J. Membrane Biol. 26, 319–333 (1976)

Fusion of Phospholipid Vesicles Reconstituted with Cytochrome *c* Oxidase and Mitochondrial Hydrophobic Protein

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Received 30 October 1975

Summary. Reconstituted cytochrome oxidase liposomes were fused with liposomes reconstituted with mitochondrial hydrophobic protein, which acts as a membrane-bound uncoupler of cytochrome oxidase. Fusion was assayed by the loss of respiratory control of cytochrome oxidase as measured by the increased rate of ascorbate oxidation induced by hydrophobic protein when both proteins shared the same vesicles. Fusion was dependent on the presence of phosphatidylserine in the liposomes and Ca^{++} in the aqueous medium. Phosphatidylcholine-phosphatidylserine liposomes required higher concentrations of phosphatidylserine and Ca^{++} than did phosphatidylethanolamine-phosphatidylserine liposomes. Cytochrome oxidase vesicles containing high concentrations of phosphatidylserine showed little or no respiratory control, while those with lower concentrations showed high respiratory control; respiratory control could be induced by fusing cytochrome oxidase vesicles containing high phosphatidylserine with protein-free liposomes containing low phosphatidylserine concentration. If cytochrome oxidase vesicles and hydrophobic protein vesicles were prefused separately for 15 min, they lost the ability to fuse upon being subsequently mixed together. The reconstituted vesicles had diameters of about 200 Å; fusion yielded vesicles with diameters in excess of 1000 Å.

Reconstitution of purified membrane proteins into liposomes has made it possible to study ion-transport systems in chemically controllable membrane environments [25, 26]. However, optimal methods of reconstitution of various membrane proteins vary considerably. For example, the cholate-dilution method, a procedure suitable for the reconstitution of several membrane proteins [27] is ineffective for the reconstitution of bacteriorhodopsin; conversely, the sonication procedure, which is the method of choice for bacteriorhodopsin reconstitution, has limited value for the reconstitution of cytochrome *c* oxidase. Thus, a serious problem arises when attempts are made to reconstitute multienzyme systems for the purpose of studying their interactions. Furthermore, it is obvious that neither of the above reconstitution techniques can be used to reconstitute any membrane proteins into planar black lipid membranes [19] upon which valuable electrical measurements could be made.

It is therefore desirable to develop a method which permits the fusion of liposomes reconstituted with membrane proteins, for the purpose either of forming hybrid reconstituted vesicles or of inserting a membrane transport protein (reconstituted into liposomes) into a black lipid membrane.

With such goals in mind, we have studied the fusion of vesicles containing cytochrome *c* oxidase and mitochondrial hydrophobic protein. Normally, reconstituted cytochrome oxidase vesicles display the phenomenon of respiratory control; they oxidize reduced cytochrome *c* at a low rate which can be stimulated over 10-fold by addition of low concentrations of uncouplers of oxidative phosphorylation, the classical example of which is 2,4-dinitrophenol. Uncouplers share the common property of making membranes permeable to protons [5, 18].

The basic principle of our assay for fusion resides in the fact that mitochondrial hydrophobic protein acts as a membrane-bound uncoupler of cytochrome oxidase when the two proteins share the same vesicle [24]. Therefore, a mixture of cytochrome oxidase vesicles and hydrophobic protein vesicles should show respiratory control, which will be lost upon fusion of the two types of vesicles. By following the loss of respiratory control, the degree of fusion can be deduced. It is probable that the hydrophobic protein (the source of which is the oligomycin-sensitive ATPase complex) exerts its uncoupling effect by acting as a proton channel or ionophore, as proposed by Mitchell [16, 17].

Papahadjopoulos and co-workers [7, 22] have recently shown rigorously that liposomes fuse in the presence of Ca^{++} ion if phosphatidylserine is a major membrane component. We now report that this Ca^{++}-induced fusion process can be used as a tool to transfer membrane-bound proteins from one membrane system to another. We shall also describe several aspects of the phospholipid requirements for fusion and of the kinetics of the process.

Materials and Methods

Cytochrome *c* oxidase [1, 28] and the hydrophobic protein fraction [9, 10] were prepared from bovine heart mitochondria. Reconstitution was carried out by the cholate-dialysis procedure as previously described [4] except that dialysis was performed against a solution containing 40 mM KCl, 0.1 mM Na-EDTA[1], and 10 mM HEPES or MOPS buffer (pH 7.0). The concentrations of the components of the reconstituted vesicles after dialysis were,

1 Abbreviations used are: EGTA, ethyleneglycol-bis (β-aminoethyl ether) N,N′-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; HEPES, hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MOPS, morpholinopropane sulfonic acid; 1799, bis-(hexafluoroacetonyl) acetone.

per ml: 1 mg cytochrome *c* oxidase, 2 to 3 mg hydrophobic protein, 25 μmoles phospholipid phosphorus.

Crude soybean phospholipids (asolectin) were obtained from Associated Concentrates, Woodside, N.Y., and were washed with acetone to remove the bulk of neutral lipids [10]. The phospholipid composition of asolectin, as determined by quantitative thin-layer chromatography, was, on a mole phosphorous basis: phosphatidylcholine 40%, phosphatidylethanolamine 33%, phosphatidylinositol 14%, lysophosphatidylcholine 5%, cardiolipin 4%. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purified from soybean phospholipids [8], and phosphatidylserine (PS) from ox brain [21]. These three phospholipids were at least 95% pure as judged by quantitative thin-layer chromatography.

Activity and respiratory control of the cytochrome oxidase vesicles were assayed in the KCl-HEPES-EDTA buffer, as described [4, 24]. The reconstituted vesicles (5–10 μl) were introduced into a 1-ml cell containing the above reaction mixture thermostated at 20 °C, and the rate of O_2 consumption was determined polarigraphically in a Gilson oxygraph. The respiratory control ratio is defined as the factor by which the oxidation rate is stimulated by addition of 1 μM valinomycin and 20 μM 1799. The control ratios ranged from 6 to 12, depending upon the preparation of cytochrome oxidase and the lipid composition of the vesicles. All rates were corrected for autooxidation in the absence of vesicles.

Fusion of cytochrome oxidase vesicles with hydrophobic protein vesicles was followed by the uncoupling effect of hydrophobic protein. The liposomes (5 μl of each type) were mixed together in 1 ml of assay buffer in the oxygraph chamber. The oxidase activity was determined by the constant slope of the oxygen trace. $CaCl_2$ (1–5 mM) was then added, and the rate of oxygen consumption, which increased over the next few minutes, was measured. At a specified time after the addition of Ca^{++}, valinomycin-1799 was added for the assay of the fully uncoupled rate of oxidation. The oxygen consumption rates immediately before and after addition of valinomycin-1799 were used to calculate the respiratory control ratio, and hence the degree of fusion, as a function of time after addition of Ca^{++}.

It should be noted that most fusion experiments were carried out directly in the oxygraph cell during measurement of the oxidation rate, and therefore in the presence of cytochrome *c* and ascorbate. Control experiments showed that the same phenomena were observed when the fusion took place in the absence of cytochrome *c*-ascorbate; i.e., when the assay was performed after, rather than during, fusion.

In order to quantify the fusion process by this assay (for instance, in studying the fusion as a function of lipid composition), it is not enough to compare the respiratory control ratios after a given time of fusion; this is because the *initial* respiratory control ratios vary under different conditions. Therefore, we define a parameter called the fractional fusion at time t, $f(t)$, as:

$$f(t)=\left(\frac{\mathrm{RCR}_0}{\mathrm{RCR}(t)}-1\right)\Big/\left(\frac{\mathrm{RCR}_0}{\mathrm{RCR}_h}-1\right). \tag{1}$$

where RCR_o is the respiratory control ratio without hydrophobic protein vesicles present, RCR(t) is the respiratory control ratio of the fusion mixture at time t, and RCR_h is the respiratory control ratio measured on hybrid vesicles made by reconstituting cytochrome oxidase and hydrophobic protein together. This definition is not arbitrary, but can be arrived at by considering the measured respiratory control ratio of an ideal mixed population of cytochrome oxidase vesicles, some not yet fused with hydrophobic protein vesicles and therefore coupled, and the rest fused and therefore uncoupled. The real situation during fusion is certainly more complicated than this simple model, and we do not wish to place undue quantitative emphasis upon the fractional fusion parameter; it merely serves as a convenient indicator by which different fusion conditions can be compared.

Electron-microscopy of the fusion process was performed by negative staining as previously described [22]. The liposomes for electron-microscopy were prepared by anaerobic sonication of 0.5 ml samples in small pyrex test tubes at 20–25 °C for 15 min in a small bath-type sonicator (model G1225P1, Laboratory Supplies Co., Hicksville, N.Y.). The liposomes were fused at a concentration of 1–2 mM phospholipid. After a given time in the presence of Ca^{++}, fusion was stopped by addition of EGTA (50% excess over Ca^{++}), and the sample was prepared for electron-microscopy. (Similar results were observed if the liposomes were prepared by cholate-dialysis [10] rather than by sonication.)

Results

Fusion of Cytochrome Oxidase Vesicles with Hydrophobic Protein Vesicles

Fig. 1 shows a fusion experiment in which cytochrome oxidase vesicles were fused with hydrophobic protein vesicles. The lipid composition of the vesicles was PS(40%)-PC(10%)-PE(50%). In the absence of Ca^{++}, but with hydrophobic protein vesicles present, the respiratory control ratio remained high and constant for at least five minutes, thus demonstrating that the proteins did not freely exchange from vesicle to vesicle through the aqueous phase. Upon addition of 2 mM Ca^{++}, a rapid decrease in the respiratory control ratio was seen, leveling off in about a minute to a low value. It is apparent from the Figure that this loss of control is not merely due to a deleterious effect of Ca^{++} since in the absence of hydrophobic protein vesicles Ca^{++} had no effect. Furthermore, if cytochrome oxidase vesicles were incubated in the presence of Ca^{++} with vesicles containing no protein at all, no loss of respiratory control was seen (data not shown).

This experiment shows that cytochrome oxidase and hydrophobic protein, initially in separate vesicles, eventually come to share the same vesicles. It does not tell us the specific mechanism by which this transfer occurs. However, vesicle-vesicle fusion is the most likely mechanism since, as mentioned in the introduction, it has already been demonstrated [7, 22] that fusion does occur under conditions similar to those used here. In addition, we have confirmed this by electron-microscopy, as shown below.

As can be seen from Fig. 1, the loss of respiratory control took place at a measurable rate; this is because of the low concentration of vesicles used in the fusion mixture (0.25 mM lipid P). When the reaction was carried out at higher vesicle concentrations, the loss was too fast to measure by this assay technique. It can also be seen that the fusion

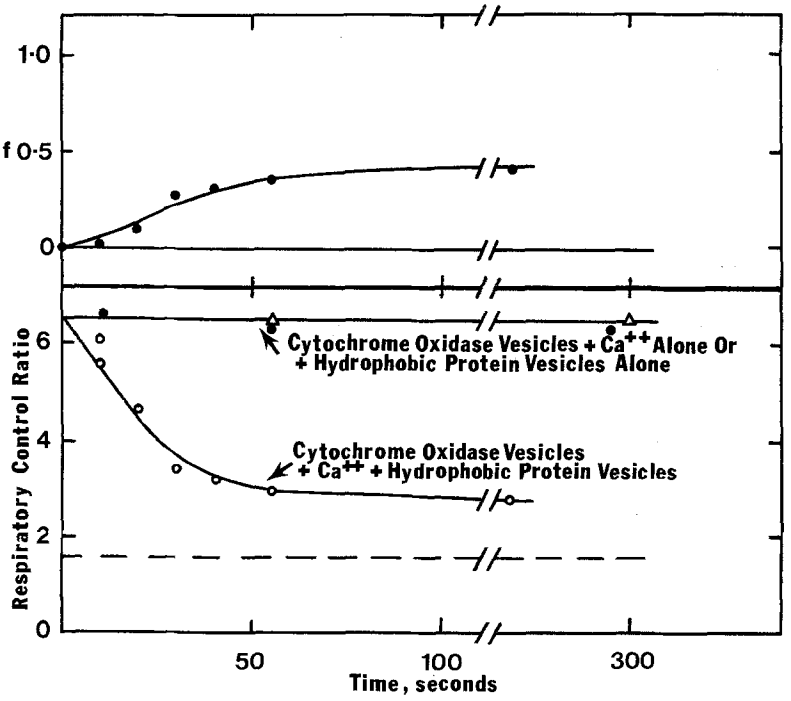

Fig. 1. Fusion of cytochrome *c* oxidase vesicles with hydrophobic protein vesicles. Vesicles were reconstituted and assayed as described in Materials and Methods using PS(40%)-PC(10%)-PE(50%) as the lipid mixture. *Lower graph*: Respiratory control ratio (RCR) is plotted as a function of time under the following conditions: (●) cytochrome oxidase vesicles+hydrophobic protein vesicles with no Ca^{++} present; (△)-cytochrome oxidase vesicles+2 mM Ca^{++}, with no hydrophobic protein vesicles present; (○)-cytochrome oxidase vesicles+hydrophobic protein vesicles+2 mM Ca^{++}. Dashed line shows RCR of hybrid vesicles formed by co-reconstitution of cytochrome oxidase and hydrophobic protein. *Upper graph*: Fractional fusion, $f(t)$, calculated from the RCR curve by Eq. (1)

did not go to completion, but leveled off before reaching complete randomization of the vesicles. (The initial rapid rate of fusion is a function of vesicle concentration, while the final level reached is not.) If the cytochrome oxidase vesicles and the hydrophobic protein vesicles were allowed to fuse separately by addition of Ca^{++}, no further fusion was observed on mixing of the vesicles (Table 1).

Finally, it should be understood that this method is not an assay of the growth in vesicle size upon fusion. Instead, it assays the fraction of cytochrome oxidase vesicles which have become uncoupled by fusion with one or more hydrophobic protein vesicles. In order to follow the increase in the vesicle size, other assay methods must be used, e.g., electron-microscopy or magnetic resonance [11, 13, 14, 22, 23].

Table 1. Loss of fusion of cytochrome oxidase vesicles with hydrophobic protein vesicles after prior fusion of separate vesicles

Sample	Respiratory Control ratio	Fractional fusion
COV	9.8±0.5	
COV+HPV	2.8±0.1	0.45±0.04
Fused COV	6.9±0.6	
Fused COV+fused HPV	6.6±0.4	<0.05

Cytochrome oxidase vesicles (COV) and hydrophobic protein vesicles (HPV) were reconstituted as described in Materials and Methods. Membrane lipid composition was PS(40%)-asolectin (60%). The vesicles labeled "fused" were fused separetely for 15 min in KCl-HEPES-EDTA buffer containing 2 mM Ca^{++}, at a concentration of 1.25 mM lipid phosphorous. One-tenth ml of each type of fused vesicle suspension was then added to the oxygraph chamber containing 0.8 ml of buffer. Ca^{++} was then added to a final concentration of 2 mM, and the respiratory control ratio was determined 3 min after addition of Ca^{++}.

Ca^{++} Requirement for Fusion. Fig. 2 shows that the fusion process, assayed as above, is controlled by the Ca^{++} concentration in the incubation medium, as expected from previous work [7, 22]. In membranes made from PS(40%)-PC(10%)-PE(50%) at Ca^{++} concentrations of 4 mM or greater, fusion proceeded to completion (f=1) within one minute. The Ca^{++}-sensitivity of the process was quite dependent upon the lipids used, with PS-PC membranes having a much lower tendency to fuse than

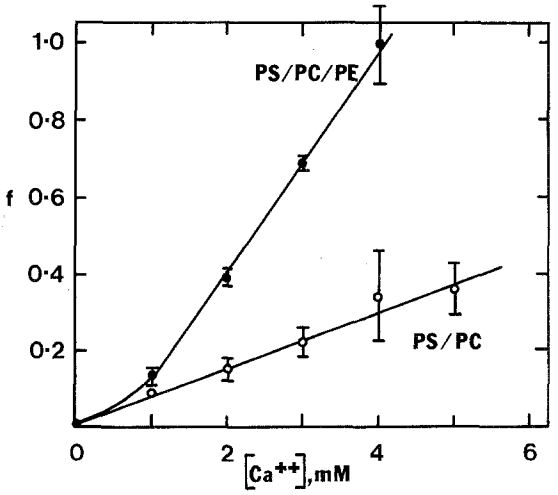

Fig. 2. Dependence of fusion on Ca^{++} concentration. Cytochrome oxidase vesicles and hydrophobic protein vesicles were allowed to fuse in the presence of varying concentrations of Ca^{++}. The fractional fusion was determined one minute after addition of Ca^{++}. Points, with standard error bars, represent triplicate determinations. Vesicle lipid compositions were (⊕) PS (40%)-PC(10%)-PE(50%); (⊖) PS(50%)-PC(50%)

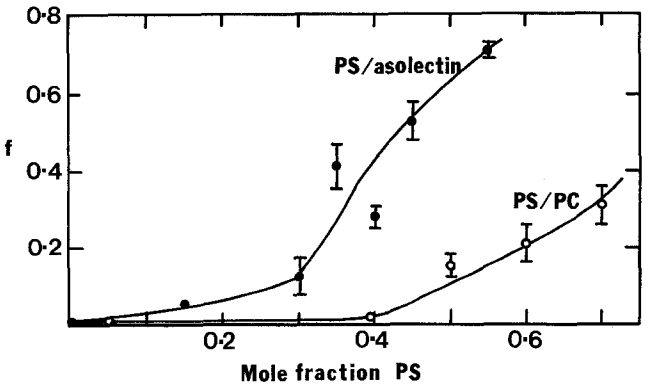

Fig. 3. Dependence of fusion on phosphatidylserine. Vesicles were made with PS-asolectin or PS-PC as described. Fusion was initiated by addition of 2 mM Ca^{++}, and the fractional fusion was determined after one minute

PS-PE-PC or PS-asolectin membranes. In all lipid mixtures containing PS, the rate and extent of fusion increased with Ca^{++} concentration, and in no case did we observe fusion in the absence of Ca^{++}. Fusion could also be induced by Mg^{++} in PS-asolectin mixtures, but Mg^{++} was required in concentrations at least twice as high as Ca^{++} for comparable results (data not shown).

Lipid requirement for Fusion. Ca^{++}-induced fusion showed a requirement for PS as a major membrane constituent as is illustrated in Fig. 3. In PS-asolectin membranes containing less than 20% PS, little if any fusion occurred in the presence of 2 mM Ca^{++}. Above 30% PS, however, fusion occurred readily in PS-asolectin mixtures, the effect becoming more pronounced with increasing PS concentration. The Figure further confirms that PS-PC membranes fuse with much greater difficulty than do PS-asolectin membranes, in agreement with the report [22] that PS-PC membranes require at least 50% PS before any fusion is discerned.

Why do PS-asolectin membranes fuse so much more readily than do PS-PC membranes? Fig. 4 demonstrates that the crucial factor in promoting the PS-asolectin fusion is PE. Here, we study the fusion of vesicles containing mixtures of PS-PC-PE, keeping the PS concentration constant at 40%. Variation of the PE and PC contents (with the sum of the two constant at 60%) showed that PE greatly facilitated the Ca^{++}-dependent fusion process. Values of fractional fusion in membranes made with PS-PE-PC to mimic the PE and PC composition of asolectin were comparable to and sometimes greater than values obtained with PS-asolectin vesicles. Unfortunately, it was not possible to examine quantitatively the fusion in PS-PE vesicles of varying PS content, because

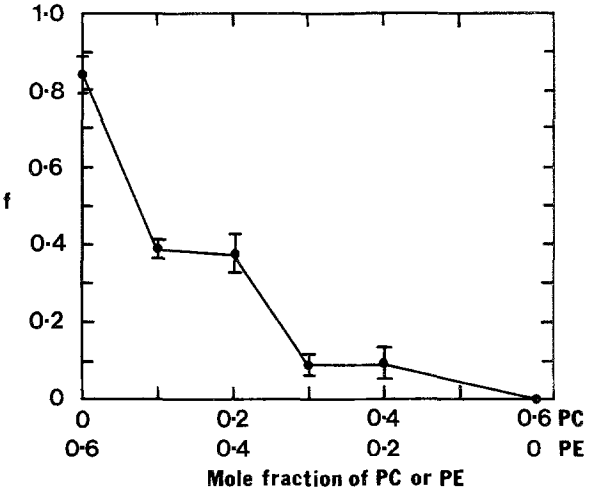

Fig. 4. Effect of phosphatidylethanolamine and phosphatidylcholine on fusion. Vesicles were made with 40% PS-60% (PE+PC) with varying amounts of PE and PC. Fusion was initiated by addition of 2 mM Ca^{++}, and the fractional fusion was determined after one minute. Points represent mean ± SE of triplicate or quadruplicate samples

the initial respiratory control ratios were rather low. At least 10% PS was still necessary for fusion, however.

Fusion of Cytochrome Oxidase Vesicles with "Naked" Liposomes

The previous assay of fusion depends upon the loss of respiratory control observed upon mixing cytochrome oxidase vesicles with hydrophobic protein vesicles under a well-defined set of conditions. Although the controls presented rule out possible uncoupling artifacts, it seemed desirable to develop an assay that depended on the development rather than the loss of respiratory control. In order to do this, we exploited an observation shown in Fig. 5. The respiratory control ratio of cytochrome oxidase vesicles is a sharp function of the fraction of PS in the membrane lipid mixture. At low PS concentrations, the cytochrome oxidase vesicles were extremely well-coupled, while as the PS concentration increased, the respiratory control ratio dropped smoothly. We were therefore in a position to perform an experiment complimentary to the cytochrome oxidase-hydrophobic protein fusion described above. Taking poorly coupled cytochrome oxidase vesicles made with 85% PS, we fused them with protein-free liposomes with 25% PS concentration. Upon fusion, the PS concentration of the cytochrome oxidase vesicles

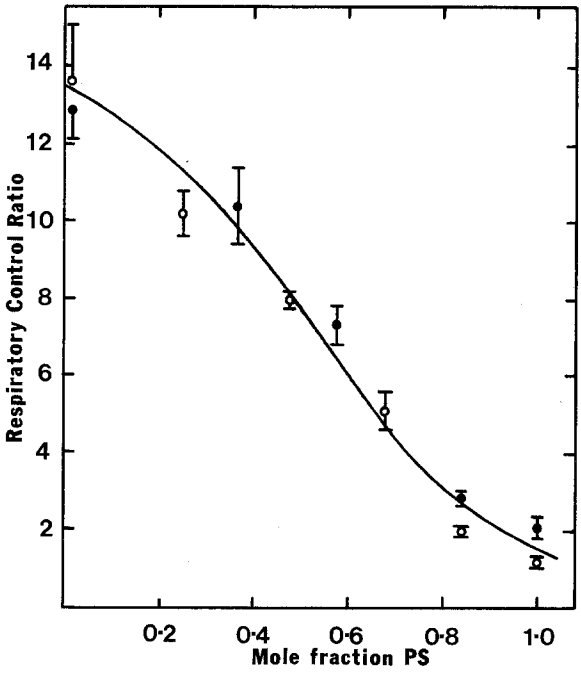

Fig. 5. Variation of respiratory control of cytochrome oxidase vesicles with phosphatidylserine concentration in phosphatidylserine-asolectin membranes. Cytochrome oxidase vesicles were made of varying PS-asolectin composition, and the respiratory control was determined in the presence of 2 mM Ca^{++}. Open and filled circles represent two different batches of cytochrome oxidase vesicles. Points plotted are mean ± SE of triplicate samples

should decrease as the lipids randomize, and an increase of respiratory control ratio should be seen. Fig. 6 gives the results of this experiment. In the absence of Ca^{++}, respiratory control remained low, as it also did in the presence of Ca^{++} but in the absence of added liposomes. When the cytochrome oxidase vesicles were incubated together with Ca^{++} and liposomes, an increase of respiratory control occurred within a few minutes. The extent of respiratory control attained increased with the amount of liposomes added.

The dependence of the final respiratory control ratio after fusion upon the ratio of liposomes to cytochrome oxidase vesicles is qualitatively that expected for fusion resulting in averaging of the membrane lipids. Quantitatively, however, the final ratios fall well below the values expected for complete fusion, i.e., total randomization of the membrane lipids. For instance, for equal concentrations of cytochrome oxidase vesicles and liposomes, the average PS concentration would be 55% on complete mixing, corresponding to a respiratory control ratio of about 7 (Fig. 5); however, the ratio in fact reached only 3, as shown

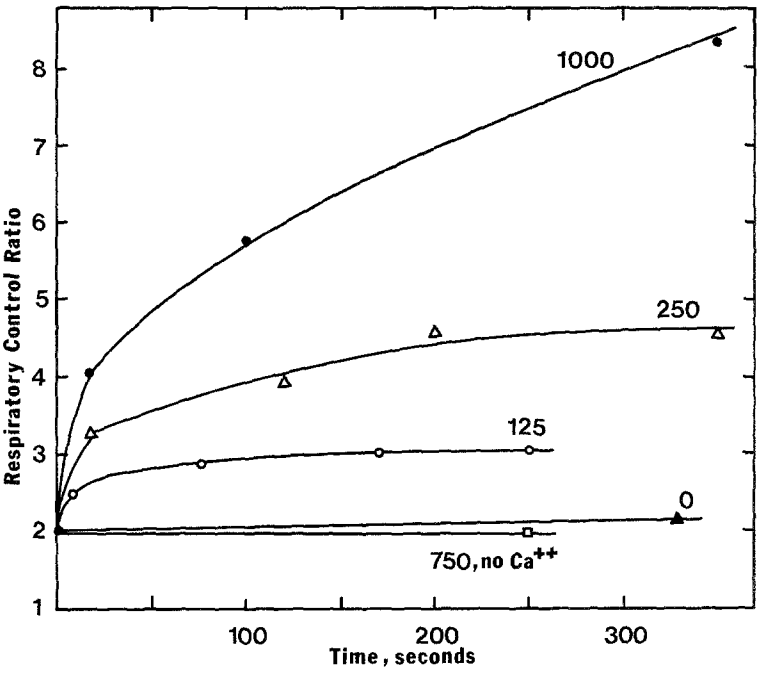

Fig. 6. Fusion of cytochrome oxidase vesicles with "naked" liposomes. Cytochrome oxidase vesicles were made with PS(85%)-asolectin(15%). Liposomes (formed by solubilization in 1% cholate followed by dialysis) were PS(25%)-asolectin(75%). Fusion was initiated with 2 mM Ca^{++}, and respiratory control ratio was determined as a function of time. Cytochrome oxidase vesicles (5 μl) containing 125 nmoles of phospholipid were mixed in the oxygraph cell with varying amounts of liposomes: (▲) no liposomes added; (○) 125 nmoles; (△) 250 nmoles; (●) 1000 nmoles; (□) 750 nmoles liposomes added, but without Ca^{++}

in Fig. 6. This is not really surprising, for at least two reasons. First, we already demonstrated (Table 1) that the fusion process slows down after an initial burst and thus fails to attain complete randomization of the lipids; second, in these experiments, it is more likely that the cytochrome oxidase vesicles will preferentially fuse with each other, since their PS concentration is much higher than that of the liposomes.

Electron-Microscopy

Figs. 7 and 8 confirm that the above assays are in fact measuring fusion. The growth in size of liposomes made from PS(40%)-PE(60%) upon addition of 2 mM Ca^{++} is apparent in Fig. 7. The liposomes increased from an average diameter of about 200 Å to diameters in excess of 2000 Å. No increase in diameter was seen between 1 hour and 24 hours

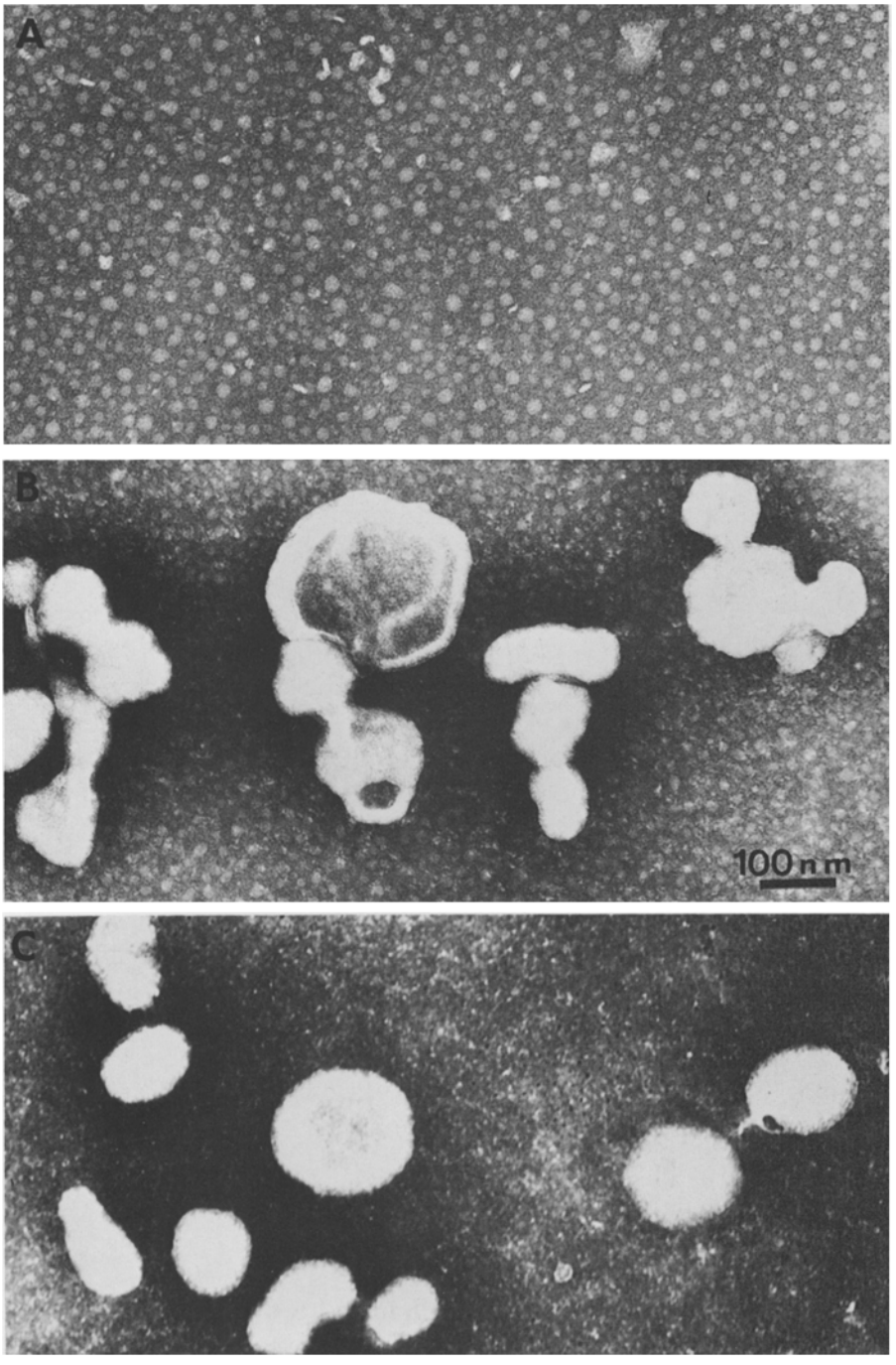

Fig. 7. Negative-stain electron-microscopy of 40% phosphatidylserine-60% phosphatidylethanolamine liposomes. Samples were prepared as described in Materials and Methods. (*A*) Control liposomes before fusion. (*B*) Liposomes fused for 1 hour in presence of 2 mM Ca^{++}. (*C*) Liposomes fused for 24 hr in presence of 2 mM Ca^{++}

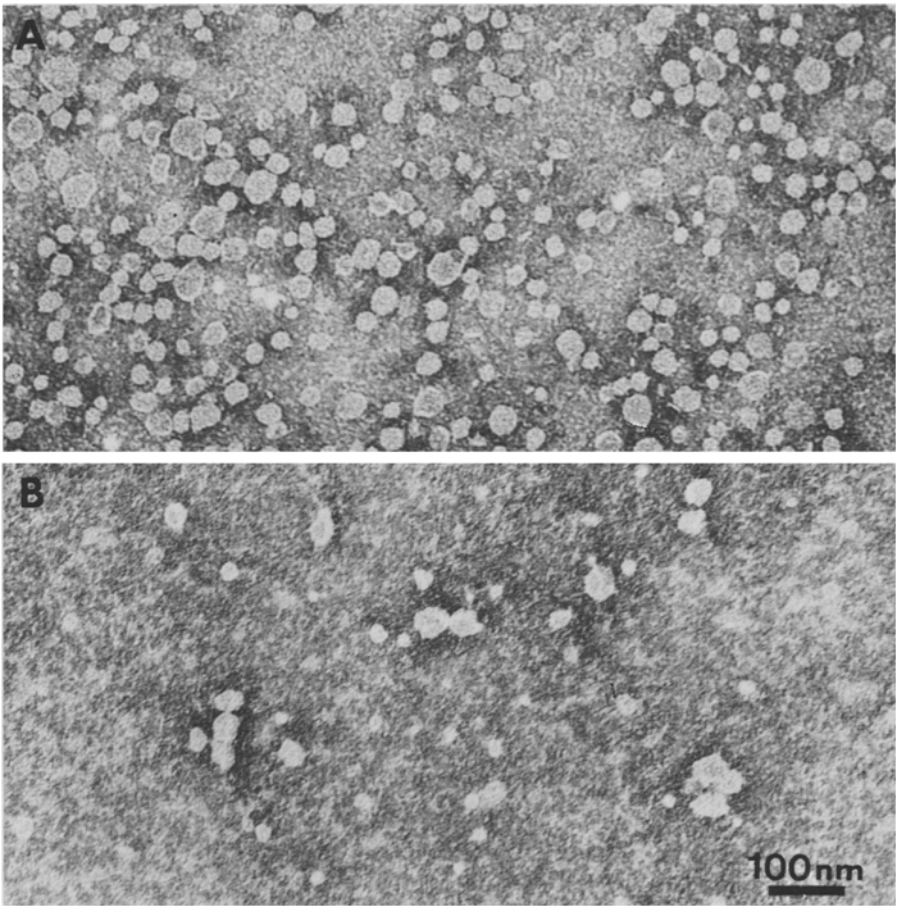

Fig. 8. Negative-stain electron-microscopy of 40% phosphatidylserine-60% phosphatidylcholine liposomes. (*A*) Control liposomes before addition of Ca^{++}. (*B*) Liposomes incubated 1 hour in presence of 3 mM Ca^{++}

of fusion. Fig. 8 confirms that PS(40%)-PC(60%) liposomes did not fuse even after 1 hour of exposure to 3 mM Ca^{++}.

Discussion

The Ca^{++}-induced fusion of PS-containing liposomes has been established by a number of workers [7, 14, 22]. However, the methods by which the fusion is assayed require specialized equipment and restrict the lipid mixture employed to fairly homogeneous, synthetic preparations. The assay technique introduced here obviates these two difficulties. How-

ever, the lipid mixtures that can be used are restricted to those which are suitable for the reconstitution of cytochrome oxidase with respiratory control. This study also shows that the Ca^{++}-induced fusion process can be used to transfer amphipathic proteins between membrane systems.

The data here raise several questions of interest, but the answers require further work. First, what is the driving force for Ca^{++}-induced fusion? The most likely explanation is that the high curvature of the initially small vesicles gives rise to a high surface energy which can be lowered by the growth of the vesicles by fusion. When the size of the vesicles increases sufficiently, fusion ceases because of the lack of a thermodynamic driving force, as has been treated theoretically [3]. A second prerequisite for fusion may be a Ca^{++}-induced lateral phase separation of the PS, as several studies have suggested [2, 6, 20, 22]. It would therefore be important to study the phase separation process in PE-PS liposomes in comparison to that in PC-PS liposomes in order to find a possible explanation for the marked difference in fusibility of the two systems.

Finally, the phenomena reported here may be useful as a general method for creating hybrid vesicles for biochemical studies of reconstituted membrane proteins. As has been discussed [26], reconstitution conditions which are suitable for one protein may not be suitable for another, making co-reconstitution difficult. The fusion procedure eliminates this problem. In addition, the creation by fusion of vesicles with large internal volumes would enhance the sensitivity of assays concerned with reconstituted membrane transport systems.

One important question which needs to be answered is the physiological relevance of all the model system fusion processes. For instance, neurotransmitter release from presynaptic vesicles involves a fusion process between membrane surfaces of high curvature [12], requiring the participation of Ca^{++} [15], in cells whose membranes contain substantial amounts of anionic lipids [21]. Such an anology is appealing but the details of the process *in vivo* are undoubtedly more complicated than those of this simple model system.

Previous studies of Ca^{++}-induced fusion of liposomes were performed exclusively with PS-PC mixtures. Since very high ($>50\%$) concentrations of PS were required for fusion to take place, the physiological importance of the model system was placed in doubt. In this report, we have shown that when PS-PE-PC lipid mixtures are used, fusion proceeds with PS concentrations that are closer to physiological levels. It may be possible to lower the PS requirement even further by using mixtures of other

lipids found in biological membranes. Thus, if membrane fusion participates in physiological processes, the complexity of the phospholipid composition of natural membranes may in part be explained.

This work was done during the tenure of a Research Fellowship (to C.M.) of the Muscular Dystrophy Association and was also supported by a grant (to E.R.) from the National Cancer Institute, D.H.E.W., #CA-08964. We are grateful to Dr. John Telford for performing the electron-microscopy.

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