Fusion of Phospholipid Vesicles with Planar Phospholipid Bilayer Membranes

I. Discharge of Vesicular Contents across the Planar Membrane

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ABSTRACT Multilamellar phospholipid vesicles are introduced into the *cis* compartment on one side of a planar phospholipid bilayer membrane. The vesicles contain a water-soluble fluorescent dye trapped in the aqueous phases between the lamellae. If a vesicle containing n lamellae fuses with a planar membrane, an $n-1$ lamellar vesicle should be discharged into the opposite *trans* compartment, where it would appear as a discernible fluorescent particle. Thus, fusion events can be assayed by counting the number of fluorescent particles appearing in the *trans* compartment. In the absence of divalent cation, fusion does not occur, even after vesicles have been in the *cis* compartment for 40 min. When $CaCl_2$ is introduced into the *cis* compartment to a concentration of $\geq$20 mM, fusion occurs within the next 20 min; it generally ceases thereafter because of vesicle aggregation in the *cis* compartment. With approximately 3×10^8 vesicles/cm^3 in the *cis* compartment, about 25–50 fusion events occur following $CaCl_2$ addition. The discharge of vesicular contents across the planar membrane is the most convincing evidence of vesicle-membrane fusion and serves as a model for that ubiquitous biological phenomenon—exocytosis.

INTRODUCTION

Exocytosis is a ubiquitous phenomenon involving the fusion of intracellular vesicles with plasma membrane and the subsequent extracellular release of the vesicular contents. It is the fundamental process in such diverse phenomena as transmitter release at chemical synapses (Katz, 1969), secretion by endocrine and exocrine glands (Douglas, 1974; Palade, 1975), mucocyst discharge in *Tetrahymena* (Satir et al., 1973), and raising of the fertilization membrane after union of sperm and egg (Epel and Vacquier, 1978). Exocytosis is one of three major schemes that cells use to transport lipid-insoluble molecules across plasma membranes; the others are pinocytosis and intramembranous proteins

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functioning as channels, carriers, or pumps. Planar phospholipid bilayer membranes incorporating peptides, proteins, or other exotica have been used extensively as model systems for this last transport scheme (Finkelstein and Mauro, 1977). In this report, we use phospholipid vesicles in combination with planar phospholipid bilayer membranes as a model for exocytosis. The area of vesicle-membrane fusion in exocytosis has been widely reported to lack intramembranous particles (Satir et al., 1973; Friend et al., 1977; Lawson et al., 1977; Pinto de Silva and Noqueira, 1977), suggesting that our model system may be biologically relevant to the normal cellular process.

Most studies of phospholipid bilayer fusion have focused on the interaction among vesicles. Fusion between vesicles can occur under appropriate conditions (Papahadjopoulos et al., 1976; Holz and Stratford, 1979), but considerable care is required to preclude such competing processes as vesicle aggregation and lipid exchange (Martin and MacDonald, 1976). Although vesicle-vesicle fusion can be studied profitably, quantitative kinetic data are difficult to obtain, and conditions across the bilayer membranes cannot be manipulated readily.

Fusion of vesicles to planar bilayer membranes, on the other hand, has presented until now its own drawbacks. Previously reported attempts to study the fusion of phospholipid vesicles to planar membranes have relied on monitoring the incorporation into the planar membrane of some membrane-associated marker from the vesicular membrane such as phospholipids (Cohen and Moronne, 1976), antibiotics (Moore, 1976), and fluorescent probes (Pohl et al., 1973). Nonfusion exchange of marker, however, can also lead to its transfer into the planar membrane. In fact, all previous reports have either concluded (Cohen and Moronne, 1976), or not ruled out that incorporation of the marker occurred via its transfer by a process other than fusion between the two membranes. Here, we present substantive proof for fusion of vesicles with planar bilayer membranes by showing transfer of vesicular contents to the aqueous compartment on the other side of the planar membrane. In the companion paper (Cohen et al., 1980) we report correlative, confirmatory observations of fusion and consider important, regulatory parameters of the process.

PRINCIPLES OF THE METHOD

We use two independent criteria for scoring fusion events. One, like those mentioned above, is the appearance in the planar membrane of a membrane-associated marker originally present in the vesicle membrane; this is considered in the succeeding paper. The second, which is the subject of this paper, is the appearance of vesicular contents on the side of the planar membrane opposite (*trans*) to that side (*cis*) on which vesicles were added. This is a new experimental criterion not previously employed, presumably because of the difficulties in defining a detectable marker.

For example, one obvious choice for a marker is an impermeant radioactive molecule, such as [^{14}C]sucrose, trapped inside the vesicle. But unless enormous numbers of fusion events occur or lead underwear is tolerated, this is not a reasonable choice, because at concentrations of 10 Ci/ml, the contents of each

0.1-μm diam vesicle contain only 0.01 dpm. A second possibility is a multiplicative system, such as an enzyme, for which small numbers of molecules can be measured. For instance, as little as one molecule of β-galactosidase is detectable (Rotman, 1961), and the solubility of the enzyme is such that several β-galactosidase molecules could be included in a 0.1-μm vesicle. The sensitivity of the detection method, however, requires a sample of at most 1 nl, whereas tens of microliters are the minimal volume feasible on the *trans* side of a planar membrane; consequently, only a minute fraction of the volume in the *trans* compartment can be assayed. Thus, again, unless enormous numbers of fusion events occur, this marker is also unsuitable. Although we have not exhausted all possible multiplicative markers in our considerations, an appropriate choice is not obvious.

We have solved the marker problem by exploiting the multilamellar nature of hand-shaken liposomes. Lipids swollen in a solution containing an aqueous-soluble fluorescent dye trap the dye in all aqueous compartments of the liposome. If an n lamellar liposome fuses with a planar bilayer, an $n-1$ lamellar liposome should be discharged into the *trans* compartment. With n on the order of 100, the "secreted" liposome would still contain large amounts of dye at the original high concentration; the contents of the aqueous phase undergo no dilution. Such a liposome appears as a readily detectable bright particle under the fluorescent microscope. We thus have an extremely sensitive assay—indeed, one capable of detecting a single fusion event. Note that any dye escaping from the vesicles in the *cis* compartment and diffusing across the planar membrane is diluted out in the *trans* compartment and undetectable there. Only dye "packaged" in liposomes is scored.

By forming multilamellar liposomes with a suitable ion-permeable channel (see following paper) incorporated in their membranes as well as a fluorescent dye trapped in their aqueous compartments, we can simultaneously measure the appearance of channels in the planar membrane and dye-containing liposomes in the *trans* compartment. Thus the two events associated with fusion can be assayed: (*a*) the incorporation of vesicular membrane into the planar membrane and (*b*) the transfer of internal vesicular contents across the planar membrane (Fig. 1).

MATERIALS AND METHODS

Planar membranes were formed at room temperature (22–24°C) by the brush technique of Mueller et al. (1963) across a 1-mm^2 hole in a Teflon partition separating two Lucite compartments. Each compartment contained 100 mM NaCl, 10 mM MES (2-[*N*-morpholino]-ethane sulphonic acid), and 0.1 mM EDTA, pH 6.0. (This salt solution is henceforth referred to as "buffer.") The membrane-forming solution was an *n*-decane solution of either 5% asolectin, or a mixture of 2% bovine phosphatidylserine + 0.5% egg phosphatidylcholine.

Multilamellar liposomes were prepared fresh daily by the method of Bangham et al. (1974): 3 mg of lipid (20% phosphatidylserine, 80% phosphatidylcholine) dissolved in ~4 ml of 2:1 chloroform-methanol was dried down by rotary evaporation. In those experiments in which the voltage-dependent anion channel (VDAC) was incorporated in the vesicle membranes, this lipid mixture, containing 4% by weight of protein, was dissolved in hexane (Cohen et al., 1980) and dried down by rotary evaporation. 6-

Carboxyfluorescein was incorporated into liposomes by adding 1 ml of dye solution (32 mM 6-carboxyfluorescein, 52 mM NaCl, 10 mM MES, and 0.1 mM EDTA, pH 6.0) containing three glass beads to the dried lipid, swirling the mixture by hand for 2 min, and setting on ice for 30 min. Liposomes were then separated from excess dye on a Sephadex G-25 (coarse) column (9-mm diam × 30-cm length) with "buffer" as eluent (Sephadex, Pharmacia Fine Chemicals, Piscataway, N.J.). The void volume was collected in 0.6-ml samples, and the three tubes with the most turbidity were pooled. We chose 6-carboxyfluorescein because of its high fluorescent quantum yield and low membrane permeability (Weinstein et al., 1977).

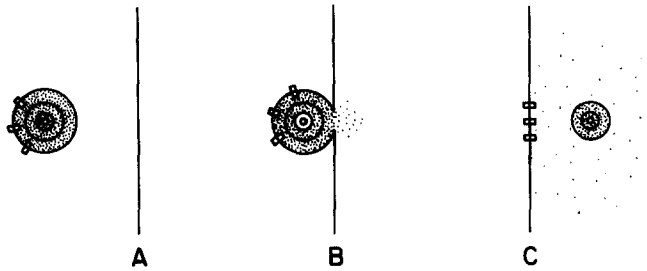

FIGURE 1. Diagram of our conception of the events associated with fusion. (A) A multilamellar vesicle containing ion-permeable channels in its membranes (open rectangles shown only in the outer lamella) and fluorescent dye (dots) trapped in the aqueous regions between the lamellae is present in one of the two aqueous compartments (*cis*) separated by a planar phospholipid bilayer membrane. (B) The outer lamella of the vesicle has coalesced with the planar membrane. (C) Fusion is complete. The ion-permeable channels originally present in the outer lamella of the vesicle are now in the planar membrane; the contents of the vesicle are discharged across the planar membrane into the opposite (*trans*) aqueous compartment. Note that dye released from between the two outermost lamellae into the *trans* compartment is diluted into a large volume and is not detectable. On the other hand, a vesicle having one fewer lamella than the original one but still containing trapped fluorescent dye at its original high concentration is now in the *trans* compartment and is observable as a fluorescent particle.

The dye-containing liposomes were counted on a fluorescence-activated cell sorter (FACS II from Becton-Dickinson & Co., Rutherford, N.J.). The sample was first surrounded by sheath fluid ("buffer") and then forced through a 60-μm ruby nozzle. The stream then passed through the path of a 1-W argon laser (488 nm). At right angles to both the laser and the stream was a microscope focused on the intersection point. A photomultiplier tube measured the fluorescent signal after scattered light was removed by a Ditric 520 interference filter (Ditric Optics, Inc., Marlboro, Mass.) in series with a Schott glass OG 495 cutoff filter (Schott America, New York). When a fluorescent particle passed through the laser beam, a pulse of light was detected. Scattered light was simultaneously measured with a photodiode. Both signals were processed by a pulse-height analyser and displayed as a histogram of frequency vs. fluorescence or light scattering intensity. By analysing a concentrated solution of liposomes, we obtained their scatter-fluorescence characteristic. This information was used to set windows on the counter to distinguish liposomes from dust particles.

Fig. 2 illustrates the fluorescence distribution of 10^7 liposomes. Because the fluores-

cence of a liposome is directly proportional to its volume, Fig. 2 also shows the distribution of the number of liposomes vs. volume. Unfortunately, we do not have absolute numbers for the volumes of the liposomes. We note, however, that the number of liposomes increases with decreasing volume. The cutoff for fluorescence, below which liposomes are not detected, is arbitrary; we have always seen increasing numbers of liposomes at the low-fluorescence end, no matter at what sensitivity we set the fluorescence threshold. In practice, the detected fluorescence was set to be as sensitive as possible without introducing counts from nonliposomal fluorescing particles.

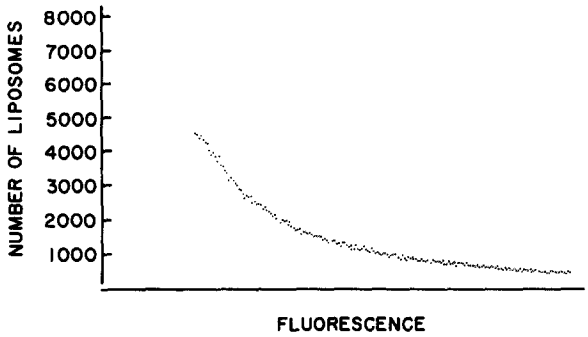

FIGURE 2. Fluorescence histogram of 10^7 vesicles (liposomes). Fluorescence is in arbitrary units; the direction of increasing intensity is from left to right. The cutoff at lowest intensity is determined by background fluorescence in the cell sorter; the pile-up of counts of liposomes whose fluorescence exceeds that measured in the last channel (i.e., at highest intensity) is not shown.

Because of the small number of particles that are transferred across the membrane into the *trans* compartment, great care had to be taken to reduce background fluorescent noise. In addition to adjusting the window settings, it was necessary to filter the buffer extensively by serially filtering through an asbestos-glass filter, a Nuclepore 0.2-μm filter, a Nuclepore 0.08-μm filter, an Amicon XM50 filter, and finally an Amicon PM 10 filter (Nuclepore Corp., Pleasanton, Calif.; Amicon Corp., Lexington, Mass.). New Teflon tubing was used in the cell sorter for each experiment, and its flow-resistor was by-passed.

In the actual experiment, the *trans* compartment (1 ml) was connected to a peristaltic pump by Teflon tubes, allowing for perfusion of the compartment at any time. Before making a membrane, the compartment was perfused with 30 ml of filtered buffer. The membrane was then formed, the *trans* compartment was again perfused with 30 ml of filtered buffer, and a 2-ml sample was taken as the blank. The chamber then sat 20 min, another 2-ml sample was removed from the *trans* compartment, and $\sim 10^9$ liposomes (0.15 mg) added to the *cis* compartment (volume $\approx$3 ml). After 20 min, a second 2-ml sample was taken (*trans*) and $CaCl_2$ was then added to the *cis* compartment to a given concentration. After another 20 min a third 2-ml sample was taken (*trans*), and additional samples were then taken at intervals throughout the course of the experiment. Control experiments were performed with liposomes in the absence of divalent cation, and with both dye and calcium added to the *cis* compartment in the absence of liposomes.

Egg phosphatidylcholine used for membrane formation was obtained from Avanti Biochemicals, Inc. (Birmingham, Ala.); egg phosphatidylcholine used for making

vesicles was either from Avanti or (type V-E) from Sigma Chemical Co. (St. Louis, Mo.); bovine phosphatidylserine was from Avanti. Asolectin, obtained from Sigma as lecithin type II-S, was washed with acetone and ether to remove neutral lipids according to the method of Kagawa and Racker (1971). Aqueous solutions were prepared from glass distilled water. 6-Carboxyfluorescein was purchased from Eastman Organic Chemicals (Rochester, N.Y.) and recrystallized. All salts were reagent grade and used as obtained.

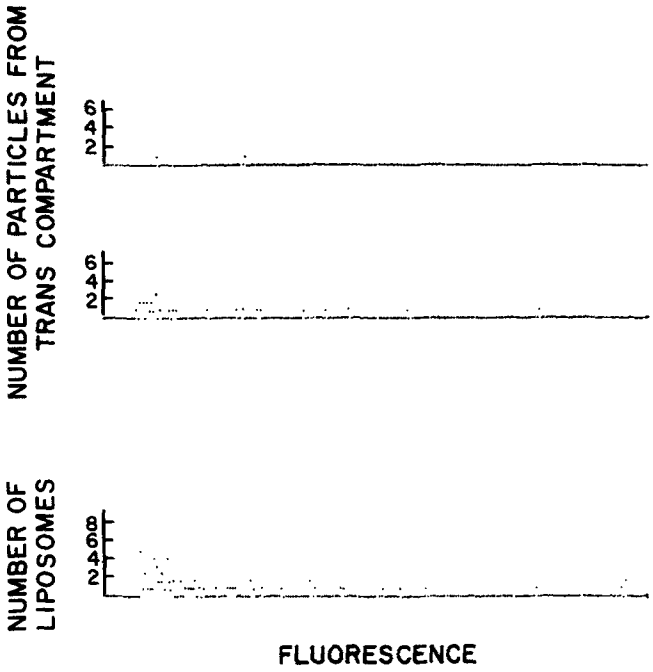

FIGURE 3. Results of a typical fusion experiment. A planar phospholipid bilayer membrane (formed from an *n*-decane solution of 2% phosphatidylserine + 0.5% phosphatidylcholine) separates symmetrical salt solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0). Vesicles (liposomes) were added to the *cis* compartment to a concentration of 4×10^8 detectable particles/milliliter. 20 min later, the 1-ml *trans* compartment was perfused with 2 ml of solution and the top histogram was obtained on the 2 ml of perfusate. Only two fluorescent particles are found, a number indistinguishable from the background value obtained before liposomes were added to the *cis* compartment. At this time $CaCl_2$ was added to the *cis* compartment to a concentration of 20 mM $CaCl_2$. 20 min later, the 1-ml *trans* compartment was again perfused with 2 ml of solution and the middle histogram was obtained on the 2 ml of perfusate. 28 fluorescent particles are now found. The bottom histogram is that of 1 ml of a 10^8 dilution of the stock liposome solution (81×10^8 liposomes/1 ml) originally added to the *cis* compartment; 72 particles are counted (instead of the theoretical 81). Note that there is no obvious difference in the distributions in the middle and bottom histograms. Since fluorescence is proportional to the volume of the liposome, this indicates that there is no striking size dependence of fusion for the liposome population in our experiments.

RESULTS

The results of a typical experiment are shown in Fig. 3. No vesicles are detected in the *trans* compartment after vesicles have been present in the *cis* compartment for 20 min. Indeed, no vesicles are seen even after 40 min. Following addition of $CaCl_2$ to a concentration of 20 mM in the *cis* compartment, vesicles appear in the *trans* compartment within the first 20 min and then cease to appear. The fluorescence histogram of the vesicles appearing in the *trans* compartment is the same as that of the vesicles in the *cis* compartment, thus indicating no obvious size dependence for fusion. Fig. 4 summarizes the results of these experiments. The number of fusion events is actually larger

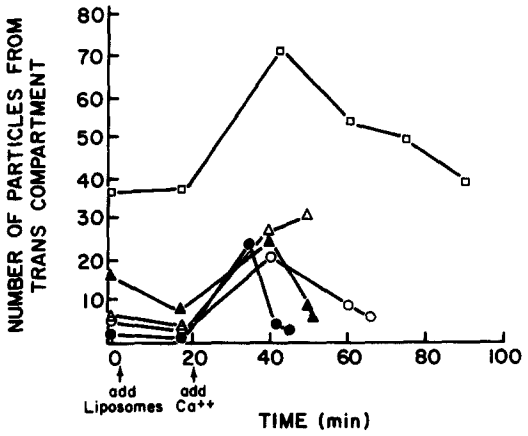

FIGURE 4. Summary of fusion experiments. The protocol for each experiment was as described in the text and in the preceeding figure. At the first arrow vesicles (liposomes) were added to the *cis* compartment to a concentration of about 3×10^8 countable particles/milliliter. Note that no significant number of particles above background are found in the *trans* compartment 18 min later. At the second arrow $CaCl_2$ was added to the *cis* compartment to a concentration of 20–40 mM. In all instances there is an increase in the number of countable fluorescent particles in the *trans* compartment. Note that since the entire contents of the *trans* compartment are sampled for each point, the subsequent decrease in countable fluorescent particles results from virtually all of the fusion events occurring within 20 min after $CaCl_2$ addition to the *cis* compartment. Filled symbols represent experiments in which the liposomal membranes contained voltage-dependent anion channels (VDAC) (see following paper [Cohen et al., 1980]); open symbols represent experiments in which the liposomal membranes did not contain VDAC.

than recorded in Fig. 4 because (*a*) the cut-off by the cell-sorter at small vesicle sizes precludes the counting of their fusions, and (*b*) control experiments in which ~100 countable vesicles were introduced into the *trans* compartment revealed that only 70% were recovered. The loss was presumably due to absorption of vesicles to chamber walls, perfusion tubing, etc.

DISCUSSION

We noted in the Introduction that there have been previous claims of successful fusion of lipid vesicles with planar membranes based on the incor-

poration into the planar film of markers originally present in the vesicle membrane, but that all of these claims were subject to an alternative, and more probable, interpretation—namely, transfer of markers by a nonfusion process. In the following paper (Cohen et al., 1980) we also employ a membrane marker, and present strong evidence that its incorporation into the planar membrane is truly a proper assay of fusion. Nevertheless, such an assay is by itself always subject to doubts; the assay described in this paper is much more convincing. The appearance in the *trans* compartment of "packaged" fluorescent material, originally contained in liposomes added to the *cis* compartment, directly argues for the occurrence of fusion events. We believe it is the most convincing evidence for vesicle-planar membrane fusion. Moreover, if the goal of vesicle-planar bilayer fusion experiments is to study the mechanism of exocytosis, the demonstration of transfer of internal vesicular contents across the planar membrane is an absolute prerequisite to further investigations. (The possibility that the liposomes traversed the bilayer without fusion is unlikely. In that case a large area of discontinuity would have to occur in the hydrocarbon region of the bilayer for the polar head groups of the liposomes to pass through. Such events would generate large *transient* increases in conductance associated with each discontinuity; we have never observed such transients.) Our success in observing transfer of internal contents resulted from the exquisite sensitivity of the assay. With only about 50 fusion events occurring, it is necessary to score individual events, as permitted by our method.

Fig. 4 summarizes the five successful[1] experiments that were performed. This small number reflects not a lack of diligence and zeal on the part of the authors, but rather their finite capacity for heroics. Reducing background fluorescent noise to an appropriate level is a tedious and prolonged procedure required for each single experiment. Membrane breakage at any time between addition of the vesicles to the *cis* compartment and subsequent sampling of total *trans* compartment contents 40 min later (20 min after Ca^{++} addition to the *cis* compartment, which in turn followed sampling of total *trans* compartment contents) aborted the experiment and required resumption of the extensive cleansing rituals. Furthermore, frequent malfunctions during the course of an experiment of the fluorescence-activated cell sorter, which was extensively modified to achieve the proper levels of sensitivity required for these experiments, spelled the demise of otherwise successfully completed experiments, inasmuch as samples could not be stored for later counting, because of losses in countable liposomes due to absorption, lysis, etc.

From this abridged account, it is apparent that the demonstration of the transfer of vesicular contents across the planar membrane was not used routinely for studying vesicle-membrane fusion, although it constitutes the most convincing proof of that phenomenon. In actual fact, we first found "fusion" using the membrane marker technique described in the following

[1] By "successful" experiments we do not mean experiments chosen because of agreement with a preconceived theory. A successful experiment is simply one that was not aborted by any of the difficulties alluded to in the following part of the text and also had a blank value less than 50 particles/2 ml.

paper (Cohen et al., 1980), and then confirmed our interpretation by the experiments described here. Once the membrane-marker technique had been thus validated, all subsequent studies of the parameters controlling fusion were performed using that method and are detailed in the account that follows.

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