

PLASMA MEMBRANES OF MAMMALIAN CELLS

A Review of Methods for Their Characterization and Isolation

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The plasma membrane surrounding living cells plays a role in intracellular metabolism, but what distinguishes this structure from other organelles is that the plasma membrane also mediates interactions between the cell and its external environment. The simplest of these interactions maintain a desirable intracellular milieu by bringing in needed substances and getting rid of waste products. The plasma membrane acts as a passive diffusion barrier to charged and large molecules, and in addition carries out facilitated diffusion, active transport, endocytosis (pinocytosis and phagocytosis), and exocytosis. Further, this membrane is involved in locomotion and chemotaxis, processes which could remove a cell from noxious surroundings or to a more nutritive medium. On a more complex level, cells must communicate with other cells; this is especially important in the formation and maintenance of multicellular organisms. The plasma membrane functions in the secretion and reception of hormones, conduction of nerve impulses, and direct cellular interactions such as adhesion and contact inhibition. Finally, on yet another level the plasma membrane may be involved in such sophisticated processes as immunological defense and information storage and retrieval.

The "isolate-and-characterize" approach was first applied to plasma membranes around 1960. Unfortunately, our present state of knowledge in this field is not as great as might have been anticipated at that time. This slow progress is partially

due to the considerable complexities inherent in working with membranes of any sort; but there are other factors as well. For example, many investigators have not been rigorous enough in their choice of plasma membrane markers and have not thoroughly analyzed their plasma membrane fractions for contamination by other subcellular particles; consequently, the true identity of many so-called plasma membrane preparations is uncertain. The vast majority of plasma membrane isolations have been preparative rather than analytical. Although the preparative approach is potentially a very valuable short-cut to an understanding of the entity being isolated, it can also lead to conclusions that are wrong unless really rigorous criteria are applied. Finally, most studies on plasma membranes are concerned simply with their chemical and enzymatic compositions; by isolating plasma membranes from the proper cell types, it should be possible to design experiments with more direct bearing on the functions of this organelle.

Isolation of plasma membranes may conveniently be divided into five sequential steps—choice of the tissue to be used, selection of markers, disruption of the tissue, fractionation, and analysis. The following discussion combines some general comments about each of these steps with a review of the literature. The literature review extends through November 1971 and is limited to studies with mammalian cells. Of course, the information derived from mammalian cells should ultimately

be considered in conjunction with data from similar studies on nonmammalian systems. The reader is referred, for example, to work from Salton's laboratory on the cell membrane of *Micrococcus lysodeikticus* (Muñoz et al., 1968 a, 1968 b, 1969) and to the work of Changeux and his co-workers on the membranes of the electroplax (Changeux et al., 1969; Bauman et al., 1970).

CHOICE OF TISSUES

Erythrocyte plasma membranes were among the first to be studied extensively (Dodge et al., 1963; Bakerman and Wasemiller, 1967). The reasons for this are obvious: a large sample of erythrocytes essentially free of other cell types is easily obtainable. The ability to form semipermeable erythrocyte ghosts by hypotonic lysis makes it a simple matter to separate these plasma membranes from the other elements of the cell (which are minimal) and yields a preparation which can be used to study essential plasma membrane functions such as transport. Much valuable information has come from investigations using erythrocyte ghosts, e.g., information about the Na^+ , K^+ -sensitive, ouabain-inhibitable adenosine triphosphatase (ATPase) now thought to be a characteristic component of most mammalian plasma membranes (Whittam, 1962; Skou, 1965; Glynn, 1968) and about the transport of such substances as glucose and other monosaccharides (Miller, 1969). However, the erythrocyte is too specialized a cell type to be of use in answering other questions about the mammalian plasma membrane such as those concerning endo- and exocytosis, locomotion, and contact inhibition. Even more nonrepresentative of mammalian plasma membranes is myelin, a specialization of the Schwann cell plasma membrane which also received early and extensive attention (Korn, 1966).

Plasma membranes were next isolated from rat liver. Because liver is easily available in large quantities and because much is already known about this tissue, its choice as starting material for such studies is not surprising; however, this choice was unfortunate. Rat liver in particular and solid organs in general have serious disadvantages as starting material for plasma membrane isolation when compared to cells such as blood cells which are not structured into permanent masses.

In the first place, solid organs are generally composed of more than one cell type (de Duve, 1964). The liver contains two different cell types, parenchymal cells and reticuloendothelial

(Kupffer) cells, which are present in approximately equal numbers (Greengard et al., 1972). The kidney contains "parietal" glomerular epithelial cells, "visceral" glomerular epithelial cells, proximal convoluted tubule epithelial cells, juxtaglomerular cells, and several other types of epithelia (Bloom and Fawcett, 1962). The small intestinal epithelium contains simple columnar cells interspersed with a variable number of mucin-secreting goblet cells; the nature of the columnar cells may vary for different parts of the small intestine (duodenum, jejunum, ileum), since these different parts serve different functions and directly below the intestinal epithelium is a layer of smooth muscle (Porteous and Clark, 1965).

To complicate things even further, solid tissues are generally vascular and contain the nerve processes innervating their blood vessels, as well as variable numbers of nerves ending on the cells of the organ itself (Bloom and Fawcett, 1962). Red blood cells may be removed by perfusion of the organ before homogenization (Coleman et al., 1967; Weaver and Boyle, 1969; Hinton et al., 1970; Touster et al., 1970), but this is seldom done. Even if the organ is perfused, the vascular endothelium, vascular smooth muscle cells, and nerve processes still remain to be broken up and fractionated with subcellular particles of the tissue cells.

Another heterogeneous element of solid organs is the connective tissue present, consisting chiefly of fibroblasts and an extracellular matrix. Some collagen, mucopolysaccharide, and the like tend to remain with the plasma membrane during isolation and thus complicate analysis of the final preparation. For instance, electron microscopy of plasma membranes prepared from rat skeletal muscle shows an outer network of collagen fibrils, an inner plasma membrane, and a basement membrane in between (Kono et al., 1964). Investigators concerned with solid tissues should note the observation by Kono and his co-workers that collagenase removed the collagen fibrils and basement membrane. Such adhering extracellular material could interfere seriously with determinations of the chemical composition of the isolated membranes and with the pattern of plasma membrane proteins revealed by polyacrylamide gel electrophoresis. In addition, it is at least possible that the extracellular matrix contains enzymatic activities which should not be attributed to the plasma membrane. For example, acetylcholinesterase has recently been

shown to be removed from the end plates of rat and frog muscle when collagenase is added to the preparation (Hall and Kelly, 1971). Indeed, deciding exactly where the plasma membrane ends and the matrix begins may be problematic in some cases.

Finally, even the plasma membrane of an individual cell in a solid organ appears to be heterogeneous. The apical, lateral, and basal membranes of a liver parenchymal cell look quite different under the microscope; for instance, the lateral plasma membrane forms junctional complexes and folds into numerous microvilli which extend into the bile canaliculi (Bloom and Fawcett, 1962). Evidence for biochemical differences between the morphologically distinct sides of the parenchymal cell plasma membrane is growing. (See below.) These morphological and biochemical differences presumably reflect functional differences.

The heterogeneity of solid tissues is thus alarming in the context of plasma membrane isolation. It is especially alarming when one considers that reported yields of plasma membrane are generally low (1–10% of theoretical) (El-Aaser et al., 1966; Song and Kappas, 1969; Song et al., 1969; Weaver and Boyle, 1969; Evans, 1970; Wolff and Jones, 1971) and that consequently the final preparations might easily be enriched in material from a very minor cell type or from a particular side of the cell. The usual approach to this heterogeneity has been to mention it in passing or, more often, to ignore it completely.

A preferable approach would be the elimination of as much of this heterogeneity as possible before beginning the isolation of plasma membranes. There are two obvious ways of accomplishing this. First, methods must be developed to make suspensions of individual cells from solid organs and to separate the different cell types present from one another. Progress is already being made in this area by Howard, Rodbell, and their co-workers, among others. Rodbell uses collagenase to prepare a suspension of rat adipose cells (Rodbell, 1964); while Howard's group combines collagenase with hyaluronidase to prepare a suspension of rat liver cells, from which they isolate the parenchymal cells (Howard et al., 1967; Howard and Pesch, 1968). de Duve (1971) points out that although enzymatic treatment can yield cell suspensions even from tough tissues like thyroid, cartilage, and aorta, such treatment may damage the cell surface. Howard and his co-workers claim that 90–

95% of their isolated liver cells are structurally intact. Substantial evidence for such statements must be provided, especially in light of the finding by Jarett and McKeel (1970) that rat epididymal adipose tissue cells which have been made into a suspension using bacterial collagenase are permeable to ATP. Electron micrographs of these cells show loss of the basement membrane and small gaps in the plasma membrane.

A second method of obtaining a more homogeneous starting material for plasma membrane isolation is to use a cell type which is not permanently structured into a solid organ. Such cells may be obtained from naturally occurring dispersed tissues, e.g. the white blood cells; or they may be cultured in suspension, as is done with Ehrlich ascites carcinoma cells. The choice of such a starting material immediately eliminates possible complications arising from the presence of vascular and nervous elements and greatly reduces contamination by extracellular material. Also, it is often an easy matter to obtain a quite homogeneous preparation of such cells, as is illustrated by preparations of small lymphocytes (Allan and Crumpton, 1970), HeLa cells (Hagopian et al., 1968; Ågren and Ronquist, 1969; Atkinson and Summers, 1971), Ehrlich ascites carcinoma cells (Wallach and Ullrey, 1962, 1962 *a*; Ågren and Ronquist, 1969), and polymorphonuclear leukocytes (Oren et al., 1963). The 16 h peritoneal exudate elicited in guinea pigs by caseinate injection contains 95% polymorphonuclear leukocytes, 2% monocytes, 2% lymphocytes, and 1% other cell types. Furthermore, good preparations of monocytes and lymphocytes can be obtained if it is necessary to do control experiments demonstrating that a 2% contamination by these cells is insignificant in the context of the study being performed on the polymorphonuclear leukocytes.

However, one type of heterogeneity will persist despite all efforts to obtain a homogeneous cell population. The differences between the apical, lateral, and basal sides of a solid tissue cell like the liver parenchymal cell will remain. Although microscopy reveals no apparent specialized regions on the plasma membrane of a free cell such as the polymorphonuclear leukocyte, other evidence for the existence of such specialized regions is growing (see below). The possibility of this type of heterogeneity must always be kept in mind when isolating plasma membranes; one way of dealing with it is discussed in the next section.

A review of the literature at this point reveals that the tissue chosen for plasma membrane isolation in the vast majority of cases is a solid organ. The single most popular tissue is

rat *liver* (Emmelot and Bos, 1962; Davidson et al., 1963; Tria and Barnabei, 1963; Skipski et al., 1965; Takeuchi and Terayama, 1965; Ashworth and Green, 1966; Lansing et al., 1967; Coleman et al., 1967; Lieberman et al., 1967; Song and Bodansky, 1967; Anderson et al., 1968; Emmelot et al., 1968; Dod and Gray, 1968; Graham et al., 1968; Neville, 1960, 1968; Stein and Stein, 1968; Berman et al., 1969; Erecinska et al., 1969; Evans, 1969; Song et al., 1969; Wattiaux-de Coninck and Wattiaux, 1969; Weaver and Boyle, 1969; El-Aaser et al., 1966, 1970; Henning et al., 1970; Hinton et al., 1970; Ray, 1970; Skidmore and Trams, 1970; Touster et al., 1970; Barancik and Lieberman, 1971; Franklin and Trams, 1971; Wright and Green, 1971).

Plasma membranes have also been isolated from the *livers* of

cows (Fleischer and Fleischer, 1969),
pigs (Lutz and Frimmer, 1970),
mice (Emmelot and Bos, 1969 *a*; Evans, 1970),
guinea pigs (Finean et al., 1966; Coleman and Finean, 1966, 1967).

Furthermore, because of the interesting changes in the plasma membrane brought about by cancer (Wallach, 1969), these structures have been isolated from a number of

solid hepatomas, chiefly in
rats (Emmelot et al., 1968; Emmelot and Bos, 1966, 1969),
mice (Emmelot et al., 1968; Emmelot and Bos, 1969 *a*).

Other solid organs from which plasma membranes have been isolated include the *small intestinal epithelium* of

rats (Forstner et al., 1968),
hamsters (Miller and Crane, 1961; Holt and Miller, 1962; Eichholz and Crane, 1965; Overton et al., 1965; Johnson, 1967; Eichholz, 1968),
guinea pigs (Hübscher et al., 1965; Finean et al., 1966; Coleman and Finean, 1966, 1967),
rabbits (Hübscher et al., 1965; Porteous and Clark, 1965),
cats (Hübscher et al., 1965),

kidneys from
rats (Davidson et al., 1963; Fitzpatrick et al., 1969; Wilfong and Neville, 1970),
guinea pigs (Finean et al., 1966; Coleman and Finean, 1966, 1967),
rhesus monkeys (Klenk and Choppin, 1969)
rat *bladder epithelium* (Hicks and Ketterer, 1970),
rat *skeletal muscle* (Kono and Colowick, 1961; McCollester, 1962; Kono et al., 1964),
rat *uterine smooth muscle* (Carroll and Sereda, 1968),
rat *white and brown adipose tissue* (Giacobino and Perrelet, 1971),
guinea pig *pancreas* (Meldolesi et al., 1971 *a*),
bovine *thyroid* (Yamashita and Field, 1970; Wolff and Jones, 1971),
bovine *mammary gland* (Keenan et al., 1970).

The list of homogeneous cell suspensions which have been used for the preparation of plasma membranes is short:

HeLa cells (Bosmann et al., 1968; Hagopian et al., 1968; Boone et al., 1969; Atkinson and Summers, 1971; Cohen et al., 1971),
mouse *ascites tumor cells* (Wallach and Ullrey, 1962, 1962 *a*; Kamat and Wallach, 1965; Wallach and Kamat, 1964, 1966; Molnar et al., 1969; McCollester, 1970),

rabbit peritoneal exudate <i>leukocytes</i>	(Woodin and Wieneke, 1966 <i>a</i> , 1966 <i>b</i> ; Wieneke and Woodin, 1967),
pig lymph node <i>lymphocytes</i>	(Allan and Crumpton, 1970),
human <i>platelets</i>	(Barber and Jamieson, 1970).

Before beginning the isolation of plasma membranes, the investigator must, of course, decide exactly what it is he wants to find out about this organelle. Indeed, this is perhaps the single most important consideration in choosing a cell type to work with. Could it be that the popularity of solid organs as a starting material for the preparation of plasma membranes has been dictated by a desire to study some special property of these particular plasma membranes? In most cases the answer is no. The great majority of the studies referred to above are primarily concerned with characterizing the chemical and enzymatic composition of mammalian plasma membranes. This characterization is unquestionably an important first step in the general understanding of plasma membranes; but such investigations could just as well be carried out with cell suspensions, with all their advantages.

With respect to the questions one can ask, the plasma membrane of the polymorphonuclear leukocyte is of particular interest. The specialized functions of this cell type intimately involve its plasma membrane and allow such studies as the following: there is ample evidence for increased lipid metabolism during phagocytosis (Sbarra and Karnovsky, 1960; Karnovsky and Wallach, 1961; Downey and Pisano, 1966; Sastry and Hokin, 1966; Elsbach, 1968; Elsbach and Farrow, 1969). A scheme for the isolation of the polymorphonuclear leukocyte plasma membrane would reveal whether or not this increased lipid metabolism is localized to the cell surface. Such a localization would suggest that the increased lipid metabolism plays a role in particle ingestion; perhaps new membrane must be synthesized to replace that part of the plasma membrane which is internalized during phagocytosis.

Furthermore, fusion of the phagocytic vacuole with lysosomes seems to be closely analogous to the process of secretion (Woodin et al., 1963). A preparation of plasma membrane vesicles together with a preparation of lysosomes from polymorphonuclear leukocytes might allow *in vitro* study of this fusion process. One of the first questions to be asked is whether the process requires soluble enzymes or other soluble factors. In addition, by comparing the enzymatic and chemical composi-

tion of the plasma membrane with that of the membranes of primary and secondary lysosomes, it should be possible to determine whether or not a specialized region of the plasma membrane is involved in phagocytosis. Such a comparison might reveal startlingly heterogeneous areas on the cell surface.

The plasma membrane of the polymorphonuclear leukocyte also functions in adhesion, locomotion (Merchant, 1950; Fukushima et al., 1954), and chemotaxis (Fukushima et al., 1954; Wilkinson et al., 1969; Horwitz and Garrett, 1971). A myosin-like contractile protein has been isolated from equine leukocytes (Senda et al., 1969). Is this protein localized in the plasma membrane and if so, how does it function there? Finally, there is evidence that ascorbic acid, which is found in rather high levels in the polymorphonuclear leukocyte, is involved somehow in maintaining the structural integrity of the plasma membrane of this cell (Nungester and Ames, 1948; Mills, 1949; Boruch et al., 1968). Comparisons between plasma membranes isolated from normal and scorbutic guinea pig polymorphonuclear leukocytes might increase our understanding of the still somewhat-mysterious vitamin C.

SELECTION OF MARKERS

To isolate one component from a mixture, it is obviously necessary to have an assay for that component; assays for the other components present are also of critical importance. In subcellular fractionation the components of a tissue homogenate are generally considered to be the morphologically distinct subcellular structures (i.e., the various organelles) or fragments of these structures. In the initial isolation two means of following the desired organelle can be used. First, if the organelle in question has a distinctive morphology, as do mitochondria, microscopy can be used to evaluate distribution. Second, if some compound or enzyme can be shown to be localized in the organelle either *in situ* or, in a homogenate or a subcellular fraction, that compound or enzyme may be used to follow the organelle throughout the fractionation. Such a localization can be established with histochemical techniques—thus,

DNA can be shown to be principally in the nucleus and glucose-6-phosphatase principally on the endoplasmic reticulum of rat liver. Once a good isolation of the desired subcellular fraction is achieved, analysis of this fraction may reveal a better marker than that used in the initial isolation, e.g., succinate dehydrogenase is used as a mitochondrial marker. However, such secondary markers are only as reliable as the fractionation scheme based on primary markers derived from morphology or histochemistry.

Historically, of course, subcellular fractionation procedures have developed in a somewhat erratic manner. Thus, many of the enzymes which are now commonly used as markers—e.g., glucose-6-phosphatase and the acid hydrolases—were first shown biochemically to be localized in specific fractions after subjecting a tissue homogenate to a certain scheme of centrifugation. Such studies, however, established only that the enzyme in question was a “marker” for the fraction it appeared in. Localizing these enzymes to distinct morphological entities required the use of morphological and histochemical criteria.

Morphological Markers

Use of phase-contrast or electron microscopy to evaluate a tissue fractionation presents serious problems. In the first place adequate criteria for identifying a given particle must be established. Intact mitochondria, rough endoplasmic reticulum, and nuclei do have distinct morphologies; but lysosomes, smooth endoplasmic reticulum, peroxisomes, and the Golgi apparatus are all smooth vesicles in a tissue homogenate and tend to look rather alike. Furthermore, if the nuclei or mitochondria are disrupted during homogenization, the membranes of these organelles also tend to form smooth vesicles.

In a similar manner the plasma membrane usually vesiculates upon disruption (Boone et al., 1969; Fitzpatrick et al., 1969; Molnar et al., 1969; Allan and Crumpton, 1970; Barber and Jamieson, 1970) and is subsequently very difficult to identify morphologically. However, this is not entirely the case with plasma membranes of liver. The cells of solid tissues, including liver, form junctional complexes—tight junctions (zonula occludens), intermediate junctions (zonula adherens), and desmosomes (macula adherens)—with neighboring cells (Bloom and Fawcett, 1962). These junctions,

which are easily identified in electron micrographs, appear to remain intact during homogenization of the liver; and as a result, large fragments of plasma membrane lying adjacent to such a complex or stretching between two junctional complexes are obtained (Neville, 1960; Emmelot et al., 1964). The microvilli extending into the bile canaliculi are also resistant to gentle homogenization. These three features—junctional complexes, large pieces of membrane, and microvilli—provide excellent morphological criteria for the identification of liver plasma membranes in homogenates and subcellular fractions. They have been utilized in virtually all isolations of plasma membranes from liver (see especially Neville, 1960; Emmelot et al., 1964; Coleman and Finean, 1966; Barclay et al., 1967; Benedetti and Emmelot, 1968; Fleischer and Fleischer, 1969; Song et al., 1969). These morphological features are undoubtedly one source of the liver's popularity as a starting material in plasma membrane studies. However, as is discussed below, there are real problems with the *quantification* of such markers. Also, one aspect of this morphological assay which is usually overlooked is the possibility or even probability that it selects for that region of the parenchymal cell plasma membrane at, or immediately adjacent to, junctional complexes. Other regions probably form smooth vesicles after disruption. These vesicles may be found among the “vesicles of unknown origin” which are invariably present in rat liver plasma membrane preparations or they may fractionate in a manner entirely unlike the distribution of junctional complexes, large membrane fragments, and microvilli. Selective isolation of specific regions of the plasma membrane need not be a disadvantage, but the investigator should not fail to consider this possibility.

Another structural specialization of the plasma membrane which resists gentle homogenization is the microvillus of the intestinal brush border (Miller and Crane, 1961; Holt and Miller, 1962). These microvilli are commonly used as a marker in isolations of the plasma membrane of the intestinal epithelium (Miller and Crane, 1961; Holt and Miller, 1962; Eichholz and Crane, 1965; Overton et al., 1965; Porteous and Clark, 1965; Coleman and Finean, 1966, 1967; Forstner et al., 1968). Again, such a marker selects for the apical surface of the plasma membrane, but the investigators involved are aware of this selection. On the other

hand, the flask-shaped invaginations which characterize the plasma membrane of rat epididymal fat cells may be more generally distributed around the cell surface; McKeel and Jarett (1970) used these invaginations as a marker in their studies.

Finally, Benedetti and Emmelot (1968) describe several fine features of the appearance of rat liver plasma membranes which can be used to distinguish them from the membranes of cytoplasmic organelles: the triple-layered appearance of the plasma membrane distinguishes it from the endoplasmic reticulum. The plasma membrane has, after negative staining, hexagonal subunits which neither the endoplasmic reticulum nor the mitochondria have. Negative staining also reveals that the globular knobs on the plasma membrane differ from those on the endoplasmic reticulum and mitochondria, and only the nuclear membrane has pores.

However, even in situations where a specialization of the plasma membrane could serve as a morphological marker for its distribution during fractionation, the question of how to quantify this marker arises. It is difficult to achieve random sampling on the minute scale used for electron microscopy, though some progress is being made in this area (de Duve, 1971). Once random samples of each of the fractions to be assayed for plasma membrane are obtained, what should be quantified? Is it the number of junctional complexes present, or the length of the membrane profiles attached to the junctional complexes, or the surface area of the membrane sheets attached to the junctional complexes? The problems are so great that it seems advisable for the present to use microscopy in plasma membrane isolation only to get some idea of the main components of the individual fractions. Unfortunately, a large number of studies have relied on morphology as the sole plasma membrane marker.

Enzymatic Markers

On the other hand, histo- and cytochemistry have their own difficulties. Years of work are required to establish optimal conditions and the necessary degree of resolution and specificity in the histochemical assay of an enzyme. As a result, there are few good staining techniques available. Only about 20 histochemical enzymatic assays have been developed to the point where they are suitable for

use with the electron microscope; most of these are for hydrolases and oxidoreductases, with no satisfactory techniques for detecting isomerases, ligases, or carboxylases (de Duve, 1964; Shnitka and Seligman, 1971).

Some of the most widely used histochemical techniques are those for assaying phosphatases (Essner et al., 1958; North, 1966; Rosenthal et al., 1966, 1969, 1969 *a*; Marchesi, 1968). The general approach involved is to allow the substrate to be split in the presence of a heavy metal ion, which precipitates with the released inorganic phosphate, and then to view the localization of this or a derived precipitate in the electron microscope. An examination of the list of problems involved with this apparently simple technique is instructive (for general discussions of these difficulties, see Essner et al., 1958; Goldfischer et al., 1964; de-Thé, 1968; Marchesi, 1968; Rosenthal et al., 1966, 1969, 1969 *a*; Shnitka and Seligman, 1971):

- (a) Fixation for microscopy may inhibit the enzyme of interest. Glutaraldehyde is the best and most commonly used fixative and a very good inhibitor of enzymes as well. When the rat kidney is perfused with glutaraldehyde, acid phosphatase is inhibited 88% and aryl sulfatase 70% within 5 min (Arbogh et al., 1971). Glutaraldehyde also inhibits mitochondrial ATPase and plasma membrane nucleoside phosphatases (de-Thé, 1968).
- (b) The capture reagent may inhibit the enzyme of interest. Pb^{++} is known, for instance, to inhibit nucleoside phosphatases, especially the Na^+ , K^+ -ATPase of red cells (Marchesi, 1968).
- (c) The substrate and/or capture reagent may not penetrate freely into the tissue or into individual cells or cytoplasmic organelles. This problem is especially serious when blocks of tissue or thick sections are used. However, inadequate penetration may occur even under the best conditions. When ATPase is examined histochemically in guinea pig peritoneal polymorphonuclear leukocytes, the reaction product is restricted to the plasma membrane; only after prolonged fixation does product appear over the mitochondria, which are known to contain an ATPase (North, 1966).

- (d) The substrates may be nonenzymatically hydrolyzed by heavy metals.
- (e) The product may diffuse some distance from its site of formation before precipitating with a heavy metal ion; Shnitka and Seligman (1971) estimate that the best localization that can be achieved is within 100 Å. In addition, it is extremely difficult to quantify staining intensities; thus, determination of substrate specificity is problematic and histochemical results are primarily qualitative and descriptive in nature. Appropriate controls can eliminate a number of these complications.

On the basis of histochemistry a plasma membrane localization has been claimed for a number of enzymes in different tissues. One of the studies referred to most often is that by Essner and his co-workers in 1958. These investigators found 5'-nucleotidase on the plasma membrane of the rat liver parenchymal cell. However, it cannot be concluded from their work that this enzyme is a good marker for the plasma membrane. No

reaction product was seen inside the cells; but this might simply be explained by lack of penetration of the substrate.

A number of other enzymes—including ATPases, alkaline phosphatase, inosine diphosphatase, and leucine aminopeptidase (Benedetti and Emmelot, 1968)—have also been demonstrated on the rat liver parenchymal cell plasma membrane *in situ*. Histochemical techniques show ATPases (including the Na⁺, K⁺-sensitive, ouabain-inhibitable ATPase) on the plasma membranes of a wide variety of cell types, including rat kidney, pancreas, and mammary glands (de-Thé, 1968), Ehrlich ascites carcinoma cells (Wallach and Ullrey, 1962), and guinea pig white blood cells (North, 1966). Alkaline phosphatase is seen at the brush borders of the small intestine (Nachlas et al., 1960; Clark, 1961) and of the proximal convoluted tubule of the kidney (de-Thé, 1968). Hormone-sensitive adenylyl cyclase appears to be localized at the plasma membrane of both reticuloendothelial and parenchymal cells in rat liver (Reik et al., 1970).

In summary, a typical plasma membrane isolation may use

<i>5'-nucleotidase</i>	(Coleman et al., 1967; Michell et al., 1967; Bosmann et al., 1968; Dod and Gray, 1968; Graham et al., 1968; Stein et al., 1968; Berman et al., 1969; Erecinska et al., 1969; Evans, 1969; Fleischer and Fleischer, 1969; Molnar et al., 1969; Nakai et al., 1969; Song et al., 1969; Wattiaux-de Coninck and Wattiaux, 1969; Allan and Crumpton, 1970; El-Aaser et al., 1970; Gahmberg and Simons, 1970; Lutz and Frimmer, 1970; Henning et al., 1970; Hinton et al., 1970; Ray, 1970; Skidmore and Trams, 1970; Touster et al., 1970; Giacobino and Perrelet, 1971; Wolff and Jones, 1971),
<i>ATPase(s)</i>	(Wallach and Kamat, 1964, 1966; Barclay et al., 1967; Coleman et al., 1967; Bosmann et al., 1968; Evans, 1969; Fleischer and Fleischer, 1969; Molnar et al., 1969; Evans, 1970; Gahmberg and Simons, 1970; Lutz and Frimmer, 1970; Ray, 1970; Yamashita and Field, 1970; Giacobino and Perrelet, 1971; Wolff and Jones, 1971),
<i>alkaline phosphatase</i>	(Hübscher et al., 1965; Porteous and Clark, 1965; Bosmann et al., 1968; Forstner et al., 1968; Graham et al., 1968; Ray, 1970; Wilfong and Neville, 1970),
<i>leucine aminopeptidase</i>	(Hübscher et al., 1965; Porteous and Clark, 1965; Dod and Gray, 1968; Forstner et al., 1968; Graham et al., 1968; Evans, 1969, 1970),
<i>adenylyl cyclase</i>	(McKeel and Jarett, 1970; Ray, 1970; Yamashita and Field, 1970; Pohl et al., 1971; Wolff and Jones, 1971),
and/or morphological criteria as markers.	

The tendency for the specific activities of these different markers to be increased in the same fraction provides additional evidence that they are all found on the plasma membrane; however, this evidence would be considerably stronger if the analytical approach were used routinely.

Assay of the plasma membrane fractions obtained using the above markers has revealed the presence of other enzymes which are in turn being used as plasma membrane markers. Examples include *nucleotide pyrophosphatase* and *phosphodiesterase* in rat liver (Lansing et al., 1967; Bosmann et al., 1968; Lutz and Frimmer, 1970; Ray, 1970; Touster et al., 1970), *invertase* and *maltase* in the intestinal epithelium (Miller and Crane, 1961; Holt and Miller, 1962; Hübscher et al., 1965; Porteous and Clark, 1965; Johnson, 1967; Eichholz, 1968; Forstner et al., 1968; Forstner, 1971), and *K⁺-stimulated p-nitrophenyl phosphatase* (which may be related to the Na⁺, K⁺-ATPase) in bovine thyroid (Wolff and Jones, 1971).

An interesting example of such "second generation" plasma membrane markers is cholesterol. The cholesterol/phospholipid ratio of isolated plasma membranes is much higher than that of other cellular membranes (Coleman and Finean, 1966; Benedetti and Emmelot, 1968). Thines-Sempoun and his co-workers (1969) have made clever use of this finding. They isolated rat liver microsomes and added digitonin to the preparation. Digitonin, which is known to complex with cholesterol, caused cholesterol, 5'-nucleotidase, and alkaline phosphatase to shift on a sucrose density gradient towards a position of higher buoyant density. The positions of monoamine oxidase, nicotinamide adenine dinucleotide (NADH)-cytochrome *c* reductase, and glucose-6-phosphatase were unchanged. The simplest explanation of this digitonin shift is that the shifted components are all found on plasma membrane vesicles present in the microsomal fraction.

Unfortunately, the reliability of such second generation plasma membrane markers is not easy to assess, again because most workers do preparative rather than analytical isolations. As emphasized above, under the best of circumstances these markers are no more reliable than the original markers based on morphological and histochemical studies.

The usual response to misgivings about the use of markers based on morphological and histo-

chemical studies is that there is no other choice. Thus, there is general agreement with Marchesi's statement (1968) that "At the present time, cytochemical methods represent the only way to determine sites of enzymatic activity in or on membranes under *in situ* or near *in situ* conditions." For the isolation of all cellular organelles other than the plasma membrane this response seems to be correct. Therefore, most investigators in the field of subcellular fractionation simply have to cope with the problems involved. However, a third approach—biochemical in nature and more reliable than morphology or histochemistry—may be used to select markers for the plasma membrane.

The plasma membrane is the one cellular structure which can be studied without disrupting the cell. With a cell suspension *in vitro* the outer surface of this membrane is accessible to biochemical investigation and chemical alteration. Probing of this outer surface with substances that do not enter the intact cells might well be expected to reveal features of the plasma membrane which could subsequently be used as markers during isolation. At least five types of such markers might emerge.

ECTOENZYMES: The plasma membrane may contain enzymes whose active sites face the external medium rather than the cytoplasm. Such enzymes will be referred to here as ectoenzymes. Claims in the literature for the presence of ectoenzymes on various cell types are not uncommon. The best known example is acetylcholinesterase which hydrolyzes the transmitter substance released into the synaptic cleft of the neuromuscular junction and which is therefore thought to have its active site facing the external medium. However, recent work suggests that this acetylcholinesterase may not be part of either the nerve or muscle plasma membrane, but may be embedded in the extracellular matrix (Hall and Kelly, 1971). On the other hand, the acetylcholinesterase of intact erythrocytes is probably a true ectoenzyme (Bishop, 1964).

Hydrolysis of ATP added to intact cell suspensions has been observed for a number of cell types, including Ehrlich ascites carcinoma cells (Wallach and Ullrey, 1962), rabbit and sheep pulmonary alveolar macrophages (Mustafa et al., 1969), rabbit and human polymorphonuclear leukocytes (Tenney and Rafter, 1968), human platelets (Chambers et al., 1967), and rat fat cells (Modolell

and Moore, 1967). Intact human platelets have also been found to dephosphorylate added adenosine diphosphate (Spaet and Lejnicks, 1966).

There is some indication of an ectopeptidase on human and dog polymorphonuclear leukocytes (Najjar and Nishioka, 1970) and of ectoserine esterases on rabbit polymorphonuclear leukocytes (Becker and Ward, 1967; Ward and Becker, 1967; Becker, 1971). Ågren and Ronquist (Ronquist, 1968; Ågren and Ronquist, 1969, 1971; Ågren et al., 1971) claim the presence of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase on the surface of human erythrocytes, Ehrlich ascites carcinoma cells, and HeLa cells.

Demonstration that intact cells act on a substrate added to the external medium is hardly sufficient evidence that the protein involved is an ectoenzyme. Nor, in experiments where a phosphorylated substrate is used, is the evidence strengthened appreciably by the often stated but generally unsubstantiated assertion that phosphorylated compounds do not get into cells. In one case an ecto-ATPase was initially thought to be present on isolated rat fat cells (Modolell and Moore, 1967). Subsequent isolation of the plasma membrane revealed little ATPase on this organelle, which strongly suggested that the isolated cells were permeable to ATP and that internal enzymes were hydrolyzing the added substrate (Jarett and McKeel, 1970). Apparently, the plasma membrane of the fat cells was damaged by the bacterial collagenase used to disperse epididymal adipose tissue into individual cells.

The kinds of evidence which must be gathered for the existence of an ectoenzyme have been described (DePierre and Karnovsky, 1972). Investigations with guinea pig polymorphonuclear leukocytes demonstrated that the intact cells hydrolyze ATP, adenosine monophosphate (AMP), and *p*-nitrophenyl phosphate. It was also shown that (a) no ATPase, AMPase, or *p*-nitrophenyl phosphatase is released into the medium during the assay for these activities; (b) the total ATPase and *p*-nitrophenyl phosphatase activities of a cell homogenate are about twice as great as those of the intact cells measured at saturating substrate levels; (c) the inorganic phosphate released by the three hydrolases of intact cells is located entirely outside the cells; and (d) when added to intact leukocytes, a protein reagent (the diazonium salt of sulfanilic acid) inhibits the three intact cell enzymes without inhibiting intracellular cyto-

plasmic enzymes. Similar evidence for ectoenzymes has been gathered by other investigators, in particular in Wallach's laboratory for an ecto-ATPase on Ehrlich ascites carcinoma cells; by Ågren and Ronquist for ectoglyceraldehyde-3-phosphate dehydrogenase and ectophosphoglycerate kinase on human erythrocytes, Ehrlich ascites carcinoma cells, and HeLa cells (Ronquist, 1968; Ågren and Ronquist, 1969, 1971; Ågren et al., 1971); and by Rothstein and his co-workers (1953) for an ectophosphatase on the epithelium of the rat intestine.

SIALIC ACID: Removal of a given substance from intact cells by treatment with an enzyme is taken as evidence that this substance is located on the outside of the plasma membrane. Quantitative removal is thought to indicate that all of the substance has this localization. The assumption is that the large size of an enzyme molecule prevents it from getting into intact cells; this assumption may be unwarranted and is in fact unnecessary.

This approach has been used chiefly with sialic acid. Eylar and his co-workers (1962) demonstrated that neuraminidase removes 95–100% of the sialic acid found in chicken, pig, lamb, calf, and human erythrocytes when the intact cells are treated with this enzyme. Treatment of intact guinea pig or human leukocytes with neuraminidase removes 40–60% of their total sialic acid (Noseworthy et al., 1972; Lichtman and Weed, 1970). Similar treatment of Ehrlich ascites carcinoma cells releases 73% of the sialic acid (Wallach and Eylar, 1961). Trypsin may also be used to release sialopeptides from intact cells (Eylar and Madoff, 1962; Seaman and Uhlenbruck, 1963; Winzler et al., 1967; Shen and Ginsburg, 1968; Pepper and Jamieson, 1969; Weis and Narahara, 1969).

Some evidence for the penetration of neuraminidase into intact cells has been reported. Wallach and Eylar (1961) maintain that neuraminidase releases more sialic acid from intact Ehrlich ascites carcinoma cells than is actually present on the plasma membrane; they claim that one-third to one-half of the sialic acid present in nuclei, mitochondria, and the soluble cytoplasm is also released by such treatment. A similar claim is made by Glick and her co-workers (1970) for treatment of intact mouse fibroblasts with neuraminidase. The most detailed study of this nature has been carried out by Nordling and Mayhew (1966). Using a variety of cell types—including human sarcoma and Ehrlich ascites carcinoma

cells—these investigators found that when fluorescent-labeled neuraminidase was added to the medium, 15–20% of the enzyme was taken up by the cells in 30 min. The nuclei and nucleoli were particularly fluorescent. This uptake did not seem to be due to damaged cells. Furthermore, Nordling and Mayhew found that the electrophoretic mobility of isolated nuclei treated directly with neuraminidase was greatly decreased and that the electrophoretic mobility of nuclei isolated from intact cells which had been treated with the enzyme was similarly decreased.

If treatment of intact cells with an enzyme is to be used to establish plasma membrane markers, one must be certain that the enzyme is not getting into the cells. One way to accomplish this would be to attach the enzyme involved covalently to a solid support which is larger than the cell being studied. Techniques for attaching proteins to solid supports are well advanced (Silman and Katchalski, 1966; Cuatrecasas et al., 1968; Cuatrecasas, 1969; Axén and Ernback, 1971; Royer and Green, 1971) and have been applied to trypsin, chymotrypsin, and papain, among other enzymes.

Neuraminidase can be covalently attached to beads of agarose measuring 40–210 μm in diameter (DePierre and Karnovsky, 1972). When intact guinea pig polymorphonuclear leukocytes, averaging 10 μm in diameter, were treated with this bead-bound enzyme preparation, all of the neuraminidase-susceptible sialic acid of the cells was released.

This approach has also been applied to biological problems by Cuatrecasas (1969). Cuatrecasas attached insulin to agarose beads measuring 60–300 μm in diameter, treated rat fat cells (50–100 μm) with these beads, and thus demonstrated that the interaction of insulin with the outside of these cells evoked all the typical effects of this hormone on adipose tissue. A similar experiment has been performed with adrenocorticotropin and adrenal cortical cells (Selinger and Civen, 1971).

Even if bound neuraminidase releases significantly less than 100% of the neuraminidase-susceptible sialic acid from intact cells, surface sialic acid might still be useful as a marker during plasma membrane isolation. One approach would be to compare the distribution of neuraminidase-susceptible sialic acid obtained when fractionation is performed on a homogenate of untreated cells with that obtained using neuraminidase-treated cells. That fraction which has lost its neurami-

dase-susceptible sialic acid by pretreatment of intact cells with the enzyme should contain the plasma membrane. Of course, evidence must be provided that removal of the surface sialic acid does not alter the manner in which the plasma membrane distributes during fractionation.

THE BINDING OF HORMONES AND OTHER BIOLOGICALLY ACTIVE SUBSTANCES: If all of the binding sites for a hormone or hormone-like substance can be shown to be present on the outside of the intact cell, then this “binding power” can be used to follow the plasma membrane during fractionation. Ideally, one would like to demonstrate that the hormone in question does not enter intact cells and that there are as many binding sites accessible to the hormone in intact cells as in a cell homogenate.

This approach has been used by Woodin and Wieneke (Woodin, 1961; Woodin et al., 1963; Woodin and Wieneke, 1966, 1966 *a*, 1966 *b*, 1968, 1970; Wieneke and Woodin, 1967). These investigators studied leucocidin, two proteins released extracellularly by *Staphylococcus* that act synergistically to kill human and rabbit leukocytes. The binding of leucocidin by rabbit polymorphonuclear leukocytes saturates at 10^5 molecules per cell, a result which suggests that leucocidin is not getting into the cells by diffusion or any other nonsaturable process. The binding of leucocidin to intact cells can be monitored by its inactivation, and this inactivation is quantitatively the same for intact cells as for homogenates. Woodin and Wieneke used the capacity to inactivate leucocidin as a marker in their isolation of the plasma membrane from rabbit polymorphonuclear leukocytes.

In a similar fashion Allan and his co-workers (1971) have investigated the binding of phytohemagglutinin to their isolated pig lymphocyte plasma membranes and have found this binding to be 22 times as effective as that by whole cells on a dry weight basis. Reports have appeared recently on the nature and kinetics of the binding of insulin (Cuatrecasas et al., 1971; Freychet et al., 1971) and glucagon (Rodbell et al., 1971) to rat liver plasma membranes. Such studies further illustrate the potential usefulness of the binding of hormones or hormone-like substances as a marker for the plasma membrane.

SURFACE ANTIGENS: The surface antigens of a cell might also provide good plasma membrane markers. Such antigens are detected through the use of specific antibodies in assays involving agglutination of, complement fixation by, supravital

dye staining of, cytotoxicity to, and chromium release from intact cells. In fact, this approach can be considered a special instance of the aforementioned approach; the specific antibodies can be thought of as hormone-like substances binding to their receptors, the surface antigens.

Many histochemical studies of surface antigens have been carried out using labeled antibodies. The specific antibodies are labeled with fluorescein or a visible marker such as ferritin or southern bean mosaic virus and can consequently be visualized under the fluorescence or electron microscope. With mouse thymocytes, erythrocytes, lymphocytes, and ascites tumor cells (Lindner, 1960; Dumonde et al., 1961; Möller, 1961; Cerottini and Brunner, 1967), as well as with rat liver cells (Benedetti and Emmelot, 1968) and numerous other cell types (e.g., Kourilsky et al., 1971), such labeled antibodies are bound primarily, if not exclusively, to the plasma membrane. Of course, this result may simply be due to inadequate penetration of the antibodies into the cells. Möller (1961) notes that when sections are used, some diffuse staining of the cytoplasm is seen.

A number of biochemical studies also suggest that the surface antigens of a cell are not found on intracellular membranes. Plasma membrane preparations from normal human lymphocytes, leukemic human lymphocytes, mouse lymphocytes, various mouse ascites tumor cells, and mouse liver are all enriched in these antigens (Herzenberg and Herzenberg, 1961; Boyle, 1967). Such evidence is, as mentioned previously, only as reliable as the fractionation procedure used.

A much more conclusive method for determining what percentage of the total "surface" antigen is actually found on the outside of the plasma membrane was developed by Haughton (1966). This investigator compared the absorption of H2-specific antibodies by intact mouse cells to the absorption by sonicates. Haughton used the ^{51}Cr cytotoxicity assay, which appears to be the most objective and reproducible assay for antibodies to surface antigens presently available (Sanderson, 1964; Wigzell, 1965; Rogentine and Plocinik, 1967). As is usually done, Haughton measured the absorption of specific antibodies as an inhibition of a standard assay for these antibodies. This approach requires evidence that antibodies do not penetrate into intact cells. In fact, indications are that antibodies do get into intact cells but only by the process of pinocytosis (Holtzer and Holtzer, 1960; Möller, 1961); this finding is consistent with

the large size of antibody molecules and the impermeability of plasma membranes to such large substances. During the short period of incubation used by Haughton, antibodies are not pinocytosed into intact mouse cells (Möller, 1961). Of the surface antigens of normal lymphocytes, lymphoma cells, and various ascites sarcoma cells, 69–97% were found to be actually localized on the surface of the intact cells.

Several plasma membrane isolations employing surface antigens as a marker have been reported. Wallach and his co-workers (Wallach and Hager, 1962; Wallach and Kamat, 1964, 1966; Wallach and Vlahovic, 1967) disrupted Ehrlich ascites carcinoma cells by nitrogen cavitation, fractionated the homogenate, and assayed the ability of the various fractions to absorb from horse antiserum those antibodies involved in agglutinating the intact cells. Nuclei, mitochondria, lysosomes, and pieces of endoplasmic reticulum were all found to be ineffective in absorbing out such antibodies. The plasma membrane fraction contained 85–91% of the total surface antigen.

Boone and his co-workers (1969) used a somewhat different approach. These investigators obtained horse antiserum to HeLa cells and labeled the globulins from this serum with ^{131}I . Intact cells were allowed to absorb the radioactively labeled antibody and the excess was washed away. Then the cells were homogenized and the distribution of ^{131}I followed during the isolation procedure. This method assumes that the antibodies remain attached to plasma membrane fragments during fractionation. The plasma membrane fraction was found to have a specific activity 49 times that of the homogenate.

Gahmberg and Simons (1970) found a 20-fold increase in the specific activity of surface antigens involved in agglutination in their plasma membrane fraction from baby hamster kidney cells. The pig lymphocyte plasma membrane fraction prepared by Allan and Crumpton (1970) was also greatly enriched in these antigens.

NONPENETRATING REAGENTS: It should be feasible to react intact cells with a reagent that does not penetrate into them, remove the excess reagent before homogenization, and use the bound reagent as a marker for plasma membrane distribution during subsequent fractionation. Such a reagent should be big and/or highly charged to prevent its getting into the cells and should react irreversibly (i.e., covalently) with groups on the cell surface under mild conditions, including neutral pH.

Detection of a relatively small number of the reagent molecules should also be possible. Attaching large numbers of reagent molecules to the plasma membrane would almost certainly inhibit enzymes present on this structure and would interfere with the analysis of the isolated membranes in other ways as well. In addition, extensive reaction with a reagent might damage the permeability barrier of the plasma membrane and allow reagent molecules to enter the cell (Berg, 1969).

A number of nonpenetrating reagents have been developed in the past few years, including 1-anilino-8-naphthalene sulfonate (Romeo et al., 1970) *p*-chloromercuribenzenesulfonic acid (Vansteveninck et al., 1965), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (Maddy, 1964), formyl methionyl sulfone methyl phosphate (Bretscher, 1971, 1971 *a*, 1971 *b*), and the diazonium salts of sulfanilic acid (Berg, 1969; Bender et al., 1971) and of the diiodo derivative of sulfanilic acid (Helmkamp and Sears, 1970; Sears et al., 1971). The first reagent does not form covalent bonds, the second forms reversible covalent bonds, but the others do react chiefly with functional groups on proteins to form irreversible covalent bonds. These compounds all possess a strong negative charge, which is presumably what prevents them from penetrating into intact cells.

Most investigators provide evidence that their reagent is not entering intact cells by demonstrating that the compound is capable of reacting with soluble intracellular proteins in a homogenate but that the reagent is not found bound to these proteins when the coupling reaction is performed with intact cells. A corollary to this finding is the observation that far fewer reagent molecules will attach to intact cells than attach to a homogenate of the same number of cells. A different approach was used by Ohta and his co-workers (1971), who coupled *p*-chloromercuribenzoic acid through the carboxyl group to aminoethyl-dextran chains with an average mol wt of 150,000 in order to keep the sulfhydryl reagent from getting into the cells they were studying. Using yet a third tactic, Phillips and Morrison (1970, 1971) added lactoperoxidase to intact human red cells and then added hydrogen peroxide and radioactive iodide. The iodide, which can be bound in covalent linkage to proteins, probably entered the cells; but the enzyme, which was responsible for making the covalent linkages, presumably could not. Consequently, only proteins exposed on the outside of the plasma membrane were iodinated. This method might be less

applicable to cell types like the polymorphonuclear leukocyte, which itself contains a myeloperoxidase capable of iodinating proteins.

These nonpenetrating reagents are being used to probe functional aspects of the outer surface of the plasma membrane of mammalian cells. Vansteveninck and his co-workers (1965) utilized *p*-chloromercuribenzenesulfonic acid to demonstrate that surface sulfhydryl groups are involved in the transport of glucose across the erythrocyte membrane. Bender et al. (1971) found that reaction of the diazonium salt of sulfanilic acid with intact human red cells inhibits the membrane-bound acetylcholinesterase as well as the facilitated diffusion of glucose.

Of particular interest is the use of such reagents to probe the architecture of the plasma membrane. Treatment of intact erythrocytes with radioactive nonpenetrating reagents and subsequent disk gel electrophoresis of the plasma membrane proteins revealed that only one or two of these proteins are exposed to the external medium (Berg, 1969; Bretscher, 1971, 1971 *a*, 1971 *b*). Furthermore, exhaustive reaction of intact erythrocytes with [³⁵S]formyl-methionyl-sulfone methyl phosphate and subsequent reaction of isolated ghosts with the same reagent has been used by Bretscher (1971, 1971 *a*, 1971 *b*) to show that a major plasma membrane glycoprotein extends all the way through the membrane in an oriented fashion.

Ohta and his co-workers (1971) are also interested in the architecture of membrane proteins. When these investigators reacted intact human red cells with a nonpenetrating sulfhydryl reagent, they observed that the Na⁺, K⁺-stimulated, ouabain-inhibitable ATPase was inhibited but that the ouabain-insensitive ATPase was unaffected. Reaction of the red cell stroma with the same reagent inhibited both enzymes. Ohta et al. conclude that only the protein involved in the ouabain-sensitive hydrolysis of ATP is exposed to the external medium in the intact cell. It is worth noting that the active site of the Na⁺, K⁺-ATPase of erythrocytes is thought to be on the inside of the plasma membrane (Whittam, 1962; Tenney and Rafter, 1968). Thus, inhibition of an enzyme by treatment of intact cells with a nonpenetrating reagent constitutes strong evidence that this enzyme is on the plasma membrane but does not reveal whether the active site is exposed intra- or extracellularly.

These nonpenetrating reagents have not yet been fully exploited as plasma membrane markers.

Fitzpatrick et al. (1969) administered 4-acetamido-4'-isothiocyanate stilbene-2,2'-disulfonic acid to rats 30 min before sacrificing the animals and subsequently found high levels of binding of this molecule only in the subcellular fraction of liver that contained the plasma membranes. However, a more quantitative and sophisticated use of such reagents in this area should be possible.

General Comments on Studies with Intact Cells

Studying the outer surface of intact cells in order to select plasma membrane markers appears to have great promise. This approach requires suspensions of cells with intact plasma membranes; but as discussed earlier, the development of methods for obtaining such cell suspensions from solid organs is to be encouraged in any case as a means of improving the homogeneity of the starting material used in plasma membrane isolations. The study of intact cells in suspension avoids many of the ambiguities and technical problems involved in morphological and histochemical approaches.

An additional advantage of this method is that it can easily provide a number of different plasma membrane markers. Two considerations emphasize the desirability of having more than one marker at hand. The first of these is the heterogeneity of the plasma membrane.

Evidence for this heterogeneity is growing. On the one hand, there is the morphological observation mentioned previously that lateral, apical, and basal surfaces of individual cells in solid organs are characterized by different structural specializations, such as microvilli and junctional complexes. On the other hand, histochemical studies also support this apparent heterogeneity. In tissues such as liver, kidney, pancreas, and mammary gland nucleoside triphosphatase appears generally to be localized in a restricted region of the plasma membrane (de-Thé, 1968). In rat liver, for instance, ATPase is seen on the plasma membrane only where the surface folds to form the microvilli of the bile canaliculus (Essner et al., 1958). 5'-Nucleotidase is localized in this same area as well as in the microvilli at the sinusoidal aspect of the liver cells (Essner et al., 1958). In the small intestine of the rat leucine aminopeptidase appears to be confined to the brush border of the epithelial cells (Nachlas et al., 1960).

More surprising is the revelation by histochemical techniques of heterogeneities in the plasma

membrane of free cells such as mouse lymphocytes, eosinophils, macrophages, red blood cells, plasma cells, and tumor cells (Cerottini and Brunner, 1967; Aoki et al., 1969; Aoki et al., 1970; Hämmerling, 1971; Stackpole et al., 1971). Antibodies to the surface antigens of these cells have been made visible by attaching fluorescein, ferritin, or southern bean mosaic virus to them. When these antibodies are then applied to the cells, the surface antigens are seen to be distributed in a non-homogeneous fashion; H2, G, θ , and TL antigens are all found in discrete areas on the plasma membrane.

Of course, such studies are subject to the uncertainties discussed above. In particular, it may be difficult for antibodies to penetrate to the basal surface of cells in solid organs; and labeled antibodies may not be able to penetrate into small foldings of the plasma membrane or through a surface coat. The occurrence of penetration artifacts with labeled antibodies attaching to cells in suspension is rendered less probable by the observation that it is often the protuberances of the cell surface, rather than infoldings, which are not labeled (Aoki et al., 1969; Kourilsky et al., 1971). In addition, a clever control was performed by Kourilsky and his co-workers (1971). These investigators worked with lymphocytes, monocytes, polymorphonuclear leukocytes, and several other cell types from humans. Using ferritin-labeled antibodies specific for HL·A antigens, they demonstrated discontinuous "patches" of these antigens on the cell surface. Moreover, if labeled polyclonal antisera (i.e., antisera containing antibodies to a number of different surface antigens) were subsequently used, they could label regions of the cell surface which had not absorbed HL·A-specific antibodies.

Biochemical evidence for the heterogeneity of plasma membranes has also been reported. A number of investigators have succeeded in separating isolated liver plasma membranes into two or three subfractions. Evans (1969, 1970) found that his light subfraction contained most of the 5'-nucleotidase, leucine aminopeptidase, and Mg^{++} -ATPase, while the heavy subfraction contained most of the Na^+ , K^+ -ATPase. House and Weidemann (1970) were partially able to separate alkaline phosphatase on the one hand from 5'-nucleotidase, ATPase, phosphodiesterase, and insulin binding on the other. Thines (cited by de Duve, 1971) has isolated rat liver plasma

membranes, broken them into smaller pieces, and demonstrated that 5'-nucleotidase, alkaline phosphodiesterase I, and alkaline phosphomonoesterase segregate with membrane fragments of different densities and cholesterol contents. Subfractions of plasma membranes from human blood platelets (Barber and Jamieson, 1970) and pig liver (Lutz and Frimmer, 1970) have also been prepared. Such studies suggest the existence of biochemically distinct areas on the plasma membrane.

The use of multiple markers for the plasma membrane may allow the investigator to deal with such heterogeneities. A priori, it seems likely that the more markers that are used, the more certain one can be of following the distribution of all the fragments of the plasma membrane.

A second reason for using more than one plasma membrane marker is revealed by consideration of a statement made by Korn and his co-workers (Ulsamer et al., 1971): "To measure the purification of plasma membranes by the purification of a specific enzyme depends on the enzyme being a true component of the plasma membrane and only of the plasma membrane." If a single marker is being used, this principle is essentially correct; if two or more are being used, it is not necessarily so.

A ratio of different enzymatic activities or other markers may be characteristic of the plasma membrane itself, even though the individual activities are not localized exclusively on this membrane. A priori, it seems likely that the more markers that are used, the more characteristic the ratio of these markers will be. The best and perhaps only means of determining such a ratio before beginning the fractionation is to select plasma membrane markers by studying the outer surface of intact cells. This approach will not work, of course, if the individual markers are segregated onto different fragments of the plasma membrane upon homogenization.

Naturally, the simplest situation arises when the marker selected is exclusively located on the plasma membrane. When this marker is chosen by studying the outside of intact cells, the possibility of such an exclusive localization can be easily tested by comparing the quantity of marker present in the intact cells to the amount measured in a homogenate or sonicate. This comparison does, however, present one problem. Upon disruption the plasma membrane may form closed semipermeable vesicles; and in some or all of these vesicles the outer surface of the plasma membrane

may be facing the inside. As a result, some amount of the marker present on the outside of the intact cell may be masked in the homogenate or sonicate. If this masked amount is equal to or greater than the quantity of the marker present on intracellular structures, the conclusion that the marker is found exclusively on the plasma membrane may be incorrectly drawn.

One way to eliminate this problem would be to perform all assays, except those on the intact cells, in the presence of something which disrupts closed vesicles, e.g., a detergent. Unfortunately, many membrane-bound enzymes, including 5'-nucleotidase (Emmelot et al., 1964), are stimulated or inhibited by detergents. The possibility of such an effect could complicate things even further.

One final advantage is to be gained by selecting plasma membrane markers through the study of intact cells in suspension. Such an investigation yields information about the "sidedness" of the plasma membrane. For example, assaying enzymes on the outside of cells and subsequently assaying enzymes on the isolated plasma membranes allows the active sites of these enzymes to be characterized as facing the external medium or the cytoplasm. Such information about sidedness will be invaluable in unravelling the mysteries of plasma membrane function.

Before leaving the subject of plasma membrane markers, it is necessary to caution against a practice which is becoming increasingly common. Nachman and his co-workers (Nachman et al., 1971) candidly state what many other investigators tacitly assume: we are "making the assumption that these marker enzymes [including 5'-nucleotidase] are distributed in macrophages as they are in hepatocytes." In addition, the macrophages referred to are from rabbits, while the hepatocytes mentioned come from rats. Such assumptions involve a dangerous lack of rigor; and they have a tendency to become self-fulfilling, since the plasma membrane fraction isolated using a given marker is bound to contain that marker.

5'-Nucleotidase is the enzyme most commonly assumed to be on the plasma membrane of mammalian cells. However, using histochemical techniques, Wallach and Ullrey (1962) found this enzyme to be localized exclusively in the nuclei of Ehrlich ascites carcinoma cells. McKeel and Jarett (1970) could not detect 5'-nucleotidase in any of their fractions obtained from rat fat cells.

Adenyl cyclase is also gaining popularity as a

plasma membrane marker, but the localization of this enzyme in different tissues is far from established. It has been found on the plasma membrane of liver, fat, and kidney cells; in the mitochondria of testis; on the endoplasmic reticulum of fat cells and cardiac muscle; and in both the mitochondria and endoplasmic reticulum of skeletal muscle, brain cortex, and pineal gland (Jost and Rickenberg, 1971). Whether these differences are real or arise from the difficulties involved in tissue fractionation remains to be seen.

Finally, studies in our laboratory have compared the hydrolysis of added ATP, AMP, and *p*-nitrophenyl phosphate by intact guinea pig polymorphonuclear leukocytes, monocytes, lymphocytes, and eosinophils. The differences are striking; for instance, the polymorphonuclear leukocytes have a very active ecto-AMPase, but intact monocytes do not hydrolyze added AMP at all. The existence of these differences in the outer surface of such closely related cells illustrates well the profound danger of assuming that plasma membrane markers for the rat liver cell will be of other cell types as well.

Markers for Intracellular Entities

In addition to markers for the plasma membrane itself, investigators must have at hand markers for the other known components of a tissue homogenate in order, among other things, to assess the contamination of the final preparation. This contamination cannot be adequately assessed with the electron microscope alone. This is clear from descriptions such as that given by Coleman and Finean (1966), who prepared plasma membranes from rat liver: "Prominent features of the preparations were bile canaliculi and large segments of opposed membranes linked by desmosomes. Also present were numbers of large and small vesicles." These vesicles of unknown origin are universally seen in electron micrographs of plasma membrane fractions. Nonetheless, some investigators use morphological criteria exclusively to assess the purity of their preparations.

A set of generally accepted markers for the various cellular organelles has been established. However, variability between tissues and species should prompt the investigator to consider carefully the applicability of these markers in any particular case (see de Duve, 1971). DNA is the marker most often used to follow the distribution of nuclei, although NAD pyrophosphorylase is also

occasionally used. Succinate dehydrogenase is the most common mitochondrial marker; but other mitochondrial enzymes such as glutamate dehydrogenase have also been used to follow the fractionation of this organelle.

Esterase and glucose-6-phosphatase activities are employed to detect membranes from the rough and smooth endoplasmic reticulum; RNA can also be used to detect rough endoplasmic reticulum. The distribution of acid phosphatase is considered a reliable indicator of the distribution of lysosomes. However, it seems advisable to assay for more than one lysosomal enzyme, since several subclasses of lysosomes separable from each other by differential and density gradient centrifugation are known to exist (e.g., Michell et al., 1970). Catalase, or urate oxidase, where appropriate, may be used as markers for peroxisomes and uridine diphosphatase or *N*-acetylglucosamine- β -galactosyltransferase as markers for membrane fragments derived from the Golgi apparatus. Finally, enzymes involved in glycolysis and the hexose monophosphate shunt are used as markers for the soluble cytoplasm.

A number of these markers are rather easily removed from the organelles with which they are associated in the intact cell. If excessive forces are applied during homogenization, organelles can be broken; and as a result, the membranes of the nucleus can be separated from the DNA, the membranous sacs of the lysosomes may lose their acid hydrolases, and peroxisomes may also lose their contents. Under these circumstances the investigator can no longer adequately follow the distribution of nuclear, lysosomal, and peroxisomal membranes.

A similar problem arises when mitochondria are disrupted. Some investigators have circumvented the problem in this case by using two or more mitochondrial markers—for instance, cytochrome *c* oxidase for the inner and monoaminoxidase for the outer membranes (Barclay et al., 1967; Erecinska et al., 1969). A better solution to all of these difficulties introduced by the fragmentation of organelles would be to avoid such fragmentation.

Finally, as de Duve (1964) points out, "We need only glance through a few randomly selected electron micrographs of tissue sections to realize how many structural components of as yet unknown biological significance are likely to be present in subcellular fractions." Unknown components may seriously contaminate plasma membrane preparations without being recognized.

DISRUPTION OF TISSUES

Disruption of tissues is still something of an art. A typical publication on plasma membrane isolation describes in great detail the composition of the homogenization medium, the ratio of tissue weight to volume of medium, the type of homogenizer employed, the clearance between the pestle and the homogenization vessel, the speed of the pestle, the number of up-and-down strokes applied, and even the extent to which the homogenate should be diluted after disruption is completed. The reader is warned that if he wants to reproduce the reported isolation scheme, he would be well advised to use exactly the same homogenization procedure.

This warning should be heeded. A relatively slight decrease in the pH of the homogenizing medium may make the cells more resistant to homogenization (Bell et al., 1971) and may increase the amount of cytoplasmic material that adheres to the plasma membrane fragments (Anderson et al., 1968). The homogenization procedure may be made more effective by adding 0.5 mM Ca^{++} to the medium (Takeuchi and Terayama, 1965); on the other hand, 5 mM ethylenediaminetetraacetate (EDTA) may be what is required (Miller and Crane, 1961). One of the most thorough studies on the effect of different homogenization methods was carried out by Wolff and Jones (1971) using bovine thyroid, a particularly difficult tissue to disrupt. These researchers found that disruption with a Potter-Elvehjem homogenizer yielded plasma membranes containing adenyl cyclase activity which would have been sensitive to thyroid-stimulating hormone but was not. In addition, yields of plasma membrane from homogenates prepared with a motor-driven Teflon pestle and a glass vessel (modified Potter-Elvehjem) were very low. Use of a Dounce homogenizer required the application of too much force, presumably resulting in fragmentation of intracellular organelles, and nitrogen cavitation gave a plasma membrane preparation with an unusually large microsomal contamination. There seems to be little doubt that many of the reported differences between various plasma membrane preparations from the same tissue are caused by the use of different homogenization procedures.

The importance of the small details of homogenization means that an investigator who wishes to isolate plasma membranes from a tissue which has

not previously been used as the starting material for such a study must devote a considerable amount of time to determining the optimal conditions for homogenization. Even if these conditions have already been determined for a tissue closely related to the one he plans to study, the investigator cannot assume that employing the reported procedure will bring him success. A dramatic example comes from studies with polymorphonuclear leukocytes. Cohn and Hirsch (1960) found that rabbit cells of this type were disrupted by one wash in cold 0.34 M sucrose followed by resuspension in the same medium with vigorous pipetting; less than 1% of the cells remained intact after this treatment. Similar treatment of guinea pig polymorphonuclear leukocytes leaves almost all of the cells intact (DePierre and Karnovsky, unpublished observations).

Criteria for Evaluating Tissue Disruption

At least four criteria should be applied in assessing the effectiveness of schemes for tissue disruption before isolation of plasma membranes. First, separation of the outer membrane of an organelle from its inner membrane or contents must be minimized. As explained in the previous section, such fragmentation renders traditional markers worthless for following the distribution of nuclear, lysosomal, and peroxisomal membranes. Nuclear and outer mitochondrial membranes are especially worrisome, since these membranes have buoyant densities close to those reported for plasma membranes (Kashnig and Kasper, 1969). It is disturbing to see how many investigators declare their plasma membrane preparations to be free of mitochondrial contamination on the basis of electron microscopy without once considering the possibility of contamination by membranes from disrupted mitochondria.

Testing biochemically for such fragmentation of organelles is relatively simple. For example, intactness of the nuclei can be evaluated by determining how much of the total DNA can be sedimented by low speed centrifugation. Thus, Allan and Crumpton (1970) demonstrated that 96% of the DNA of pig lymphocytes could still be sedimented at 300 *g* after homogenization. Similarly, it has been shown in our laboratory that after careful disruption of 99% of guinea pig polymorphonuclear leukocytes, 95% of the DNA was still pelleted by low gravitational forces (DePierre and Karnovsky, unpublished data).

Since nuclear membranes could conceivably be stripped off without seriously fragmenting the nuclear contents, the test for nuclear intactness by sedimentability should ideally be used in conjunction with electron microscopy. The intactness of lysosomes and peroxisomes can be evaluated by measuring the activity of an acid hydrolase and of catalase or urate oxidase, respectively, in the presence and absence of a detergent, i.e., by measuring the latency of these enzymes. However, unless one is specifically interested in obtaining intact lysosomes or peroxisomes, sedimentation of these enzymes is probably a better criterion to use. It is quite possible that the membranes of lysosomes and peroxisomes could be damaged slightly enough to allow the entrance of substrates without allowing the release of enclosed enzymes. After disruption of guinea pig polymorphonuclear leukocytes using our procedure (DePierre and Karnovsky, unpublished data), lysosomal enzymes demonstrated 60–70% latency and 85–95% sedimentability, showing that the lysosomes survived the homogenization procedure relatively intact. We used a similar approach to demonstrate the intactness of mitochondria in the homogenate: the activity of glutamate dehydrogenase, which is located exclusively inside the mitochondria (Ashwell and Work, 1970) and cannot be detected if the mitochondria are intact, was five times as great in a sonicate as it was in the homogenate, i.e., glutamate dehydrogenase was 80% latent. These biochemical tests for the fragmentation of organelles are valuable enough and simple enough to be performed routinely on tissue homogenates. Unfortunately, they are virtually never performed in studies whose object is the isolation of plasma membranes.

A second criterion for evaluating the effectiveness of different homogenization procedures is the size of the plasma membrane fragments which are produced. In general, large fragments are easier to separate from other components of the homogenate than are small fragments. Microsomes, the major contaminant of almost all plasma membrane preparations (Finean et al., 1966; Benedetti and Emmelot, 1968; Boone et al., 1969; McKeel and Jarrett, 1970), are especially difficult to separate from small plasma membrane vesicles. The ideal homogenization procedure would yield large "ghosts" which would sediment in the nuclear fraction and could then be separated from the nuclei on the basis of their lower buoyant density. However, excluding results obtained through the use of chemical agents, ghosts have been obtained

from only a very few cell types, including the erythrocyte and HeLa cells (Boone et al., 1969; Atkinson and Summers, 1971). In most cases the investigator must work with smaller plasma membrane fragments.

A third criterion for the effectiveness of a homogenization scheme is the minimization of various aggregation artifacts which arise when cells are disrupted. It is possible, and perhaps to some extent even unavoidable, that plasma membrane fragments will coaggregate with other structures in the homogenate; this makes the separation of plasma membrane from the component to which it is stuck extremely difficult, if not impossible. Entrapment of cytoplasm and perhaps even of cytoplasmic organelles inside plasma membrane vesicles may also occur. Another phenomenon which may lead to erroneous conclusions is the adsorption of soluble proteins onto plasma membrane fragments. Such adsorption can be quite substantial; Benedetti and Emmelot (1967) found that 18–33% of the protein in their isolated rat liver plasma membranes was readily solubilized by physiological saline and was therefore probably adsorbed soluble protein. Apparently, positively charged soluble proteins show considerable affinity for the negatively charged plasma membrane fragments. The opposite phenomenon, i.e. loss of material which properly belongs to the plasma membrane due to homogenization and isolation procedures, may also occur.

Finally, as many of the cells should be disrupted as possible. The larger the percentage of cells that are broken, the larger is the potential yield of plasma membrane. However, this consideration is distinctly less important than the other three criteria for effective homogenization, especially if relatively large amounts of homogeneous starting material can be obtained without undue difficulty. The extent to which the cells have been disrupted is usually ascertained by phase-contrast microscopy.

Conditions for Tissue Disruption

The two major variables in tissue disruption are the composition of the medium and the type of force or forces used to break the cells. The major factors in the composition of the medium include the nature (i.e., ionic or nonionic) of the major solute, the concentration of this solute, the pH, and the presence of small amounts of additional substances, such as divalent cations or EDTA.

The major solute in virtually all homogenization media used in plasma membrane isolation procedures is sucrose. For some as yet unexplained reason, cells are often more easily disrupted in sucrose than they are in an ionic medium such as Krebs-Ringer phosphate solution. Furthermore, salts cause aggregation of the particles in liver homogenates (de Duve, 1967). It should be noted, however, that sucrose is not always the solute of choice. Sucrose, not salts, causes aggregation in homogenates prepared from the spleen (de Duve, 1967), and 0.25 M sucrose disrupts the nuclei of pig lymph node cells (Allan and Crumpton, 1970).

Should the medium be isotonic or hypotonic? This remains a controversial question. The most commonly used homogenization medium was developed by Neville (1960) for rat liver and later used by Emmelot and his co-workers (1964) in their studies on the same tissue. These investi-

gators homogenized in ice-cold water buffered with 1 mM NaHCO₃. The apparent advantage of using such a hypotonic medium is that it makes use of osmotic forces to disrupt the cells and thereby reduces the amount of mechanical force which must be applied. This procedure might thus be expected to result in larger plasma membrane fragments. However, the dangers are great, especially since many investigators carry out subsequent steps of their isolation scheme in the same hypotonic medium employed for homogenization. Nuclei, mitochondria, and lysosomes are all known to lyse under hypotonic conditions. Coleman and Finean (1966), who used hypotonic homogenization and isolation conditions to obtain plasma membranes from various tissues of the guinea pig, state: "That the fragmentation of other subcellular organelles had taken place under the hypotonic isolation conditions was apparent from electron micrographs."

Despite the dangers, ice-cold water containing 1 mM NaHCO₃ has been used as the homogenization medium in preparing plasma membranes from the *liver* of

rats	(Neville, 1960; Davidson et al., 1963; Tria and Barnabei, 1963; Skipski et al., 1965; Ashworth and Green, 1966; Coleman and Finean, 1966, 1967; Emmelot and Bos, 1962, 1966, 1969; Finean et al., 1966; Higgins and Green, 1966; Barclay et al., 1967; Lansing et al., 1967; Lieberman et al., 1967; Song and Bodansky, 1967; Dod and Gray, 1968; Graham et al., 1968; Erecinska et al., 1969; Evans, 1969; Song et al., 1969; Wattiaux-de Coninck and Wattiaux, 1969; Evans, 1970; Skidmore and Trams, 1970; Ray, 1970; Barancik and Lieberman, 1971; Franklin and Trams, 1971),
guinea pigs	(Coleman and Finean, 1966, 1967),
mice	(Evans, 1970),
pigs	(Lutz and Frimmer, 1970),
the <i>kidneys</i> of	
rats	(Davidson et al., 1963),
guinea pigs	(Coleman and Finean, 1966, 1967),
bovine <i>thyroid</i>	(Yamashita and Field, 1970),
bovine <i>mammary gland</i>	(Keenan et al., 1970),
rat <i>brown and white adipose tissue</i>	(Giacobino and Perrelet, 1971).

Strongly hypotonic conditions with Tris replacing the bicarbonate have been used in the homogenization of

<i>HeLa Cells</i>	(Atkinson and Summers, 1971).
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Many investigators, notably Takeuchi and Terayama (1965), have expressed concern over the lysis of organelles which might result from hypotonicity and have developed homogenization procedures using an isotonic sucrose medium. With rat liver as a starting material, Berman and his co-workers (1969) tried both Neville's hypotonic isolation procedure and a modification of the procedure developed by Takeuchi and Terayama; they then compared the gel electrophoresis patterns of the proteins from the two plasma membrane preparations and found marked differences. Other investigators have also used isotonic homogenization media in the preparation of plasma membranes from

rat <i>liver</i>	(Ashworth and Green, 1966; Higgins and Green, 1966; Graham et al., 1968; Erecinska et al., 1969; Henning et al., 1970; Hinton et al., 1970),
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rat fat cells	(McKeel and Jarett, 1970),
Ehrlich ascites carcinoma cells	(Wallach and Ullrey, 1962 <i>a</i> ; Wallach and Kamat, 1964, 1966),
rabbit small intestinal epithelium	(Porteous and Clark, 1965),
rat kidney	(Fitzpatrick et al., 1969),
baby hamster kidney	(Gahmberg and Simons, 1970),
rabbit polymorphonuclear leukocytes	(Wieneke and Woodin, 1967; Woodin and Wieneke, 1966 <i>a</i> , 1966 <i>b</i> , 1970).

An interesting compromise involves homogenizing the cells rapidly in a somewhat hypotonic medium, thus utilizing osmotic forces to disrupt the cells, and then adding an aliquot of concentrated sucrose or salt to return the medium to isotonicity, thus saving the intracellular organelles from any more osmotic damage than they have already suffered. For example, Boone and his co-workers (1969) homogenized HeLa cells in 10 mM Tris and immediately afterwards added enough 60% sucrose to give a final concentration of 0.25 M. These investigators found no morphological evidence for disruption of nuclei; unfortunately, they performed no other tests for the fragmentation of organelles. On the other hand, in experiments in our laboratory, guinea pig polymorphonuclear leukocytes were disrupted in 75 mM sucrose-5 mM NaHCO₃. Immediately afterwards, enough 9.0% NaCl was added to make the solution isotonic, and extensive biochemical tests for the fragmentation of organelles were performed. The results indicate good preservation of organelle structure.

A strong prejudice exists for buffering the homogenization medium at pH 7.4. Some evidence arguing against the use of lower pH's has been reported. Using dog liver cells, Bell and his collaborators (1971) found that pH 6.9 rendered about one-half of the cells resistant to homogenization conditions which disrupted all the cells at pH 7.4; the effect was reversed by raising the pH again. With rat liver Anderson and his co-workers (1968) found that as the pH of the homogenization medium was raised from 5.8 to 7.8, the efficiency of homogenization under controlled conditions increased while the amount of cytoplasm attached to the isolated cell membrane decreased. In our studies (DePierre and Karnovsky, unpublished data) guinea pig polymorphonuclear leukocytes were disrupted in a medium buffered at pH 8.0-8.2 by bicarbonate with no ill effects. Indeed, this high pH seemed to increase the percentage of cells which were disrupted and to de-

crease the coaggregation of different organelles.

Finally, a number of special ingredients have been added to the homogenization medium by various investigators. Hunter and Commerford (1961) found that Ca⁺⁺ was necessary to keep the nuclei intact during pressure homogenization of the livers of various animals. Hinton and his collaborators (1970) also found that divalent cations preserved nuclear structure and obviated the need for homogenizing rat liver under hypotonic conditions. Ray (1970) reports that 0.5 mM Ca⁺⁺ increased his yield of plasma membranes from rat liver. Divalent cations have also been added to the media used for homogenizing HeLa cells (Boone et al., 1969), rat hepatomas (Emmelot and Bos, 1966, 1969), Ehrlich ascites carcinoma cells (Wallach and Ullrey, 1962 *a*; Wallach and Kamat, 1964, 1966), and rat white and brown adipose tissues (Giacobino and Perrelet, 1971). On the other hand, EDTA in concentrations of 1-5 mM was helpful in disrupting dog liver cells (Bell et al., 1971), hamster small intestinal epithelial cells (Miller and Crane, 1961; Holt and Miller, 1962), rabbit small intestine (Porteous and Clark, 1965), rat intestinal epithelium (Forstner et al., 1968), rat fat cells (McKeel and Jarett, 1970), rat kidney (Fitzpatrick et al., 1969), rabbit polymorphonuclear leukocytes (Woodin and Wieneke, 1966 *a*, 1966 *b*, 1970; Wieneke and Woodin, 1967), and HeLa cells (Bosmann et al., 1968). The effectiveness of both divalent cations and EDTA may be related to the importance of divalent cations in holding tissues together and in maintaining the structural integrity of membranes in vivo.

The chief forces employed to disrupt tissues for plasma membrane isolation are osmotic forces, mechanical forces, and the force produced when a sudden decrease in pressure causes a gas to come out of solution ("the bends"). The use of hypotonic media, as discussed above, is not the only way to produce osmotic forces across a plasma membrane. Barber and Jamieson (1970) wanted

to isolate plasma membranes from human blood platelets; but they discovered that the small volume of the platelets rendered them resistant to disruption by "no clearance" tissue grinders, the nitrogen bomb, and even sonication. These investigators decided to load the platelets with glycerol. When the inside concentration of glycerol reached 4.3 M, the loaded platelets were placed in 0.25 M sucrose-0.01 M Tris, pH 7.5, and 80-90% of them lysed. Barber and Jamieson report that electron microscopy revealed no extensive damage to intracellular organelles from this procedure.

Mechanical disruption of tissues is most commonly achieved with a Potter-Elvehjem or Dounce homogenizer (de Duve, 1971). The favorite current version of the Potter-Elvehjem is a motor-driven Teflon pestle inserted into a smooth-walled glass tube. The Dounce homogenizer is operated manually and the pestle has a spherical head. Of the two, the Dounce device is more gentle and works well for free cells like HeLa cells (Bosmann et al., 1968; Boone et al., 1969). However, solid organs have a tough connective tissue framework which requires use of the rougher Potter-Elvehjem homogenizer for effective disruption.

In general, investigators attempt to apply mechanical forces in such a way that large fragments of plasma membrane are obtained and intracellular organelles are damaged minimally. For example, a loose-fitting pestle is often employed and only a few up-and-down strokes are performed. Such precautions are successful in producing large fragments from solid organs, where the plasma membranes are reinforced by junctional complexes and perhaps by the extracellular matrix as well; but de Duve (1971) points out that even the gentle disruption of rat liver using a Potter-Elvehjem homogenizer damages at least 15% of the lysosomes and strips the outer membrane off 10% of the mitochondria.

A third force for disrupting tissues was first employed by Hunter and Commerford in 1961 and has subsequently been used by Wallach and Kamat (1964, 1966) in their isolation of plasma membranes from Ehrlich ascites carcinoma cells. If a cell suspension is subjected to a gas (usually nitrogen) under high pressure and then returned suddenly to atmospheric pressure, the cells break. This disruption probably results from the transient formation of bubbles as the gas comes out of solution. Wallach and Kamat praise this procedure as giving quantitative disruption of

cells under isosmotic conditions in an inert atmosphere without danger of local heating. These investigators state that if divalent cations are present, no nuclear breakage occurs, but they do not examine the condition of other organelles in the homogenate. After all, bubbles might form inside the organelles as well as within the cytoplasm. A serious disadvantage of this technique is that the plasma membrane is fragmented into rather small pieces which cannot be separated from microsomes without some manipulation. Nitrogen cavitation or the nitrogen bomb, as this procedure is called, has also been used to homogenize rat liver (Ashworth and Green, 1966; Higgins and Green, 1966; Graham et al., 1968; Erecinska et al., 1969) and baby hamster kidney cells (Gahmberg and Simons, 1970).

One final danger involved in homogenization is worth noting. When cells are broken, the plasma membrane may be exposed to enzymes to which it is not normally accessible. Meldolesi and his co-workers (1971) express misgivings that proteases may become activated during fractionation of pancreatic cells. More alarmingly, Atkinson and Summers (1971) find that their HeLa cell ghosts can be degraded by enzymes in the homogenate. Sulfhydryl reagents are required to prevent this degradation; low temperature alone was not enough. This finding is not totally unexpected, since some enzymes, e.g. pancreatic lipase (Meldolesi et al., 1971), are known to be active in the cold. The investigator should be on the lookout for such degradation.

FRACTIONATION

The properties of a subcellular particle that can be used as a basis for separating it from other particles are extremely limited in number. They include mass, volume, the ratio of mass to volume (i.e., density), shape, and the ratio of electrostatic charge to volume, which determines the electrophoretic mobility. Two fractionation techniques, together involving all but one of these properties, are commonly used to separate plasma membrane fragments from the other components of a homogenate. Investigators usually start with differential centrifugation and then subject that fraction most enriched in plasma membrane to isopycnic density gradient centrifugation.

Separation of subcellular particles by differential centrifugation is based on differences in the sedimentation coefficients of these particles.

The sedimentation coefficient is a complex function of the volume, shape, and density of the particle, as well as of the viscosity and density of the medium (de Duve et al., 1959). The medium used in differential centrifugation has a density less than the buoyant density of all the components of the homogenate; in the case of plasma membrane isolation, it is most often the same as the homogenization medium. As a result, all of the subcellular particles present can be pelleted. The object is to determine what combination of gravitational force and time of centrifugation will selectively pellet each particle type.

A number of investigators have based their plasma membrane isolations on differential centrifugation alone (Miller and Crane, 1961; Holt and Miller, 1962; Hübscher *et al.*, 1965; Porteous and Clark, 1965; Carroll and Sereda, 1968). Generally, the sedimentation coefficients of the various cellular organelles are not dissimilar enough to allow their clean separation simply by differential centrifugation. Usually, one or more isopycnic density gradient centrifugations are subsequently performed.

The medium used in such centrifugation forms a fairly steep density gradient extending over the range of densities of the particles to be separated. This gradient in density is usually established by a gradient in sucrose concentration. However, this approach establishes an osmolarity as well as a density gradient; the extra complication can be avoided by using a high molecular weight solute like Ficoll (Wallach and Kamat, 1966). Centrifugation is continued until each particle reaches the position corresponding to its own density.

A number of variations on this theme are possible (de Duve et al., 1959; Anderson, 1955, 1966; de Duve, 1964, 1967, 1971; Anderson et al., 1968). For instance, the density gradient may be continuous or discontinuous. A discontinuous gradient creates the illusion of clear-cut separation and is thus very popular with investigators using the preparative approach. However, a continuous gradient would be expected to give the best separation (Wallach and Kamat, 1966).

Isopycnic density gradient centrifugation has proven rather successful in isolating plasma membrane fragments from the other components of tissue homogenates (e.g., Emmelot et al., 1964; Eichholz and Crane, 1965; El-Aaser et al., 1966; Barclay et al., 1967; Neville, 1967; 1968; Stein et al., 1968; Boone et al., 1969; Molnar et al.,

1969; Barber and Jamieson, 1970; McKeel and Jarrett, 1970). In the case of rat liver the plasma membrane fragments bands at a buoyant density of 1.16–1.19 (e.g., Weaver and Boyle, 1969; Ray, 1970).

It should not be forgotten that the plasma membrane often fragments into semipermeable vesicles and that the density of such vesicles is affected by the osmotic activity, the pH, the ionic strength, and the composition of the medium they are in. Wallach and Kamat (Wallach and Kamat, 1964; Kamat and Wallach, 1965; Wallach et al., 1966 *a*) did a systematic study of the effects of such factors on the buoyant density of plasma membrane vesicles from Ehrlich ascites carcinoma cells in order to determine the conditions for optimal separation of these vesicles from microsomes. Similarly, de Duve (1964) has demonstrated that mitochondria, lysosomes, and peroxisomes are separable by isopycnic density gradient centrifugation in certain media but not in others.

Electrophoresis has not yet been applied extensively to the isolation of plasma membranes or of other cellular organelles. Sellinger and Borens (1969) have performed a zonal density gradient electrophoresis on membranes of brain cortex. These investigators obtained a single, homogeneous electrophoretic peak containing the highest specific activities of both *N*-acetylneuraminic acid and acetylcholinesterase. This fraction presumably contained plasma membranes from synaptic regions.

ANALYSIS: COMPARISON OF THE PREPARATIVE AND ANALYTICAL APPROACHES

Once a homogenate has been separated into a number of distinct fractions, the investigator interested in studying the plasma membrane has two alternatives. He may discard all of the fractions except the one he considers to be most enriched in plasma membrane and concentrate his analysis on this single fraction; this is called the *preparative approach*. Or he may choose the *analytical approach*, which involves analysis of all the fractions obtained and makes no initial assumptions about the identity of these fractions.

As has been stressed time and time again by de Duve (1964, 1967, 1971), the analytical approach is much to be preferred over the preparative one. In theory, it is perfectly acceptable to

make a pure preparation of a given organelle and to conclude that the properties of this preparation are those of the organelle. For bulk properties such as total nitrogen content, total lipid content, and light scattering, this approach is probably valid in practice as well. However, the enzymatic activities and individual chemical constituents of a fraction cannot be assigned unequivocally to the major component of that fraction, because in practice it has simply been impossible to obtain a preparation of a given organelle which is free enough from contamination by other organelles. This fact is essentially ignored by the preparative approach, with the result that many cytoplasmic enzymes were once thought to be localized in the nucleus and mitochondria were once thought to contain what are now known to be lysosomal and peroxisomal enzymes (de Duve, 1964).

The analytical approach to subcellular fractionation is characterized by a balance sheet wherein are given measurements of marker and other enzymes, chemical constituents, and other things of interest for the homogenate and for all fractions subsequently obtained. Such a balance sheet is very useful in a number of ways. In the first place, the percentage recovery of an enzyme or chemical constituent can easily be determined by comparing the amount present in the homogenate to the total amount present in all the various fractions. This recovery value tells the investigator if he has lost material at some point and reveals the inactivation of enzymes during the fractionation procedure, should this occur. The last point is particularly important. If the plasma membrane fraction is found to contain no succinate dehydrogenase, for instance, the investigator must be certain that this finding is not simply due to inactivation of succinate dehydrogenase. An investigator using the preparative approach has no control for this possibility. Also, the recovery values may reveal unexpected but important phenomena; recoveries of well over 100% for the acid hydrolases eventually led to the discovery of lysosomal latency (de Duve, 1967).

The greatest value of a balance sheet is that it can be used to determine the subcellular localization of an enzymatic activity or a chemical constituent. This is done by comparing the percentage of the activity found in the various fractions with the percentage distributions of the different markers; if the activity in question distributes

in a manner identical to that of one of the markers, the two are probably located on the same particle. Using this approach, Michell and his collaborators (1967) demonstrated that the phosphatidylinositol kinase of rat liver distributes in the same manner as 5'-nucleotidase, a presumptive plasma membrane marker. Studying the same tissue Erecinska and his co-workers (1969) discovered that phosphodiesterase I distributed in a manner very similar to that of 5'-nucleotidase.

This approach can even be used to identify new organelles: if an enzymatic activity or chemical constituent does not distribute in a manner identical to that of any marker, it may be localized on an as yet unknown subcellular particle. It was in this way that lysosomes and peroxisomes were discovered (de Duve, 1964). Thus, the analytical approach even helps the investigator to avoid assigning the properties of unknown contaminants to his plasma membranes. Of course, this approach requires reasonable confidence in the markers being used, and ideally, two activities should be shown to distribute together in several fractionation schemes before it is finally concluded that they are localized on the same particle.

Clearly, the analytical approach should be used routinely in subcellular fractionation studies. Unfortunately, only a very few plasma membrane studies have been analytical (e.g., Touster et al., 1970; Erecinska et al., 1969). In most cases investigators do not even determine the percentage recovery of the presumptive plasma membrane marker in their presumptive plasma membrane fraction. Determination of this recovery is at least as important as determination of the specific activity. For instance, the usual procedure for isolating rat liver plasma membranes gives a preparation with a specific activity of 5'-nucleotidase about 20 times that of the homogenate (Touster et al., 1970; Wattiaux-de Coninck and Wattiaux, 1969; Weaver and Boyle, 1969; Evans, 1970); but the recovery of this enzyme is generally 5–10% or less (Weaver and Boyle, 1969; Song and Kappas, 1969; Evans, 1970). In evaluating such a preparation, the investigator must consider two possibilities: the yield of plasma membrane could be very low, in which case a certain subpopulation of plasma membrane fragments may have been selectively isolated; or most of the 5'-nucleotidase could be located on other structures and this enzyme may simply not be a good marker for rat liver plasma membranes.

SPECIAL APPROACHES TO THE ISOLATION OF PLASMA MEMBRANES

A number of special methods for the isolation of plasma membranes have been developed, and three of these should be mentioned. In 1966 Warren, Glick, and Nass reported a technique which has become relatively popular. Working with mouse fibroblasts, these investigators swelled the cells in a hypotonic medium and then "stabilized" the plasma membrane in a "stretched" state. The stabilizers used included fluorescein, mercuric acetate, 5,5'-dithiobis(2-nitrobenzoic acid), Zn^{++} , acetic acid, and Tris. The first two of these are sulfhydryl reagents; but what exactly any of these substances does to the plasma membrane is unclear. When the treated cells were disrupted with a Dounce homogenizer, the plasma membrane came off in large fragments. The size of these pieces provided a morphological assay for the plasma membrane and made its separation from other components of the homogenate comparatively easy.

In addition to its use with mouse fibroblasts (see also Warren and Glick, 1968; Weinstein, 1968), this approach has been applied to rat bladder epithelium (Hicks and Ketterer, 1970) and kidney cells from rhesus monkey and hamster (Klenk and Choppin, 1969). A variation of the Warren method, using glutaraldehyde as the stabilizing agent, was developed by Nachman and his collaborators (1971) to isolate plasma membranes from rabbit alveolar macrophages.

This approach is worth exploring further, but warily. Whatever the stabilizing agents do to the plasma membrane may result in inactivation of enzymes localized on this structure. As discussed previously, glutaraldehyde fixation certainly results in such inactivation. The stabilizing agents may also strengthen the adsorption of cytoplasmic elements to the plasma membrane. Warren and his co-workers (1966) saw material attached to the undersurface of their isolated plasma membranes in electron micrographs. Manson and his collaborators (1968) also saw intracellular material attached to the plasma membranes they isolated using fluorescein mercuric acetate.

Heine and Schnaitman (1971) applied to mammalian cells a method originally developed by Wetzel and Korn (1969) for *Acanthamoeba*. Heine and Schnaitman allowed human L cells to phagocytize polystyrene latex beads, during which

process 30% of the plasma membrane was internalized, according to their calculations. The cells were then disrupted using nitrogen cavitation, and the phagosomes could be cleanly separated from other components of the homogenate on a discontinuous sucrose density gradient because of the beads they contained. Heine and Schnaitman maintain that the membranes of the isolated phagosomes represent a relatively pure plasma membrane preparation.

This approach must be used with serious reservations. In the first place, the membrane of the isolated vesicles may derive from lysosomal membranes as well as from the plasma membrane. Fusion between phagocytic vacuoles and lysosomes occurs rapidly (Zucker-Franklin, 1968). Phagosomes isolated from *Acanthamoeba* (Wetzel and Korn, 1969), human polymorphonuclear leukocytes (Stossel et al., 1971, 1971 a; Smolen et al., 1971), guinea pig polymorphonuclear leukocytes (Stossel et al., 1971, 1971 a), and rabbit alveolar macrophages (Stossel et al., 1971; Nachman et al., 1971) all contained considerable amounts of lysosomal acid hydrolases. Heine and Schnaitman (1971) did not assay their phagosomes for lysosomal enzymes. Another consideration in using this method is the distinct possibility that phagocytosis may involve specialized regions of the plasma membrane. In this case membranes isolated from phagosomes would not be representative of the plasma membrane as a whole.

A similar approach, involving exocytosis instead of endocytosis, was developed by Dowben and his co-workers (1967). Electron microscopic investigation demonstrates how fat droplets are secreted from the bovine mammary gland (Patton and Trams, 1971): the droplets are surrounded by pieces of plasma membrane and little if any cytoplasm is included. Dowben and his collaborators isolated these droplets, formed ghosts from them by freeze-thawing or sonication, and characterized the resulting membranes, concluding that the properties they found were those of the plasma membrane.

Again, objections arise. Better evidence that cytoplasmic components are not also packaged into the droplets or if so, that these components are later totally separated from the ghosts is required. Furthermore, this secretory process may also involve specialized regions of the plasma membrane, although the results of another study

on bovine mammary gland plasma membranes make this unlikely (Keenan et al., 1970). Finally, this approach is applicable to only an extremely small number of cell types.

Along similar lines, de-Thé (1968) suggested that the envelope of "budding viruses" should be studied as representing a piece of the plasma membrane.

CONCLUSION

Present understanding of the structure of plasma membranes and its relationship to function is disappointing in view of the effort that has been made in this field during the past decade. This slow progress is partially due to the considerable complexities inherent in working with membranes of any sort. This discussion has attempted to indicate weaknesses in current methodology and to point to the more promising approaches, especially in the area of selecting plasma membrane markers.

The authors are deeply grateful to Marion Stubbs who checked the text and made many valuable criticisms and suggestions.

This work was supported by United States Public Health Service Grant No. A1-03260 from the National Institute of Allergy and Infection Disease.

Received for publication 5 July 1972, and in revised form 10 October 1972.

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