

# Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids

Emmanuel Farge and Philippe F. Devaux

Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

**ABSTRACT** The influence of a phospholipid transmembrane redistribution on the shape of nonspherical flaccid vesicles was investigated at a fixed temperature by optical microscopy. In a first series of experiments, a transmembrane pH gradient was imposed on egg phosphatidylcholine (EPC)-egg phosphatidylglycerol (EPG) (100:1) giant vesicles. The  $\Delta$ pH induced an asymmetric distribution of EPG. Simultaneously, discoid vesicles were transformed into tubular or a series of connected small vesicles. The fraction of phospholipid transfer necessary for a shape change from discoid to two connected vesicles was of the order of 0.1% of the total phospholipids. Additional lipid redistribution was accompanied by a sequence of shape changes. In a second series of experiments, lyso phosphatidylcholine (L-PC) was added to, or subtracted from, the external leaflet of giant EPC vesicles. The addition of L-PC induced a change from discoid to a two-vesicle state without further evolution, suggesting that lipid transfer and lipid addition are not equivalent. L-PC depletion from the outer leaflet generated stomatocyte-like vesicles. Whenever possible, we have determined whether the giant vesicles undergoing shape changes were unilamellar or multilamellar by measuring the elastic area compressibility modulus,  $K$ , by the micropipette assay (Kwok and Evans, 1981). Shape transformations triggered by phospholipid modification of the most external bilayer were indeed influenced by the presence of other underlying membranes that played a role comparable to that of a passive cytoskeleton layer. It appears that in real cells, invaginations of the plasma membrane or budding of organelles could be triggered by a phospholipid transfer from one leaflet to the other caused, for instance, by the aminophospholipid translocase which is present in eukaryotic membranes.

## INTRODUCTION

Giant liposomes spontaneously exhibit different shapes that are reminiscent of biological cell morphologies. Mechanical stress, temperature or pH variation as well as osmotic shocks are capable of inducing drastic changes from nearly spherical shapes to flattened discoids or stomatocytes. Under particular conditions large vesicles can give rise to local membrane protrusions resembling the budding phenomena that takes place in natural membranes. These local deformations are triggered on apparently homogenous surfaces, in the absence of any protein or polymers forming a cytoskeleton meshwork by the intercalation of amphiphiles or by a temperature shift. Theoretical grounds for these shape changes were already proposed in 1970 by Canham who showed that the discoid shape corresponds to a minimum value of the membrane bending energy (Canham, 1970). In 1974, Evans showed that alterations in the surface tension on either face can produce curvature (Evans, 1974). The concept of spontaneous curvature was introduced by Deuling and Helfrich (1976). More recent articles by Svetina and Zeks (1989), Berndt et al. (1990), and Lipowsky (1991) generalize the bilayer couple hypothesis introduced by Sheetz and Singer to explain erythrocyte shape changes which follows the intercalation of a small percentage of amphiphiles in the outer or inner

monolayer of the cell membrane (Sheetz and Singer, 1974). The basic idea is that, because phospholipids do not diffuse transversally (i.e., no flip-flop), the liposome, or cell surface, adapts to a difference in surface area of the two monolayers by generating local invaginations. A theoretical phase diagram of cell shapes was established by Svetina and Zeks, and is characterized by a discrete number of equilibrium shapes (Svetina and Zeks, 1989). Sequential shape changes have also been obtained experimentally with giant liposomes. See for example the articles by Sackman et al. (1986) and Berndt et al. (1990). In the experiments of Sackman and collaborators with DMPC liposomes, only the temperature was varied. The authors explained the sequence of shape changes they observed by a small asymmetry in the thermal expansivities of the two monolayers. In fact they predict that a relative difference of the order of  $10^{-3}$  in the thermal expansivities due to local impurities or that a difference in composition of both monolayers of  $10^{-4}$  would be sufficient to explain the temperature induced shape change. Morphological change of tube-like liposomes were also observed by interaction with an amphiphilic polymer which selectively intercalates into the outer monolayer of the liposomes (Ringsdorf et al., 1988).

Here we have induced shape changes of giant liposomes by manipulating the fraction of phospholipids in each monolayer at a fixed temperature. For this purpose two different procedures were used: (a) a small quantity of egg phosphatidylglycerol (EPG), initially mixed with EPC, was redistributed through the bilayer by means of a pH gradient. The efficiency of this technique for phospholipid translocation was demonstrated by Cullis and co-workers in large unilamellar vesicles (Hope et al., 1989; Redelmeier et al., 1990); (b) lyso-phosphatidylcholine (L-PC) was either added to or removed from the outer monolayer of EPC liposomes. Optical observation with a phase contrast microscope allowed us to discriminate between multilayered liposomes and vesicles made with one or a few stacked membranes. However, the unilamellarity of a giant liposome is not revealed by simple optical inspection. Because unilamellar vesicles and multilamellar vesicles may respond differently when subjected to the same phospholipid manipulation, it was necessary to determine the unilamellarity of the giant liposomes from the value of the elastic area compressibility modulus by the micropipette aspiration technique developed by Kwok and Evans (1981).

One of the conclusions of this investigation is that the transfer of a very small fraction of phospholipids from one monolayer to the other is sufficient to induce important shape changes in giant vesicles and occasionally shedding. Thus, in real cells, the membrane invaginations, which take place during all the membrane traffic proceeding during vesicle formation, could be triggered by protein controlled lipid redistribution between the two monolayers (Devaux, 1991).

## MATERIALS AND METHODS

### Materials

Egg phosphatidylcholine (EPC) was purified according to Singleton et al., 1965. Egg phosphatidylglycerol (EPG) was purchased from Sigma Chemical Co. (St. Louis, MO). The purity of the phospholipids was checked by thin layer chromatography. No lyso compounds could be detected initially, i.e., before incubation. Lyso phosphatidylcholine (L-PC) was obtained by phospholipase A<sub>2</sub> treatment. Fatty acid free BSA was purchased from Sigma Chemical Co.

### Giant vesicles preparation

Two types of giant lipid vesicles were made: vesicles of pure EPC and vesicles of EPC mixed with 1% of EPG. The first type of vesicles (pure EPC) were made following the method of Needham and Evans, 1988. Briefly, 50  $\mu$ l of chloroform-methanol containing EPC at a concentration of 10 mg/ml, was deposited on a teflon disk and the solvent evaporated under argon while maintaining the disk at 45°C. The dried film was then submitted to a stream of argon saturated in water, at 45°C, during 20 min in order to prehydrate the lipid film. 8 ml of a 100 mM sucrose solution at a nonbuffered pH of 5.5 was then added to the film and the mixture incubated overnight at 45°C under argon

atmosphere. This technique led to the formation of lipid vesicles with a significant fraction of relatively spherical giant vesicles with a diameter of 20 to 30  $\mu$ m. More elongated giant vesicles, as well as discocytes and stomatocytes, were obtained by avoiding the prehydration of the lipid film. The long incubation at 45°C resulted in the hydrolysis of a small fraction of the lipids. TLC of the lipids followed by phosphorus determination of the percentage of lyso versus phospholipids at the end of the incubation period revealed that the percentage of lyso compound was of the order of 0.1–0.2%.

The second type of vesicles (EPC + 1% EPG) were prepared by a different method. While 1% of EPG considerably decreased the yield of formation of giant liposomes when using the former method, a simpler approach gave a good yield with these lipids: 50  $\mu$ l of chloroform-methanol containing a mixture of EPC and EPG (100:1 mol/mol), at a concentration of 10 mg of lipids per ml, were dried under an argon stream in a glass tube. The lipidic film was then prehydrated during 20 min by an argon stream saturated in water at 45°C. Sucrose solution (100 mM) was then added at 45°C and incubated 5 min only. During this incubation, long "lipid wires" attached to the bottom of the glass tube grew. The suspension was then vortexed for 1 min, which reproducibly transformed these long lipid wires in a few giant vesicles with the appearance of unilamellarity. Because the latter technique necessitated only a brief incubation at 45°, no lyso compounds were formed, as revealed by TLC.

### Micropipette manipulation and vesicle observation

The vesicles were examined at room temperature with an inverted E. Leitz, Inc. Labovert phase contrast microscope (Rueil-Malmaison, France). A CCTV Hitachi (Paris, France) camera allowed us to record the shape changes on a video system composed of a U-Matic SP VO 9600P (Sony, Paris, France) provided with a black and white monitor and a video graphic printer (Sony model UP 850). In order to enhance the phase contrast the vesicles were transported to a medium containing 100 mM glucose and 10 mM NaCl before observation (Needham and Evans, 1988). The difference in density between internal sucrose and external glucose allowed the vesicles to sediment on the glass plates that permitted an easier observation. Micropipettes with an internal diameter of 40–20  $\mu$ m were used to carry selected vesicles from one medium to another and also to inject L-PC or BSA in the medium surrounding the vesicles. Injection of L-PC was achieved by imposing a local flux of 5 mM L-PC in glucose (100 mM) in front of the vesicle under observation. Similarly, depletion of L-PC and free fatty acids from the external leaflet of vesicles was achieved with a local flux of fatty acid free BSA (1 g/100 ml) in a glucose solution.

The transmembrane redistribution of EPG was obtained by transferring a selected vesicle initially in an environment at pH 5.5, containing 100 mM glucose, into a medium of same osmolarity but at pH 9.5 (Tris 10 mM). The transfer was achieved with the help of a large micropipette (30 to 50  $\mu$ m diameter). Control experiments showed that such micromanipulation did not provoke shape change if the external pH was unchanged. The kinetics of vesicle shape change which accompanied EPG transmembrane redistribution (Redelmeier et al., 1990) was followed with the video recorder immediately after transfer to the high pH medium. Attempts to transfer the vesicles into a medium of low pH were unsuccessful because the liposomes stuck to the glass micropipette at low pH. Adjusting the pH directly in the medium already containing the liposomes was also unsuccessful because the liposomes disappeared from the field of observation during such manipulation.

The unilamellarity of the giant vesicles was determined after a cycle of shape changes by measuring the elastic area compressibility modulus,  $K$ , by the micropipette suction technique of Kwok and Evans, 1981. For these experiments, a rather spherical or an inflated discoid

vesicle is required. In practice the length,  $L$ , of the vesicle fraction aspirated into the micropipette must be of the order of magnitude of the diameter of the vesicle,  $D$ , or smaller. If  $L \gg D$ , which happens with flat discocytes, the vesicle is unstable when aspirated and generally collapses. To overcome this difficulty, the vesicles selected for the micropipette assay were always first transferred to a medium of low osmolarity in order to provoke their swelling. The diameter of the micropipettes was 6 to 8  $\mu\text{m}$ . The pressure in the micropipette was controlled by a variable U shape tube device driven by a micromanipulated rack (Prior, Cambridge, UK). The micropipette suction pressure was measured via an in-line pressure transducer (Sedeme, Paris, France) which permitted us to explore the range  $\pm 200$  mbar. The elastic modulus of dilation  $K$  was determined from the slope of the line obtained by plotting the micropipette suction pressure versus relative area change of the vesicle. Measurements were carried out with approximately 30 EPC vesicles. The histogram showed two well separated peaks, one at 125 dyn/cm and the other one at 248 dyn/cm. These values are close from the values reported by Kwok and Evans,

respectively, for unilamellar and bilamellar vesicles (Kwok and Evans, 1981). Unfortunately it was not possible to verify the unilamellarity of all the vesicles whose shape changes were monitored by microscopy. Indeed, in several instances, the vesicles either collapsed after prolonged periods of shape changes or irreversibly disappeared from the field of observation.

## RESULTS

### Phospholipid redistribution between inner and outer monolayers

Fig. 1 shows a typical sequence of shape transformations which took place in less than 2 min after the transfer of a discocyte EPC lipid vesicle containing 1% of EPG from a medium at low pH (5.5) to a medium at high pH (9.5).

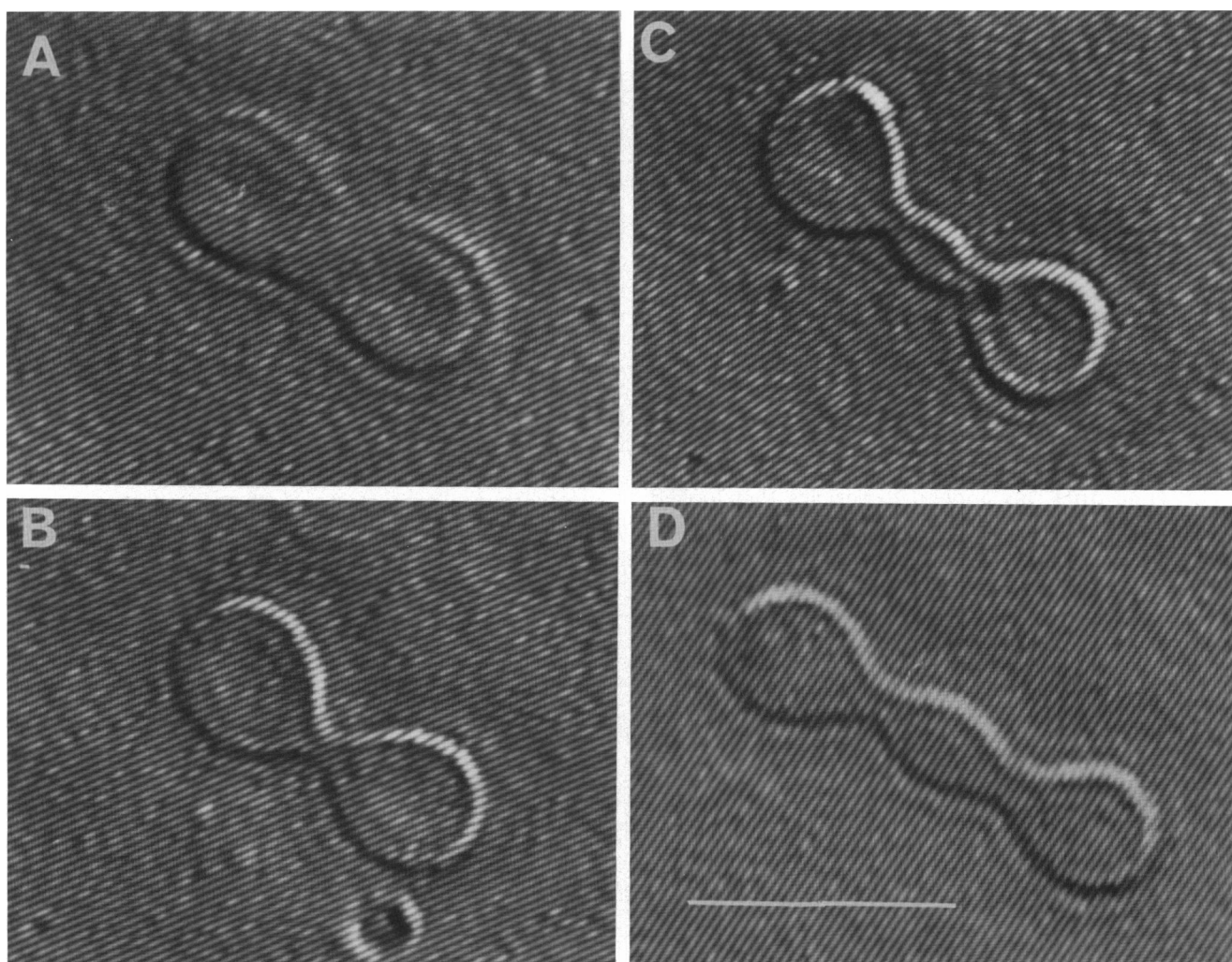


FIGURE 1 Sequence of shapes observed by phase contrast microscopy with a unilamellar EPC-EPG (100:1) vesicle ( $K = 135$  dyn/cm), originally at pH 5.5 in 100 mM glucose and submitted to an external pH of 9.5 in 100 mM glucose. The time elapsed after transfer to high pH medium is, respectively: (A)  $t = 5$  s; (B)  $t = 30$  s; (C)  $t = 55$  s; (D)  $t = 80$  s. Temperature, 20°C. The bar corresponds to 20  $\mu\text{m}$ .

This vesicle was selected because its appearance in the phase contrast microscope made it a plausible candidate for a single walled vesicle, an hypothesis that was confirmed afterwards (see below). The sequence of shape transformations shown in Fig. 1 was continuous. The passage from a discocyte to a two-vesicle shape and then a three-vesicle shape, was obtained in many similar experiments. Maintaining the external pH at 9.5 was accompanied by further shape transformations. But in the experiment shown in Fig. 1, the external pH was brought back to pH 5.5 after 80 s and the external osmolarity reduced to permit  $K$  measurement; the vesicle reverted to a swollen discoid shape in approximately 3 min. The vesicle was then aspirated into a small diameter micropipette and the elastic modulus  $K$ , found to be 135 dyn/cm, thereby indicating the signature of a single walled vesicle (Kwok and Evans, 1981).

Figs. 2 and 3 show other examples of shape transformations induced by pH in an EPC/EPG vesicle. In Fig. 2 the duration of the exposure to high pH was 5 min. At this time, the external osmolarity was reduced to 10 mM glucose and the transmembrane gradient abolished: this instantaneously provoked the swelling and a compartmentation of the giant liposome which transformed into eight nearly perfect spheres (Fig. 3*A*), while annealing the  $\Delta$ pH, i.e., flipping back EPG to the inner monolayer, caused the sequential decrease of the number of connected bubbles (Fig. 3, *A* to *F*). Note that during these various processes, there was no vesicle release. In particular, the transfer of the vesicle from high to low external osmolarity implied creation of shear forces that did not result in the separation of the spheres. Thus, it appears that the spheres are always interconnected.

The same  $\Delta$ pH was applied to vesicles that looked multi-walled because of their very thick membranes or those that were revealed as double-walled after micropipette assay. These vesicles did not transform like the former ones. Instead of a series of connected vesicles they gave rise to several protrusions emerging from an almost spherical large vesicle (Fig. 4). The small protrusions did not give rise to shedding.

Control experiments were carried out with multi-walled vesicles that did not contain PG nor detectable amounts of free fatty acid. In such cases, the change of pH did not induce the formation of protrusions, at least during a 20-min observation period.

### Lipid addition to the outer monolayer

In a second series of experiments, giant vesicles of EPC were submitted to a gentle stream of L-PC injected in the vicinity of their outer envelope. Fig. 5 shows a typical sequence of shape changes. This result was obtained

repeatedly with unilamellar vesicles and involved a shape transformation which stopped after the formation of two contiguous spheres of unequal volume. In a few instances, such "8"-shape vesicles were manipulated with a narrow bore micropipette ( $\sim 4 \mu\text{m}$ ) and their shape modified. In particular, vesicles with two smaller protrusions were obtained by this mechanical treatment. However, addition of L-PC alone to unilamellar vesicles, always generated vesicles with the shape seen in Fig. 5*D*. With the multilamellar giant vesicles, addition of L-PC to the external leaflet first created ripples at the surface of the giant vesicles. These, subsequently, transformed into multiple protrusions and eventually small vesicles separated out (see Fig. 6). After this shedding process the large multilamellar vesicle seemed unchanged, as it had simply peeled off one layer of membrane.

### Lipid depletion from the outer monolayer

Selective lipid depletion from the outer monolayer of a large vesicle containing one or several bilayers was achieved by L-PC and free fatty acid extraction with BSA injected near the outer surface of the vesicle. Control experiments showed that only those vesicles that contained L-PC and fatty acids underwent a shape change characterized by a single invagination. The vesicle then adopted a stomatocyte shape (Fig. 7).

## DISCUSSION

Shape changes of giant liposomes, under conditions of constant osmolarity, are generally attributed to a variation of the surface areas between inner and outer leaflets,  $\delta S$ , imposing membrane bending. Berndt et al. (1990) have predicted that a value of  $\delta S/S \sim 10^{-4}$  would suffice to trigger an overall shape change of a giant phospholipid vesicle. However, in the experiments carried out by Sackman and collaborators (Sackman et al., 1986 and Berndt et al., 1990), the shape changes were triggered by a temperature variation and could only be indirectly correlated to  $\delta S/S$ . Furthermore, in previous experiments on shape changes, the unilamellarity of the giant vesicles was not determined. Here we show that at a fixed temperature, a redistribution of lipids, as well as the addition to or depletion of lipids from one monolayer, generate important shape changes of nonspherical EPC vesicles. These experiments confirm the general nature of Sheetz and Singer's bilayer couple hypothesis (Sheetz and Singer, 1974). They prove that the existence of a cytoskeleton meshwork is not a requisite for shape change and that the asymmetrical modifications of the

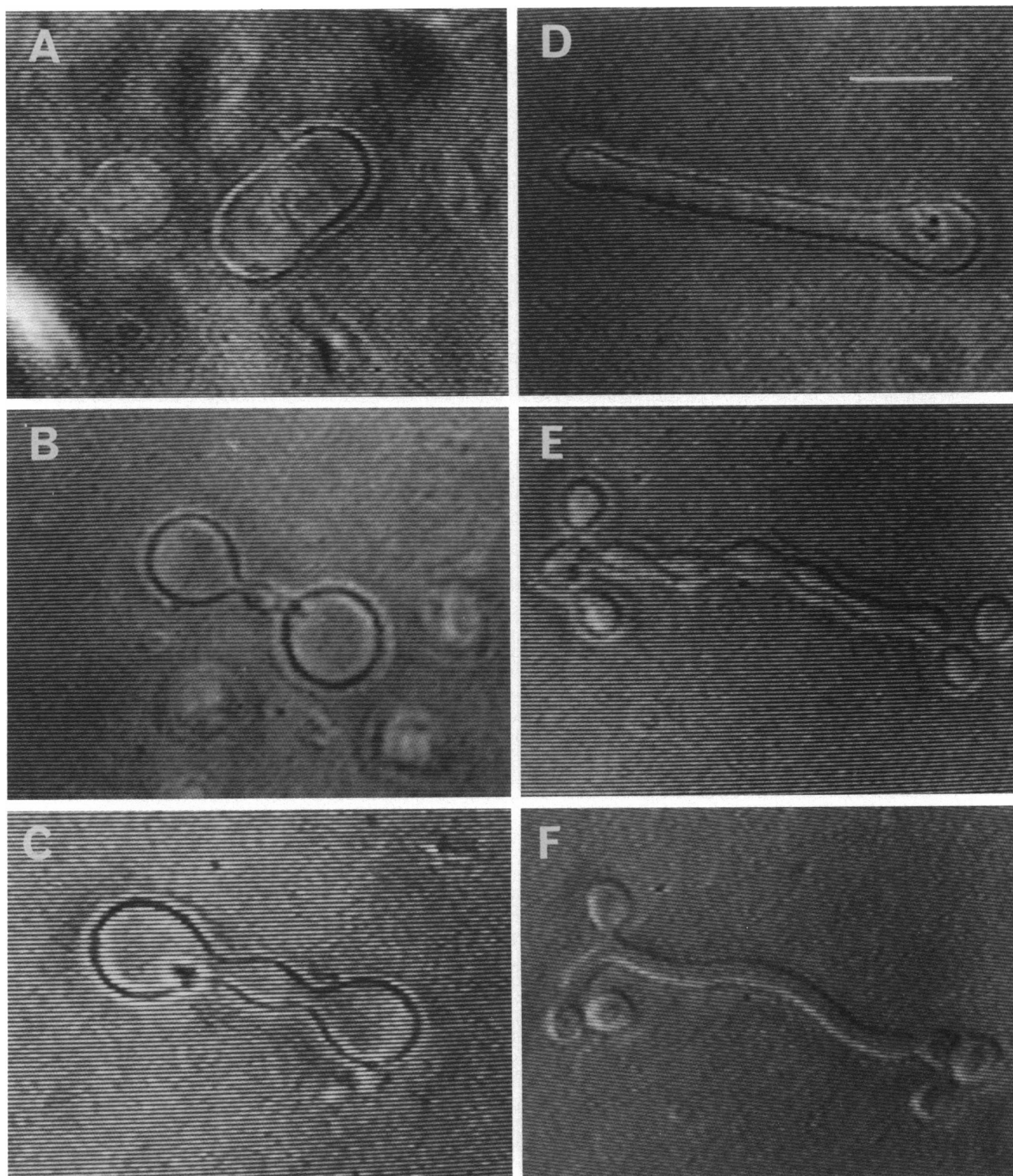


FIGURE 2 Shape transformations obtained with a giant EPC-EPG (100:1) vesicle submitted to a high external pH. (A) before transfer; (B)  $t = 50$  s; (C)  $t = 120$  s; (D)  $t = 160$  s; (E)  $t = 4$  min; (F)  $t = 5$  min. Temperature, 20°C. The bar corresponds to 20  $\mu\text{m}$ .



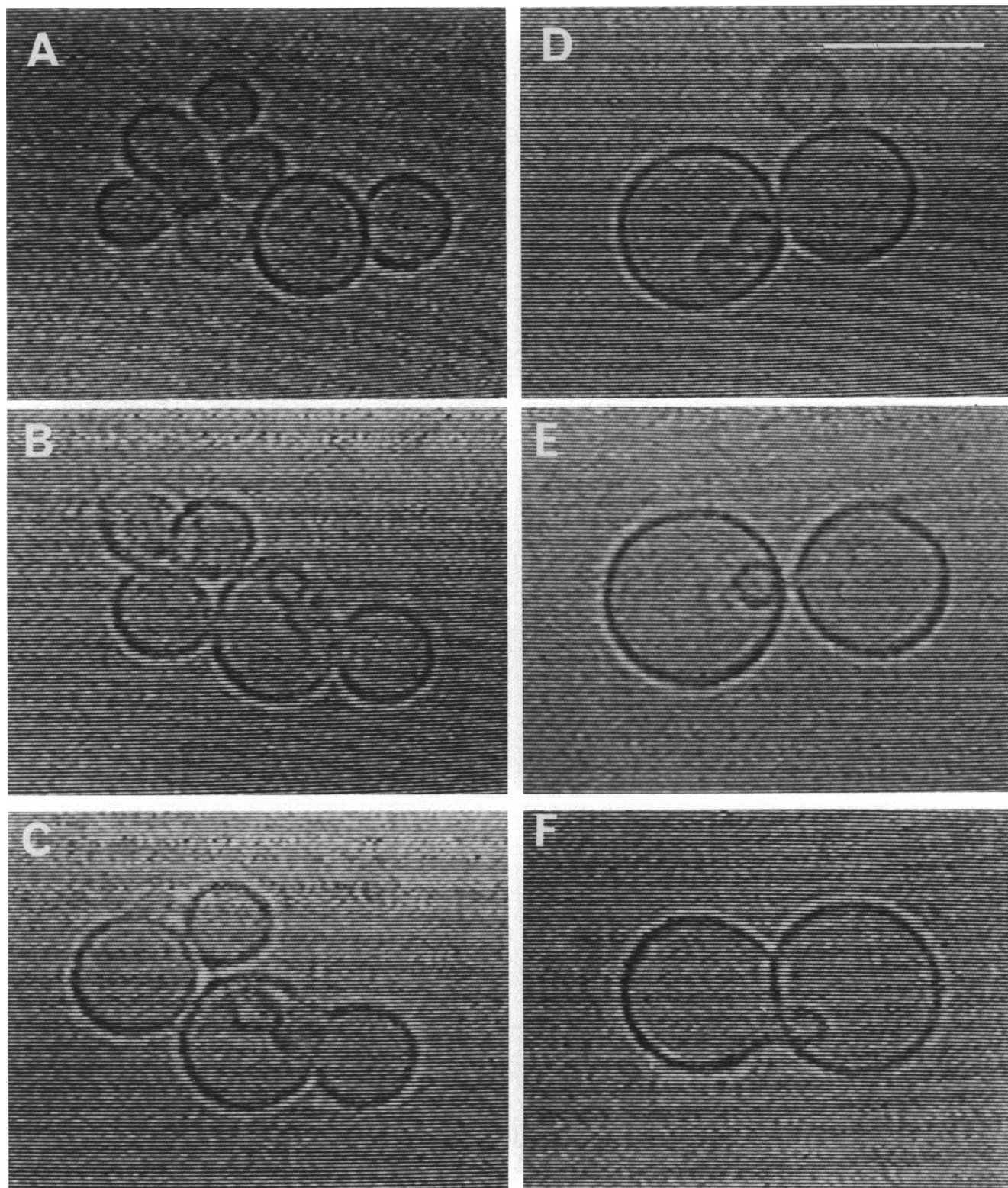


FIGURE 3 Same vesicle as in Fig. 2. After 5 min in pH 9.5, 100 mM glucose, the vesicle is transferred with a micropipette to a medium at pH 5.5, with 10 mM glucose. (A) immediately after transfer; (B)  $t = 20$  s; (C)  $t = 1$  min; (D)  $t = 2$  min; (E)  $t = 4$  min; (F)  $t = 5.5$  min. Temperature,  $20^{\circ}\text{C}$ . The bar corresponds to  $20\ \mu\text{m}$ .

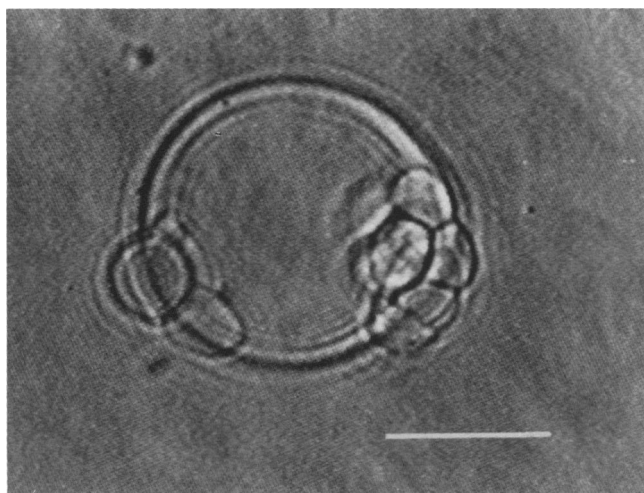


FIGURE 4 Bilamellar vesicle ( $K = 248$  dyn/cm) of EPC-EPG (100:1) vesicle after transfer from a medium at pH 5.5 to a medium of the same osmolarity at pH 9.5. Before transfer, the vesicle had a slightly discoid shape with no external protrusions. Temperature, 20°C. The bar corresponds to 20  $\mu$ m.

phospholipid bilayer suffice to trigger sequential shape changes which mimic plasma membrane or organelle shape modifications in cells. Furthermore the discreteness of the authorized shapes predicted by theoreticians is fully verified (Deuling and Helfrich, 1976; Svetina and Zeks, 1989).

Our experiments with a  $\Delta$ pH induced shape change enable us to evaluate the fraction of lipids which must be redistributed in order to induce the shift from a discoid shape to a two-vesicle shape with a giant EPC-EPG vesicle. The estimate is based on the work of Redelmeier et al. (1990). These authors showed that EPC transmembrane distribution in a matrix of EPC is  $\Delta$ pH dependent with a time constant for EPG distribution of the order of 16 min at 45°C for a  $\Delta$ pH of 5. They also measured the temperature dependence as well as the  $\Delta$ pH dependence of the rate constant. Using their data, we deduced that the passage from a discoid vesicle (Fig. 1A) to a two sphere vesicle (Fig. 1B), which took place 30 s after raising the external pH to pH 9.5 (i.e., with a  $\Delta$ pH of  $\sim 4$ ), involved the redistribution of  $\leq 0.1\%$  of the total phospholipids of the membrane. Thus  $\delta S/S \leq 10^{-3}$ . This value is to be compared with the theoretical value of  $10^{-4}$  obtained by Berndt et al. (1990). Although the agreement is not perfect, it clearly shows that the redistribution of a very small fraction of phospholipids can induce important shape changes.

More surprising is our finding that the addition of L-PC to a unilamellar discoid vesicle leads invariably to a "8"-shape double vesicle which does not evolve when

more L-PC is added (Fig. 5). Thus, the net addition of lipid to one monolayer is not equivalent to the lipid transfer from one monolayer to the other. In the following lines, we propose an explanation to this reproducible phenomenon. The basis of the model is that the addition of L-PC to one lipid leaflet of a bilayer creates a tension  $\tau$ . In a previous publication (Farge et al., 1990), we have shown that the addition of a surface element  $\delta S_o$  to the external leaflet leads to an actual surface increase  $\delta S_e$ , such that

$$\frac{\delta S_e}{S_o} = \frac{1}{2} \frac{\delta S_o}{S_o}.$$

This is accompanied by the creation of a surface tension  $\tau$ . In the case of a transition from a discoid vesicle to a two sphere vesicle  $\delta S/S \sim 10^{-3}$  (see above) and the magnitude of  $\tau$  can be deduced from the relation:  $\tau = K(\delta S/S)$ . Here we find  $\tau \approx 0.12$  dyn/cm. According to Evans and Rawicz (1990) such a tension should inhibit the thermal fluctuation (or flickering) of the membranes and hence cancel the repulsive forces associated with these motions (Helfrich, 1978). As a consequence, the surface of the two spheres forming the "8"-shape vesicle should be attracted to each other by van der Waals forces. The actual observations of the sequence of events leading from Fig. 5, A to D indeed suggest that this is the case because a contraction of the giant vesicle along its axis of symmetry took place as soon as the two spheres were formed. Our hypothesis is that the attraction of the two parts seals them and prevents further shape change. This seems to be an example of the "unbinding-adhesion transition" described by Lipowsky and Leibler (1986). Note that one may question whether the different results obtained with L-PC and EPG could be due to the difference in charge of the two molecules. However, liposomes of "pure" EPC contained after incubation at high temperature  $\sim 0.1\%$  of free fatty acids (see Materials and Methods) which are negatively charged. So the charge distribution is similar, with  $\approx 0.1\%$  of charged lipids in both cases.

In several instances, multilayered vesicles gave shape transformations different from those with unilamellar vesicles. In our experiments, the phospholipid asymmetry was always induced solely in the most external bilayer. Thus, in the case of multilamellar vesicles, the propensity towards curvature of the external bilayer had to overcome the resistance of other layers of lipids, which play a passive role comparable to that of a cytoskeleton. But clearly this resistance is not symmetrical. Excess curvature directed towards the exterior of a bilayer, stacked on the top of other membranes, was satisfied by budding of several small vesicles and gave

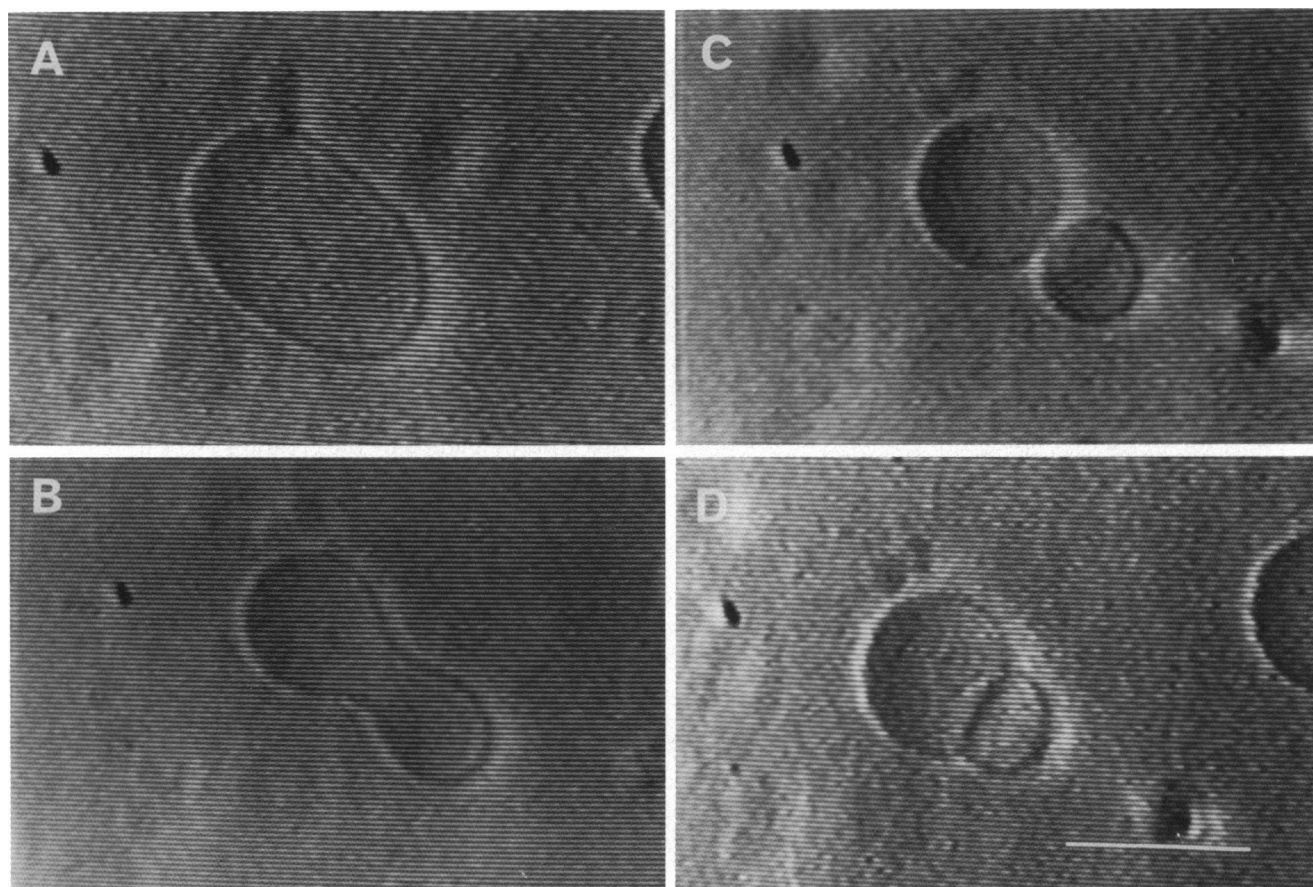


FIGURE 5 EPC vesicle to which L-PC is added externally through a micropipette (not visible). (A)  $t = 0$  s; (B)  $t = 40$  s; (C)  $t = 60$  s; (D)  $t = 65$  s. Temperature, 20°C. The bar corresponds to 20  $\mu\text{m}$ .

very different shapes compared to unilamellar vesicles. See Figs. 2 and 4. By contrast, lipid depletion (by BSA) of the most external leaflet gave, in all instances, a single invagination regardless of the number of lipid layers. In the latter case, the bending of the external bilayer can only be satisfied by a simultaneous bending of the underlying membranes.

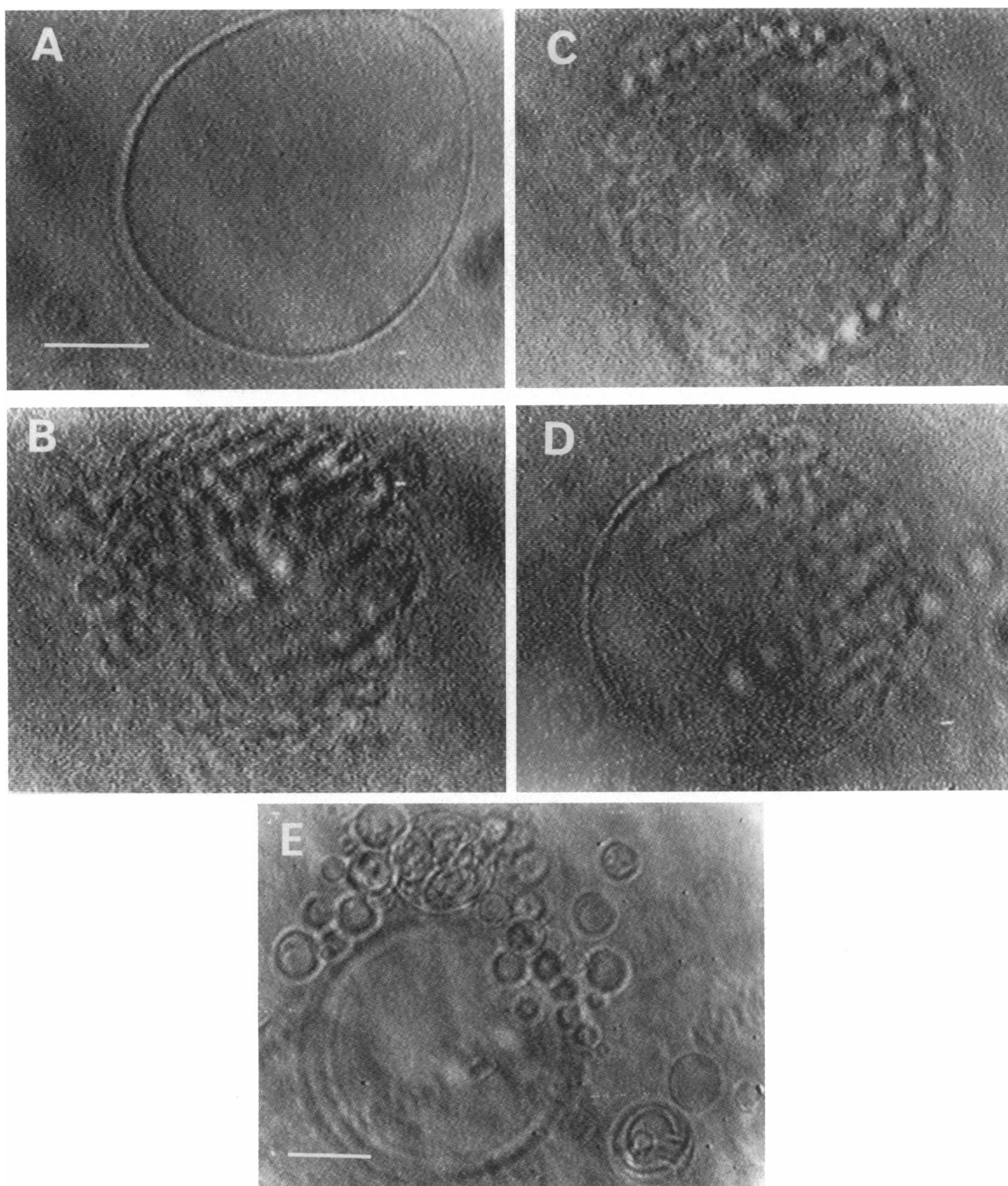
In conclusion, the shape transformation of a discocyte multilamellar giant vesicle resembled the shape transformation of erythrocytes more closely than did unilamellar vesicles shape changes. Thus, erythrocyte shape changes, as emphasized already by several authors, can only be accounted for in a model that includes a bilayer and a cytoskeleton (see for example Elgsaeter et al., 1986). The question that is still debated is the role of ATP in these shape changes. Here, we see that if the external bilayer alone had an ATP driven lipid pump comparable to that existing in human erythrocytes and named "aminophospholipid translocase" (Seigneuret and

Devaux, 1984; Devaux, 1991), it would suffice to modify the cell shape of a multilayered vesicle.

Finally, it is noteworthy that these experiments gave only exceptional shedding. Even the presence of L-PC, which is supposedly a fusogenic agent, did not seem to be sufficient to trigger vesicle separation when the vesicles were formed from a unilamellar liposome. On the other hand, L-PC added to relatively spherical multilamellar giant liposomes did give shedding (Fig. 6). Presently, we have no molecular explanation to propose in order to explain this reproducible result.

Of course, it would be interesting to investigate the influence of the overall lipid composition. These experiments were conducted with EPC as the major lipid component. Changing the lipid composition brings about serious difficulties when trying to form giant unilamellar vesicles. In this article our objective was to determine the characteristic features of the shape transformation that followed a phospholipid asymmetry imposed on





**FIGURE 6** Evolution of the shape of a bilamellar EPC vesicle ( $K = 234 \text{ dyn/cm}$ ) after addition of L-PC externally. The sequence of shape transformations corresponds to: (A)  $t = 0 \text{ s}$ ; (B)  $t = 8 \text{ s}$ ; (C)  $t = 30 \text{ s}$ ; (D)  $t = 1 \text{ min}$ ; (E)  $t = 2 \text{ min}$ . Temperature,  $20^\circ\text{C}$ . The bar corresponds to  $20 \mu\text{m}$ . Same scale for A, B, C and D; different scale for E.

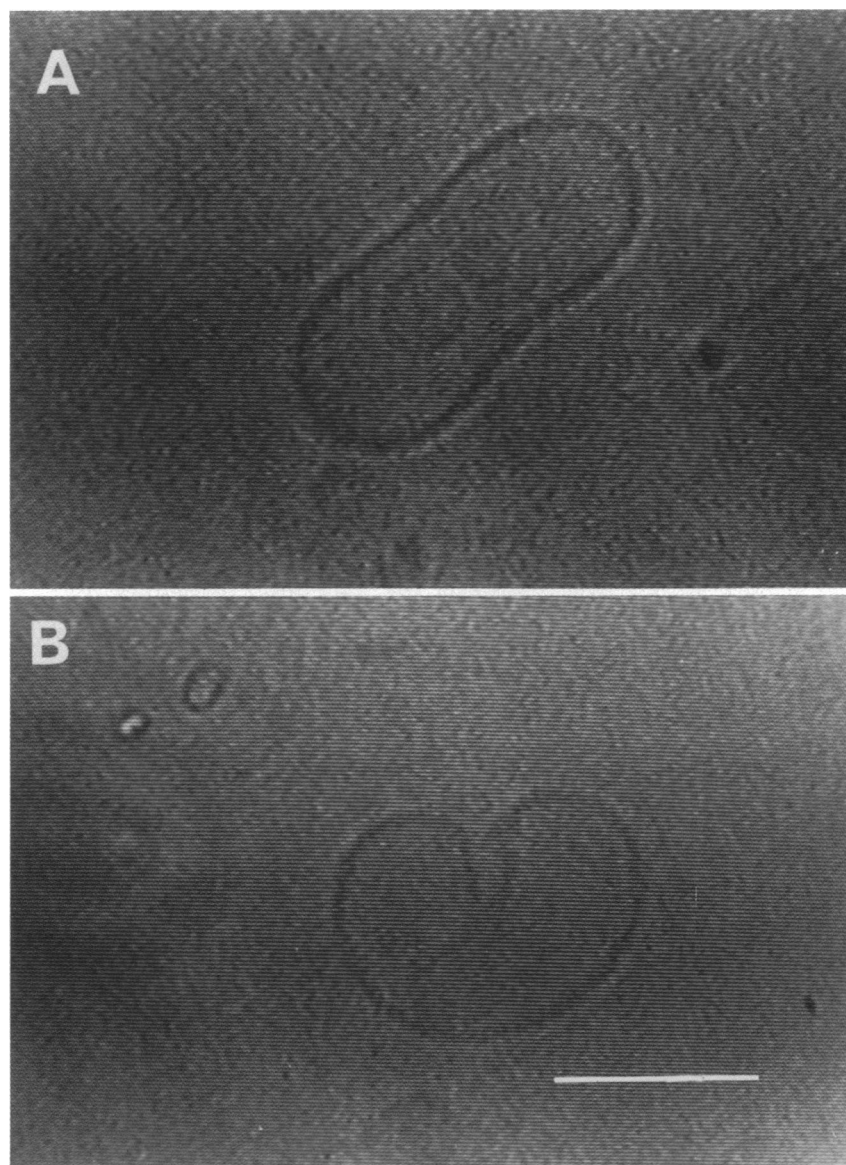


FIGURE 7 Depletion of L-PC from the outer leaflet of a vesicle of EPC/L-PC by incubation with fatty acid free BSA: (A) before BSA addition; (B) after BSA addition. Temperature, 20°C. The bar corresponds to 20  $\mu$ m. The vesicle contained 1% of L-PC at the beginning of the experiment.

vesicles with the size of real cells. This study should be extended to vesicles containing other lipids, in particular it is conceivable that phosphatidylethanolamine would accumulate in the region of low curvature. Local nonbilayer structure could then be responsible for vesicle separation. Alternatively, one would have to postulate that in biological cells specific proteins are responsible for the separation of membrane invaginations created by budding or endocytosis. However, the precise role of proteins in endocytic pathways is yet to be demonstrated.

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