

VOLUME THREE

**MEMBRANE
STRUCTURE AND
FUNCTION**

Edited by

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CHAPTER TWO

Fusion of Isolated Biological Membranes

A Tool to Investigate Basic Processes of Exocytosis and Cell-Cell Fusion

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1 INTRODUCTION

An important function of cellular and subcellular membranes is to create and maintain cytoplasmic and extracytoplasmic compartments. However, dynamics of cellular activities require exchange of material between the different compartments. This is achieved in several ways:

- 1 Carriers, channels, or special membrane junctions (nexuses) transport ions and small molecules (see also Chapter 4 of this volume on intercellular communication).
- 2 Cotranslational transfer apparently is responsible in several cases for compartmentation of secretory proteins.
- 3 Membrane fusion permits the transfer of even high molecular weight substances as known for the exocytotic secretion of hormones, neurotransmitters, and enzymes by a variety of cells. Membrane fusion is also involved in the development of cytoplasmic continuity between cells (e.g., myoblasts).

The complexity of events in cells during secretion or muscle development has prevented major progress in the understanding of the mechanism of

fusion of biological membranes. Even the trigger for this reaction was unknown up to now. The strategic role of Ca^{2+} during secretion is well documented (for review see ref. 1) and the initiation of exocytotic membrane fusion has been attributed to this ion (for review see ref. 2).

Alternative hypotheses for the action of Ca^{2+} during secretion have also been suggested. These include (i) reduction of the viscosity of the cytoplasm, (ii) interaction with the microtubular-microfilamentous system, (iii) modification of the activity of enzymes, and (iv) change of the surface charge of membranes (for reviews see refs. 3–5). To overcome the difficulties arising in work with intact cells, several model systems for membrane fusion have been developed such as liposome-liposome fusion, liposome-cell fusion, and artificially induced cell-cell fusion (see Section 3 of this chapter).

Membrane fusion during exocytosis is experimentally accessible using isolated membranes which are involved in the physiological fusion reaction. Thus fusion could be studied in the absence of other cytoplasmic components. Another cellular system where membranes undergo fusion is the formation of multinucleated myotubes from mononucleated myoblasts during embryonic myogenesis. The formation of this syncytium (myotubes) has been investigated using myoblasts in culture (for review see ref. 6). However, these experiments did not lead to a ready interpretation of the basic process of membrane fusion because of interfering processes such as motility, aggregation, and metabolism of the cells. We have therefore used isolated cell membranes of myoblasts in order to study the mechanism of cell-cell fusion. As a simplification of the natural membranes, we have also used liposomes, prepared from lipids extracted from isolated secretory vesicle membranes.

In this chapter the fusion of various biological membranes as well as liposomes is described and compared with model systems. The close similarities between fusion of isolated biological membranes and exocytotic secretion or myotube formation are emphasized.

2 EXPERIMENTAL APPROACH

Exocytotic release of cell products involves the fusion of a secretory vesicle with the cell membrane, thereby allowing the vesicle contents to communicate with the extracellular fluid.

The investigation of attachment and fusion of secretory vesicles with isolated cell membranes (“*in vitro* exocytosis”) involves several pitfalls. Excluding the problem of low yield and moderate purity of isolated cell membrane fractions, these membranes tend to form closed vesicles which are predominantly rightside out. Thus the chance for secretory vesicles to

gain access to the cytoplasmic surface of the cell membrane is limited. Even if it were possible to obtain cell membrane vesicles which are mainly oriented inside out, the assay of membrane fusion would be difficult. No morphological methods to discriminate unequivocally between the different membranes are yet available. The most straightforward indicator of fusion would be the release of the content of secretory vesicles to the surrounding medium, as suggested recently (7). However, if secretory vesicles undergo fusion with inside-out cell membrane vesicles, these substances would be trapped within the interior of the fusion product.

In an increasing number of cell types, after stimulation, serial fusions of secretory vesicles with the cell membrane have been observed. By this process, termed *compound exocytosis*, the content of several secretory vesicles can be released through one orifice in the cell membrane (Figure 1). Compound exocytosis is most prominent in mast cells (8) where it can even be followed cinematographically in the light microscope (1). Compound exocytosis also occurs in the acinar cells of the parotid gland (9), in exocrine (10) and endocrine pancreatic cells (11), in the adeno-hypophyseal and neurohypophyseal cells (12, 13), as well as other neurosecretory cells (14). All these examples clearly indicate that during compound exocytosis secretory vesicles interact with each other in the same way as the secretory vesicles interact with the cell membrane. Thus exocytotic membrane fusion can be followed with isolated secretory vesicles if the trigger acting in stimulated cells is added to the membranes (Figure 1). Consequently, secretory vesicles have been isolated from a variety of cells and their interaction has been studied under different experimental conditions (13, 15-19).

Membrane fusion is also involved in the development of skeletal muscle. In this process cell membranes of myoblasts fuse with each other (Figure 2). In contrast to differentiated cells, myoblasts are advantageous for the isolation of cell membranes. Due to their large surface-to-volume ratio and their minute amount of intracellular membranes, a cell membrane fraction of high purity can be obtained (20-22).

As outlined, the tendency of isolated cell membranes to form rightside out vesicles hinders the analysis of exocytosis *in vitro*. By contrast, this orientation of the cell membranes is useful in studying myoblast fusion with isolated membranes (Figure 2). In fact, cell membrane vesicles isolated from myoblasts in culture have been found to be rightside out when comparing the distribution of membrane particles in isolated cell membrane vesicles and cell membranes of intact cells (21, 22).

Fusion of secretory vesicles as well as of isolated myoblast membranes depends on the presence of Ca^{2+} in the incubation medium. Other cations have also been tested for their ability to trigger fusion. In this way the process has been characterized in terms of its requirement of divalent

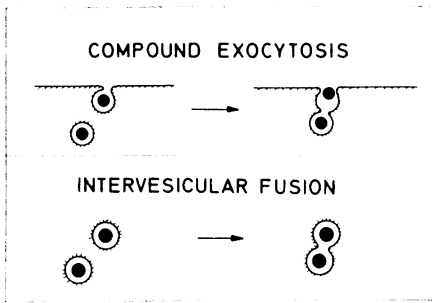


Figure 1 Schematic representation of compound exocytosis and intervesicular fusion of isolated secretory vesicles. The cytoplasmic surfaces of membranes are marked with strokes.

cations, which possibly reflects the specificity of their binding sites on the membranes.

Information concerning temperature dependence of membrane processes provides clues about the nature of and the changes in the physical state of the microenvironment of the membrane sites involved. Abrupt changes in the function-temperature relationships can be correlated with phase transitions and phase separations of the phospholipid matrix surrounding the functional site. Thus fusion was investigated at different temperatures in order to obtain information about the fusion site and its embedding into the membrane.

Specific modifications of the membrane components can be carried out with the aid of enzymes. Therefore the isolated membranes were pretreated with proteases and neuraminidase in order to identify the chemical nature of membrane components involved in the process of fusion. Furthermore, the techniques of glutaraldehyde fixation or the pretreatment of myoblasts with inhibitors of protein biosynthesis were applied to analyze the possible participation of proteins in the mechanism of membrane fusion. For comparative reasons we also investigated the fusion of pure phospholipid vesicles reconstituted from extracted lipids of isolated secretory vesicle membranes.

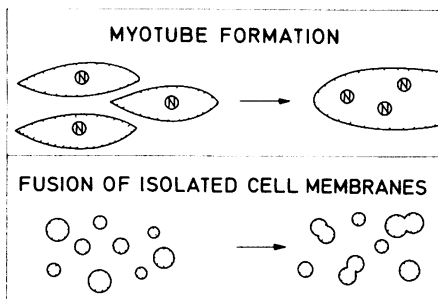


Figure 2 Schematic representation of myotube formation and fusion of isolated myoblast cell membranes. The cytoplasmic surfaces of membranes are marked with strokes (N = nucleus).

2.1 Fusion of Isolated Secretory Vesicles

In freeze-fracture electronmicrographs the morphology of secretory vesicles isolated from different tissues is similar. If the concentration of the divalent cation Ca^{2+} in a suspension of isolated secretory vesicles is raised, the vesicles become attached to each other and assemble in groups. The attachment of vesicles is accompanied by an aggregation of membrane particles which is often observed at the area of membrane contact. Most important is the fact that vesicle fusion can be observed in suspensions containing Ca^{2+} . In freeze-fracture, fused vesicles are characterized by a continuous cleavage plane in both membrane faces, that is, from one vesicular membrane to the other (for interpretation of vesicle fusion in freeze-fracture electronmicrographs, see ref. 18). Fusion of isolated secretory vesicles triggered by Ca^{2+} was demonstrated with secretory vesicles from mouse pancreatic islets (15), rat liver (16, 18), bovine adrenal medulla (17, 19), and bovine neurohypophysis (13).

As an example, a suspension of bovine adrenal medulla secretory vesicles in buffered sucrose medium containing EGTA is shown in Figure 3. The membrane faces [denoted EF face or PF face (23)] are studded with randomly distributed particles, which are more frequent in the concave PF faces than in convex EF faces. Addition of Ca^{2+} results in the formation of fused vesicles. In thin section electronmicrographs secretory vesicles isolated from the adrenal medulla exhibit electron-dense cores. After fusion, one continuous membrane enclosing two electron-dense cores can be observed (19). Intermixing of vesicle contents during fusion of secretory vesicles from rat liver has been demonstrated. If the conversion of proalbumin to albumin within these vesicles was blocked by an inhibitor of the converting enzyme, this effect could be compensated by addition of untreated vesicles and $10^{-4} M \text{Ca}^{2+}$ (24).

2.1.1 Influence of Cations

The percentage of fused vesicles counted after addition of Ca^{2+} in $10^{-3} M$ concentrations varies between 6 and 10%, depending on the tissue. These percentages, found in freeze-fractured suspensions of isolated secretory vesicles, appear to be low compared to the purity of the cell fractions investigated. It must be taken into consideration that only those structures whose long axis deviates from the cleavage plane by a certain angle can be identified as fusion products (twinned vesicles). Obviously all other twinned vesicles and those which are arranged more or less perpendicular to the plane of observation cannot be detected in the electron microscope. Further-

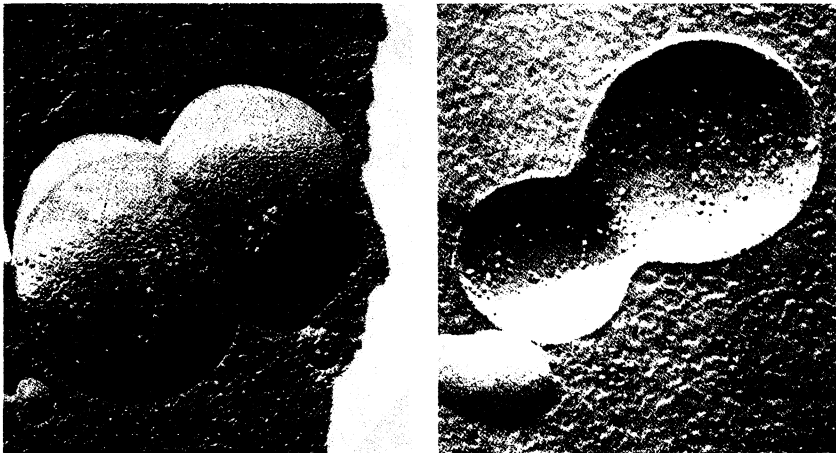
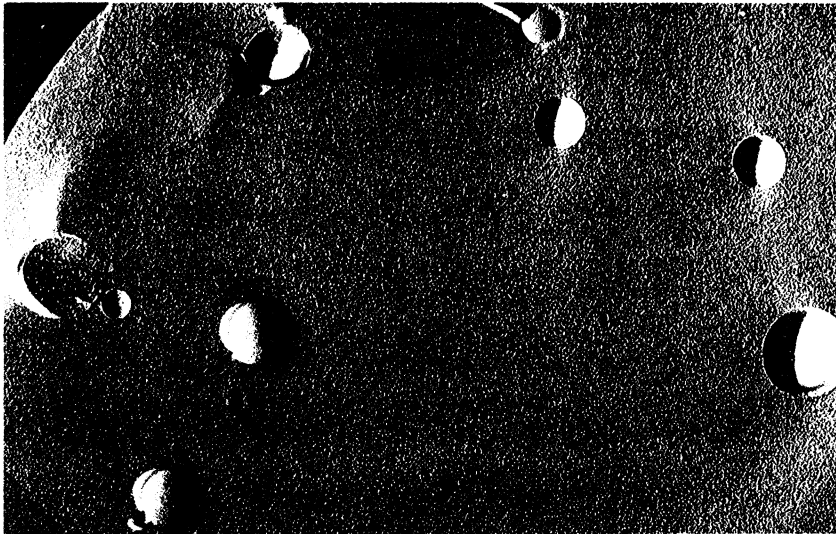


Figure 3 Freeze-fracture electronmicrographs of isolated secretory vesicles from adrenal medulla in a cacodylate buffered sucrose medium containing 1 mM EGTA (top, $\times 25,000$). Incubation with 10^{-4} M Ca^{2+} (5 min, 37°C) results in the formation of fused vesicles which are characterized by a continuous cleavage plane in the membrane PF faces as well as in the membrane EF faces (bottom, $\times 80,000$).

more, immature and damaged vesicles are likely to occur in preparations of isolated secretory vesicles.

If the percentage of fused vesicles is plotted as a function of the free Ca^{2+} concentration, sigmoidal curves are obtained for all membranes investigated (Figure 4). The steepest part of these curves is at approximately 10^{-6} M Ca^{2+} , at which point 50% of the fusion obtained in 10^{-4} M Ca^{2+} has occurred.

Is the requirement of vesicle fusion for Ca^{2+} compatible with the Ca^{2+} concentration occurring in the cytoplasm? This question must be answered in the positive because of the findings of several workers who determined free Ca^{2+} concentrations in different cells. Free Ca^{2+} concentrations in various resting cells have been estimated to be about 10^{-7} M or less (25-30). Fusion of the intracellular membranes described above is low under these conditions. The observation that membrane fusion increases at higher Ca^{2+} concentrations is pertinent since there is abundant evidence that secretion by exocytosis is paralleled by a rise in free intracellular Ca^{2+} (see Section 2.2).

The fact that Ca^{2+} promotes fusion of isolated secretory vesicles requires that Ca^{2+} act specifically because there is a large excess of free Mg^{2+} inside the cell. The concentration of free Mg^{2+} in different cells was determined to be in the millimolar range (31-36). In fact, comparable concentrations of Mg^{2+} as well as other divalent cations failed to induce fusion of secretory vesicles from pancreatic islets (15), liver (16, 18), neurohypophysis (13), and adrenal medulla (17, 19). Mg^{2+} , simultaneously added with Ca^{2+} to the

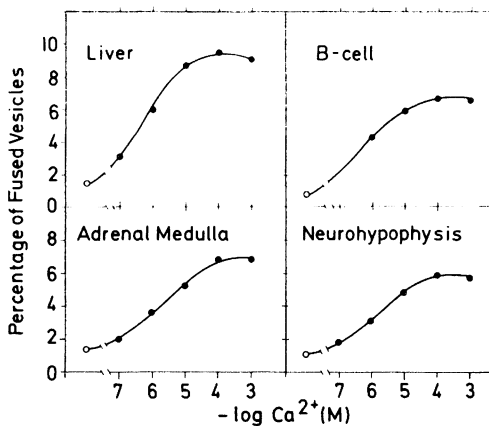


Figure 4 Fusion of isolated secretory vesicles from different tissues as a function of the free Ca^{2+} concentration. Unpublished data and from Gratzl and Dahl (18), Gratzl et al. (13).

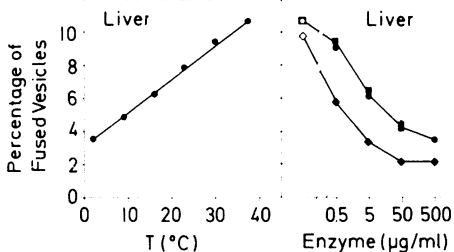


Figure 5 Temperature dependence of the fusion of rat liver secretory vesicles induced by $10^{-4} M$ Ca^{2+} (left) and effect of proteases and neuraminidase (right). Before addition of Ca^{2+} (final concentration $10^{-4} M$) vesicles were preincubated ($0^{\circ}C$, 30 min) with various concentrations of trypsin (solid square), pronase (solid circle), neuraminidase (solid diamond), or with 500 $\mu g/ml$ heat-inactivated enzymes (open square, circle, and diamond). From Gratzl and Dahl (18).

membranes investigated, inhibited Ca^{2+} -induced fusion in a concentration-dependent manner (13, 18, 19).

2.1.2 Influence of Temperature

Temperature-dependent changes in the properties of fluorescent probes or spin labels bound to adrenal medulla secretory vesicle membranes and the discontinuities of temperature-versus-activity plots of certain integral proteins of the same membranes have been interpreted as indicating the occurrence of structural changes in the lipid phase of these membranes (37, 38). Since such processes might strongly influence or even be prerequisite for membrane fusion (see Sections 2 and 3.1), we have investigated the temperature dependence of secretory vesicle fusion triggered by $10^{-4} M$ Ca^{2+} . However, fusion of secretory vesicles isolated from liver as well as adrenal medulla was found to be linear in the temperature range of 2–37°C (18, 19). This is shown for rat liver secretory vesicles in Figure 5.

The linear increase of fusion of secretory vesicles with temperature contrasts with the nonlinear fusion of liposomes prepared from certain phospholipids. This fusion increases greatly if the lipids are in the fluid state (Section 3.1). Unfortunately, no data are available on the temperature dependence of the initial velocity of fusion of both secretory vesicles and liposomes. Fusion of artificial and biological membranes deserves further investigation before the importance of thermotropic transitions for both processes can be judged.

2.1.3 Involvement of Glycoproteins or Gangliosides

Ca^{2+} -induced fusion of secretory vesicles isolated from rat liver is decreased after pretreatment with neuraminidase, pronase, or trypsin (Figure 5). Con-

comitantly, a certain percentage of radioactivity is lost from the surface of vesicular membranes labeled *in vivo* with (^3H) leucine or (^3H) glucosamine (18), which is efficiently incorporated into protein-bound glucosamine and sialic acid (39). Thus the membrane components attacked by the hydrolytic enzymes used seem to be of particular importance for the fusion of these biological membranes. Since neuraminidase (*Clostridium perfringens*) catalyzes the hydrolysis of gangliosidic and protein-bound sialic acid, an effect on glycolipids cannot be excluded. In this context, it is of interest that the membranes investigated in fact contain gangliosides (40). On the other hand, glycoproteins localized at the cytoplasmic surface of secretory vesicles from rat liver (41) may be a substrate for both neuraminidase and the proteases.

Fusion of secretory vesicles isolated from adrenal medulla is also abolished if the vesicles are preincubated with neuraminidase, proteases, or glutaraldehyde (17, 19). Sialic acid can be removed from the cytoplasmic surface of adrenal medulla secretory vesicles with neuraminidase (42–44), which, in addition, reduces the electrophoretic mobility of these vesicles (42, 43). Since gangliosides as well as glycoproteins are present in the membranes of adrenal medulla secretory vesicles (45–47), the target of the hydrolytic enzymes in these membranes is not known.

The role of glycoproteins or gangliosides in membrane fusion of secretory vesicles is difficult to assess. They may act as recognition sites for interacting membranes or as receptors for Ca^{2+} , specifically triggering fusion of secretory vesicles. Actually, sialic acid is able to bind Ca^{2+} and other divalent cations (48, 49). On the other hand, Ca^{2+} binding can also occur at certain carboxyl groups of proteins. Carboxyl groups present in the membranes of adrenal medulla are substrates for carboxymethylase, an enzyme which has been suggested to have a regulatory function in exocytotic secretion (50).

Ca^{2+} -induced fusion occurring at low Ca^{2+} concentrations is a specific property of secretory vesicles. For example, microsomal vesicles isolated from rat liver do not fuse under these conditions (16). The amount of sialic acid residues present on the surface of secretory vesicles, which can be regulated by sialyltransferases and neuraminidase in the Golgi apparatus (51–53) could be critical in making secretory vesicles competent for fusion and thereby could control exocytotic secretion.

2.2 Comparison of the Properties of Intervesicular Fusion and Exocytosis

The chain of events linking stimulation of a secretory cell and release of secretory product has been termed *stimulus-secretion coupling* (54). During this process the membranes of secretory vesicles fuse with the cell

membrane (exocytosis) and the vesicle content is released into the extracellular fluid. Many recent reviews have surveyed the available data on stimulus-secretion coupling of cells (1, 4, 55–63). One exception is the hepatocyte for which a secretory stimulant is unknown, and therefore it appears to lack coupling of stimulus to secretion. Only those reviews which deal with cell types used for studying exocytotic membrane fusion *in vitro* are considered here. In the following account the ionic events underlying stimulus-secretion coupling are described. For further details and references the review articles cited should be consulted.

In order to stimulate a secretory cell the presence of Ca^{2+} in the *extracellular* fluid is required. This ion can, to some extent, be replaced by Sr^{2+} or Ba^{2+} , but not by Mg^{2+} , Co^{2+} , or Mn^{2+} , which even inhibit stimulus-evoked secretion. However, measurements of $^{45}\text{Ca}^{2+}$ in secretory cells point to the possibility that *intracellular* Ca^{2+} is redistributed after stimulation. Without stimulation secretion can be triggered by Ca^{2+} and the addition of ionophores, which are thought to introduce divalent cations into cells. The experiments which provide the most convincing evidence that Ca^{2+} can trigger secretion are those in which Ca^{2+} is introduced directly into the cell. Ca^{2+} injected into the presynaptic nerve terminal of the giant synapse evokes transmitter release (64); Sr^{2+} has effects similar to those of Ca^{2+} . Intracellular application of Mg^{2+} and Mn^{2+} is practically ineffective. Mg^{2+} and Mn^{2+} lead to slight reduction in the amount of transmitter released by injection of Ca^{2+} . In addition, it was reported that injection of Ca^{2+} , but not Mg^{2+} , into mast cells elicited extrusion of secretory granules (80). Microinjection of Ca^{2+} into amphibian oocytes similarly resulted in exocytosis of cortical vesicles; Mg^{2+} was ineffective (65).

Does the intracellular free Ca^{2+} concentration increase during stimulation? That it does was shown with aequorin-loaded squid giant synapses in which aequorin luminescence paralleled stimulation and transmitter release (29). Moreover, exocytosis of cortical vesicles in fertilized eggs was preceded by an increase in free Ca^{2+} from $10^{-7} M$ or less to $3 \times 10^{-5} M$ (27). Taken together, these studies support the concept that exocytosis is triggered by a rise in the intracellular concentration of free Ca^{2+} but leave open the question concerning the site of action of this cation.

Is the ionic requirement of intervesicular fusion of isolated secretory vesicles compatible with the data obtained from studies on secretion by intact cells? In other words, is it probable that Ca^{2+} induces the final step of membrane fusion during exocytosis? Intervesicular fusion of secretory vesicles isolated from liver, pancreatic islets, neurohypophysis, and adrenal medulla is low in media containing less than $10^{-7} M$ Ca^{2+} , the concentration estimated in resting cells. Vesicle fusion, however, increases (Figure 4) as the Ca^{2+} concentration is increased to the level found in stimulated cells.

No other divalent cations have yet been found which are able to induce fusion of these biological membranes in concentrations up to $10^{-3} M$. This is in contradiction to the finding described earlier that extracellular Ba^{2+} or Sr^{2+} , or intracellularly injected Sr^{2+} , can elicit the release of secretory product. One explanation that could account for this discrepancy follows.

As is generally accepted, the regulation of low intracellular free Ca^{2+} concentrations is governed by extracellular Ca^{2+} , cation fluxes through the cell membrane, membranous and nonmembranous cytoplasmic binding sites, as well as various energy-dependent intracellular Ca^{2+} -sequestering systems. In intact cells, a change in the intracellular or extracellular concentration of any cation will probably affect the intracellular Ca^{2+} concentration. Since some pools have high capacities, the impairment of their balance will result in an inundation of the small amount of free Ca^{2+} . Actually, Ca^{2+} , as well as Sr^{2+} and other divalent cations, are avidly taken up by mitochondria by an energy-dependent mechanism (66). Sr^{2+} shares the same site as Ca^{2+} , and hence competes with this ion (67). Also, Sr^{2+} (as well as Ba^{2+}) markedly interferes with Ca^{2+} fluxes across cell membranes (68). Thus injection of Sr^{2+} or replacement of extracellular Ca^{2+} by Sr^{2+} or Ba^{2+} could very well result in a redistribution and enhancement of free intracellular Ca^{2+} , which triggers exocytosis.

Release of secretory product by the interaction of a secretagogue with a variety of cells in the presence of extracellular Ca^{2+} is inhibited by Mg^{2+} , Co^{2+} , and Mn^{2+} . This inhibition might simply be due to an interference of these ions with the Ca^{2+} channels of the cell membrane (for review see ref. 62). Interestingly, a similar antagonism is found if Mg^{2+} or Mn^{2+} is injected intracellularly together with Ca^{2+} (64), an observation which agrees with the inhibition by Mg^{2+} of Ca^{2+} -induced fusion of secretory vesicles isolated from different tissues (see Section 2.1.1).

With the few exceptions already discussed, it can be summarized that the ionic requirements for fusion of secretory vesicles are consistent with those of secretion by intact cells. Both processes require a low concentration of Ca^{2+} , are specific for this cation, and are inhibited by Mg^{2+} . It is therefore proposed that the final intracellular trigger in stimulus-secretion coupling is Ca^{2+} .

In pancreatic islet cells (69) and neurohypophysial cells (61) stimulus-evoked secretion decreased with temperature and ceased at $15-20^{\circ}C$. Further reduction of temperature resulted in an increase of insulin release by pancreatic islets (70). This was thought to be due to cell damage. Under these conditions, the neurohypophysis also released vasopressin (71, 72). However, a biochemical and morphological investigation of this process revealed that secretion induced by cold was accomplished by exocytosis (13, 72, 73). Neurosecretosomes (13, 74), as well as synaptosomes (75), when

exposed to cold also release secretory product. Similar effects of cold have been described in other secretory systems (76, 77). The mechanism of hormone release from pancreatic islets induced by low temperatures has since been reinvestigated (69). After cooling the islets to 2°C, insulin release in the absence of the physiological stimulus was paralleled by an increase in the appearance of exocytotic profiles. It has been suggested that this is related to a redistribution of Ca^{2+} from intracellular stores. It is significant that membrane fusion of isolated secretory vesicles induced by Ca^{2+} was reduced but still present at low temperatures (see Section 2.1.2). Thus membrane fusion during exocytosis in intact cells and in the *in vitro* system can occur at low temperature.

Mast cell histamine secretion induced by polymyxin B exhibits a biphasic Arrhenius plot with a transition temperature near 16°C (78). These data were interpreted as indicating a phase change in the mast cell membranes. Unfortunately, no direct study of the effect of temperature on the membranes of mast cells is available. In experiments with isolated secretory vesicles from liver and adrenal medulla, a linear relationship between fusion and temperature was observed. This does not exclude a phase change, because a kinetic analysis of the fusion of these membranes is still lacking. However, at present we would interpret the nonlinearity of secretion exhibited by intact cells with temperature as reflecting the different temperature sensitivities of various processes involved in exocytosis (e.g., Ca^{2+} handling of the cells, membrane fusion) rather than as a phase change in the interacting membranes.

Membrane fusion of isolated secretory vesicles is sensitive to enzymatic attack (see Section 2.1.3). Only one investigation has described the action of neuraminidase on a secretory system (79): injection of neuraminidase into a presynaptic neuron progressively reduced postsynaptic responses. This may suggest a functional role of gangliosides or protein-bound sialic acid in synaptic transmission and points to another striking similarity between exocytotic secretion and the fusion of membranes involved in this process.

The direct approach to the interaction between isolated secretory vesicles and cell membranes *in vitro* poses several difficulties, as outlined in Section 2 and confirmed by recent investigations (13). In the presence of cell membranes an increase of vasopressin release from isolated neurohypophyseal secretory vesicles in response to a rise in Ca^{2+} concentration was observed. The specificity of the Ca^{2+} effect and the antagonism by Mg^{2+} suggested that the release of hormone is not the result of a general leakiness. Few electronmicroscopical observations of interactions between secretory vesicles with open sheets of cell membranes have led to the interpretation that in this case an *in vitro* exocytosis type of reaction could have contributed to the observed release.

The total release of intravesicular secretory product by the addition of a cell membrane fraction was claimed to be the result of vesicle membrane-cell membrane fusion (7). This implies that the cell membrane fraction consists mainly of open sheets instead of closed vesicles. However, in published electronmicrographs of the cell membrane fraction used by these authors, sheets are virtually absent (81). On the other hand, total release of secretory vesicle contents can occur in several ways, for example, as a consequence of proton translocation (82). In studies, therefore, following the release of vesicle contents *in vitro* simple leakage must be excluded and secretory vesicle cell membrane fusion must be demonstrated.

2.3 Fusion of Cell Membranes Isolated from Myoblasts

Myoblasts are the embryonic precursor cells of adult skeletal muscle. During development, which can be followed *in vitro*, myoblasts are seen to fuse and form myotubes which, following differentiation, become striated muscle fibers (83, 84). During the first two days in culture, myoblasts divide and then withdraw from the cell cycle and build up myotubes. Myotube formation *in vitro* can be prevented by low Ca^{2+} concentrations in the culture medium (85). This can be achieved either by addition of EGTA to the culture medium or by the use of Ca^{2+} -depleted media. Myoblasts grown in Ca^{2+} -depleted media fuse after addition of Ca^{2+} . This shows that myoblasts grown in low Ca^{2+} media have acquired the capability of fusing, hence the term *fusion-competent myoblasts*.

Cell membranes can be isolated from fusion-competent myoblasts (20). Isolated cell membranes form rightside-out vesicles (21) and are randomly distributed when dispersed in an isotonic sodium chloride or sucrose medium (Figure 6). If divalent cations (Ca^{2+} , Mg^{2+} , or Sr^{2+}) are added, vesicles attach to each other and membrane particles redistribute. Aggregations of membrane particles are frequently observed in areas of membrane contact (21).

With a medium containing 1.4 mM Ca^{2+} , isolated cell membrane vesicles fused (Figure 6) and the same concentration was found to be optimal for myotube formation. In the waste of fused vesicles a ringlike aggregation of membrane particles was often observed (21). Except for greater variations in diameter of the cell membrane vesicles, the morphology of these membranes in freeze-fracture electronmicrographs is similar to that of secretory vesicles (Section 2.1). The changes in the distribution of membrane particles during fusion also are comparable. Secretory vesicles and cell membranes, however, differ with respect to the concentrations of Ca^{2+} required for fusion (see Sections 2.1.1 and 2.3.1).

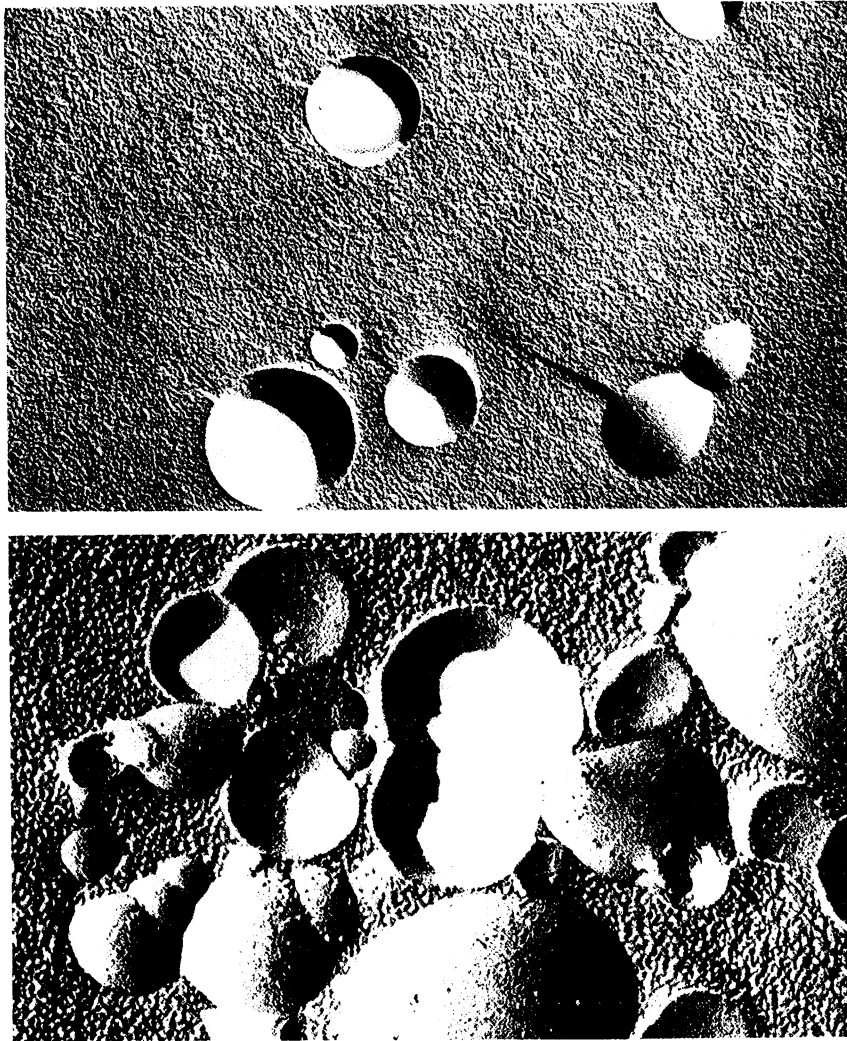


Figure 6 Freeze-fracture electronmicrographs of isolated myoblast cell membranes before (top, $\times 40,000$) and after addition of Ca^{2+} (bottom, $\times 80,000$). Cell membrane vesicles incubated with $1.4 \text{ mM } \text{Ca}^{2+}$ (5 min, 37°C) contact each other and fuse (bottom).

2.3.1 Influence of Cations

With various concentrations of Ca^{2+} , Mg^{2+} , and Sr^{2+} , two types of vesicle fusion with different ion specificities can be distinguished (Figure 7) (22). The first type is that observed in the physiological range of Ca^{2+} concentrations (up to 2.8 mM) which was not triggered by Mg^{2+} or Sr^{2+} . At higher concentrations of Ca^{2+} , an additional increase of fusion was observed. This could also be triggered by Sr^{2+} or Mg^{2+} . This second type of fusion is discussed in detail in Section 3.1.

The Ca^{2+} concentration of the extracellular fluid *in vivo* is known to be relatively constant. Thus although Ca^{2+} is required for fusion, it cannot have a regulatory function in myoblast fusion. This contrasts sharply with its role in secretory processes (see Section 2.2). However, this statement is valid only if Ca^{2+} in fact interacts with that side of the myoblast cell membrane exposed to the extracellular fluid. If intracellular Ca^{2+} were necessary for the induction of myoblast fusion, a rise in the Ca^{2+} concentration of the cytoplasm of these cells during development could trigger myotube formation. Actually, the presence of ionophore A23187, which probably increases the intracellular Ca^{2+} concentration, did not result in myoblast fusion (86). Moreover, fusion of cell membrane vesicles which exhibit the same orientation as the cell membranes of intact cells (rightside out) was not increased at suboptimal Ca^{2+} concentrations in the presence of A23187 (22). Both findings are consistent with a Ca^{2+} binding site in myoblast cell membranes which faces the extracellular space.

The extent of fusion of cell membrane vesicles isolated from myoblasts at different developmental stages (20–60 hr in culture) parallels the fusion capacity of the corresponding myoblasts (22). Thus membrane components incorporated into the myoblast cell membranes during development are obviously responsible for the acquisition of fusion competence during growth in Ca^{2+} -depleted media. These experiments, furthermore, are consistent with the concept that fusion of myoblasts is controlled not by

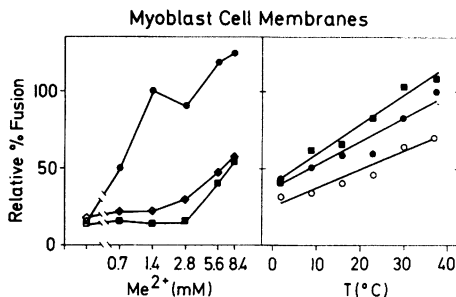


Figure 7 Fusion of isolated myoblast cell membranes as a function of Ca^{2+} (solid circle), Sr^{2+} (solid diamond), or Mg^{2+} (solid square) (left). Temperature dependence of the fusion of the myoblast cell membranes induced by 1.4 mM Ca^{2+} after 1 (open circle), 5 (solid circle), and 30 min (solid square) of incubation (right). From Dahl et al. (22).

Ca^{2+} but by a specific cell membrane component which is supposed to be proteinaceous in nature.

2.3.2 Influence of Temperature

The kinetics of vesicle fusion are not linear, but rather exhibit a fast and slow phase (22). After various incubation periods the relationship between fusion and temperature proved linear (22). No abrupt transition from a fusible to a nonfusible state of the membranes was obvious in a temperature range of 2–37°C. (Figure 7). This meant that thermotropic phase transitions or phase separations linked to the fusion process do not take place. Actually no thermotropic transitions have been observed in isolated myoblast cell membranes between 12 and 54°C based on measuring fluorescence polarization in the presence or absence of Ca^{2+} (88). However, a transient increase of fluorescence polarization which reaches its maximum after 5–10 min is induced by the addition of Ca^{2+} . This effect, and that of Ca^{2+} on fusion of isolated myoblast cell membranes (21), is inhibited by Mg^{2+} and lysolecithin (88). The transient change in polarization was interpreted as being production of local phase separations, which could be relevant to the mechanism of membrane fusion. The concept that the existence of different membrane domains is essential for membrane fusion is further supported by the sensitivity of fusion toward cholesterol (22) and lysolecithin (21). Both agents inhibit fusion of myoblasts as well as myoblast cell membranes and interfere with phase transitions and phase separations (89, 90).

2.3.3 Involvement of Proteins or Glycoproteins

Studies on the fusion of cell membrane vesicles isolated from myoblasts during different developmental stages indicate that not Ca^{2+} but a membrane component accessible to this ion from the extracellular space plays a regulatory function in the fusion of myoblasts. Since fusion of isolated cell membrane vesicles induced by 1.4 mM Ca^{2+} is sensitive to treatment with glutaraldehyde or proteases (Figure 8) (22), this membrane component obviously is proteinaceous in nature. Moreover, fusion of isolated secretory vesicles induced by comparatively low concentrations of Ca^{2+} is abolished by glutaraldehyde as well as proteases (see Section 2.1.3). Neuraminidase is another enzyme which reduces the ability of isolated cell membrane vesicles to fuse (Figure 8) (22). A parallel finding has been described with isolated secretory vesicles (Section 2.1.3). The essential role of proteins in the fusion of myoblast cell membranes is further supported by the reduction of fusion following inhibition of protein biosynthesis by cycloheximide (22). From the experiments described above it is concluded

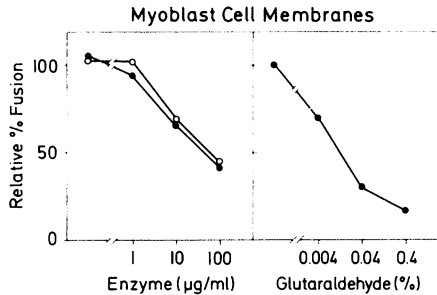


Figure 8 Effect of trypsin (solid circle) and neuraminidase (open circle) (left) as well as glutaraldehyde (right) on fusion of myoblast cell membranes induced by 1.4 mM Ca^{2+} . From Dahl et al. (22).

that during maturation of the myogenic cell, special proteins are synthesized and inserted into the cell membrane, thereby rendering this cell membrane competent for fusion.

2.4 Comparison of Myotube Formation and Fusion of Isolated Myoblast Cell Membranes

Myotube formation has been investigated with the electron microscope (91). With this method only late stages of fusion (myotubes) could be detected. Ultrastructural studies combined with electrophysiological techniques revealed that myoblasts, coupled by gap junctions, lose their junctional resistance within milliseconds. This suggests that the establishment of cytoplasmic continuity between two adjacent cells is a very fast process (92). This may explain why it is difficult to show the formation of the primary pore in electronmicrographs. In fact, no such figures have yet been published. Cell contacts, however, represented by gap junctions can easily be found (Figure 9). A correlation between cell-cell communication via gap junctions and fusion has been suggested (92, 93), but in a recent report it was concluded that cell communication by itself is not a sufficient property for fusion (94).

The contact of isolated membrane vesicles is frequently characterized by an aggregation of membrane particles (21), indicating that membrane particles may eventually participate in the formation of close membrane apposition. However, in contrast to fusion, membrane contact *and* aggregation of membrane particles can be evoked by Ca^{2+} as well as by other divalent cations (21). Addition of Ca^{2+} is sufficient to induce fusion of isolated cell membrane vesicles. This supports findings obtained with intact myoblasts that extracellular factors other than Ca^{2+} and even the supply of cellular energy are not essential for the fusion process itself (95, 96). The cytoplasmic concentration of cyclic nucleotides has been shown to change simultaneously with the appearance of fusion competence (97, 98). Experi-

ments with isolated cell membrane vesicles indicate that it is unlikely that these substances control membrane fusion directly (21, 22).

Cell motility apparently is essential for myotube formation, since this process is affected by cytochalasin B (99, 100). Fusion of vesicles was not impaired by similar concentrations of cytochalasin B (22), suggesting that

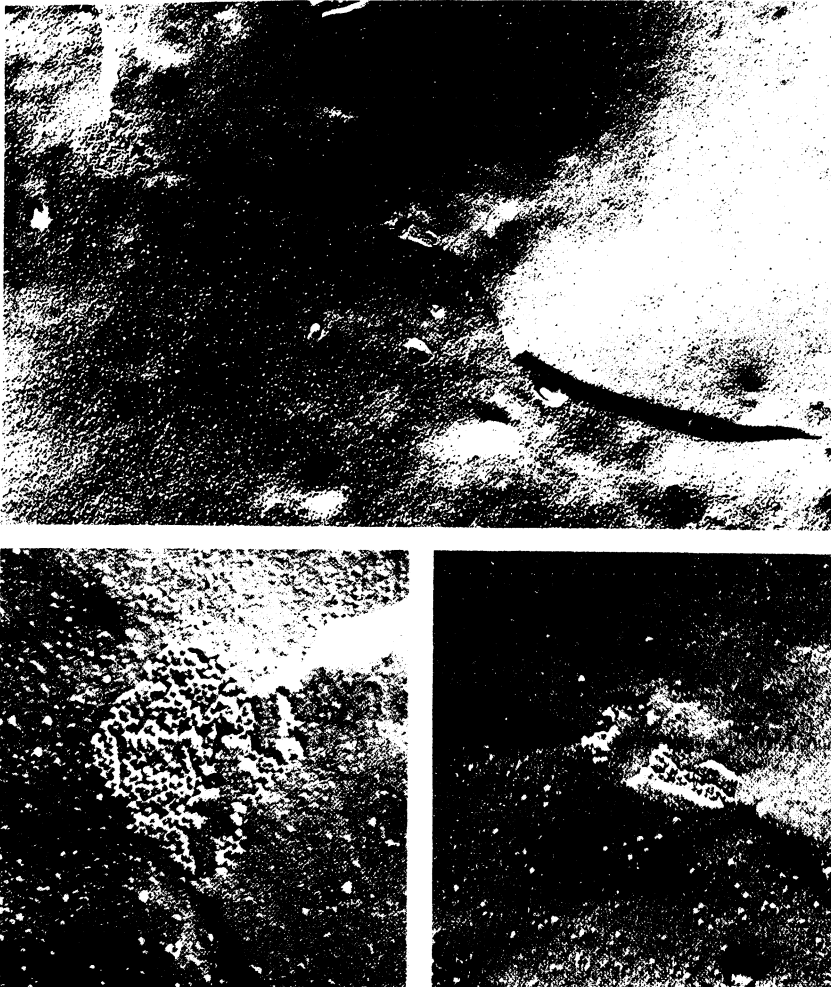


Figure 9 Freeze-fracture electronmicrographs of myoblasts (50 hr in culture) 15 min after addition of 1.4 mM Ca^{2+} . Cell membranes of myoblasts contact each other (top, $\times 30,000$). At higher magnification gap junctions between neighboring cells can be identified (bottom, $\times 100,000$).

elements sensitive to cytochalasin B are not necessary for membrane rearrangement during pore formation.

Ca^{2+} in physiological concentrations has been shown to specifically trigger fusion of myoblasts (87) as well as cell membrane vesicles (21, 22). For myoblasts a slight fusion-promoting effect of 2.4 mM Sr^{2+} has been reported (101). All other cations investigated were found unable to replace Ca^{2+} . Furthermore, a competitive interaction between Ca^{2+} and Mg^{2+} (101) and Ca^{2+} and H^+ (87) was disclosed by kinetic studies on myotube formation. In contrast to vesicle fusion, myotube formation was inhibited at Ca^{2+} concentrations higher than 2.8 mM (87). All these data show that Ca^{2+} is bound specifically to the membrane and triggers fusion. The precise molecular nature of the membrane component interacting with Ca^{2+} remains to be elucidated.

The phospholipid pattern and fatty acid compositions of whole cells and membranes isolated from myoblasts have been determined (102, 103). No differences were detected between fused and unfused myoblasts. The phospholipid composition has been changed by incubation of myoblast cultures in media containing lysolecithin (104), cholesterol (87, 103), dipalmitoyl lecithin (87), and phosphatidylethanolamine (103). Only the enrichment of membrane oleate content resulted in a slight increase in myotube formation (103). All other treatments produced a diminution in myotube formation. Similarly, incorporation of lysolecithin or cholesterol into isolated cell membrane vesicles reduced fusion (21, 22). This indicates that the overall change of membrane fluidity in either direction interferes with fusion.

The results derived from fluorescence polarization studies of myoblasts indicate a high fluidity for myoblast membranes after 50 hr in culture. No thermotropic transition between 5 and 41°C could be detected in myoblasts after 1–4 days in culture (105). A similar lack of thermotropic transition, as demonstrated by fluorescence polarization, was reported for isolated cell membranes between 28 and 45°C (88). The observed abrupt change of myotube formation rate at 35°C (87) may be due to a temperature-dependent rate-limiting process which differs from membrane fusion. Thus it appears from fusion experiments and physical measurements that high membrane fluidity may be a requirement for fusion. Ca^{2+} , however, seems to create membrane domains of different fluidity (see Section 2.3.2). It is possible that the latter process is disturbed by addition of any lipid in excess.

Lectins and glycoproteins may be important for membrane recognition as well as for fusion. Correlation between the presence of lectin and myotube formation has already been described (106–108). The function of these lectins remains unclear.

A parallel increase of myoblast sialic acid content and fusion competence has been described (95). An inhibition of myotube formation at high Ca^{2+}

concentrations after removing membrane sialic acid was reported (95). Fusion of myoblast cell membrane vesicles is similarly inhibited by removal of sialic acid from the vesicular membrane (22). The exact function of this compound with respect to fusion is not yet known.

3 MODEL SYSTEMS FOR MEMBRANE FUSION INDUCED BY DIVALENT CATIONS

Membrane fusion has been studied with membranes exhibiting a natural ability to fuse, with biological membranes or cells which do not fuse *in vivo* but can be induced to fuse under nonphysiological or pathological conditions, and with pure lipid membranes (liposomes). In addition, liposome-cell fusion has been investigated as a model system for membrane fusion. For comparative reasons, those fusion processes in which divalent cations play an essential role will now be examined.

3.1 Liposome-Liposome Fusion

Membrane proteins are critical for the fusion of secretory vesicles and myoblast cell membranes. Since biological membranes are composed of proteins *and* lipids, the function of lipids during membrane fusion should be elucidated as well. Owing to the heterogeneity of natural membrane lipids, a drastic simplification in several laboratories was performed as follows. Artificial membranes (liposomes) were prepared from different well-defined lipids, and their interaction was followed under various experimental conditions. In this way the effects on fusion of the lipid head groups, the physical state of the lipid acyl chains (fluidity), and other basic properties of the membrane bilayer could be examined. Thus far, fusion of liposomes has been studied in three different ways: by monitoring (*a*) the mixing of vesicle contents, (*b*) the mixing of membrane components, and (*c*) the change in size and number of liposomes detected by electronmicroscopy. With regard to *a*, although the most desirable assay for fusion would be the interaction of substances included in the content of liposomal vesicles and a detection of their interaction after fusion, this possibility, to our knowledge, has been explored by few groups of workers (109, 110). As for *b*, most investigators have determined the mixing of membrane components to assess fusion of liposomes. This can be done by using ultracentrifugation or gel filtration (90), electron spin resonance, or nuclear magnetic resonance techniques (111-114). However, mixing of membrane components need not exclusively result from fusion, but can occur by exchange of the membrane components during collision or by diffusion. A method by which intermixing of

membrane components by mechanisms other than fusion can be distinguished seems to be differential scanning calorimetry. This technique makes use of two populations of liposomes prepared from lipids displaying different transition temperatures (90, 115–117). If fusion of these two classes of liposome occurs, membranes are formed with the same transition temperature as that of an equimolar mixture of the two lipids. If the transition temperature of the original liposomes gradually shifts to that of the equimolar mixture, exchange of lipids by mechanisms other than fusion is more likely.

Interaction of membrane components can be monitored with the aid of membrane proteins incorporated into liposomes. In this way electron transport between two populations of liposomes under certain conditions can be followed, indicating close apposition or fusion of vesicular membranes (118).

With regard to *c*, the enlargement of liposomes and the reduction of their number can also be followed by electronmicroscopy. Qualitatively, this has been shown with negative staining and freeze-fracture techniques (90, 114, 116, 117, 119). Provided other mechanisms leading to an increase in liposome size are excluded, fusion of liposomes can also be evaluated quantitatively in freeze-fracture electronmicrographs (17, 19). Another morphological approach to studying fusion of lipid bilayers involves the use of phospholipid spherical membranes. Their interaction is observed through a low-power microscope (120, 121).

On the basis of these various experimental approaches, the following basic requirements for liposome fusion in terms of cations, lipid composition, and physical state of the bilayer have been found essential. In the presence of divalent cations, liposomes fuse if they carry a net negative charge contributed by phosphatidylserine, phosphatidic acid, and phosphatidylglycerol. Liposomes prepared from neutral phospholipids do not fuse when divalent cations are added (90, 113, 116–118, 121). The concentration of divalent cations (e.g., Ca^{2+} or Mg^{2+}) used to induce fusion of liposomes with a net negative surface charge is in the millimolar range. The lowest reported concentration of cations sufficient for fusion of liposomes is 0.2 mM. This is the case with phosphatidic acid liposomes where Ca^{2+} and Mg^{2+} are equally effective. Other phospholipid vesicles (e.g., those prepared from phosphatidylglycerol) appear to be more susceptible to fusion by Ca^{2+} than by Mg^{2+} but require higher concentrations of Ca^{2+} (10 mM) (90, 113, 116–118, 121). In mixtures containing neutral phospholipids, a threshold concentration of lipids with net negative charge must be present in liposomes to be fused with divalent cations. The lowest mole percentage reported is 40% for phosphatidylserine (118).

It has been established that a significant degree of liposome fusion occurs only when the lipid molecules in the vesicle membrane are either at or above their transition temperature (117, 122). Accordingly, phase transitions, simultaneous existence of differently composed membrane domains, and high fluidity of membranes are prerequisites for fusion. It is well known that divalent cations can produce isothermal phase transitions (123), phase separation, and formation of domains (124, 125), as well as a decrease in surface charge leading to a reduction in electrostatic repulsive forces of these membranes (126). By correlating some or all of these phenomena with the process of cation-induced membrane fusion, several mechanisms relating to liposome fusion and fusion of biological membranes have been proposed (117, 127).

Liposomes can also be prepared using lipids extracted from biological membranes. With these vesicular membranes fusion of membrane lipids of biological membranes in the absence of proteinaceous components can be investigated.

Several laboratories have provided evidence that lipid composition of adrenal medulla secretory vesicle membranes is complex (45, 47). The lipids of these membranes characterized by a net negative charge are phosphatidylserine (about 8% of total lipid phosphorus), phosphatidylinositol (2%), and phosphatidic acid (1%). Another negatively charged lipid that might be relevant for liposome fusion induced by divalent cations is the ganglioside G_{M3} (for ganglioside nomenclature, see ref. 128). The presence of gangliosides (46) in the adrenal medulla secretory vesicle membranes and their identification (45) have recently been reported.

We have extracted the lipids from bovine adrenal medulla secretory vesicles with organic solvents (129) and prepared liposomes by sonication. Using this extraction procedure all membrane lipids with the exception of gangliosides can be recovered. Figure 10 is a representative electronmicrograph of the liposomes we obtained. The diameter of these liposomes is small (about 400 Å) but increases dramatically after incubation with high concentrations ($>2.5 \times 10^{-3}M$) of divalent cations (Figure 10). Concomitantly, the number of liposomes decreases. To determine the degree of fusion, the membrane area per liposome was evaluated quantitatively. Compared with controls, no increase in the size of liposomes was observed with 10^{-4} or $10^{-3}M$ divalent cations after 5 min of incubation (Figure 11). A gradual increase in vesicle size occurred with 2.5 mM divalent cations. Higher concentrations (5–10 mM) result in a dramatic enlargement of liposome size as evidenced in the electronmicrograph presented in Figure 10 and in a quantitative evaluation given in Figure 11. The fact that enlargement of these liposomes starts within milliseconds (19) and is complete

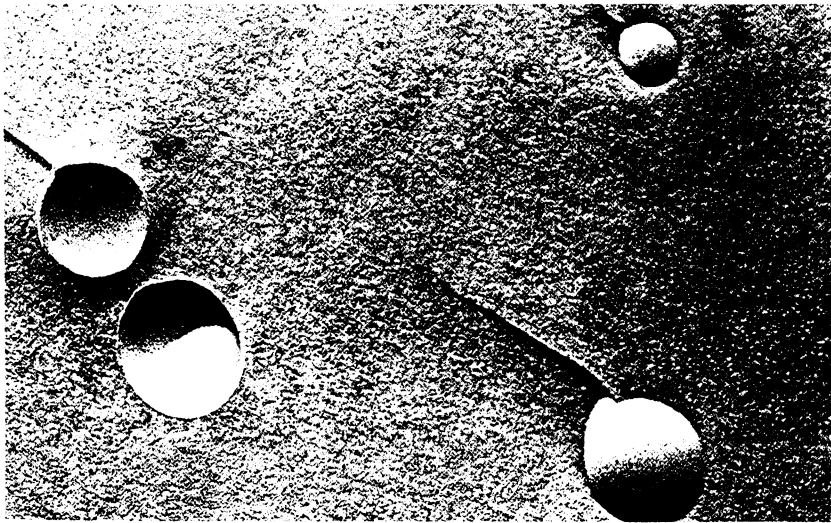
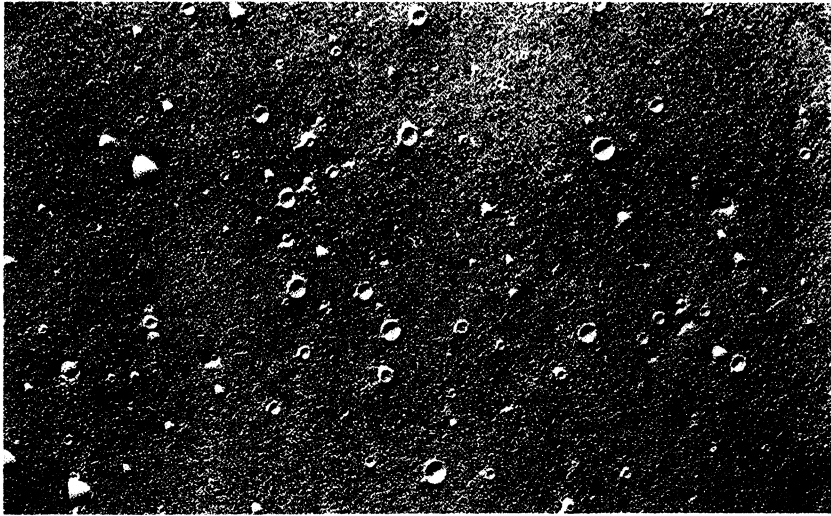


Figure 10 Freeze-fracture electronmicrograph of liposomes prepared from lipids extracted from bovine adrenal medulla secretory vesicles (top, $\times 40,000$). After incubation with $10^{-4} M$ Ca^{2+} (5 min, $37^{\circ}C$) the number of liposomes is reduced and their size is increased (bottom, $\times 40,000$).

within 5 min excludes mechanisms other than fusion. The requirement of millimolar concentrations of the divalent cations Ca^{2+} , Mg^{2+} , and Mn^{2+} and the nondiscriminatory role of these ions in the process of membrane fusion of liposomes is in accordance with findings using liposomes prepared from pure phospholipids as described earlier in this chapter.

If the fusion of liposomes prepared from extracted natural lipids is compared with those prepared from pure phospholipids, some salient differences become apparent, in addition to the similarities which have already been described. Clearly any model for the fusion of biological membranes should be comparable with the physiological process particularly in regard to its velocity. In most reports the process of liposome fusion is several orders of magnitude too slow to fulfill this requirement. Only in one case (118) has fusion been shown to occur within seconds. Preliminary experiments with liposomes prepared from the lipids of adrenal medulla secretory vesicles revealed a very fast time course (milliseconds) for fusion (19).

Although lysolecithin has been used as a fusogenic agent (109, 120), it was shown in several studies to elicit intermixing of membrane components by a mechanism other than fusion (115). Despite the high concentration of lysolecithin present in the phospholipid extract from adrenal medulla vesicle membranes [roughly 17% of total phospholipid (45, 47)], the liposomes prepared from the extract of these membranes do not fuse with $\leq 2.5 \text{ mM}$ divalent cations.

Cholesterol was shown to inhibit fusion of liposomes prepared from pure phospholipids (90). This is significant since the concentration of cholesterol

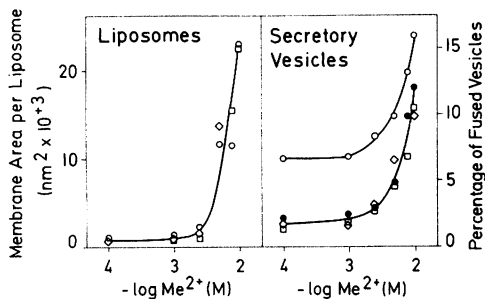


Figure 11 Fusion of adrenal medulla secretory vesicles (right) and of liposomes (left) prepared from their membrane lipids as a function of the divalent cation concentration. The membrane area per liposome is obtained by dividing the areas of all membranes exposed in $480 \mu\text{m}^2$ of a freeze-fracture replica by the number of liposomes observed. Liposomes or secretory vesicles were incubated (5 min, 37°C) with various concentrations of Ca^{2+} (open circles), Mg^{2+} (open diamonds), and Mn^{2+} (open squares). Ca^{2+} -induced fusion of secretory vesicles was also investigated after pre-treatment with $500 \mu\text{g/ml}$ neuraminidase for 30 min at 0°C (solid circles).

in the extracts from adrenal medulla secretory vesicle membranes is approximately 30% (45, 47). Yet liposomes prepared from these extracts are still able to fuse. Fusion of liposomes prepared from pure phospholipids requires a high content of negatively charged phospholipids. This is not the case with adrenal medulla membranes and other physiologically fusing membranes, since the concentration of charged lipids in such membranes is low (45, 47, 102, 103). A membrane component present in intact adrenal medulla secretory vesicles is the ganglioside G_{M3} (45, 46). Gangliosides, despite their similarities to charged phospholipids in respect of binding of divalent cations (48, 130) and formation of domains (130), have not yet been investigated insofar as membrane fusion is concerned.

The fusion of isolated secretory vesicles obtained from various tissues (see Section 2.1) differs from that of liposomes in several fundamental respects. First, isolated secretory vesicles specifically fuse in the presence of low concentrations of Ca^{2+} . Above $10^{-3} M$ Ca^{2+} a further increase of secretory vesicle fusion is observed starting from the plateau reached at $10^{-4} M$ and $10^{-3} M$ Ca^{2+} . (compare Figure 11 and Figure 2). With other divalent cations in millimolar concentrations, isolated secretory vesicles also fuse, starting at the control level (Figure 11). Second, Ca^{2+} -specific fusion of intact secretory vesicles is abolished if vesicles are incubated with proteases, neuraminidase, or glutaraldehyde before addition of Ca^{2+} . After pretreatment with neuraminidase, the curve obtained with high concentrations of Ca^{2+} is similar to that obtained with other divalent cations without pretreatment (Figure 11). Thus two types of fusion with different properties can be observed with isolated secretory vesicles. One type, specific for Ca^{2+} at concentrations lower than $10^{-3} M$, is abolished after enzymatic pretreatment. A second type of fusion, reminiscent of the fusion of liposomes (Figure 11) occurs if a high concentration of divalent cations is present. This second type, like liposome fusion (data not shown), is increased if Ca^{2+} plus Mg^{2+} is added. This is in contrast to the first type of fusion which is inhibited by Mg^{2+} .

Interaction of adrenal secretory vesicles when incubated with millimolar concentrations of Ca^{2+} or Mg^{2+} has also been reported by other investigators (131, 132). This interaction was interpreted in terms of a reversible aggregation of vesicles. Attachment of vesicles can be discriminated from fusion by withdrawal of divalent cations with an excess of EGTA. This leads to a disappearance of membrane contacts, but the fusion products (twinned vesicles) persist (19).

An analogous situation, as seen with secretory vesicles, is described for isolated cell membranes purified from myoblasts in culture (see Section 2.3 and ref. 22). One type of fusion which increased at Ca^{2+} concentrations up to $2.8 mM$ is specific for Ca^{2+} , is abolished by pretreatment with enzymes

or glutaraldehyde, and is reduced after inhibition of protein biosynthesis. A second type of membrane fusion of isolated myoblast cell membranes can be induced with several divalent cations (Ca^{2+} , Mg^{2+} , Sr^{2+}) at concentrations greater than 2.8 mM.

3.2 Liposome-Cell Fusion

The study of interaction between lipid vesicles of defined composition provides information about the involvement of charge and structure, as well as the chemical nature of phospholipid molecules in fusion. To elucidate whether these lipid properties similarly influence the fusion of lipids with biological membranes, it has been necessary to investigate the interaction of liposomes with cells. However, the interpretation of the results obtained is hampered by the fact that liposomes are incorporated by cells either by fusion or other mechanisms such as endocytosis, surface adsorption, or molecular exchange. Furthermore, the methods used to discriminate between these various routes are problematic. Unfortunately, one basic requirement for liposome-liposome fusion—the effect of divalent cations—has not yet been investigated in connection with liposome-cell fusion. Hence only the properties of the lipids incorporated by cells can be reviewed here briefly.

Cationic liposomes or anionic liposomes coated with heat-aggregated immunoglobulin are taken up by Hela cells (133) or phagocytes (134) via the endocytotic route. Experiments reported recently suggest that liposomes prepared from neutral phospholipids may enter cells both by fusion with the cell membrane and by endocytosis (135–142). The contribution of each of the two mechanisms is assessed by means of inhibition of endocytosis by cytochalasin B, glutaraldehyde, or inhibitors of cellular energy metabolism. Uptake of “solid” negatively charged vesicles is also affected by such treatments. It has therefore been concluded that these liposomes are largely incorporated by endocytosis. By contrast, incorporation of “fluid” charged vesicles is not influenced greatly by such treatment, suggesting that fusion of vesicles with the cell membrane predominates (138–140). It would thus seem that liposome fusion with cells bears a close resemblance to fusion of liposomes with each other in respect of surface charge and physical state of the membranes (see Section 3.1).

3.3 Cell-Cell Fusion

Several types of cell fuse when incubated with liposomes, lipophilic compounds, or viruses. These cells, mainly erythrocytes or monolayer and suspension cell cultures, do not exhibit natural fusion capacity as do myo-

blasts (see Section 2.4). Such experiments are useful for inducing cell-cell fusion (e.g., for hybridization) but may be less satisfactory regarding their physiological relevance.

3.3.1 Cell-Cell Fusion by Liposomes

Fusion of cells by liposomes bears some striking similarities to the fusion of liposomes with cells. This is not surprising since fusion of cells by liposomes is most likely initiated by an attachment of liposomes with cells creating a bridge. Fusion of the bridging liposomes with adhering cells leads to cytoplasmic continuity of the cells (143). Neutral phosphatidylcholine liposomes are unable to bring about cell fusion. However, extensive cell fusion is elicited by negatively charged or positively charged vesicles (143–145). In addition to surface charge, the ability of liposomes to fuse cells requires that lipids in the vesicle membrane be “fluid” at the experimental temperature (143). Thus both vesicle surface charge and the physical state of vesicle lipids are of major importance for cell-cell fusion by liposomes as well as liposome-cell fusion and fusion of liposomes with each other. Although divalent cations might also play some role in cell-cell fusion by liposomes, few experimental data suggest this possibility (143–145).

3.3.2 Cell-Cell Fusion by Lipophilic Compounds

Avian erythrocytes and their interaction have been investigated by several groups as a model for membrane fusion. Erythrocytes fuse upon addition of various lipophilic compounds (fusogenic lipids). This process demands Ca^{2+} in millimolar concentrations. Mg^{2+} and Ba^{2+} can replace Ca^{2+} (146). Desialized or ATP-depleted chicken erythrocytes form polykaryons in the presence of Ca^{2+} and the ionophore A23187 at neutral pH (147, 148). Preincubation of chicken erythrocytes at pH 10.5 and subsequent addition of Ca^{2+} also causes fusion of the cells (149). Thus it appears that pretreatment of avian erythrocytes is obligatory for fusion. As found in other model systems, divalent cations in millimolar concentrations are essential for erythrocyte fusion.

3.3.3 Cell-Cell Fusion by Viruses

Binding of viruses to cells is achieved by the interaction of the virus envelope with receptor substances present on the cell surface. These receptors contain sialic acid, which can be removed by applying neuraminidase (150). Cells treated with this enzyme cannot be agglutinated by viruses (151, 152). The first step of viral binding to cells is independent of divalent

cations (152–155). However, subsequent fusion of nucleated cells requires the presence of divalent cations (156). Accordingly in this model system for membrane fusion, virus-cell interaction and virus-mediated cell-cell fusion differ with respect to the requirement of divalent cations. Fusion of human or avian erythrocytes by a low concentration of virus seems to be an exceptional case, considering the fact that fusion can proceed in the absence of divalent cations (155, 157).

Although fusion of cells by viruses is seemingly a complex process (for review see ref. 158), basic properties such as involvement of divalent cations are shared with those of other model systems. The participation of neuraminidase-sensitive structures in the binding of viruses to cells as well as in fusion of biological membranes (see Sections 2.1.3 and 2.3.3) are matters of particular interest.

4 SUMMARY AND CONCLUSION

Membrane fusion involved in exocytosis and skeletal muscle development can be investigated experimentally using isolated secretory vesicles and myoblast cell membranes. Each of the two processes occurs under conditions reflecting the physiologic ion environment of the membrane. Secretory vesicles fuse with Ca^{2+} at low (cytoplasmic) concentrations and myoblast cell membranes fuse after addition of Ca^{2+} in concentrations found in the extracellular fluid. Ca^{2+} cannot be replaced by other divalent cations in this “physiological” range of concentration.

At higher concentrations, a second type of fusion of isolated biological membranes has been observed which can be triggered by Ca^{2+} as well as other divalent cations. This type of fusion in its nonspecific requirement for high concentrations of divalent cations strongly resembles the properties of various fusion model systems such as liposome-liposome fusion, liposome-cell fusion, and cell-cell fusion.

Proteinaceous membrane components participate in the fusion of secretory vesicles and myoblast cell membranes induced by Ca^{2+} in physiological concentration. This is supported by the effect on this process of proteolytic enzymes, neuraminidase, glutaraldehyde, and inhibitors of protein biosynthesis.

Membrane phospholipid composition is important in fusion of biological membranes insofar as it defines the physical state of the membrane. Membrane lipids have to be at least in part in the liquid crystalline state. The requirement for acidic phospholipids has been shown for lipid-lipid and lipid-cell fusion. In these processes they are possibly the site of divalent cation interaction. Since acidic phospholipids hardly discriminate between

different cations and interact with them only at concentrations greater than 10^{-4} M, these phospholipid-ion interactions are not sufficient to explain the ion dependence of various types of biological fusion.

Obviously, membrane proteins are responsible for the unique properties of myoblast cell membrane or secretory vesicle fusion. However, the mechanism of their action is not yet clear. They may act as recognition sites of interacting membranes, bind Ca^{2+} specifically, or be involved in the production of structural changes comparable to those presumably taking place during fusion of pure lipid bilayers. No doubt studies of the fusion of biological membranes *in vitro* are promising and will lead to an understanding of the molecular mechanism underlying exocytosis and cell-cell fusion.

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