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Polyhistidine Mediates an Acid-Dependent Fusion of Negatively Charged Liposomes†

Chen-Yen Wang and Leaf Huang*

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 the 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 and phosphatidylethanolamine (PE), but not sphingomyelin, showed high levels (about 80%) of fusion competency. For liposomes made of PE/phosphatidylserine (PS) (1:1), fusion at pH 5.2 and 2.5 μg/mL polyhistidine resulted in an increase in the average liposome diameter from 296 to 2400 Å, 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.

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 (Maeda et al., 1981; White et al., 1981; Marsh et al., 1982). Although the mechanism of membrane fusion is still obscure, it is clear that the viral

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[1] Abbreviations: PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; CL, cardiolipin; Sph, sphingomyelin; *N*-NBD-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).

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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. Polyhistidine 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 paper, 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.

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 M_r 9000), poly(L-glutamic acid) (average M_r 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, 10 μmol of the appropriate lipid mixture was dried to a film under N_2 and vacuum dessicated for at least 30 min. The dry lipid was resuspended in 1 mL of phosphate-buffered saline (PBS), pH 7.4, and sonicated for 20 min in a bath sonicator (Laboratory Supplies, Hicksville, NY). To label the liposomes, 0.5 mol % of *N*-NBD-PE and 2 mol % of *N*-Rh-PE were included in the lipid mixtures, and the liposomes were prepared by using a procedure identical with that used to prepare the unlabeled 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 of HCl of appropriate concentration. The suspension immediately became turbid. After about 5 min at room temperature, an 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 & Haugland, 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 530 nm of *N*-NBD-PE when the samples were excited at 470 nm. 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 labeled 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, and n is the concentration ratio of unlabeled to labeled liposomes in the mixture. To check the validity of eq 1, labeled and unlabeled 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 Figure 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 eq 1 is valid. For $n = 2$, the fluorescence enhancement was less than the theoretical value. All experiments were done with $n = 2$.

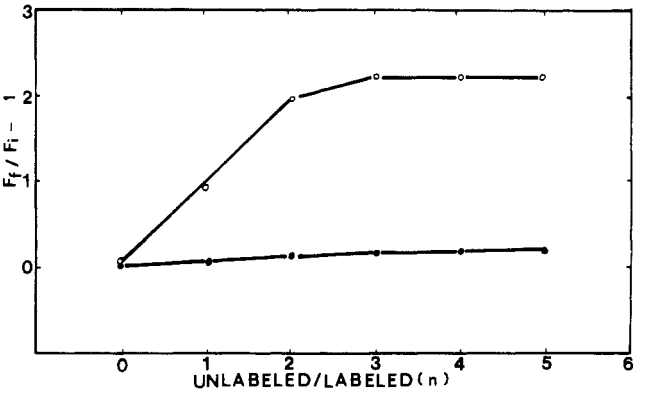

FIGURE 1: Test of the validity of eq 1. Unlabeled and fluorescently labeled (0.15 mM total lipid) liposomes were mixed at different ratios (n). The initial fluorescence, F_i, and the final fluorescence, F_f, after thorough sonication of the mixture were measured (○). To estimate the contribution of light scattering in this experiment, the fluorescently labeled liposomes were replaced with the unlabeled ones (●). PE/PS (1:1) liposomes were used.

Gel Filtration. One milliliter of liposome suspension in PBS was applied to a Bio-Gel A-50M column (30 × 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 the 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 the 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 1% potassium phosphotungstate and were viewed with a Zeiss or JEM6C electron microscope. Photographs were taken at 40000–50000× magnification and

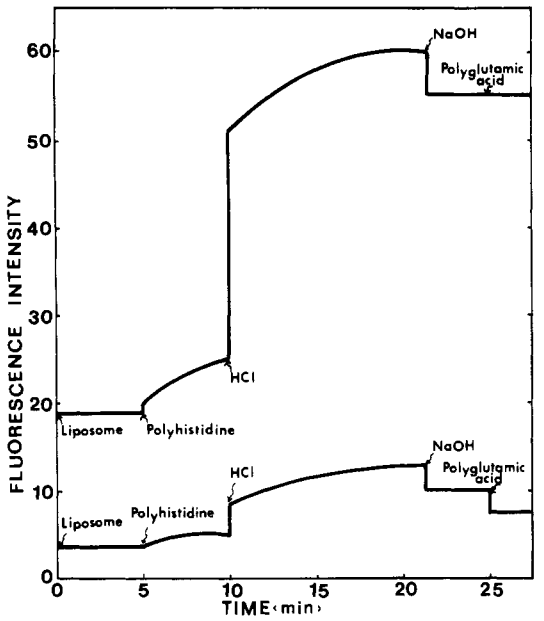

FIGURE 2: Time course of fluorescence enhancement during polyhistidine-induced liposome fusion at room temperature (upper curve). PE/PS (1:1) liposomes were used at [lipid] = 0.15 mM and [polyhistidine] = 5 μg/mL. Addition of HCl reduced the pH to 5.2 which was returned to 7.4 by the addition of NaOH.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

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. The fluorescence intensity of calcein-entrapped liposomes was measured in 2 mL of PBS. At high concentration, the fluorescence of calcein is efficiently self-quenched. Enhancement of fluorescence occurs when the dye leaks from the liposomes (Allen & Cleland, 1980). Calcein fluorescence was measured at λ_{ex} = 490 nm and λ_{em} = 530 nm. Liposome leakage is defined as

$$\% \text{ leakage} = \frac{I_f - I_i}{I_t - I_i} \times 100 \qquad (2)$$

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.

Results

Fusion Measured by Resonance Energy Transfer. The time course of fusion for PE/PS (1:1) liposomes is shown in Figure 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 total fluorescence 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 Figure 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 disaggregated by the addition of polyanion.

Table I: 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.

To ascertain that the observed fluorescence increase is truly induced by polyhistidine at acidic conditions, an experiment was performed in which the order of addition of various reagents was altered. As can be seen in Table I, a large fluorescence increase, expressed as the percent of fusion according to 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 a 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 fluorometric measurements, and to demonstrate liposome fusion by using a different technique, we fractionated various pretreated liposomes on the basis of particle size by gel filtration. Mixtures of 2 parts of unlabeled and 1 part of labeled 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-X-100. 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

$$E = 1 - F/F_t \qquad (3)$$

according to Struck et al. (1981). As labeled liposomes fuse with unlabeled ones, the quenching of *N*-NBD-PE fluorescence decreases which results in larger F values and hence smaller E values. Chromatograms of the untreated liposomes (Figure 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 (Figure 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 *N*-NBD-PE

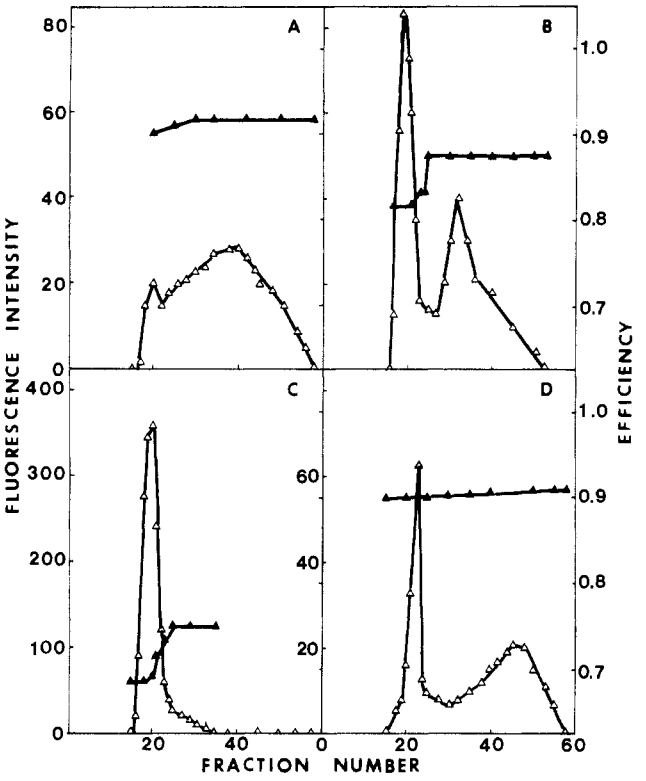

FIGURE 3: Gel filtration of liposomes. Fluorescently labeled and unlabeled PE/PS (1:1) liposomes were mixed and chromatographed on an A50M column (A) or chromatographed after treatment with polyhistidine (5 μg/mL) (B), with polyhistidine and then acid (pH 5.2 and then returned to pH 7.4) (C), or with 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.

fluorescence and a total shift of liposome elution to the void volume fractions (Figure 3C). Furthermore, the efficiencies of energy transfer in these fractions were much lower than those for 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 (Figure 3D). There was little or no liposome fusion detected since the efficiencies of energy transfer across the chromatogram were 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 negative-stain electron microscopy failed to reveal the aggregated liposomes (data not shown).

It is worth noting that liposomes eluted in fractions 30–38, shown in Figure 3B, after polyhistidine treatment could be induced to fuse by 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_r 9000), 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 II: Dependence of Liposome Fusion on Lipid Composition[a]

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

[a] [Polyhistidine] = 5 μg/mL, [lipid] = 0.15 mM, and pH of acid treatment = 5.2. [b] [Polyhistidine] = 10 μg/mL; other conditions unchanged.

(Table II). It is clear that fusion requires the presence of acidic lipid in the liposomes; no fusion was observed with neutral liposomes. PC liposomes did not fuse even at a polyhistidine concentration of 25 μg/mL. Although liopsomes 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 the highest fusion activities. Liposomes composed of acidic lipids alone (e.g., PS, PG, or CL) only had low activities. This observation cannot 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 III). Before the addition of polyhistidine, sonicated liposomes were unilamellar and rather homogeneous in size with an average diameter ranging from 170 to 400 Å, 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, which increased about 7-fold.

The electron micrographs of the PE/PS (1:1) liposomes are shown in Figure 4. Aggregation occurred on polyhistidine-treated liposomes (Figure 4B,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 (Figure 5A) showed the size range of the original liposomes was about

Table III: Change of Liposome Diameter with Polyhistidine and Acid Treatments As Revealed by Negative-Stain Electron Microscopy[a]

lipid composition	diameter (Å) liposomes	polyhistidine-treated liposomes[b]	polyhistidine- and acid-treated liposomes[c]
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 examination by electron microscopy.

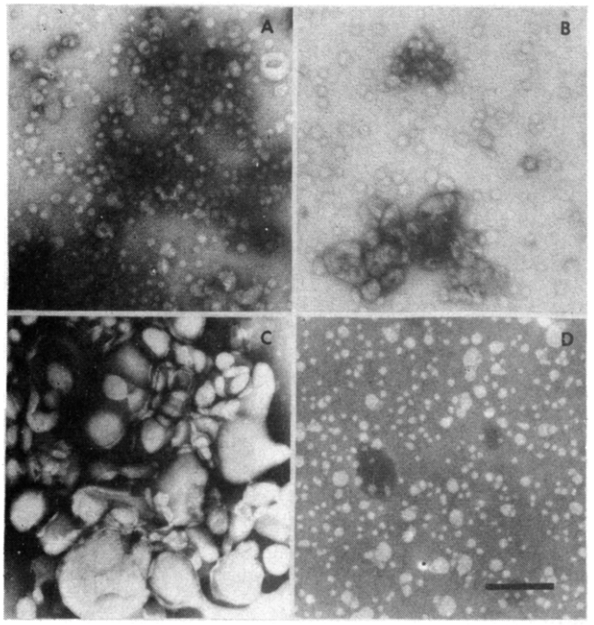

FIGURE 4: Negative-stain electron micrographs of PE/PS (1:1) liposomes: original liposomes (A); liposomes after addition of polyhistidine (2.5 μg/mL) (B); liposomes after sequential treatment of polyhistidine and acid (pH 5.2 and then returned to pH 7.4) (C); liposomes treated only with acid (D). The bar is 0.1 μm.

174–522 Å with a large proportion being about 260–270 Å in diameter. After treatment with 2.5 μg/mL polyhistidine, the size range was about 260–650 Å with a mean diameter of 424 Å. In a comparison of the elution profile of PE/PS (1:1) liposomes in Figure 3B with the size distribution in Figure 5B, the results are consistent. It indicated that some of the polyhistidine-treated liposomes fused having lower efficiency of energy transfer and some of the liposomes aggregated whose efficiency was close to those of the original liposomes. The size distribution of the fused liposomes shown in Figure 5C presented a large shift. Most of the liposomes were in the range of 1800–3000 Å in diameter. The mean diameter of fused liposomes was 2084 Å which was 7 times the size of the original ones. The broad distribution also indicated that the extent of liposome fusion varied. Some liposomes had only undergone one round of fusion, but others had several rounds. The size distribution of acid-treated liposomes (Figure 5D)

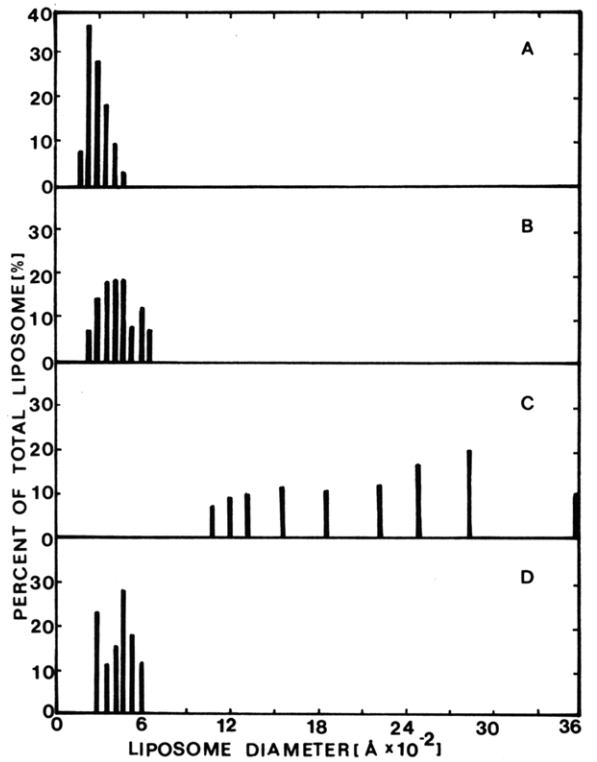

FIGURE 5: Size distribution of PE/PS (1:1) liposomes measured by negative-stain electron microscopy. Panels A, B, C, and D correspond to the panels in Figure 4.

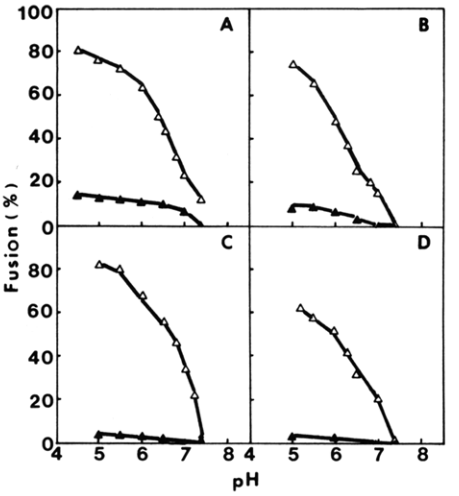

FIGURE 6: Effect of medium pH on liposome fusion. Fusion of PE/PS (1:1) (A), PC/PS (1:1) (B), PC/PG (1:1) (C), and PC/CL (2:1) (D) liposomes was measured by the resonance energy transfer method at [polyhistidine] = 0 (▲) or 5 μg/mL (△).

showed that they appeared in the range of 170–570 Å with a mean diameter of 339 Å. These results clearly showed that the acid treatment alone could not induce significant fusion of the PE/PS liposome.

Effect of Medium pH. The extent of liposome fusion was critically dependent on the pH of the acid treatment. Figure 6 shows the effect of the medium pH on the 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, approximatey 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 a lower level

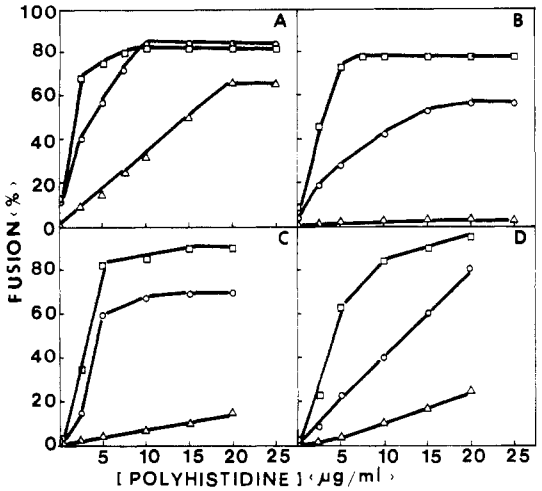

FIGURE 7: Effect of polyhistidine concentration on liposome fusion. Fusion of PE/PS (1:1) (A), PC/PS (1:1) (B), PC/PG (1:1) (C), and PC/CL (2:1) (D) liposomes was measured by the resonance energy transfer method at pH 7.4 (△), 6.5 (○), and 5.2 (□).

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 Figure 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 concentrations 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 a comparable level of fusion. In some cases such as the PC/PS (1:1) and PC/PG (1:1) liposomes, the 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, a low level of fusion or no fusion was observed for other types of liposome. No fusion was observed when polyhistidine was replaced by histidine, polyglutamic acid, or a random polymer of Glu, Ala, and Tyr, even at 20 μg/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. The curves in Figure 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 a 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.

Discussion

In the present study, we have shown that polyhistidine

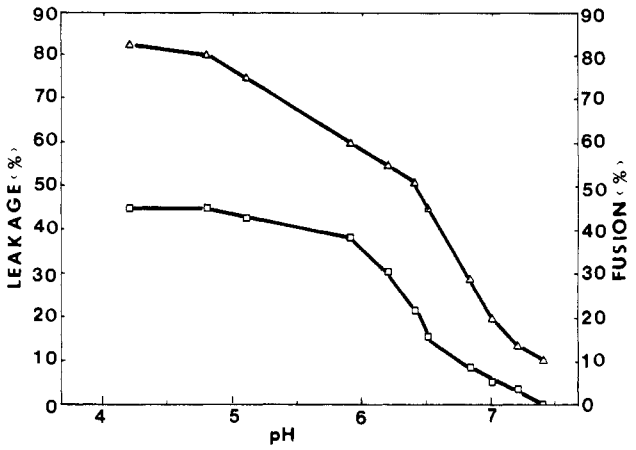

FIGURE 8: Liposome leakage during fusion. The percent leakage of calcein (□) encapsulated in the PE/PS (1:1) liposomes (0.15 mM lipid) was measured in the presence of polyhistidine (5 μg/mL) with acid treatment at the indicated pH as described under Materials and Methods. In a parallel experiment, liposome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

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

lipid composition	pH	α^a	[polyhistidine] for 50% max fusion[b] (μg/mL)	protonated His residue concn (μM)	protonated His residues per negative charge
PE/PS	7.4	0.04	10.0	2.6	0.07
(1:1)	6.5	0.15	2.5	2.4	0.06
	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 $pH = pK_0 + \log [(1 - \alpha)/\alpha] - 0.868n\alpha w$ (Patchornik et al., 1957) where pK_0 = the intrinsic pK_a for histidine (pK_0 = 6.15 used for this calculation), α = the fraction of protonated histidine residues, n = the average number of histidine residues per polyhistidine molecule (n = 58 for an average molecular weight of 9000), and w = the electrostatic interaction factor (w = 0.0575 used for this calculation). [b] Data taken from Figure 7A. [c] Total concentration of negatively charged phospholipid = 75 μM. If it is assumed that half of the phospholipid residues are on the outer monolayer of the unilamellar liposomes, the effective concentration = 37.5 μM. The concentration of CL was half of this value, but the concentration of the negative charges was the same, since CL carried two negative charges per molecule. [d] Estimated by assuming maximum fusion was 100%.

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 IV, at concentrations of polyhistidine which caused 50% of maximal fusion of liposomes of a given lipid composition, approximately the same amounts 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.

It is useful to compare the liposome fusion induced by polyhistidine with those induced by other fusogens. For example,

polyhistidine-mediated liposome fusion at low pH is a more efficient process than the fusion of liposomes induced by Ca^{2+} or polylysine. Ca^{2+} does not induce fusion of PS 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). Maximum fusion of the negatively charged liposomes induced by polylysine occurs at a charge ratio of nearly 1 (Walter et al., 1984). Polyhistidine, on the other hand, can effectively fuse liposomes at a charge ratio of 0.2 or less (Table IV). 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+}-induced (Papahadjopoulos et al., 1977; Ohnishi & Ito, 1974) and polylsyine-induced (Galla & Sackmann, 1975; Gad et al., 1979) 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 a high degree of local destabilization. In the case of PE/PS (1:1) liposomes, domains enriched with PE may undergo a bilayer to hexagonal (H_{II}) phase transition or the formation of the inverted micelles or lipidic particles [for a review, see Cullis & De Kruijff (1979)]. Such nonlamellar structures of the phospholipids have been postulated to be a strong driving force for membrane fusion (Verkleij et al., 1979, 1980). Indeed, although the resonance energy transfer assay showed that liposomes containing PE/PS and PC/PS had approximately an equal degree of fusion under the same conditions (Table II), electron microscopic observations revealed that PE/PS liposomes fused to become much larger liposomes than the PC/pS liposomes (Table III). 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.

Binding of polyhistidine to the negatively charged liposomes is unlikely to bring about a total charge neutralization of the liposomes even at saturating concentrations of polyhistidine (Table IV). This is due to the steric hindrance for the binding of polymeric molecules to the liposome surface. The residual negative charges on the liposomes tend to decrease the liposome aggregation and fusion. 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 at saturation.

The present study bears some relevance to the acid-dependent fusion of the viral envelopes with the target membranes as in the cases of the influenza (Maeda et al., 1981; 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). Although the interactions of the hydrophobic segment in the viral envelope glycoproteins with the target membranes have been considered to be an important step in the fusion (White & Helenius, 1980), the results of the present study provide an additional possibility. The protonation of the histidine residues of the viral glycoproteins at the acidic pH may trigger a lipid phase separation of the target membrane and eventually lead to the fusion event. 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 out hypothesis.

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Registry No. Poly(L-histidine), 26062-48-6; poly(L-histidine), SRU, 26854-81-9.

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Analysis of the Ternary Interaction of the Red Cell Membrane Skeletal Proteins Spectrin, Actin, and 4.1†

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ABSTRACT: Spectrin dimers interact weakly with F-actin under physiological solvent conditions (with an association constant of about 5×10^3 M^{-1} at 20 °C). In the presence of the membrane skeletal constituent, protein 4.1, strong binding is observed; an analysis of the profiles for formation of a ternary complex leads to an association constant of about 1×10^{12} M^{-2}. This association becomes weaker at low ionic strength, whereas the opposite applies to the spectrin–actin interaction. The stability of the ternary complex is maximal at physiological ionic strength and somewhat above. The effect of temperature in the range 0–20 °C on the formation of the ternary complex is small, whereas the spectrin–actin interaction almost vanishes at low temperature. There is no detectable calcium sensitivity in either the binary or the ternary system within the limits of precision of our assay. The ternary complex resembles the natural system in the membrane in that the actin is resistant to dissociation and unavailable in the deoxyribonuclease assay; after selective proteolytic destruction of spectrin and 4.1, all the actin becomes available. In the absence of 4.1, spectrin dimers do not measurably protect the actin against dissociation.

The shape and mechanical properties of the mammalian red blood cell are determined by a network of proteins on the cytoplasmic surface of the membrane. The constituents of this complex are spectrin, actin, and proteins 4.1 and 4.9, the latter being a minor component. For the formation of a highly stable continuous network under physiological salt conditions it was shown (Ungewickell et al., 1979) that the actin must be polymerized, the spectrin must be tetrameric, and 4.1 must be present. In the absence of 4.1, interaction of spectrin with F-actin is still observed (Brenner & Korn, 1979) but is clearly very much weaker (Cohen & Foley, 1980). The spectrin occurs in the cell predominantly as a tetramer (Ungewickell & Gratzer, 1978; Liu & Palek, 1980), made up of two heterodimers associated head-to-head. The 4.1 binding sites are near the distal ends of the tetramer (Tyler et al., 1979), and the tetramer is thus divalent with respect to 4.1 and actin (Ungewickell et al., 1979). The binding of 4.1 to spectrin has been studied (Tyler et al., 1980; Wolfe et al., 1982), but that of red cell 4.1 to actin, which by implication should occur, appears not yet to have been detected under physiological solvent conditions and must thus be supposedly weak in the absence of spectrin.

Red cell spectrin is the archetypal member of a class of elongated proteins, all of which bind to F-actin (Levine & Willard, 1981; Goodman et al., 1981; Davis & Bennett, 1982; Repasky et al., 1982; Glenney et al., 1982); protein 4.1 has similarly been found in various cell types (Cohen et al., 1982; Spiegel et al., 1982). The binding of brain spectrin (fodrin) to actin is also promoted by 4.1 (Burns et al., 1983). We have studied the nature of the interaction between actin, red cell spectrin, and 4.1 and the thermodynamic characteristics of what appears still to be the only known ternary protein system of this kind to have been characterized.

Materials and Methods

Protein Preparation. Proteins were prepared from human red cells that had been stored for less than 1 week. Spectrin

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