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POLYCATION-INDUCED FUSION OF NEGATIVELY-CHARGED VESICLES

ALEXANDER E. GAD [a,*], BRIAN L. SILVER [b] and GERA D. EYTAN [a,**]

[a] *Department of Biology, and* [b] *Faculty of Chemistry, Technion, Israel Institute of Technology, Haifa 32000 (Israel)*

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Sonicated vesicles of 20–50 nm in diameter consisting of neutral phospholipids and a variety of acidic phospholipids were interacted with polylysine, cytochrome *c*, Ca^{2+} and Mg^{2+}. The addition of polycations caused massive aggregation accompanied by an increase of membrane permeability as determined by leakage of flurorescent dye. Aggregation was followed by fusion of the vesicles into structures that in some cases exceeded 1 μm in diameter. Polylysine induced aggregation and appreciable fusion at charge ratios (polylysine/phospholipid) of 0.5–2, while divalent cations did so only at charge ratios (cation/phospholipid) greater than 10. Aggregation and fusion induced by polylysine were dependent also on the size of the polycation, i.e., the longer the molecule the less needed to induce similar aggregation. It appears that, due to the concentration of charges on a single molecule, polylysine is at least an order of magnitude more effective than divalent cations at inducing fusion of membranes. Cytochrome *c* induced fusion of similar vesicles at moderately acidic pH (pH 4.2).

Introduction

Cations such as Ca^{2+} and to a lesser extent Mg^{2+} have been reported to cause phase separation [1–4] and fusion of negatively-charged vesicles [5,6,7]. The effects that divalent cations exerted on membranes seemed relevant to physiological processes, especially in view of the finding that exocytosis which involved fusion of vesicles with plasma membrane depended on Ca^{2+} [8]. Later on, the mechanism of exocytosis turned out to be more sophisticated with the discovery of synexin, a protein which specifically complexed with Ca^{2+} and catalyzed aggregation of chromaffin granules [9].

Myelin sheath is known to contain a basic protein. Palmer and Dawson [10] have reported that this basic protein formed complexes with several acidic phospholipids. Steck et al. [11] showed that the basic protein-lipid complex was sensitive to changes in ionic strength, suggesting that the interaction was indeed electrostatic. Recently, Brady et al. [12] reported that while normal human basic protein caused dense packing of PG membranes, the protein from multiple sclerotic patients failed to do so, a phenomenon corresponding to pathological findings.

We expected polylysine to induce fusion of vesicles containing acidic phospholipids based on reports according to which it caused change in transition temperature like Ca^{2+} [1]. It formed separate domains of PA and PC [13] and it also

* Present address: Thimann Laboratories, UCSC, Santa Cruz, CA 95064, U.S.A.

** To whom correspondence and requests for reprints should be addressed.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; LysoPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

caused a shift of acidic phospholipids from the liquid to the crystalline state [14]. Papahadjopoulos et al. [15] stated, based on studies of divalent cation interaction with PS vesicle, that an obligatory condition for fusion to occur was the shift of the phospholipid from the liquid to the crystalline state following aggregation and formation of separate domains in the bilayer. In addition, polylysine has been reported to cause aggregation and precipitation of bacterial membranes [16], negatively-charged vesicles, submitochondrial particles, sarcoplasmic reticulum vesicles and erthrocyte vesicles [17]. Polylysine was reported to have induced fusion of PE vesicles [18].

In the present work we studied the effect of polylysine on artificial membranes consisting of PC, PE and acidic phospholipids, thus imitating the composition of native membranes Polylysine and cytochrome *c* induced fusion of vesicles to some extent with all the acidic phospholipids tried and were more effective than divalent cations.

Materials and Methods

Cardiolipin, dicetyl phosphate, lysophosphalidylcholine (lysoPC), egg yolk phosphatidylcholine (PC, type VI) and beef heart cytochrome *c* (type VI) were purchased from Sigma Chemical Co. Polylysines and poly(aspartic acid) were purchased from Chemalog. 6-Carboxyfluorescein was purchased from Eastman Kodak. Cholestane spin label (3-doxylcholestane) was purchased from Syva.

Phosphatidylethanolamine (PE) was purified from soy bean phospholipids according to Kagawa et al. [19]. Phosphatidylserine (PS) was purified from bovine brain according to Papahadjopoulos and Miller [20]. Phosphatidic acid (PA) and phosphatidylglycerol (PG) were prepared from egg yolk phosphatidylcholine (PC) by treatment with cabbage phospholipase D essentially as described by Papahadjopoulos and Miller [20], except that an equal volume of glycerol was added to the mixture used in order to prepare phosphatidylglycerol. Phospholipid concentration was determined as previously described [21] and was expressed as mM phosphate. All phospholipids were checked for purity on thin-layer chromatography.

Small unilamellar vesicles were prepared by drying under N_2, 25 μmol of the desired mixture of phospholipids. The mixture was redissolved in ether and dried again under N_2. When prepared with ESR label the mixtures were redissolved in petrol ether. The dry mixture was suspended in 1 ml 20 mM Hepes (pH 7.6), 128 mM KCl and 32 mM NaCl. This buffer was used throughout the work unless otherwise mentioned. The suspension was sonicated to clarity in bath type sonifier (80 W).

For turbidity measurements, vesicles (4 mM P_i) were incubated in the presence of different amounts of polylysines, Mg^{2+} or Ca^{2+}, at room temperature for 1 h. Polylysine was neutralized by addition of double amounts of poly(aspartic acid) and 8 vol. of equimolar phosphate buffer (pH 7.6). Mg^{2+} and Ca^{2+} were removed by double concentration of EDTA after which 8 vol. of buffer were added. Suspensions were shaken for 10 min and turbidity was measured at 340 nm in a Zeiss spectrophotometer. The continuous change in turbidity was recorded in a Cary 113 C as follows: 0.5 μmol vesicles were incubated with 1 ml buffer and desired concentration of cation in a 1 ml cuvette and continuous change in turbidity was measured at 340 nm.

For turbidity measurement of cytochrome *c*-treated vesicles, 0.5 μmol of vesicles were mixed with varying amounts of cytochrome *c* in 0.2 M NaH_2PO_4 (pH 4.2) for 1 h at room temperature. Reaction was stopped by titrating the suspension with equimolar Na_2HPO_4 to pH 7.6. Equimolar phosphate buffer (pH 7.6) was added to complete 9 vol. and turbidity was meaured at 340 nm.

ESR spectra were recorded at 21°C on a Bruker X-band spectrometer taking care to avoid modulation broadening.

For fluorimetric measurements, the vesicles (50 mM) were prepared as already described, with the addition of 6-carboxyfluorescein (0.1 M, final concentration). After sonication the suspension was passed through a 25×1 cm Sephadex G-50–150 column in order to separate free dye from entrapped. 0.5 μmol of vesicles were mixed with varying amounts of polylysine and the change in fluorescence was monitored in a MPF-44B Perkin Elmer fluorimeter (320 nm excitation, 510 nm emission).

Samples for thin sectioning were prepared as

follows: Having completed the reaction with polylysine, the vesicles were incubated at 37°C, mixed with 5% melted agarose and rapidly cooled on ice. The resulting gel was sliced into 1 × 1 mm cubes and the material was fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate for 1 h at room temperature followed by overnight fixation at 4°C with 1% OsO_4 in 0.2 M phosphate buffer (pH 7.6). Excess OsO_4 was washed away by cold saline solution and the samples were incubated on ice with an aqueous solution of uranyl acetate (2.5%) for 15 min. Dye was washed away with cold saline. Samples were dehydrated in 30%, 60%, 90% and twice in 100% cold acetone, then transferred to 100% acetone at room temperature. The samples were incubated for 30 min with acetone/epon (1:1, v/v) and 30 min in acetone/epon (1:2, v/v). Several cubes of material were dipped in 100% epon for at least 10 min in order to allow complete evaporation of the remaining acetone. Finally, the samples were heated at 60°C for 48 h. Sectioning and post-staining were executed as previously described [21].

Results and Discussion

When saturating amounts of Ca^{2+} were added to vesicles containing an acidic phospholipid and large amounts of PE the suspension turned extremely turbid. Upon removal of the cations the suspension remained turbid (Fig. 1a). It has been shown previously that under these conditions such vesicles fused to form large structures that were not dispersed by chelating agents [21]. The remaining turbidity was used as an assay for fusion. Polylysine induced a drastic increase in the turbidity, which was not reversed by removal of the reagent, suggesting, that polylysine too was capable of inducing fusion.

When similar amounts of either Ca^{2+} or polylysine were added to vesicles not containing PE, a marked difference in cation effect was observed. While Ca^{2+} did not cause aggregation, confirming previous results [21], polylysine induced a great increase in the turbidity (Fig. 1b). However, upon the removal of polylysine, almost all the turbidity disappeared. In other words, most of the change was due to reversible aggregation rather than fusion of the vesicles.

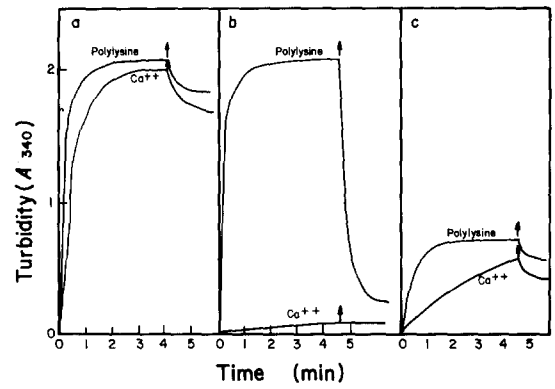


Fig. 1. Effect of Ca^{2+} and polylysine on vesicle aggregation and fusion. 0.5 μmol vesicles consisting of PC/PE/CL at a molar ratio of 1:6:3 (a, c) or PC/CL at a molar ratio of 7:3 (b) were incubated with either 20 mM $CaCl_2$ or 100 μg polylysine (a, b) or with 5 mM $CaCl_2$ or 25 μg polylysine (c). The change in the turbidity was recorded. CL, cardiolipin.

Vesicles that undergo massive fusion after addition of Ca^{2+}, i.e. containing large amounts of PE, were incubated with varying amounts of short (1.5–8 kDa), medium-sized (30–70 kDa) and long (70 kDa) polylysine. All the suspensions became turbid. In samples containing larger amounts of polycations, visible aggregation appeared as a white precipitate (Fig. 2). Less of the longer polylysine was needed to form the precipitate. The amounts

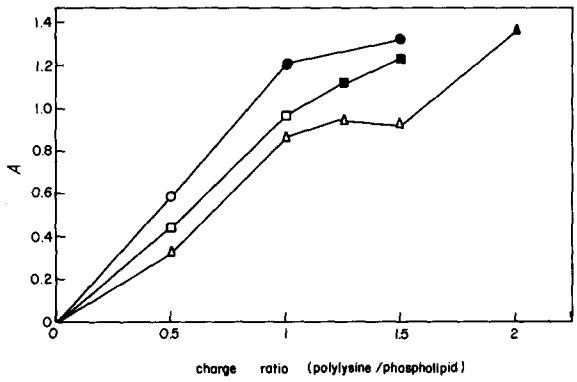


Fig. 2. Effect of polylysine concentration on fusion of vesicles. Vesicles containing 30 mol% cardiolipin were prepared and incubated as described under Methods with different concentrations of short (△), medium (□) or long (○) polylysine. Turbidity was measured at 340 nm after removal of polylysine. Precipitates are represented by full symbols (▲, ■, ●, respectively).

of polylysine required for precipitation were similar to those required for precipitation of membrane vesicles of various sources [17]. Upon neutralizing the polylysine by poly(aspartic acid), the clumps partially disaggregated and the suspension became highly turbid instead. Smaller amounts of the longer species were required to induce the same turbidity (Fig. 2). The difference in the potential to induce aggregation and fusion could be attributed to the capacity to create in the suspension a locally higher concentration of charge. This was supported by the comparison of the effect of polylysine to that of Ca^{2+} or Mg^{2+} (Fig. 3). Ca^{2+} and Mg^{2+} induce visible aggregation and fusion of dicetyl phosphate containing vesicles only when present at concentrations higher than 10 mM, i.e., when being at least in 10-fold excess of charge as compared to polylysine. The number of charges on polylysines, Mg^{2+} and Ca^{2+} was related to the number of charges on the vesicles, thus obtaining a charge ratio of 1–2 for polylysine and at least 10 for both Mg^{2+} and Ca^{2+}. The charge ratio was calculated assuming that at the pH used, PE was not charged and not taking into account the distribution of the acidic phospholipid between the layers.

Taking into consideration the size of the sonicated vesicles, the number of vesicles per μmol organic phosphate and the number of polylysine molecules per mg, we calculated that 2–4 molecules of medium-size polylysine (~50 kDa) per vesicle were enough to cause precipitation of the vesicles. While divalent cation-induced aggregation involves independent binding of many atoms to the vesicles, the association of the first charge of polylysine to the vesicle surface increases the probability of association of the next charge on the same molecule in a cooperative manner.

Further demonstration of the higher efficiency of polylysine as compared to divalent cations is seen in Fig. 1c and Fig. 4. At low concentrations of Ca^{2+} the change in turbidity (Fig. 1c) and permeability of the vesicles (Fig. 4b) was gradual. In contradistinction, addition of small amounts of polylysine induced a rapid change that was completed within 2 min (Fig. 1c, Fig. 4a). This observation could be explained by the rapid cooperative binding of polylysine to the bilayer.

Experiments were carried out with spin-labelled vesicles and they were broadly consistent with the conclusions derived from other methods. Cholestane spin-label (3-doxylcholestane) was incorporated into vesicles of various compositions at a concentration of 12 mol%. At this concentration considerable line-broadening occurs due to exchange (Fig. 5a). These vesicle suspensions were mixed with an excess of unlabeled vesicles of the same composition. No change in the ESR spectrum occurred except for a loss of intensity due to dilution of the labelled vesicles in the suspension.

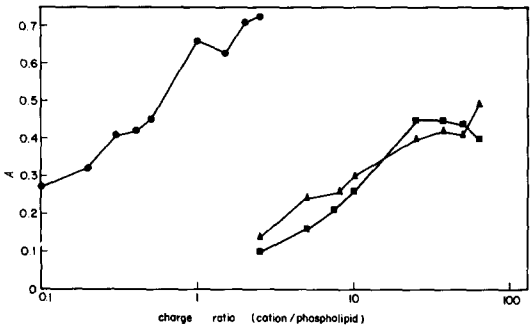


Fig. 3. Comparison of the effect of polylysine, Ca^{2+} and Mg^{2+} on fusion of vesicles. Dicetyl phosphate-containing vesicles were treated with different concentrations of medium polylysine (●), Ca^{2+} (▲) and Mg^{2+} (■). After removal of the cations, turbidity was measured at 340 nm. The concentration of cations was related to the charge of the vesicles (approx. 150 nequiv.) thus defining charge ratio of cation to phospholipid.

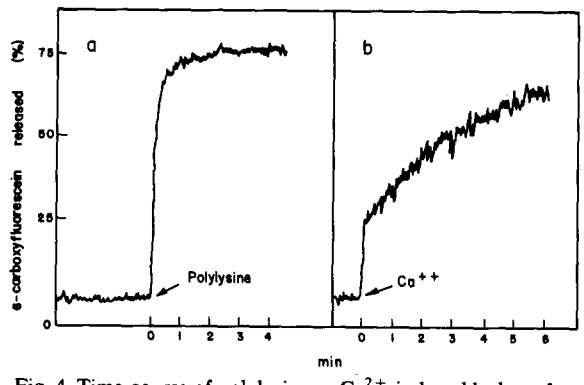


Fig. 4. Time-course of polylysine or Ca^{2+}-induced leakage from vesicles. Vesicles prepared with cardiolipin, containing 6-carboxyfluorescein were incubated with 20 μg medium polylysine (a) and 10 mM Ca^{2+} (b) as described under Methods. The time-course of change in fluorescence was recorded.

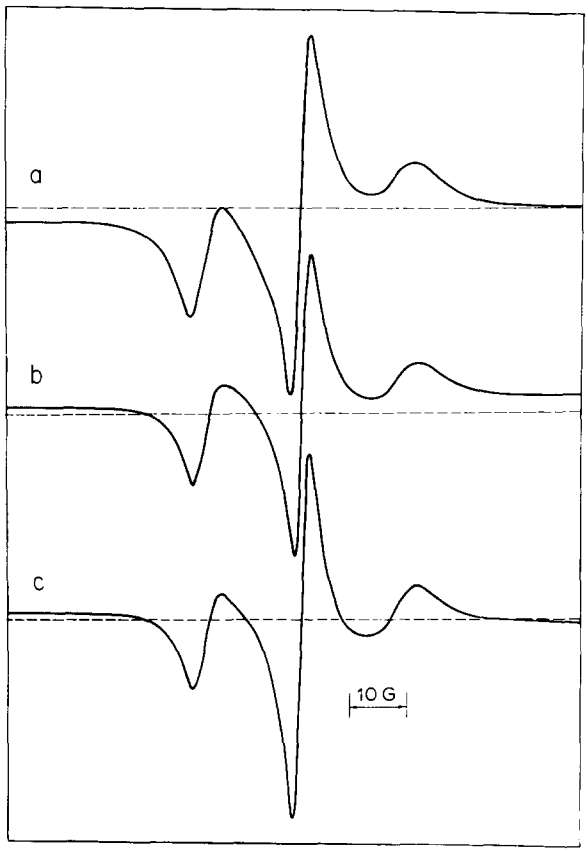


Fig. 5. Comparison of the effect of polylysine and Ca^{2+} on fusion of vesicles, ESR study. Vesicles consisting of PC, PE and cardiolipin at a molar ratio of 1:6:3 were prepared with or without 12 mol% spin-label (3-doxylcholestane). X-band ESR spectra of labelled vesicles incubated with 10-fold excess of vesicles not containing the label were recorded before the addition of cations (a) and after the addition of Ca^{2+} (b) or medium-size polylysine (c). The cations were added to give a charge ratio of 1 (cation to phospholipid). Measurements were carried out at room temperature.

Subsequent addition of Ca^{2+} or polylysine to this mixed system caused a narrowing of the lines in the ESR spectra for vesicles composed of PC, PE and cardiolipin in a molar ratio of 1:6:3 (Fig. 5b, c). No changes were observed with pure PC vesicles. Narrowing is a consequence of a reduction in the concentration of spin-label in the labelled vesicles. This can arise from fusion of labelled and unlabelled vesicles and subsequent lateral diffusion of the spin label in the fused membrane. PC vesicles do not fuse under these experimental conditions and no dilution and resulting narrowing are expected or observed. This also indicates that polylysine has no effect on either the label or on uncharged vesicles. Polylysine caused a greater narrowing (Fig. 5c) than an equivalent charge ratio of Ca^{2+}, again in agreement with their expected relative effectiveness. Vesicles consisting of PC and cardiolipin in a molar ratio of 7:3 undergo massive aggregation but no appreciable fusion upon addition of polylysine (Fig. 1b), and consistently they show only a slight line-narrowing under these conditions indicating that the effect observed is not due to transfer between aggregated vesicles.

If polylysine is to be used as fusion inducer between vesicles and cells it must cause minimal spillage of entrapped material from the vesicles. One should bear in mind the fact that in native systems, fusion between, for example, exocytotic vesicles and the plasma membrane occurs without spillage of the contents. The dependence of leakage on the size and concentration of polylysine (Fig. 6) was similar to the dependence of fusion on the same factors (Fig. 2). At concentrations causing precipitation, at least 75% of the material leaked within the first two minutes. An exception was long polylysine, that at concentrations higher than

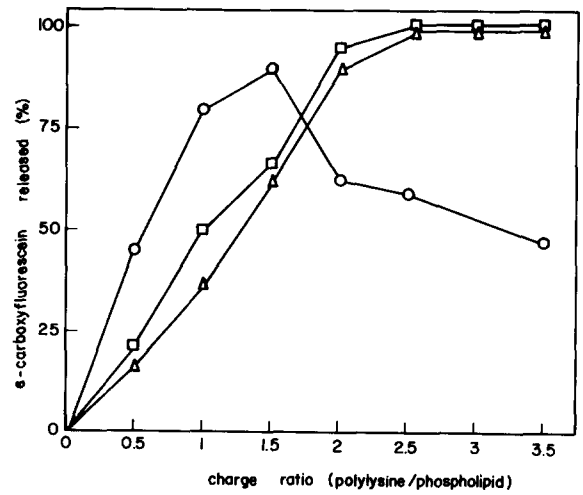


Fig. 6. Effect of polylysine concentration on permeability of vesicles. Fluorescence of vesicle suspension containing carboxyfluorescein was measured as described under Methods, 2 min after the addition of short (△), medium (□) or long (○) polylysine. The fluorescence change was converted to % of dye that had leaked out of the vesicles.

60 μg per μmol phospholipid (charge ratio > 1.5) induced rapid fusion with only partial leakage of contents (Fig. 6). The dramatic change in permeability, observed with the shorter polylysines, seems to be in contrast with a report according to which 17 kDa polylysine changed the permeability of PG vesicles only slightly [22]. However, this is not the case since the minimal concentration used with the PG vesicles was 800 μg per μmol phospholipid, i.e., charge ratio of 20. In view of these findings, it should be worthwhile trying to use excess of long polylysine in order to introduce vesicle-entrapped material into cells.

The use of lysoPC instead of PC, added insight to data already obtained [21]. The presence of 20 mol% lysoPC reduced the permeability change after addition of polylysine by a factor of 4–5 (Fig. 7). Furthermore, vesicles containing lysoPC formed visible aggregates just like the PC-containing ones, but unlike the latter, upon neutralizing the polylysine in the suspension of the lysoPC-containing vesicles turned translucent, i.e. less fusion took place. A similar effect was seen in vesicles containing only PC and up to 40 mol% of cardiolipin. Divalent and trivalent cations did not induce aggregation while polylysine induced essentially reversible aggregation with little fusion.

One can therefore attribute two roles to lysoPC:

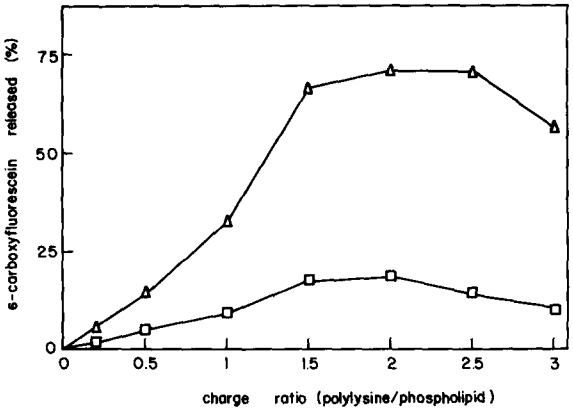


Fig. 7. Effect of lysophosphatidylcholine on polylysine-induced permeability of vesicles. Vesicles, consisting of 30 mol% cardiolipin, 50 mol% PE and 20 mol% of PC (△) or lysoPC (□), containing 6-carbyxyfluorescein were prepared as described under Methods. Vesicles were checked for permeability in the presence of different charge ratios of medium-size polylysine to phospholipids.

(a) LysoPC alters the distribution of cardiolipin so that less is in the outher layer, thus enabling polylysine, but not Ca^{2+}, to induce aggregation.

(b) LysoPC inhibits the shift of PE from bilayer to hexagonal state, since in its presence polylysine induced a much smaller charge in permeability.

Litman [23] reported that with large PE to PC ratios, as used in this work, PE conferred instability on the bilayer, not permitting entrapment of material inside. An lysoPC had greater heat to tail ratio than PC [24], it would better restrict the bilayer to hexagonal shift of PE.

In addition to cardiolipin as the acidic component of vesicles, several acidic phospholipids were tried. Vesicles containing 30 mol% of dicetyl phosphate (Fig. 3), PA, PG and PS were interacted with medium-sized polylysine. All the vesicles used were induced to fuse. While dicetyl phosphate, like cardiolipin precipitated at a 1–2 charge ratio, the others did so at lower ratios, i.e. with less polylysine. This observation may be due to the different affinities for polylysine or to differences in the distribution of the acidic phospholipids between the inner and the outer layers.

Several structures, sometimes exceeding 1 μm in diameter were seen in thin sections of vesicles treated with polylysine. Small amounts of polylysine (charge ratio < 0.5) induced formation of large unilamellar structures in vesicles containing PA (Fig. 8a), dicetyl phosphate or cardiolipin. Similar amounts induced formation of large loose oligolamellar structures in PS containing vesicles (Fig. 8b). Large amounts of polylysine (charge ratio > 0.5) induced formation of large dense multilamellar structures in PS containing vesicles (Fig. 8c) and small dense multilamellar structures in PA and PG containing vesicles. Similar amounts of polylysine induced formation of large unilamellar structures and amorphous aggregates of membranes in vesicles containing dicetyl phosphate (Fig. 8d) and cardiolipin. Structures resembling the last two described above had already been seen in a Ca^{2+}-treated cardiolipin-containing vesicles [21].

Cytochrome *c* induced massive fusion of negatively-charged visicles only at moderately acidic pH (pH 4.2). Since the molecule possesses 23 charges when fully protonated at extremely low

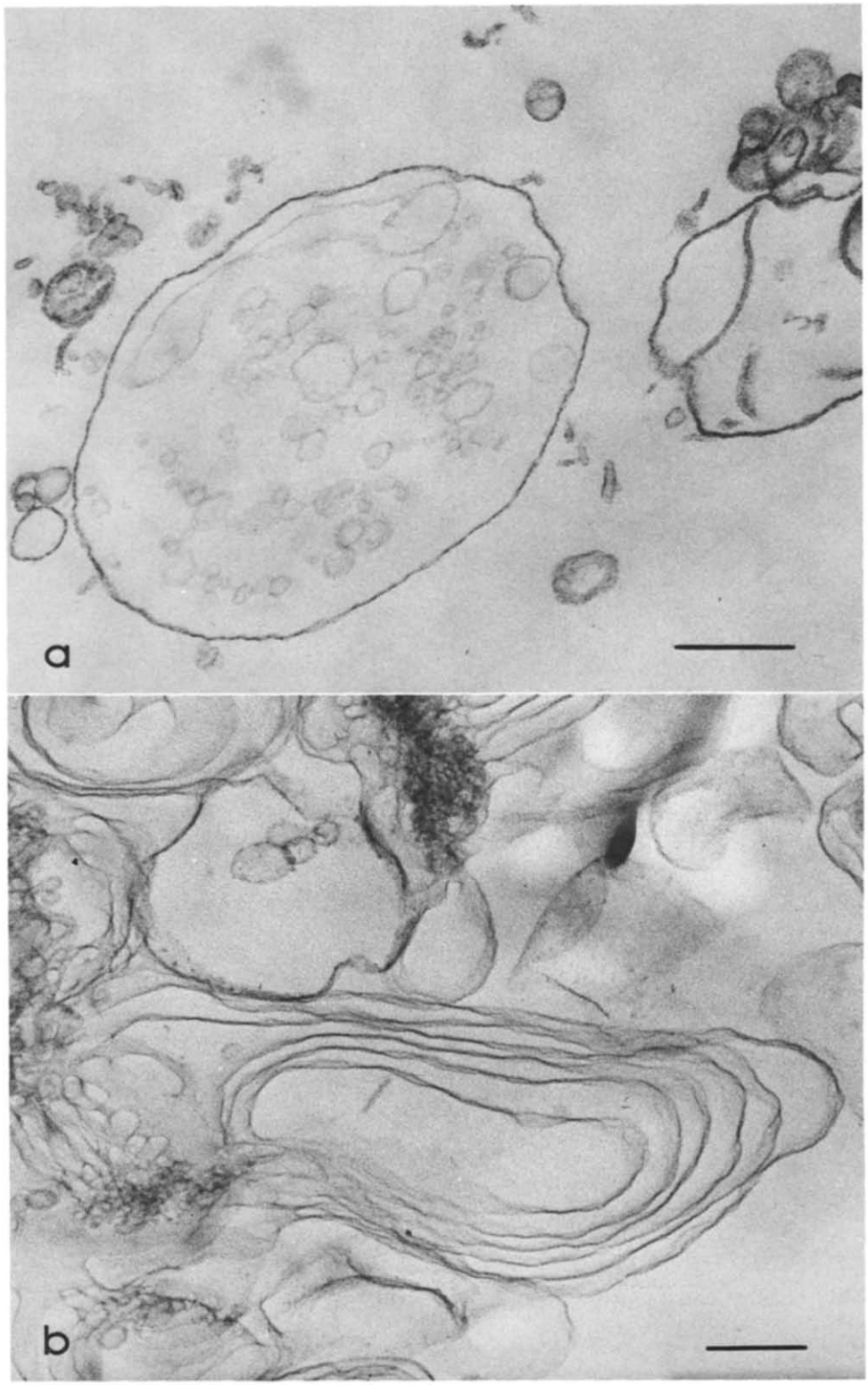
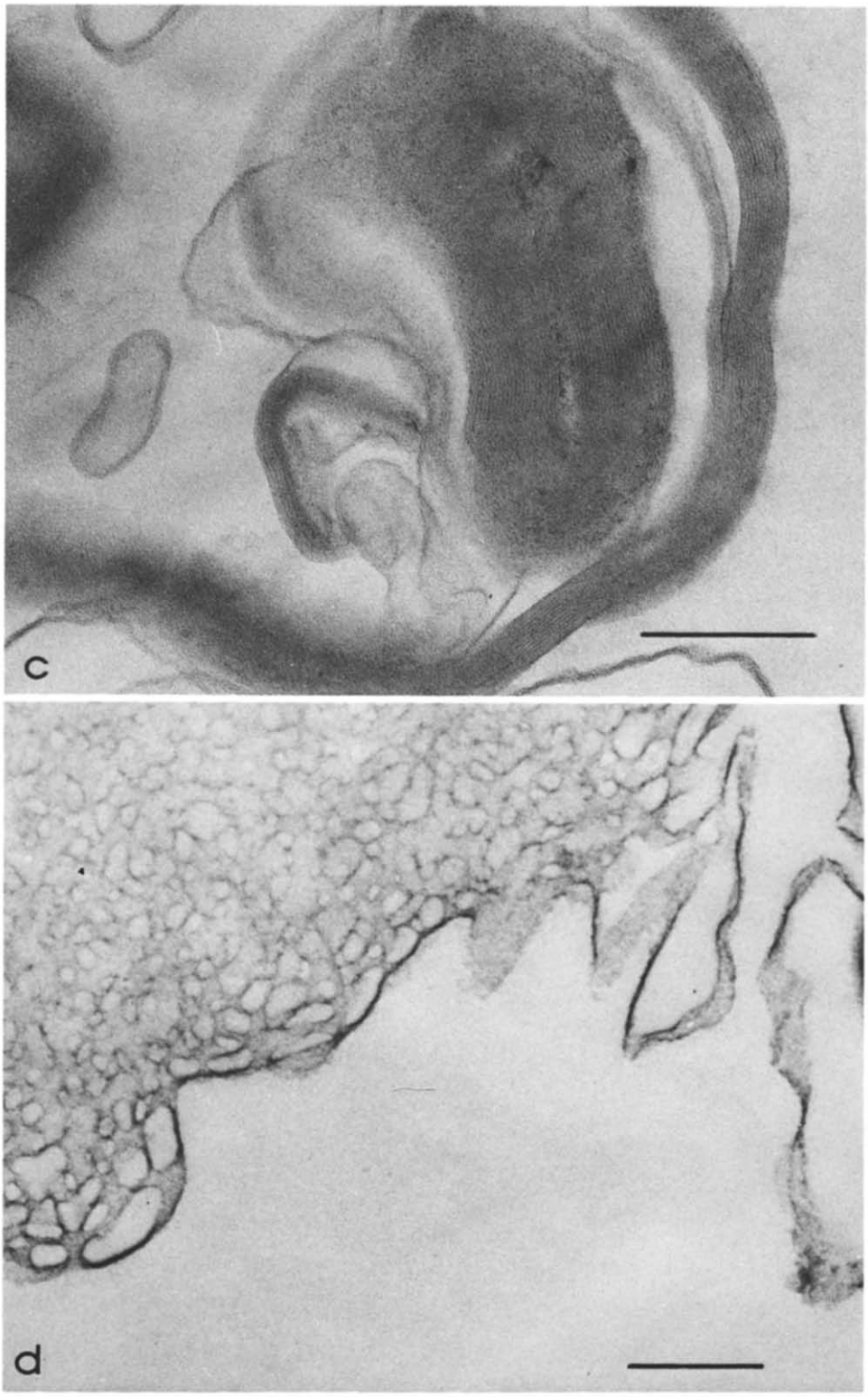


Fig. 8. Thin sections of polylysine-treated vesicles. Vesicles containing 30 mol% of acidic phospholipids were prepared, treated with polylysine and processed for electron microscopy as described under Methods. (a) PA-containing vesicles were treated with polylysine (10 μg/mol phospholipid). (b) PS-containing vesicles treated with polylysine (10 μg/μmol phospholipid). (c) PS-containing vesicles



treated with polylysine (40 μg/μmol phospholipid). (d) Decetyl phosphate-containing vesicles treated with polylysine (40 μg/μmol phosphate). The bars represent 0.2 μm.

pH we may safely assume that at pH 4.2 it carries about one charge per 1 kDa. This would imply that cytochrome *c* induced an effect similar to polylysine at charge ratios of 2–4, slightly higher than polylysine, but still much lower than divalent cations. Our results are in some conflict with an earlier report describing cytochrome *c*-induced fusion of PE vesicles in slightly alkaline pH [18].

In summary, polylysine and cytochrome *c* induce vesicle aggregation following by fusion into structures of more 1 μm in diameter. Using a technique that will encapsulate materials at high yield, e.g., reverse phase evaporation [25] and using larger amounts of long polylysine, there is a good chance that polylysine will turn out advantageous over divalent cations in introducing solutes into cells, because of its capacity to induce fusion at lower concentrations. Polylysine may prove superior in introducing membrane components to cells, used instead of divalent cations as already described [26,27]. In this case, the change in the permeability of the vesicles does not interfere with the process of incorporating a new component into the target membrane.

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References

1 Galla, H.-J. and Sackmann, E. (1975) Biochim. Biophys. Acta 401, 509–529
2 McDonald, R.C. Simon, S.A. and Baer, E. (1976) Biochemistry 15, 885–891
3 Van Dijck, P.W.M., De Kruijff, B., Verkleij, A.J., Van Deenen, L.L.M. and De Gier, J. (1978) Biochim. Biophys. Acta. 512, 84–96
4 Vail, W.J. and Stollery, J.G. (1979) Biochim. Biophys. Acta 551, 74–84
5 Papahadjopoulos, D., Vail, W.J. Jacobson, K. and Poste, G. (1975) Biochim. Biophys. Acta 394, 483–491
6 Miller, C. and Racker, E. (1976) J. Membrane Biol. 26, 319–333
7 Kantor, H.L., Mabrey, S., Prestegard, J.H. and Sturtevant, J.M. (1977) Biochim. Biophys. Acta 466, 402–410
8 Douglas, W.W. and Rubin, R.P. (1961) J. Physiol. (London) 159, 40–53
9 Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858–2866
10 Palmer, F.B. and Dawson, R.M.C. (1969) Biochem. J. 111, 629–636
11 Steck, A.J., Siegrist, P., Zahler, P., Herschkowitz, N.N. and Schaefer, R. (1978) Biochim. Biophys. Acta 509, 397–409
12 Brady, G.W., Fein, D.B., Wood, D.D. and Moscarello, M.A. (1981) FEBS Lett. 125, 159–160
13 Hartmann, W., Galla, H.-J. and Sackmann, E. (1977) FEBS Lett. 78, 169–172
14 Hartmann, W. and Galla, H.-J. (1978) Biochim. Biophys. Acta 509, 474–490
15 Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579–598
16 Lancaster, J.R. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7657–7661
17 Rosier, R.N., Gunter, T.E., Tucker, D.A. and Gunter, K.K. (1979) Anal. Biochem. 96, 384–390
18 Stollery, J.G. and Vail, W.J. (1977) Biochim. Biophys. Acta 471, 372–390
19 Kagawa, Y., Kandrach, A. and Racker, E. (1973) J. Biol. Chem. 248, 676–684
20 Papahadjopoulos, D. and Miller, N. (1967) Biochim. Biophys. Acta 135, 624–638
21 Gad, A.E., Broza, R. and Eytan, G.D. (1979) Biochim. Biophys. Acta 556, 181–195
22 Papahadjopoulos, D., Moscarello, M.A., Eylar, E.H. and Isac, T. (1975) Biochim. Biophys. Acta 401, 317–335
23 Litman, B.J. (1972) Biochemistry 13, 2545–2554
24 De Kruijff, B., Van den Besselaar, A.M.H.P. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 443–453
25 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198
26 Gad, A.E., Broza, R. and Eytan, G.D. (1979) FEBS Lett. 102, 230–234
27 Eytan, G.D. and Eytan, E. (1980) J. Biol. Chem. 255, 4992–4995