J. Membrane Biol. 30, 271–282 (1976)

Ca^{++}-Induced Fusion of Proteoliposomes: Dependence on Transmembrane Osmotic Gradient

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Received 24 May 1976

Summary. The fusion of cytochrome oxidase liposomes with liposomes reconstituted with mitochondrial hydrophobic protein is dependent on the presence of an acidic phospholipid in the liposomes and on the addition of Ca^{++} ions. Liposomes which have grown, by fusion, to diameters in excess of 1000 Å lose the ability to fuse further, unless an osmotic gradient across the liposome membrane is established, with the internal osmotic pressure higher than the external. At a given Ca^{++} concentration, the extent to which this second fusion step takes place is determined by the ratio of internal to external osmolarity. Single-walled liposomes with diameters exceeding 1 μm have been produced by this technique. The data suggest that the thermodynamic driving force for the Ca^{++}-induced fusion is an excess surface free energy which can be supplied by membrane curvature or transmembrane osmotic gradients.

A system has recently been developed in which unilamellar liposomes (Papahadjopoulos, Poste, Schaeffer & Vail, 1974) or liposomes reconstituted with membrane proteins (Miller & Racker, 1976) can be made to fuse with each other. The fusion process is absolutely dependent on two conditions: an acidic phospholipid as a major membrane constituent, and Ca^{++} (>1 mM) in the aqueous medium. Under these conditions, the liposome diameters grow from about 300 Å to values in the range 1000–1500 Å within 5 min. A curious aspect of the fusion is that after this initial growth in liposome size, fusion ceases (Miller & Racker, 1976); no further increase in liposome size is observed even up to 24 hr. One possible explanation for this refractoriness is a decrease in the curvature of the fused liposomes. An excess surface free energy may be associated with the curvature of the initially small liposomes, providing the thermodynamic driving force for fusion. Once the liposomes have grown to diameters of negligible curvature, the surface free energy would no longer

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be reduced by further fusion and the process would stop, as has been treated theoretically (Gent & Prestegard, 1974).

If such an explanation applies to the fusion system under study, it should be possible to cause further fusion of initially fused liposomes by inducing an excess surface free energy via a positive osmotic gradient across the liposome membrane (i.e., with the internal osmolarity greater than the external). In this report, we demonstrate that prefused liposomes can indeed be fused further in the presence of Ca^{++} by the imposition of a positive transmembrane osmotic gradient.

Materials and Methods

Biochemicals

Cytochrome *c* oxidase (Eytan, Carrol, Schatz & Racker, 1975) and mitochondrial hydrophobic protein "crude F_0" (Kagawa & Racker, 1966; 1971) were prepared from bovine heart mitochondria as described. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purified from soybean phospholipids (Kagawa, Kandrach & Racker, 1973), and phosphatidylserine (PS) from bovine brain (Papahadjopoulos & Miller, 1967); crude soybean phospholipids (asolectin) were treated with acetone as described (Kagawa & Racker, 1971).

Unilamellar liposomes were prepared either by anaerobic sonication of the lipids in (in mM) 40 KCl-10 HEPES[1]-0.1 EDTA, pH 7.0 (hereafter referred to as "buffer") as described (Miller & Racker, 1976), or by dissolving the lipids in buffer containing 25 mM cholate, followed by overnight dialysis against cholate-free buffer (Hinkle, Kim & Racker, 1972; Racker, 1972). All liposomes were prepared at a lipid P concentration of 25 mM. Cytochrome oxidase vesicles or hydrophobic protein vesicles were made at protein concentrations of 1 mg/ml and 2.5 mg/ml, respectively.

Liposome Fusion

Fusion of cytochrome oxidase vesicles with hydrophobic protein vesicles was assayed at 20°, as described (Miller & Racker, 1976). The method is based on the fact that the hydrophobic protein, by virtue of its ability to make the liposome membranes permeable to protons (Racker, 1972; Shchipakin, Chuchlova & Evtodienko, 1976) stimulates the rate of oxygen consumption catalyzed by cytochrome oxidase vesicles. This stimulation takes place only when the two proteins share the same vesicle membrane. The rate of oxygen consumption in buffer containing cytochrome oxidase vesicles (5 μg protein), cytochrome *c* (Sigma, Type VI, 1.2 mg/ml), and K ascorbate (25 mM) was measured in a 1 ml cell of a Gilson oxygraph, with or without hydrophobic protein vesicles present (12 μg protein). To induce fusion, $CaCl_2$ was added (1 to 5 mM), and the rate of oxygen consumption was followed for several minutes. Fusion of cytochrome oxidase vesicles with hydrophobic protein vesicles is indicated by an increase in the rate of oxygen consumption upon addition of Ca^{++}. After the new rate of oxygen consumption was established, valinomycin (1 μM) and 1799 (15 μM) were added to measure the fully uncoupled rate. Fusion is measured

1 Abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid; EDTA, (Ethylenedinitrilo)-tetraacetic acid; 1799, bis (hexafluoroacetonyl) acetone.

quantitatively by the decrease in the respiratory control ratio, which is defined as the ratio of the rate of oxygen consumption in the presence of valinomycin-1799 to that in its absence. An estimate of the fraction, f, of cytochrome oxidase vesicles which have become uncoupled by fusion with hydrophobic protein vesicles is given by:

$$f = \left(\frac{RCR_0}{RCR_f} - 1\right) \Big/ \left(\frac{RCR_0}{RCR_h} - 1\right), \tag{1}$$

where RCR_0, RCR_f, and RCR_h represent the respiratory control ratios of cytochrome oxidase vesicles alone, cytochrome oxidase vesicles fused with hydrophobic protein vesicles, and hybrid vesicles made by reconstituting cytochrome oxidase and hydrophobic protein together, using the same mixture of proteins and lipids that was used in the fusion mixture.

In some of the experiments to be reported, we assay the fusion of vesicles which had already been fused once. We call the first step of fusion the "primary fusion", and the subsequent step, the "secondary fusion". Assays of secondary fusion were carried out as above, except that the cytochrome oxidase vesicles and hydrophobic protein vesicles were made to undergo primary fusion *separately*, by exposure to Ca^{++} (at a lipid concentration of 2.5 mM) for 5–15 min before being mixed together in the oxygraph cell. In these experiments, it was necessary to load the inside of the primarily fused vesicles with an osmotically active solute (glucose in most experiments). This was accomplished in one of two ways. Either the vesicles were formed originally in the usual buffer containing the appropriate concentration of glucose and primarily fused in the same buffer; or the vesicles were made in buffer without glucose and then primarily fused in buffer containing glucose. This latter technique is adequate for glucose-loading since it has been shown that during fusion, the vesicles become transiently leaky to small molecules (C. Miller and G. Eytan, *unpublished*). Identical results were obtained regardless of the method used for glucose loading.

Electronmicroscopy

A suspension of liposomes or proteoliposomes (0.5–2 mM lipid P) was applied to a 400-mesh carbon-coated copper grid, and negatively stained with 2% Na-phosphotungstate. In some experiments, the liposomes were fixed with 0.05–0.5% OsO_4 before negative staining. For thin-sectioning, liposome suspensions were embedded in Epon-Anasolete epoxy resin, sectioned, and positively stained with uranyl acetate and lead citrate (Telford & Matsumura, 1970). Grids were viewed in an Allied Electronics Industries Electron Microscope, Model EM6B, operating at 60 kV.

Results

Primary Fusion

The basic assay of fusion of cytochrome oxidase vesicles with hydrophobic protein vesicles is shown in Fig. 1. A mixture of the two types of vesicles in the absence of Ca^{++} displays a low, constant rate of oxygen consumption; upon addition of 3 mM Ca^{++}, the rate rises to a new level after a few minutes. Addition of valinomycin-1799 increases the rate further. Since Ca^{++} does not accelerate the oxidation rate of

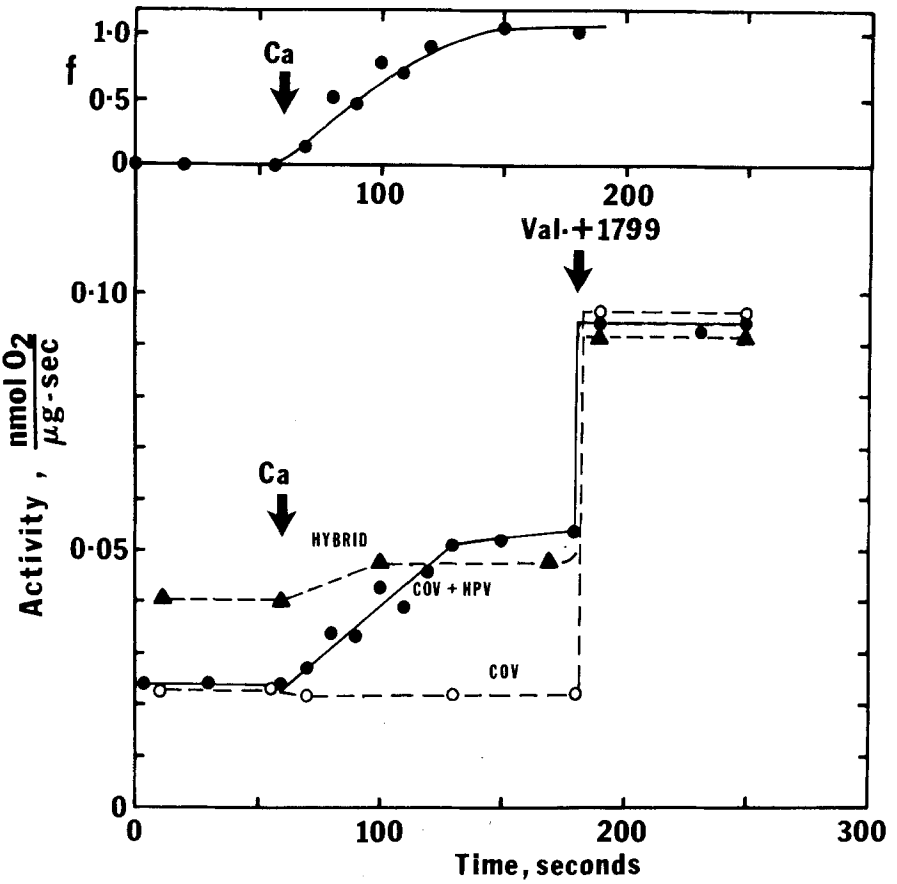

Fig. 1. Assay of fusion of cytochrome oxidase vesicles with hydrophobic protein vesicles. The rate of oxygen consumption catalyzed by cytochrome oxidase vesicles (*COV*) in the presence or absence of hydrophobic protein vesicles (*HPV*) was measured as described under *Materials and Methods*, and is plotted as specific activity. Zero time is defined by addition of cytochrome oxidase vesicles to the oxygraph cell. After one min, 3.2 mM Ca^{++} was added, and two minutes later valinomycin and 1799 were added to uncouple the vesicles fully. Phospholipid composition of the vesicles was 40% PS-60% asolectin. (○)—cytochrome oxidase vesicles alone (5 μg protein); (●)—mixture of cytochrome oxidase vesicles (5 μg) and hydrophobic protein vesicles (12 μg); (▲)—hybrid vesicles made by reconstituting together cytochrome oxidase and hydrophobic protein

cytochrome oxidase vesicles alone, and since hydrophobic protein vesicles do not affect the cytochrome oxidase vesicles in the absence of Ca^{++}, the change in respiration rate is taken as the result of fusion of the two types of vesicles (Miller & Racker, 1976).

The data of Fig. 1 are calculated by Eq. (1) to yield the time course of the fractional fusion parameter shown in the insert above Fig. 1. Under these conditions the fractional fusion parameter attains a value of unity

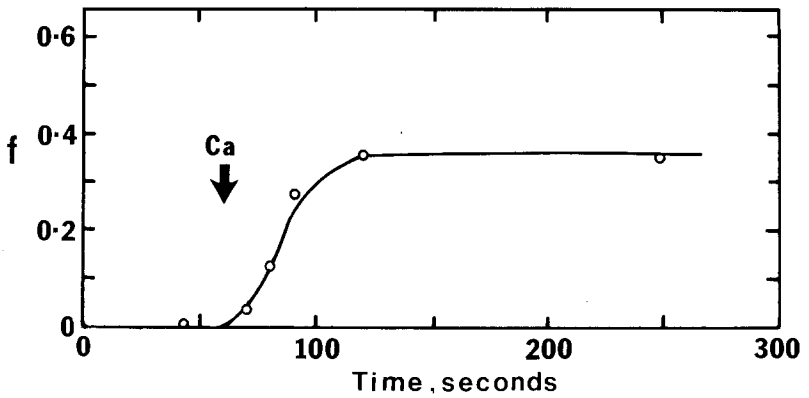

Fig. 2. Refractoriness of fused vesicles to further fusion. A time course of the fractional fusion parameter is determined as described in the legend of Fig. 1, except under milder fusion conditions. Lipid composition of the vesicles was 40% PS-10% PC-50% PE; at the arrow, 2 mM $CaCl_2$ was added

within 2 min, indicating that all the cytochrome oxidase vesicles have become uncoupled by fusion with hydrophobic protein vesicles to the same extent as the hybrid vesicles. Fig. 2 shows a similar experiment under milder fusion conditions, i.e., with a lower Ca^{++} concentration and different lipid composition; here, the fractional fusion parameter levels off at a value well below unity, indicating that fusion of the vesicles ceases before all the cytochrome oxidase vesicles can become uncoupled, even though Ca^{++} is still present. Thus, fusion causes the vesicles to become refractory to further fusion.

Secondary Fusion

This refractoriness can be eliminated by imposing a transmembrane osmotic gradient during the second fusion step. It can be seen from Fig. 3 that liposomes which have undergone primary fusion do not fuse further unless the osmotic strength inside the vesicles is made higher than that outside by adjustment with sucrose. The data also show that in contrast to the secondary fusion, the primary fusion is not inhibited by external sucrose; in fact a slight stimulation was observed in this experiment.

The dependence of the secondary fusion process on an osmotic gradient is shown more fully in Fig. 4. Here the fractional fusion parameter for secondary fusion is measured as a function of external osmotic strength in vesicles loaded with different *internal* osmotic strengths. Osmotic

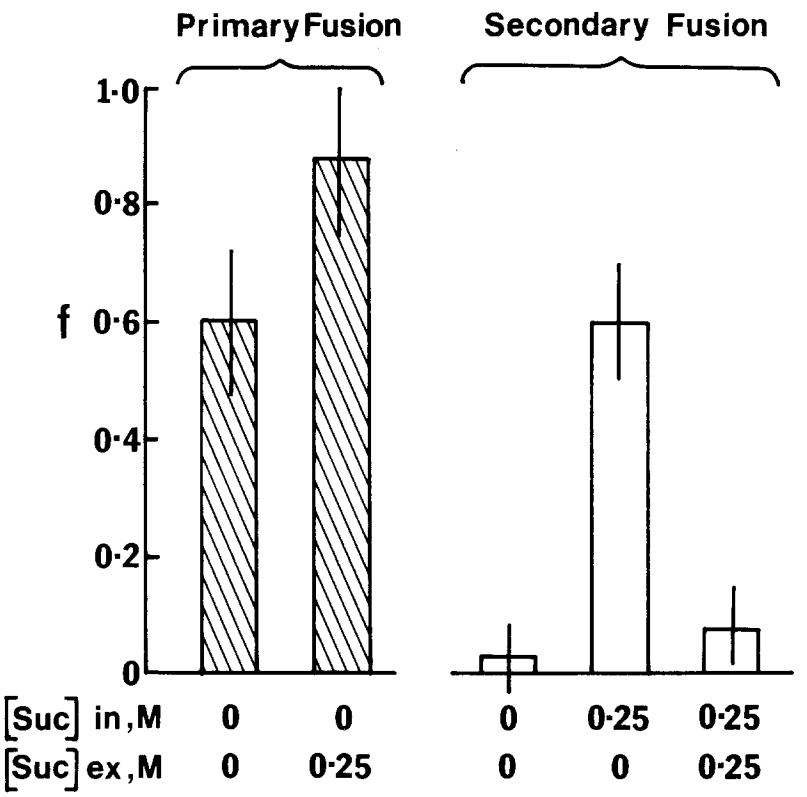

Fig. 3. The dependence of secondary fusion on osmotic gradient. Cytochrome oxidase vesicles and hydrophobic protein vesicles (40% PS-60% asolectin) were tested for fusion in the presence of 3 mM Ca^{++} for 2 min as described under *Materials and Methods*. The osmotic conditions are indicated in the figure by internal and external sucrose concentrations, $[Suc]_{in}$ and $[Suc]_{ex}$. The cross-hatched bars show the fractional fusion parameters *f*, for the *primary* fusion step. The unfilled bars show the *f*-parameters for the *secondary* fusion step. These latter samples underwent the primary fusion step by separate exposure to 3 mM Ca^{++} for 15 min, in the presence or absence of 0.25 M sucrose. Each *f*-value reported represents the mean ± standard error of 3 determinations

strength is adjusted with glucose in this experiment, and the vesicles are loaded by primary fusion in buffers containing either 0.25 M or 0.5 M glucose. It can be seen that the fusion of the vesicles loaded with 0.5 M glucose is inhibited at external glucose concentrations above 0.4 M; only 0.2 M external glucose is required for comparable inhibition of fusion of vesicles loaded with 0.25 M glucose. The data from both sets of vesicles, when replotted against the ratio of internal-to-external osmotic pressure, fall on the same curve, within the error of the measurements. This observation represents our strongest evidence that the secondary fusion process requires an osmotic gradient, with the internal osmolarity higher than the external. Assuming that the vesicles behave like perfect osmometers,

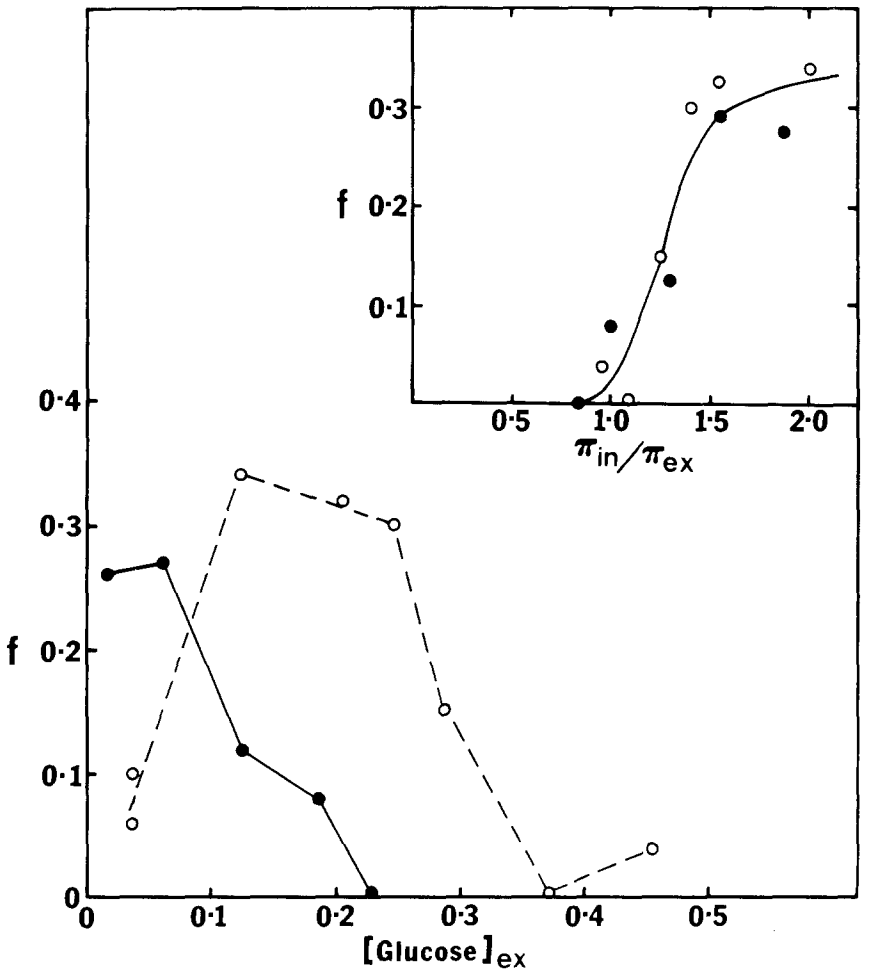

Fig. 4. Effect of osmotic gradients on the secondary fusion process. Cytochrome oxidase vesicles and hydrophobic protein vesicles were separately fused at 2.3 mM lipid P concentration, in buffer containing 5 mM Ca^{++} and either 0.25 M (●—●) or 0.5 M (○—○) glucose. After 5 min of this primary fusion, 7 mM EDTA was added and the mixture was placed on ice. Samples were then assayed as usual for fusion in buffer containing 5 mM Ca^{++} and varying concentrations of glucose. The fractional fusion parameter *f*, was determined after 2 min of fusion. Vesicles were prepared with 40% PS-60% asolectin. The data were replotted against the ratio of internal osmotic pressure, π_{in}/π_{ex}, taking into account the osmotic contribution from glucose, KCl, HEPES, $CaCl_2$, and K ascorbate

the data show that the secondary fusion process does not begin to occur until the vesicles have swollen, on the average, to 1.2–1.5 times their initial volume. This is an upper limit since ideal osmotic behavior is unlikely. It should be noted that loss of respiratory control is seen *only* in the presence of Ca^{++} and hydrophobic protein vesicles (data not shown). Uncoupling of the cytochrome oxidase can therefore not have been caused by the osmotic gradient itself. It can be seen from Fig. 4 that fusion

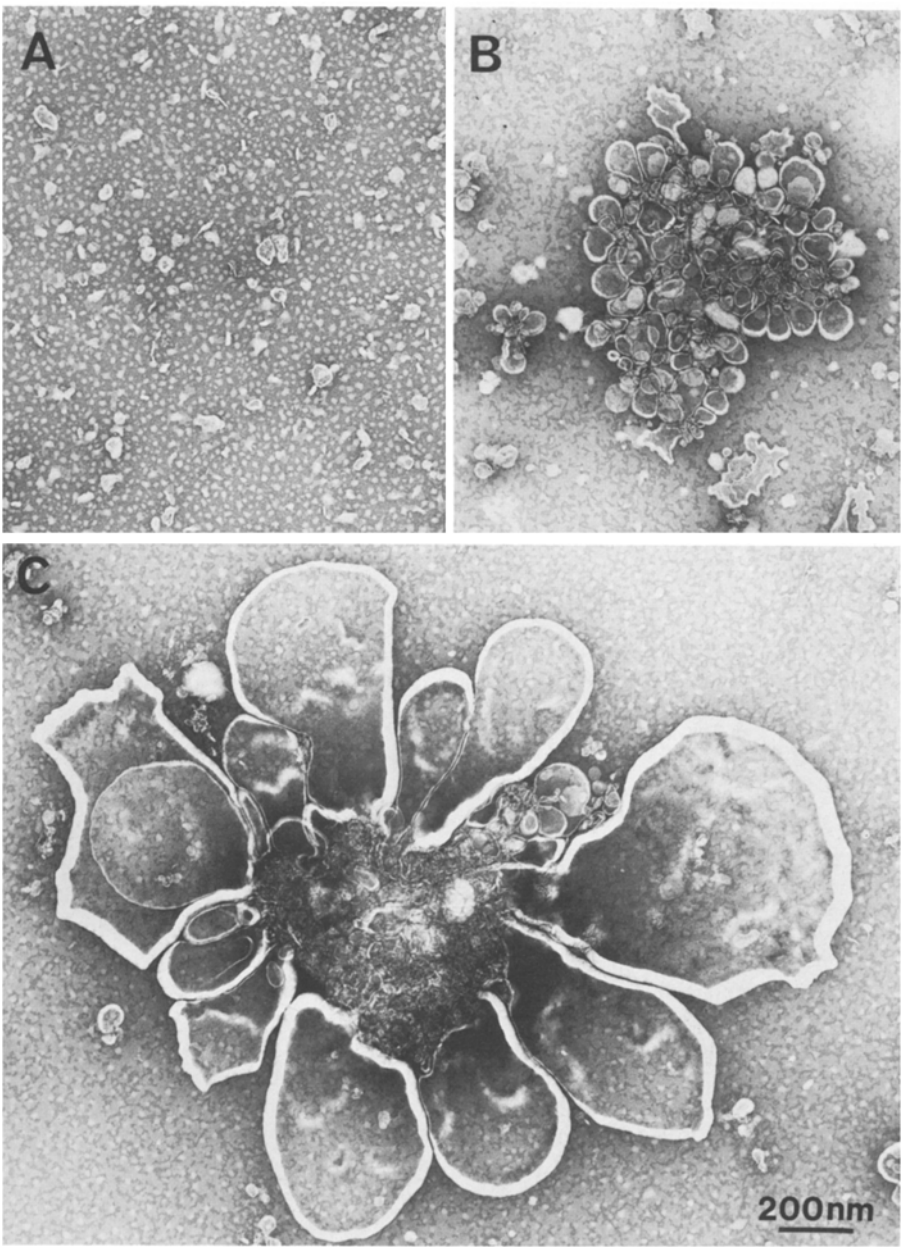

Fig. 5. Growth in vesicle size during two rounds of fusion. Cytochrome oxidase vesicles and hydrophobic protein vesicles were made by the cholate dialysis procedure as described under *Materials and Methods*, in buffer containing 0.25 M glucose. The lipid composition was 40% PS-60% asolectin. The first round of fusion was initiated by adding 4 mM $CaCl_2$ to the mixed vesicles (2 mM lipid P concentration) suspended in buffer containing 0.25 M glucose. After 15 min, 5 mM EDTA was added, the vesicles were centrifuged for 30 min at 100,000 × *g* and were resuspended in glucose-containing buffer at a lipid concentration

of vesicles loaded with 0.5 M glucose is also inhibited at low external glucose concentration. Since at 40 mM external glucose concentrations, the osmotic ratio inside to outside is about 2.5, it is possible that under such drastic conditions the vesicles become transiently leaky and dissipate the osmotic gradient before they can fuse.

Electronmicroscopy

Fig. 5 presents electronmicrographs of fusion between vesicles of cytochrome oxidase and of hydrophobic protein. It can been seen (Fig. 5 *A, B*) that during primary fusion the initially small vesicles (average diameter 400 Å) grow to diameters in the range 1000–2000 Å, as reported previously with protein-free liposomes (Papahadjopoulos *et al.*, 1974; Miller & Racker, 1976). These vesicles which had been loaded with 0.25 M glucose during the primary fusion process were then exposed to a low osmotic strength medium containing Ca^{++} for a second round of fusion. Fig. 3 *C* shows that they grow further, exhibiting diameters up to 5000 Å. There is some indication that the growth may proceed from nucleation sites consisting of aggregated smaller vesicles, but this aggregation may be an artifact of the negative staining procedure. By using sequential osmotic steps in this way, we have been able to observe vesicles with diameters up to 2 μm (as seen in both light and electronmicroscope). However, after several such steps, the vesicle size distribution becomes very heterogeneous.

Fig. 6 shows a thin-section electronmicrograph of liposomes that have undergone three rounds of fusion, a primary fusion followed by two osmotically-induced fusions. It can be seen that not all of the vesicles are single-walled, but many multilayer regions are seen. These multilayered regions may be analogous to the "cochleate figures" seen by Papahadjopoulos *et al.* (1975) as an intermediate state of the Ca^{++}-induced fusion

of 25 mM P. (All the lipid was pelleted by the centrifugation.) A sample of this suspension was prepared for electronmicroscopy, and the remainder was fused a second time by 10-fold dilution into buffer without glucose, containing 4 mM $CaCl_2$. After 15 min, 5 mM EDTA was added and the sample was prepared for electronmicroscopy. In parallel to this experiment, samples were assayed for 2 min of fusion in the oxygraph cell, as described in the text. For the first round of fusion, $f=0.75$; for the second round of fusion, $f=0.35$. (*A*) Vesicles before fusion. (*B*) Vesicles after the first round of fusion. (*C*) Vesicles after the second (osmotically-induced) round of fusion

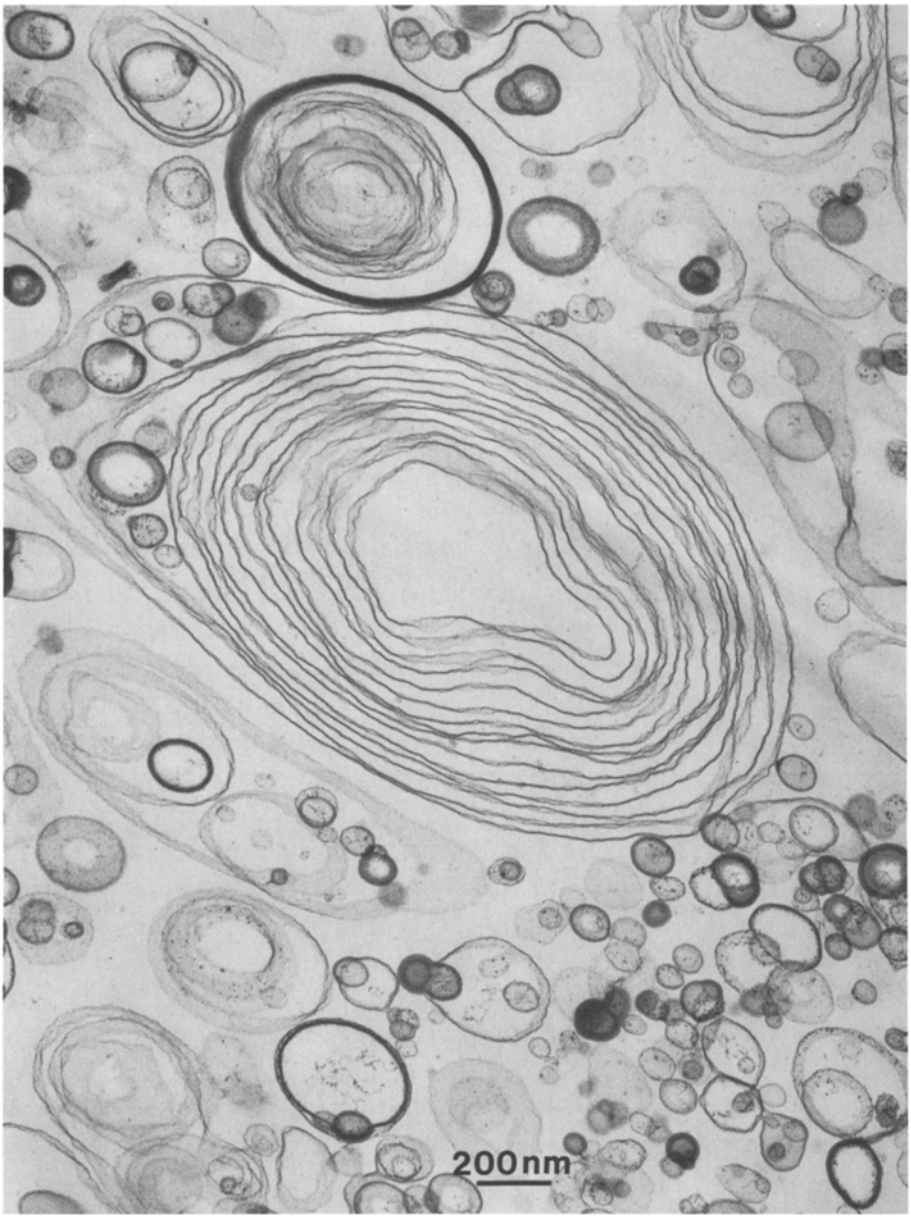

Fig. 6. Thin-section electron micrograph of multiply-fused liposomes. Liposomes (40% PS-60% asolectin) were made to undergo three rounds of fusion by an extension of the procedure described in the legend of Fig. 3. The liposomes were formed by sonication in buffer containing 0.5 M glucose and were initially fused at a concentration of 5 mM lipid P by addition of 4 mM $CaCl_2$. After 15 min, they were fused a second time by 2-fold dilution with glucose-free buffer, also containing 4 mM Ca^{++}. After 15 min they were then fused a third time by 10-fold dilution in glucose-free buffer containing 4 mM Ca^{++}. In this way, the vesicles were subjected to two sequential osmotic steps, each of which induced a round of fusion. The sample was then pelleted by a 30 min centrifugation at $100{,}000 \times g$, and prepared for thin-sectioning

of phosphatidylserine liposomes. However, it is also apparent that a large portion of the lipid exists in single-walled bilayer form. In fact, most of the vesicles must be unilamellar, since enzyme activity measurements show that over 80% of the cytochrome oxidase in the fused vesicles is accessible to reduced cytochrome *c* added externally.

Discussion

This work was originally stimulated by the observation that the Ca^{++}-induced fusion of PS-containing liposomes comes to a stop after the liposomes grow to diameters of about 1000 Å. We have now shown that these liposomes which have become refractory to fusion can be induced to fuse further by a positive osmotic gradient. This suggests that the driving force for fusion is an excess surface free energy which can be reduced by fusion. The membrane curvature of the initially small liposomes would supply this excess free energy. After the vesicles have grown, fusion ceases because of the negligible curvature of the large liposomes. By supplying an external source of excess surface free energy in the form of membrane stress caused by a positive osmotic pressure difference across the membrane, the Ca^{++}-induced fusion process is made to proceed again; the fusion itself would dissipate the osmotic gradient, since the liposomes become transiently leaky during fusion. These formulations are consistent with the observation that high external osmotic strength inhibits only the *second* round of fusion; it does not inhibit the first round of fusion, with vesicles that have a high surface curvature.

The considerations also apply to the observation (Ahkong *et al.*, 1973*a, b*) that swelling seems to be a necessary condition for the fusion of avian erythrocytes, regardless of the specific agent which induces the cell fusion. In addition, Taupin and co-workers (1975) have shown that positive osmotic gradients induce the formation of pores in dipalmitoyllecithin liposomes; these pores, the authors suggest, could serve as nucleation sites for fusion. On the other hand, Papahadjopoulos *et al.* (1974) have presented evidence that multilayered liposomes undergo Ca^{++}-induced fusion even though they have negligible curvature and are without osmotic gradients. It is difficult to assess the relevance of these findings to our work, since these experiments were done on a much longer time scale (tens of hours) and with much higher concentrations of acidic phospholipid (80–100% phosphatidylserine) than used in our studies.

This work was performed during the tenure (of C.M.) of a grant from the Muscular Dystrophy Association.

This work was supported by Grant #CA-08964, awarded by the National Cancer Institute, Department of Health, Education & Welfare; Grant #BC-156 from the American Cancer Society; Grant #BMS-75-17887 from the National Science Foundation.

References

Ahkong, Q.F., Cramp, F.C., Fisher, D., Howell, J.I., Tampion, W., Verrinder, M., Lucy, J.A. 1973*a*. Chemically-induced and thermally-induced cell fusion: Lipid-lipid interactions. *Nature New Biol.* **242**:215

Ahkong, Q.F., Fisher, D., Tampion, W., Lucy, J.A. 1973*b*. The fusion of erythrocytes by fatty acids, esters, retinol, and α-tocopherol. *Biochem. J.* **136**:147

Eytan, G., Carrol., R.C., Schatz, G., Racker, E. 1975. Arrangement of the subunits in solubilized and membrane-bound cytochrome *c* oxidase from bovine heart. *J. Biol. Chem.* **250**:8598

Gent, M.P.N., Prestegard, J.H. 1974. Cholesterol-phosphatidylcholine interactions in vesicle systems. Implication of vesicle size and proton magnetic resonance line-width changes. *Biochemistry* **13**:4027

Hinkle, P.C., Kim, J.J., Racker, E. 1972. Ion transport and respiratory control in vesicles formed from cytochrome oxidase and phospholipids. *J. Biol. Chem.* **247**:1338

Kagawa, Y., Kandrach, A., Racker, E. 1973. Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXVI. Specificity of phospholipids required for energy transfer reaction. *J. Biol. Chem.* **248**:676

Kagawa, Y., Racker, E. 1966. Partial resolution of the enzymes catalyzing oxidative phosphorylation: IX. Reconstruction of oligomycin-sensitive adenosine triphosphatase. *J. Biol. Chem.* **241**:2467

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXV. Reconstitution of particles catalyzing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* **246**:5477

Miller, C., Racker, E. 1976. Fusion of phospholipid vesicles reconstituted with cytochrome *c* oxidase and mitochondrial hydrophobic protein. *J. Membrane Biol.* **26**:319

Papahadjopoulos, D., Miller, N. 1967. Phospholipid model membranes: I. Structural characteristics of hydrated liquid crystals. *Biochim. Biophys. Acta* **135**:624

Papahadjopoulos, D., Poste, G., Schaeffer, B.E., Vail, W.J. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim. Biophys. Acta.* **352**:10

Papahadjopoulos, D., Vail, W.J., Jacobson. K., Poste, G., 1975. Cochleate lipid cylindars: formation by fusion of unilamellar lipid vesicles. *Biochim. Biophys. Acta* **394**:483

Racker, E. 1972. Reconstitution of cytochrome oxidase vesicles and conferral of sensitivity to energy transfer inhibitors. *J. Membrane Biol.* **10**:221

Shchipakin, V., Chuchlova, E., Evtodienko, Y. 1976. Reconstruction of mitochondrial H^+-transporting system in proteoliposomes. *Biochem. Biophys. Res. Commun.* **69**:123

Taupin, C., Dvolaitzky, M., Sauterey, C. 1975. Osmotic pressure induced pores in phospholipid vesicles. *Biochemistry* **14**:4771

Telford, J.N., Matsumura, F. 1970. Dieldrin binding in subcellular nerve components of cockroaches. An electron microscopic and autoradiographic study. *J. Econ. Entomol.* **63**:795