome Fusion.

Lipid composition	pH	∂[a]	polyhist. for 50% max. fusion[b]	Protonated his. res.	Protonated his. res. negative[c] charge
			μg/ml	μM	
	7.4	0.04	10.0	2.6	0.07
PE/PS	6.5	0.15	2.5	2.4	0.06
(1:1)	5.2	0.39	1.5	3.8	0.10
PC/PS	6.5	0.15	5.0	4.9	0.13
(1:1)	5.2	0.39	2.0	5.0	0.13
PC/PG	6.5	0.15	3.5	3.4	0.09
(1:1)	5.2	0.39	3.0	7.6	0.20
PC/CL	6.5	0.15	11.5[d]	9.9	0.20
(2:1)	5.2	0.39	4.0	10.0	0.20

a. calculated according to $pH = pk_o + \log \frac{1 - \partial}{\partial} - 0.868n\,\partial\,w$ (Patchornik et al. 1957) where pK_o = intrinsic pK_a for histidine, (pK_o = 6.15 used for this calculation), ∂ = fraction of protonated histidine residues , n = average number of histidine residues per polyhistidine molecule (n = 58 for average m.w. = 9,000), w = electrostatic interaction factor (w = 0.0575 used for this calculation).

b. data taken from Fig. 7A

c. total conc. of negatively charged phospholipid = 75 μM. Assuming that half of them resides on the outer monolayer of the unilamellar liposomes, effective concentration = 37.5 μM. Concentration of CL was half of this value, but concentration of the negative charges was the same, since CL carried 2 negative charges per molecule.

d. estimated by assuming maximum fusion was 100%

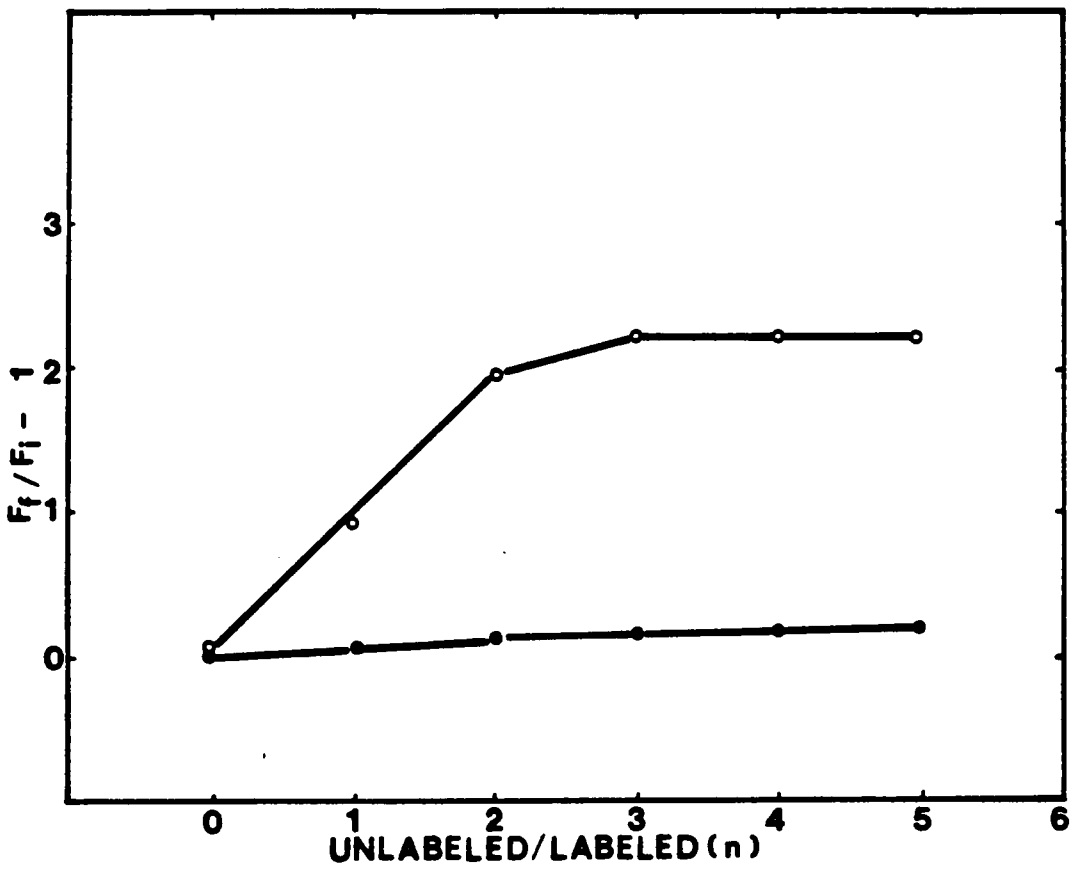

Fig. 1. Test of the validity of eq. (1). Unlabelled and fluorescently labelled (0.15 mM total lipid) liposomes were mixed at different ratios (n). The initial fluorescence, F_i, and the final fluorescence, F_f, after thorough sonication of the mixture were mearued (o-o). To estimate the contribution of light scattering in this experiment, the fluorescently labelled liposomes were replaced with the unlabelled (●-●). PE/PS (1:1) liposomes were used.

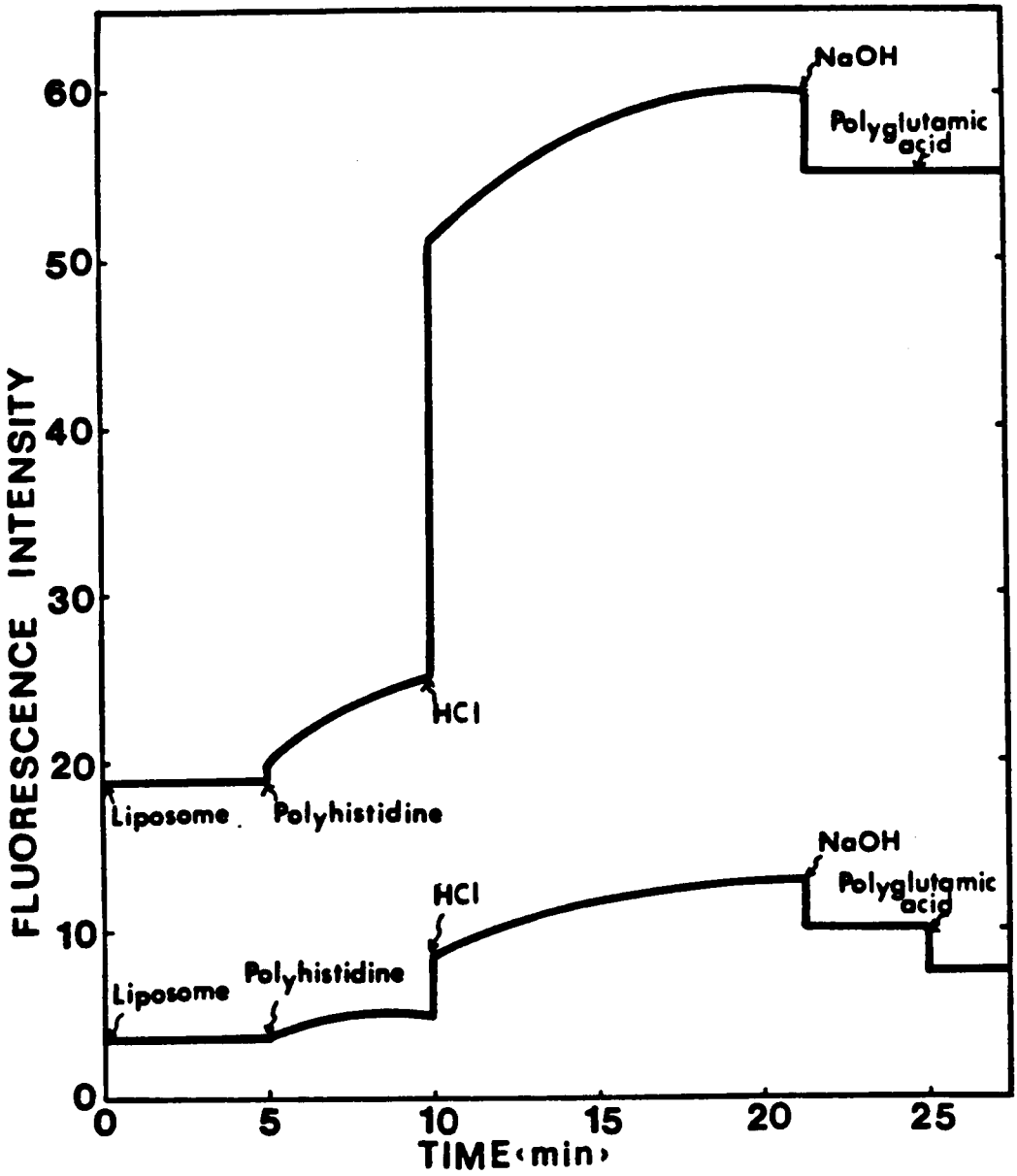

Fig. 2. Time course of fluorescence enhancement during polyhistidine induced liposome fusion (upper curve). PE/PS (1:1) liposomes were used at [lipid] = 0.15 mM, [polyhistidine] = 5 μg/ml. Addition of HCl reduced pH to 5.2 which was returned to 7.4 by the addition of NaOH. Addition of polyglutamic acid (10 μg/ml) was to neutralize the effect of polyhistidine. Bottom curve, identical conditions were used except the fluorescently labelled liposomes were replaced with the unlabelled to estimate the contribution of light scattering.

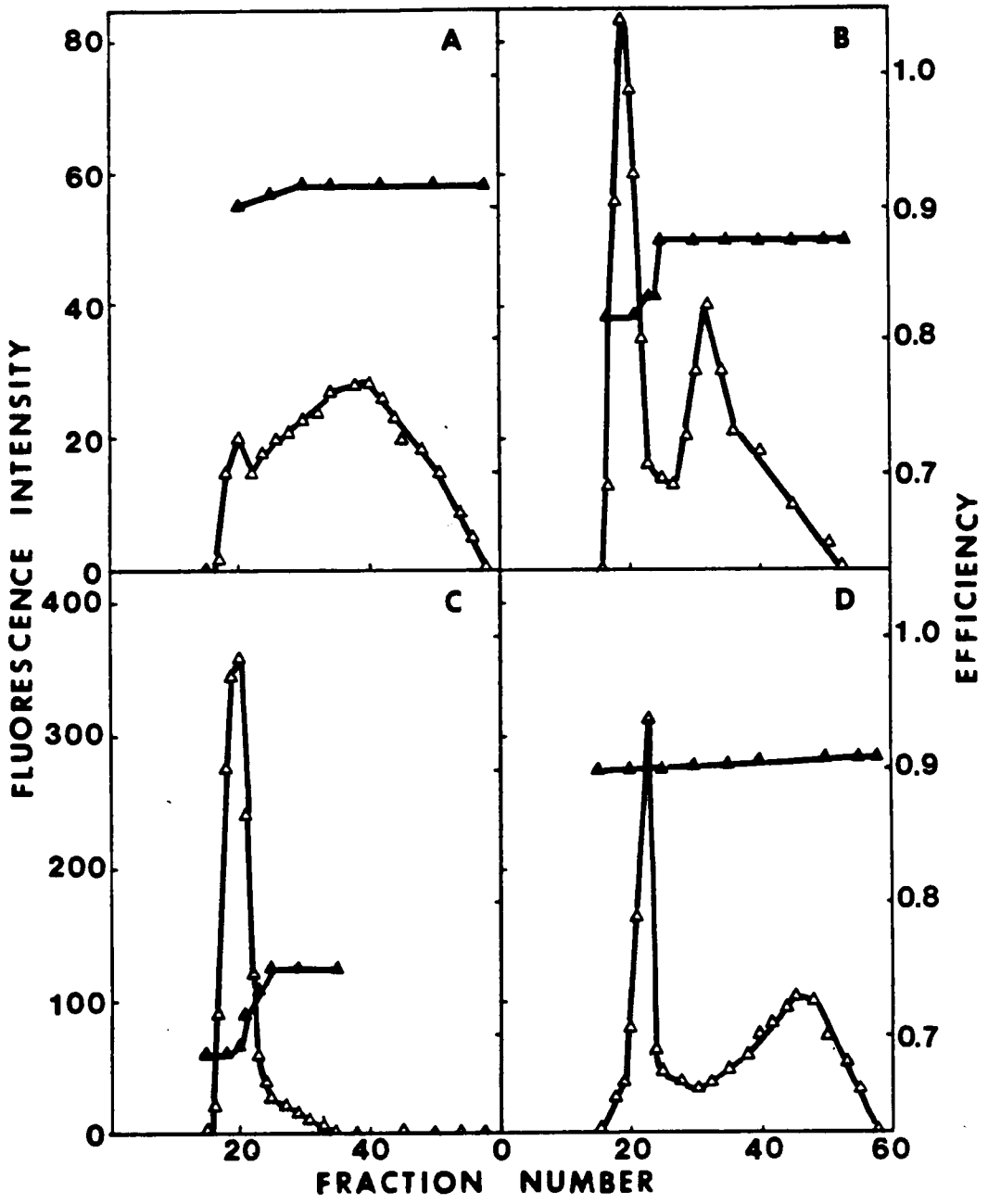

Fig. 3. Gel filtration of liposomes. Fluorescently labelled and unlabelled PE/PS (1:1) liposomes were mixed and chromatographed on an A50M column (A), or chromatographed polyhistidine (5 μ g/ml) (B), polyhistidine and then acid (pH 5.2 then returned to 7.4) (C), or acid alone (D). Fractions were assayed for N-NBD-PE fluorescence (△-△). The total fluorescence was also measured in the presence of 0.2% triton X-100, and the efficiency of the resonance energy transfer (▲-▲) of each fraction was calculated according to eq. (3).

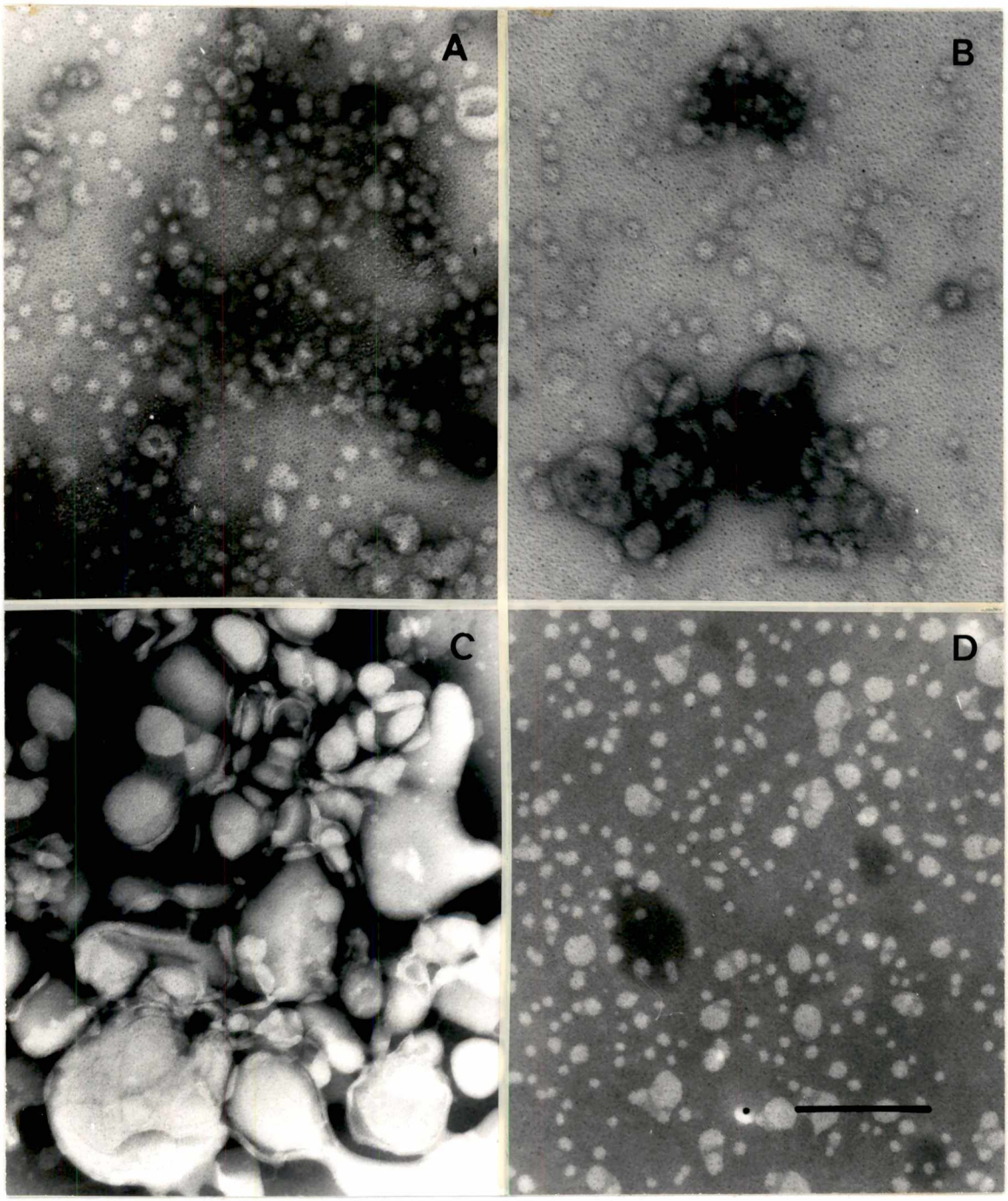

Fig. 4. Negative stain electron micrographs of PE/PS (1:1) liposomes. Original liposomes (A); liposomes after addition of polyhistidine (2.5 g/ml) (B); liposomes after sequential treatment of polyhistidine and acid (pH 5.2 then returned to 7.4) (C); liposomes treated only with acid (D). Bar is 0.2 μm.

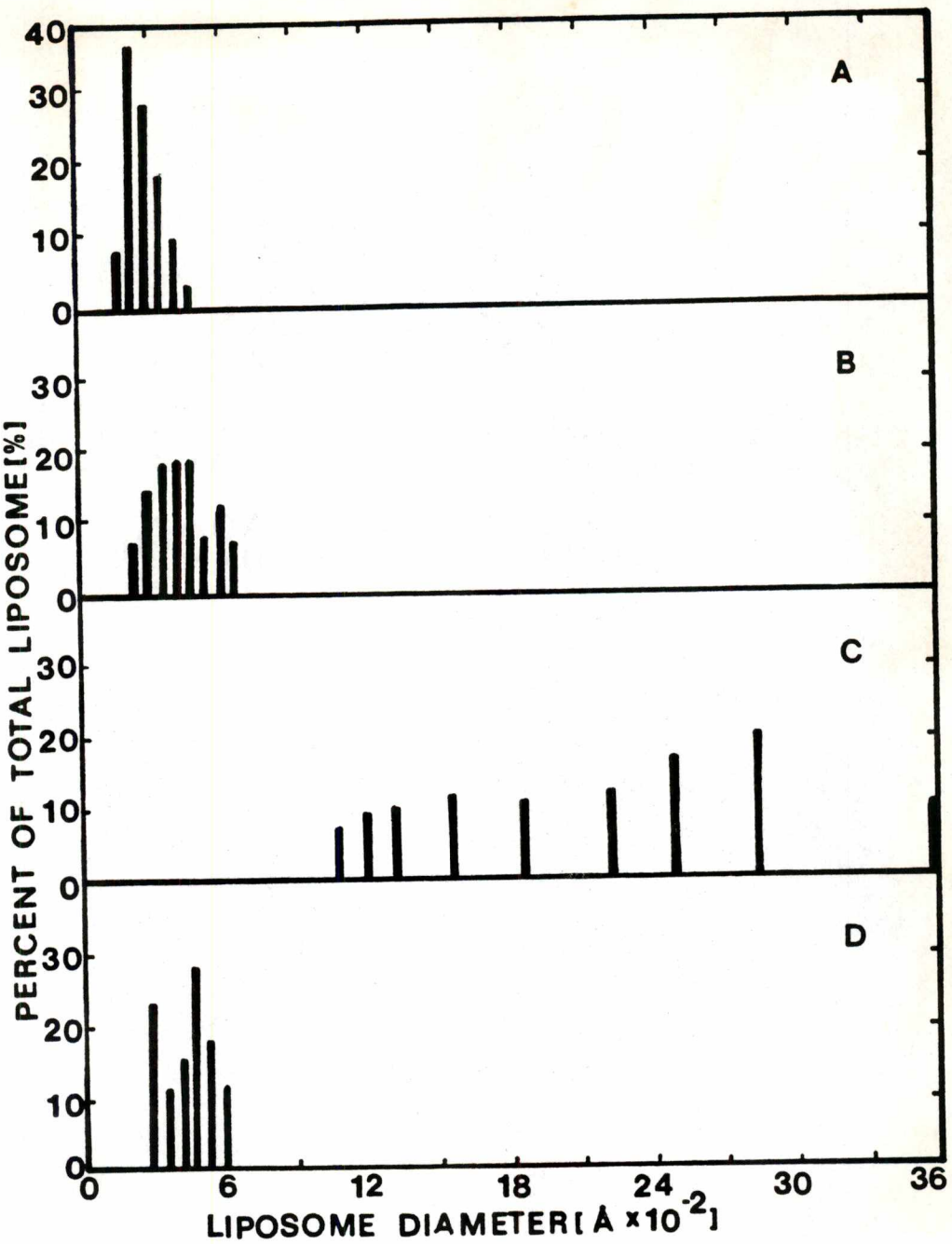

Fig. 5. Size distribution of PE/PS (1:1) liposomes measured by negative stain electron microscopy. Panels A, B, C, D correspond to panels in Fig. 4.

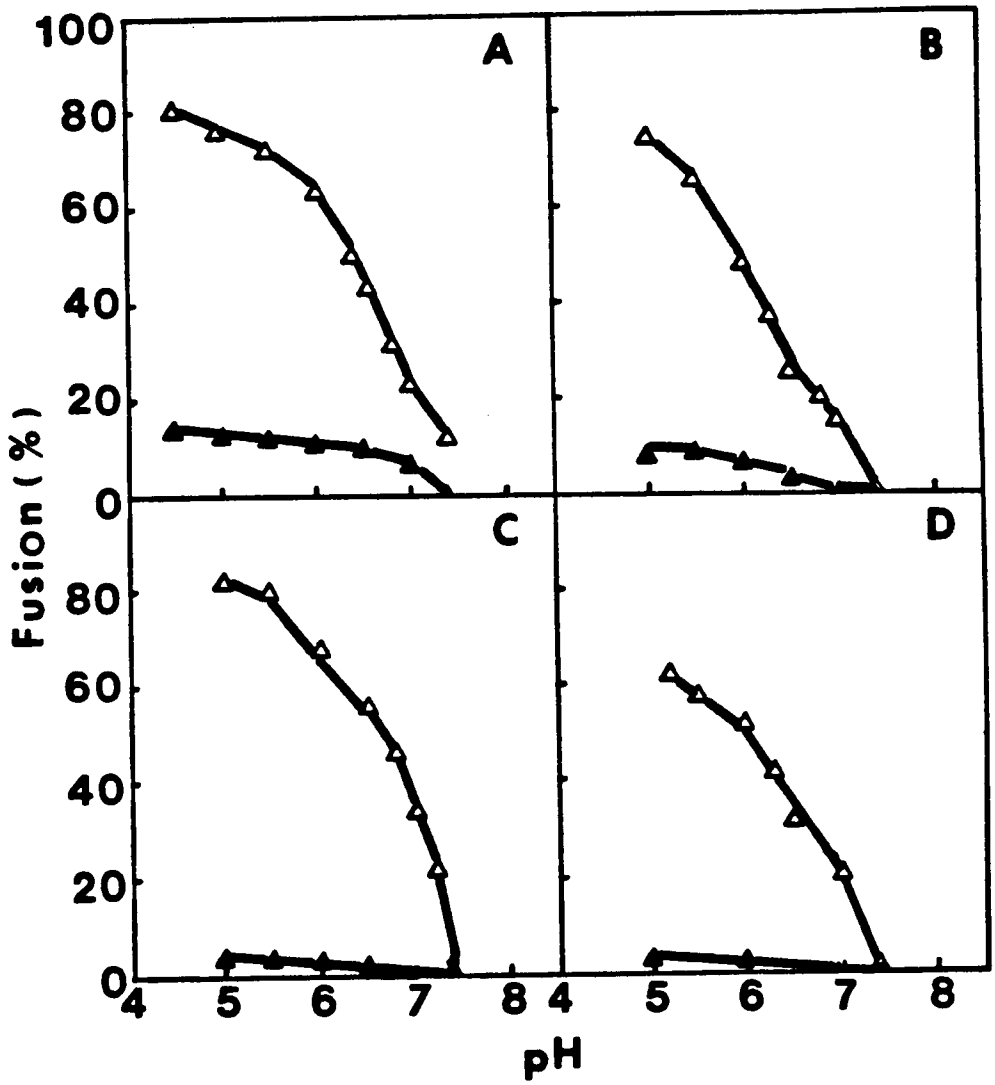

Fig. 6. Effect of medium pH on liposome fusion. Fusion of PE/PS (1:1) (A), PC/PS (1:1) (B), PC/PG (1:1) (C) and PC/CL (2:1) (D) liposomes were measured by the resonance energy transfer method at [polyhistidine] = 0(▲-▲) or 5 μg/ml (Δ-Δ).

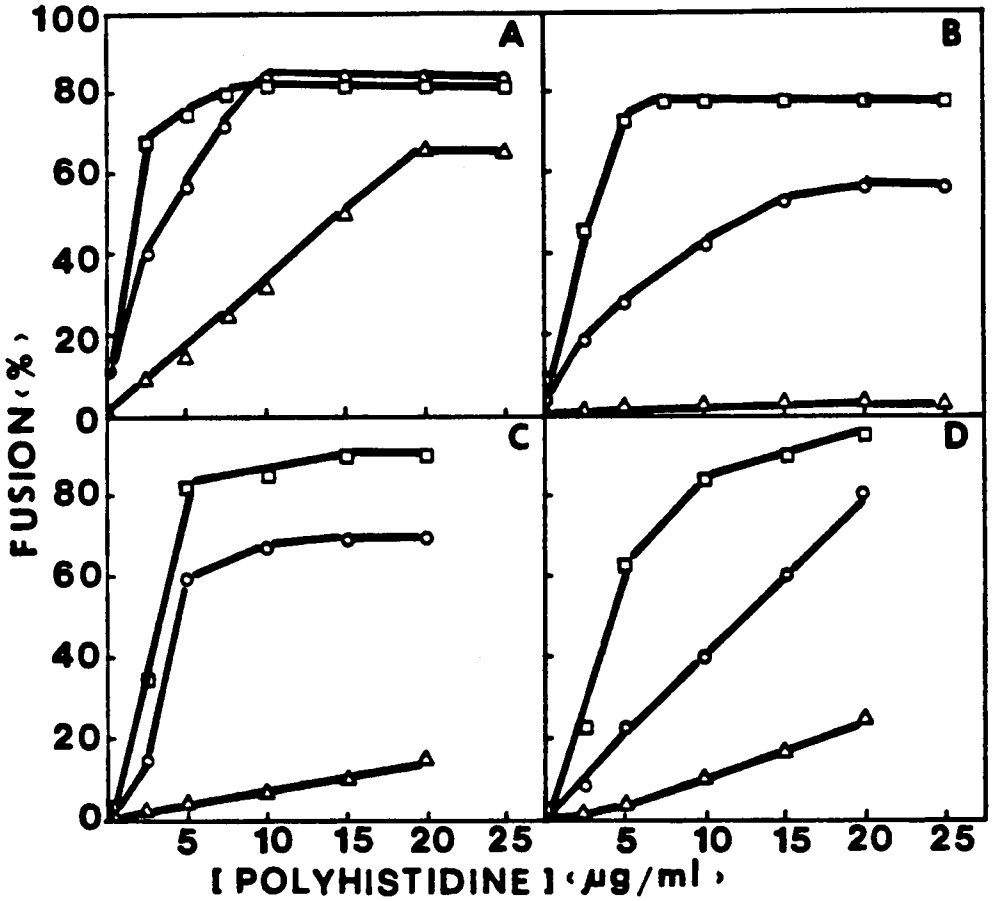

Fig. 7. Effect of polyhistidine concentration on liposome fusion. Fusion of PE/PS (1:1) (A), PC/PS (1:1) (B), PC/PG (1:1) (C) and PC/CL (2:1) (D) liposomes were measured by the resonance energy transfer method at pH 7.4 (Δ-Δ), 6.5 (o-o) and 5.2 (□-□).

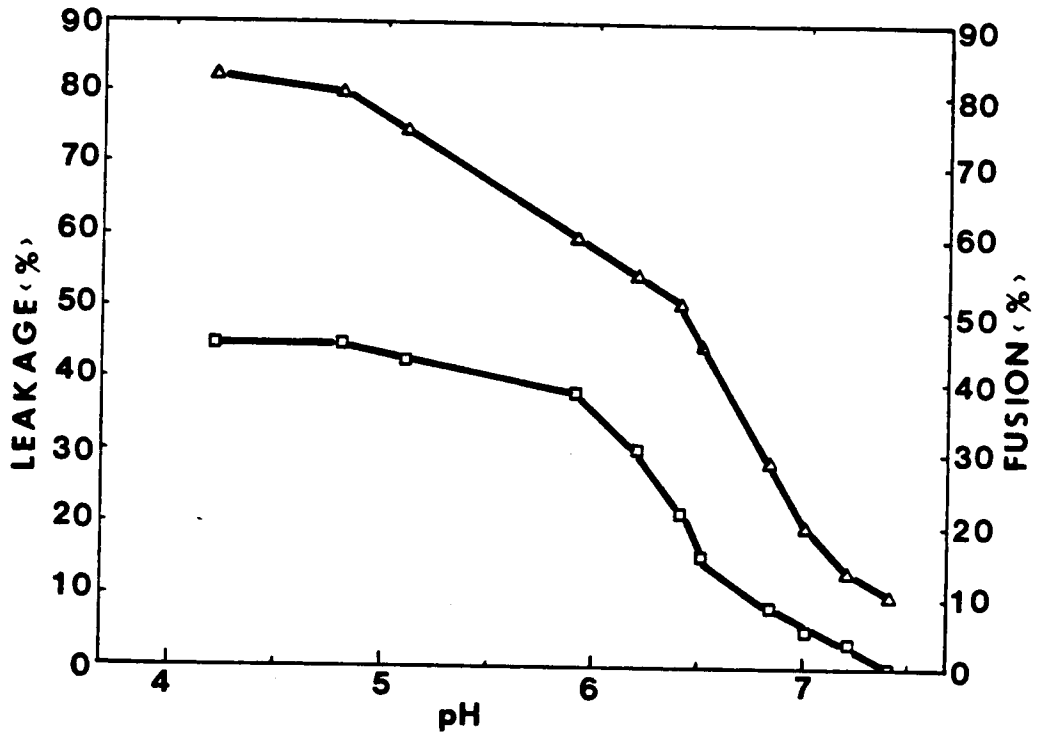

Fig. 8. Liposome leakage during fusion. Percent leakage of calcein (□-□) encapsulated in the PE/PS (1:1) liposomes (0.15 mM lipid) was measured in the presence of polyhisitidne (5 μg/ml) with acid treatment of the indicated pH as described in Materials and Methods. In a parallel experiment, liposome fusion (△-△) was measured under the same conditions with the resonance energy transfer method.

VITA

Chen-Yen Wang was born in Taiwan, Republic of China, on April 5, 1954. She graduated from the University of Chinese Culture in June of 1976 with a Bachelor of Science, and in June of 1978 with a Master of Science in Horticulture.

In July of 1978 she was employed by Dr. Shang-Shyng Yang to work in the Department of Agricultural Chemistry of the National Taiwan University. In one project she carried out various physical and chemical studies of red algae while in a second project she helped select and characterize several yeast fermentation mutants.

In September of 1982, Ms. Wang enrolled at The University of Tennessee, Knoxville. In March of 1984, she received the degree of Master of Science in Biochemistry.