

Chapter 1

Studies on Membrane Fusion with Natural and Model Membranes

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1. INTRODUCTION

Fusion of membranes is a common and highly important event in the biology of eukaryotic cells. Membrane fusion is required for the uptake by endocytosis and the intracellular digestion of extracellular material and also for the transport of intracellular materials to the extracellular space by exocytosis. The formation of endocytotic vesicles at the cell surface involves invagination of a segment of the plasma membrane, which must then fuse with itself in order to form a closed vesicle. Subsequently, the digestion of the contents of the endocytotic vesicles involves a series of membrane fusion sequences between these vesicles and lysosomes and Golgi vesicles (review, Edelson and Cohn, 1978). Membrane fusion also plays a prominent role in the reverse process of exocytosis in which fusion takes place at the cell surface between the membrane of the exocytotic vesicle and the plasma membrane. Exocytotic discharge is basic to the processes of cell excretion and secretion and is involved in the release of a wide variety of enzymes, hormones, and neurotransmitter substances from such cells as the newly fertilized egg, blood

platelets, leukocytes, mast cells, nerve cells, cells participating in the formation of kinins, angiotensin, and erythropoietin, and hormone-producing cells in the adrenal medulla, neurohypophysis, anterior pituitary, thyroid, and pancreas (reviews, Ceccarelli et al., 1974; Douglas, 1975; Carafoli et al., 1975).

The localization of cellular secretory products within membrane-bound vesicles and their export from the cell by fusion of the secretory granule with the cell surface offer a number of advantages over other methods of secretion. The products stored within secretory granules are mostly proteins and are therefore protected from destruction by cytoplasmic enzymes. This mode of storage also enables the secretion products to be transported over long distances within the cell without significant decay. A notable example is found in nerve axons, where secretory granules containing neurotransmitter substances are transported for distances of up to several centimeters before being released by fusion at the nerve terminal. The major significance of the storage of secretory products within vesicles lies not so much in the storage process, however, as in the process by which these products are released from the cell by exocytosis, because this enables the entire contents of the granule to be exported from the cell without loss or degeneration within the cell. Finally, exocytosis offers a further advantage over other methods of secretion in that the stimulus for the release of the secretory product can act directly on the plasma membrane, which is also the site of fusion and release. This enables the exocytotic secretion of material to be restricted to specific regions of the plasma membrane by means of differences in the ability of various areas of the plasma membrane to respond to the release stimulus and by differences in the capacity of regions of the plasma membrane to fuse with the secretory granule membrane.

Although it is established that membrane fusion plays an important role in the release of cellular secretory products that are essential for homeostasis, it now seems likely that intracellular material packaged into membrane-bound vesicles by the Golgi apparatus is released by fusion and exocytosis at the cell surface in many cell types which would not formally be classified as secretory cells. The fusion and incorporation of vesicles from the Golgi apparatus into discrete regions of the plasma membrane may be a common occurrence in most, if not all, cells and could serve as a possible mechanism for achieving quantitative and qualitative variations in the distribution of material at the cell surface and for determining the specificity of cell surfaces (reviews, Morré, 1977; Holtzman et al., 1977). Similarly, budding of segments of membrane and their release from parent structures such as the endoplasmic reticulum followed by their fusion with other organelles such as the Golgi apparatus may provide a mechanism for the transfer of membranes and other materials within the cell (see discussion by Morré, 1977).

Events requiring membrane fusion are equally common in the interaction

between cells. The zona occludens formed between the surfaces of apposed cells, both *in vivo* and *in vitro*, involves "partial" fusion of a limited area of the plasma membranes of the two cells to form a single five-layered membrane (review, McNutt, 1977). The fused membranes present at these sites may function as areas of cell-to-cell ionic and macromolecular exchange, and communication of this type may be important in maintaining spatial "polarity" and social "order" within cell communities (review, Sheridan, 1976).

Complete fusion between cells to form multinucleate cells (polykaryocytes) is also well documented, and much of our current understanding of the membrane fusion reaction is derived from the study of cell fusion. Spontaneous cell fusion occurs *in vivo* as a normal process in fertilization of ova by spermatozoa (review, Gwatkin, 1976) and in the genesis of the polykaryocytes found in bone, muscle, and the placental syncytiotrophoblast (review, Poste and Allison, 1973). Cell fusion is also an important response in pathological situations. Fusion of fixed and free macrophages is a common cellular response in a large number of chronic inflammatory conditions induced by a wide range of chemical, physical, and biological agents (review, Papadimitriou, 1978). Recent experiments have also established that in certain tumors fusion can occur between host and tumor cells (review, Ringertz and Savage, 1976), although the functional significance of this phenomenon is presently unclear.

Cell fusion also occurs as a cytotoxic or cytopathic response to infection with a number of DNA- and RNA-containing viruses (reviews, Poste, 1970a, 1972). The ability of viruses to induce cell fusion has also been exploited extensively over the last decade as a potent tool for producing hybrid cells and heterokaryons for the experimental study of genomic and phenotypic regulation in mammalian cells (review, Ringertz and Savage, 1976). This powerful experimental approach has further emphasized the need for an understanding of the membrane fusion reaction so that fusion between highly different types of cells can be engineered more accurately.

It is thus clear that membrane fusion is of vital significance in the economy of the cell. The integrity of many cellular organelles and certain features of the functional coordination between cells are linked closely to this aspect of membrane behavior. The sequential and temporal organization of the membrane fusion reaction plays an important part in the overall balance of cellular activity, and may be of prime importance as a mechanism by which the differentiated cell state is maintained. Equally important, it must also be acknowledged that pathological dysfunction in the various digestive, excretory, and secretory activities of the cell might result from failure or alteration of the membrane fusion reaction (Armstrong and D'Arcy Hart, 1971; Jones and Hirsch, 1972), and the value of certain therapeutic modalities for such conditions might stem from their ability to affect membrane

fusion (see Armstrong and D'Arcy Hart, 1975; Oliver et al., 1976; Edelson and Cohn, 1978). These important questions are now beginning to receive the attention they deserve.

No attempt will be made in this chapter to review each of these examples of membrane fusion in detail. We will confine our comments largely to experiments on spontaneous and experimentally induced cell fusion and the fusion of lipid vesicles of defined composition with cultured cells and with other vesicles. Although this emphasis largely reflects our own research interests, experiments using these particular systems have provided most of the available information on the mechanism(s) of membrane fusion.

Despite the widespread importance of membrane fusion in both cellular and subcellular functions, surprisingly few studies have been done with the specific aim of identifying the mechanism(s) of membrane fusion and the factors that regulate this phenomenon. Thus much of our understanding on this aspect of membrane behavior has come indirectly from experimental observations on the factors that control the various cellular and subcellular phenomena in which membrane fusion occurs. This situation is changing rapidly, however, as more investigators from a wide variety of scientific disciplines become interested in defining the molecular events involved in membrane fusion. Over the last 3 years there has been a dramatic increase in the number of publications concerned with various aspects of membrane fusion. The increased interest in this phenomenon is also reflected in the diversification of experimental strategies now employed in analyzing highly different examples of membrane fusion behavior at both the cellular and subcellular levels.

Finally, membrane fusion cannot be considered in isolation from other aspects of membrane activity. Any analysis of the mechanism(s) of membrane fusion must take into account the basic properties of the membranes involved and, wherever possible, identify the relationship between events in membrane fusion and other aspects of membrane function. In this way, the experimental study of membrane fusion can also provide useful information on basic membrane organization. At this level, the study of membrane fusion begins to overlap with other major areas of contemporary membrane research concerned with the dynamic properties of biological membranes and their role in specialized cellular functions.

2. SPONTANEOUS CELL FUSION

Events in biology which are classified as "spontaneous" can be defined as those for which a cause is not known. In this section we will briefly review examples of cell fusion occurring *in vivo* and *in vitro* which can occur without the deliberate addition of exogenous fusing agents such as viruses or chemicals. Most examples of spontaneous cell fusion nonetheless represent strictly

programmed events in which fusion occurs during the ontogeny of particular tissues, during specific developmental cycles or in response to specific pathological states.

The one general characteristic of spontaneous cell fusion is its rarity. Of the many different cell types present in higher eukaryotes, only a very small number of types can fuse spontaneously. Even in those cells which are able to fuse, there are barriers to fusion with inappropriate cells. For example, although gametes fuse during fertilization, fusion is not only highly species specific but also tissue specific in the sense that fusion with somatic cells does not take place. In contrast, species barriers do not appear to restrict the spontaneous fusion of myoblasts from different species or the fusion of monocytes, but tissue-specific barriers to fusion exist which dictate that neither of these cells will fuse spontaneously with cells of a different histiotypic origin.

No attempt will be made to provide an exhaustive review of all aspects of cell fusion behavior. We will confine our comments largely to the role of changes in plasma membrane organization in determining cell fusion behavior and the nature of the events responsible for triggering cell fusion. In most instances, however, detailed information on these questions is still lacking. Although a large literature exists for subjects such as fertilization and myogenesis, most experimental observations have been concerned with the physiological or developmental events which follow cell fusion and few experiments have been undertaken with the specific aim of elucidating the membrane events involved in fusion *per se*.

2.1. Fertilization

There is a voluminous literature dealing with various aspects of fertilization, but only in the last few years have detailed studies begun to be made on the surface properties of male and female gametes and the complex series of membrane interactions by which these cells fuse. Experimental analysis of the membrane changes accompanying fusion between sperm and egg poses a number of technical problems which are not encountered with other examples of cell fusion discussed in this chapter. First, the techniques for the handling and maintenance of gametes *in vitro* are in general more demanding than those used for most somatic cells. Second, while large numbers of spermatozoa can be easily obtained, the isolation and *in vitro* manipulation of eggs are more difficult. In the case of mammals, the small numbers of eggs which can be obtained from donor females (even after superovulation) dictate that it is often difficult to obtain sufficient amounts of cellular material for certain biochemical analyses. Although this problem can be avoided by using amphibians or invertebrate species which produce large numbers of eggs, restrictions in the breeding season of many of these species limit the availability of material at certain times of the year. Finally, the major drawback to fertilization as a system for studying membrane fusion is that the number

of fusion events is limited. In both vertebrates and invertebrates, only a single sperm fuses with the egg. This contrasts with the other examples of cell fusion discussed in this chapter in which fusion is not restricted to two cells but involves multiple sequences of cell-to-cell fusion. However, there are a number of similarities between gamete fusion and other examples of cell fusion which warrant brief discussion. Except where stated otherwise, the following remarks refer to the fusion of mammalian gametes.

Unlike the situation in many invertebrates and certain nonmammalian vertebrates, the mammalian spermatozoon is not functionally competent when released from its association with the Sertoli cells in the germinal epithelium of the testis and must undergo further maturation in the epididymus before it acquires the ability to fuse with the egg. Inherent in much of the work that has been done on sperm maturation, although infrequently stated as such, is the concept that the three gross anatomical segments of the epididymis, the caput, corpus, and cauda, also constitute distinct physiological compartments in which specific maturational changes occur in the sperm. It is now clear from both ultrastructural and biochemical observations that this is an oversimplification (review, Hamilton, 1973). For descriptive purposes, however, the three anatomical divisions of the epididymis are of some value and are still widely used in the literature to define the source of spermatozoa used in experiments.

The maturation of spermatozoa within the epididymis and the development of competence to penetrate the egg involve changes in several different organelles of the spermatozoon as well as changes in the behavior of the intact cell. A number of phenomenological observations have been made concerning alterations in spermatozoa during their transport through the epididymis. These include caudal migration and loss of the cytoplasmic (kinoplasmic) droplet; changes in the response of spermatozoa to heat, cold, and alkaloids; increased specific gravity in mature sperm; and increased capacity of mature sperm for sustained motility (review, Bedford, 1972). Apart from changes in sperm mobility which are of importance for penetrating the outer vestments of the egg, it is difficult to define meaningful functional correlations between the above alterations and development of the ability to fuse with the egg.

Transit of spermatozoa through the epididymis is also accompanied by a number of alterations in plasma membrane properties. These include changes in the number and distribution of negatively charged groups on different regions of the sperm surface; alterations in adhesiveness and agglutinability; changes in the affinity of the surface for histochemical dyes; changes in lipid composition; and binding of macromolecular components from epididymal secretions to the sperm surface (for references, see the review by Gwatkin, 1976). The functional importance of these changes in allowing the sperm to fuse with the egg is unknown. However, from our

knowledge of the factors that affect other examples of membrane fusion it is not unreasonable to suggest that changes in membrane lipid composition and coating of the sperm surface with high molecular weight epididymal secretions might affect the fusion capacity of the sperm.

Significant changes in lipid composition have been shown to occur in several species as spermatozoa pass through the epididymis (Dawson and Scott, 1964; Crogan et al., 1966; Quinn and White, 1967; Scott et al., 1967; White, 1973; Turner et al., 1975). The membranes of testicular sperm have a higher lecithin and cholesterol content and a higher ratio of saturated to unsaturated fatty acids than mature sperm from the cauda epididymis or ejaculated sperm. These findings suggest that the membrane lipids in mature sperm would be more "fluid" than those of testicular sperm. These data refer, however, to the bulk lipid composition, and localization of different classes of phospholipid to different regions of the sperm has not yet been attempted. Nonetheless, in general terms, these differences in membrane lipid composition may be important in determining the ability of the sperm plasma membrane to fuse with other membranes during the acrosome reaction and subsequent fusion with the eggs. Recent studies on the *in vitro* fusion of model membranes of defined composition have shown that an essential requirement for fusion is that the lipids in the interacting membranes be "fluid" and that increases in membrane lecithin and cholesterol content reduce the capacity of membranes to fuse (see Section 7). If similar conditions apply to fusion involving the sperm plasma membrane, then the changes in lipid composition accompanying sperm maturation in the epididymis would be expected to exert an important influence on the fusion capacity of the sperm.

It is now recognized that spermatozoa must reside in the female tract for several hours before they acquire the capacity to penetrate the zona pellucida and fertilize the ovum. The need for a physiological change in mammalian spermatozoa resulting from their residence in the female tract as a prerequisite for successful fertilization was first recognized by Austin (1951) and Chang (1951), and this change was termed "capacitation" by Austin (1952). Since capacitation was first recognized, an enormous amount of research has been done on this phenomenon with the aim of elucidating the factors involved (reviews, Bedford, 1970, 1972; Gwatkin, 1976).

There is now considerable evidence that the process of capacitation involves the removal from the sperm surface of "coating factors" which were absorbed to the surface during transit of sperm through the epididymis (review, Gwatkin, 1976). The binding of extrinsic "coating factors" from epididymal secretions to the sperm surface has been proposed to "stabilize" the sperm plasma membrane and reduce its capacity to fuse. Membrane stabilization of this kind would presumably facilitate the storage of mature sperm in the cauda epididymis and prevent premature activation of the acrosome reaction. Once the stabilizing factors are removed, the sperm plasma membrane

appears to be highly susceptible to fusion. This is quickly manifest with the onset of the acrosome reaction, which involves fusion between the outer acrosomal membrane and the overlying plasma membrane (see below). The need for the removal of coating material(s) from the sperm surface before fertilization can take place is reminiscent of the situation in virus-induced cell fusion of somatic cells where digestion and removal of the cell surface coat are necessary prerequisites for fusion (see Poste, 1970a,b, 1972).

The mechanism(s) responsible for loss of coating factors from the sperm surface during capacitation is unknown. *In vitro* treatment of spermatozoa from several species with hypertonic salt solutions has been shown to induce capacitation and elution of material from the sperm surface (references, Gwatkin, 1976). The induction of capacitation *in vitro* by high ionic strength tissue culture media and physiological salines raises the possibility that increases in the osmolarity of the secretions in the female genital tract might induce elution of coating material from the cell surface *in vivo*. There is presently no information available to support or refute this possibility. The other possible mechanism for removal of coating material from the sperm surface involves digestion by enzymes present in female tract secretions. For example, treatment of sperm *in vitro* with pronase, trypsin, β -glucuronidase, β -amylase, or neuraminidase has been shown to induce capacitation (see Gwatkin, 1976). It is tempting to speculate that the capacitating action of these enzymes is mediated via their ability to remove surface-associated materials from the sperm. The high levels of proteolytic activity found in the estrous uterus (Joshi and Murray, 1974) compared with the low enzyme activity and high protease inhibitor content of the progestational uterus (Hammer et al., 1968) provide additional circumstantial evidence compatible with this interpretation. Conditions in the estrous uterus would thus be expected to favor enzymic removal of surface materials from spermatozoa, but definitive evidence on this point is still lacking.

As a result of the changes in the sperm plasma membrane induced by capacitation, spermatozoa next undergo the acrosome reaction (Fig. 1). This involves fusion at several points between the outer acrosomal membrane and the overlying plasma membrane (Barros et al., 1967; Bedford, 1968; Franklin et al., 1970; Thompson et al., 1974). Fusion between these membranes creates a series of connecting ports which presumably allow the release of the acrosomal enzymes. The latter are believed to facilitate the passage of spermatozoa through the various investments of the egg (review, Gwatkin, 1976).

Insight into the mechanism of membrane fusion in the acrosome reaction is limited. However, in view of the central role of Ca^{2+} in controlling numerous other examples of membrane fusion (review, Poste and Allison, 1973), it is of interest that the acrosome reaction can be controlled *in vitro* by manipulating the Ca^{2+} concentration in the incubation medium. Yanagi-

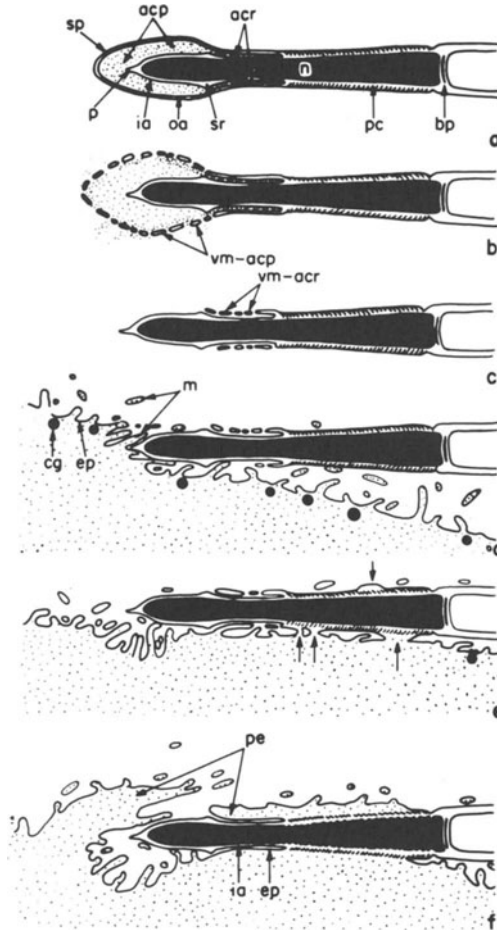


Fig. 1. Diagrammatic representation of a sagittal section of the head region of a hamster spermatozoon showing the various membrane fusion events involved in the interaction of the spermatozoon with the egg. (a) In the oviduct. No obvious morphological change in sperm morphology is observed at this stage. (b) In the cumulus oophorus, showing vesiculation of the outer acrosomal membrane and the overlying plasma membrane (start of the acrosome reaction) and proposed release of hyaluronidase from the acrosome. (c) Penetration of the spermatozoon through the zona pellucida (substance of the zona not shown). (d) After penetration of the zona, showing entrapment of the head of the spermatozoon by microvillous projections on the surface of the vitellus. (e, f) Fusion between the spermatozoon and the egg. Note that fusion occurs between the postacrosomal region of the sperm plasma membrane and the closely apposed plasma membrane of the vitellus (shown by arrows). Key: acp, acrosomal cap (anterior portion of acrosome); acr, acrosomal collar (equatorial segment of the acrosome); bp, base plate; cg, cortical granule; ep, egg plasma membrane; ia, inner acrosomal membrane; n, nucleus; oa, outer acrosomal membrane; pc, postacrosomal region of the plasma membrane; sp, sperm plasma membrane; pe, perivitelline extension; vm, vesiculated membrane. Reproduced with permission from Gwatkin (1976).

machi and Usui (1974) and Rogers and Yanagimachi (1975) found that incubation of guinea pig spermatozoa in a simple modified Krebs-Ringer or Tyrode's solution containing 0.1% bovine serum albumin resulted in capacitation and the subsequent onset of the acrosome reactions in about two-thirds of the spermatozoa after 10–12 hr. If Ca^{2+} was omitted from the incubation medium, the acrosome reaction did not take place, but it could be induced within minutes by the addition of Ca^{2+} to the preincubated spermatozoa. A full preincubation period was necessary to obtain this rapid response to Ca^{2+} , indicating that capacitation, presumably involving loss of surface coating materials, was an essential prerequisite for the acrosome reaction. Ca^{2+} has also been shown to be essential for the acrosome reaction in several marine invertebrates (Dan, 1967). It is conceivable therefore that the natural inducer of the acrosome reaction might act by altering the permeability of the plasma membrane to Ca^{2+} . It is perhaps significant therefore that the acrosome reaction can be induced in uncapacitated sperm by the carboxylic acid ionophore A23187, which facilitates Ca^{2+} transport across the plasma membrane (Summers et al., 1975). The induction of the acrosome reaction by A23187 shares obvious similarities with reports of ionophore-induced triggering of exocytosis in somatic cells in which secretory granules undergo rapid fusion with the plasma membrane after treatment with this compound (see Cochrane and Douglas, 1974; Eimerl et al., 1974; Kagayama and Douglas, 1974; Garcia et al., 1975; Nordmann and Currell, 1975; Williams and Chandler, 1975).

Completion of the acrosome reaction involves sloughing of the vesiculated fused acrosome-plasma membrane complex from the head of the sperm. In most species the vesiculated products of membrane fusion are lost from the sperm head before penetration of the zona pellucida of the egg begins (Bedford, 1972; Thompson et al., 1974). Following loss of the vesiculated membrane elements, continuity of the surface membrane over the sperm head is maintained by fusion at the anterior limit of the equatorial region between the plasma membrane and the remaining fragment of the outer acrosomal membrane. Thus at the end of the acrosome reaction the sperm head is covered by the inner acrosomal membrane anteriorly and the original plasma membrane posteriorly.

Before sperm which have undergone the acrosome reaction can fuse with the egg plasma membrane they must first attach to and penetrate through the zona pellucida around the egg. A number of elegant experiments have been done on this aspect of fertilization, but they fall beyond the scope of this chapter and the interested reader is referred to the comprehensive review by Gwatkin (1976) for further details.

Once through the zona pellucida, the head of the sperm makes immediate contact with the plasma membrane of the egg and fusion follows quickly. Electron microscopic evidence of fusion between the plasma

membrane of the egg and the penetrating spermatozoon was first described in mammals by Szollosi and Ris (1961) in rat gametes. This study was important in that it established that the plasma membranes of the two gametes did in fact fuse and that the spermatozoon was not phagocytosed by the egg. Electron microscopic evidence of fusion between gamete plasma membranes has since been obtained for the hamster (Barros and Franklin, 1968; Yanagimachi and Noda, 1970), the mouse (Stefanini et al., 1969), the rat (Pikó, 1969), and the rabbit (Bedford, 1970, 1972). Yanagimachi et al. (1973) have also obtained electron histochemical evidence for intermixing of plasma membrane components from male and female cells following gamete fusion.

These ultrastructural studies also established that fusion of the sperm with the egg always involves the postacrosomal region of the sperm plasma membrane. The role of the postacrosomal plasma membrane in initiating fusion between mammalian gametes is in striking contrast to gamete fusion in marine invertebrates in which initial contact with the egg membrane is made by the apical region of the sperm head (reviews, Epel, 1975; Summers et al., 1975).

Fusion between mammalian gametes is initiated by the development of point fusions between the postacrosomal plasma membrane of the sperm and microvillus-like processes on the vitelline surface. Scanning electron microscopy of this process shows that the vitelline microvilli wrap around the sperm head (Yanagimachi and Noda, 1972). It is of interest to note that microvilli are also prominent in fusion occurring between various somatic cells (see Poste, 1970b). The low radius of curvature of such structures may be of value in achieving close contact between interacting cells, since microvilli would encounter significantly less electrostatic repulsion to contact than larger cellular projections (see Pethica, 1961; Poste, 1970b). The plasma membrane around the postacrosomal region of the sperm head next disappears and this region of the nucleus then becomes enveloped by ooplasm. In contrast, the more anterior region of the sperm head covered by the inner acrosomal membrane and the equatorial segment (if it persists) does not fuse with the egg plasma membrane and is instead phagocytosed as a separate vesicular structure which gradually disintegrates within the egg (Barros and Franklin, 1968; Pikó, 1969; Yanagimachi and Noda, 1970). The factors that dictate these different uptake mechanisms for specific regions of the spermatozoon are presently unknown. The induction stimulus for initial fusion between the postacrosomal plasma membrane and the egg is also unknown. The specific nature of this interaction is indicated by the fact that tated spermatozoa are unable to fuse with eggs (Yanagimachi and Noda, 1970). The maturity of the oocyte can also affect the fusion reaction and sperm are unable to penetrate immature oocytes (Barros and Munoz, 1973; Overstreet and Bedford, 1974). The importance of species specificity in

determining the ability of sperm and egg membranes to fuse has not been investigated in detail, although there is some evidence to suggest that it is not a major limiting factor. Hanada and Chang (1972) found that capacitated mouse spermatozoa readily entered zona-free eggs of both homologous and heterologous species *in vitro*. This apparent lack of species specificity in sperm-egg fusion is perhaps not surprising in the light of experiments with somatic cells in which interspecific hybrid cells have been created by fusion of cells from diverse species and even different taxonomic orders. However, even though fusion between gametes from heterologous species can occur, the overall fusion response displayed by gametes appears to be highly specific since spermatozoa do not appear to be able to fuse spontaneously with somatic cells *in vitro* and are instead incorporated by phagocytosis (references, Gwatkin, 1976). The present lack of information on the biochemical events accompanying the fusion of sperm and egg reflects our general ignorance of the macromolecular organization of the membranes involved. However, there is some evidence that the structural organization of the postacrosomal plasma membrane may differ from that of other regions of the sperm plasma membrane. The translational mobility of lectin receptors within this region of the plasma membrane appears to be higher than in other regions of the membrane (Nicolson and Yanagimachi, 1974). Clustering of integral membrane proteins has been proposed as a necessary event in membrane fusion (see Poste and Allison, 1973), and it is tempting to speculate that the higher mobility of components in the postacrosomal membrane dictates that this region may be more appropriately organized to allow redistribution and clustering of membrane proteins prior to fusion with the egg plasma membrane.

Nicolson *et al.* (1975) have used similar lectin-binding techniques to monitor the translational mobility of components within the plasma membrane; they found a uniformly high degree of lectin receptor mobility throughout the egg membrane. This observation suggests that fusion could probably occur at any point on the egg surface, assuming of course that correct apposition with the postacrosomal region of the sperm had first occurred. However, recent observations by Johnson *et al.* (1975) suggest that in mouse egg the attachment of sperm *in vitro* rarely occurs to the membrane region overlying the second metaphase spindle. The functional significance of this finding has yet to be established. However, it may well be a protective mechanism to ensure that extrusion of the second polar body can take place unimpaired, or, alternatively, it may serve to prevent fusion of the sperm with the egg at a region in which the sperm head itself might be subsequently extruded.

Following successful fusion of the fertilizing spermatozoon with the egg, penetration of the egg by additional sperm is inhibited by the rapid

onset of the so-called block to polyspermy. The block to polyspermy in mammalian fertilization operates at both the zona pellucida and the egg plasma membrane, although significant species variation exists concerning which of these two sites is more important (for details, see review by Gwatkin, 1976). In the case of the block imposed at the level of plasma membrane it is not clear whether this involves a change in the fusion susceptibility of the egg plasma membrane or whether sperm penetration is inhibited at an earlier step such as attachment to the membrane.

A number of studies have also been done on the interaction of gametes with cultured somatic cells. As mentioned earlier, *spontaneous* fusion between gametes and somatic cells has not been detected. However, by using a cell-fusing agent such as Sendai virus or lysolecithin, fusion of both sperm (Brackett et al., 1971; Gabara et al., 1973; Bendich et al., 1974) and eggs (Graham, 1969; Baranska and Koprowski, 1970; Lin et al., 1973) with somatic cells has been achieved. Evidence has been obtained to show that sperm nuclei can undergo limited activation, including initiation of DNA synthesis, within the cytoplasm of somatic cells (Johnson et al., 1970; Croce et al., 1972; Zelenin et al., 1974). However, the equivalent of syngamy, namely the formation of a viable hybrid cell combining both sperm and somatic cell genomes within a single nucleus, has yet to be achieved. There are indications, however, that nuclei at earlier stages of spermiogenesis may be easier to activate. For example, Nyormoi et al. (1973) have reported that proliferating hybrid cells could be produced by fusing rat spermatids with an established mouse cell line.

Despite the general failure to achieve true germ cell-somatic cell hybrids, there is some evidence that sperm can nonetheless act as vectors for the transfer of genetic information to somatic cells (Higgins et al., 1975). These investigators showed that treatment of Chinese hamster DON cells with rat sperm resulted in subsequent expression of rat fetal antigens by the DON cells. Karyotypic analysis of these cells failed, however, to reveal the presence of any rat chromosomes, indicating that an extremely limited amount of genetic material had been transferred. It was not established in this study whether sperm fused with somatic cells or were endocytosed. However, this type of experiment may well open the way for the experimental use of mammalian sperm as tools in somatic cell genetics. The experimental transfer of eukaryotic genetic information by nuclear transplantation and virus-induced cell fusion techniques has already emerged as a powerful approach in the genetic analysis of mammalian somatic cells *in vitro*, and a similarly bright future might well await a method for transferring very small amounts of genetic material. A particular attraction in transferring genetic information to somatic cells from spermatozoa, as opposed to any other cell, is that if the technique could be refined it would

enable direct mapping of genes in a haploid genome and would also offer novel opportunities for genetic and biochemical analysis of a genome which has not yet been modified by the process of tissue differentiation.

2.2. Myoblast Fusion

The formation of multinucleated skeletal muscle fibers involves the fusion of mononucleated cells called "myoblasts" (reviews, Konigsberg, 1965; Yaffe, 1969; Fischman, 1972). This phenomenon has been studied in considerable detail using cultured myoblasts, usually of avian or rodent origin (for detailed description of methods, see Yaffe, 1973). Few experimental studies have been done, however, with the specific aim of identifying the mechanism of myoblast fusion. Most of the work on myogenesis has been concerned with the role of cell fusion in triggering other steps in muscle cell differentiation. Fusion of myoblasts to form multinucleate myotubes is accompanied by extensive alterations in nucleic acid metabolism, energy metabolism, changes in plasma membrane receptors, and the synthesis of myosin, actin, and a number of enzymes.

The temporal relationship between myoblast fusion and biochemical differentiation has stimulated numerous experiments to define whether fusion is a necessary prerequisite for the expression of the differentiated phenotype. One experimental approach to this question has been to arrest myoblast fusion to see if unfused myoblasts are able to synthesize the various gene products expressed by differentiated myotubes. In addition to offering information on cell differentiation, such experiments have provided some insight into the factors which influence the cell fusion process and thus warrant brief review.

When mononucleated myoblasts are placed in culture, they first proliferate at an exponential rate, but on reaching a critical cell density they stop dividing and enter a nonmitotic state in which they become susceptible to fusion. The relationship between the onset of cell fusion and cell population density does not depend simply on an increased frequency of cell contact and appears to require modification of the culture medium by material released from the myoblasts (Konigsberg, 1971). Fusion of myoblasts is initiated only during the G_1 phase of the cell cycle, and cells in S, G_2 , or M are refractory to fusion (see Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1969; O'Neill and Stockdale, 1972; Buckley and Konigsberg, 1974). Bischoff and Holtzer (1969) have reported that following completion of mitosis a period of 2–5 hr is required before myoblasts are able to fuse, but O'Neill and Stockdale (1972) suggest that fusion susceptibility may be acquired in as little as 30 min after mitosis.

There is presently no information available on changes in cell surface

properties that might accompany the transition of myoblasts from a rapidly dividing state to a nondividing fusion competent state. It seems reasonable to conclude that myoblast fusion must be preceded by some form of specific cell recognition and adhesion between fusion competent myoblasts. For example, various nonmuscle cells fail to be incorporated into myotubes when co-cultivated with rapidly fusing muscle cell cultures (Yaffe, 1969; Bischoff and Holtzer, 1970). Cardiac myoblasts are able to fuse with skeletal myoblasts but smooth muscle cells are not incorporated (Bischoff and Holtzer, 1970).

Apart from the obvious requirement for the acquisition of cell surface properties compatible with fusion, the interaction of myoblasts with myotubes involves additional factors that regulate the final size of myotubes. For example, in a population of 1000 fusion-competent myoblasts, only two or three myotubes containing several hundred nuclei are formed rather than many myotubes containing small numbers of nuclei. This suggests that myogenesis does not proceed simply via the chance collision of fusion-competent cells. One mechanism by which the size and number of myotubes could be controlled is if the adhesive affinity of myoblasts for myotubes is greater than for other myoblasts. Thus, following the initial fusion of myoblasts to form early myotubes, fusion-competent myoblasts would adhere preferentially to myotubes and undergo fusion with existing myotubes rather than chance fusion with other myoblasts. There is also evidence that beyond a certain stage of maturation the myotube surface becomes refractory to fusion (Bischoff and Holtzer, 1969; Bischoff and Lowe, 1974). Fusion-competent myoblasts adhering to such myoblasts would be forced to seek other fusion-competent myoblasts and thus initiate a new generation of myotubes.

The nature of the cell surface components responsible for cell recognition and adhesion during myoblast fusion is not known. It has been shown that constant changing of the culture medium prevents myoblast fusion, suggesting that either cell adhesion or fusion (or both) requires cellular factors released into the medium (Konigsberg, 1971; Schudt and Pette, 1976). The nature of the active component(s) in conditioned medium is unknown. Recent experiments with substituted media have shown that nucleoside monophosphates can overcome the block to fusion imposed by medium depletion (Schudt and Pette, 1976). The relevance of this finding for myogenesis occurring under natural conditions is not clear, however, and information on the mechanism(s) by which nucleotides influence myoblast adhesion and fusion is also lacking.

Some insight into the nature of the molecules which are exposed on the surface of myoblasts and which presumably participate in cell recognition, adhesion, and fusion has come from studies on the effect of enzymic modification of the myoblast surface. Schudt and Pette (1976) reported that incubation of chick myoblasts with various glycosidases and monosaccharides did not inhibit their ability to fuse. This suggests that cell surface oligosac-

charides are not of major importance in the fusion process. Electron histochemical studies have also shown that the cell coat, which is composed of a variety of cell surface glycoproteins, is rarely detected in fusion-competent myoblasts (Fischman, 1972). As pointed out by Schudt and Pette (1976), this may be an important factor in determining the fusion competence of these cells. For example, electron histochemical studies have shown that the only membranes in *Echinospaerium nucleofilum* which undergo fusion are free of coat material (Vollet and Roth, 1974). Enzymic reduction of the cell coat has also been shown to be required for virus-induced fusion of a number of nonmuscle cells (Poste, 1972; Reeve et al., 1972). Also, as mentioned in the previous section, removal of coating antigens from the sperm surface is required before fusion with the egg can take place.

However, the recent isolation of a β -D-galactosyl-specific lectin with cell agglutination properties from the surface of developing muscle cells (Teichberg et al., 1975) dictates that the involvement of cell surface oligosaccharides in myoblast adhesion cannot be totally discounted. Maximum production of this lectin coincides with the period of greatest cell fusion activity (Nowak et al., 1976), raising the possibility that the interaction of cell surface-associated lectin molecules with oligosaccharides on adjacent cells could promote cell adhesion. Precedent for lectin-mediated cell adhesion exists in slime molds, where developmentally regulated lectins associated with the cell surface have been shown to promote specific cell adhesion during the differentiation of these organisms from vegetative to colonial forms (Rosen et al., 1975). However, initial observations on the involvement of the β -D-galactosyl-specific lectin in myogenesis have produced conflicting results. Gartner and Podleski (1975) reported that incubation of L6 rat myoblasts with specific haptenic inhibitors for the lectin blocked their ability to fuse, but Den et al. (1976) have been unable to confirm this observation using chick myoblasts.

Hynes et al. (1976) have reported that transformation of rat myoblasts with avian sarcoma viruses abolishes their ability to fuse. These investigators also found differences in the cell surface proteins expressed by untransformed and virus-transformed myoblasts. External labeling methods revealed that untransformed myoblasts expressed a high molecular weight ($>200 \times 10^3$) surface protein which resembled the so-called LETS cell surface protein found in various nonmuscle cells arrested in the G_1 phase of the cell cycle. This protein was not detected in virus-transformed myoblasts. It is unclear, however, whether the absence of the LETS-like protein in transformed myoblasts is causally related to their inability to fuse. Since this protein was not detected in untransformed myoblasts until *after* extensive fusion had occurred, it seems unlikely that the two phenomena are tightly coupled. Rather, the onset of fusion competence and expression of this protein would appear to be un-

related traits which are expressed by myoblasts as a result of entering the G₁ stage of the cell cycle. Furthermore, loss of fusion competence does not always accompany the transformation of myoblasts by tumor viruses (Fogel and Defendi, 1967; Simons et al., 1971).

The fusion of myoblasts requires a higher level of Ca²⁺ in the culture medium than that required to maintain cell replication (Shamberg et al., 1969; Van der Bosch et al., 1972, 1973; Cox and Gunter, 1973). Myoblasts grown in Ca²⁺-deficient media can enter the nonmitotic state which precedes fusion but do not undergo fusion. Addition to Ca²⁺ to such cultures results in the initiation of cell fusion, and multinucleated myotubes are detectable within 1–2 hr. Conversely, lowering the Ca²⁺ concentration in myogenic cultures in which fusion has started immediately inhibits further cell fusion. The extreme sensitivity of myoblast fusion to Ca²⁺ thus provides a useful experimental tool for synchronizing and/or arresting cell fusion.

The minimum Ca²⁺ concentration required for myoblast fusion varies between species ranging from 6.5×10^{-4} M for lizard myoblasts (Cox and Gunter, 1973) to 3×10^{-5} M for embryonic chick myoblasts (Shamberg et al., 1969). However, maximum cell fusion in which up to 90% of the myoblasts fuse to form myotubes requires a fivefold greater Ca²⁺ concentration in both systems (Van der Bosch et al., 1972, 1973; Cox and Gunter, 1973). The effect of Ca²⁺ on myoblast fusion is pH dependent (Van der Bosch et al., 1972). Optimum fusion at the Ca²⁺ concentration found in most complete synthetic media (1.8×10^{-3} M) occurs at pH 7.8. Lowering the pH decreases the amount of fusion at any given Ca²⁺ concentration, and higher concentrations of Ca²⁺ are required to induce maximum cell fusion. Sr²⁺ can substitute for Ca²⁺ in promoting myoblast fusion, but Mg²⁺, Mn²⁺, Zn²⁺, Cu²⁺, and La³⁺ are all inhibitory (Schudt et al., 1976).

Reporter and Raveed (1973) have shown that treatment of myoblasts with lysolecithin abolishes their ability to fuse. The formation of lysolecithin within the myoblast plasma membrane also might provide an explanation for the inhibitory effects of phospholipase A on myoblast fusion (Schudt and Pette, 1976). The inhibition of myoblast fusion by lysolecithin is in contrast to the well-documented action of this agent in promoting the fusion of a wide range of nonmuscle cells (see Section 4). The reason for this marked variation in cellular response to lysolecithin is unclear.

The spontaneous fusion of myoblasts is also inhibited by cytochalasin B (Sanger and Holtzer, 1971; Delain and Wahrmann, 1975). As discussed later in Section 3, cytochalasin B also inhibits virus-induced cell fusion, but in this system evidence has been obtained which suggests that cytochalasin does not impair membrane fusion per se but affects membrane-associated cytoskeletal elements involved in the redistribution of plasma membrane proteins immediately prior to fusion. The effect of cytochalasin on myoblast fusion may be

similar since this drug does not inhibit fusion of isolated vesicles prepared from the plasma membrane of chick myoblasts (C. Shudt, personal communication).

The lipid composition of the myoblast plasma membrane may also be important in determining the ability of these cells to fuse. Studies on the fusion of model membranes (Section 7) indicates that membranes composed of neutral phospholipids such as phosphatidylcholine are unable to fuse, although their fusion susceptibility can be enhanced by enrichment with acidic phospholipids such as phosphatidylserine. It is of interest to note therefore that acidic phospholipids constitute nearly 20% of the total lipids in the chick myoblast plasma membrane whereas sarcoplasmic reticulum membranes from differentiated skeletal muscle cells which are not known to fuse contain more than 70% phosphatidylcholine and less than 2% acidic phospholipids (Weidekamm et al., 1976). The reduction in fusion seen after incubation of myoblasts with lipid vesicles composed of neutral lipids and cholesterol (Van der Bosch et al., 1973) might also result from changes in plasma membrane lipid composition. Although analyses of membrane lipids were not done in these experiments, it is conceivable that exchange diffusion of phospholipids and cholesterol between vesicles and the plasma membrane altered the composition of the latter to a sufficient extent to impair fusion.

The fusion of cultured myoblasts can also be reversibly arrested by drugs that block nucleic acid metabolism. However, there is no evidence to indicate that the inhibition of myoblast fusion by such agents as halogenated pyrimidines (Bischoff and Holtzer, 1970) and rifampicin and ethidium bromide (Brunk and Yaffe, 1976) results from a direct effect on the membrane fusion reaction. Rather, it seems more likely that such agents impair synthetic processes which must be completed in order for myoblasts to enter the fusion-competent state.

A potentially useful system for analyzing membrane changes in myoblast fusion has been described recently by Schudt et al. (1976). These investigators have isolated intact vesicles from myoblast plasma membranes and have demonstrated that fusion of the vesicles can be induced by Ca^{2+} and inhibited by Mg^{2+} , lysolecithin, and phospholipases in identical fashion to intact cells. This system thus offers new opportunities for studying membrane fusion in a cell-free system under controlled conditions.

Another possible area for future research on myoblast fusion involves the use of myoblast variants which lack the capacity to fuse. Comparison of the surface properties of variants and wild-type fusion-competent myoblasts would be particularly instructive. Spontaneously arising myoblast variants which cannot fuse (Tarikas and Schubert, 1974) and mutagen-induced temperature-sensitive variants which fuse only at permissive temperatures (Loomis et al., 1973) have already been described. To the best of our knowl-

edge, however, such cells have yet to be used to investigate the mechanism of myoblast fusion.

2.3. Fusion of Macrophages

The term "giant cell" is widely used in the clinical literature to describe the multinucleate cells found in a wide variety of chronic inflammatory conditions (review, Papadimitriou, 1978). In vivo studies using macrophages labeled with radioisotopes or particulate matter have established that these multinucleate cells are formed by the fusion of macrophages (see Papadimitriou, 1978). The parental macrophages which form these polykaryons migrate from the circulatory system to sites where foreign particulate matter has accumulated and they undergo fusion there. The function of these multinucleate cells is believed to be to ingest foreign material by phagocytosis and to effect tissue debridement by the release of lysosomal enzymes. Fusion of macrophages to produce multinucleate cells with an identical morphology to those seen in granulomatous lesions in vivo has also been described in cultured macrophage populations (references, Papadimitriou, 1978).

Although a large amount of work has been done on the ultrastructure and enzyme cytochemistry of fused macrophages both in vivo and in vitro, information on the factors which control the fusion between these cells is scant. Recently, Papadimitriou and Sforcina (1975) have examined whether a range of pharmacological agents known to modify virus-induced cell fusion in vitro might influence the spontaneous fusion of mouse macrophages. These investigators found that the tertiary amine local anesthetic lignocaine and the antihistaminic phenothiazine derivatives trifluoperazine and diphenhydramine significantly reduced the extent of macrophage fusion in foreign body granulomas. This resembles the inhibitory effect of these drugs on virus-induced cell fusion (Poste and Reeve, 1972) and a number of examples of membrane fusion behavior involving subcellular membranes (see Poste and Allison, 1973). Papadimitriou and Sforcina (1975) also found that Verapamil HCl, a drug which has been shown to block the entry of Ca^{2+} into excitable cells (Nayler and Szeto, 1972), produced marked inhibition of macrophage fusion.

The nature of the factor(s) responsible for triggering spontaneous fusion of macrophages is unknown. There is some evidence, however, to suggest that inflammatory exudates may contain a factor which specifically promotes macrophage fusion. Gallindo (1972) observed that cultures of alveolar macrophages harvested from the lungs of rabbits previously sensitized with heat-killed BCG could be induced to fuse and form multinucleate giant cells by the addition of specific antigen to the culture fluid. It was shown subsequently (Gallindo et al., 1974; Parks and Weiser, 1975) that the supernatant fluids

from cultures of antigen-stimulated lymph nodes taken from BCG-sensitized animals contained a factor(s) which promoted the fusion of *normal* alveolar macrophages in culture. This factor was considered to be a lymphokine and was termed "macrophage fusion factor." This factor may be related to the one described by Godal et al. (1971) in supernatant fluids obtained from mixed leukocyte cultures which induced normal rabbit monocytes to fuse in vitro. Gallindo et al. (1974) have shown that the "macrophage fusion factor" released by sensitized lymphocytes has a molecular weight of 60,000, is non-dialyzable, and is resistant to heating at 80°C for 30 min, indicating that it probably does not correspond to macrophage migration inhibition factor.

It is presently unclear how this factor promotes macrophage fusion. If it acts directly on the plasma membrane to render it fusion susceptible, it might be of interest to test the effect of lymphoid cell supernatants containing this factor on the fusion susceptibility of cells other than macrophages and on fusion of model membrane systems of the kind discussed later in Section 7.

2.4. Other Examples of Spontaneous Cell Fusion

Spontaneous cell fusion also takes place in the formation of the syncytiotrophoblast during blastocyst implantation in certain species of mammals (see Enders and Schlafke, 1971; Sherman and Wudl, 1976), during the formation of multinucleate osteoclasts (Ham, 1974), and during plasmogamy in a number of higher fungi (review, Ling and Ling, 1974); and fusion between tumor cells and normal host cells has been detected in a number of animal tumors (review, Ringertz and Savage, 1976). We remain completely ignorant, however, of the events accompanying these examples of fusion. None of these phenomena has been studied in detail, and their usefulness as test systems for the experimental study of membrane fusion is limited.

3. VIRUS-INDUCED CELL FUSION

The first report of a probable example of virus-induced cell fusion was in 1873 when Luginbuhl described the presence of multinucleated cells at the periphery of smallpox pustules. Subsequently, Unna (1896) and Warthin (1931) observed similar multinucleate cells in tissue lesions produced by vari-cella and measles viruses, respectively. Similar examples of multinucleate cell formation have been reported in tissues infected with many other viruses (review, Roizman, 1962), and these too probably arise via cell fusion since the viruses in question have all been shown to induce extensive fusion of cells cultured in vitro (reviews, Poste, 1970a, 1972). Enders and Peebles (1954), using measles virus, were the first to demonstrate virus-induced cell fusion in a tissue culture system, and this was soon followed by reports of fusion of cells

in vitro by mumps virus (Henle et al., 1954), human parainfluenza virus (Chanock, 1956), and Sendai (HVJ*) virus (Okada, 1958). Subsequently, a large number of enveloped viruses have been shown to produce fusion in vitro (see below). In addition to any intrinsic interest that virologists may entertain for virus-induced cell fusion, this phenomenon has been widely exploited by investigators in many areas of cell biology, virology, immunology, and genetics as a method for producing hybrid cells and heterokaryons whose novel properties provide valuable insight into numerous aspects of cellular organization (reviews, Harris, 1970; Poste, 1972; Davidson, 1974; Watkins, 1974; Appels and Ringertz, 1975; Gordon, 1975; Zeuthen, 1975; Ringertz and Savage, 1976).

The fusion of cultured cells by viruses has also attracted interest as a possible system for defining the factors involved in membrane fusion (reviews, Poste, 1972; Poste and Allison, 1973). The fusion of cells in vitro by viruses is easily controlled, and the well-developed specificities of viruses for particular cell types offer a reliable and highly reproducible method for inducing extensive cell fusion of a wide range of cell types. The experimental study of the mechanism(s) of virus-induced cell fusion is facilitated by the availability of virus variants which display differing capacities to fuse cells, thus permitting identification of the viral components responsible for fusion. Conversely, variation in the susceptibility of different cell types to fusion when challenged by the same virus (reviews, Poste, 1972; Poste and Waterson, 1975) provides useful material for investigating the cellular response(s) during fusion.

The major disadvantage of virus-induced cell fusion as a system for studying membrane fusion is the shortcoming shared by all forms of cell fusion; namely, it is extremely difficult to obtain rapid and synchronized fusion of large numbers of cells. Although the actual fusion event between any two cells may take place very rapidly (probably on a millisecond time scale; see Poste and Allison, 1973), the number of cells undergoing fusion at any given time is low, and fusion, assessed in terms of the response of the entire cell population, is extended over several hours. This dictates that it is difficult to conduct detailed biochemical and biophysical studies on the membrane changes accompanying fusion in such systems, because any changes occurring in the few cells that are actually fusing at any particular time often cannot be detected against the large "background" population of nonfusing cells. Consequently, the study of factors that influence the overall extent of cell fusion by viruses (or any other fusion agent) can in general provide information only on the gross regulation of the fusion process and does not offer insight into the molecular events responsible for the fusion reaction. However, with the recent isolation of the macromolecular components associated with induction of fusion by certain viruses (see Section 3.4) new opportunities arise

*Synonym: hemagglutinating virus of Japan.

where it may be possible to use isolated viral macromolecules to induce rapid fusion of natural and model membranes in cell-free systems.

3.1. Two Kinds of Virus-Induced Cell Fusion

It is now recognized that viruses induce cell fusion by two distinct mechanisms. Some confusion still persists in the literature concerning this concept, and it is pertinent to outline briefly the differences between these two kinds of cell fusion.

The first type of virus-induced cell fusion, called "fusion from without" (FFWO), occurs independently of virus replication, is induced equally well by infective and noninfective virus, and does not require the synthesis of new virus-specific or host cell-specific material. FFWO takes place only after infection with very high doses of virus and occurs rapidly, being detectable even at the light microscope level as soon as 5 min after addition of the virus, with maximum fusion occurring 1–3 hr after exposure to the virus. The best-known example of FFWO is the use of inactivated Sendai virus to induce cell fusion (Okada, 1962; Harris, 1970), but large doses of other RNA-containing lipid-enveloped viruses can induce FFWO including Newcastle disease virus (NVD) (Poste et al., 1972), mumps (Henle et al., 1954), measles (Cascardo and Karzon, 1965), canine distemper (Rankin et al., 1972), and SV5 (Holmes and Choppin, 1966). Among the DNA-containing viruses, FFWO has been reported only with strains of herpes simplex virus (Tokumaru, 1968) and vaccinia virus (Kaku and Kamahora, 1964; Magee and Miller, 1968).

The second type of virus-induced cell fusion, called "fusion from within" (FFWI), is characterized by fusion beginning in the later stages of the virus replicative cycle following infection of cells with moderate or low doses of virus. Intracellular replication of the virus and the synthesis of new virus-specific macromolecules are necessary for this type of fusion to occur. The dependence of fusion from within on virus multiplication and expression of a functional viral genome dictates that maximum cell fusion coincides with the most intensive phases of virus multiplication and thus occurs several hours, or even days, after infection. FFWI does not require synthesis of complete new virions, since fusion can still occur in cells infected with conditional lethal mutants under restrictive conditions and in cells in which the later stages of virus replication have been selectively blocked by metabolic inhibitors (review, Poste, 1972). FFWI is induced by a large number of DNA-containing viruses (herpesviruses, poxviruses, lentiviruses, spumaviruses) and RNA-containing viruses* (paramyxoviruses, morbilliviruses, pneumoviruses, coronaviruses, rhabdoviruses). (For detailed list of individual viruses and references, see Table 1 of the review by Poste, 1972.)

*International Committee on Taxonomy of Viruses, classification and nomenclature; see *Virology* 71:371–378 (1976).

The terms "fusion from without" and "fusion from within," introduced by Kohn (1965) and Bratt and Gallaher (1969), respectively, are derived by analogy from the terms "lysis from without" and "lysis from within" used to distinguish between rapid lysis of *Escherichia coli* by high multiplicities of bacteriophage (lysis from without) and lysis occurring after productive infection produced by intracellular accumulation of lysozyme (lysis from within).

Here we will be concerned primarily with FFWO induced by inactivated viruses. This offers a more convenient experimental system than FFWI since it not only produces rapid cell fusion but also avoids the problem of alterations in cellular function imposed by intracellular virus replication. However, as discussed later (Section 3.4) in the case of cell fusion induced by certain paramyxoviruses, both FFWO and FFWI may result from modification of the host cell plasma membrane by the same virus envelope glycoprotein.

3.2. Cell Fusion by Inactivated Viruses: Techniques

Among the many viruses capable of inducing cell fusion, Sendai virus (a member of the paramyxovirus group) has achieved a near monopoly as the agent of choice for fusing cells. The widespread use of Sendai virus as a tool for fusing cells has been a somewhat arbitrary but nonetheless successful decision. The virus can be grown conveniently to high titers in a short time in embryonated eggs, and the fusion activity of such preparations is relatively stable (see below). A second attractive feature, particularly to nonvirologists, is that Sendai virus is considered nonpathogenic for man and is thus easier to handle than certain other viruses with equal or even greater cell fusion activity. For example, virulent and mesogenic strains of Newcastle disease virus (NDV) (also a paramyxovirus) are able to induce very marked FFWO, fusing up to 60–80% of cells in a population within 2 hr (compared with 30–40% for Sendai virus) (see Poste et al., 1972), but their extreme pathogenicity for avian species dictates that they be used only where strict virus containment facilities are available (and even then usually only under strict licensing supervision from the appropriate National Agencies responsible for Animal Disease Control). The range of cell types susceptible to fusion by inactivated Sendai virus and NDV is extremely wide. Susceptibility extends over a considerable range of the vertebrate phylum, and the type of cell used for fusion studies is thus virtually limited only by the requirements and the ingenuity of the investigator.

Detailed technical descriptions of methods for the fusion of cultured cells using inactivated Sendai virus and/or other paramyxoviruses are available in numerous publications (reviews, Okada, 1969; Watkins, 1971; Giles and Ruddle, 1973; Poste, 1973), and only a few general comments will be given here.

Sendai virus for use in cell fusion experiments is normally grown in eggs since virus propagated in many mammalian cell cultures lacks fusion activity (Hosaka, 1962; Okada, 1969; Homma, 1971, 1972). The chorioallantoic

membrane (CAM) is inoculated with 0.1 hemagglutinating unit (HAU; 1 HAU = 2×10^7 virus particles = 0.02 μ g viral protein) of virus and the egg incubated (point end of the egg down) for 3 days at 37°C and then maintained at 4°C overnight. Storage at 4°C serves to kill the chick embryo and also limits contamination of the virus-containing allantoic fluids by blood from ruptured blood vessels. Heavy contamination by blood is a serious problem since the virus particles adsorb to erythrocytes and are lost during the initial centrifugation (see below). The pooled allantoic fluids from infected eggs are first centrifuged at 500g for 10 min to remove contaminating cells and other particulate debris, after which the number of HAU per milliliter of allantoic fluid is determined by a standard sheep red blood cell agglutination assay (for details of method, see Rosen, 1969). The virus-containing fluids are then concentrated by centrifugation at 30,000g for 30 min, the supernatant being discarded and the deposited virus pellet being resuspended in balanced salt solution as stock virus with a known HAU titer. A convenient concentration for stock virus preparations is 40,000 HAU/ml, which can be diluted in balanced salt solution for cell fusion experiments. Stock virus can be stored at -70°C after rapid freezing, and addition of 0.5% bovine serum albumin before freezing avoids the rapid decay of cell fusion activity (Neff and Enders, 1968). Virus can be stored routinely for 6-9 months under these conditions without significant loss of fusion activity.

One aspect of the growth of Sendai virus in eggs for cell fusion studies that is rarely mentioned in the literature concerns the problem of emergence of variants with reduced cell fusion capacity when the virus is passaged repeatedly through eggs at high virus concentrations (see Watkins, 1971). This phenomenon, which appears to involve the emergence and eventual domination of incomplete (noninfective) particles, is not peculiar to Sendai virus and has been seen with other cell-fusing paramyxoviruses (see Poste, 1972; Choppin and Compans, 1975).

Most investigators have chosen to inactivate Sendai virus for cell fusion experiments by means of UV irradiation. However, both the dose and duration of UV irradiation exert an important influence on the cell fusion capacity of the virus. Harris et al. (1966) inactivated Sendai virus by exposure for 3 min to a calculated intensity of UV radiation of 3000 ergs/cm²/sec, but most subsequent reports have involved 10 min UV inactivation of the virus. Guggenheim et al. (1968) examined this problem in detail and showed that a UV dose of 1500 ergs/cm²/sec for 10-15 min was optimal for obtaining maximum inactivation of virus infectivity without impairing fusion capacity. Greater doses caused a significant reduction in the fusion activity. Interestingly, nonirradiated Sendai virus was found to have a significantly lower fusion capacity than UV-irradiated virus. Empirically, a dose of 1500 ergs/cm²/sec can be achieved by placing a dish or watch glass containing virus 25 cm from a 15-W germicidal UV lamp (the lamp should be changed regularly, however, to ensure significant emission at 2600 Å).

One possible complication of the UV inactivation procedure is that virus infectivity may not be destroyed completely. Neff and Enders (1968), recognizing this difficulty, showed that β -propiolactone (BPL) destroyed Sendai virus infectivity without affecting its cell fusion capacity. Pedreira and Tauraso (1969), in a detailed comparison of inactivation procedures for preparing Sendai virus for cell fusion experiments, showed that BPL was superior to UV irradiation for this purpose. However, a major drawback is that concentrated solutions of BPL are carcinogenic and mutagenic (Roberts and Warwick, 1963). Also, contamination of BPL with metals or metal salts may lead to a violent chemical reaction.

More recently, Apostolov and Damjanovic (1973) found that the infectivity of freeze-dried Sendai virus could be destroyed after heating at 100°C for 20 min, while the hemagglutinating, hemolytic, and cell fusion activities were unaffected. Heating had largely been overlooked as a possible method for inactivating viruses since it had been shown to be accompanied by protein denaturation and loss of biological function. However, the important additional factor exploited by Apostolov and Damjanovic is that the heat stability of proteins is greatly increased after freeze-drying. This method therefore offers a highly reliable method for inactivating Sendai virus for cell fusion studies (no information is available, however, on the effect of similar heat treatment on the cell fusion activity of other viruses).

3.3. Optimum Conditions for Virus-Induced Cell Fusion

The reader is referred to the articles by Okada (1962) and Harris et al. (1966) for details of methods for fusing cells in suspension and to Kohn (1965) and Poste et al. (1972a) for fusion of cells in monolayers. Combinations of these two systems have also been described in which a suspension of cells is added to a monolayer of cells at the same time as the virus (Davidson, 1969; Klebe et al., 1970; Poste and Reeve, 1972).

The degree of cell fusion that takes place after treatment with inactivated viruses is related to the concentration of virus used, the number of cells and their inherent susceptibility to fusion, and, to a lesser extent, the conditions of incubation during the interaction of virus with the cells. The effect of these factors on the efficiency of cell fusion has been reviewed fully elsewhere (Poste, 1970a, 1972; Watkins, 1971; Giles and Ruddle, 1973), and only a few general comments are necessary.

Cell types differ markedly in their susceptibility to virus-induced cell fusion (both FFWO and FFWI). This is probably the single most important factor determining both the extent of cell fusion and the size of the polykaryocytes formed. Evidence from many reports (review, Poste, 1970a) indicates that cells from established cell lines and malignant cells have a greater fusion capacity than primary and secondary cell strains. Fibroblasts fuse less well than epithelial cells, and both leukocytes and lymphoid cells are relatively resistant to virus-induced cell fusion (see Poste, 1970a). The reasons for these differences

are not clear. The number, availability, and type of virus receptors on the cell surface (Poste and Waterson, 1975) and differences in plasma membrane lipid composition (Klenk and Choppin, 1969; Poste et al., 1972) may affect cellular susceptibility to fusion. However, detailed information on the mechanism(s) by which these factors and other aspects of cell surface organization can influence the cell fusion reaction is still lacking. Cellular susceptibility to fusion is also influenced by a number of ill-defined factors such as cell passage level, the length of time since subcultivation before exposure to virus, and the stage in the cell cycle (see Poste, 1972).

Within general limits it can be stated that both the extent of cell fusion and the size of individual polykaryocytes increase with increases in the virus dose. The concentration of virus required to induce cell fusion depends in large part on the fusion susceptibility of the cell(s) in question. Some cell types undergo extensive fusion when exposed to relatively low doses of virus (100–1000 HAU/ml) while other cells routinely require higher virus doses (4000–20,000 HAU/ml) and certain cell types may need to be treated with doses of virus as high as 80,000 HAU/ml before fusion will occur (see Watkins, 1971; Poste, 1972, for examples). Generalization is difficult, however, and a number of cell types may show marked cytotoxicity when exposed to low (<1000 HAU/ml) or to intermediate (5000 HAU/ml) doses of inactivated Sendai virus while others show no evidence of injury after incubation with virus concentrations as high as 80,000 HAU/ml for 2 hr (see Watkins, 1971; Poste, 1972). Consequently, it may be stated with reasonable confidence that methods which are successful in producing extensive fusion of one cell type may not work with other cells or may induce cytotoxicity. Trial-and-error methods are therefore usually required to establish the optimal virus dose, cell numbers, and incubation conditions to obtain efficient fusion of each cell type.

The susceptibility of a wide range of cells to virus-induced cell fusion (FFWO and FFWI) can be increased significantly, however, by pretreatment with phytohemagglutinin (PHA) (Poste et al., 1974, 1976a; Sullivan et al., 1975; Yoshida and Ikeuchi, 1975). Commercially available preparations of PHA from Difco Laboratories Inc. (Detroit, Michigan) and the Burroughs Wellcome Co. (Triangle Park, North Carolina, and Beckenham, England) are able to induce significant enhancement of virus-induced cell fusion (Poste et al., 1976a). Preparations from both manufacturers are supplied as a lyophilized powder, which can be reconstituted and diluted in phosphate-buffered saline (PBS) or culture medium. Large amounts of PHA with cell fusion-enhancing properties can also be isolated directly from red kidney beans by affinity chromatography on a thyroglobulin-Sepharose column (for details of method, see Poste et al., 1976a).

The mechanism by which PHA enhances virus-induced cell fusion remains to be elucidated. Since PHA-conjugated to Sepharose is equally as effective as the free lectin in enhancing cell fusion (Reeve et al., 1974), it is clear

that the effect of PHA on the fusion process is mediated entirely at the cell surface and does not require entry of lectin into the cells. The most straightforward explanation for the ability of PHA to enhance virus-induced cell fusion is that agglutination of cells by PHA creates conditions of close cell-to-cell contact that facilitate subsequent fusion. Despite the attractive simplicity of this interpretation, the role of agglutination in promoting cell fusion is confused by the observation that while PHA enhances fusion, other lectins such as Con A, wheat germ agglutinin, and soybean agglutinin inhibit virus-induced cell fusion (Poste et al., 1974) even though they are equally as effective as PHA in agglutinating cells. A possible explanation for the conflicting effects of different lectins on virus-induced cell fusion concerns the question of whether or not a particular lectin is able to bind to virus-receptor sites on the cell surface. Lectins, such as Con A and wheat germ agglutinin, that inhibit paramyxovirus-induced cell fusion prevent binding of these viruses to the cell surface whereas PHA does not alter either the rate or the extent of virus particle binding (Poste et al., 1976a). These results suggest that the enhancement of virus-induced cell fusion by PHA might therefore result merely from its ability to agglutinate cells without affecting the subsequent binding of virus to its receptors on the cell surface. If this interpretation is correct, then other agents that promote cell agglutination without impairing virus binding might also be effective in enhancing virus-induced cell fusion. It is therefore of interest to note that De Boer and Loyter (1971) found that agglutination of erythrocytes by pretreatment with polylysine enhanced their subsequent fusion by inactivated Sendai virus or phospholipase C.

PHA is a mixture of five isolectins, which possess varying degrees of erythroagglutinating, leucoagglutinating, and mitogenic activities (see Miller et al., 1973). Ion-exchange chromatography and polyacrylamide gel electrophoresis have been used to isolate two major biological fractions, designated L-PHAP and H-PHAP (Miller et al., 1973). The former is a single homogeneous protein with potent leucoagglutinin activity but low erythroagglutinin activity. The H-PHAP fraction is a complex protein mixture with high erythroagglutinin activity but little or no leucoagglutinin activity. Both L-PHAP and H-PHAP possess mitogenic activity. Characterization of the cell fusion-enhancing activity of these various PHA fractions isolated from affinity chromatography-purified PHA indicates that cell fusion enhancing activity resides largely in the L-PHAP fraction, which contains a single protein corresponding to the most anodal protein of the five PHA proteins resolved by gel electrophoresis (Poste et al., 1976a).

3.4. Identification of the Viral Components Associated with Induction of Membrane Fusion

The virological literature on FFWO abounds with proposals for the existence of "fusion factors" within virus particles which can act directly on membranes to render them susceptible to fusion. These have been variously termed

"cytolysin" (Henle et al., 1954), "cell wall-destroying enzyme" (Zhadanov and Bukrinskaya, 1962), "cell fusion substances" (Okada et al., 1964), "fusion factor" (Cascardo and Karzon, 1965), and "syncytium-producing toxin" (Tokumaru, 1968). In none of these examples has the nature of the fusion factor or its relationship to virus structure and the macromolecular subunits of the virus been defined. The very nature of the terms chosen to describe these hypothetical factors implies that they function as lytic enzymes which modify the structural integrity of cellular membranes. Despite extensive efforts to identify such factors by fractionation of viruses causing FFWO (for references, see review by Poste, 1972), fusion of cells by a single molecular (or macromolecular) component isolated from disrupted virus particles has yet to be achieved.

Lucy (1970) and Barbanti-Brodano et al. (1971) have proposed that the ability of paramyxoviruses such as Sendai virus to induce FFWO results from the presence of lysolecithin within the virus envelope which acts on cell membranes, at the site(s) of virus attachment to induce membrane fusion in similar fashion to fusion induced by exogenous lysolecithin (Poole et al., 1970). This hypothesis is not convincing for several reasons.

First, the lysolecithin content of lipid-enveloped cell-fusing viruses such as Sendai virus is lower than that of other lipid-enveloped viruses such as the influenza viruses which do not induce FFWO (Neurath et al., 1973). Similarly, analysis of lyso- compounds in a number of NDV strains has failed to reveal any correlation between lysolecithin content and cell fusion activity, similar lysolecithin concentrations being found in both fusing and nonfusing strains (Parkes and Fox, 1975). In addition, attempts to identify the formation of lyso- compounds in cell membranes during virus-induced cell fusion (both FFWO and FFWI) have been unsuccessful (Hosaka, 1962; Falke et al., 1967; Elsbach et al., 1969; Diringer and Rott, 1976). Even assuming that the lysolecithin associated with paramyxovirus particles can gain access to cell membranes, the amount of lysolecithin is probably inadequate to induce cell fusion. A thousand HAU of egg-grown Sendai virus contains approximately $0.1 \mu\text{g}$ of lysolecithin (calculated from the data of Blough and Lawson, 1968). This dose of virus is sufficient to induce FFWO in a wide range of cell types, yet the induction of cell fusion by addition of exogenous lysolecithin is achieved only at concentrations of $200 \mu\text{g}$ or greater. Even then, the extent of cell fusion induced by exogenous lysolecithin is far less than that produced by the virus and is often accompanied by extensive cell lysis, which is not seen in virus-treated cell populations.

Second, experiments showing that treatment of Sendai virus particles with phospholipase B (lysolecithinase) eliminates their ability to induce FFWO cannot be interpreted (Barbanti-Brodano et al., 1971) as evidence that lysolecithin is responsible for the cell fusion activity. In the experiments described by Barbanti-Brodano et al., virions were not analyzed for their lysolecithin content, so it is impossible to tell whether the inhibitory effects of phospholi-

pase treatment on fusion activity were accompanied by removal of lysolecithin from the virus. It seems equally likely that elimination of cell fusion activity by this type of treatment results merely from enzymic perturbation of the structural integrity of the virus envelope. Similar inhibition of cell fusion activity in lipid-enveloped viruses has been reported after treatment with phospholipases A, C, and D (Kohn, 1965; Tokumaru, 1968; Poste and Allison, 1973). If lysolecithin in the virus particle were functioning as a "fusion factor," then by the same logic used by Barbanti-Brodano et al. treatment of virus particles with phospholipase A to generate lysolecithin would be expected to enhance rather than abolish their fusion activity.

The best evidence concerning the identity of the viral components responsible for cell fusion has come from recent studies on two paramyxoviruses, Sendai virus and NDV. Independent studies in several laboratories over the past 5 years have established that the ability of these viruses to induce FFWO (and also probably FFWI) is correlated with the presence of a specific glycoprotein in the virus envelope.

Several of the biological activities of paramyxoviruses are associated with the virus envelope. These include hemagglutinating, hemolytic, and cell fusion activities and the ability to attach and penetrate into susceptible cells. The virion envelope consists of lipids derived from the plasma membrane of the host cell arranged in the form of a bilayer (review, Rott and Klenk, 1977). The external surface of the lipid bilayer is covered with spikelike projections which are composed of glycoproteins (see Rott and Klenk, 1977). The inner side of the lipid bilayer of the virus envelope is coated with a nonglycosylated protein, the so-called membrane (M) protein. The available evidence suggests that the M protein plays a major role in maintaining the structure of the virus envelope and that it serves as the recognition site for the interaction of the virus nucleocapsid with the putative virus envelope during virus assembly, when, before budding, the nucleocapsid aligns beneath areas of the cellular plasma membrane which contain the viral envelope of proteins (review, Rott and Klenk, 1977). The virus envelope, in turn, encloses the RNA genome and its associated nucleoprotein (NP) subunit. For a more detailed discussion of paramyxovirus structure and the synthesis and assembly of viral subunits into intact virions, the reader is referred to the recent reviews by Choppin and Compans (1975) and Rott and Klenk (1977). The remainder of this section will be limited to a discussion of the functional properties of the two envelope glycoproteins since these are involved in the initial attachment of virions to the cell surface and the subsequent series of events that culminate in cell fusion.

The envelope glycoproteins of three paramyxoviruses, Sendai, NDV, and simian virus 5 (SV5), have now been successfully isolated by extraction with nonionic detergents, and the individual glycoproteins have been separated by sucrose density gradient centrifugation and affinity chromatography

(methods, Scheid et al., 1972; Scheid and Choppin, 1973, 1975; Urata and Seto, 1975). In each of these viruses the larger (molecular weight 69,000–76,000) of the two envelope glycoproteins has been found to possess both neuraminidase and hemagglutinating activities (Scheid et al., 1972; Scheid and Choppin, 1973, 1976; Seto et al., 1973; Tozawa et al., 1973). This protein is called the HN glycoprotein. The smaller envelope glycoprotein (molecular weight 53,000–56,000), referred to as the F (fusion) protein, is devoid of neuraminidase and hemagglutinating activities and is involved in virus-induced cell fusion and hemolysis.

The clue that the ability of paramyxoviruses to induce FFWO was associated with the F glycoprotein came from studies in which the structural proteins of virions grown in different host cells were compared. As mentioned earlier, both Sendai virus and NDV exhibit significant differences in their cell fusion activity when grown in different host cells. For example, Sendai virus grown in eggs (Fukai and Suzuki, 1955; Okada, 1958, loc. cit.), primary chick embryo fibroblasts (Blair and Robinson, 1968), primary chick embryo lung cells (Darlington et al., 1970), and primary calf kidney cells (Tozawa et al., 1973) is able to induce FFWO of a wide variety of cell types and also lyse erythrocytes. However, when the same strain of virus is grown in established mammalian cell lines such as bovine MDBK cells (Scheid and Choppin, 1974, 1975) or mouse L cells (Ishida and Homma, 1960; Homma, 1971) the progeny virions lack cell fusion and hemolytic activities and are also noninfective.

Comparison of the envelope glycoproteins in Sendai virions grown in embryonated eggs and with those of virions grown in MDBK or L cells has revealed that while the content of the HN glycoprotein is similar, virions grown in MDBK and L cells lack the F protein found in egg-grown virus and instead contain large amounts of a higher molecular weight component, F_0 (Fig. 2) (Homma and Ouchi, 1973; Scheid and Choppin, 1974, 1975). F_0 has since been shown to be a structural precursor for F, and posttranslational cleavage of F_0 to F is produced by cellular proteases. Cleavage occurs within the plasma membrane of infected cells during final assembly of new virions (Scheid and Choppin, 1975, 1976). In host cells such as MDBK and L cells, proteolytic cleavage of F_0 to F does not occur and progeny virions are released from infected cells containing F_0 rather than F. The presence of F_0 instead of F in these virions therefore correlates with their inability to induce FFWO.

Similar host-dependent variation in the cleavage of the F_0 precursor to the F protein and a correlation between possession of the F protein and the ability to induce FFWO have been demonstrated for NDV (Samson and Fox, 1973; Poste, 1975; Hightower et al., 1975; Nagai et al., 1976a,b). NDV differs from Sendai virus, however, in that cleavage of F_0 to F occurs in a much wider range of host cells. Infectious virions with cell fusion activity can be obtained not only by growing NDV in eggs but also in a variety of established mammalian cell lines (see Poste and Reeve, 1972; Poste et al., 1972; Alexander et al., 1973;

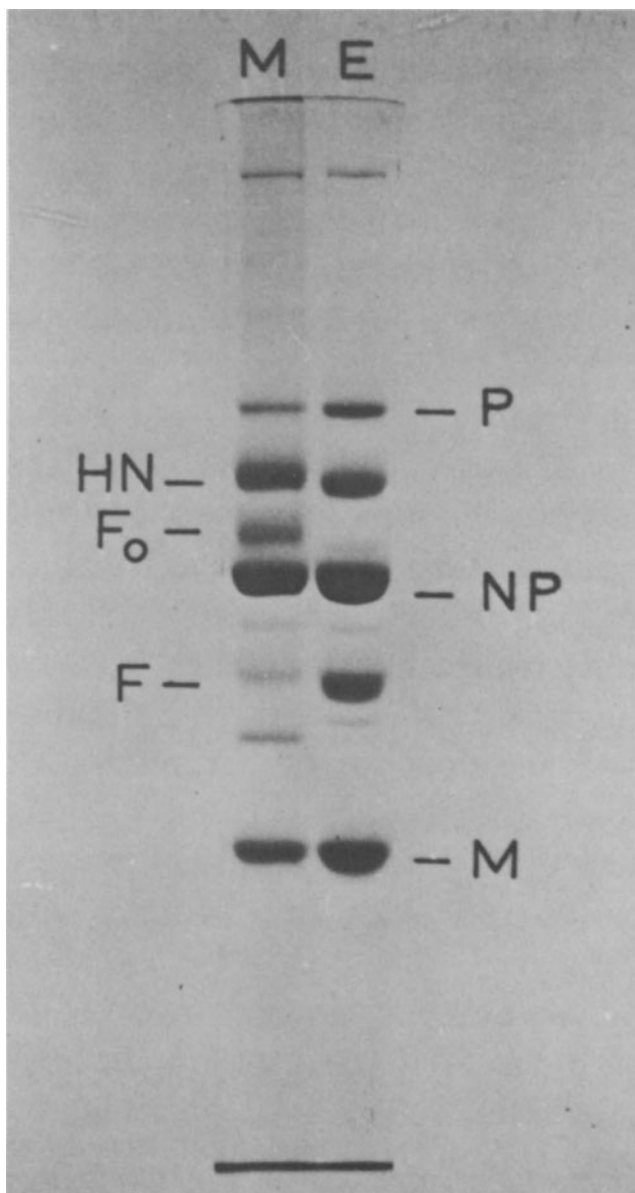


Fig. 2. Polypeptides of Sendai virions grown in embryonated eggs (E) and bovine MDBK cells (M) after SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue. The nonglycosylated proteins P, NP, and M are identified on the right, and the envelope glycoproteins HN, F₀, and F, with molecular weights of 69,000, 65,000, and 53,000, respectively, are identified on the left. Reproduced with permission from Scheid and Choppin (1975).

Poste, 1975; Nagai et al., 1976a,b). However, individual strains of NDV differ in this regard. Virulent and mesogenic strains yield infective progeny with cell fusion activity when grown in eggs and a variety of avian and mammalian cells. In contrast, these properties are expressed only by avirulent NDV strains grown in embryonated eggs or cultured chick chorioallantoic membrane cells, and avirulent strains grown in mammalian cells are in general noninfective and unable to induce cell fusion.

Cleavage of the F_0 precursor to produce F can also be accomplished in vitro. Treatment of Sendai virus grown in MDBK or L cells with 1 $\mu\text{g}/\text{ml}$ trypsin for 10 min at 37°C converts F_0 to F, and this is accompanied by restoration of infectivity and cell fusing and hemolytic activities (Homma and Ouchi, 1973; Scheid and Choppin, 1974, 1975). Activation of these biological activities in Sendai virions by in vitro cleavage of the F_0 precursor to F can be achieved with trypsin but not with plasmin, chymotrypsin, or elastase. However, Scheid and Choppin (1976) have isolated a series of Sendai virus mutants in which the F_0 precursor is not cleaved by trypsin but is cleaved by chymotrypsin or elastase. In contrast to wild-type Sendai virus, these mutants do not yield infective virions when grown in eggs. The egg-grown mutant virions contain the uncleaved F_0 precursor and thus lack cell fusion activity. However, cleavage of F_0 by treating these virions with chymotrypsin or elastase in vitro restores infectivity and cell fusion activity.

The ability to select for virus mutants in which the cleavage of F_0 and activation of cell fusion activities can be induced by different proteases raises the possibility that in the natural host or during adaptation of a virus to a particular host within the laboratory, variants may emerge which are susceptible to the specific proteases present in the host in question. Differences in the susceptibility of the F_0 glycoprotein to cleavage by the available range of host proteases may explain the observation that Sendai virions grown in MDBK or L cells contain the uncleaved F_0 precursor, are noninfective, and lack cell-fusing activities, while the related paramyxoviruses SV5 and NDV grown in these cells contain the cleaved F protein, are infective, and display cell-fusing activities (Scheid and Choppin, 1973, 1974; Poste, 1975; Nagai et al., 1976a,b).

Additional evidence that the F glycoprotein is necessary for paramyxovirus-induced FFWO has come from studies of virus particles lacking this glycoprotein. Shimizu and Ishida (1975) have reported that the F glycoprotein spikes can be selectively digested from egg-grown Sendai virus by trypsin (20 $\mu\text{g}/\text{ml}$ for 1 hr at 35°C), leaving the HN spikes intact on the surface of the virus. F-deficient particles produced by this method exhibit normal hemagglutinating and neuraminidase activities, but their infectivity and cell fusion and hemolytic activities are markedly reduced. Shimizu and Ishida (1975) also reported that the HN spikes could be selectively removed from Sendai virions by a fungal semialkaline protease, producing virions devoid of neuraminidase and hemagglutinating activity but still retaining a population of surface spikes

which presumably correspond to the F glycoprotein. Unfortunately, the crucial question of whether such virions exhibited cell fusion activity was not tested since HN-deficient particles were unable to adsorb to cells (work discussed later suggests, however, that this problem could be overcome by provision of an "artificial virus receptor" on the cell surface by coating cells with plant lectins). The behavior of virions enzymically depleted of the HN glycoprotein is analogous to the results described by Portner et al. (1975) for conditional lethal mutants of Sendai virus with a temperature-sensitive mutation affecting HN synthesis. When grown at nonpermissive temperatures these mutants lack HN. Although the F glycoprotein is present, these virions are unable to induce FFWO since they cannot attach to cells due to the absence of the HN glycoprotein.

Comparable observations have also been made using virus particles reconstituted from isolated Sendai virus subunits (Hosaka and Shimizu, 1972a,b, 1977; Hosaka, 1975). These studies have established that reconstituted particles lacking the F glycoprotein are devoid of cell fusion activity, but the more interesting experiments on the fusion activity of particles containing only the F glycoprotein were again frustrated by failure of the particles to attach to cells.

The attachment of paramyxoviruses to the cell surface via the HN envelope glycoprotein is an essential preliminary step in virus-induced FFWO. Paramyxoviruses are believed to bind to neuraminidase-sensitive sialic acid residues on cell surface glycoproteins in similar fashion to the orthomyxoviruses (influenza viruses) (reviews, Gallaher and Howe, 1976; Bächli et al., 1977). Treatment of cells with neuraminidase abolishes their susceptibility to FFWO by Sendai virus (see Bächli et al., 1977), presumably by destroying the receptor needed for virus adsorption. Similarly, preinfection of cells with neuraminidase-containing paramyxoviruses renders them resistant to FFWO when treated subsequently with Sendai virus (Wainberg and Howe, 1973a,b). The loss of cellular susceptibility to FFWO occurs rapidly after infection with the first virus and probably results from cleavage of the receptors for Sendai virus by neuraminidase present in the first virus. However, the neuraminidase activity associated with paramyxovirus particles does not appear to play a direct role in either virus attachment or the subsequent attachment of FFWO. Indeed, the ability of Sendai virus to induce FFWO has been reported to be enhanced after selective inactivation of viral neuraminidase (Neurath et al., 1972). Also, measles virus which lacks neuraminidase is able to induce extensive FFWO (Cascardo and Karzon, 1965). Viral neuraminidase may, however, facilitate the fusion process by promoting structural alterations in the cellular plasma membrane.

The strict requirement for attachment of virus particles to neuraminidase-sensitive receptors as a preliminary to cell fusion is not seen with certain strains of NDV. Bratt and Gallaher (1969) and Poste and Waterson (1975) have shown that virulent strains of NDV are able to attach to and induce FFWO of neuro-

minidase-treated cells. The nature of the receptors used by NDV on neuraminidase-treated cells has not been identified. Poste and Waterson (1975) suggested that neuraminidase-resistant sialic acid moieties associated with glycolipids (see Weiss, 1973) might serve as receptors. Some support for this possibility is provided by recent studies showing that paramyxoviruses can attach to ganglioside receptors in model membranes (Haywood, 1975).

A number of other observations suggest that the initial adsorption of paramyxoviruses to the cell surface prior to cell fusion need not be mediated solely by neuraminidase-sensitive sialoglycoproteins. Horse erythrocytes contain the *N*-glycolyl form of neuraminic acid instead of *N*-acetylneuraminic acid and are not susceptible to agglutination and fusion by Sendai virus (Yamamoto et al., 1974) or certain strains of NDV (Burnet and Lind, 1950). However, after being coated with concanavalin A, horse erythrocytes adsorb Sendai virus and become susceptible to fusion by this virus (Yamamoto et al., 1974; Hosaka and Shimizu, 1977). Similarly, human erythrocytes rendered unsusceptible to agglutination and fusion by Sendai virus by desialation can be fused by Sendai virus after coating with concanavalin A as an "artificial virus receptor" (Hauri and Bachi, 1976, unpublished; cited in Bachi et al., 1977). However, the concentration of concanavalin A (and other lectins) used in experiments of this kind is critical. Other studies have shown that pretreatment of cells with concanavalin A prevents virus adsorption and inhibits FFWO induced by paramyxoviruses (Okada and Kim, 1972; Poste et al., 1974).

The creation of "artificial virus receptors" on the cell surface may, however, offer a potentially useful method for studying the functional properties of paramyxovirus envelope glycoproteins. As mentioned previously, evaluation of the cell fusion activity of virus particles lacking the HN glycoprotein has been hindered because of the failure of such particles to adsorb to cells. It might be of interest, therefore, to test whether virus particles lacking HN but containing the F glycoprotein (naturally occurring *ts* mutants, enzymically depleted particles, or reconstituted particles) could induce agglutination and fusion of lectin-treated erythrocytes. Unpublished data by Shimizu et al. cited by Hosaka and Shimizu (1977) suggest that this approach may indeed be feasible. They reported that HN-deficient Sendai virus particles were able to induce agglutination and FFWO of concanavalin-A-treated chick erythrocytes but had no effect on untreated erythrocytes.

Although it is now established that the ability of paramyxoviruses to induce FFWO is correlated with the presence of the F glycoprotein in the virus envelope, it has not yet been possible to induce cell fusion by adding the pure preparations of the isolated F glycoprotein to cell cultures. Expression of the cell fusion activity of the F glycoprotein appears to require the additional presence of lipid(s) (Hosaka and Shimizu, 1972a,b, 1977), presumably in some form of ordered structural interaction with the F glycoprotein. Fusion was greatest when the viral glycoproteins were mixed with phosphatidylethanol-

mine, while phosphatidylcholine and sphingomyelin were relatively ineffective. Interpretation of this work is complicated, however, by the lack of control experiments on the fusion activities of the lipids alone. This question is crucial in the light of more recent studies showing that lipid vesicles (liposomes) prepared from certain phospholipid(s) can induce cell fusion (see Section 6).

It would therefore be of considerable interest to determine whether the incorporation of the isolated F glycoprotein into lipid vesicles which lack the ability to induce cell fusion or to fuse with other vesicles (Section 7) would result in the vesicles' acquiring fusion activity.

3.5. Virus-Cell Interactions in Virus-Induced Cell Fusion

There is now a general consensus that FFWO induced by paramyxoviruses and other lipid-enveloped viruses requires fusion of virus particles with the cellular plasma membrane. Differences in opinion remain, however, as to whether cell fusion occurs at the same time that fusion of virus particles with the plasma membrane is taking place or whether fusion between the virus envelope and the plasma membrane and cell-to-cell fusion occur as related but separate processes.

Fusion between the virus envelope and the cellular plasma membrane (Fig. 3) is the normal mechanism by which paramyxoviruses and many other lipid-enveloped viruses infect cells (Meiselman et al., 1967; Morgan and Howe, 1968; Heine and Schnaitman, 1969; Howe and Morgan, 1969; Zee and Talens, 1971; Apostolov and Poste, 1972; Bächli and Howe, 1972; Bächli et al., 1973; Wolinsky and Gilden, 1975; Okada et al., 1975). The relationship of FFWO to the normal process of virus penetration thus explains the correlation discussed

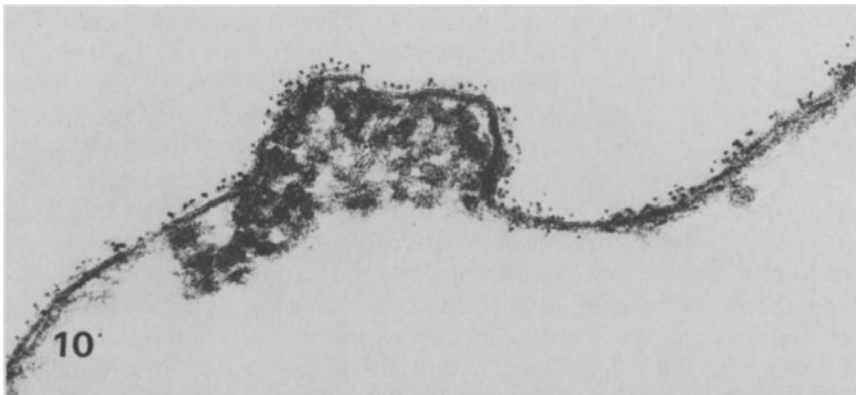


Fig. 3. Negatively stained electron micrograph showing fusion of a Sendai virion with the plasma membrane of a human erythrocyte. Reproduced with permission from Bächli et al. (1977).

earlier in which paramyxoviruses lacking the F envelope glycoprotein were not only unable to fuse cells but also noninfective.

Attachment of virus particles to specific receptors on the cell surface is not sufficient to "trigger" the necessary membrane changes for subsequent fusion between the virus envelope and the cellular plasma membrane. As mentioned in the previous section, Sendai virus particles lacking the F protein adsorb normally to cells via the HN envelope glycoprotein yet are noninfective and do not induce FFWO. The separate nature of virus attachment and the processes of fusion between the virus envelope and the plasma membrane and cell-to-cell fusion is also indicated by experiments showing that modification of paramyxoviruses by heat (Okada, 1969), glutaraldehyde (Toister and Loyter, 1972), or antibodies against the F glycoprotein (Seto et al., 1973) does not impair virus attachment but inhibits virus penetration and FFWO.

Virus attachment and the subsequent steps of virus penetration and cell-to-cell fusion can also be distinguished on the basis of their temperature dependence. Virus adsorption, although temperature independent, is usually done at 2–4°C. However, fusion between the virus envelope and the cell membrane and cell-to-cell fusion cannot occur at this temperature (Okada, 1962; Harris and Watkins, 1965; Apostolov and Poste, 1972; Gallaher and Bratt, 1972, 1974; Poste and Allison, 1973; Wainberg and Howe, 1973a). The optimum temperature for these processes is 37°C; the frequency of both drops rapidly with the reduction in temperature, and at temperatures below 18°C they are completely inhibited (Okada, 1962; Apostolov and Poste, 1972; Gallaher and Bratt, 1972; Pasternak and Micklem, 1973; Okada et al., 1975). This is a general characteristic of most, if not all, examples of membrane fusion (review, Poste and Allison, 1973) and is probably due to the effect of temperature on the physical state of membrane lipids. Membrane fusion appears to require that phospholipids in the interacting membranes be in a "fluid" state (Papahadjopoulos et al., 1974, 1976a,b; Papahadjopoulos and Poste, 1975) and lowering the temperature reduces membrane "fluidity."

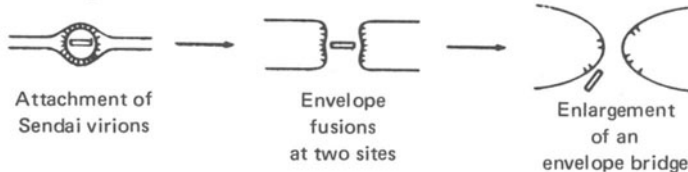
Apostolov and Almeida (1972) have proposed that FFWO occurs when a virus particle in close association with the membranes of two different cells fuses simultaneously with the plasma membrane of both cells (Fig. 4, scheme 2), permitting the virus to act as a connecting "bridge" between the two cells. Progressive enlargement of such "bridges" results in complete cell fusion.

The virus bridge hypothesis is weakened substantially, however, by the fact that most electron microscopic observations of virus-induced FFWO indicate that the fusion of plasma membranes on adjacent cells occurs more commonly at sites where neither intact virus particles nor viral fragments can be recognized (Bächi and Howe, 1972; Bächi et al., 1973, 1977; Cassone et al., 1973; Hosaka and Shimizu, 1972a,b, 1977). In addition, immunoelectrohistochemical studies have shown that viral envelope antigens are not present on the membrane over the cytoplasmic bridges formed between cells (Hosaka and

1. Cell-cell bridge formation:



2. Cell-envelope-cell bridge formation:



3. Fused envelope-cell bridge formation:

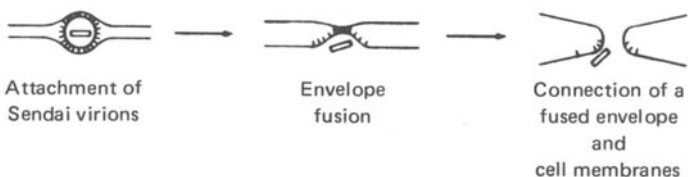


Fig. 4. Three hypotheses proposed to explain cell fusion from without by Sendai virions. Reproduced with permission from Hosaka and Shimizu (1977).

Shimizu, 1977). Both sets of observations argue strongly against the virus bridge hypothesis.

Additional evidence against the virus bridge hypothesis has come recently from the work of Maeda et al. (1977b). These investigators have been able to separate fusion between the Sendai virus envelope and the cellular plasma membrane from the process of cell-to-cell fusion. They found that incubation of Ehrlich ascites cells with Sendai virus in medium supplemented with high concentrations of saccharides (> 0.38 M glucose, mannose, or galactose or > 0.25 M sucrose) did not affect initial fusion of virus particles with the cellular plasma membrane but blocked cell-to-cell fusion. Although the mechanism underlying this phenomenon remains to be identified, these results indicate that virus envelope-plasma membrane fusion and cell-to-cell fusion are separate events. These results, together with the electron microscopic data mentioned above, support the concept that FFWO occurs after the integration of viral envelope proteins into the cell plasma membrane and that cell fusion probably results from an interaction between an area of plasma membrane on one cell containing viral envelope glycoproteins and unmodified plasma membrane on an adjacent cell (Fig. 4, scheme 3).

A similar sequence of events to those in scheme 3 of Fig. 4 may also be responsible for virus-induced FFWI. In contrast to FFWO, in which viral glycoproteins are inserted into the plasma membrane during virus penetration, modification of the plasma membrane during FFWI would occur when newly synthesized, viral glycoproteins are inserted into the plasma membrane during final assembly of new progeny virions at the cell surface. It is known that FFWI does not occur before newly synthesized viral glycoproteins have been inserted into the plasma membrane (Poste, 1975; Rott and Klenk, 1977) and FFWI can be inhibited by ligand-induced cross-linking of the viral glycoproteins within the plasma membrane (Ludwig et al., 1974; Poste et al., 1974).

In the case of the paramyxoviruses, the parallels between FFWO and FFWI can be extended to include a requirement for the presence of the F glycoprotein within the plasma membrane in order for FFWI to occur. FFWI occurs only in those host cells in which cleavage of the F_0 precursor to F occurs. FFWI is not seen during virus replication in host cells in which cleavage of F_0 to F does not take place (Poste, 1975; Nagai et al., 1976a,b). However, in the latter situation FFWI can be induced by brief treatment of infected cells with proteases (Nakamura and Homma, 1974; Nagai et al., 1976b).

The role of viral envelope glycoproteins in causing FFWI induced by paramyxoviruses is also indicated by experiments showing that FFWI can be inhibited by agents that block glycosylation of viral proteins. Cultivation of cells infected with NDV [Gallaher et al., 1973; Poste et al., 1977 (unpublished)] or SV5 (Rott et al., 1975) in the presence of 2-deoxy-2-fluoro-D-glucose or 2-deoxy-2-fluoro-D-mannose and glucosamine prevents complete glycosylation of the newly synthesized virus envelope proteins. Although the partially glycosylated proteins are inserted into the plasma membrane of infected cells, FFWI does not occur. Poste et al. [1977 (unpublished)] have also found that NDV virions released with incompletely glycosylated envelope proteins cannot induce FFWO. Inhibition of FFWI by fluorosugars has also been reported in cells infected with herpesviruses (Ludwig et al., 1974; Knowles and Person, 1976; Schmidt et al., 1976). However, the identity of the (glyco)-protein(s) involved in FFWI induced by these viruses has yet to be established.

If similar modification of the host cell plasma membrane by viral glycoproteins is responsible for both FFWO and FFWI, it is necessary to ask why the capacity of membrane-integrated viral glycoproteins to induce FFWO is lost soon after initial virus penetration even though they persist in the plasma membrane for up to 4 hr following the initial penetration of the virus via fusion with the plasma membrane (Durand et al., 1975; Maeda et al., 1977b). It might reasonably be expected that such membrane-integrated viral glycoproteins would retain the capacity to induce cell fusion. This is clearly not the case. Following the early period of FFWO that accompanies initial virus penetration, cells become resistant to fusion until *newly synthesized* envelope glycoproteins are introduced into the plasma membrane which results in FFWI.

One possible explanation for the rapid decline of FFWO activity following viral penetration is that a "critical concentration" of viral envelope glycoproteins per unit area of plasma membrane might be required to induce the necessary degree of membrane perturbation for fusion to take place. For example, following initial fusion of the virus envelope with the plasma membrane, the viral glycoproteins quickly undergo lateral diffusion within the membrane (Bächi and Howe, 1972; Bächi et al., 1973; Okada et al., 1974), with resulting dilution of their concentration at the original site(s) of penetration.

The possible requirement for a "critical concentration" of viral envelope glycoproteins within the plasma membrane to induce cell fusion (both FFWO and FFWI) might also account for the finding that antiviral antibodies can enhance virus-induced FFWI in certain situations. For example, using parainfluenza virus type 2 propagated in a cell line in which FFWI did not normally occur, Wainberg and Howe (1972) found that antiviral antibodies, added at a stage in infection at which newly synthesized envelope glycoproteins had been inserted into the plasma membrane, caused the cells to fuse. A possible interpretation of this observation is that the antibodies served as a ligand to cross-link the viral glycoproteins into "patches" of the necessary size required to induce membrane destabilization and permit fusion to occur. However, as mentioned earlier, in experiments using ligands to cross-link cell surface components both the number of ligand receptors and the valency and concentration of the ligand are crucial in determining the extent of cross-linking and the resulting alteration in cell surface properties. Thus, other studies using multivalent ligands to cross-link surface components on paramyxovirus-infected cells have produced marked inhibition of both FFWO and FFWI (Poste et al., 1974; Rott et al., 1975).

Before finishing this discussion of the structural basis of FFWO induced by lipid-enveloped viruses, brief mention should be made of the hypothesis for FFWO proposed by Okada (1962, 1969), since this is often cited in the cell fusion literature even though there is little experimental evidence to support it. Okada's hypothesis proposes that FFWO induced by Sendai virus results from the action of a membrane lytic factor associated with the virus particle. This hypothesis was advanced largely on the basis that Sendai and related viruses causing FFWO were able to lyse erythrocytes. In Okada's scheme, cell fusion is considered to occur via the fusion of areas of plasma membranes on adjacent cells which have been modified by the viral "lysin." Perturbation of the plasma membrane by the "lysin" is proposed as occurring in the membrane surrounding attached virus particles so that fusion occurs solely between plasma membranes and the virus particles do not form part of the bridge (Fig. 4, scheme 1). In this scheme, fusion of the virus envelope with the plasma membrane is not required. Although this hypothesis for FFWO would accommodate the fact that virus antigens are not usually detected on the intercellular bridges (see above), there is little other evidence in support of it.

As mentioned before, no evidence has been obtained for chemical modification of host membranes during FFWO or for the existence of virus-associated molecules with membrane lytic properties. Indeed, even hemolysis does not involve overt lysis of the erythrocyte membrane but results from colloid osmotic swelling of the erythrocyte following alterations in plasma membrane permeability produced by fusion of virus particles with the plasma membrane (reviews, Bächli et al., 1977; Hosaka and Shimizu, 1977; also see below).

Although there is a large body of evidence suggesting that the ability of paramyxoviruses to induce hemolysis is closely related to their cell fusion activity and that hemolysis requires fusion of the virus envelope with the erythrocyte plasma membrane, recent work has shown that the cell fusion and hemolytic activities of Sendai virus can be separated. Homma et al. (1976) and Shimizu et al. (1976) have found that Sendai virions harvested immediately after a one-step growth cycle in eggs are devoid of hemolytic activity yet are fully infective and can induce FFWO. However, treatments such as freeze-thawing, sonication, prolonged incubation at 37°C, or complement-mediated immune damage to the virus envelope activate the hemolytic activity of such virions. Shimizu et al. (1976) have shown that the envelope of nonhemolytic virions is much less permeable to uranyl acetate (UA) than that of hemolytic virions (so-called UA⁺ virions). Hemolysis occurred only after fusion of UA⁺ virions with the plasma membrane of erythrocytes. This finding thus suggests that hemolysis is confined to erythrocytes that have undergone fusion with virus particles which possess permeable (damaged?) envelopes. The leakage of ions through the region of the cellular plasma membrane containing the integrated permeable UA⁺ virus envelope then presumably results in colloid-osmotic swelling of the erythrocyte with eventual rupture of the plasma membrane and release of hemoglobin.

3.6. Mechanisms

The molecular mechanism(s) responsible for fusion between the virus envelope and the plasma membrane and fusion between the plasma membranes of adjacent cells in FFWO and FFWI is still unknown. In the case of FFWO and FFWI induced by paramyxoviruses a reasonable case can be made that the viral F protein is involved in the process responsible for perturbing cellular membranes to render them susceptible to fusion, but details of the mechanism(s) involved are again unknown.

In common with other examples of membrane fusion, electron-microscopic studies have revealed structural alterations in the plasma membranes of cells during FFWO. Freeze-etch studies of the plasma membranes of erythrocytes incubated with Sendai virus at 37°C have revealed redistribution and clustering of intramembranous particles at sites of virus attachment (Bächli et al.,

1973, 1977). This effect was not induced merely by virus adsorption since attachment of virus at 4°C did not alter membrane structure. In addition, incubation of erythrocytes at 37°C with influenza virus, which does not fuse with the plasma membrane, also failed to modify membrane ultrastructure.

The significance of the redistribution of intramembranous particles during virus-induced FFWO, as well as in other examples of membrane fusion (see Poste and Allison, 1973), is not understood. Studies on the fusion of phospholipid vesicles with cells (Section 6) or other vesicles (Section 7) indicate that phospholipid bilayers possess the capacity to fuse spontaneously, provided that the lipid composition and physical state of lipids within the bilayer are appropriate and certain ionic requirements are met. Fusion between natural membranes might therefore occur via a similar interaction between phospholipid bilayers in adjacent membranes. Poste and Allison (1973) proposed that the redistribution and aggregation of intramembranous particles seen in fusing membranes might provide a mechanism whereby areas of the membrane become devoid of integral proteins, with fusion taking place between the particle-free areas on adjacent membranes (this proposal assumes that the intramembranous particles are integral membrane proteins or oligomeric complexes of integral proteins). Ahkong et al. (1975a) have extended Poste and Allison's hypothesis to include the additional proposal that agents causing membrane fusion promote the redistribution of intramembranous particles by increasing the "fluidity" of membrane liquids. While the requirement for a "fluid" membrane for fusion to take place is not disputed (see Section 7), there is presently no experimental evidence to support the view that agents causing cell fusion increase the fluidity of membrane lipids. Indeed, there is evidence to the contrary since agents such as lysolecithin, DMSO, and myristic acid which induce fusion of both natural (Section 4) and model membranes (Section 7) actually produce effects on phospholipid bilayers which are indicative of a decrease in fluidity (Papahadjopoulos et al., 1976a,b).

There is an increasing amount of evidence which suggests that the mobility and distribution of many integral proteins within the plasma membrane are controlled by cytoskeletal elements (microfilaments and microtubules) associated with the cytoplasmic face of the membrane (reviews, Nicolson, 1976; Poste and Nicolson, 1976, 1977). This raises the question of whether the redistribution of intramembranous particles seen in membrane fusion requires the participation of these cytoskeletal elements. Unfortunately, there is little information available concerning this potentially important question.

Circumstantial evidence that membrane-associated cytoskeletal systems might participate in membrane fusion has come from studies showing that drugs acting on microfilaments and microtubules can alter virus-induced cell fusion. Sendai virus-induced FFWO is inhibited by cytochalasin B (an inhibitor of microfilaments) (Pasternak and Micklem, 1973; Ohki et al., 1975) but is enhanced by drugs such as colchicine which disrupt microtubules (Ohki

et al., 1975). However, cytochalasin B does not inhibit initial fusion of Sendai virus particles with the plasma membrane (Poste and Allison, 1973; Ohki et al., 1975). This indicates that cytochalasin B does not impair membrane fusion *per se* and suggests that the inhibitory effects of this drug on FFWO result from its effect on membrane-associated microfilaments. The recent finding that tertiary amine local anesthetics impair microfilaments in a similar fashion to cytochalasin B (Poste et al., 1975a,b) may explain earlier observations showing that these drugs inhibited virus-induced FFWO and FFWI (Poste and Reeve, 1972).

Assuming that the redistribution of integral membrane proteins seen during FFWO and other examples of membrane fusion does involve changes in the interaction between the proteins and cytoskeletal elements in the cytoplasm, it remains to be established how such changes occur.

Treatment of erythrocytes with neuraminidase has been shown to produce redistribution and aggregation of intramembranous particles within the plasma membrane (Elgsaeter and Branton, 1974) and to increase their susceptibility to chemically induced fusion (Ahkong et al., 1975b). These observations prompt the obvious question as to whether the neuraminidase associated with paramyxovirus particles might facilitate structural changes in cell membranes during FFWO. Although the viral enzyme may facilitate such structural rearrangements, it is clear that neuraminidase alone cannot induce the full degree of membrane perturbation required for fusion to occur. As mentioned earlier, neuraminidase activity is associated with the HN glycoproteins in the viral envelope, yet particles containing HN are unable to induce cell fusion unless the F glycoprotein is also present. Also, neuraminidase activity can be selectively inactivated without impairing the cell fusion activity of the virus (Neurath et al., 1972) and vice versa (Calio et al., 1973). Furthermore, lipid-enveloped viruses such as measles and canine distemper lack neuraminidase yet induce extensive cell fusion.

Evidence has been obtained recently which suggests that the function of the membrane-associated cytoskeletal elements controlling the mobility of plasma membrane proteins is influenced by changes in intracellular calcium concentration (Nicolson and Poste, 1976; Poste and Nicolson, 1976; Schreiner and Unanue, 1976). Elevation of intracellular calcium has been shown to promote the redistribution of integral membrane proteins by ligands bound to the cell surface. These effects have been interpreted as resulting from Ca^{2+} -induced depolymerization of microtubules and activation of the contractile microfilament systems (see Poste and Nicolson, 1976; Schreiner and Unanue, 1976). Since fusion of paramyxoviruses with the plasma membrane is accompanied by a significant increase in plasma membrane permeability to ions and intracellular metabolites (Fuchs and Giberman, 1973; Katzman and Wilson, 1974; Pasternak and Micklem, 1973, 1974), the possibility must be considered that increased entry of Ca^{2+} into cells

following virus penetration might raise the intracellular calcium concentration to a sufficient extent to alter the cytoskeletal elements controlling protein mobility and produce redistribution of membrane proteins.

Experimental evidence for changes in intracellular calcium concentration in virus-induced cell fusion has yet to be obtained. However, some support for the concept that changes in intracellular calcium can induce redistribution of integral membrane proteins and promote cell fusion is provided by recent studies on erythrocytes. The mobility of integral proteins in the erythrocyte plasma membrane is restrained by their "linkage" to a network of spectrin molecules on the inner face of the plasma membrane (Nicolson and Painter, 1973; Elgsaeter et al., 1976). The spectrin network can be precipitated by Ca^{2+} (Elgsaeter et al., 1976), and cationic aggregation of spectrin molecules in erythrocyte ghosts is accompanied by redistribution and aggregation of intramembranous particles (Pinto da Silva and Nicolson, 1974; Elgsaeter et al., 1976). Ca^{2+} -induced redistribution of plasma membrane proteins may explain the finding that fusion of erythrocytes can be induced simply by incubation with the Ca^{2+} ionophore A23187, which increases the permeability of the plasma membrane to Ca^{2+} (Ahkong et al., 1975c).

The effects of cations on virus-induced cell fusion have been studied in some detail. In common with other examples of membrane fusion, virus-induced FFWO and FFWI both require Ca^{2+} (for references, see reviews by Okada, 1969; Poste and Allison, 1973), Mn^{2+} , Sr^{2+} , and Ba^{2+} can substitute for Ca^{2+} with varying degrees of efficiency (see Poste and Allison, 1973).

There are a number of mechanisms by which Ca^{2+} and other divalent cations may influence virus-induced cell fusion. These include effects on the fluidity of membrane lipids, induction of lateral phase separation in membrane lipids, and alterations in the function of the membrane-associated cytoskeletal elements controlling the mobility and distribution of integral membrane proteins. These mechanisms are not mutually exclusive and their possible roles in membrane fusion will be discussed in more detail later in Section 8 when discussing the central role of Ca^{2+} in *all* forms of membrane fusion.

4. CHEMICALLY INDUCED CELL FUSION

The first report of chemically induced cell fusion, although rarely acknowledged in the literature, is that of Michel (1937), who induced fusion of isolated plant protoplasts by incubating them for 10–20 min in 0.5 M sodium nitrate. Michel's use of sodium nitrate was motivated only by the need for a nontoxic hypertonic medium and not by direct appreciation of its fusion activity. However, later experiments by Power et al. (1970) comparing the ability of a range of inorganic salts to fuse plant protoplasts established that 0.25 M sodium nitrate is a highly effective and consistent agent in pro-

ducing both homo- and heterokaryons from protoplasts from several species. Other studies have shown that protoplast fusion can be further enhanced by the presence of calcium ions and high pH. Keller and Melchers (1973) found that up to 50% of *Nicotiana* protolasts could be fused by incubation in a medium containing sodium nitrate, 0.05 M calcium chloride, and 0.4 M mannitol buffered to pH 10.5. The high levels of fusion obtained in this system are comparable to those achieved in experimentally induced fusion of animal cell populations, and this has opened the way for exciting experiments on the formation of entirely new hybrid plants by fusion of protoplasts from different species (review, Carlson and Polacco, 1975).

Studies in several laboratories have also shown that 0.2–0.3 M polyethylene glycol (PEG) is highly effective in fusing isolated plant protoplasts (Bonnett and Eriksson, 1974; Constabel and Kao, 1974). Following this finding, PEG has been investigated for its ability to fuse animal somatic cells in vitro (Pontecorvo, 1975). Compared to most of the other chemicals able to induce fusion of cultured animal cells (see below) PEG not only induces extensive cell fusion but also is considerably less cytotoxic. PEG is now emerging as a popular alternative to inactivated Sendai virus in achieving cell fusion for the establishment of somatic cell hybrids (see Davidson and Gerald, 1976; Steplewski et al., 1976; Vaughn et al., 1976). Norwood et al. (1976), have also shown recently that the efficiency of PEG-induced fusion of mammalian cells can be further enhanced by the addition of dimethylsulfoxide.

The majority of observations on chemically induced cell fusion have been made by Lucy and his colleagues at the University of London. These investigators have screened a large number of lipophilic molecules for their ability to induce fusion of avian erythrocytes and, in certain instances, mammalian somatic cells. Among the many surface-active lipophilic substances now identified as being able to fuse animal cells, the best known is probably lysolecithin. Lucy (1969) first proposed on theoretical grounds that lysolecithin might possess fusion activity, and this was demonstrated experimentally by Howell and Lucy (1969), who showed that avian erythrocytes could be fused within as little as 30 sec after exposure to 100–1000 $\mu\text{g/ml}$ lysolecithin. The cell fusion activity of lysolecithin is not restricted to erythrocytes, and fusion of various mammalian somatic cells by this agents has been reported (see Koprowski and Croce, 1973). A major drawback of lysolecithin, however, is its extreme cytotoxicity. Although its cytotoxic activity can be reduced by including defatted serum proteins or albumin in the incubation medium (Poole et al., 1970; Croce et al., 1971) or by using it as an emulsion (Ahkong et al., 1972), comparison of the effectiveness of different cell-fusing agents on identical cell populations has revealed that lysolecithin is significantly more cytotoxic than inactivated Sendai virus or lipid vesicles (see

below) and also produces less cell fusion than the latter two systems (Kataoka and Koprowski, 1975; Poste and Papahadjopoulos, 1976a). Indeed, treatment of cells with lysolecithin has been shown in certain cases to abolish completely their ability to fuse (Reporter and Norris, 1973; Kataoka and Koprowski, 1975).

As of late 1976, Lucy and his colleagues had identified more than 30 different fat-soluble substances able to fuse avian erythrocytes in vitro. These include various unsaturated (C_{16} and C_{18}) or medium chain length (C_{10} to C_{14}) saturated fatty acids and their methyl and glyceryl esters (Ahkong et al., 1973a); the fat-soluble vitamins retinol and α -tocopherol (Ahkong et al., 1973a); the nonionic detergents Arlacel A (mannide monooleate), Span 20 (sorbitan monolaurate) and Span 80 (sorbitan monooleate) (Ahkong et al., 1974); immunological adjuvants such as Freund's (Ahkong et al., 1974); and dimethylsulfoxide (Ahkong et al., 1975a). Some of these agents such as glycerol monooleate (Cramp and Lucy, 1974), oleylamine (Bruckdorfer et al., 1974), and retinol (Lucy, 1975) have also been shown to fuse mammalian somatic cells in vitro. Kosower et al. (1975) have described a cyclopropane fatty acid ester, A_2C [(2-(2-methoxy)ethoxyethyl-9,10-metholene octanoate)], with cell fusion activity.

In addition to direct induction of cell fusion, incubation of cells with certain surface-active substances at concentrations which do not induce fusion can nonetheless modify their susceptibility to fusion by other cell-fusing agents. Hart et al. (1975) have shown that pretreatment of avian erythrocytes with detergents (50 $\mu\text{g}/\text{ml}$) of the octylphenyl series (Triton X-45, X-100, X-114, and Nonidet P40) enhanced Sendai virus-induced fusion of the cells by fifteen- to twentyfold. Triton WR-1339 was less efficient producing a seven- to tenfold enhancement in virus-induced cell fusion, and similar pretreatment with this detergent completely abolished their susceptibility to subsequent fusion by oleic acid or glyceryl monooleate. Two macrocyclic octylphenyl detergents, Macrocydon and HOC-60, inhibited both viral and chemically induced fusion.

The mechanism(s) by which this diverse group of membrane-active substances induces and/or promotes cell fusion is presently unclear. Lucy proposed originally that the action of lysolecithin, and perhaps other cell-fusing chemicals, resulted from their ability to induce structural changes in membrane lipids, converting the normal planar bilayer into globular micelles. Micelle formation was proposed as producing a highly unstable membrane which would fuse on contact with any other similarly perturbed membrane. It is now clear, however, that micelle formation alone is insufficient to induce membrane fusion. For example, even though lysolecithin at high concentrations induces extensive micellization of model membranes prepared from phosphatidylcholine, it does not promote fusion of such membranes at lower concentra-

tions, but only molecular exchange (see Papahadjopoulos et al., 1976a; and Section 4). In addition, conversion of lipid bilayers to micelles is not a consistent feature of the many lipophilic agents which induce fusion (see Howell et al., 1973). In the face of this evidence, Lucy and his colleagues now propose that the fusion activity of various lipophilic substances results from their common ability to increase the "fluidity" of membrane lipids. This is proposed as leading to membrane instability and rearrangements in membrane structure, including the redistribution of integral membrane proteins (see Ahkong et al., 1975a). As in the case of the earlier "micelle" hypothesis, simple perturbation of this type is considered sufficient to render membranes susceptible to fusion. The simple notion that an increase in the fluidity of membrane lipids is sufficient to trigger fusion would not, however, explain the differences in the fusion capacity of neutral and charged membranes mentioned above and discussed in detail in Section 7. While evidence has been obtained to support the view that membrane lipids must be at or above their T_c for fusion to occur (see Papahadjopoulos et al., 1973b, 1976a,b; Poste and Papahadjopoulos, 1976a,b; and Sections 6 and 7), there is no evidence to support Lucy et al.'s proposal that the lipophilic substances causing cell fusion can modify the fluidity of phospholipids. Indeed, there is evidence to the contrary. For example, agents such as lysolecithin myristic acid and DMSO actually increase the T_c of phospholipids (see Papahadjopoulos et al., 1976a). An alternative explanation for the fusion activity of these compounds will be discussed in Section 8, where it is proposed that they induce membrane instability by promoting transbilayer "flip-flop" of phospholipid molecules.

Fusion of cultured mammalian somatic cells can also be induced by small unilamellar lipid vesicles prepared by the sonication of phospholipids dispersed in aqueous solutions (Papahadjopoulos et al., 1973a; Martin and MacDonald, 1976a; Poste and Papahadjopoulos, 1976a). Papahadjopoulos et al. (1973a) showed that fusion activity depended on vesicle surface charge and the physical state of phospholipids in the vesicle membrane. Fusion was observed only with negatively charged vesicles in which the phospholipids were above their T_c at 37°C. Neutral vesicles prepared from egg yolk lecithin and negatively charged vesicles composed of "solid" phospholipids were unable to induce cell fusion. Positively charged vesicles are devoid of cell fusion activity even when their lipids are "fluid" (Martin and MacDonald, 1976a), but incorporation of lysolecithin into the vesicle membrane confers cell fusion activity. However, incorporation of similar amounts of lysolecithin into neutral lecithin vesicles does not result in the ability to fuse cells (Martin and MacDonald, 1976a).

A discussion of the possible mechanism by which lipid vesicles induce cell fusion and the differing fusion properties of negative, positive, and neutral vesicles will be given later in Section 6.

5. FUSION OF SUBCELLULAR MEMBRANES

Some of the most dramatic examples of membrane fusion are seen in the phenomena of endocytosis and exocytosis. For example, in specialized phagocytic cells such as the macrophage, endocytosis may result in the internalization of the equivalent of as much as twice the surface area of the cell per hour in the form of membrane-bound endocytotic vesicles which fuse with lysosomes within the cytoplasm (review, Edelson and Cohn, 1978). Similarly, in exocytosis, massive synchronized fusion of large numbers of membrane-bound secretory granules can occur within as little as 30 sec of exposure of secretory cells to the appropriate secretagogue (review, Holtzman et al., 1977). Endocytosis and exocytosis also provide striking examples of the highly specific nature of membrane fusion. In endocytosis, for example, endocytotic vesicles can fuse with each other and with lysosomes but never fuse with other membrane-bound structures such as mitochondria, the nucleus, or the endoplasmic reticulum. Similar specificity is manifest in exocytosis in which secretory granules, apart from fusion with each other, fuse only with the plasma membrane and do not fuse with other membrane-bound organelles. Also, in many instances the fusion of secretory granules is confined to specific regions of the plasma membrane (for examples, see Poste and Allison, 1973; Holtzman et al., 1977).

To date, however, neither endocytosis nor exocytosis has received much attention from investigators interested in membrane fusion. This neglect is probably due to the technical problems associated with studying these examples of membrane fusion. In both endocytosis and exocytosis, the fusion reaction is merely one step in a rapid multistep process which often makes it too difficult to "isolate" the fusion reaction from events which precede or follow fusion and which may have little or no relevance to fusion per se. Nonetheless, a vast literature has accumulated on diverse aspects of endocytosis and exocytosis from which at least some indirect insight can be gained into the factors influencing membrane fusion in these phenomena. A number of comprehensive reviews of the membrane interactions occurring in endocytosis (Edelson and Cohn, 1978) and exocytosis (Ceccarelli et al., 1974; Douglas, 1975; Normann, 1976) have been published recently and we will therefore not discuss these phenomena further other than to point out that there are similarities between membrane fusion in these phenomena and many other examples of membrane fusion. In particular, the important role of Ca^{2+} in regulating endocytosis and exocytosis emerges as a major feature shared with all other forms of membrane fusion. This will be discussed in more detail in Section 8 when examining the possible role of Ca^{2+} -mediated changes in membrane organization as a "key" event common to all forms of membrane fusion.

It seems likely, however, that the relative neglect of endocytosis and

exocytosis as experimental systems for studying events in membrane fusion will soon be remedied.* In an effort to better understand the membrane changes accompanying fusion of intracellular secretory granules with the plasma membrane during exocytosis, several investigators have begun to isolate secretory granules and test their fusion capacity in cell-free systems *in vitro*. Rapid and synchronized fusion of secretory granules from the pancreas (Dahl and Gratzl, 1976), the adrenal medulla (Schober et al., 1977), and invertebrate eggs (Vacquier, 1976) can be induced simply by addition of differing concentrations of Ca^{2+} to the medium surrounding the vesicles. This ability of Ca^{2+} to induce fusion between natural membranes in these systems is of considerable interest in view of similar observations of Ca^{2+} -induced fusion of model lipid membranes discussed later in Section 7. The fusion of isolated secretory granules under defined conditions *in vitro* may thus offer a particularly instructive system for studying membrane fusion since the various questions concerning the role of Ca^{2+} -induced phase changes and lateral phase separations in membrane lipids in triggering membrane fusion which have emerged from work on the fusion of simple lipid membranes (Section 7) can now be tested directly on natural membranes.

6. INTERACTIONS AND FUSION OF LIPID VESICLES WITH CULTURED CELLS

Studies done in several laboratories over the last 5 years have shown that cells cultured *in vitro* can incorporate large numbers of lipid vesicles (liposomes) added to the culture medium. Although much of the work on the interaction of lipid vesicles with cells has been concerned primarily with evaluating the usefulness of lipid vesicles as carrier vehicles for introducing biologically active materials into cells (review, Poste et al., 1976b), observations on the mechanisms by which vesicles are incorporated into cells have revealed that fusion of vesicles with the cellular plasma membrane could be an important pathway for vesicle uptake. The ability of vesicles to fuse with the cellular plasma membrane is influenced, however, by both the surface charge characteristics of the vesicle and the physical state of the phospholipids in the vesicle membrane. Some data indicate that fusion is restricted to vesicles bearing a net surface charge (positive or negative) and composed of phospholipids that are "fluid" at the experimental temperature(s) used (Poste and Papahadjopoulos, 1976a; Martin and MacDonald, 1976a) although there are reports indicating fusion of "neutral" vesicles (Pagano and Huang, 1975; Weinstein et al., 1977). In contrast, vesicles composed of phospholipids that are "solid" appear not to fuse with the cellular plasma membrane and are instead incorporated by endocytosis (Poste and Papahadjopoulos, 1976a) or adsorption to the cell surface (Pagano and Takeishi, 1977; Szoka et al., 1978).

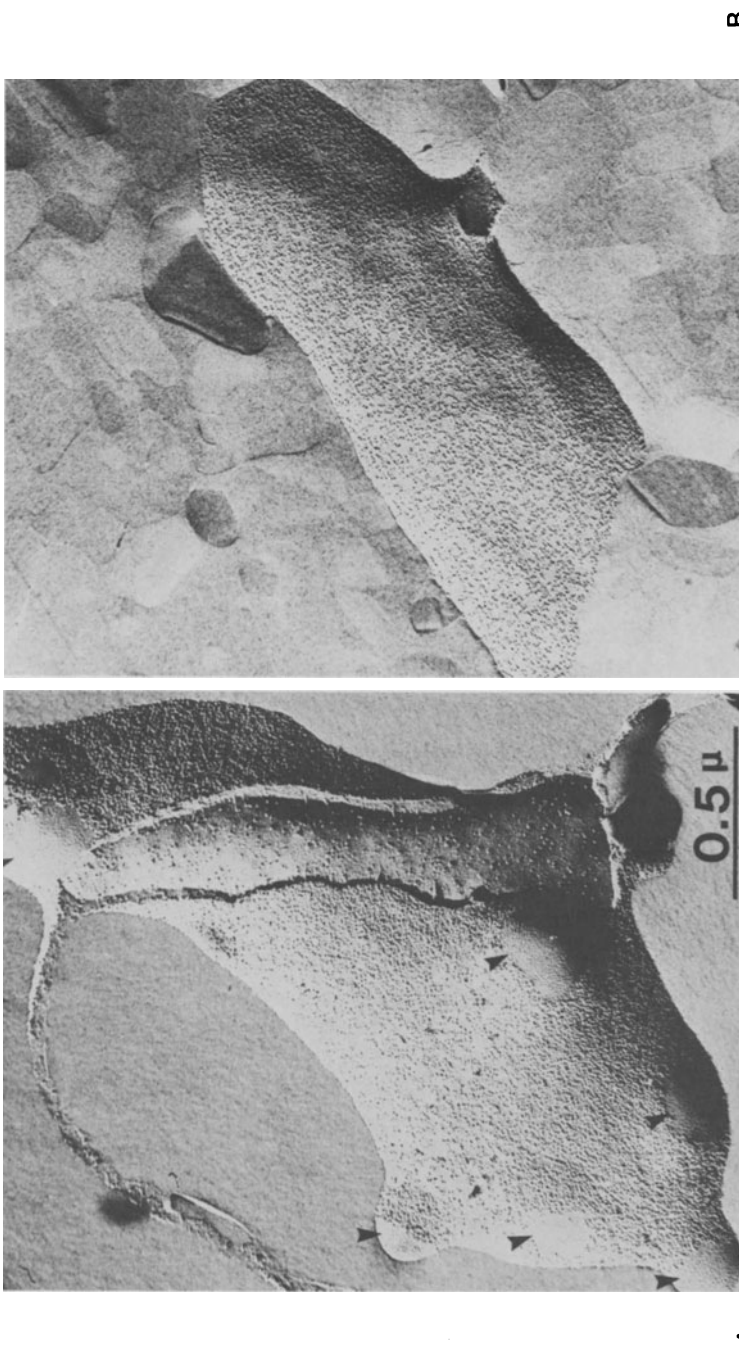
*A recent report (Oates and Touster, 1978) on the development of improved methods for following *in vitro* fusion of phagolysosomes is of considerable interest.

There is a report, however, indicating fusion of "solid" vesicles irrespective of surface charge (Batzri and Korn, 1975). This difference in the fusion behavior of "fluid" and "solid" vesicles parallels observations on vesicle-vesicle fusion discussed later in Section 7) in which fusion has been found to occur only between charged vesicles whose lipids were at or above their gel-to-liquid crystalline transition temperature.

Martin and MacDonald (1976d) have provided evidence which suggests that small unilamellar vesicles composed of fluid phospholipids and bearing a positive surface charge are able to fuse with the plasma membrane of human erythrocytes *in vitro*. These investigators showed that vesicles containing 10% dinitrophenylaminocaproylphosphatidylethanolamine (DNPPE), 5% stearylamine, 20% lysolecithin, and 65% lecithin were incorporated by erythrocytes and the vesicle-derived DNPPE could be demonstrated in the erythrocyte plasma membrane by fluorescein-conjugated anti-DNP antibodies and by electron microscopy using ferritin-conjugated anti-DNP antibodies. Vesicle-treated erythrocytes were also susceptible to immune lysis by anti-DNP serum plus complement. Fluorescence microscopic observations also revealed that the anti-DNP-DNPPE complexes were able to diffuse within the plasma membrane eventually aggregating in a single region of the membrane.

Martin and MacDonald pointed out that their results, as well as those in the studies described above, do not formally rule out the possibility that vesicle uptake might proceed not by membrane fusion but rather by a non-energy-requiring form of endocytosis driven by surface tension effects. No evidence is available, however, to support the existence of such a mechanism. Further support for the view that such vesicles can fuse with the plasma membrane has been obtained in ultrastructural studies showing vesicle-induced changes in plasma membrane morphology (Magee et al., 1974; Papahadjopoulos et al., 1974; Batzri and Korn, 1975). Morphological evidence for fusion of negatively charged vesicles with erythrocyte ghosts is shown in Fig. 5.

Evidence for fusion of small unilamellar vesicles with the plasma membrane of cultured cells has also come from recent observations on cellular uptake and intracellular distribution of vesicle-associated fluorescent dyes. Weinstein et al. (1977) have shown that treatment of mammalian lymphocytes with phosphatidylcholine vesicles containing water-soluble 6-carboxyfluorescein (6CF) exhibited a diffuse cytoplasmic staining pattern, suggesting that the vesicles had fused with the plasma membrane releasing 6CF into the cytosol. Since a high concentration of 6CF was used during initial formation of the vesicles, the fluorescence emission from 6CF within intact vesicles is markedly quenched, but when the dye is diluted by release within the cytosol (or by vesicle lysis extracellularly) a marked increase in fluorescence occurs. This system however tends to obscure the possible contribution of both endo-



A

B

Fig. 5. A: Electron micrograph of freeze-fractured human erythrocyte ghost after incubation with multilamellar lipid vesicles, showing areas (arrows) devoid of intramembranous particles. Vesicles (PS/PC, 1/9 molar ratio) were dispersed by mechanical (vortex) agitation in 50 mM NaCl, pH 8.2. Incubation with ghosts was performed in the same buffer with 1.3 nmol phospholipid per 10⁶ cells, for 30 min at 37°C. The cells were spun down at room temperature, resuspended in 30% (v/v) glycerol, and frozen in Freon 22. Large arrow indicates direction of shadowing. B: Same as in A, except the ghosts were incubated without lipid vesicles. From Papahadjopoulos et al. (1974).

cytotic processes and adsorption of intact vesicles to the cell surface, as discussed in a recent study (Szoka et al., 1978).

The vesicles used by Weinstein et al. (1977) were composed exclusively of phosphatidylcholine. However, as mentioned below, neutral phosphatidylcholine vesicles in certain other studies do not appear to fuse with cells or with other vesicles. As Weinstein et al. acknowledge, 6CF may bestow a negative surface charge on the vesicles which would thus enhance their ability to fuse with the cellular plasma membrane (see above).

Poste and Papahadjopoulos (1976a) have presented data showing that neutral vesicles prepared from egg phosphatidylcholine do not fuse with the plasma membrane and, like the solid charged vesicles described above, are incorporated via endocytosis. This inability of neutral vesicles to fuse with the plasma membrane is consistent with other data showing that neutral membranes display a poor fusion capacity. For example, neutral lecithin vesicles are unable to induce cell-to-cell fusion—a process that is believed to require fusion between vesicles and the cellular plasma membrane (see below). Neutral vesicles are also highly resistant to fusion with other vesicles (see Section 7). Finally, it is of interest to note that Haywood (1974b) was unable to detect fusion of the lipid envelope of Sendai virus with neutral vesicles prepared from egg phosphatidylcholine, but fusion of the virus envelope with vesicles was readily observed after interaction of virus with phosphatidylcholine vesicles containing egg phosphatidylethanolamine. Batzri and Korn (1975), on the other hand, presented evidence indicating that vesicles composed of egg phosphatidylcholine were incorporated by amoebae via endocytosis irrespective of surface charge (positive, negative, or neutral).

In contrast to the above negative observations on the fusion of neutral lecithin membranes, Pagano and Huang have claimed that neutral lecithin vesicles are able to fuse with the cellular plasma membrane of cultured cells. They claim that vesicle-plasma membrane fusion, and not endocytosis, represents the predominant pathway for cellular uptake of such vesicles (Huang and Pagano, 1975; Pagano and Huang, 1975). This apparent discrepancy between the results of Pagano and Huang and the studies mentioned above in which no evidence of fusion was detected merits further examination since the inability or ability of neutral membranes to fuse is crucial in evaluating the proposal discussed later that charge-charge interactions are required for all forms of membrane fusion (see Section 8). Accurate definition of the fusion behavior of neutral vesicles is also relevant to their experimental use as carrier vehicles for introducing materials into cells, since the question of whether such vesicles are endocytosed or fuse with the plasma membrane will determine the final intracellular distribution of vesicles and any material(s) encapsulated within them. Pagano and Huang's proposal that neutral lecithin vesicles can fuse with the cellular plasma membrane rests mainly on data showing that treatment of cells with inhibitors of *either* respiration *or* gly-

colysis did not abolish vesicle uptake. This is by no means certain since inhibition of glycolysis or respiration *alone* is insufficient to block endocytosis in many cell types and inhibition of *both* pathways of energy production is required for complete inhibition of endocytosis (Oren et al., 1963; Steinman et al., 1974; Poste and Papahadjopoulos, 1976b).

An alternative interpretation of Pagano and Huang's data is that the metabolic inhibitors used in their experiments did in fact block endocytosis and that the continued uptake of vesicles in the presence of the inhibitors was due to fusion of vesicles with the plasma membrane but fusion was possible either because of the presence of negatively charged contaminants or because of differences in cell type. For example, vesicles prepared from dimyristoyllecithin as used by Pagano and Huang are highly resistant to fusion with other vesicles but readily fuse when small amounts of negatively charged impurities such as myristic acid are present in the vesicle membrane (Kantor and Prestegard, 1975; Papahadjopoulos et al., 1976a). Myristic acid can be produced during prolonged sonication of pure dimyristoyllecithin with a probe-type sonicator even in the presence of nitrogen and adequate cooling, resulting in the formation of vesicles with a negative ζ -potential (Hauser, 1971). Since Pagano and Huang employed a prolonged probe sonication (30 min at 41°C) to produce their unilamellar vesicles and did not characterize the composition of their final preparation, it remains a real possibility that negatively charged myristic acid was produced which enabled the vesicles to fuse with cells and thus be incorporated by cells treated with metabolic inhibitors.

Pagano and Huang also reported that cellular uptake of dimyristoyllecithin vesicles was higher than that of dioleoyllecithin vesicles at 37°C. More surprising, however, was the finding that at 2°C the uptake of dimyristoyllecithin vesicles was double that at 37°C. They interpreted these data as indicating that cellular uptake of saturated lipids or lipids that are below their T_c (i.e., solid) involves extensive adsorption on the cell surface (Pagano and Takeishi, 1977). This conflicts with data presented elsewhere showing that uptake of both fluid and solid vesicles is quantitatively similar and also with data showing that uptake of both fluid and solid vesicles is substantially reduced at low temperatures (Papahadjopoulos et al., 1974; Poste and Papahadjopoulos, 1976a; Poste et al., 1976b).

This conflict between results with "solid" vesicles, prepared from an equimolar mixture of dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine having a T_c at 43°C by Poste and Papahadjopoulos (1976a), and those obtained with "solid" dimyristoyllecithin vesicles by Pagano and Huang (1976) can perhaps be accounted for by the inherent instability of dimyristoyllecithin vesicles, and also by the more recent observations indicating a considerable degree of adsorption to the cell surface with various types of vesicles (Szoka et al., 1978).

As shown in detail by Hauser and Barratt (1973), sonicated dimyristoyl-

lecithin vesicles are relatively unstable, as indicated by their high cation diffusion rates and their tendency to aggregate into larger structures. In contrast, vesicles composed of longer-chain saturated lecithins, such as dipalmitoylphosphatidylcholine and distearylphosphatidylcholine, are much more stable and are impermeable to cations (Papahadjopoulos et al., 1973), and when appropriately annealed neither aggregate (Sheetz and Chan, 1972) nor fuse with other vesicles (Papahadjopoulos et al., 1974, 1976a; Lawaczek et al., 1975) at temperatures below their T_c . The increased uptake of dimyristoyl-lecithin vesicles at 2°C compared with 37°C seen by Pagano and Huang, although somewhat paradoxical, could well reflect the instability and anomalous behavior displayed by vesicles at temperatures close to the T_c (Prestegard and Fellmeth, 1975; Jacobson and Papahadjopoulos, 1976; Martin and MacDonald, 1976d). Although the incubation temperature of 2°C is considerably lower than the T_c of nonsonicated dimyristoyl-lecithin bilayers (Hinz and Sturtevant, 1972), it has been shown that sonication in a bath-type sonicator or sonication with a probe sonicator produces a dramatic lowering of the onset for the transition of pure dipalmitoylphospholipids (Hinz and Sturtevant, 1972; Jacobson and Papahadjopoulos, 1976) and that prolonged probe sonication can produce vesicles with a very ill-defined phase transition, which probably reflects extensive broadening of the endothermic peak (Hinz and Sturtevant, 1972). Since Huang and Pagano did not characterize the thermotropic properties of their final preparations, the possibility must be considered that their vesicles were within the broad phase transition range even at 2°C. The anomalous uptake pattern could reflect the inherent instability of sonicated DML vesicles at temperatures close to their T_c . Since both endocytosis (review, Edelson and Cohen, 1978) and membrane fusion (review, Poste and Allison, 1973) are substantially reduced (and probably completely inhibited) at 2°C, it becomes necessary to consider the possibility that the enhanced uptake of radiolabeled phospholipids from DML vesicles at 2°C might result from the exchange diffusion of individual phospholipid molecules between vesicles and cellular membranes, as well as adsorption of intact vesicles (Pagano and Takeishi, 1977).

The molecular events involved in the fusion of lipid vesicles with the cellular plasma membrane remain to be established. Pagano and Huang (1975) have proposed a model for this process based on the well-known interaction and incorporation (so-called snapping) of oil droplets into the plasma membrane of living cells (Chambers and Kopac, 1937). Pagano and Huang propose that uptake of vesicles by fusion with the plasma membrane involves the formation of an "intermediate" structure in which the vesicle is incorporated entirely within the lipid bilayer of the plasma membrane in an analogous fashion to the formation of oil droplet lenses within bilayers (Henn and Thompson, 1968). We consider this model to be interesting but unlikely on thermodynamic considerations and believe that the analogy with the "snap-

ping" of oil droplets into cells may not be entirely appropriate. As Pagano and Huang acknowledge, the driving force for the interaction of an oil droplet with a lipid bilayer is the large difference in surface energy of the two systems due to the comparatively high surface energy of oil droplets in water (Davis and Rideal, 1961; Thompson and Henn, 1970). However, phospholipid bilayers have very low surface energies (Thompson and Henn, 1970) and the difference in surface energy between a sonicated phospholipid vesicle and a planar lipid bilayer in the plasma membrane should not be expected *a priori* to provide a sufficient driving force to perturb the stability of the two membranes and lead to fusion. Indeed, published data are available showing that sonicated neutral vesicles do not fuse with relatively flat, large multilamellar vesicles (Papahadjopoulos et al., 1975a, 1976a; Papahadjopoulos and Poste, 1975).

It is impossible at present to make generalizations concerning the mechanism of uptake of lipid vesicles by cells. The various groups that have studied such interactions have produced data which have led to conflicting conclusions. It seems possible that the differences are partly due to varying methodologies, differences in chemical composition and physical properties of the vesicles, and cell type used. It is also possible that the various mechanisms of uptake such as fusion, endocytosis, adsorption, and molecular exchange all contribute to varying degrees depending on the conditions. Clearly, the quantitation of the contribution of each mechanism will be obtained only with the development of experimentation that can distinguish unequivocally between the various pathways of uptake.

In addition to fusion with the plasma membrane of cultured cells, small unilamellar phospholipid vesicles have been shown to induce cell fusion and the formation of polykaryocytes (Papahadjopoulos et al., 1973a; Martin and MacDonald, 1976c; Poste and Papahadjopoulos, 1976b). The ability of vesicles to induce cell-to-cell fusion shows a number of striking parallels to the fusion of vesicles with the plasma membrane outlined above. Thus formation of polykaryocytes via cell-to-cell fusion has been observed only following the treatment of cells with vesicles that bear a net negative (or positive) surface charge and are composed of phospholipids that are "fluid" at 37°C. In contrast, both neutral vesicles in which the phospholipids are "fluid" at 37°C and also negatively charged vesicles composed of "solid" phospholipids are devoid of cell fusion activity.

The exact mechanism by which lipid vesicles induce cell-to-cell fusion is unknown. It is not unreasonable to suggest, however, that cell-to-cell fusion in this situation is a by-product of fusion of vesicles with the cellular plasma membrane. In this sense, cell fusion induced by lipid vesicles could be viewed as analogous to the FFWO induced by lipid-enveloped viruses discussed in Section 3. However, there is presently no evidence available to indicate whether vesicles might create a "fusion bridge" by fusing simultaneously

with the plasma membranes of adjacent cells or whether fusion of vesicles with the plasma membrane precedes cell-to-cell fusion as appears to be the case for virus-induced FFWO. In either situation the failure of neutral and solid vesicles to induce cell fusion would be explained by their inability to fuse with the plasma membrane. The inability of positively charged fluid vesicles without lysolecithin to induce cell fusion (Martin and MacDonald, 1976c) is surprising since there is evidence that they can fuse with the plasma membrane (Martin and MacDonald, 1976d). However, evidence presented in detail in Section 8 indicates that Ca^{2+} -induced lateral phase separation of membrane phospholipids and the formation of domains of acidic phospholipids may well be a crucial factor in membrane fusion. Since positively charged phospholipid species would not undergo this type of phase separation, this might explain their inability to render the plasma membrane susceptible to fusion. This possibility, as well as other possible events in vesicle-plasma membrane fusion and vesicle-induced cell-to-cell fusion, will be discussed in more detail in Section 8.

7. FUSION OF MODEL LIPID MEMBRANES (VESICLES AND BLACK LIPID FILMS)

7.1. Definitions, Systems of Study, Relative Advantages

Studies on membrane fusion with lipid model membranes such as vesicles and black lipid films provide unique opportunities for studying the mechanism of membrane fusion at the molecular level. Phospholipid vesicles and black lipid films are stable structures of limited permeability to small ions and large molecules (Thompson and Henn, 1970; Papahadjopoulos and Kimelberg, 1973; Bangham et al., 1974). Their chemical composition can be varied considerably from pure one-component systems to various mixtures of phospholipids differing in head-group or hydrocarbon chain configuration. Thus questions relating to head-group specificity, bilayer fluidity, phase transitions, and separations, etc., can be studied in great detail. In addition, other membrane components such as cholesterol, glycolipids, various proteins, and glycoproteins can be incorporated into the phospholipid bilayer, and their effects on fusion can be studied under controlled conditions. Finally, various membrane-active molecules and drugs that have been implicated in membrane fusion can be added externally to a well-defined system and their role on the mechanism of fusion can be assessed in detail. The results from the above simple systems can then be correlated with the evidence on fusion with natural membranes.

During the last few years several laboratories have undertaken to study membrane fusion with model membranes. The methods and systems that have been used to detect fusion vary widely. The evidence for fusion has been based on observations such as transfer of labeled lipid molecules between vesicles

(Taupin and McConnell, 1972; Maeda and Ohnishi, 1974; Papahadjopoulos et al., 1974), changes in NMR spectra and size of vesicles (Prestegard and Fellmeth, 1974; Lau and Chan, 1975; Papahadjopoulos et al., 1974; Poznansky and Weglicki, 1974), increase in optical density of vesicle suspensions (Martin and MacDonald, 1976a; Lansman and Haynes, 1975), mixing of lipid molecules from two vesicle populations (Papahadjopoulos et al., 1974; Martin and MacDonald, 1976a), transfer of fluorescent probes or ionophores between vesicles and black lipid films (Pohl et al., 1973; Moore, 1976), mixing and reactivity of two membrane proteins incorporated initially in different lipid vesicles (Miller and Racker, 1976a), optical microscopic observations on spherical bilayer black lipid films (Breisblatt and Ohki, 1976), and electrical conductance measurements with apposed hemispherical bilayer black lipid films (Liberman and Nenashev, 1972; Neher, 1974). The lack of a consistent definition of the phenomenon of membrane fusion has led to disagreement as to whether a particular experimental observation provides conclusive evidence for fusion. For example, transfer of spin-labeled lipid molecules (Van der Bosch and McConnell, 1975; Maeda and Ohnishi, 1974) and fluorescent probes (Pohl et al., 1973) from one type of membrane to another is consistent with fusion but can also be interpreted as molecular exchange or diffusion of single molecules or clusters of molecules (Martin and MacDonald, 1976a; Papahadjopoulos et al., 1976a). Similar alternative interpretations can be used for evidence (for references, see above) involving changes in proton magnetic resonance spectra, also for evidence on increase in vesicle size and even for evidence indicating complete mixing of the lipid molecules between two different populations of vesicles.

The most rigorous and incontrovertible evidence for fusion between two vesicles should involve stoichiometric mixing of the vesicle membrane components, as well as mixing of the vesicle contents enclosed within their interior aqueous space. Positive evidence for either of the above mixing reactions would constitute strong indication for fusion since the occurrence could not be explained by any of the alternative mechanisms proposed up to now. Stoichiometric (one-to-one) mixing of vesicle lipids has been unequivocally demonstrated for the case of calcium-induced fusion of acidic phospholipid vesicles (Papahadjopoulos et al., 1974, 1976b). Evidence for mixing of the water-soluble vesicle contents has not been reported as yet* except for an early observation (Taupin and McConnell, 1972) involving a small degree of reactivity of spin-labeled compounds trapped within lecithin vesicles. In any case, lack of evidence for mixing of water-soluble vesicle contents cannot be taken as an indication that fusion has not occurred. This is because the vesicles may become leaky during fusion, in which case their contents would be released into the outside medium. Such an occurrence

*See Notes Added in Proof, note 1, p. 99.

could be particularly important for the fusion of small sonicated lipid vesicles. Release of vesicle contents has been observed to occur during calcium-induced fusion of unilamellar phosphatidylserine vesicles (Papahadjopoulos et al., 1977).

7.2. Methods for Studying Fusion in Model Membranes

Mixing of the vesicle contents has not been studied in detail as yet, although a preliminary report has been published (Taupin and McConnell, 1972). Such mixing could be followed by several spectroscopic techniques for observing the spectral changes of a compound trapped inside one vesicle, which could be induced only after collision with another compound trapped inside another vesicle. Appropriate controls would be needed to establish the extent of reaction occurring outside the vesicles, since this could be reflecting only vesicle lysis without fusion.*

Mixing of the vesicle lipid components has been reported from two laboratories, followed by either differential scanning calorimetry (Papahadjopoulos et al., 1974, 1976a,b) or changes in optical density at different temperatures (Martin and MacDonald, 1976a). In both cases the system was based on the use of two different populations of phospholipid vesicles, differing only in their transition temperatures. Each of the individual components and their mixtures had characteristic transition temperature (T_c). Figure 6 shows an example of such a system consisting of hand-shaken dispersions of dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylglycerol. The individual peaks represent the endothermic transitions as observed by differential scanning calorimetry. Molecular mixing can thus be followed by the shifts in the transition temperatures following incubation of the two vesicle populations. Since the stoichiometry of any mixing can be established unequivocally, this method provides the opportunity to differentiate between true fusion and molecular exchange or diffusion between vesicles (Papahadjopoulos et al., 1976a,b).

Transfer of labeled molecules has been followed by observing the disappearance of spin-spin exchange broadening when a population of phospholipid vesicles containing a spin-labeled lipid molecule is incubated with a population of unlabeled phospholipid vesicles (Van der Bosch and McConnell, 1975; Maeda and Ohnishi, 1974). Transfer of isotopically labeled phospholipid molecules between small and large vesicles has also been followed, following separation by centrifugation (Papahadjopoulos et al., 1974). The reservations concerning the above methods involve the possibility that the observed molecular transfer is due to a molecular exchange or diffusion and not true fusion of the whole vesicle.

*See Notes Added in Proof, note 1, p. 99.

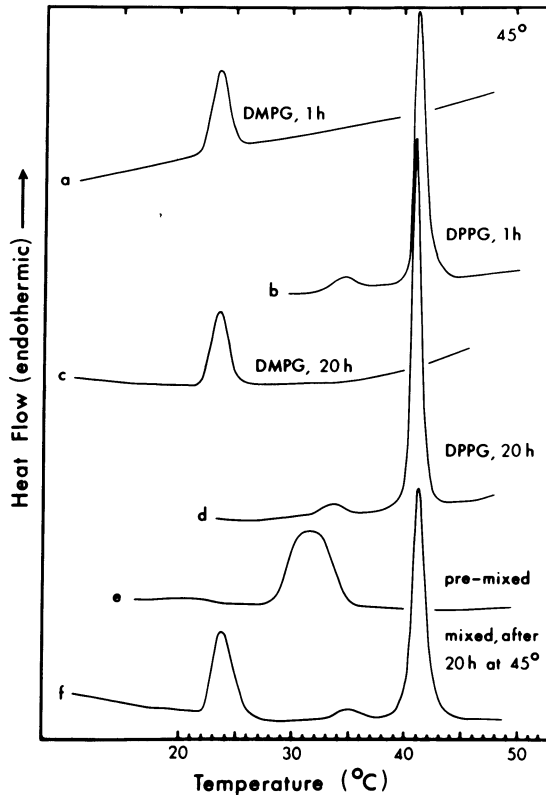


Fig. 6. Differential scanning calorimetry of pure and mixed phosphatidylglycerol vesicles. In all cases, the phospholipids were suspended by shaking in 100 mM NaCl buffer, pH 7.4, at 45°C at a concentration of 5 μ mol/ml. They were then incubated further at 45°C for the indicated times: (a) dimyristoylphosphatidylglycerol (DMPG), 1 hr; (b) dipalmitoylphosphatidylglycerol (DPPG), 1 hr; (c) dimyristoylphosphatidylglycerol, 20 hr; (d) dipalmitoylphosphatidylglycerol, 20 hr; (e) mixture of dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylglycerol suspended together and incubated for 1 hr at 45°C; (f) vesicles of dimyristoylphosphatidylglycerol and vesicles of dipalmitoylphosphatidylglycerol as in (a) and (d) above mixed after incubation for 20 hr at 45°C and analyzed immediately. From *Biochim. Biophys. Acta* 448:245–264. (1976).

Increase in vesicle size has been followed by increases in optical density (Lansman and Haynes, 1975; Martin and MacDonald, 1976a), by gel filtration chromatography (Papahadjopoulos et al., 1974; Prestegard and Fellmeth, 1974), and also by electron microscopy involving negative stain (Lau and Chan, 1976; Miller and Racker, 1976a), freeze-fracture (Papahadjopoulos et al., 1975a, 1976b), and thin sections (Miller et al., 1976). Of these methods, increase in turbidity and decrease in elution volume through sepharose column would not differentiate between vesicle aggregation and fusion. On the other hand, turbidity changes can be followed in a stopped-

flow apparatus for studying the kinetics of vesicle-vesicle interactions (Lansman and Haynes, 1975). All of the electron microscopy methods are capable, in principle, of distinguishing aggregation from fusion, although the technique of freeze-fracture would be preferable because of the lack of artifacts involved in drying (negative stain), fixing and dehydrating (thin sections).

Figures 7 to 11 represent typical examples of freeze-fracture electron micrographs obtained with different phospholipid vesicle preparations. They indicate the ability of the technique to preserve and reveal the morphological characteristics of each vesicle preparation. Figure 7 was obtained from a

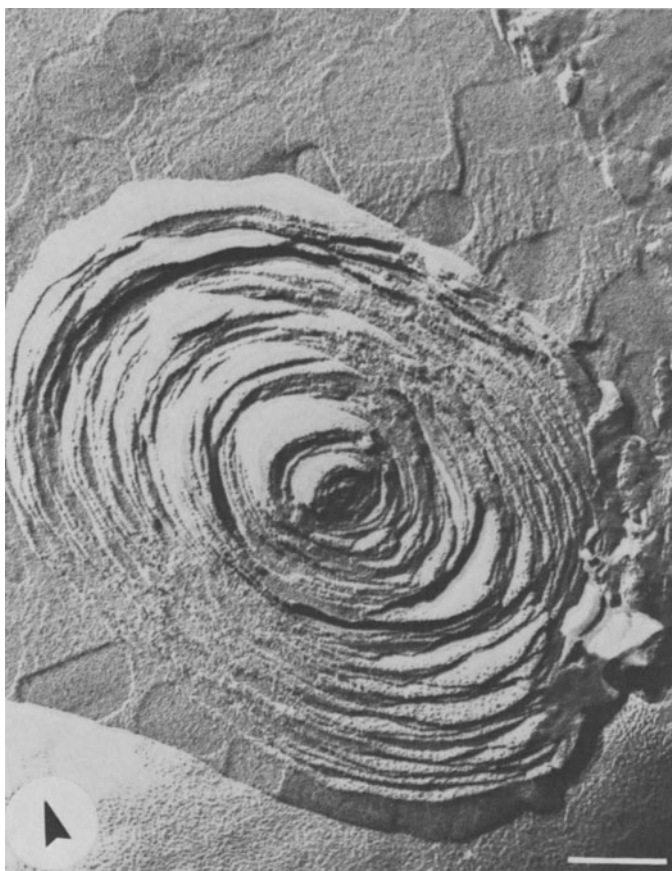


Fig. 7. Freeze-fracture electron micrograph of a multilamellar phospholipid vesicle prepared from 10% phosphatidylserine (bovine brain) and phosphatidylcholine (egg yolk) by vortex shaking of the lipid mixture in 100 mM NaCl buffer at pH 7.4. The direction of shadowing is indicated by the arrowhead in the lower left-hand corner. Scale bar: 0.1 μm (1000 \AA).



Fig. 8. (A) Freeze-fracture electron micrograph of dimyristoylphosphatidylcholine (DMPC) suspended by vortex shaking in 100 mM NaCl buffer at 37°C and then equilibrated at 25°C before freezing to low temperature. Scale bar: 0.25 μm (2500 Å).

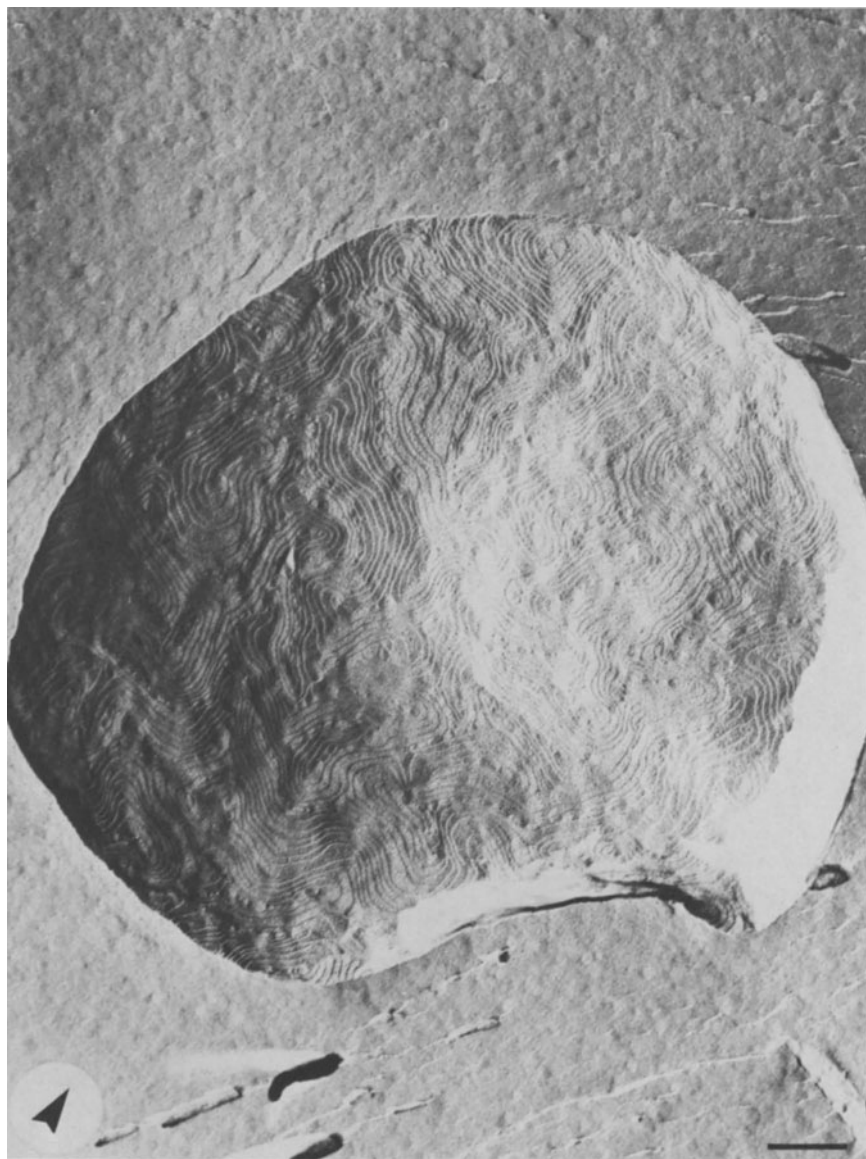


Fig. 8 (B). Same as in A except that the DMPC suspension was equilibrated at 0°C before freezing.

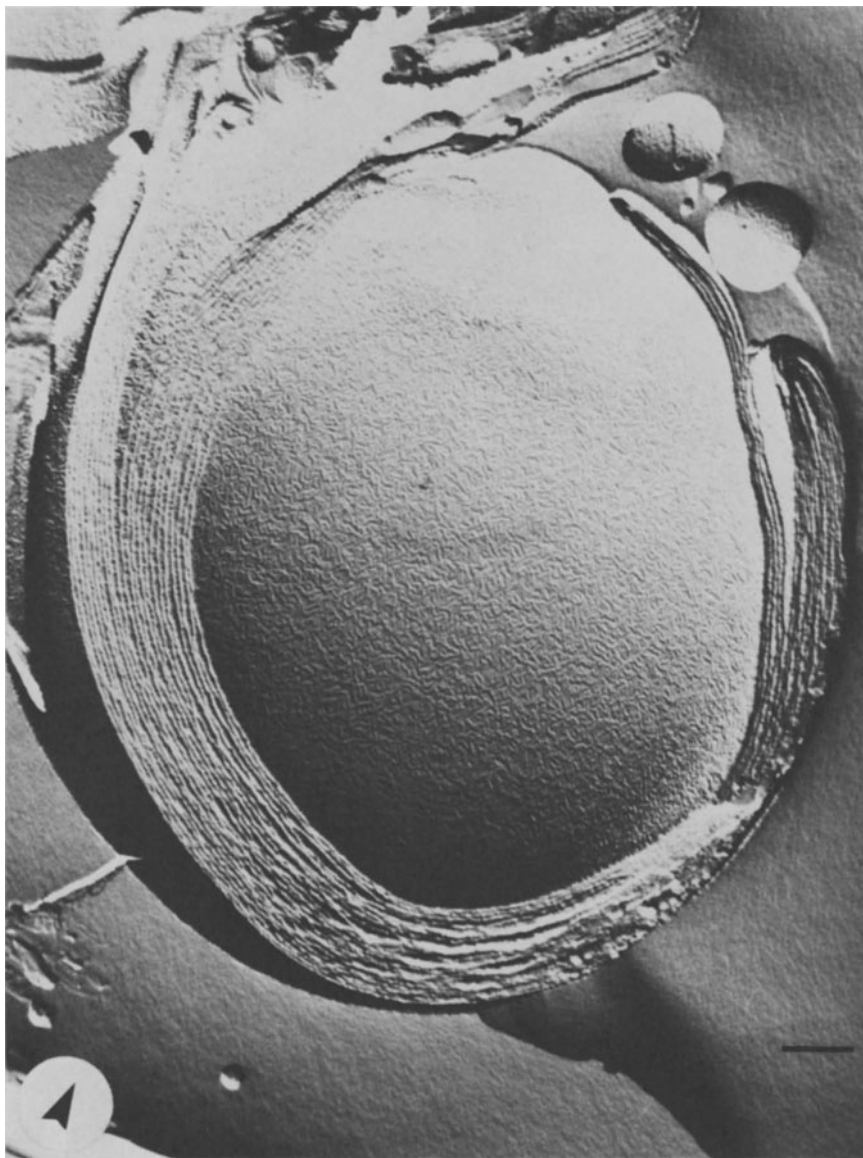


Fig. 8 (C). Same as in A except that the DMPC suspension was equilibrated at 45°C before freezing.

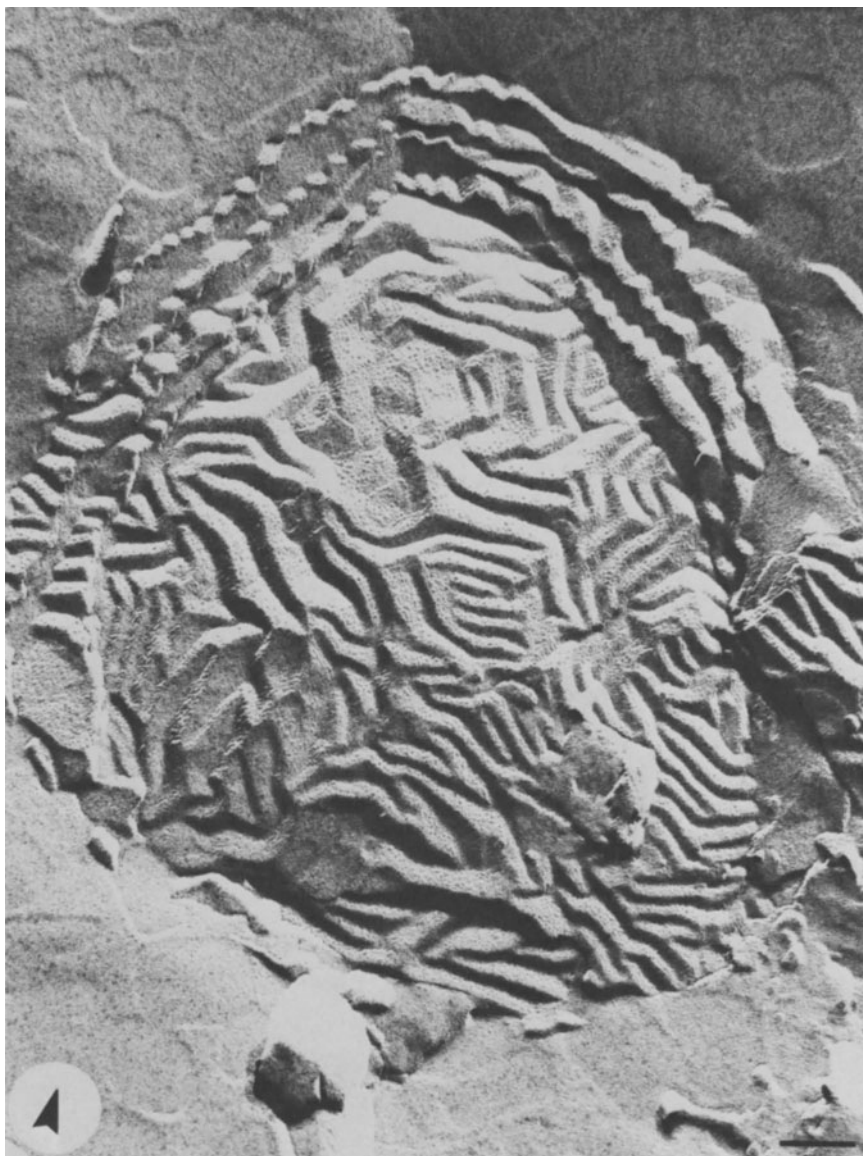


Fig. 9. Freeze-fracture electron micrograph of dimyristoylphosphatidylglycerol (DMPG) suspended by vortex shaking at 37°C in 100 mM NaCl buffer, pH 7.4, and equilibrated at 25°C before freezing. Scale bar: 0.1 μm (1000 Å).

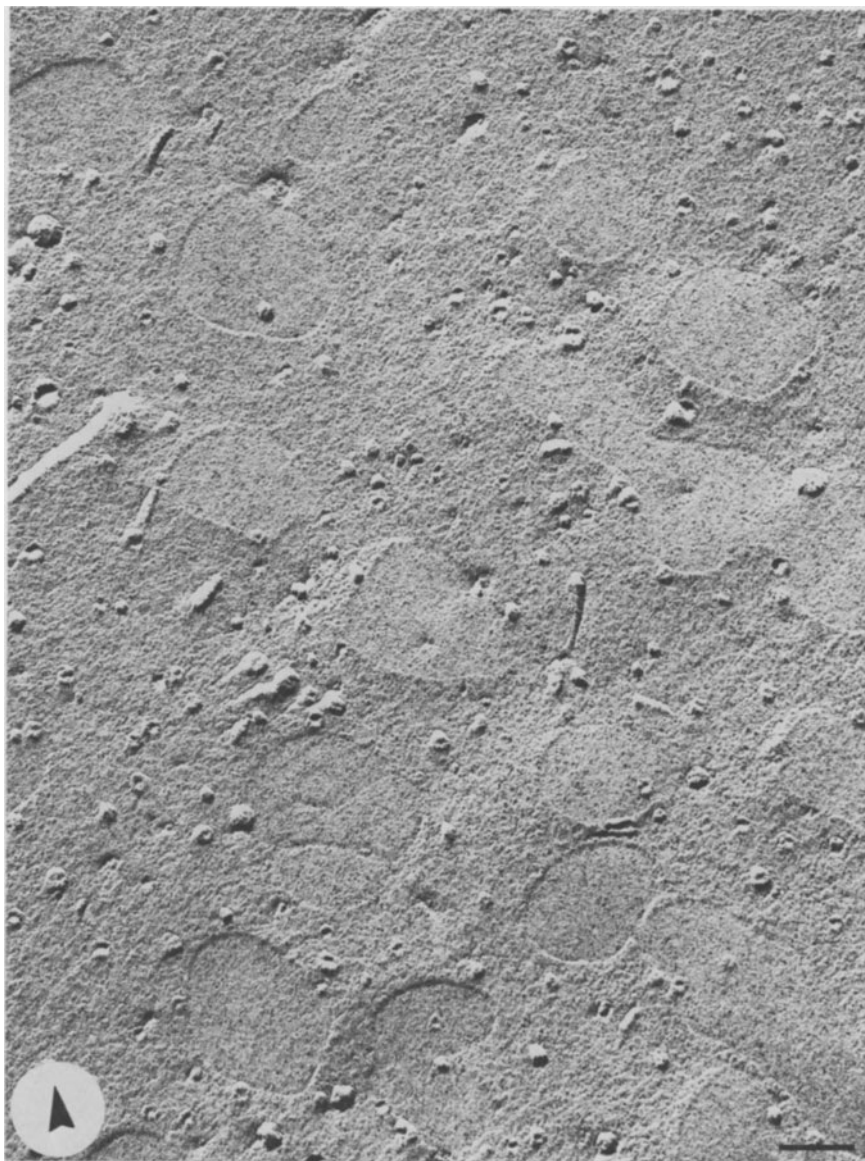


Fig. 10. Freeze-fracture electron micrograph of DMPC after sonication for 1 hr at 37°C in a bath-type sonicator. Details and temperatures as in Fig. 8A. Scale bar: 0.1 μm (1000 Å).

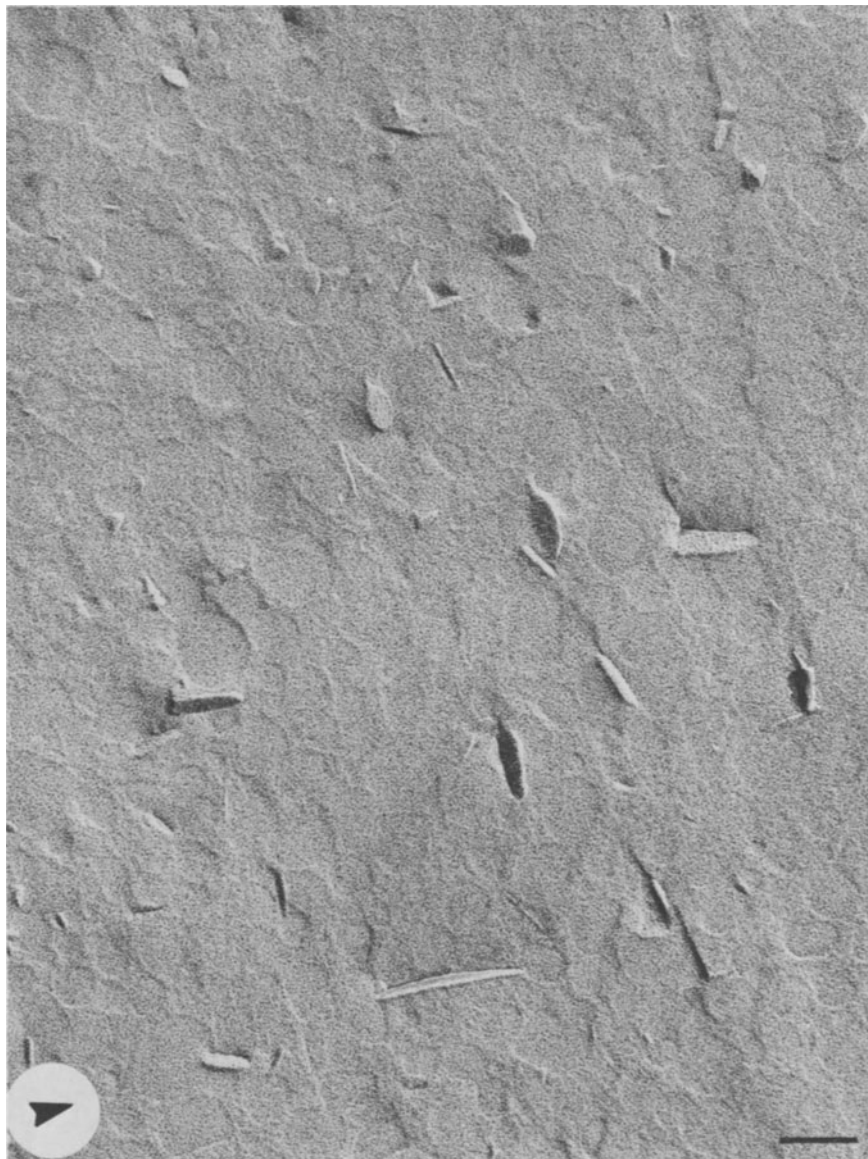


Fig. 11. Freeze-fracture electron micrograph of DMPG after sonication for 1 hr at 37°C in a bath-type sonicator. Other details as in Fig. 9. Scale bar: 0.1 μm (1000 Å).

hand-shaken dispersion showing a fracture through a multilamellar vesicle (liposome) composed of 10% phosphatidylserine in phosphatidylcholine. All the fracture planes are smooth, as is usual for fluid phospholipid membranes quenched from temperatures above their T_c (Deamer et al., 1970; Ververgaert et al., 1973). Figure 8 was obtained from a hand-shaken dispersion of dimyristoylphosphatidylcholine at 22°C, showing the typical striated fracture planes (Ververgaert et al., 1973). Such striations are in agreement with crystallographic evidence (Tardieu et al., 1973) for a "pleated" (or corrugated) membrane structure below the T_c . Recent evidence indicates that the pleated structure is related to the premelt transition observed by calorimetry (Janiak et al., 1976). Figure 8B,C presents evidence in good agreement with the X-ray diffraction studies, showing that the "striations" are prominent only between the premelt and the main transitions. Figure 9 was obtained under similar conditions as Fig. 8A, except that the lipid was dimyristoylphosphatidylglycerol suspended in NaCl buffer (Papahadjopoulos et al., 1976b). In this case the striations appear much more angular, deeper and with a larger bandwidth. Figures 10 and 11 were obtained from the same lipid preparations as in Figs. 8 and 9, respectively, except that the suspensions were sonicated before microscopy (Papahadjopoulos et al., 1976a). It can be seen that while the phosphatidylcholine gave fractures showing spherical vesicles of approximately 200–300 Å diameter similar to those obtained with various phospholipids sonicated at temperatures above their T_c (Papahadjopoulos and Miller, 1967; Huang, 1969; Johnson et al., 1971; Hauser et al., 1973), the phosphatidylglycerol vesicles appeared as flattened spheroidal disks.

Increase in vesicle size has been followed indirectly by two other techniques, namely nuclear magnetic resonance (Prestegard and Fellmeth, 1974; Lau and Chan, 1975) and differential scanning calorimetry (Suurkuusk et al., 1976; Papahadjopoulos et al., 1976a). In both cases the starting material is a preparation of small sonicated vesicles which show distinctive properties [narrow, well-resolved proton paramagnetic resonance (PMR) spectrum; also a broadened thermotropic transition at a lower temperature]. As the size increases, the PMR signal broadens and the endothermic transition sharpens, both properties being associated with the unsonicated multilamellar vesicles. Although combination of PMR and gel filtration (Prestgard and Fellmeth, 1974), PMR and electron microscopy (Lau and Chan, 1975), or DSC and electron microscopy (Papahadjopoulos et al., 1976a) indicates that the spectral or thermotropic changes are associated with an increase in vesicle size, the evidence still does not exclude the possibility of growth in vesicle size through molecular diffusion (Martin and MacDonald, 1976; Papahadjopoulos et al., 1976a).

An ingenious new method for monitoring fusion was introduced (Miller and Racker, 1976a), involving the interaction of two membrane-embedded

proteins. Thus cytochrome oxidase is reconstituted into lipid vesicles and interacted with another population of vesicles which contain a hydrophobic protein. It was established that when both proteins were reconstituted into the same vesicles, the cytochrome oxidase was "uncoupled" and thus could not retain proton gradients. Thus, by following the changes in the permeability properties of the cytochrome oxidase vesicles, vesicle fusion could be detected by the presence of both proteins in the same vesicle. Miller and Racker (1976b) also studied the fusion of fragmented sarcoplasmic reticulum with planar lipid bilayer membranes by following changes in conductance.

7.3. Fusion and Molecular Exchange with Pure Phospholipid Vesicles and Bilayers

Fusion (coalescence) of oil droplets in water is driven by the high interfacial tension of the system and results in an overall decrease of the oil-water interphase, thus minimizing the free energy of the system. Since it is generally accepted that phospholipid vesicles and black lipid films are very stable structures with very low interfacial tension (Thompson and Henn, 1970; Bangham, 1972), it would be surprising to find that they tend to fuse extensively. A critical evaluation of the studies published recently on this subject permits now a reasonable description of the ability of pure phospholipid membranes to fuse.

An early report on sonicated lecithin vesicles indicated a small but measurable degree of fusion (Taupin and McConnell, 1972). A later study with sonicated dimyristoylphosphatidylcholine (DMPC) vesicles revealed that extensive and rapid fusion occurred only at the temperature region of the expected transition from solid to liquid crystalline (Prestegard and Fellmeth, 1974). The occurrence of fusion in this case was concluded from the observed increase in vesicle size. However, it was reported subsequently (Kantor and Prestegard, 1975) that such fusion (increase in vesicle size) could occur only in the presence of myristic acid, which was present as an impurity in the original DMPC preparations. Other studies using similar methodology have indicated that the observed changes in PMR spectra and increase in vesicle size could be the result of incomplete "annealing" of the vesicles (Lawaczek et al., 1975, 1976).

Evidence for a slow, time-dependent increase in the size of sonicated DMPC and also DPPC vesicles incubated at temperatures near or below the T_c has been obtained with chromatographically pure lipids, under conditions of preparation that would ensure adequate annealing of the vesicles, by Martin and MacDonald (1976a), Suurkuusk et al. (1976), and Papahadjopoulos et al. (1976a). The authors of these three studies were in general agreement that the existing evidence is not definitive enough concerning the mechanism of the observed increase in vesicle size. Although fusion is not

ruled out, other processes such as molecular diffusion could account for the observed phenomena.

The interaction of vesicles composed of pure phospholipids was also studied by mixing of their lipid components. Using unsonicated lecithin vesicles (Papahadjopoulos et al., 1974, 1976a), it was observed that no appreciable mixing was occurring at temperatures a few degrees above the phase transition of the vesicles, indicating no fusion even during long incubation times (20 hr). A study with sonicated lecithin vesicles observed considerable mixing between two populations of lecithin vesicles (Martin and MacDonald, 1976a). However, the kinetics of mixing indicated a process of gradual transfer of the more soluble molecules to the more stable vesicles. This appears to be compatible with a diffusion process rather than true fusion. Similar observations and conclusions were reported in another study involving the interaction of multilamellar phosphatidylglycerol vesicles (Papahadjopoulos et al., 1976a) suspended in 0.1 M NaCl, pH 7.4.

Interaction between pure phospholipid vesicles was also studied by measuring the transfer of radioactive lipid from small sonicated vesicles to large multilamellar vesicles (Papahadjopoulos et al., 1974). It was observed that no transfer above background was occurring—even during incubation at 37°C for 20 hr in 0.1 M NaCl, pH 7.4—between egg lecithin vesicles or brain phosphatidylserine vesicles or several mixtures of these lipids.

Fusion of spherical bilayer black lipid films has been studied recently (Breisblatt and Ohki, 1975, 1976) by direct optical observations. These large spherical bilayers are allowed to float in a density gradient, and the percentage of fusion (one larger sphere) is scored among spheres that have formed a common interface. Although the percentage of fusion events is low at low temperature, there is a large increase at higher 30–40°C temperatures with both lecithin and phosphatidylserine bilayers. Although the system is interesting, it is not possible at present to exclude the problems of lipid impurities and mechanical instability. Enhancement of fusion by lysolecithin and a decrease of extent of fusion by cholesterol have already been reported with this system (Breisblatt and Ohki, 1976).

In conclusion, the studies to date with pure phospholipid vesicles suspended in water or monovalent salt solutions reveal that fusion between fluid vesicles (above their transition temperatures) is very slow, if it occurs at all. This appears to be true for both sonicated and nonsonicated neutral or negatively charged vesicles. Extensive growth in size of the sonicated vesicles at temperatures close to the T_c has now been observed in several laboratories. However, the interpretation of the phenomenon has varied from a mechanism involving diffusion (Martin and MacDonald, 1976a) to incomplete annealing (Lawaczek et al., 1975) to a myristic acid impurity (Kantor and Prestegard, 1975). The refractoriness of the pure phospholipid vesicle

membranes to fusion could be reflecting the innate stability of these systems, and also the existence of large repulsive forces at the interface of even neutral (lecithin) membranes (Le Neveu et al., 1976), possibly due to structured water (Marcelja and Radic, 1976), which do not allow close contact between colliding membranes.

7.4. Effects of Lysolecithin, Fatty Acids, DMSO, etc.

The role of lysolecithin in membrane fusion has been of considerable interest since the demonstration by Howell and Lucy (1969) that this compound can induce fusion of erythrocytes (see Section 4). Since then, this method has been used in several laboratories for the production of cell hybrids (Croce et al., 1971), although its use is limited by the considerable cell lysis (see Section 4). The possible role of lysolecithins in membrane fusion under physiological conditions has been discussed (Poste and Allison, 1973; also see Section 4). The most interesting proposal in this respect involved the production of endogenous lysolecithin by the action of calcium-activated phospholipase, which would make these membranes susceptible to fusion (Lucy, 1975). Although this is still a possibility, its importance as a major mechanism has been diminished greatly by several subsequent observations. First, acidic phospholipids can cause extensive cell fusion without lysolecithin and without appreciable cell lysis (Papahadjopoulos et al., 1973a). Indeed, neutral (lecithin) vesicles containing up to 20% lysolecithin are unable to induce cell fusion (Martin and MacDonald, 1976b). Second, calcium alone can induce phospholipid membrane fusion without any concomitant enzymic hydrolysis (Papahadjopoulos et al., 1974, 1976b). Third, extensive hydrolysis of intact red cells by phospholipases can be achieved without cell lysis, indicating that endogenous lysolecithin per se is not lytic for cell membranes (Zwaal et al., 1973). It has also been shown that the lytic effects of lysolecithin on model membranes are reduced substantially in the presence of cholesterol, which may be related to the ability of lysolecithin to form a stoichiometric complex with cholesterol, which has a lamellar configuration rather than micellar (Inoue and Kitagawa, 1974; Klopfenstein et al., 1974; Mandersloot et al., 1975; Rand et al., 1975).

The effect of exogenous lysolecithin on the thermotropic properties and fusion of phospholipid vesicles was studied recently in some detail (Papahadjopoulos et al., 1976a). It was shown that addition of lysolecithin produced an increase in the T_c of individual phospholipids even when added at a 20% molar ratio (Fig. 12A). This was an unexpected result, and it does not support the impression derived from the literature that lysolecithin is able to increase the fluidity of membrane lipids (Ahkong et al., 1973a; Howell et al., 1972). A fluidization of the bilayer following addition of lysolecithin would be

expected to result in a decrease in the T_c of the membranes. If lysolecithin is added to two populations of preformed multilamellar lecithin vesicles (Papahadjopoulos et al., 1976a), considerable enhancement of mixing of the lipid components is observed (Fig. 12A,B; compare curve Ae with curves Ba and Bb). However, the kinetics of mixing indicate a gradual enrichment of each of the lecithins with the other, which is compatible with an exchange diffusion process rather than fusion. True fusion should have resulted in the formation of a third component with an intermediate melting point, reflecting the formation of a unique mixture with the stoichiometry of the initial two populations of lecithins. As shown in Fig. 12B, lysolecithin induces the formation of such third component when added to two populations of phosphatidylglycerol vesicles.

It was concluded from these data that lysolecithin can produce some fusion, at least with acidic phospholipid membranes, but the initial effect on lecithin membranes is an enhancement of the rate of molecular mixing, probably via exchange diffusion rather than fusion. It was also concluded that, at relatively low concentrations of lysolecithin, before solubilization of the phospholipid membranes becomes pronounced (up to 20% mole ratio!) its effect on lipid bilayers is not fluidization but rather an apparent "stabilization" as evidenced by an increase in the T_c .

As mentioned earlier, myristic acid has been shown to enhance the rate of growth in size of sonicated DMPC vesicles, possibly indicating an effect on vesicle fusion (Kantor and Prestegard, 1975). It is not clear, however, whether this effect is due to improper annealing (Lawaczek et al., 1975) or whether it relates to structural fluctuations induced by the bilayer phase transition (Kantor and Prestegard, 1975) or to the properties of the sonicated vesicles containing myristic acid. In order to provide insight into this problem, we recently studied the effect of myristic acid on the rate of mixing of the lipid components between two lecithin populations, DMPC and DPPC (Papahadjopoulos et al., 1976a). It was first established that the presence of myristic acid (premixed with the phospholipid in a 10% mole ratio before vesicle formation) produced an increase in the T_c and a broadening of the main transition, while the premelt transition was not apparent at all. These effects were similar to those observed with lysolecithin discussed above. When such multilamellar vesicles each composed of myristic acid and either one of the two lecithins were incubated together, no appreciable mixing was observed. The conditions included incubation for 4 hr at 50°C at pH from 4.5 to 8.5 in 0.1 M NaCl solution (Papahadjopoulos et al., 1976a). The same type of experiment was performed with sonicated preparations of the same lipids. In this case, incubation of the two populations for 3 and 20 hr at 50°C produced a gradual shift of the two endothermic peaks toward intermediate temperatures. This result demon-

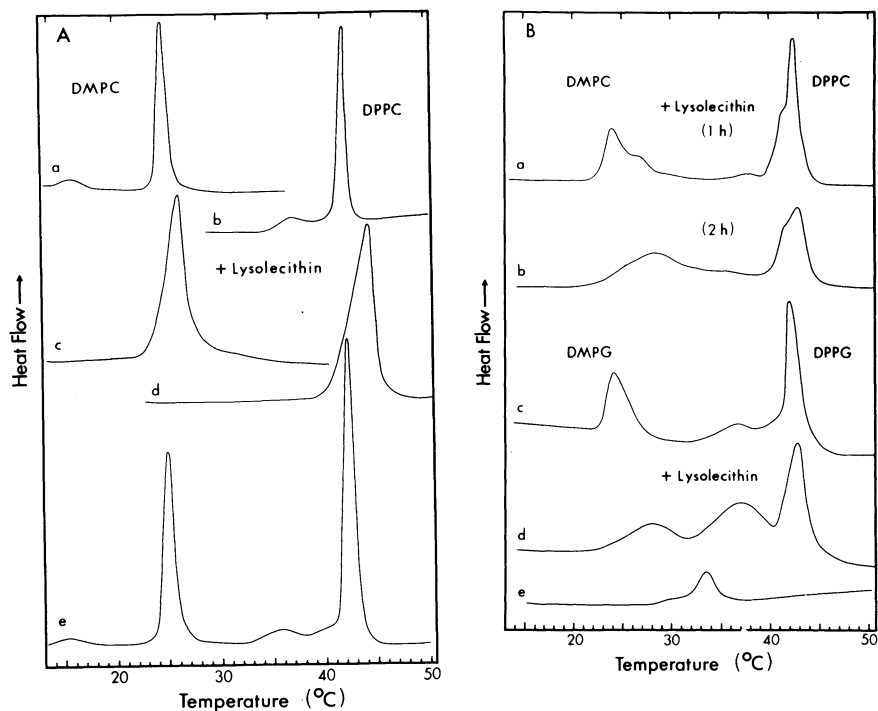


Fig. 12. Effect of lysolecithin on the thermotropic properties and molecular mixing in phospholipid vesicles. **A:** Individual phosphatidylcholine components were suspended separately in 100 mM NaCl buffer, pH 7.4, at 45°C and 10 $\mu\text{mol/ml}$. Lysolecithin was dissolved in the same buffer and was added in a ratio of 1.0 μmol to 4.0 μmol lipid in 0.5 ml total volume. Samples were incubated for 1 hr at 45°C: (a) dimyristoylphosphatidylcholine alone, (b) dipalmitoylphosphatidylcholine alone, (c) dimyristoylphosphatidylcholine plus lysolecithin, (d) dipalmitoylphosphatidylcholine plus lysolecithin, and (e) dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine formed separately and incubated for 1 hr at 45°C. **B:** Individual phospholipid vesicles prepared as in A and incubated as follows: (a) dimyristoylphosphatidylcholine incubated with dipalmitoylphosphatidylcholine in the presence of lysolecithin as in A (c and d) at a molar ratio of 1.0 to 4.0 total lipid for 1 hr at 45°C; (b) same as in (a) except 2 hr at 45°C; (c) dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylglycerol formed separately and incubated together in equimolar amounts for 1 hr at 45°C; (d) as in (c) above except for the addition of 2 μmol lysolecithin to 4 μmol of dimyristoylphosphatidylglycerol and 4 μmol dipalmitoylphosphatidylglycerol in 5 ml volume of 100 mM NaCl buffer; (e) as in (d) except for the addition of 5 μmol lysolecithin to μmol dimyristoylphosphatidylglycerol and 4 μmol dipalmitoylphosphatidylglycerol. From *Biochim. Biophys. Acta* 448:245–264 (1976).

strates extensive mixing between the two populations of vesicles, but the shift of the two peaks is consistent with the kinetics of exchange diffusion rather than fusion.

It can be concluded from the above results that myristic acid can have a considerable effect on phospholipid mixing between bilayer membranes of different vesicles. However, the extent of its effect and the type of mixing observed depend largely on the condition of the phospholipid bilayers. For example, it has no effect (no enhancement of mixing) on the large multilamellar lecithin vesicles, but it induces extensive exchange diffusion of lecithin molecules between sonicated vesicle preparations at temperatures above their T_c . The effect of myristic acid on the growth of sonicated DMPC vesicles at a temperature identical to their T_c could be related to the structural fluctuations that the bilayer is undergoing at the T_c . Since this phenomenon is dependent on the phase transition but greatly enhanced by myristic acid at the T_c , it can be concluded that myristic acid is providing the electrostatic attractive forces necessary to bring two vesicles into close apposition. Fusion or molecular exchange then can occur at points of structural discontinuities induced by the phase transition. This interpretation is based on recent observations concerning the existence of large repulsive forces between lecithin bilayers (Le Neveu et al., 1976) and the importance of phase changes during the calcium-induced fusion events (Papahadjopoulos et al., 1977), which will be discussed in detail below.

Dimethylsulfoxide (DMSO) has been shown to produce cell fusion (Ahkong et al., 1975a) as well as particle redistribution in cell membranes (McIntyre et al., 1974) and drastic changes in the phase transition characteristics of phospholipid membranes (Lyman et al., 1976a,b). The interest in the possible effects of DMSO on membranes is augmented because of its well-known properties and as an inducer of leukemic cell differentiation (Preisler et al., 1973). Its effect on the ability of phospholipid membranes to fuse was examined recently (Papahadjopoulos et al., 1976a). This study involved the addition of high concentrations of DMSO into suspensions of two populations of multilamellar vesicles composed of pure phospholipids. It was found that 30% DMSO has no appreciable effect on the mixing of lecithins (incubation for 20 hr at 45° C) as detected by differential scanning calorimetry. However, under similar conditions, incubation of two populations of phosphatidylglycerol vesicles, produced a high degree of mixing (~50%) following incubation for 1.5 hr at 45° C. Furthermore, the characteristics of this mixing indicated that it was due to true fusion rather than exchange diffusion (Papahadjopoulos et al., 1976a).

The effect of DMSO in promoting fusion between phosphatidylglycerol but not phosphatidylcholine vesicles seems to be related to its ability to induce a new phase transition with a T_c at much higher temperatures (Lyman

et al., 1976a). This remarkable effect of DMSO in stabilizing acidic phospholipid membranes in the frozen (solid) state was shown recently to be correlated with leukemic cell differentiation (Lyman et al., 1976b). Although further studies with DMSO are needed before its mechanism of action is understood, its effect in promoting both fusion and a large increase in T_c is reminiscent of the effect of Ca^{2+} , which will be discussed later. A synergistic effect between Ca^{2+} and DMSO has already been noted (Lyman et al., 1976a), and the phenomenon is now being investigated in this laboratory in more detail.

7.5. Effects of Proteins and Polypeptides

The phospholipid vesicle system presents excellent opportunities for studying the possible role of specific proteins involved in natural membrane fusion phenomena such as secretion and fusion of viruses with cells. Although it is possible that the key step in the mechanism of membrane fusion involves only calcium and acidic phospholipids (see below), specific proteins may play an important role in conferring specificity or in organizing the lipid bilayer in microdomains favoring or not favoring fusion. It is also possible that specific proteins may induce fusion between membranes that are not otherwise susceptible to fusion. The reports that have appeared to date indicate that proteins or peptides may play a significant role, although the ones used so far are not known to be involved in natural membrane fusion phenomena.

Alamethicin is a cyclic oligopeptide (Payne et al., 1970) that is known to form voltage-dependent ion channels in black lipid films (Mueller and Rudin, 1968). Its interaction with phospholipid vesicles had been studied before (Hauser et al., 1970), and its effect in broadening the lipid proton resonance spectra had been interpreted as immobilization of the hydrocarbon chains by the peptide. More recently, evidence has been presented which was interpreted as indicating that alamethicin induces fusion of lecithin vesicles at temperatures well above the T_c (Lau and Chan, 1975). The evidence was based partly on the observed increase in vesicle size, the translocation of external ions and alamethicin molecules into the interior of the vesicles during incubation, and the NMR data indicating that alamethicin interacts primarily with polar head-group region of the lecithin vesicles (Lau and Chan, 1974, 1976). However, it is not entirely clear whether Lau and Chan have excluded the possibility that the observed phenomena are due to an increased diffusion of lecithin molecules from smaller to larger vesicles, which is mediated by alamethicin.

Concanavalin A is a protein that has been used extensively to char-

acterize cell surface phenomena because of its strong binding to specific glycoproteins (Review, Nicolson, 1974). In a recent study with lecithin vesicles, it was observed that the presence of concanavalin A induced a large increase in the transfer rate of spin-labeled lipid molecules between vesicles (Van der Bosch and McConnell, 1975). Moreover, it was noted that this effect was pronounced only at the temperature of the phospholipid transition. It was concluded that concanavalin A caused fusion between lecithin vesicles, although a rigorous exclusion of diffusion mechanisms was not attempted. The phenomenon is interesting, although the mechanism is at present not clear. In some respects, this effect of concanavalin A is reminiscent of the effect of myristic acid, discussed earlier.

The effect of a hydrophobic membrane protein on fusion of lecithin vesicles was reported recently (Papahadjopoulos et al., 1976a). The protein was a purified major component of myelin proteolipid (Folch and Lees, 1951; Gagnon et al., 1971), and its interaction with phospholipid vesicles had been studied already in some detail (Papahadjopoulos et al., 1975b,c). Briefly, it had been shown that this protein can be incorporated into phospholipid bilayers up to 50% by weight, without affecting the T_c of the main endothermic transition. The resultant lipoprotein membranes have an intermediate buoyant density and contain intramembrane particles in freeze-fracture electron micrographs (Vail et al., 1974; Papahadjopoulos et al., 1975c). Low-angle X-ray diffraction of such membranes indicated that the incorporation of the protein did not increase the thickness compared to that of the pure lipid, indicating that a good part of the protein must be embedded within the lipid bilayer (Rand et al., 1976).

Figure 13 shows the thermotropic transition of two lecithins (DMPC and DPPC), dispersed in NaCl buffer as multilamellar vesicles, containing 15% of the proteolipid apoprotein (lipophilin). Incubation of these two populations of vesicles together for 0.5 and 1.5 hr at 45°C produced a large degree of mixing (Fig. 13, curves c and d). Under similar conditions, no mixing at all was observed with the same two lipids as multilamellar vesicles in the absence of protein (curve e). The kinetics of mixing indicate that increasing amounts of DPPC were mixing with all the available DMPC. It was concluded that the data are best explained by a mechanism involving fusion, assuming a decreasing propensity for fusion with increasing content of DPPC (Papahadjopoulos et al., 1976a). However alternative mechanisms involving diffusion could not be excluded.

It is still too early to generalize on the possible effects of various proteins on phospholipid membrane fusion. The existing evidence indicates that proteins and oligopeptides do enhance molecular mixing between vesicles and that this effect may be related to fusion. Very little can be said concerning the mechanism(s) involved in such interactions.

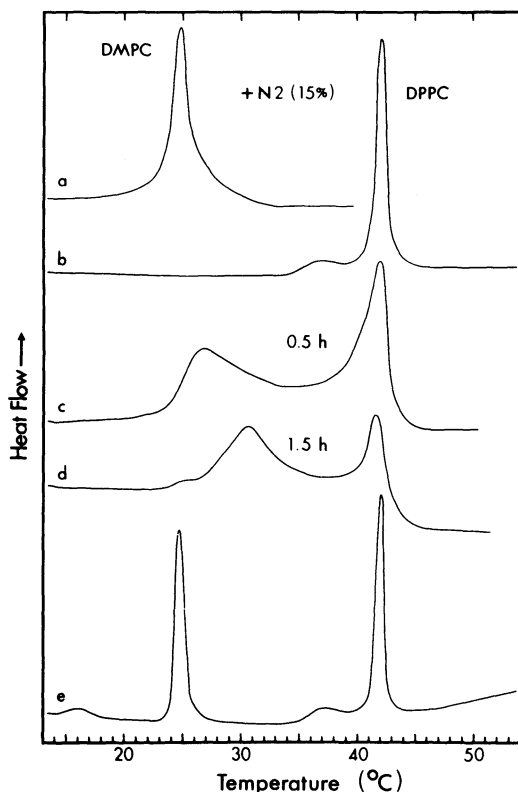


Fig. 13. Effect of a hydrophobic membrane protein on molecular mixing between phosphatidylcholine vesicles. Phospholipid and the major proteolipid apoprotein from myelin (N2) were mixed in chloroform-methanol-water, evaporated together, and suspended in 10 mM NaCl buffer, pH 6.5, by shaking with the aid of glass beads at 45°C. 2.7 mg of the apoprotein was mixed with 20 μ mol of each phosphatidylcholine and suspended in 4 ml buffer. The suspension was layered over a cushion of 60% sucrose solution and centrifuged at 10^5g for 1 hr at 20°C. The material collected at the interface between sucrose and buffer was pipetted off, diluted to 4 ml of buffer, and then incubated alone or after mixing at 45°C as follows: (a) dimyristoylphosphatidylcholine with N2 alone, 1 hr; (b) dipalmitoylphosphatidylcholine with N2 alone, 1 hr; (c) dimyristoylphosphatidylcholine with N2 and incubated together with dipalmitoylphosphatidylcholine with N2, 0.5 hr; (d) same mixture as in (c), 1.5 hr; (e) pure dimyristoylphosphatidylcholine vesicles incubated with pure dipalmitoylphosphatidylcholine vesicles, 1.6 hr at 45°C. From *Biochim. Biophys. Acta* 448:245–264 (1976).

7.6. Effects of Bivalent Metals (Calcium and Magnesium)

Particular attention has been given to the role of calcium ions (Ca^{2+}) in regulating the many cellular processes in which membrane fusion occurs (Poste and Allison, 1973; Rubin, 1975). Although the involvement of Ca^{2+} in

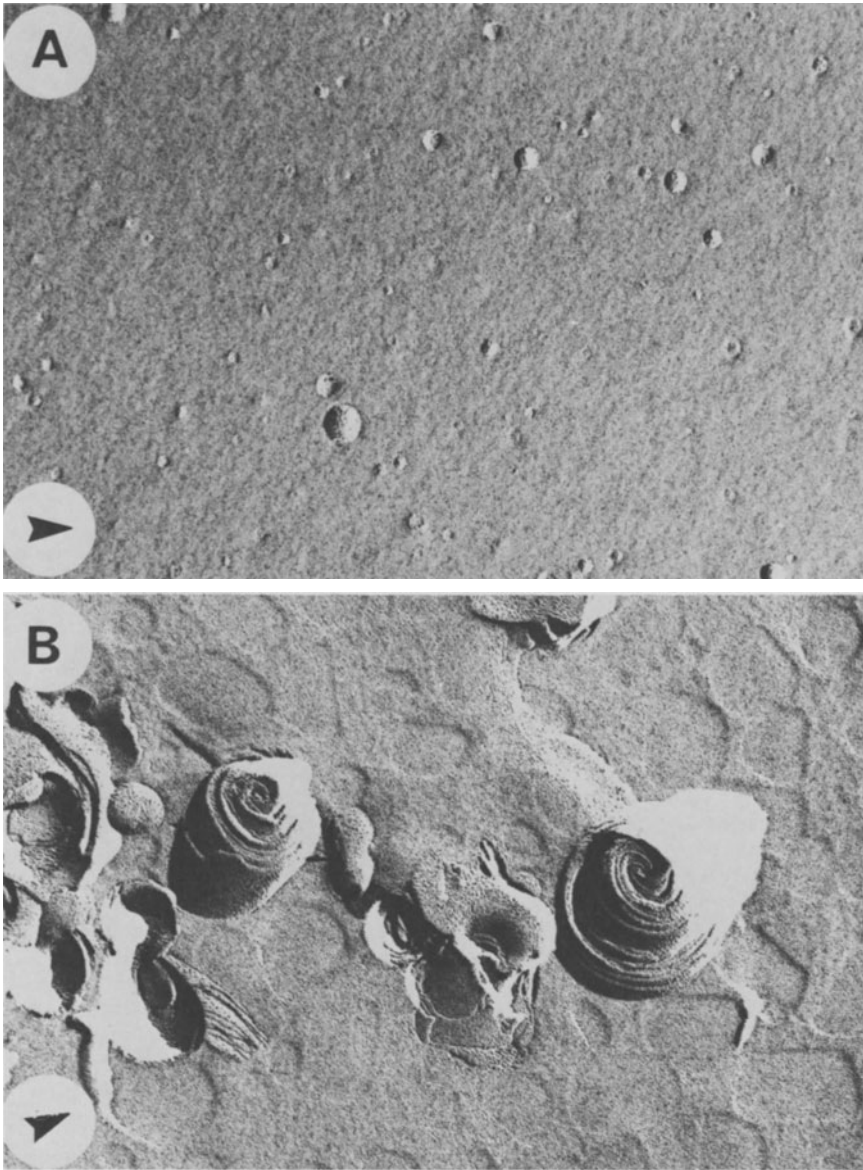


Fig. 14. Freeze-fracture electron micrograph of phosphatidylserine (PS) vesicles before and after addition of Ca^{2+} (and EDTA). A: After sonication in 100 mM NaCl buffer at pH 7.4 for 1 hr at 25°C in a bath-type sonicator. B: After dialysis of the initial sonicated suspension against same buffer containing 1.5 mM CaCl_2 at 25°C. C: After addition of excess EDTA to the Ca^{2+} -treated PS vesicles from above. This figure indicates the morphology of various PS vesicles from (A) sonicated unilamellar vesicles (SUV) to (B) cochleated cylinders to (C) large unilamellar vesicles (LUV). Other preparation details as in Papahadjopoulos *et al.* (1977). Scale bar: 0.1 μm (1000 Å).

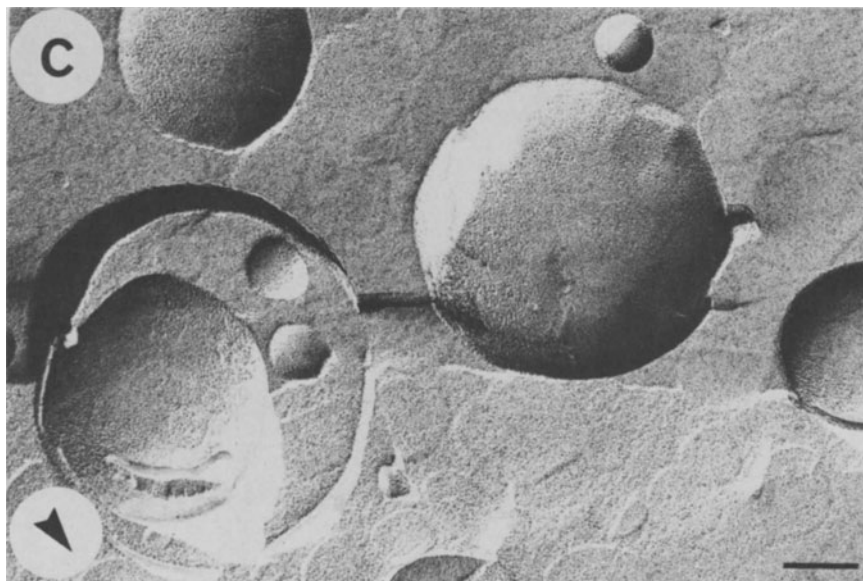


Fig. 14. (Continued)

such phenomena may include participation in diverse reactions with several cellular components, its interaction with membrane phospholipids seemed to us to be a reasonable ground for further investigation. The reason for this optimism was based on observations made several years ago that Ca^{2+} could induce abrupt changes in the permeability of phosphatidylserine (PS) vesicles (Papahadjopoulos and Bangham, 1966). Later it was shown that this effect was related to the Ca^{2+} asymmetry across the PS membranes (Papahadjopoulos and Ohki, 1969) which were very stable and impermeable in the absence of Ca^{2+} or when Ca^{2+} was present on both membrane sides. Other details of the interaction of PS with Ca^{2+} , which were reviewed recently (Papahadjopoulos et al., 1977), include the formation of a stoichiometric (2/1, PS/Ca mole ratio) complex at 1 mM Ca^{2+} in the presence of 100 mM NaCl, pH 7.4 (Bangham and Papahadjopoulos, 1966), the condensation of the area per molecule in PS monolayers (Bangham and Papahadjopoulos, 1966; Papahadjopoulos, 1968), and the crystallization of the acyl chains and disappearance of the normal PS endothermic transition (Jacobson and Papahadjopoulos, 1975).

The first report on the role of Ca^{2+} in the fusion of phospholipid vesicles (Papahadjopoulos et al., 1974) included evidence on the increase in size of sonicated PS vesicles not reversible by excess EDTA, which was demonstrated by both gel filtration and negative-stain electron microscopy. It was later demonstrated by freeze-fracture electron microscopy that the addition of Ca^{2+} to sonicated PS vesicles (1–10 mM) induces the formation of large

cochleated cylinders (Papahadjopoulos et al., 1975a) which become large closed spherical vesicles following addition to excess EDTA (Fig. 14). Calcium-induced fusion was also demonstrated with vesicles containing mixtures of PS and PC as shown by the mixing of the lipid components (Papahadjopoulos et al., 1974a). The same technique was used more recently to demonstrate fusion between PG vesicles at 10 mM Ca^{2+} and between phosphatidic acid (PA) vesicles at 0.2 mM Ca^{2+} (Papahadjopoulos et al., 1976b). The evidence on the mixing of two populations of multilamellar PG vesicles is shown in Fig. 15. It can be seen that at 5 mM Ca^{2+} the vesicles aggregate but no mixing is observed. However, at 7.5 mM Ca^{2+} the aggregation is followed by the formation of a new component with a melting point identical to that of the equimolar mixture of the two PGs. This third component is becoming the predominant membrane present at 10 mM Ca^{2+} with very small amounts of the two initial pure PG membranes remaining (Fig. 15, curve e).

One of the most remarkable aspects of these interactions is the specificity for divalent metal. It was observed with a PG system similar to that shown in Fig. 15 that the addition of Mg^{2+} (up to 20 mM) produces no mixing, although it produces aggregation of the PG vesicles (Papahadjopoulos et al., 1975b). Similarly, incubation of PS vesicles in the presence of 2–5 mM Mg^{2+} at 37°C produces slow aggregation of intact vesicles (Papahadjopoulos et al., 1977) instead of the cochleated cylinders which are produced by Ca^{2+} under similar conditions. It thus appears that Mg^{2+} does not induce fusion of PS or PG vesicles under the conditions used for Ca^{2+} -induced fusion. On the other hand, both Ca^{2+} and Mg^{2+} are equally effective in inducing fusion of PA vesicles (Papahadjopoulos et al., 1976b). Earlier studies on the binding of Ca^{2+} and Mg^{2+} to PS and also PA vesicles indicated similar binding constants (Hendrickson and Fullington, 1965; Abramson et al., 1966), although considerable differences between Ca^{2+} and Mg^{2+} were noted in their effects on the condensation of PS monolayers and the decrease in surface potential (Papahadjopoulos, 1968). Because of the differences between Ca^{2+} and Mg^{2+} discussed above, it can be concluded that the effects of Ca^{2+} on fusion of PS and PG vesicles must be due to specific binding to the head-group charges (Papahadjopoulos, 1968; Papahadjopoulos et al., 1977) rather than to simple neutralization and screening of the negative charges relating to double layer electrostatics (McLaughlin et al., 1971), which would not be expected to exhibit specificity between bivalent metal ions.

The following general conclusions have been drawn concerning the characteristics of Ca^{2+} -induced fusion of phospholipid vesicles (Papahadjopoulos et al., 1976b). First, the vesicles should carry a net negative surface charge, and the bilayers must be fluid (above the T_c) at the experimental temperature. Second, there is a definite "threshold" concentration at which

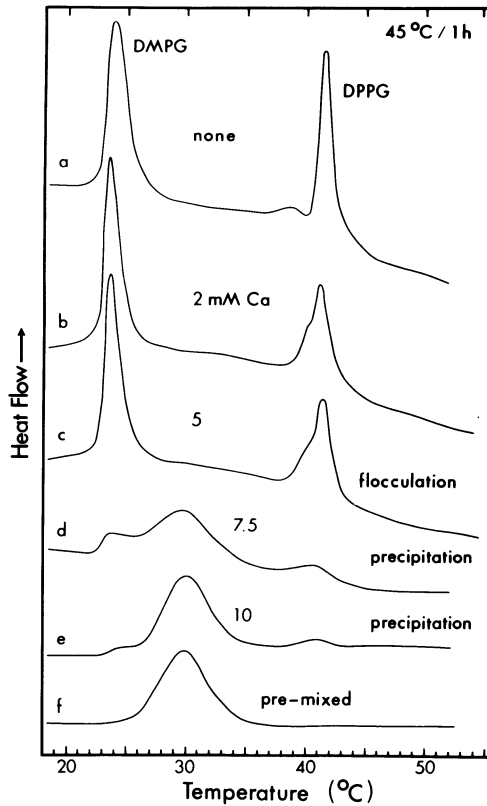


Fig. 15. Effects of different Ca^{2+} concentrations on the mixing of preformed vesicles of dimyristoylphosphatidylglycerol (DMPG) and dipalmitoylphosphatidylglycerol (DPPG). Conditions as described in caption of Fig. 1 except that the samples were incubated at 45°C for 1 hr under the following conditions: (a) no additions; (b) 2 mM CaCl_2 , 1 hr, then 3 mM EDTA, 0.5 hr, 45°C ; (c) 5 mM CaCl_2 , 1 hr, then 6 mM EDTA, 0.5 hr, 45°C ; (d) 7.5 mM CaCl_2 , 1 hr, then 8.5 mM EDTA, 0.5 hr, 45°C ; (e) 10 mM CaCl_2 , 1 hr, then 12 mM EDTA, 0.5 hr, 45°C ; (f) equimolar mixture of the two lipids suspended in 100 mM NaCl buffer. From *Biochim. Biophys. Acta* 448:265–283 (1976).

Ca^{2+} becomes effective. This threshold varies with different phospholipids suspended in 0.1 M NaCl, pH 7.4, from 0.2 mM for PA to 0.1 mM for PS to 10 mM for PG. All the above vesicles become leaky to their contents at approximately the same threshold concentrations of Ca^{2+} . Third, although fusion is accompanied by aggregation, it is clear that aggregation as such is not enough to induce fusion. Fourth, Ca^{2+} -induced fusion is accompanied by a drastic change in the thermotropic properties of the vesicle membranes, resulting in the crystallization of the acyl chains and a shift of the phase transition to a much higher temperature. It has been concluded recently that

such phase changes are a key event for the mechanism of Ca^{2+} -induced fusion (Papahadjopoulos et al., 1977).

7.7. Role of Phase Transitions and Phase Separations

The correlation between Ca^{2+} -induced phase changes and membrane fusion is illustrated in Fig. 16. Here the addition of 1 mM Ca^{2+} to PG vesicles produces only a shift of the two peaks to slightly higher temperatures, as would be expected from considerations of simple double layer electrostatics (Trauble and Eibl, 1974). Addition of EDTA reverses the process, giving back the original components without any mixing or fusion (curve c). When, however, the Ca^{2+} concentration is raised to 10 mM both transitions disappear completely (curve d) and as shown earlier they appear at much higher temperatures (Verkleij et al., 1974). The bilayers have therefore undergone an isothermal phase transition under these conditions. Addition of excess EDTA to the mixture (curve e) indicates that the two populations of PG vesicles are now completely mixed, presumably because of fusion.

The same phenomenon of an isothermic phase transition occurs during the interaction of PS with Ca^{2+} , in which case crystallization of the acyl chains is obtained at 1 mM Ca^{2+} (Jacobson and Papahadjopoulos, 1975). A recent study on the mechanism of calcium-induced fusion of phospholipid (PS) vesicles (Papahadjopoulos et al., 1977) indicates that the phase change from a fluid to a solid state is a key event during fusion induced by cations. For example, it was shown that fusion by Ca^{2+} is inhibited at 0° C, a temperature at which the bilayers would be crystallized before Ca^{2+} is added. In this case the vesicles were observed to aggregate only, without fusion. Addition of Mg^{2+} up to 10 mM at 37° C induced immediate aggregation of vesicles with only a minimal degree of fusion. In this case the T_c was increased from 7° C to 18° C, but the vesicles remained fluid at the experimental temperature, 37° C. Furthermore, it was shown that decreasing the temperature from 37° C to 12° C greatly enhanced the degree of fusion induced by 10 mM Mg^{2+} . In this latter case the PS vesicles are fluid initially ($T_c = 7^\circ \text{C}$ in 0.1 M NaCl, pH 7.4) but become solid following addition of Mg^{2+} ($T_c = 18^\circ \text{C}$ in 0.1 M NaCl/5 mM MgCl_2 , pH 7.4). It was therefore concluded that the difference between Ca^{2+} and Mg^{2+} in their effectiveness to induce fusion of PS vesicles at 22–37° C is due to the fact that Ca^{2+} induces a phase transition (crystallization) while Mg^{2+} does not.*

It is relevant to ask at this point whether Ca^{2+} can induce similar phase transitions in mixed phospholipid membranes, and whether such transitions could account for the fusion of PS/PC vesicles mentioned earlier. This question has been studied independently in two laboratories with different techniques, one involving electron spin resonance probes (Ohnishi and Ito,

*See Notes Added in Proof, note 2, p. 99.

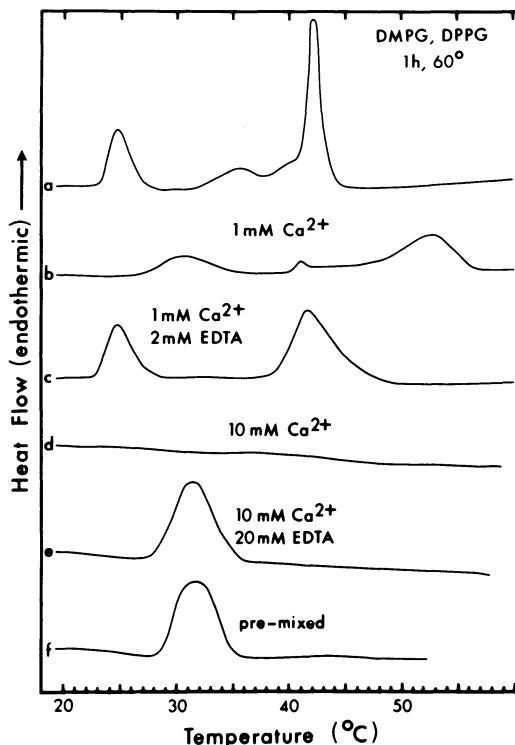


Fig. 16. Effects of Ca^{2+} on the thermotropic phase transitions and mixing of preformed vesicles of dimyristoylphosphatidylglycerol (DMPG) and dipalmitoylphosphatidylglycerol (DPPG). Here and also in Fig. 15, the phospholipids were suspended separately by shaking in 100 mM NaCl buffer, pH 7.4, at 45°C at a concentration of $5\text{ }\mu\text{mol/ml}$ and then equilibrated for 30 min at 45°C . Aliquots from each suspension containing approximately $2\text{ }\mu\text{mol}$ of each lipid were mixed, diluted to a total concentration of $1\text{ }\mu\text{mol/ml}$ with 100 mM NaCl buffer, pH 7.4, and incubated (for this experiment) at 60°C for 1 hr under the following conditions: (a) no other additions; (b) 1 mM CaCl_2 ; (c) 1 mM CaCl_2 for 1 hr, then 2 mM EDTA and additional incubation for 0.5 hr at 60°C ; (d) 10 mM CaCl_2 ; (e) 10 mM CaCl_2 for 1 hr, followed by 20 mM EDTA for 0.5 hr; (f) equimolar quantities of dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylglycerol, mixed in chloroform and suspended in 100 mM NaCl buffer at 45°C as above. After the end of each of the above incubations the suspensions were centrifuged at $100,000g$ for 15 min at 20°C . The wet pellets were then transferred to the sample pans of the differential scanning calorimeter and analyzed within 1–2 hr. From *Biochim. Biophys. Acta* 448:265–283 (1976).

1973, 1974; Ito and Ohnishi, 1974) and the other involving DSC and X-ray diffraction (Papahadjopoulos et al., 1974; Jacobson and Papahadjopoulos, 1975). The evidence indicates that Ca^{2+} can induce a lateral phase separation, resulting in the formation of separate domains of PS-Ca and PC. Figure 17 shows data obtained by DSC which indicate the appearance of a pure PC component from vesicles composed of a PS/PC mixture, following the

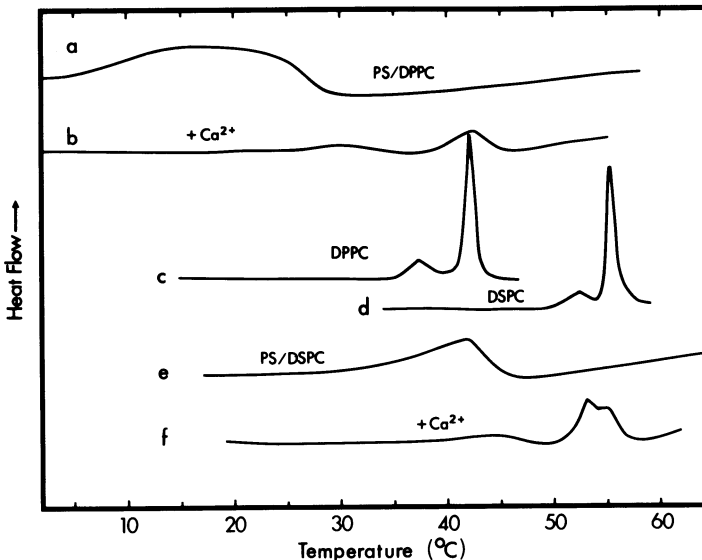


Fig. 17. Differential scanning calorimetry thermogram of mixed lipid membranes before and after addition of Ca^{2+} . Phospholipid dispersions were made in 100 mM NaCl buffer, pH 7.4, at concentrations of 4 $\mu\text{mol/ml}$. (a) PS/DPPC (2/1) mixture, dispersed without sonication for 30 min at 37°C; (b) PS/DPPC (2/1) mixture, dispersed without sonication for 30 min at 37°C, then incubated for 30 minutes with Ca^{2+} (10 mM) at 37°C; (c) pure DPPC nonsonicated dispersions dispersed at 42°C; (d) pure DSPC, nonsonicated, dispersed at 58°C; (e) PS/DSPC (2/1) mixture dispersed without sonication at 42°C; (f) PS/DSPC (2/1) mixture, dispersed by sonication for 1 hr at 42°C, then incubated for 1 hr with Ca^{2+} (10 mM) at 42°C. If EDTA (equimolar to Ca^{2+}) is added after the above Ca^{2+} treatment, the calorimeter scan of the sample is identical to that shown in (e). All the above samples were centrifuged at room temperature for 10 min at 100,000g, and the pellets were transferred into the calorimeter sample pans with a Pasteur pipette. Amount of phospholipid per sample was approximately 1 μmol . From *Biochemistry* 14:152-161 (1975).

addition of Ca^{2+} to the sonicated vesicles. Similar phase separation has been induced by Ca^{2+} with PA/PC mixtures (Galla and Sackmann, 1975) but not with PG/PC mixtures (Verkleij et al., 1974; Van Dijk et al., 1975).

Freeze-fracture electron microscopy has been used recently to visualize the separate domains of PS and PC in the presence of Ca^{2+} . Figure 18 was obtained from multilamellar vesicles composed of a mixture of 2/1 PS/DPPC following addition of Ca^{2+} (10 mM) and incubation (and quenching) at 45°C. The fracture surfaces show extensive "patched" appearance, while the same vesicles before addition of Ca^{2+} appeared generally "smooth," as is usual for fluid bilayers. Measurements of the relative area between "raised" and nonraised regions in the micrograph showing patches gave a ratio of approximately 2/1, which coincides with the molar ratio of the two components, PS and PC. It is concluded, therefore, that the patched structure shown in Fig. 18 is due to the formation of PS and PC domains, although



Fig. 18. Freeze-fracture electron microscopy of phospholipid vesicles composed of phosphatidylserine and phosphatidylcholine at a molar ratio of 2/1. The vesicles were suspended initially in 100 mM NaCl buffer, pH 7.4, as in Fig. 17, and then incubated for an additional 1 hr after addition of CaCl_2 to 20 mM final concentration. The temperature was kept at 45°C during incubation with Ca^{2+} and quenching. Scale bar: $0.1\ \mu\text{m}$ ($1000\ \text{\AA}$). Other details as in Fig. 14.

the details relating to the localization of the fracture planes within the different domains is not clear at present (Vail and Papahadjopoulos, 1977).

It has been pointed out (Papahadjopoulos et al., 1974, 1977) that the occurrence of a phase separation in PS/PC vesicles correlates with fusion of the same vesicles. Thus both phenomena are observed only above a threshold concentration of Ca^{2+} whose magnitude is dependent on the initial molar ratio of PS/PC; neither is observed with ratios of PS/PC less than 1/1, and neither can be induced by Mg^{2+} even at high concentrations. The same conditions as above were found more recently to be effective in inducing fusion of PS/PC vesicles by following the mixing of two membrane-embedded proteins initially reconstituted in separate vesicles (Miller and Racker, 1976a). This study also established that calcium was more effective in inducing fusion between PS/PE vesicles compared to PS/PC vesicles. It is not known, however, whether this effect is related to a higher effectiveness in inducing phase separations in a PS/PE membrane system.

It is evident from the experiments discussed above that there is a strong correlation between aggregation of vesicles, an increase in their permeability, and the onset of phase changes and membrane fusion. All these events are initiated in a highly cooperative fashion by increasing Ca^{2+} (and only in certain cases of Mg^{2+}) concentrations. The Ca^{2+} concentrations needed for these effects vary widely with different phospholipids or mixtures, generally requiring a high surface charge density (negative). Charge neutralization, which generally leads to aggregation of vesicles, is not sufficient in itself to induce fusion. The additional key event required for fusion of lipid bilayer membranes seems to be the phase transition from fluid to crystalline (condensed acyl chain packing) which is induced by Ca^{2+} isothermally at concentrations above a threshold. It has recently been proposed (Papahadjopoulos et al., 1977) that the addition of Ca^{2+} induces a transient unstable state during which the lipid bilayer is highly susceptible to fusion but becomes stable again after Ca^{2+} equilibration or removal.

The molecular events responsible for creating the abovementioned unstable state were discussed in detail recently (Papahadjopoulos et al., 1977) and could involve the following: (1) Ca^{2+} asymmetry across the phospholipid bilayer membrane, (2) the structural discontinuities (phase boundaries) between two different phospholipid domains (solid and fluid) on the plane of the membrane, and (3) the transient local release of heat liberated by the exothermic crystallization of the phospholipid acyl chain. Figure 19 represents diagrammatically the concepts and proposals outlined above. In this diagram, A represents a phospholipid vesicle in its resting (stable) fluid state at temperatures above the T_c of the bilayer. The vesicle is composed either of pure acidic phospholipid (PS, PA, PG) or a mixture of an acidic and a neutral phospholipid (PS, PC). Addition of Ca^{2+} in the external aqueous space can produce a phase transition (B) or a phase separation (B'), thus inducing a complete (B) or a partial (B') crystallization of the outer mono-

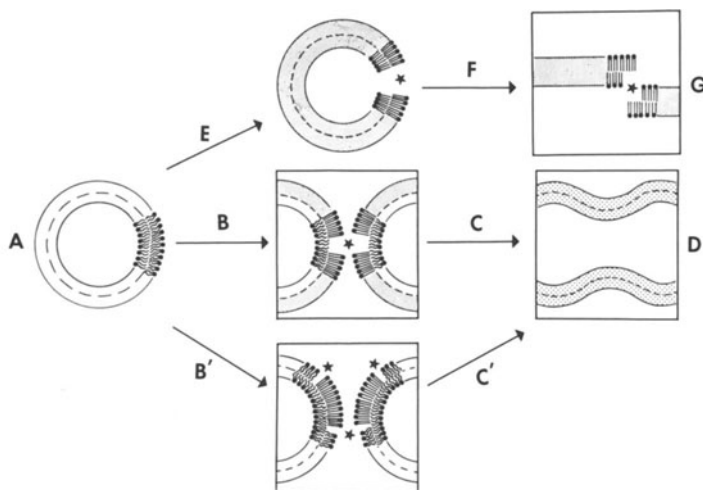


Fig. 19. Proposed events in fusion between phospholipid vesicles resulting from Ca^{2+} -induced phase changes. The stippled areas of the vesicle membranes indicate crystallized domains, the broken lines indicate the position of the bilayer midplanes, and the asterisks indicate structural defects, domain boundaries, or regions of transient hydrocarbon-water contact. As indicated in the text, these transient energy states may be generated by the mismatch of monolayer regions which have undergone a Ca^{2+} -induced phase transition (separation) with the remaining fluid regions of the vesicle. Phospholipid molecules in fluid domains are represented with "wavy" acyl chains and in solid domains with straight-line acyl chains. From *Biochim. Biophys. Acta* 465:579–598 (1977).

layer of the vesicle membrane. This molecular condensation may well create structural defects with resulting exposure of the hydrocarbon chains to water. The instability of such structures would make them susceptible to fusion, which would occur at such points of hydrocarbon-water contact (C and C') with resultant fused vesicles (D). An additional role for Ca^{2+} , implicit in the above scheme, is its ability to aggregate acidic phospholipid vesicles and thus facilitate close contact between adjacent membranes. Evidence for an anhydrous "intermembrane" Ca -PS complex was reported recently.*

8. SIMILARITIES BETWEEN DIFFERENT FORMS OF MEMBRANE FUSION: A POSSIBLE UNIFYING MECHANISM

Despite the bewildering diversity of the many examples of membrane fusion phenomena discussed in this review, certain similarities compel us to consider the possibility that there is an underlying key mechanism common to most if not all forms of membrane fusion, although the stimulus (or stimuli) responsible for triggering this key reaction will, of course, vary in different examples of membrane fusion.

*See Notes Added in Proof, note 3, p. 99.

Earlier proposals for the mechanism of membrane fusion such as the induction of membrane instability via removal of calcium from membranes (Poste and Allison, 1973) or via an increase in the fluidity of membrane lipids (Ahkong et al., 1975a; Lucy, 1975) do not provide enough detailed information at the molecular level and/or are not consistent with the data now available. The most detailed information available so far concerning molecular mechanism of membrane fusion has come from recent work on the fusion of model membranes in cell-free systems. This work, reviewed in Section 7, has permitted the relative contribution of membrane lipids, proteins, and metal ions in influencing the fusion reaction to be defined in greater detail than has hitherto been possible with natural membranes. The key event in the fusion of model membranes is presently interpreted (Papahadjopoulos et al., 1977) as involving the interaction of Ca^{2+} with membrane lipids to create rigid, crystalline domains of phospholipids following Ca^{2+} -induced lateral phase separations of acidic phospholipids within a mixed lipid membrane. The formation of such domains is proposed to induce an unstable, highly permeable membrane which is susceptible to fusion with adjacent similarly perturbed membranes.* Although the structural organization of model membranes is acknowledged as considerably less complex than that of natural membranes, it is instructive to examine whether similar Ca^{2+} -induced phase separation of acidic phospholipids into separate rigid domains might provide the mechanism underlying fusion occurring in natural membranes.

In summarizing the work discussed in Section 7 on the fusion of phospholipid vesicles, the following general points can be made regarding the factors that influence fusion (Papahadjopoulos et al., 1976a,b, 1977).

1. In the absence of Ca^{2+} , purified phospholipid membranes in the form of closed vesicles are highly stable at temperatures both above and below the gel-to-liquid crystalline transition temperature of the lipids in the vesicle membrane. Such vesicles neither fuse nor undergo changes in size and composition via the exchange of phospholipid molecules between adjacent vesicles.

2. Modification of the structural organization of the lipid bilayer of such vesicles by the introduction of exogenous amphipathic molecules such as lysolecithin or myristic acid (at temperatures when the vesicles are fluid, $T > T_c$) can increase the rate of phospholipid exchange between vesicles, but in the absence of Ca^{2+} fusion does not occur within a significant time scale.

3. Aggregation and fusion of vesicles prepared from pure acidic phospholipids (PS, PA, PG) can be induced simply by the addition of Ca^{2+} at temperatures where the vesicle lipids are "fluid." The concentration of Ca^{2+} required to induce fusion ranges from 10^{-2} to 10^{-4} , depending on the phospholipid head group involved.† However, in contrast to the high susceptibility

*See Notes Added in Proof, note 3, p. 99.

†See Notes Added in Proof, note 4, p. 100.

of vesicles containing acidic phospholipids to Ca^{2+} -induced fusion, vesicles composed of neutral phospholipids do not fuse under these conditions.

4. The fusion of vesicles containing acidic phospholipids (PS, PG) is highly specific for Ca^{2+} . Mg^{2+} causes rapid vesicle aggregation, but fusion does not occur except under special conditions.

5. The presence of proteins and other amphipathic molecules can increase phospholipid exchange between vesicles and also perhaps induce fusion, particularly at a temperature where the lipid bilayers are undergoing a phase transition.

Several general conclusions can be drawn from these observations: It is obvious that the close apposition of two membranes is an essential prerequisite for fusion. However, the finding that vesicles can be aggregated without concomitant fusion indicates that the close apposition of two lipid bilayers does not automatically lead to fusion even when the lipids in both membranes are in a fluid state. The phospholipid bilayer is thus viewed as having a very high structural stability. Consequently, fusion of apposed membranes *requires an additional step* in which the stability of the membranes is altered to render them susceptible to fusion. We consider that this additional step, *and the crucial event in fusion*, is a Ca^{2+} -induced lateral phase separation which results in the formation of rigid crystalline domains of acidic phospholipids within a mixed lipid membrane. It is proposed that membrane fusion is initiated between closely apposed membranes at the boundaries between crystalline domains of acidic phospholipids and the surrounding noncrystalline lipid. Such boundaries represent structurally unstable points of high free energy and thus offer focal points for mixing of molecules from the apposed membranes (Papahadjopoulos et al., 1977).

Ca^{2+} is therefore envisaged as playing a dual role in membrane fusion. First, Ca^{2+} will promote the close apposition of adjacent membranes by enhancing electrostatic interactions between them.* Second, Ca^{2+} will induce destabilization of the apposed membranes by inducing the formation of domains of crystalline acidic phospholipids whose boundaries represent sites at which fusion can occur. If Ca^{2+} -induced phase separation of acidic phospholipids is indeed the key event underlying membrane fusion then the fusion susceptibility of membranes will be determined by (1) the content of acidic phospholipids in the membrane and also their distribution within the bilayer (see below) and (2) the concentration of Ca^{2+} in the vicinity of the membrane.

The distribution of acidic phospholipids in the two halves (monolayers) of the lipid bilayer would be expected to influence whether a cellular membrane would be able to fuse with other membranes from only one side or from both sides. For example, in the most extreme situation, insufficient acidic phospholipids might be present in either the inner or the outer half of

*See Notes Added in Proof, note 5, p. 100.

the bilayer so that Ca^{2+} -induced phase separation of acidic phospholipids could not occur and the membrane would remain resistant to fusion.

Since acidic phospholipids vary in their ability to undergo Ca^{2+} -induced phase separation, the fusion susceptibility of any given membrane may be regulated not only by the absolute content of acidic phospholipids but also by the specific distribution within the bilayer of those acidic phospholipid species that undergo phase separation in the presence of Ca^{2+} . For example, PS and PA undergo lateral phase separation to form rigid crystalline domains in mixed lipid membranes at Ca^{2+} concentrations similar to those found under physiological conditions. In contrast, PG (and perhaps PI) does not undergo lateral phase separation at Ca^{2+} concentrations in the physiological range. Thus the content of PS (and also perhaps PA) would be a major factor in determining the fusion susceptibility of a particular membrane, while the preferential distribution of PS in either the outer or the inner half of the bilayer would further dictate that different sides of the same membrane could vary significantly in their fusion susceptibility.

This point is particularly pertinent to the fusion behavior of the plasma membrane. Studies of plasma membrane lipid composition in mammalian erythrocytes have revealed that PS is present almost exclusively in the inner (cytoplasmic) half of the bilayer while the outer (external) lipid monolayer is composed predominantly of neutral phospholipids (PC and sphingomyelin) (review, Rothman and Lenard, 1977). While it is not known whether similar lipid asymmetry is present in the plasma membrane of other cells, this type of asymmetry in the distribution of acidic phospholipids, if widespread, has major implications for the fusion behavior of the plasma membrane. The hypothesis outlined above predicts that the relative absence of PS in the outer lipid monolayer of the plasma membrane would dictate that Ca^{2+} in the external medium could not induce lateral phase separation of acidic phospholipids into fusion-susceptible domains. The membrane would thus be resistant to fusion at the outer membrane face as in cell-to-cell fusion (see Section 3).

In contrast, the inner monolayer of the plasma membrane with its higher content of PS would be expected to form such domains when exposed to the necessary concentration of Ca^{2+} and thus be highly susceptible to fusion. Under normal conditions, however, the concentration of Ca^{2+} in the cytoplasm ($<10^{-5}$ M) is at least 2 orders of magnitude (and often more) lower than that in the external medium (10^{-3} M). This lower intracellular calcium concentration would thus be insufficient to induce the lipid phase separation required for fusion. Although Mg^{2+} is present intracellularly at higher concentrations (10^{-3} M), Mg^{2+} cannot substitute for Ca^{2+} in inducing phase separation and the membrane thus remains resistant to fusion.

The fusion susceptibility of the two faces of the plasma membrane would change, however, if the distribution of acidic phospholipids within the bilayer were altered and/or the concentration of Ca^{2+} in the cytoplasm were in-

creased. For example, if the content of acidic phospholipids in the outer lipid monolayer of the plasma membrane were to increase as a result of structural rearrangements within the bilayer, then Ca^{2+} in the external medium could induce the formation of crystalline acidic phospholipid domains and the membrane would become susceptible to fusion with other similarly perturbed membranes. This proposal that fusion initiated at the outer face of the plasma membrane might require enrichment of the outer lipid monolayer with acidic phospholipids will be examined in more detail later when discussing the possible mechanism underlying cell-to-cell fusion induced by lysolecithin and other amphipathic molecules and also in the fusion of lipid-enveloped viruses and lipid vesicles with the plasma membrane.

The major assumption made above is that the degree of transbilayer asymmetry in the distribution of acidic phospholipids is so extreme that the outer face of the plasma membrane is inherently resistant to fusion and that fusion can occur only when the content of acidic phospholipids in the outer monolayer is increased via some form of redistribution of acidic phospholipids from the inner to the outer monolayer or via the transfer of acidic phospholipids from adjacent membranes by exchange processes.

An alternative possibility, however, is that although lipid asymmetry may exist, the difference in the distribution of acidic phospholipids between the outer and inner monolayers is not so extreme as that suggested above and acidic phospholipids are in fact present in the outer monolayer in sufficient amounts to undergo Ca^{2+} -induced lateral phase separation. For example, acidic phospholipids are now known to be present as an annulus or shell around certain integral membrane proteins (Warren et al., 1975a,b; Armitage et al., 1977; Boggs et al., 1977), and it seems likely that at least some of these molecules would be present in the outer monolayer. Consequently, it may well be that sufficient acidic phospholipids are already present in the outer monolayer of the plasma membrane to permit their separation into domains by Ca^{2+} without the need for enrichment of the acidic phospholipid content in the outer monolayer.

Since the Ca^{2+} concentration of the external medium is sufficient to induce lateral phase separation of acidic phospholipids, the plasma membrane must be in a "fusion-susceptible" state. At first sight this is difficult to reconcile with the apparent lack of spontaneous fusion processes involving the plasma membrane. However, the fusion resistance of the plasma membrane could be more apparent than real and the low frequency of spontaneous fusion be determined by some additional restraint. For example, simple steric factors could be operating to frustrate the establishment of the required initial close contact between the lipid bilayers of the plasma membrane and other membranes. In cell-to-cell fusion this type of steric hindrance could perhaps be mediated by the various glycoproteins and glycosaminoglycans present in the "cell coat" which would provide a "barrier" to a surface projection by one cell readily achieving direct apposition of its bilayer with that on another cell.

In this respect, it is of interest that enzymic treatment of cells to remove surface glycoproteins and/or glycoaminoglycans renders cells with a fusion-resistant phenotype susceptible to fusion by viruses (review, Poste, 1972). Similarly, enzymic removal of glycoprotein "coating factors" from the surface of uncapacitated sperm enables them to fuse prematurely with or without undergoing capacitation. Also, the observation (Diacumakos, 1973) that cultured mammalian somatic cells will fuse spontaneously if the cells are merely "brought together" by micromanipulation (in Ca^{2+} -containing medium) suggests that the plasma membrane may indeed be in a fusion susceptible state.

The possibility that the outer monolayer of the plasma membrane may contain preexisting Ca^{2+} -segregated domains of acidic phospholipids and thus be in an inherently fusion-susceptible state will be examined again later (Section 8.1) when considering the possibility that lipid-enveloped viruses or lipid vesicles that fuse with the plasma membrane may interact specifically with such domains.

In the case of intracellular fusion events involving membrane-bound organelles, either with each other or with the inner face of the plasma membrane (e.g., exocytosis), we consider that changes in intracellular Ca^{2+} concentration would be sufficient to trigger fusion *without* the need for any redistribution of acidic phospholipids within the bilayer. The inner face of the plasma membrane and the outer face of the membranes surrounding the various organelles that undergo fusion could both be characterized by a high content of acidic phospholipids such as PS (see Bretscher, 1972; Zwaal et al., 1973; Gordesky and Marinetti, 1973; Marinetti and Love, 1974; Nilsson and Dallner, 1977; Rothman and Lenard, 1977) and when exposed to the initial concentration Ca^{2+} would be expected to undergo *immediate* lateral phase separation to create fusion-susceptible crystalline domains of acidic phospholipids. The reason such membranes are not continually undergoing fusion is that the intracellular Ca^{2+} concentration is too low for phase separation and domain formation to take place. However, under conditions where the Ca^{2+} concentration in the vicinity of such membranes is raised to a level sufficient to trigger phase separation, they will then immediately become fusion susceptible. This scheme is compatible with the very rapid fusion of secretory granules with each other and with the plasma membrane induced in secretory cells by treatment with the Ca^{2+} -ionophore A23187, which increases intracellular Ca^{2+} levels. Similarly, fusion of isolated secretory granules in cell-free systems occurs following addition to Ca^{2+} to the solution surrounding the granules (see Section 5).

We therefore propose that the crucial event responsible for triggering most, and perhaps all, membrane fusion phenomena is Ca^{2+} -induced separation of acidic phospholipids such as PS into rigid crystalline domains, with fusion occurring at domain boundaries between adjacent membranes. Under normal conditions most membranes do not fuse. This is clearly necessary to

prevent uncontrolled spontaneous fusion of different cells within tissues and also to ensure that the various membrane-bound organelles retain their structural identity, thus enabling different functional activities to be localized within specific compartments of the cell. We consider that membrane fusion does not occur under normal conditions either because of (1) steric hindrance which frustrates close apposition between two “fusion-susceptible” membranes containing Ca^{2+} or (2) preexisting asymmetry in the distribution of acidic phospholipids within the membrane or (3) an insufficient Ca^{2+} concentration in the vicinity of the membrane. The latter two factors constitute a “forbidding asymmetry” which ensures that acidic phospholipids cannot be segregated into rigid domains to render membranes unstable and susceptible to fusion. Fusion can be initiated, however, by (1) removal of steric restraints to the close apposition by two “fusion-susceptible” membranes (i.e., with preexisting phase-separated domains), (2) changes in the distribution of acidic phospholipids within the bilayer which permit the existing (extracellular) concentration of Ca^{2+} in the vicinity of the membrane to induce separation of acidic phospholipids (this is proposed as the mechanism operating in certain fusion events initiated at the outer face of the plasma membrane such as cell-to-cell fusion; see below), and (3) changes in the Ca^{2+} concentration (intracellular) in the vicinity of membranes that contain sufficient preexisting amounts of acidic phospholipids to undergo immediate phase separation to create the fusion-susceptible acidic phospholipid domains (this is proposed as the mechanism underlying fusion of intracellular organelles with each other and/or with the inner face of the plasma membrane following influx of Ca^{2+} into the cell; see below).

This proposal for the induction of membrane fusion via Ca^{2+} -induced phase separation of acidic phospholipids offers a single mechanism which can explain both extracellular fusion (cell-to-cell, enveloped virus-to-cell, lipid vesicle-to-cell) as well as intracellular fusions (endocytosis, exocytosis).

In the remainder of this chapter we will attempt to define how our proposal might apply to specific examples of membrane fusion. Our treatment is, admittedly, extrapolative and hypothetical, but hopefully may stimulate further experimentation.

8.1. Fusion Initiated at the Outer Face of Plasma Membranes

8.1.1. Virus-Induced Cell Fusion from Without (FFWO)

As discussed in detail in Section 3.5, an important functional separation can be made in the case of FFWO induced by paramyxoviruses between initial fusion of the viral envelope with the cellular plasma membrane and the subsequent step of cell-to-cell fusion. This can be demonstrated experimentally using cytochalasin B, which permits initial fusion of the virus to take place but blocks subsequent cell-to-cell fusion. This suggests that the

latter step requires participation of the membrane-associated microfilament system. As proposed above, we envisage that microfilaments are responsible for producing redistribution of integral membrane proteins over a sufficiently large area of the plasma membrane so that the subsequent step of cell-to-cell fusion occurs through the interaction between lipid bilayers of adjacent cells at regions depleted of membrane proteins. The central issue is therefore the mechanism by which the virus fuses with the plasma membrane, which can be considered separately from the problem of how the virus-modified plasma membrane subsequently fuses with the plasma membrane of another cell. Several recent publications have described biochemical and morphological changes during virus fusion.*

We consider that lipid-enveloped viruses could be in a preexisting fusion-susceptible state. This conclusion comes from recent evidence indicating no pronounced asymmetry in the distribution of acidic phospholipids between the inner and outer monolayers of the virus envelope (Rothman and Lenard, 1977). This situation would allow for the presence of sufficient acidic phospholipids in the outer monolayer to permit charge-charge interactions and therefore to achieve close contact with the bilayer of the cellular plasma membrane in the presence of Ca^{2+} . The question still remains, however, as to how the virus can render the closely apposed cell plasma membrane susceptible to fusion.

Recent data obtained with electron spin resonance probes (Maeda et al., 1977a) may shed some light on this crucial question. It was observed that during incubation of HVJ (Sendai) virions with erythrocytes considerable exchange diffusion of phospholipid molecules between the virus envelope and the cellular plasma membrane occurred, without accompanying fusion of the two membranes. This was shown clearly by the fact that lipid molecules but not proteins were transferred between the two membranes (Maeda et al., 1977a). It is therefore possible that during the close contact of the viral envelope with the cellular plasma membrane, the outer monolayer of the plasma membrane is enriched with acidic phospholipids which were originally present in the external monolayer of the virus. Soluble proteins that enhance the exchange of phospholipid molecules have been isolated from various sources, and although some of them are specific for certain head-group configuration, others are nonspecific (Wirtz et al., 1976; Bloj and Zilversmit, 1977). The attachment of virus to receptors on the surface of the cell could also produce other alterations such as aggregation of receptor sites, which, in turn, could produce redistribution of other membrane components and a general decrease in membrane stability. This might result in an increase (transbilayer) flip-flop of phospholipids and further enrichment of the outer monolayer with acidic phospholipids. It is therefore possible to envisage that the appearance of acidic phospholipid molecules either by (1)

*See Notes Added in Proof, note 6, p. 100.

enhanced exchange diffusion or (2) enhanced inside-outside flip-flop rate, or both. The presence of Ca^{2+} in the medium at concentrations in the range of 1–4 mM would induce phase separation in both the virus envelope and the cellular plasma membrane and thus initiate fusion.

Alternatively, as mentioned earlier, the plasma membrane may contain preexisting small domains of acidic phospholipids, and this raises the possibility that virus particles that fuse with the plasma membrane could interact specifically with these domains. Thus in this situation both the virus envelope and the cellular plasma membrane are considered as possessing preexisting domains of acidic phospholipids so that fusion between them would take place *automatically*, provided, of course, that sufficiently close contact between the bilayers of the two membranes could be achieved with accompanying apposition of domain boundaries. Virus particles, by virtue of their small size, would be expected to encounter minimum repulsive energy in achieving this type of close contact with the plasma membrane (review, Poste, 1972).

It is known, however, that the ability of paramyxoviruses to fuse with the plasma membrane is influenced by the nature of the glycoproteins present in the virus envelope (Section 3.5). Virions containing the F glycoprotein are able to fuse, but virus particles containing the F_0 glycoprotein cannot fuse. It has hitherto been assumed that the F protein is *directly responsible for inducing fusion* [i.e., the F glycoprotein is somehow able to induce the membrane perturbation(s) required to destabilize the membrane and permit fusion to occur]. It is equally possible, however, that the role of the F and F_0 proteins in determining whether fusion takes place does not involve a direct effect on fusion per se but on the preceding step of virus attachment which determines the opportunities for achieving close contact between the bilayers of the virus envelope and the plasma membrane required for fusion.

The possible role of virus envelope glycoproteins in virus attachment is indicated by the finding that paramyxoviruses with the F_0 glycoprotein bind only to neuraminidase-sensitive moieties that are usually associated with cell surface glycoproteins, while infective F-containing viruses can bind to both the above receptors and to an unidentified class of receptors found on neuraminidase-treated cells (Poste and Waterson, 1975). The latter receptors could be neuraminidase-resistant sialic acid moieties associated with glycolipids, since infective F-containing paramyxoviruses have been shown to bind ganglioside-containing lipid vesicles (Haywood, 1974b). The ability to attach to glycolipid receptors would have the advantage that it would bring the virus envelope into much closer contact with the lipid bilayer than if attachment were to receptors on glycoproteins.

This concept that envelope glycoproteins influence virus attachment rather than fusion is a departure from the generally held belief (albeit without experimental proof!) that the F protein is directly responsible for fusion of the

virus envelope with the plasma membrane. Thus in our present state of knowledge it might be more appropriate to merely say that presence of the F protein is "correlated with" the ability of the virus to fuse with cells.

Irrespective of whether fusion of enveloped viruses with the plasma membrane requires an initial step of enrichment of acidic phospholipids in the outer monolayer of the plasma membrane or proceeds automatically because both the virus envelope and the plasma membrane contain pre-existing domains of acidic phospholipids, the fusion of numerous virus particles with the plasma membrane might be expected to increase the overall acidic phospholipid content of the outer monolayer of the plasma membrane, thus creating more acidic phospholipid domains, which, in turn, would be expected to increase membrane permeability because of the enhanced permeability of the domain boundaries. Such permeability changes have indeed been detected. As discussed in detail above, the resulting increased rate of Ca^{2+} entry into the cytoplasm under these conditions would be expected to produce a number of changes, including (1) breakdown of the microtubular "anchoring" proteins which would facilitate the redistribution of integral membrane proteins by free diffusion and/or mechanically assisted processes involving the microfilament system, and (2) activation of the actomyosin microfilament system, which would redistribute the now "unanchored" membrane proteins. These two events would be expected to produce aggregation of integral proteins and thus create large areas in the bilayer free of integral membrane proteins. Such protein-depleted regions would provide the sites for subsequent fusion with adjacent plasma membrane, which could proceed via (1) apposition of similar protein-depleted bilayers at sites of simultaneous ionic penetration containing acidic phospholipid domain boundaries or (2) modification of the plasma membrane of an adjacent uninfected cell by viral glycoproteins integrated within the plasma membrane via the same mechanism responsible for modifying the fusion susceptibility of the plasma membrane during initial fusion of virus particles with the plasma membrane. Virus-induced fusion from within (Section 3.1) could be viewed as analogous to the events just described for fusion from without, except that newly synthesized viral F glycoproteins are inserted into the plasma membrane after transport from the smooth endoplasmic reticulum rather than by penetration from the outside.

8.1.2. Fusion of Lipid Vesicles with the Plasma Membrane and Vesicle-Induced Cell Fusion

We consider that the membrane interactions in these two examples of membrane fusion are similar to those discussed in the preceding section for the fusion of lipid-enveloped viruses with the cellular plasma membrane and the accompanying process of cell fusion from without. Thus we consider that

while fusion of vesicles with the plasma membrane can occur without necessarily causing cell-to-cell fusion (as discussed below, this seems to be the case for positively charged vesicles), the process of cell-to-cell fusion must always be preceded by fusion of vesicles with the plasma membrane. By analogy with virus-induced FFWO, we consider that vesicle-induced cell-to-cell fusion requires an additional step involving reorganization of the cellular plasma membrane after initial fusion of vesicles with the plasma membrane has taken place. This additional step is considered identical to that operating in virus-induced FFWO and involves microfilament-induced redistribution of integral membrane proteins, with cell-to-cell fusion occurring via the interaction of lipid bilayers at membrane sites depleted of integral proteins.

Fusion of lipid vesicles with the plasma membrane seems to be restricted to vesicles bearing electrostatic charges on their surface, which can be either negative (Poste and Papahadjopoulos, 1976b) or positive (Martin and MacDonald, 1976c). Neutral vesicles are unable to fuse with the plasma membrane (Poste and Papahadjopoulos, 1976b) and also they do not induce cell-to-cell fusion (Papahadjopoulos et al., 1973a) even when they contain up to 20% lysolecithin (Martin and MacDonald, 1976c). This behavior could be related to the large repulsive forces that two neutral (lecithin) lipid bilayers have to overcome before they can come into close contact (Le Neveu et al., 1976). However, incorporation of as little as 5% stearylamine into neutral vesicles containing lysolecithin permits them to induce cell-to-cell fusion (Martin and MacDonald, 1976c). This indicates that stearylamine by conferring a surface charge enhances electrostatic interactions so that close apposition can be achieved between the vesicle and cellular membranes leading to fusion. When lysolecithin is present in the vesicle membrane, the newly introduced lysolecithin (now within the bilayer of the plasma membrane) initiates further perturbation in the membrane, rendering it susceptible to fusion with similarly perturbed membranes on an adjacent cell, leading to cell-to-cell fusion. This subsequent role of lysolecithin is indicated by experiments showing that positively charged vesicles alone do not induce cell-to-cell fusion (Martin and MacDonald, 1976c), although they can be shown to fuse with the plasma membrane.

The following sequence of events is proposed for the fusion of both negative and positive vesicles.

1. Close apposition of vesicles and cellular membranes. A surface charge seems to be required in order to achieve electrostatic interaction and close apposition between the vesicle and plasma membrane lipid bilayer. For negatively charged vesicles, Ca^{2+} could be important at that stage. Electrostatic charges in the plasma membrane could be provided by proteins (negative or positive) or by a small amount of acidic (PG or PI) phospholipids (negative). The prerequisite should be very close apposition (within a few angstroms) to the lipid bilayer of the plasma membrane.

2. Bilayer stability. The bilayer of both vesicles and plasma membrane must be in a fluid state. The importance of membrane fluidity has been established as a requirement for fusion for the cell membrane (Poste and Allison, 1973) and for vesicles fusing with vesicles (Papahadjopoulos et al., 1974) and cells (Poste and Papahadjopoulos, 1976b). It is also clear, however, that close apposition of the fluid bilayers is not enough for fusion since the structures are intrinsically stable.

3. Destabilization of bilayer structure. Several factors may be operating at this point depending on the type of vesicle. In general, we would expect that the induction of phase separations would be instrumental in producing the unstable domain boundaries needed for initiation of fusion. Proteins could be involved as nucleation sites for such events either by providing an annulus of acidic phospholipids in the immediate vicinity of the protein (Armitage et al., 1977; Boggs et al., 1977) or by the expected structural discontinuities between the annular lipids and the bulk bilayer. Evidence that fusion between vesicles can be enhanced by various proteins and peptides has been discussed earlier (Section 7). Furthermore, proteins could be acting at the plasma membrane surface as catalysts for exchange diffusion of lipid molecules between vesicles and cell membrane. Evidence for such an exchange has been reported recently (Pagano and Huang, 1975). Such exchange diffusion processes might be expected to enrich the outer monolayer of the plasma membrane with PS. Once sufficient PS had been introduced into the outer monolayer, Ca^{2+} in the medium would induce separation of the PS into crystalline domains and thus render the plasma membrane susceptible to fusion.

4. Fusion of destabilized membranes. Negatively charged vesicles would be fusing within a region of the membrane which carries acidic phospholipid domains created partly through the interaction with the vesicles (exchange diffusion) and the presence of Ca^{2+} . Calcium also provides the cohesive forces between the two matching domains.* Fusion between domains will be initiated at domain boundaries. Positively charged vesicles could also be interacting with negatively charged phospholipids (such as PI or PG) which may preexist in the external monolayer. However, such molecules (PG) do not undergo Ca^{2+} -induced phase separation from mixtures with PC (Verkleij et al., 1974; Van Dijck et al., 1975) and pure PG vesicles fuse only at very high (unphysiological) Ca^{2+} concentrations (Papahadjopoulos et al., 1976b) in the absence of proteins. It is possible, however, that as the positively charged stearylamine in the vesicle interacts with negatively charged PI or PG on the plasma membrane, the interaction propagates in a zipping type of mechanism which creates matching domains in the adjacent membranes. The domain boundaries would again be regions of instability where fusion can be initiated. In both of the above cases, proteins could be playing an important role in

*See Notes Added in Proof, note 3, p. 99.

nucleating small acidic phospholipid domains around them and thus enhancing the likelihood of the final fusion events.

5. Recovery and further fusion events. Fusion of negatively charged vesicles with the plasma membrane would create an enrichment of negative charges in the outer monolayer of the plasma membrane. In the event that these molecules can be induced to undergo phase separation by Ca^{2+} , the cell membranes will become much more permeable. As discussed earlier for the fusion of lipid-enveloped viruses with the plasma membrane, Ca^{2+} influx into the cytoplasm will lead to effects on membrane-associated cytoskeletal elements, producing topographic redistribution of integral membrane proteins. As in the case of virus-induced FFWO, such redistribution phenomena are probably required for cell-to-cell fusion and we consider that vesicle-induced cell-to-cell fusion involves a similar mechanism whereby cell fusion is initiated between the lipid bilayers in membranes depleted of integral proteins. In contrast, fusion of positively charged vesicles will not result in increase in acidic phospholipid in the outer monolayer and Ca^{2+} -induced phase separations will thus not occur. In this situation, membrane permeability is not altered sufficiently to permit entry of Ca^{2+} and the subsequent intracellular effects on the cytoskeleton. Topographic redistribution of integral proteins will not therefore take place, frustrating the subsequent fusion between cells. However, the inclusion of lysolecithin within positively charged vesicles permits them to induce cell-to-cell fusion (Martin and MacDonald, 1976c). In this case, initial fusion of the vesicles with the plasma membrane serves to introduce lysolecithin directly into the lipid bilayer of the plasma membrane. As discussed in more detail in Section 8.1.3, we consider that in this situation lysolecithin can promote structural reorganization within the bilayer, leading to lipid redistribution and enrichment of acidic phospholipids such as PS within the external monolayer of the plasma membrane. Once sufficient amounts of PS are present in the external monolayer, Ca^{2+} in the medium will induce separation of PS into crystalline domains, at which point not only is the membrane susceptible to fusion with other membranes but also the enhanced permeability of the membrane to Ca^{2+} will initiate the cytoskeletal-assisted redistribution of membrane proteins necessary for cell-to-cell fusion. We propose that the reason neutral vesicles containing lysolecithin fail to induce cell-to-cell fusion (Martin and MacDonald, 1976c) is that such vesicles are unable to fuse with the cellular plasma membrane and lysolecithin is thus frustrated from insertion into the lipid bilayer of the plasma membrane.

8.1.3. Cell-to-Cell Fusion Induced by Lysolecithin and Other Amphipathic Molecules

Several proposals have been made in relation to the possible "fluidizing" effect of lysolecithin and other amphipathic molecules that induce cell fusion and lysis (Howell et al., 1972; Ahkong et al., 1973b). Recent evidence

indicates, however, that contrary to expectations the addition of lysolecithin to lecithin vesicles does not "fluidize" but actually stabilizes the rigid crystalline state of the bilayers (Papahadjopoulos et al., 1976a). Moreover, lysolecithin has been shown to enhance exchange diffusion between lecithin vesicles, but true fusion was not observed (Papahadjopoulos et al., 1976a). These data dictate that there is a need for critical reconsideration of the much-cited view (Ahkong et al., 1975a) that lysolecithin-induced membrane fusion results from its "fluidizing" effect on membrane lipids.

We instead propose that the role of lysolecithin in cell fusion is also related to Ca^{2+} -induced phase separations. An *absolute* requirement for Ca^{2+} during cell-to-cell fusion when induced by lysolecithin and other amphipathic molecules has been reported (see Lucy, 1975).

We propose that the incorporation of exogenous lysolecithin (or other similar amphipaths) into the cellular plasma membrane will result in the accumulation of new molecules within the outer monolayer. This accumulation can proceed either via the introduction of free molecules or via the fusion of whole micelles, and is driven by the excess free energy of the initial system. Such accumulation asymmetrically across the bilayer will decrease the stability of the bilayer and would be expected to lead to changes in shape, which have actually been observed as echinocytosis or crenations in erythrocytes treated with lysolecithin (La Celle et al., 1976), and to an increase in the rate of flip-flop motion of molecules from one monolayer to the other. This process would tend to equilibrate lipid composition in the two monolayers and thus reduce any lipid asymmetry, resulting in the appearance of negatively charged phospholipids such as PS in the outer monolayer. This could be followed by a lateral phase separation of PS induced by the extracellular Ca^{2+} and the membranes would now be in a fusion susceptible state.

It is therefore possible to relate the documented effect of lysolecithin on vesicles where it enhances exchange diffusion (Papahadjopoulos et al., 1976a) and increases permeability (Lee and Chan, 1977) with a subsequent Ca^{2+} -induced phase separation in the outer monolayer, which renders the membranes fusion susceptible. It should be noted, however, that the above sequence of events is valid only for the action of *exogenous* lysolecithin and its *asymmetrical accumulation* within the membrane. The presence of *endogenous* lysolecithin originating from the action of pure phospholipases seems to have no lytic effects and does not alter membrane lipid asymmetry in the plasma membrane of erythrocytes (Zwaal et al., 1973). This could also be related to the ability of lysolecithin to interact with cholesterol to form stable bilayers (Rand et al., 1975).

8.2. Fusion Initiated at the Inner (Cytoplasmic) Face of the Plasma Membrane and Fusion of Intracellular Organelles

In common with all other forms of membrane fusion behavior discussed in this chapter, the fusion of intracellular organelles and the fusion of diverse

types of secretory granules with the inner face of the plasma membrane during exocytosis both exhibit an obligatory requirement for Ca^{2+} . There is now a vast body of experimental evidence (review, Carafoli et al., 1975) showing that these specific examples of fusion are "triggered" by a rise in the intracellular Ca^{2+} concentration which usually results from the increased influx of Ca^{2+} into the cell, although in a few instances mobilization of Ca^{2+} previously bound to organelles such as mitochondria and the endoplasmic reticulum may also contribute. More recently, studies in several laboratories have shown that exocytosis and the intracellular fusion of secretory granules with each other can be induced experimentally by increasing intracellular Ca^{2+} using the cation ionophores A23187 and X537A (review, Carafoli et al., 1975). In exocytosis induced by physiological inducers, as well as that induced experimentally by ionophores, the fusion of large numbers of secretory granules with the plasma membrane (and with each other) occurs as a very rapid and highly synchronized process.

We consider that these examples of membrane fusion require only that the intracellular Ca^{2+} concentration be raised to the level necessary to induce phase separation of acidic phospholipids, and that substantial changes in the transbilayer distribution of acidic phospholipids are not required. As mentioned earlier, the inner face of the plasma membrane and the membranes of secretory granules are characterized by the presence of acidic phospholipids such as PS. Such membranes are thus able to undergo *immediate* lateral phase separation and assume a "fusion-susceptible" condition as soon as the concentration of Ca^{2+} required to induce phase separation is reached in the cytoplasmic areas near the plasma membrane.

NOTES ADDED IN PROOF

1. Two recent reports have described such systems in some detail. Dunham et al. (1977) have studied the interaction of a dye, Arsenazo III, with Ca^{2+} —both encapsulated initially in separate vesicle populations—by following changes in the spectrum of Arsenazo. Ingolia and Koshland (1978) have studied the interaction of firefly extracts with ATP by following the luminescence response. In both cases, significant interaction was observed, which was interpreted as due to fusion, although a considerable degree of lysis was also found to occur.

2. Evidence obtained from experiments with a mixing microcalorimeter Papahadjopoulos et al., 1978; Portis et al., 1979) indicate that the reaction of PS vesicles with Ca^{2+} (at concentrations of 1–5 mM) is exothermic, releasing 5.5 kcal/mol. On the other hand, the reaction of PS vesicles with Mg^{2+} (5 mM) and Ca^{2+} at subthreshold concentrations (0.7 mM) is endothermic (approx. 0.2 kcal/mol). This provides direct evidence for the crystallization of the acyl chains by Ca^{2+} .

3. Recent studies (Newton et al., 1978; Portis et al., 1979) have revealed

that although both Ca^{2+} and Mg^{2+} form stoichiometric (2:1) complexes with PS, the Ca-PS complex contains no appreciable amounts of free water, in contrast to the Mg-PS complex which indicates the presence of 7 Å of water space between bilayers. Kinetics of vesicle aggregation and release of vesicle contents by Ca^{2+} and Mg^{2+} , along with binding studies, have produced evidence in favor of two types of complexes, which we have designated *cis* and *trans*. The former involves binding of the metal ions to one membrane only and occurs at subthreshold concentrations of Ca^{2+} and at all concentrations with Mg^{2+} . The latter (*trans*) complex involves binding of the metal ion to two (adjacent) membranes, is accompanied by elimination of free water, and occurs only with Ca^{2+} at concentrations above threshold. It was concluded that the formation of such an intermembrane complex could be the key event that induces the crystallization of the acyl chains, phase separation, and subsequent fusion at domain boundaries (Portis et al., 1979).

4. Recent studies (Portis et al., 1979) indicate that there is a synergistic effect between Ca^{2+} and Mg^{2+} in inducing fusion of PS vesicles. Thus, in the presence of 3 mM Mg^{2+} , aggregation and fusion of PS vesicles can be induced by 0.2 mM Ca^{2+} . This unexpected observation was explained on the basis of two (*cis* and *trans*) types of complexes with PS (see note 3).

5. Recent studies indicate that Ca^{2+} -induced aggregation of PS vesicles is accompanied by removal of most of the water between bilayers (Newton et al., 1978). Such close apposition may therefore involve molecular contact, and the formation of specific "intermembrane" complexes (Portis et al., 1979).

6. Any detailed mechanism of the molecular events associated with fusion of lipid-enveloped virus with plasma membrane would have to take into account the biochemical changes that have been observed to occur, such as those described recently by Volsky and Loyter (1978).

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