proximately 3 days duration. We have also found that cats given a dose of LSD within the range employed in human studies (2.5 μg/kg) (*10, 11*) show a significant increase in limb flicks ($\bar{X}$ = 4.7 per hour; N = 5) in the absence of any other dramatic behavioral changes, a result that demonstrates the sensitivity of these measures to low doses of LSD.

In previous studies of the effects of hallucinogens in animals, investigators have utilized nonspecific behavioral measures, such as the disruption of either rope climbing or bar pressing in rats (*12*). The behaviors we describe here appear to be specific to hallucinogenic drugs and also have face validity in the sense that the constituent behaviors can be described as bizarre or inappropriate to the context in which they occur. The limb flick and abortive grooming behaviors are ideal for use as a model since they are sensitive, robust (occurring in every animal tested), reliable (stable across test sessions), quantifiable, and easy to score. They also reflect some of the major effects of LSD in humans, such as long-lasting psychological and perceptual effects, and long-lasting tolerance following a single dose.

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Plasma Membrane Vesiculation: A New Technique for Isolation of Plasma Membranes

Abstract. *Monolayer cell cultures of macrophages, monocytes, myoblasts, and density-inhibited and transformed fibroblasts form and release cell surface membrane vesicles following exposure to formaldehyde, related low-molecular-weight aldehydes, and disulfide blocking agents. Vesicles have a unique composition of proteins and lipids. They show enrichment of cholesterol and sphingomyelin content and a seven- to tenfold enrichment of 5′-nucleotidase activity. Vesicles also contain intramembranous particles and show a trilamellar unit membrane and no ultrastructural evidence of contamination with other cytoplasmic organelles. The technique is proposed as a novel method for isolating plasma membrane vesicles from cells in culture.*

A variety of techniques for the isolation of cell surface membrane fragments have been reported. These involve cell homogenization (*1*) or cell disruption by nitrogen cavitation (*2*) followed by differentiation and isopycnic centrifugation of native or "stabilized" (*1*) cells. Plasma membrane fragments have also been partially purified by affinity chromatography (*3*). We report here the development of a new procedure for the isolation of plasma membrane vesicles (PMV's) which may avoid some of the disadvantages of traditional techniques (*4*). It is based on the observation that a variety of aldehydes and disulfide blocking agents promote the formation and release of plasma membrane vesicles from cells in culture. As early as 1919 a variety of such agents were reported to produce cell surface "blebs" (*5*). We have extended these observations and have

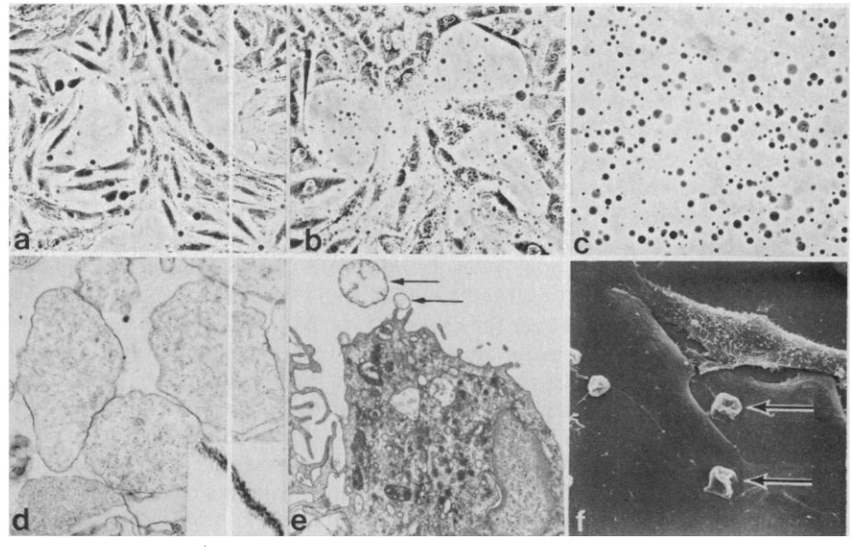

Fig. 1. (a) Phase micrograph of L_6 myoblasts exposed to 250 m*M* formaldehyde in calcium-magnesium PBS for 15 minutes, showing the formation of small cell surface membrane vesicles. (b) More extensive cell surface vesiculation with vesicles in suspension is apparent after 60 minutes incubation in 3T3 cells. (c) Vesicles decanted from 3T3 cultures represent plasma membranes. (d) Thin sections of 3T3 vesicles show no subcellular membrane contamination and (inset) a trilamellar membrane structure. (e) Analysis of macrophage vesiculation by electron microscopy also shows that vesicles are derived from the plasma membrane (arrows). (f) Scanning electron microscopy of L_6 myoblasts shows that multiple vesicles are released from individual cells (arrows). Magnifications: (a) × 140; (b) × 140; (c) × 140; (d) × 17,000; (inset) × 224,000; and (e) and (f) × 360.

developed a procedure to induce the release of cell surface blebs or vesicles into the medium. Plasma membrane vesicles have been isolated from guinea pig macrophages; from various cell lines, including human leukemic monocytes and rat L_6 myoblasts; and from a variety of density-inhibited and transformed cells, including mouse embryo cells transformed by simian virus 40 and methylcholanthrene (*6*).

Plasma membrane vesicles form on the cell surfaces of monolayer cultures within 15 to 30 minutes after exposure in situ to low concentrations of reagents, including formaldehyde (*7*) and *N*-ethylmaleimide (Fig. 1a). Vesicles are released into the medium (Fig. 1b) after incubation for 1 to 2 hours at 37°C. Vesicles can then be decanted to yield a PMV preparation (Fig. 1c) (*8*).

The PMV's released by these procedures show a wide size variation, from approximately 0.5 to 15 μm in diameter. All vesicles that are visible by phase microscopy can be sedimented by centrifugation at 30,000*g* for 30 minutes at 4°C. Submicroscopic vesicles that require sedimentation at higher forces have not all been studied in detail. Vesicles examined by electron microscopy (*9*) show no evidence of significant contamination with cytoplasmic organelles or nuclear fragments (Fig. 1d). Both large (5 to 10 μm) and small (1 μm) vesicles show a comparable ultrastructural appearance. The PMV's have a trilamellar unit membrane structure and contain a fine microreticular network (*10*). Intramembranous particles are also identified when PMV's are examined by the freeze-fracture technique. Transmission (*9*) and scanning electron microscopy (*11*) demonstrate that multiple vesicles are released from individual cells (Fig. 1, e and f).

BALB/c 3T3 cells, SV3T3 cells, and rat L_6 myoblasts shed PMV's in abundance. Approximately 500 μg of vesicle protein is recovered from 10^8 cells (*12*). Vesicles from such cells have been found to contain approximately 15 major polypeptides of different size classes when examined by polyacrylamide disk gel electrophoresis (Fig. 2) (*13*). Plasma membrane vesicles derived from cells labeled in situ with ^{125}I by the lactoperoxidase method contain ^{125}I-labeled glycoproteins and glycolipid (*14, 15*). Additional evidence supporting the plasma membrane derivation of PMV's is the observation that they contain specific and nonspecific membrane receptors. For example, SV3T3 PMV's are agglutinated by wheat germ agglutinin and by concanavalin A. Macrophage vesicles are also agglutinated by a variety of plant lectins and have been found, in addition, to contain receptors for T lymphocytes and for the Fc fragment of immunoglobulin (*16*).

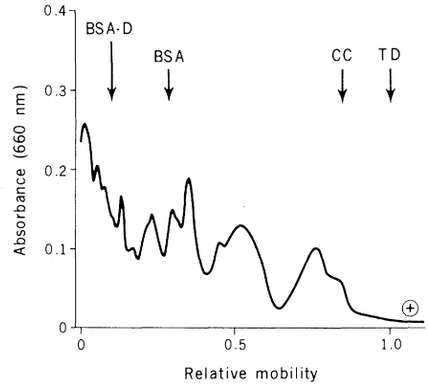

Fig. 2. Densitometric tracing of solubilized SV3T3 PMV proteins subjected to electrophoresis on a 7.5 percent polyacrylamide gel in 0.1 percent SDS, fixed and stained with Coomassie blue. The SV3T3 PMV's contain approximately 15 major proteins of different size classes with estimated molecular weights from 8,000 to more than 150,000 daltons. Standards are cytochrome c (CC), bovine serum albumin (BSA), and the dimer of BSA (BSA-D); TD signifies the position of the tracking dye.

Plasma membrane vesicles contain all major classes of phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, and show enrichment in sphingomyelin and cholesterol, a characteristic of most plasma membrane preparations (*17, 18*). The PMV's of SV3T3 cells, for example, contain approximately 15 percent sphingomyelin, compared to 3 to 5 percent observed in whole cells, and PMV's show a cholesterol/phospholipid ratio of approximately 0.80, compared to less than 0.30 in whole cell preparations.

A seven- to tenfold enrichment in 5′-nucleotidase activity is observed in PMV's (*12, 19*). For example, in PMV's from SV3T3 cells the 5′-nucleotidase activity is 65 nmole of inorganic phosphorus released per milligram of protein per minute, whereas in whole cell controls the activity is 7 nmole mg^{-1} min^{-1}. Evaluation of glucose-6-phosphatase activity shows that PMV's from fibroblasts and myoblasts contains less activity than those in whole cells. For example, L_6 PMV contains as little as 10 percent of the activity in whole cell controls; that is, 2 compared to 18 nmole of Pi mg^{-1} min^{-1}. Activity of NADH-cytochrome c reductase, a marker for endoplasmic reticulum, cannot be detected in isolated PMV's. The results obtained to date support the ultrastructural observations showing a lack of contamination of PMV's by cytoplasmic organelles, in particular with fragments of endoplasmic reticulum. The validity of these marker enzymes has previously been established in such cells, and this topic has been critically reviewed (*4*). To exclude the possibility that any of our observations may reflect mycoplasma contamination, only cultures shown to be free of mycoplasma contamination by electron microscopy and by duplicate cultural analysis have been used. In addition, PMV's have been prepared from sterile peritoneal macrophages within 2 hours after isolation. These cells were not exposed to extended culture and were free of mycoplasma contamination.

The conditions necessary for the production of PMV's from different monolayer cell lines vary in minor ways. In general, 25 to 250 m*M* formaldehyde or 1 to 10 m*M* *N*-ethylmaleimide in phosphate-buffered saline (*p*H 7.4) or in tissue culture medium induces plasma membrane vesiculation. Pyruvic aldehyde, acetaldehyde, glyoxal, pyridoxal, glutaraldehyde, iodoacetate, *p*-chloromercuribenzoate, and related compounds also promote some degree of vesiculation at appropriate concentrations. Ethanol, methanol, acetone, formic acid, acetic acid, pyridoxine, ascorbic acid, 2-mercaptoethanol, dithiothreitol, and many other nonaldehyde and nonsulfhydryl blocking reagents fail to induce significant vesiculation, although toxic concentrations of some reagents do cause cellular swelling and lysis. Plasma membrane vesiculation is a dose-, time-, and temperature-dependent phenomenon. Maximum vesiculation typically occurs within 2 hours at 37°C. Vesiculation is enhanced by the inclusion of 0.5 m*M* calcium in the vesiculating medium. Dithiothreitol (2 m*M*) also potentiates the ability of cells to produce vesicles in response to formaldehyde treatment. Under the conditions we have used, vesiculation is not blocked by pretreatment of cells for 3 hours at 37°C with either colchicine (10^{-6}*M*) or cytochalasin b (5 μg ml^{-1}); however, vesiculation is blocked by reducing the temperature to 4°C. Plasma membrane vesiculation appears to result from a change in the permeability of the plasma membrane associated with the binding of vesiculants to free sulfhydryl or free amino groups in the cell.

Further studies are required to establish that PMV's produced by this procedure are uniformly representative of the entire cell surface. An antiserum against PMV's has been produced and used to study PMV derivation in intact whole cells. Preliminary results show uniform

labeling of the cell surface. This suggests that PMV's contain at least certain antigens that are represented over the entire cell membrane. In addition, no significant staining of cytoplasmic or nuclear components can be observed in fixed cell preparations (*20*). The results reported here, therefore, establish that PMV's have the general characteristics of plasma membranes.

Plasma membrane vesicles isolated by this new method should provide a valuable source of material to study changes in membrane composition and in the modulation of linkages of cell surface receptors and membrane enzymes. It should also be possible to study differences in the phosphorylation of membrane proteins of various cell populations, in view of our recent observation that L_6 myoblast PMV's contain an adenosine 3′,5′-monophosphate-dependent protein kinase (*21*). This technique may also serve as a model system to study the physiological shedding of the cell surface reported in lymphocytes and other mammalian cells (*22*).

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6. Guinea pig macrophages from sterile peritoneal exudates, induced by Marcol 50, were washed and incubated in RPMI 1640 for 1 hour before vesiculation to allow the cells to attach and spread. The J-111 leukemic human monocytes were obtained from the American Tissue Culture Collection. Rat L_6 macrophages were kindly provided by D. Shubert and were grown in Dulbecco's modified essential medium (DMEM) containing 10 percent fetal calf serum (FCS). The 3T3 and SV3T3 cells were obtained from G. Todaro, and AKR mouse embryo cells and methylcholanthrene-transformed AKR cells were obtained from H. Moses. The 3T3 cells were grown in DMEM and 10 percent FCS and AKR cells were grown in McCoy's medium in 10 percent FCS. Continuous cell cultures were routinely performed at 37°C in a humidified atmosphere containing 10 percent CO_2. All cell lines were negative for mycoplasma as determined by duplicate culture by Flow Laboratories and the Mayo Microbiology Laboratory and by electron microscopy.
7. Formaldehyde was prepared fresh by dissolving paraformaldehyde in H_2O at 65°C with continuous stirring, followed by the addition of NaOH to clear the solution. This 10 percent formaldehyde solution was cooled and then added to either phosphate-buffered saline or tissue culture medium to achieve the desired concentration.
8. Intact cells dislodged from the cell monolayer can be removed by filtration through a glass wool column or by centrifugation on a Ficoll or sucrose gradient adjusted to a density at which PMV's band at the interface and whole cells form a pellet at the bottom of the tube.
9. Plasma membrane vesicle pellets were fixed in 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer for 12 hours at 4°C. Pellets were then washed and postfixed in 2 percent osmium tetroxide in 0.1*M* phosphate buffer for 1 hour. Specimens were dehydrated in ethanol, stained en bloc in 2 percent uranyl acetate, and embedded in Epon. Sections were than stained in uranyl acetate and lead citrate. Thin sections were examined in a Philips EM 201 electron microscope.
10. Preliminary results suggest that vesicles can be disrupted by nitrogen cavitation and that the contents of the vesicles can be removed by washing in 10 m*M* EDTA under appropriate conditions.
11. Cells for scanning electron microscopic studies were grown on glass cover slips, treated with 25 m*M* formaldehyde and 2 m*M* dithiothreitol for 30 minutes at 37°C, then either fixed in 1 percent calcium permanganate in 0.1*M* phosphate buffer for 10 minutes at 4°C and postfixed in 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer for 12 hours at 4°C or fixed solely in 2.5 percent glutaraldehyde–0.1*M* cacodylate buffer for 90 minutes at 37°C. Specimens were coated with gold-palladium and examined in an ETEC scanning electron microscope.
12. Vesicle protein content was routinely determined as described by O. Lowry, J. S. Rosebrough, A. C. Farr, and R. J. Randall [*J. Biol. Chem.* **193**, 265 (1951)]. Since dithiothreitol (DTT) interferes with the Lowry reaction, vesicle protein determinations by the Lowry procedure were performed only on specimens that had been extensively dialyzed. To further alleviate this problem, protein assays were also performed by the fluorescamine method. Vesicles isolated from approximately 10^8 cells and centrifuged at 30,000*g* for 30 minutes contain up to 500 μg of protein. Some of this protein represents intravesicle content. Plasma membrane enzyme activities expressed per milligram of protein, therefore, represent minimal values.
13. Plasma membrane vesicles to be analyzed by gel electrophoresis were dialyzed against water or 10 m*M* tris buffer, *p*H 7.4, for 36 hours at 4°C to remove bound formaldehyde. Our studies have shown that this treatment removes all detectable bound [^{14}C]formaldehyde from PMV's. Similar reversible binding of formaldehyde to RNA and DNA has been reported by E. J. Ering and J. Ofengand [*Biochemistry* **6**, 2500 (1967)]. Vesicles were then centrifuged at 30,000*g* at 4°C for 30 minutes, and the wet pellet was solubilized in 1 percent sodium dodecyl sulfate, 10 m*M* DTT, and 8*M* urea and heated at 90°C for 5 minutes. In occasional samples some material remained insoluble. It was removed by centrifugation at 30,000*g* for 15 minutes at room temperature. Aliquots were then placed on 7.5 percent acrylamide gels. A 0.1 percent SDS–0.1*M* PO_4 buffer (*p*H 7.4) was used. Gels were subjected to electrophoresis at 8 ma per tube. They were then fixed overnight and subsequently stained with Coomassie blue for protein or with periodic acid–Schiff reagent for carbohydrate (not illustrated).
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17. Plasma membrane vesicle phospholipid composition was analyzed by two-dimensional thin-layer chromatography of the vesicles extracted in chloroform and methanol (2 : 1). Elution in the first dimension was in chloroform, methanol, and 28 percent aqueous ammonia (130 : 70 : 10) and in the second dimension in chloroform, acetone, methanol, acetic acid, and H_2O (100 : 35 : 25 : 20 : 10). The percentages of individual phospholipids were determined by inorganic phosphorus assay of individual thin-layer chromatography spots according to the method of L. F. Eng and E. P. Noble [*Lipids* **3**, 157 (1968)].
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Inhibition by Anions of Human Red Cell Carbonic Anhydrase B: Physiological and Biochemical Implications

In a study of inhibition by anions of the enzymatic activity of human erythrocyte carbonic anhydrases B and C, Maren *et al.* (*1*) measured inhibitory behavior for both hydration of CO_2 and dehydration of HCO_3^-, tabulating their results as I_{50}, the inhibitor concentrations that halve the observed velocities of the enzymatic reactions. With but a single exception, values of I_{50} for the dehydration reaction were reported to be substantially greater than for the hydration reaction; for Cl^-, the ratio of values is 16. The experimental conditions were stated to be such that the I_{50} values should not be much different from the true K_I values. Maren *et al.* say that these results do not violate the Haldane relation (*2*): "Our finding of different inhibition constants between hydration and dehydration would only violate considerations of equilibria, that is, the Haldane relation, if the inhibiting mechanism were the same in the two directions."

However, the results of Maren *et al.* do violate the principle of microscopic reversibility, that is, the Haldane relation. That this is so can be seen from the following. For a solution of CO_2 and HCO_3^- in buffer at *p*H 7.2—the mean value used in (*1*)—at 25°C, the ratio of $[CO_2]$ to $[HCO_3^-]$ is 1:10, from the known pK_E of 6.2 (*3*). The lifetime of these two species must be in the same ratio to maintain equilibrium; for example, in the absence of enzyme, the mean time of hydration of a CO_2 molecule is about 20 seconds and the time is independent of *p*H below *p*H 9; the mean time of dehydration of a HCO_3^- anion must then be about 200 seconds (*3*). The addition of enzyme to the solution can only decrease the lifetimes—that is, increase the rates of interconversion of CO_2 and HCO_3^-—by the identical factor for each direction. This is