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CATIONIC AMPHIPHILES INDUCE FUSION OF ACIDIC LIPOSOMES

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Decylamine, dodecylamine and tetradecylamine induced aggregation and fusion of acidic liposomes at concentrations of about 1 mM, 75 μM and 75 μM, respectively. Aggregation was assayed as increase in turbidity. Fusion was assayed as intermixing of membranes and contents, and was observed in the electron-microscope to form large liposomes. Only at higher concentrations did these amphiphiles induce massive leakage of the liposomes' contents. Similar effects were caused by hexadecylpyridinium bromide (CP) and hexadecyltrimethylammonium bromide (CTAB). The trivalent cation 4-dodecyldiethylenetriamine and the more hydrophobic amphiphile, trioctylmethylammonium chloride, induced fusion at concentrations of about 10–20 μM. Octylamine and heptylamine induced size increase at mM concentrations. They induced membrane intermixing but little or no content intermixing. Thus, these amphiphiles seem to promote size increase either by transfer of lipid or mainly by 'cracking and annealing'.

Introduction

Liposome-liposome and liposome-cell fusion have been subjects of intensive research for the past decade (for reviews, see Ref. 1–3). Membrane fusion is an ubiquitous and essential mechanism in cell biology. Valuable information leading to better understanding of fusion phenomena occurring in cells has been obtained from studies of artificial lipid model systems; e.g. liposomes. Liposomes have been envisaged as delivery vehicles for drugs and macromolecules into cells both in vivo and in vitro (for reviews, see Refs. 4 and 5). Fusion of liposomes and reconstituted proteoliposomes with planar lipid membranes is a procedure gaining wider popularity for insertion of proteins and antigens into planar membranes [6,7].

A widely used fusion system is the cation-induced fusion of acidic liposomes [1–3]. Calcium ions in the mM range induce fusion of acidic liposomes containing a variety of acidic lipids. La^{3+} and polycations (e.g. polylysine) induce fusion at lower concentrations [3,8]. A variety of proteins including synexin [9], clathrin [10], a viral protein [11] and albumin [12] have been shown to participate in fusion processes. It has been suggested that combination of exposed hydrophobic surfaces and electrostatic charges are general features of proteins capable of inducing membrane fusion [13]. The polypeptides, polymyxin B [14,15] and melittin [16], are extremely efficient fusogens. They induce fusion of liposomes at concentrations two to three orders of magnitude lower than those needed when calcium or magnesium was used. Both polymyxin B and melittin are amphiphiles composed of a hydrophobic moiety and a hydrophilic sequence of amino acids bearing a few positive charges. In the present paper, we attempted to define the molecular characteristics allowing amphiphiles to induce fusion of membranes. For this purpose the fosogenic capacity of various multi- and univalent cationic amphiphiles has been tested. It turned out that a combination of one cationic charge and a hydrophobic moiety seems to satisfy the requirements for fusogenic capacity.

Materials and Methods

Cardiolipin and egg yolk phosphatidylcholine (type VII) were purchased from Sigma. The cationic amphiphiles were purchased from Fluka AG. except for 4-dodecyldiethylenetriamine which was purchased from Eastman.

Small unilamellar liposomes were prepared by sonication [13]. The buffer used throughout the work consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.6) and 150 mM NaCl. All assays and incubations were performed at 37 °C. The assay of membrane intermixing was based on dilution of chlorophylls and was performed as described [14]. Local concentrations of chlorophylls in the liposome membranes were determined as described [14]. Intermixing of vesicle contents was determined by a modification of the procedure of Wilschut et al. [18] as modified in Ref. 19. Leakage of vesicle contents was determined with the fluorescent dye carboxyfluorescein as in Ref. 20. Samples were prepared for electron-microscopy as described [8]. Turbidometric assays were performed with a Beckman DU-8 spectrophotometer.

In some experiments, the cationic amphiphiles were removed by adsorption to Bio-Beads SM 2. For this purpose the Bio-Beads SM 2 were pretreated as described [21] and aliquots containing 5 ml drained beads were resuspended in conical glass centrifuge tubes. The beads were washed three times by resuspensions in 8 ml buffer, centrifugations and removal of the supernatant. A sample of liposome suspension (2 ml) containing a cationic amphiphile was added to the drained beads and mixed thoroughly for 1 min. After centrifugation for 1 min at $1000 \times g$, the supernatant was removed. The concentration of amphiphiles remaining in the sample was determined with fluorescamine as described [22].

Results

Upon addition of decylamine to acidic liposomes, their suspension became turbid. The turbidity increased with decylamine concentrations up to 0.5–1 mM. At higher concentrations, the turbidity of the suspension was less marked (Fig. 1a). In order to assay membrane intermixing, two liposome populations were incubated together, one contained chlorophylls and the other was non-pigmented. Upon addition of decylamine, intermixing of liposome membranes occurred with dilution of chlorophylls in the membranes. The dilution was monitored as reduction in energy transfer from chlorophyll *b* to chlorophyll *a* [14]. As shown in Fig. 1b, maximal membrane intermixing occurred at concentrations exceeding 1.0 mM.

Liposome fusion resulting in intermixing of vesicle contents was monitored using the assay developed by Wilschut et al. [18,19]. Two liposome populations were mixed, one containing terbium ions and the other dipicolinic acid. Upon addition of decylamine, intermixing of liposome contents occurred, with formation of the fluorescent complex terbium-dipicolinic acid. As shown in Fig. 1c, addition of decylamine concentrations of 0.1–0.5 mM caused intermixing of the liposome contents. At higher concentrations, decylamine induced rapid intermixing of the liposome contents, but the resulting fluorescence was quenched, presumably as a result of contents leakage.

The fluorescence of the terbium-dipicolinic complex could have reflected leakage of the contents from aggregated liposomes and intermixing in the volume trapped within the aggregate. In order to exclude this possibility, the decylamine was removed at various times by adsorption to Bio-Beads SM2. This treatment effectively removed the decylamine. The remaining concentration in the liposome suspension was below the detection limit of the amine assay which was 1 μM. Moreover, preincubation of decylamine with the Bio-Beads SM2 completely prevented subsequent fusion of liposomes (Fig. 2). Removal of decylamine from a suspension of fusing liposomes caused disaggregation evident as a partial reversal of the turbidity. The removal of the amphiphile did not quench the fluorescence of terbium dipicolinate. This indicates that the fluorescent complex was sequestered within the fused vesicles and not trapped among them. The removal of decylamine prevented the decay of the fluorescence due to leakage of vesicles' contents.

Leakage of vesicle contents was monitored as dequenching of trapped carboxyfluorescein [20]. High concentrations of the dye were trapped within the liposomes. Under these conditions, the fluores-

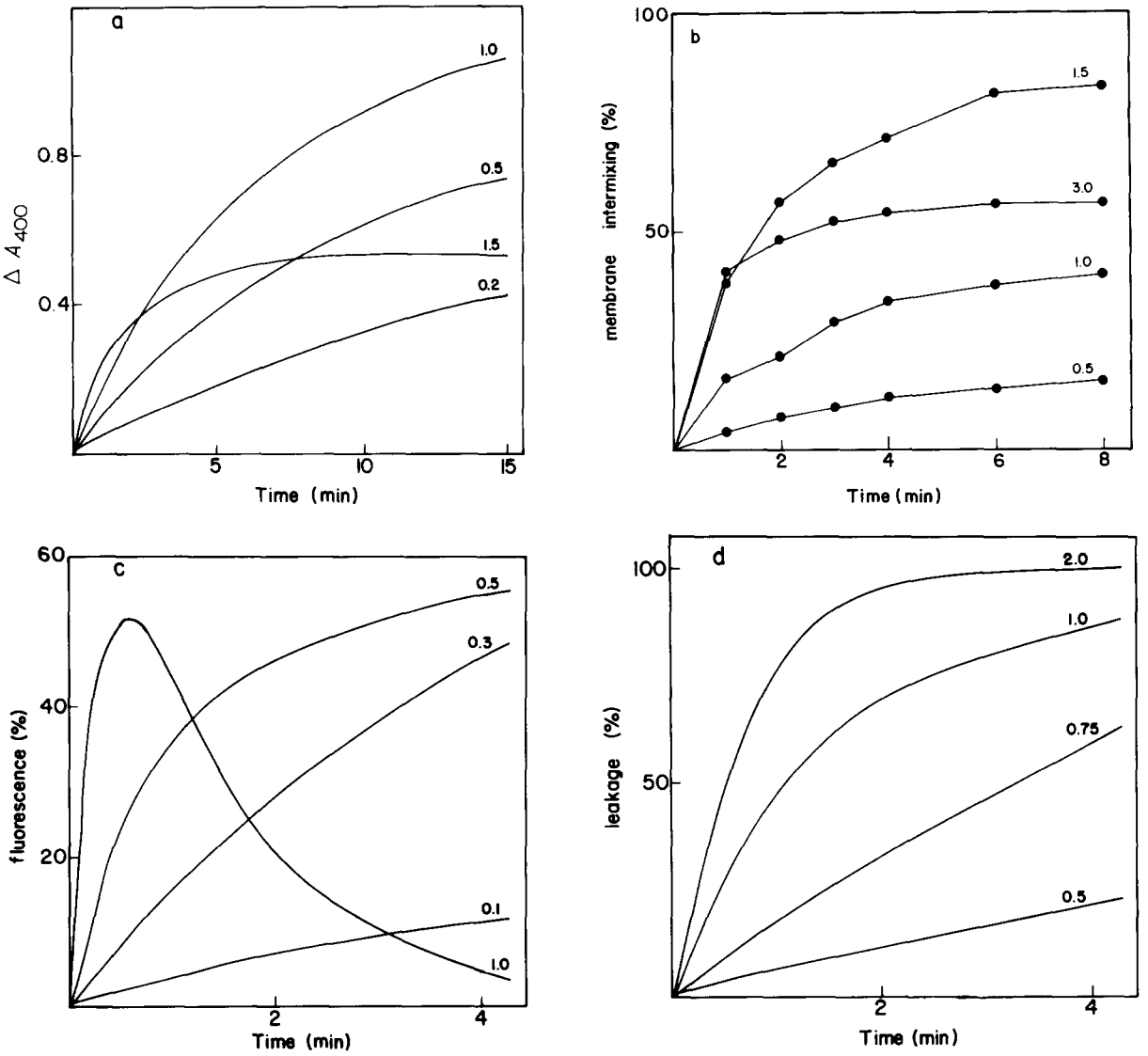

Fig. 1. Effects of decylamine on acidic liposomes. Small unilamellar liposomes containing phosphatidylcholine and cardiolipin (50 and 50% (w/w), respectively) were prepared and incubated at 37 °C at a final concentration of 50 μg/ml. The mM concentrations of decylamine indicated in the figures were added and their effects were monitored as described under Materials and Methods. Part a: aggregation of membranes was monitored as increase in absorbance at 400 nm. Part b: intermixing of liposome membranes. Part c: intermixing of liposome contents was assayed as fluorescence of Tb-dipicolinic acid complex. Part d: leakage of liposome contents was assayed as dilution of trapped carboxyfluorescein. The intermixing and leakage results are expressed as % of maximal amount.

cence of the dye was almost completely quenched. Upon leakage, the dye was diluted and became highly fluorescent. As shown in Fig. 1d, at concentrations of up to 0.5 mM, decylamine-induced leakage of contents was limited. At higher concentrations, decylamine caused rapid leakage of the liposome contents.

Fluorescence energy transfer between chlorophylls can also be used to monitor phase separation phenomena in the liposomes' membranes. It has been shown with other fluorescent compounds that Ca^{2+} induces phase separation of acidic from neutral lipids [23]. As shown in Fig. 3, addition of calcium ions to acidic liposomes containing chlo-

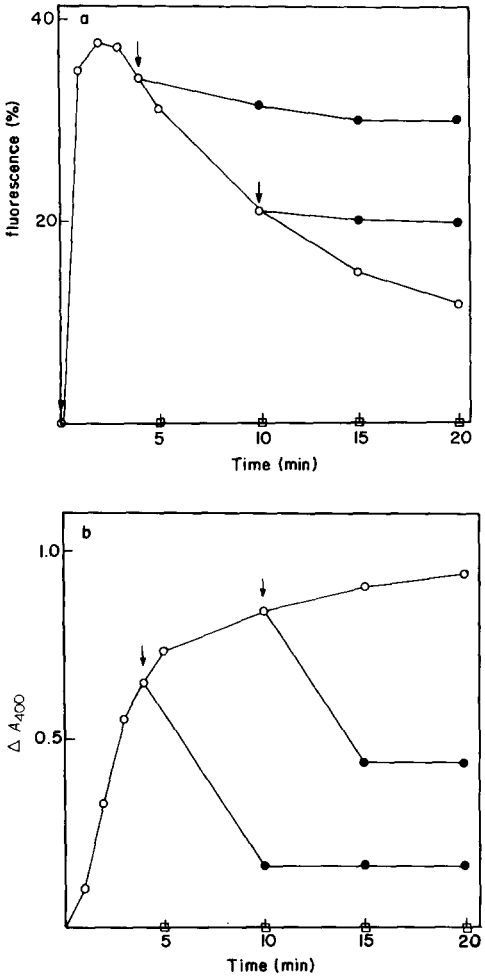

Fig. 2. Removal of decylamine after incubation with acidic liposomes. Small unilamellar liposomes containing either terbium or dipicolinate were prepared as described in the legend to Fig. 1. Equal concentrations of the liposomes were incubated at a final concentration of 50 μg/ml. Decylamine (0.75 mM) was added and, after various intervals, samples were withdrawn and treated with Bio-Beads SM2 in order to remove the decylamine as described under Materials and Methods. The liposome suspension was separated from the beads, further incubated and assayed after various intervals for contents intermixing and turbidity (●———●). In addition, a decylamine solution in buffer (0.75 mM) was mixed thoroughly with Bio-Beads SM2 for 1 min. Liposomes containing either terbium or dipicolinate were added to a final concentration of 50 μg/ml and further incubated. After various time intervals samples were separated from the beads and assayed for contents intermixing and turbidity (□———□).

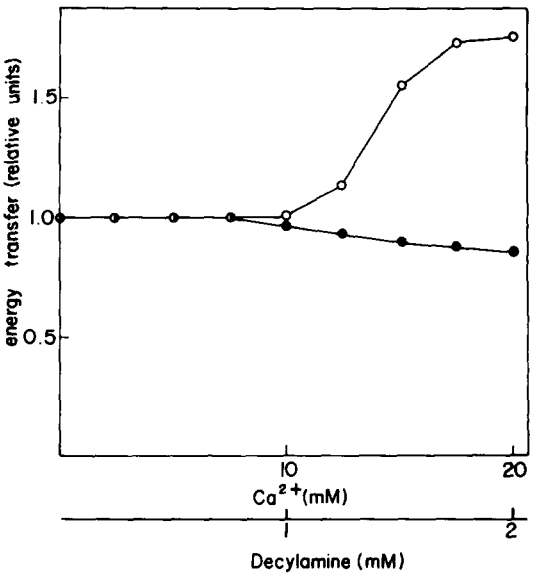

Fig. 3. Effect of Ca^{2+} and decylamine on phase separation. Small unilamellar liposomes containing phosphatidylcholine, cardiolipin and chlorophylls (49:49:2 w/w/w, respectively) were prepared and incubated at a final concentration of 50 μg/ml. Various Ca^{2+} (○———○) and decylamine (●———●) concentrations were added and after 15 min incubation the efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* was estimated. The results were expressed as ratio of transfer efficiency in the sample to that measured in the original liposomes.

rophylls led to increased efficiency of energy transfer from chlorophyll *b* to chlorophyll *a*. This increase reflected, presumably, the higher local concentrations of chlorophylls in the fluid phase after separation-out of the acidic phospholipids. In contradistinction, dodecylamine did not increase the transfer efficiency and at higher concentrations even reduced it. It seemed that no phase separation occurred. At higher concentrations, decylamine molecules, inserted into the membrane, interfered with energy transfer.

Electron-microscopy confirmed that decylamine induced massive size-increase of acidic liposomes. The liposomes were prepared by sonication. Their diameter was in the range of 20–40 nm (Fig. 4a). After incubation, in the presence of decylamine, aggregates of large liposomes with diameters of 0.2–1.0 μm were observed (Fig 4d).

Primary amines with longer aliphatic chains such as dodecylamine and tetradecylamine had similar effects on acidic liposomes. However, their effective concentration was much lower and the liposomes formed by fusion were not as large.

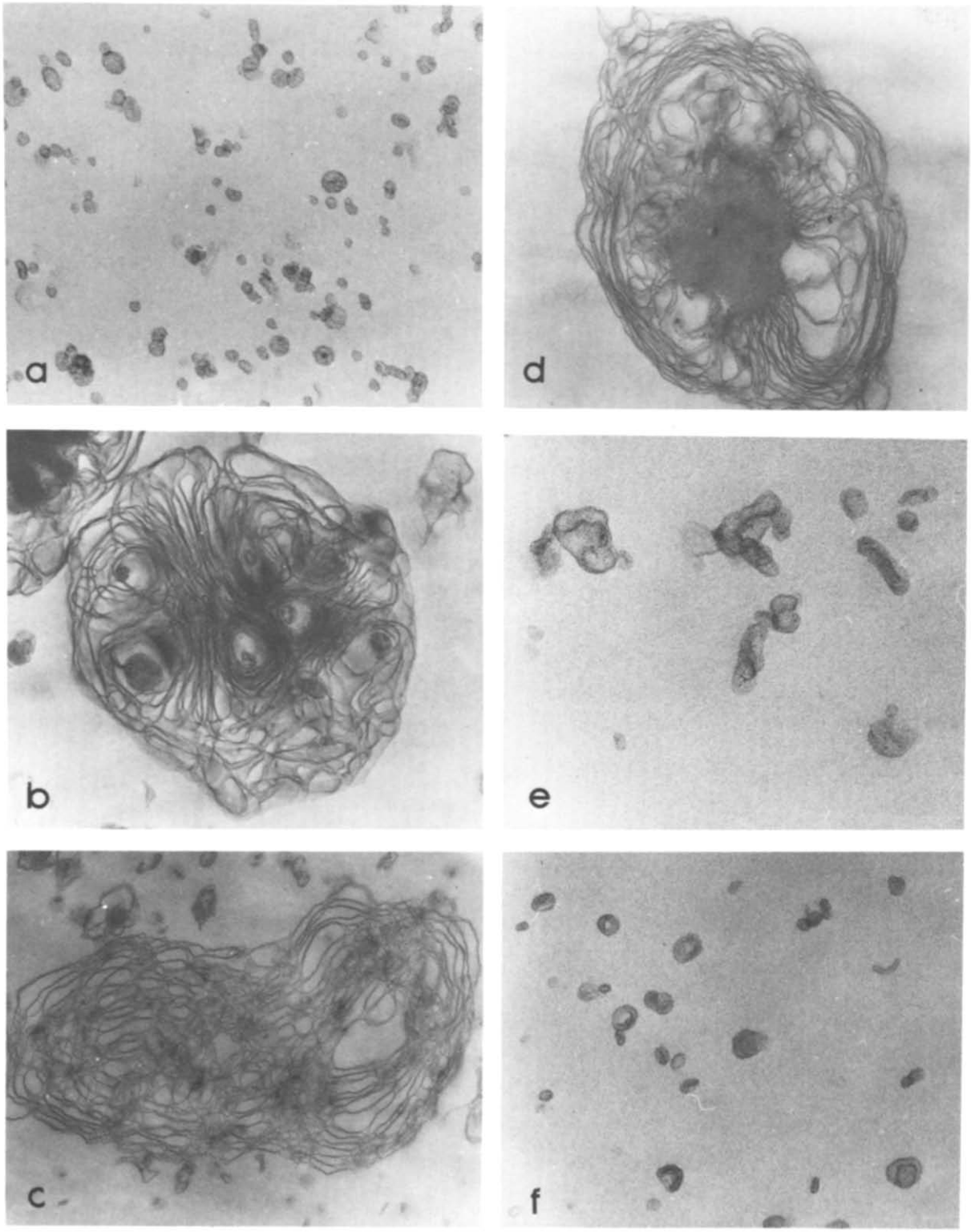

Fig. 4. Electron microscopy of liposomes incubated in presence of various amphiphiles. Small unilamellar liposomes were prepared as described in the legend to Fig. 1 and incubated for 15 min at a concentration of 100 μg/ml either in absence (part a) or presence of heptylamine (5 mM, part b), octylamine (5 mM, part c), decylamine (1 mM, part d), dodecylamine (100 μM, part e) or tetradecylamine (100 μM, part f). Samples were processed for electron microscopy and thin-sections were examined and photographed at final magnifications of a, e, f: $\times$100000; b: $\times$30000; c,d,: $\times$15000.

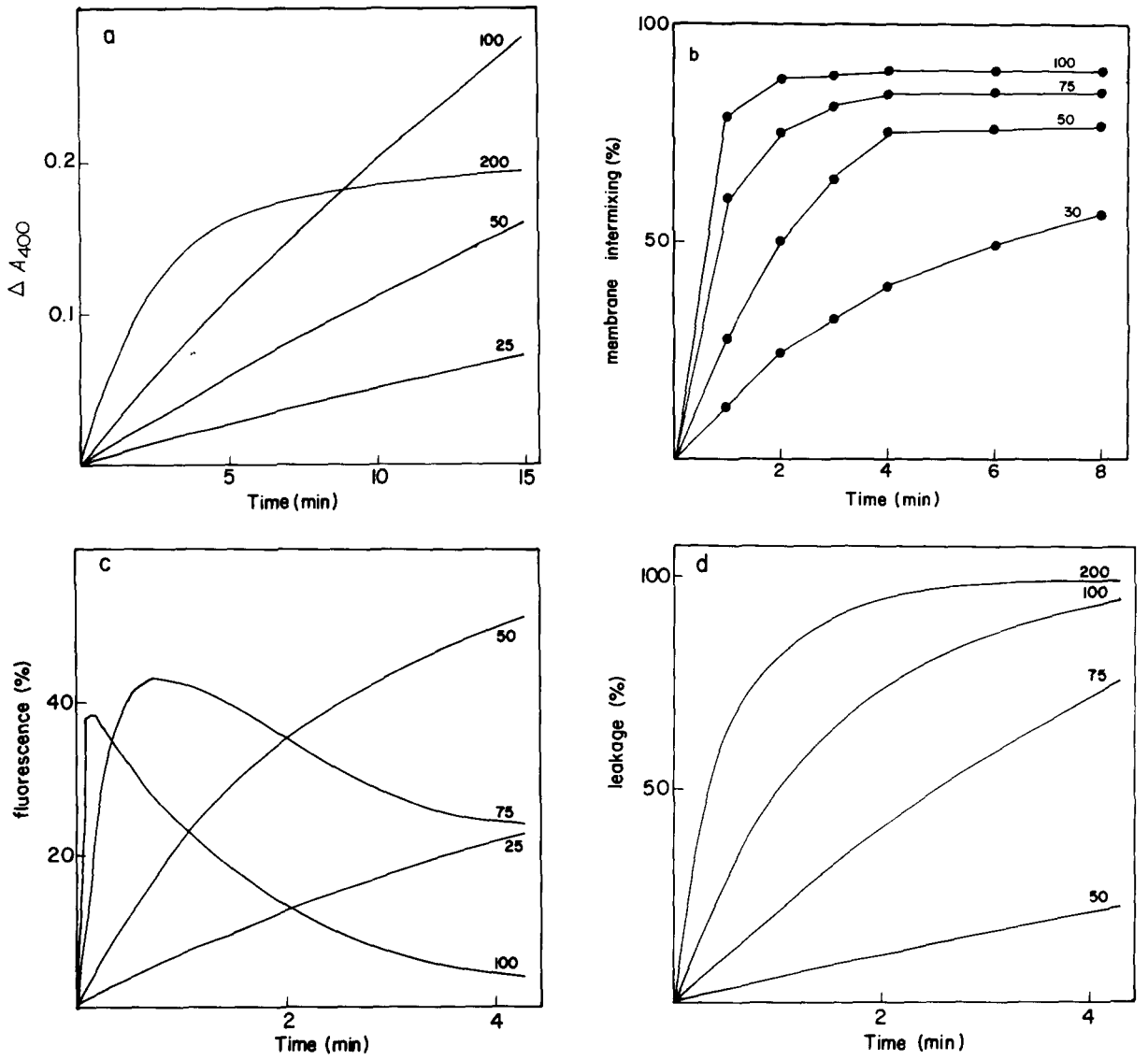

Fig. 5. Effects of tetradecylamine on acidic liposomes. Samll unilamellar liposomes were prepared and incubated as described in the legend to Fig. 1. The μM concentrations of tetradecylamine indicated in the figures were added and their effects were monitored as described under Materials and Methods. Part a: aggregation of membranes was monitored as increase in absorbance at 400 nm. Part b: intermixing of liposome membranes. Part c: intermixing of liposome contents was assayed as fluorescence of Tb-dipicolinic acid complex. Part d: leakage of liposome contents was assayed as dilution of trapped carboxyfluorescein. The intermixing and leakage results are expressed as % of maximal amounts.

Maximal membrane intermixing was already observed at 75–100 μM tetradecylamine (Fig. 5b). Maximal intermixing of contents with little leakage occurred at 50–75 μM (Fig 5c). Leakage of liposome contents was limited at concentrations of up to 50 μM. Maximal turbidity was observed in the presence of 100 μM tetradecylamide (Fig. 5a). However, this turbidity was low compared with the maximal turbidity obtained with decylamine. Electron-microscopy of liposomes incubated in the presence of tetradecylamine and dodecylamine revealed vesicles with average diameters of 45 and 80 nm, respectively, compared with 25 nm diameter of the original liposomes (Fig. 4). The

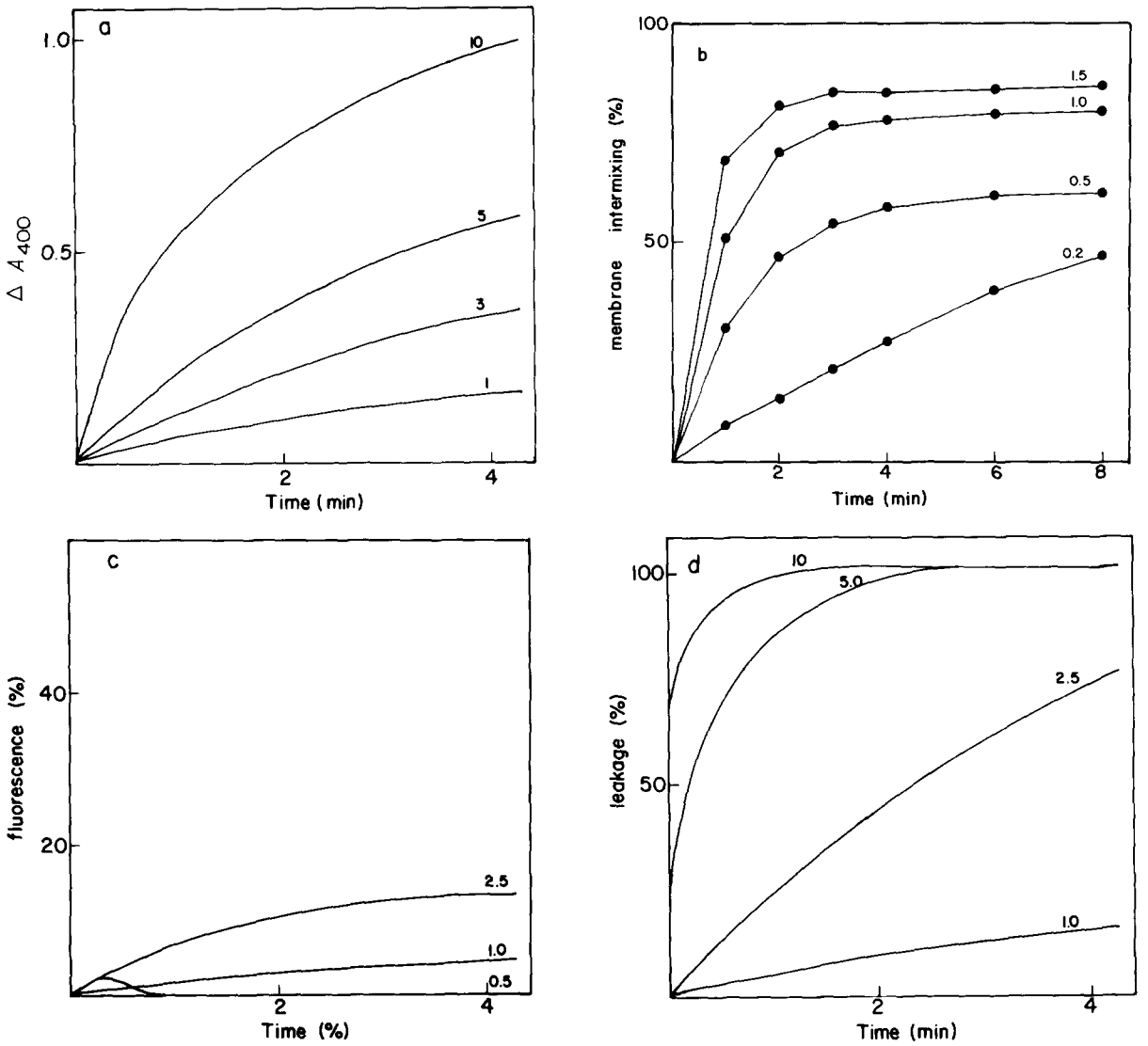

Fig. 6. Effects of octylamine on acidic liposomes. Small unilamellar liposomes were prepared and incubated as described in the legend to Fig. 1. The mM concentrations of octylamine mentioned in the figures were added and their effects were monitored as described under Materials and Methods. Part a: aggregation of membranes was monitored as increase in absorbance at 400 nm. Part b: mixing of liposome membranes was assayed as dilution of chlorophylls included in part of the liposome population. Part c: intermixing of liposome contents was assayed as fluorescence of the Tb-dipicolinic acid complex. Part d: leakage of liposome contents was assayed as dilution of trapped carboxyfluorescein. The intermixing and leakage results are expressed as % of the maximal amounts.

electron-micrographs indicate that dodecylamine and tetradecylamine induced a few rounds of fusion events leading to extensive intermixing of liposome membranes and contents, but only limited size increase. Removal of the decylamine or tetradecylamine almost completely reversed the turbidity increase but had little effect on the fluorescence of terbium dipicolinate formed upon fusion of suitable liposomes (results not shown).

Octylamine induced maximal aggregation and membrane intermixing at concentrations of 10 and 1.5 mM, respectively. While extensive membrane intermixing occurred with octylamine (Fig. 6b), the extent of contents intermixing was low (Fig.

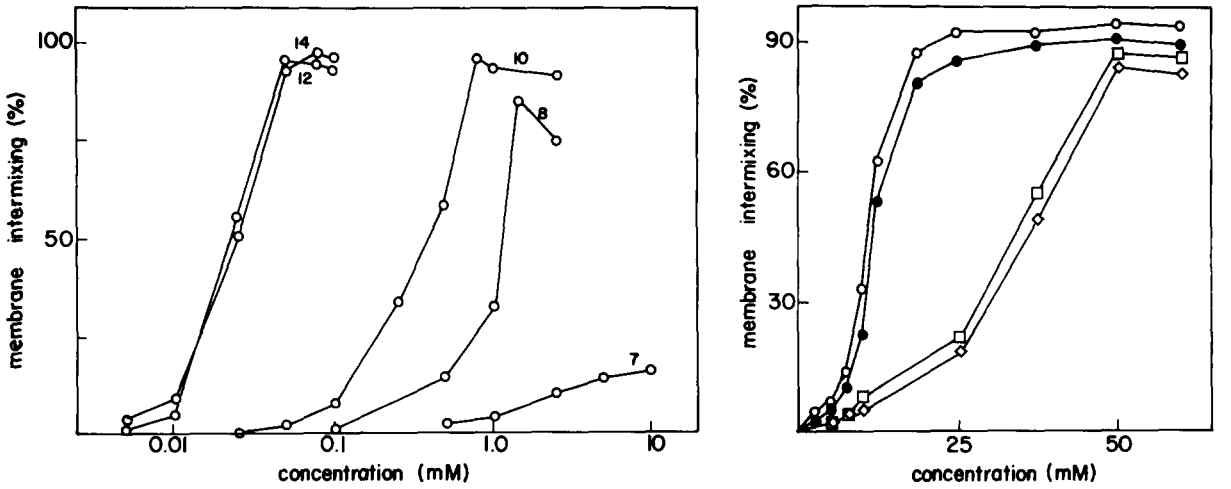

Fig. 7. Effect of amphiphilic amines on membrane intermixing. Small unilamellar liposomes were prepared and incubated as described in the legend to Fig. 1. Part of the liposome population also contained chlorophylls (1% w/w). Various concentrations of heptyl-, octyl-, dodecyl- and tetradecylamines were added. the liposomes were incubated and membrane intermixing was assayed after 15 min incubation as described under Materials and Methods. The numbers in the figure express the number of carbon atoms in the aliphatic chains of the amines.

Fig. 8. Effect of various amphiphiles on membrane intermixing. Small unilamellar liposomes were prepared and incubated as described in the legend to Fig. 6. Various concentrations of hexadecylpyridinium bromide (□——□), hexadecyltrimethylammonium bromide (◇——◇) trioctylmethylammonium chloride (○——○) and 4-dodecyldiethylenetriamine (●——●) were added. The samples were incubated for 15 min and membrane intermixing was assayed as described under Materials and Methods.

6c). Leakage of the contents at concentrations of over 5 mM was rapid and complete. Addition of high concentrations of octylamine to chlorophyll-containing liposomes resulted in reduction in the efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* (results not shown). No phase separation was observed at any concentration tested. Electron microscopy of liposomes incubated in the presence of octylamine revealed large structures composed of membranes. Heptylamine induced limited membrane intermixing only at high concentrations (Fig. 7). No intermixing of contents was observed, but it did cause leakage. Electron microscopy of heptylamine-treated liposomes revealed large membranous structures (Fig. 4).

The apparent contradiction between results obtained with the membrane intermixing assay and the electron-microscopy data could have risen from a large increase in size of a part of the liposome population. The heptylamine concentration used in the electron microscopy experiment caused only partial leakage of liposome contents. A similar concentration, added to chlorophyll-containing

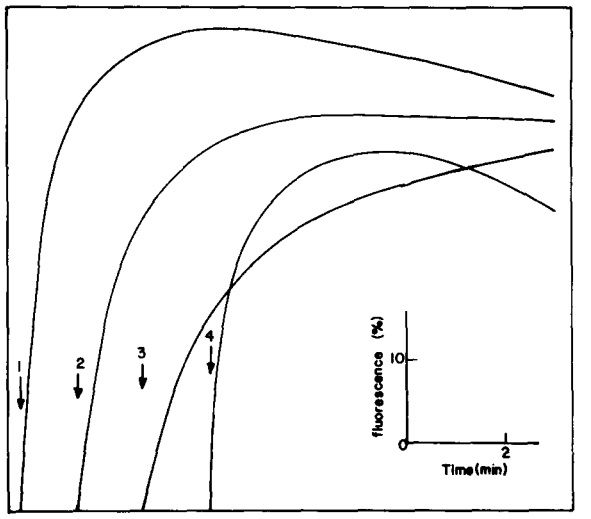

Fig. 9. Effect of various amphiphiles on contents intermixing. Small unilamellar liposomes were prepared for assay of contents intermixing as described in the legend to Fig. 1. At the time points indicated by the appropriate arrows the following amphiphiles were added: 1, hexadecyltrimethyl ammonium bromide (30 μM); 2, hexadecylpyridinium bromide (30 μM); 3, trioctylmethylammonium chloride (10 μM) and 4, dodecyldiethylenetriamine (10 μM). Fluorescence of the Tb-dipicolinic acid complex was monitored.

liposomes, caused partial reduction in efficiency of energy transfer between the chlorophylls. The reduction could be due to insertion of amphiphiles into a part of the liposomes population. Amines with shorter aliphatic residues had no effect on the liposomes. No change in size, membrane or contents intermixing was observed.

The effects described above were not limited to amphiphilic primary amines. Cationic amphiphiles such as hexadecylpyridinium bromide and hexadecyltrimethylammonium bromide (CTAB) induced membrane and content intermixing at concentrations in the range of 25–50 μM (Figs. 8,9). The trivalent cationic amphiphile 4-dodecyldiethylenetriamine was effective at lower concentrations. Maximal membrane intermixing occurred at around 20 μM and optimal content intermixing was observed at around 10 μM. The amphiphile with three aliphatic chains trioctylmethylamine induced membrane and contents intermixing at similar concentrations.

At concentrations of up to 10 mM, the amphiphilic cations did not induce intermixing of either membrane or contents of neutral liposomes composed of phosphatidylcholine.

Discussion

Size-increase of lipid vesicles can occur by one of the following mechanisms: (a) fusion of vesicles (resulting in intermixing of both membrane and contents); (b) 'cracking and reannealing' in which case the contents are spilled; (c) transfer of lipid molecules. It has been claimed that all these mechanisms are fusion events [24]. However, in analogy to fusion events in cells, the term fusion should be restricted to cases where intermixing of both membrane and contents occurs [18,25]. The size-increase induced by amphiphilic amines with aliphatic chains longer than eight carbon atoms was characterized by membrane and contents intermixing with limited leakage of contents. Massive leakage was only induced at higher concentrations. Thus, the major operative mechanism seems to be fusion of the liposomes. On the other hand, octylamine and hexylamine induced membrane intermixing leading to massive size increase with little or no contents intermixing. thus, these compounds seem to induce size increase by 'cracking or annealing' or by transfer of individual molecules.

Cationic amphiphiles such as decylamine induced liposome fusion in the absence of added divalent or trivalent ions. Fusion was not inhibited by the presence of chelators. This is in contrast to the effect exerted by synexin [9] and polyamines, such a spermine [26], that induced aggregation of acidic liposomes and lowered the threshold concentrations of calcium ions needed to induce fusion.

Two features seem to render the amphiphiles effective in inducing fusion: a cationic hydrophilic group and a hydrophobic moiety. As opposed to hydrophilic cations, where only multivalent cations are effective, univalent cationic amphiphiles are as effective as trivalent ones (on a charge basis). For example, the concentrations of the trivalent amphiphile, 5-dodecyldiethylenetriamine, needed to induce fusion are about three times lower than those of dodecylamine. However, on a charge basis, the trivalent amphiphile is as effective as the univalent one. The determining factor characterizing the apolar moiety seems to be the degree of the hydrophobicity and not the chain-length of the aliphatic chain. Thus the most effective amphiphile in terms of concentrations needed to induce liposome fusion was trioctylmethylammonium chloride despite the fact that its chains were composed solely of 7 carbons. Concentrations of trioctylmethylammonium chloride of less than 10 μM induced fusion of acidic liposomes. These low concentrations were comparable to the concentrations of melittin [16] and polymyxin B [14] required to induce fusion. They were much lower than the concentrations of hydrophilic cations such as Mg^{2+} and Ca^{2+} needed to induce fusion [1–3].

Lucy's group [27–29] has studied the effect of amphiphiles on cell-cell-fusion. They have shown that a variety of amphiphiles such as glycerolmonooleate, lysophosphatidylcholine, some fatty acids and oleylamine induce extensive fusion of cells. Some of these amphiphiles are detergents capable of lysing cells and dissolving membranes at high concentrations. It has been shown that at least some of these amphiphiles do not have an effect on liposome-liposome fusion. Not only does lysophosphatidylcholine not promote liposome fu-

sion, it actually inhibits Ca^{2+}-induced fusion. At least, some of these detergents such as Triton X-100, octylglycoside, lysophosphatidylcholine and myristic acid cause a size increase of liposomes either by 'cracking and annealing' or by transfer of individual molecules [30–33]. The contents of the liposomes were spilled at much lower Triton X-100 concentrations that those required for promotion of size increase [31]. The effect of hexylamine seems to be similar to that of other detergents.

Amphiphiles such as decylamine promote fusion of acidic liposomes probably by formation of intermembrane complexes leading to close apposition of membranes. The hydrophobic tail of the amphiphile is buried in the lipid matrix of one liposome while the cationic hydrophilic moiety interacts electrostatically with an acidic phospholipid present in the membrane of another liposome. For fusion of liposomes to occur, the fusogen has to induce not only close apposition but also destabilization of their membrane. There is still controversy as to the nature of the factors leding to this destabilization. Among the factors suggested are phase separation of lipids in the membranes [34], dehydration of the bilayers [35] and transition from bilayer to inverted hexagonal structures [36,37]. It has been suggested that under certain conditions Ca^{2+} induces phase separation of lipids [34]. Recently, doubt has been cast whether phase separation is indeed a prerequisite for fusion [23]. Since monovalent amphiphiles do not form '*cis*' complexes with the phospholipids, they are not expected to, and indeed do not induce extensive phase separation (Fig. 3). However, it is possible that at the sites of membrane apposition a high concentration of '*trans*' complexes is formed leading to a localized phase separation event. At this focal point, membrane destabilization might occur by one of the mechanisms suggested earlier.

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