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Interactions at the Surface of Plant Cell Protoplasts; An Electrophoretic and Freeze-etch Study*

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Introduction

The physical limitation of the cell protoplast has been demonstrated both mechanically, and microscopically, as a tangible barrier between cell cytoplasm and the external environment. In higher plant cells the isolation afforded by this limiting membrane is enhanced by the normally cellulosic cell wall, usually very close to the membrane outer surface. The work to be described is an attempt to illustrate aspects of structure and some functions of the outer protoplast membrane, or plasmalemma, and from this information, to discern something of the dynamic nature of this plasma membrane.

The experiments were performed upon spherical, naked higher plant cells (isolated protoplasts) released from tissue pieces into suspension by digestion of the cell wall with the appropriate degrading enzymes in a plasmolysing solution.

Earlier work from this laboratory on the endocytotic uptake of latex particles by tomato fruit locule protoplasts[1,2] led to a proposed mechanism for the process which has certain similarities to the situation in animal and protistan cells.[3–5] The parameters to be met for uptake to occur were an initial surface binding of the particle to the protoplast, followed by membrane stretch over the area of particle contact. This localized stretch brought about vesiculation of the particle and the final uptake of the vesicle–particle complex into the cytoplasm. The membrane-stretch stage of this process has been well illustrated by freeze-etch replicas which supply quantitative data on changes in the number of granules per unit area of membrane.[2]

To enquire further into the phenomenon of surface adhesion, the initial stage in the uptake process, both particles and protoplasts were studied using the techniques of microelectrophoresis as applicable to whole cells and large particles. Valuable information was gained

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concerning electrical charges near the relevant surfaces, and also the effects of various substances believed to affect endocytosis or surface adhesion in such systems.

The techniques of freeze-etching and microelectrophoresis were also applied to aspects of membrane fusion in plant protoplast systems under study in our laboratory. From information gained by freeze-etch and thin section studies[6] it would appear that an essential pre-requisite for membrane fusion is an intimate contact, sustained for a considerable length of time.

The membrane stretch hypothesis was developed from observations of endocytosis. It has been extended, on the basis of freeze-etch observations, to a general concept applicable to other membranes.

Other theories of endocytosis are considered.[7, 8]

Materials and Methods

(1) *Protoplast Isolation*

Protoplasts were prepared from tomato fruit locule tissue as has been previously described.[2]

Protoplasts from tobacco leaf mesophyll tissue were prepared using pieces cut from the leaf of 50–60 day-old *Nicotiana tabacum* var Xanthi nc plants, after the lower epidermis had been removed.[9] Peeled leaf pieces were incubated in a mixture of 5% w/v cellulase (Onozuka p. 1500), and 0·5% macerozyme (all Japan Biochemicals Co. Ltd., Nishinomiya, Japan), and 25% sucrose at 20°C for 4 h. The material was gently swirled at intervals during the treatment. The protoplasts thus released were washed twice by flotation in 25% sucrose. The plasmolyticum (sucrose) was made up in distilled water except where stated otherwise.

(2) *Incubation of Isolated Protoplasts with Latex Spheres*

Incubations of tomato fruit protoplasts with polystyrene latex spheres were carried out using methods previously described.[2]

The spheres used were Dow-Latex, 0·109 μ in diameter. The latex suspensions were added to the experimental media without prior dilution.

(3) *Induced Fusion of Petunia Leaf Protoplasts*

Protoplasts were isolated from pieces of leaf (of 50–60 day-old plant) from which the lower epidermis had been removed. These were incubated in a mixture of 0·3% macerozyme and 1% cellulase (Onozuka P 1500) in 13·5% sorbitol for approximately 18 h. Liberated protoplasts were washed once by centrifugation in fresh 13·5% sorbitol and then by flotation in 20% sucrose. The sucrose was replaced by 0·47 M $NaNO_3$ and the protoplasts were immediately centrifuged

for 5 min at approximately 150 × g. Following this centrifugation, the protoplasts were quickly washed in 20% sucrose before freeze-etching.

(4) *Freeze-etching*

Specimens for freeze-etching were variously pretreated. For deep-etching, protoplasts were glutaraldehyde-fixed and washed in several changes of distilled water. In some cases, fixed protoplasts were re-suspended in 20–25% glycerol/water as described previously.[2] Otherwise protoplasts were frozen directly while suspended in the culture medium, which was generally 20% sucrose. Drops of the suspending medium, with protoplasts, were mounted on collared gold specimen holders and frozen by immersion in Freon 22 at its freezing point. Freeze-etching was performed using the now standard technique[10] utilizing a Balzers 360M freeze-etch apparatus. Platinum-carbon for shadowing was evaporated from an electron beam source (Balzers EVM 052) in most cases. Replica cleaning was as previously described.[2]

(5) *Electrophoresis*

The microelectrophoresis was carried out using a flat cell[11] with platinum black electrodes, which prevented any electrolysis or polarization. The effective inter-electrode distance was determined from measurements of the resistance of a solution of known specific conductance in the cell. The electrophoresis cell was thermostatted in an oil bath attached to the microscope, the whole apparatus being in a constant temperature room maintained at 25 ± 1°C. A known potential difference was applied across the electrodes using a Gelman Hawksley stabilized D.C. power supply. The distribution of particle mobilities across the cell was found to be parabolic and the other criteria for true electrophoresis also held. The mobility of a total of approximately 40 particles was recorded by observation of their movement against a calibrated eyepiece graticule for each current direction at each stationary level. The stationary levels were predicted using Komagata's equation.[12]

The addition of substances under investigation was made directly to the protoplast suspension in amounts to give a final concentration as stated (see Tables). Suspensions were incubated with the additives at 25°C for 20 min and then placed directly into the electrophoresis cell.

For aqueous colloidal systems the correct equation to calculate zeta potential (ζ) from electrophoretic mobility is that due to J. Th. G. Overbeek.[13] For the biological cells studied in these experiments this reduces to the Smoluckowski equation[14] as

$$u_E = \frac{\zeta D}{4\pi\eta}$$

In electrophoretic experiments with latex particles where for various ionic considerations this equation cannot be used, then the equation attributed to Henry[15] has been used

$$u_E = \frac{\zeta D}{6\pi\eta} f(ka)$$

where u_E is mobility (microns per sec per volt cm); D the dielectric constant; η the viscosity (centipoise) and $f(ka)$ is Henry's constant.

Results

Freeze-etching

1. *Changes in granule densities during endocytosis.* Our recent work on the endocytosis of latex spheres by isolated tomato fruit protoplasts[2] showed that adhering latex spheres were taken up singly in involutions of the plasmalemma which closely adhere to the latex sphere at all stages. It was proposed that the mechanism involved some form of membrane stretch. This suggestion was based on changes in the density of the granules found on frozen-etched plasmalemmae. These earlier observations were made on glutaraldehyde-fixed material and it seemed advisable to check that the observations were not induced by the fixative. Similar uptake of latex spheres were found in freeze-etched material which was unfixed and untreated with glycerol as a cryoprotective (Fig. 1). Direct comparison of granule density changes is complicated, however, by the fact that the distribution of granules between the complementary fracture faces of the membrane is altered by the medium in which the material is frozen. All the equivalent stages of endocytosis previously reported for fixed material are found in the unfixed material. The closing of the neck of the invagination may be affected to some extent by fixation since it is more usual to find invaginations with wide necks in unfixed material (arrow, Fig. 1) than in fixed material.

2. *Other changes in granule density associated with membrane "stretch".* Variations in granule density at the plasmalemma surface exposed by freeze-etching are not limited to invaginations associated with latex uptake. Occasionally evaginations of the plasmalemma of the isolated protoplast are found (Fig. 2). These have been observed light-microscopically and in unfixed freeze-etched material and cannot be considered a fixation artefact. The number of granules per unit area on the surface of the "blow-out" (Fig. 2) and on the surface of the background plasmalemma have been measured and compared. The change in granule density between plasmalemma and evaginated plasmalemma is roughly proportional to the change in membrane area which would occur if a circle of plasmalemma of the diameter of the neck of

the "blow-out" had been extended to form the "blow-out". The overall "stretch" in this case is approximately 33 times.

Extended plasmalemma of the above sort may come in contact with latex spheres (Fig. 3). Binding of the latex occurs, and it appears that the membrane folds in an attempt to accommodate the sphere. It seems probable that the membrane has reached the limit of its "stretch" capacity.

It should be noted that granule density variations are commonplace in plant cells both at the plasmalemma and in other membranes. In cytoplasmic vesicles localized granule density changes may be found which suggest that membrane "stretch" is occurring (Figs. 4 and 5). Changes of this sort may be found in both fixed and unfixed material.

3. *The fate of latex spheres following uptake.* Latex spheres enter the isolated protoplast closely bounded by plasmalemma. However, within the protoplast they are commonly found in relatively large vesicles which contain several latex spheres within their interior (Fig. 7). Most likely, these arise by fusion of the small latex-containing vesicles with pre-existing cytoplasmic vesicles. It is of interest to note that cytoplasmic vesicles can be differentiated by the freeze-etch technique. Isolated protoplasts freeze-etched while still in their native plasmolyticum usually demonstrate distinct differences in ice-crystal size between external medium, cytoplasm, and vacuole (Fig. 6). The vacuole always contains ice-crystals which are considerably larger than those in the external medium, presumably as a result of the exclusion of the plasmolyticum from the vacuole itself. These ice-crystal size differences, however, are not limited solely to the vacuole. Cytoplasmic vesicles contain ice of different sizes (Fig. 6) which tend to be characteristic of vacuole or external medium. It appears that when latex spheres are found within large cytoplasmic vesicles these vesicles contain ice which is characteristic of the external medium.

4. *The plane of fracture in freeze-etched membranes.* There is considerable evidence to suggest that frozen-fractured membranes fracture along an internal plane which is probably the hydrophobic interior of the membrane.[16] Isolated protoplasts were fixed, mounted in distilled water, and freeze-etched using the deep-etching technique.[17, 18] Deep-etching involves sublimation from the surface of the ice-crystals surrounding the specimen. Thus, both the outer surface of the membrane and the fracture face are exposed. The observations (Fig. 8) of deep-etched isolated protoplast plasmalemma support the concept of internal membrane fracture. The granules of the membrane are clearly centrally located and appear to extend beyond the outer surfaces of the membrane.

5. *Additional observations of frozen-fractured membranes.* In glutaraldehyde-fixed material all membranes appear as a non-structured continuum variously covered with granules. However, occasionally in

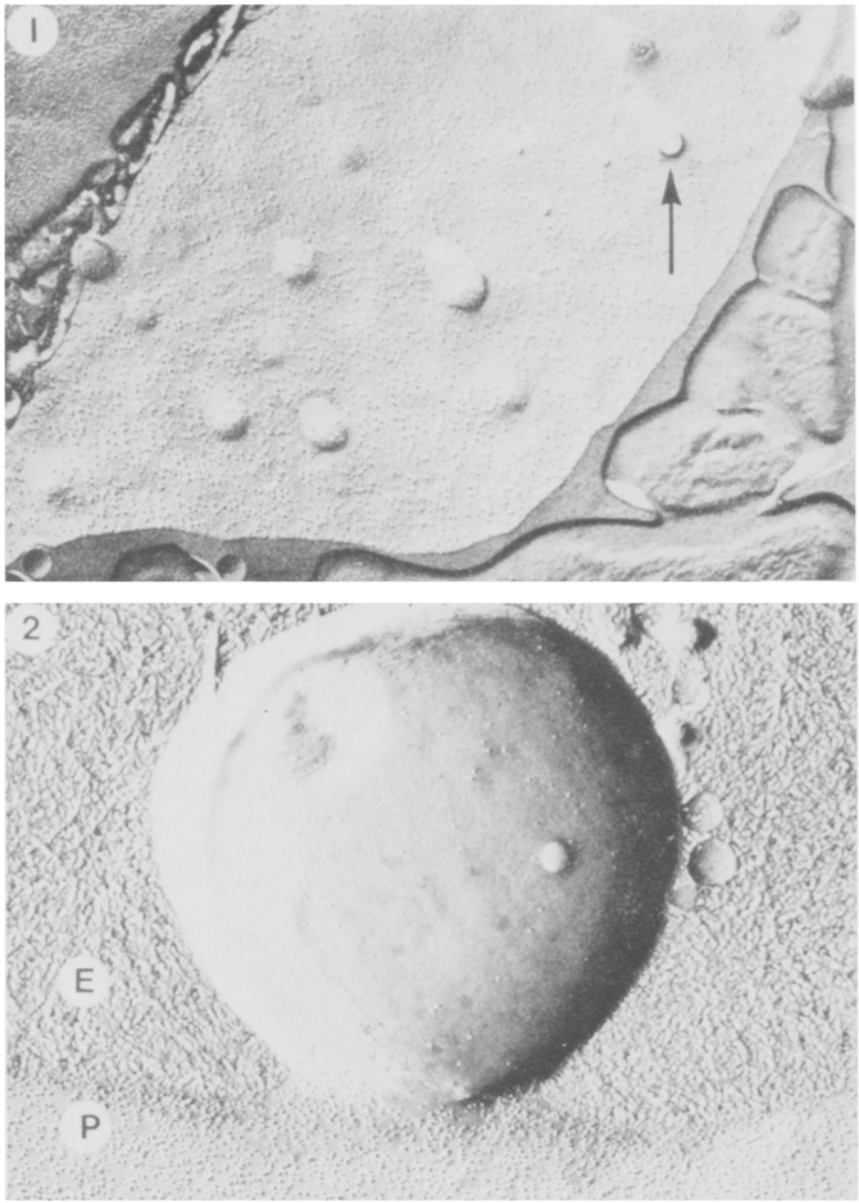

Figure 1. Endocytosis of latex spheres by an isolated tomato fruit protoplast. The plasmalemma is viewed from the inside. A wide-necked invagination has been snapped off at the neck (arrow). The specimen was prepared without chemical fixation and without the addition of a cryoprotective agent. (Magnification ×50,000.)

Figure 2. An evaginated region of tomato fruit protoplast plasmalemma. The evagination has been formed by expansion of a localized region of the plasmalemma. The specimen was glutaraldehyde-fixed and treated with glycerol. Glycerol/water eutectic (E), plasmalemma (P). (Magnification ×66,000.)

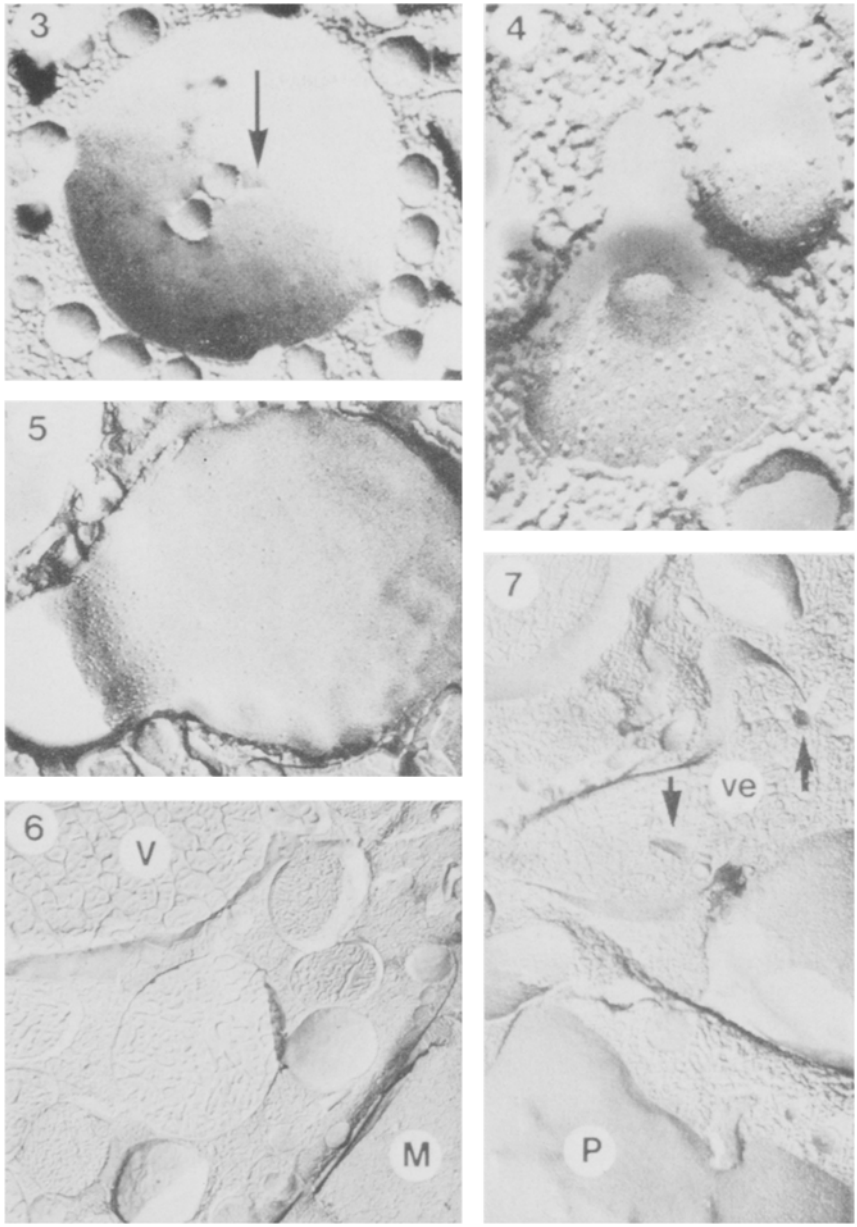

Figure 3. Binding of latex spheres to a region of expanded plasmalemma. Folding of the plasmalemma has occurred (arrow). Specimen glutaraldehyde-fixed, glycerol treated. (Magnification ×74,000.)

Figures 4 and 5. Cytoplasmic vesicles revealing membrane expansion which is indicated by changes in granule density on the fractured face of the membranes. The membrane "stretch" phenomenon. Specimens unfixed and frozen in native plasmolyticum. (Magnification ×105,000 (Fig. 4) ×64,500 (Fig. 5).)

Figure 6. Differences in ice-crystal size reveal the permeability barriers to entry of the sucrose plasmolyticum of isolated protoplasts. Cytoplasmic vesicles retain the permeability characteristics of the membranes from which they derived. Vacuole (V) frozen sucrose plasmolyticum (M). Specimen unfixed and frozen in native plasmolyticum. (Magnification ×10,500.)

Figure 7. Several latex spheres found in a relatively large cytoplasmic vesicle following endocytosis by a tomato fruit protoplast. The latex has entered the vesicle by fusion of the small latex-containing endocytotic vesicles with a pre-existing cytoplasmic vesicle. Specimen unfixed and frozen in native plasmolyticum. Cytoplasmic vesicle (Ve), plasmalemma (P), Latex spheres (arrows). (Magnification ×33,000.)

unfixed material alterations in this basic structure may be seen. These may consist of a regular pattern in the background continuum (Fig. 9); or of a series of ridges or flow-lines (Fig. 10). The latter, when found on the plasmalemma, usually seem to be associated with underlying organelles. Sometimes similar structures may be found on the fractured face of cytoplasmic vesicles (Fig. 11). Although of a similar structure these may have a different functional significance. We have seen structures of the type shown in Fig. 10 in protoplast material which has been both glycerinated and unglycerinated in sucrose plasmolyticum. It seems unlikely that these are artefacts. The tobacco leaf material (Fig. 10) was partially infiltrated with 20% glycerol for less than 1 min, and was frozen as quickly as possible after removal from the plant.

Electrophoresis

The differing mobilities of various plant protoplasts and of polystyrene latex spheres was recorded as a function of the time taken to travel a known distance as measured by a graticule. From these mobilities a value for the zeta potential has been calculated.

TABLE I. Measurement of zeta potential of tomato fruit protoplasts and polystyrene latex spheres in 20% sucrose with various added compounds

(i) Tomato fruit protoplasts

Additive	Electrophoretic mobility (U_e) μ sec^{-1} V cm^{-1}	Zeta potentials Mv
None	−0·77	−22·44
Poly-L-ornithine 2 μg/ml	Barely negative unmeasurable within error of experiment	
Poly-D-lysine 2 μg/ml	−0·44	−12·83
Sodium nitrate 8·5 mg/ml	−0·48	−13·99
DEAE Dextran 4 μg/ml	−0·47	−13·71
Glycerol monoleate 250 μg/ml	−0·79	−23·04

(ii) Polystyrene latex spheres 0·109μ diameter

Additive	Electrophoretic mobility	Zeta potential
None	−1·88	−54·82
Poly-L-ortnithine 2 μg/ml	−0·90	−26·24
Sodium nitrate 17·0 mg/ml	→0	
Glycerol monoleate 250 μg/ml	−1·78	−51·90

In experiments using sodium nitrate and poly-L-ornithine washing the protoplasts three times with 20% sucrose brought the measurements of mobility (in 20% sucrose) back to the two values for untreated material.

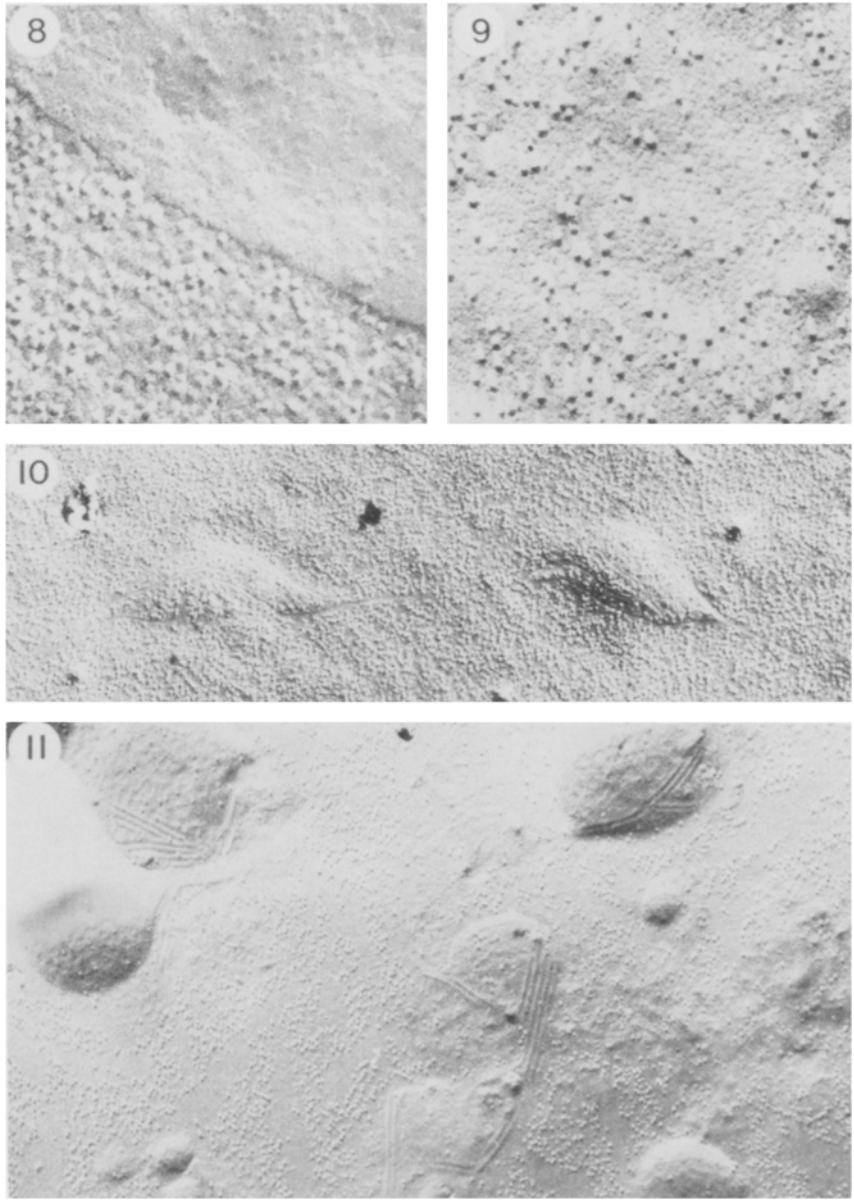

Figure 8. Deep-etched plasmalemma of tomato fruit protoplast. The fracture face of the membrane (bottom) and the real outer surface (top) are revealed. (Magnification ×130,000.)

Figure 9. A fractured membrane revealing structure in the non-granular continuum. Specimen unfixed and not treated with a cryoprotective. (Magnification ×135,000.)

Figure 10. Fractured plasmalemma of tobacco leaf cell frozen immediately after removal from the plant. 20% glycerol/water was used to mount the tissue piece but had only partially infiltrated this cell. (Magnification ×50,000.)

Figure 11. Fracture face of a cytoplasmic vesicle in a tomato fruit protoplast. No fixation or cryoprotective was used. (Magnification ×50,000.)

TABLE II. Measurement of the zeta potential of tobacco leaf protoplasts in 23% Sucrose with various added compounds

Additive	Electrophoretic mobility	Zeta potential
None	−0·90	−26·24
Poly-L-ornithine 2 μg/ml	−0·42	−12·24
Poly-L-ornithine 10 μg/ml	+0·45	+13·12
Sodium nitrate 8·5 mg/ml	→0	
Glycerol monoleate 250 μg/ml	−0·90	−26·24

Discussion

Introduction

The endocytotic uptake of polystyrene latex spheres by tomato fruit protoplasts was first observed in an electron microscope study using ultrathin sections.[1] This result has since been verified using the now standard method of freeze-etching;[2] the results of which point towards a possible mechanism for the process.

Uptake is initiated by adhesion of the latex sphere to the membrane resulting in a depression that extends solely over the area of contact of the sphere. The membrane bound particle continues to travel towards the cytoplasm and eventually comes to lie in a plasmalemma invagination closely surrounded by the membrane. The neck of the vesicle then fuses to liberate the small tightly membrane bounded vesicle into the cytoplasm. The intimacy of contact between sphere and plasmalemma at all stages of endocytosis has important implications with respect to the mechanism of endocytotic initiation (see later). Large vesicles are often observed within the cell containing several spheres, but uptake has not been seen to occur other than singly. It is probable, therefore, that the endocytotic vesicles fuse with others of similar nature, and probably also with non-sphere containing vesicles which originate from the plasmalemma (see later).

The membrane-stretch mechanism which we have proposed for the invagination process was based upon the freeze-etching observation of a reduction of granules on the membrane face in the endocytosing region. A mechanism for endocytosis by lymphocytes, macrophages and mast cells has been proposed by Allison[7] which would involve a clumping of granules. He proposed that involution then occurs by cytoplasmic microfilaments pulling on the granules. Although the two mechanisms would appear contradictory it is not improbable that the two systems are quite different, having differing mechanisms. The endocytosis of large solids by higher plant cells is an unusual process whereas the phagocytes studied by Allison are specially adapted for this process.

Freeze-etching

The phenomenon of membrane "stretch". It is not necessary to discuss at length in this paper the precise nature of freeze-etch fracture of biological membranes. This has already been treated at length by various authors, notably Branton.[19,20] Our evidence (Fig. 8) supports the view that fracture of the tomato fruit protoplast plasmalemma is along an internal plane. The similarity in appearance of other membranes suggests that internal fracture is a general phenomenon. The granules of freeze-etched membranes commonly have been presumed to have a functional significance, although this significance is often biased towards the interests of the author. The possibility that they might represent lipo-protein micelles has been proposed.[16] However, whatever their significance we have regarded them simply as markers for the state of the membrane. In the case of the endocytosis of latex spheres and of the evaginated plasmalemma (Fig. 2) there is good reason to believe that granules are neither synthesized nor do they disappear to provide extra membrane but simply that the membrane between the granules expands. The expansion of 3 to 4 times required for the endocytosis of latex spheres could, on some membrane models,[21,22] be a process for which all the additional membrane material is contained within the membrane. However, an expansion of 33 times or more (Fig. 2) is unlikely to be by such a process. More likely additional membrane material (lipo-protein or possibly lipid alone) is added by intussusception from a cytoplasmic pool.[23] The observation of folding in a highly "stretched" membrane in association with a bound latex sphere (Fig. 3) suggests that the stretching process has a limit. Presumably this limit is determined by the size of the sink of potential membrane wherever this may be.

We propose that the membrane expansions characterized by granule density changes are rapid processes which enable most cytoplasmic membranes to be highly dynamic in terms of the area which they can cover. In the case of latex sphere uptake it is probable that membrane expansion is initiated by localized changes in the electrical field impinging on the membrane (see discussion later). It seems reasonable that other membrane "stretch" phenomena are also electrically induced. In these cases, however, the motive force is not necessarily produced in the region undergoing change. A localized weakening of the membrane could enable existing forces, previously in equilibrium, to produce the change. The possibility that energetically active proteins are present in the membrane cannot be ignored. Membrane stretch mechanisms could be controlled, via the local electrical environment, by membrane bound ion pumps.

The membrane model which has the best supporting evidence is that of Robertson.[24] This is derived from the myelin sheath of nerves. Myelin forms by extension of the plasmalemma of the Schwann cell.

The membranes of freeze-etched myelin are virtually granule-free.[25,26] It seems probable, on our evidence, that myelin forms by the membrane stretch process described above. This would make myelin an untypical membrane with regard to lability and lack of variation in structure, but nevertheless having the same backbone structure as most other membranes.

Bennett's concept of membrane flow[8] is, in our view, limited by regarding the membrane as a relatively rigid sheet in which membrane turnover requires the addition, by fusion, of fully synthesized membrane. A highly labile membrane capable of considerable, rapid, localized stretch is more capable of fulfilling the multifarious transformations which typify biological membranes.[21]

Vesicle fusion. The observation that vesicles fall into distinct categories on the basis of ice crystal size suggests that the permeability properties of the tonoplast and plasmalemma from which these vesicles presumably arise are retained by the vesicles themselves. The results show clearly that the effective permeability barrier to plasmolytica is the tonoplast. Of particular interest is the observation that the latex spheres are found in large vesicles which contain ice characteristic of the external medium, not of the vacuole. These vesicles are presumably bounded by plasmalemma-type membrane. The small vesicles which contain the latex are also bounded by plasmalemma and there appears to be a recognition capacity. This specificity of recognition is probably based on electrical charge characteristics since an essential prerequisite for fusion is membrane contact and this is probably electrically determined (see discussion later).

The effect of fixation. The alterations in membrane structure revealed in Figs. 9 and 11 and the observations that glutaraldehyde may cause the closing of the necks of endocytotic invaginations show the value of the freeze-etch technique for electron-microscopic examination of membranes. The effect of glutaraldehyde on the neck of the endocytotic invaginations could account for the widespread observation of closed plasmodesmata between plant cells. From a purely teleological point of view it is in the interests of a cell to defend itself against the onslaught of fixatives by closing intercellular connections.

Other alterations in membrane structure revealed by freeze-etching. The substructure seen in the background continuum of the membrane (Fig. 9) could represent the lipoprotein micelles envisaged by Lucy.[22] Regions such as these are localized and the orientation of the substructure differs between adjacent patches. The spacing periodicity is approximately 12–13 nm. No cryoprotective was used in the instances where this substructure was revealed and it is possible that it has arisen as a result of the growth of ice-crystals close to the membrane.

Similar structures to those shown in Fig. 10 have been previously reported[27] and deserve careful assessment. They usually appear to be

associated with underlying organelles closely associated with the plasmalemma and they give the impression of flow lines. The sharp angles and junctions suggest that they are not formed in association with underlying microtubules. It is tempting to believe that this is a manifestation of membrane induced cytoplasmic flow perhaps of the sort envisaged by Hejnowicz.[28] Clearly, conformational changes of a transitory nature are occurring in these regions of the membranes.

Electrophoresis

Electrical nature of latex adhesion. The most plausible explanation for the initial adhesions of latex to plasmalemma are that surface properties, probably electrical in nature, are involved. Electrophoretic

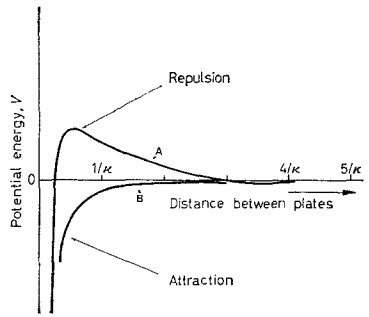

Figure 12. Total interaction energy curves for two charged surfaces, as a summation of the attraction curve and repulsion curves, showing variation with distance. (Adapted from *Introduction to Colloid and Surface Chemistry*, D. J. Shaw, Butterworth (Publ.).)

measurements were made on both protoplasts and latex spheres to investigate the nature and magnitude of these charges. Effects of various additives, all believed to promote endocytosis or cell adhesion, were studied. It was observed that both protoplasts and latex spheres had negative electrophoretic mobility and negative zeta potential, the magnitude of those of the latex being greater.

The forces which act against particle aggregation are a consequence of repulsive interactions between similarly charged electrical double layers at the surfaces. The aggregation is brought about by the universal inter-molecular London and Van der Waals attractive forces. A summation of these two forces, expressed in terms of potential energy, and their variation with the distance between the surfaces, is shown in Fig. 12(A). It can be seen that there is a peak of repulsive energy which will act as a barrier to the forces of attraction. The amount of adhesion in a mixed suspension of protoplasts and latex spheres will relate directly

to the number of particles which are at a sufficiently high energy level to overcome this barrier.

The compounds under investigation appear to reduce the magnitude of the zeta potential, and in extreme cases to reverse the sign. It can be postulated, therefore, that the effects of the additives are probably due to their cationic components. Reduction of zeta potential towards zero affects the interaction of two approaching charged surfaces and will lower the repulsive "barrier" as depicted in Fig. 12(B). More particles in the suspension will therefore have sufficient kinetic and thermodynamic energy to approach within range of attractive forces. More latex spheres would, therefore, in the presence of one of these additives, adhere to the protoplast plasmalemma. This necessarily will increase the number of spheres in a membrane bound, potentially endocytotic, situation. Experiments to determine increased rates of uptake in this system are not yet complete; however, support for the tenures above is offered by experiments in which endocytotic uptake of tobacco mosaic virus by tobacco leaf protoplasts is enhanced by pre-incubation with poly-L-ornithine.[29] It is possible, however, because of their relatively large size (mol. wt. 120,000) that these polycations act as bridges between the two surfaces, part of the molecule binding to each of them. If the polycations bind flat to the surface for the whole of their length, as may well happen at low concentrations, then their mode of action will be directly comparable to that of the inorganic materials.

When considering the "membrane-stretch" of the developing endocytotic vesicle that follows the adhesion of the latex sphere (see earlier) it would seem reasonable to propose that the contact is directly responsible for initiation of the stretch. As the two charged surfaces come within a small distance of one another it is proposed that considerable energetic perturbance results, giving rise to a localized instability within the membrane architecture. The forces responsible for this disturbance would be the Van der Waals and London's attractive forces; changes in surface potential caused by electrical double-layer interaction;[30] energy redistribution resulting from elimination of charged species at the surfaces; expulsion of water, and steric molecular interferences. It is believed that the energies described above would be large enough to alter protein and lipid configurations. Our hypothesis is continued by suggesting that the resultant instability is compensated for by the rearrangement of molecules within the matrix of the membrane. This is manifested as the phenomenon of "membrane-stretch" described previously. It is possible that the instability induced in the membrane by latex sphere contact facilitates incorporation of fresh components from the cytoplasm into the membrane framework. Freeze-etch evidence would dispute the notion that stretch derives from reduction in granule number (see earlier).

As has been previously described the plasmalemma membrane at the neck of the endocytotic vesicle fuses with itself to isolate the endocytotic vesicle and repair the plasmalemma. The endocytotic vesicle is also known to fuse with various cytoplasmic vesicles. The ease and regularity with which these fusions occur led to a consideration of the events necessary for membrane/membrane fusion, and the energetic status of the membrane required for completion of the process.

Fusion. Fusion of higher plant protoplasts with one another is a manipulation under intensive investigation in our laboratories, and surface properties are of prime importance.

Electrophoretic investigation of tobacco leaf protoplasts, a system in which fusion is commonly studied, demonstrated a negative mobility and zeta potential. Compounds such as sodium nitrate, used to induce aggregation of protoplasts, reduced the value for both these properties, as did various substances used elsewhere in attempts to promote endocytosis, e.g. poly-L-ornithine. Glycerol mono-oleate, one of a group of compounds variously related to lipids, and commonly used in fusion experiments, appeared to have little or no effect on surface electrical properties.

Certain parallels between experiments aimed at inducing fusion, and concerned with endocytosis thus became apparent. In both cases one is dealing with a suspension of colloidal, or near colloidal negatively charged particles. The attractive and repulsive interactions between these particles can again be summarized by Fig. 12. For successful protoplast fusion it is necessary, as in endocytosis, to bring these protoplasts into intimate and sustained contact (Figs. 13 and 14). Substances which lower the surface charge on the particles will lower the barrier of repulsive energy represented on the interaction diagram. This increases the number of particles with sufficiently high energy to approach within the distances of London's and Van der Waals' attractive forces.

As the two membranes come within molecular distances a great deal of potential perturbing energy is available, derived from atomic and molecular interactions, steric interferences, surface potentials of the original membrane, and ionic displacements, as previously inferred for endocytosis. This again results in molecular instability within the area of membrane contact. We propose that this instability is "reduced" by fusion of the membranes at the edges of the area of contact. The membrane within the original area of contact may well re-order to form a series of vesicles, which could be considered as similar to the endocytotic vesicles with respect to their stability. The unstable membrane may, however, by dispersed into the cytoplasm. Previously it was postulated that the "inducers" of fusion acted by facilitating the initial contact phase. It is possible that these compounds may also have molecular effects on membrane instability, as under certain circumstances they appear to cause bursting of non-fused protoplasts. The very large

polycationic materials, e.g. poly-L-ornithine may also have a bridging action during membrane fusion, adsorbing at different regions on the molecule to both protoplasts.

Lipid related compounds such as glycerol mono-oleate that appear not to affect surface electrical properties may well only act directly on stability of the molecular architecture of the membrane. It may, therefore, be necessary to use these compounds in combination with other means for aggregating protoplasts.

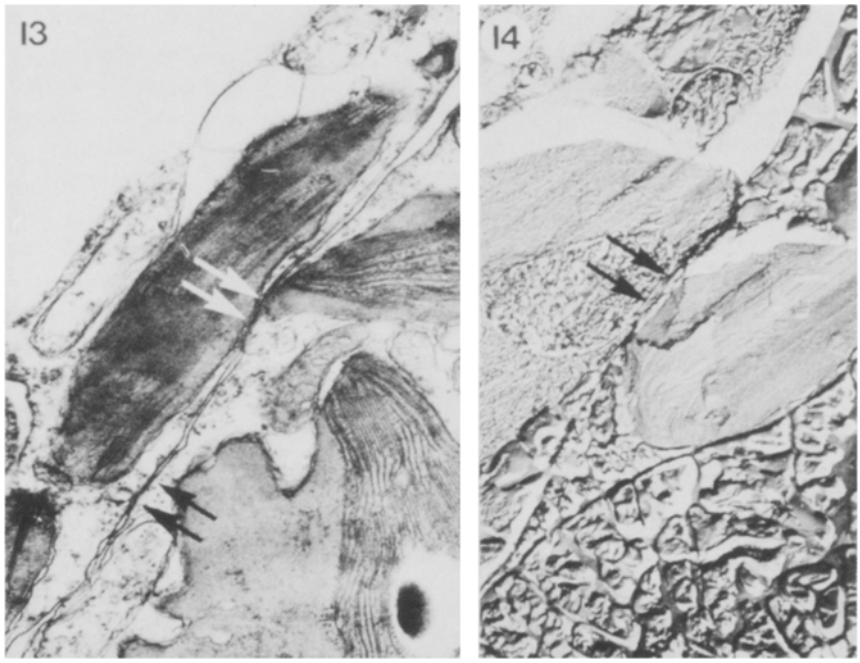

Figure 13. A thin-section micrograph of the junction between two fusing *Petunia* protoplasts. Protoplasts were induced to fuse using sodium nitrate. There are localized regions in which the two plasmalemmae come in very close contact (arrows). These are believed to be the regions of actual membrane/membrane fusion. (Magnification ×45,000.)

Figure 14. Freeze-etch micrograph of the equivalent region ot Fig. 13. This specimen was prepared without chemical fixation or prior treatment with a cryoprotective. The region of close contact (arrows) cannot be a preparative artefact. (Magnification ×8,500.)

These proposed criteria for membrane fusion can be used to derive a theory relating to membrane fusions within the cell cytoplasm. Fusion of cell organelles with other organelles, or with plasmalemma or tonoplast, may be inhibited if they are not at a sufficiently high energy level to overcome the peak of their repulsive interactions.

The plan for fusion which has been derived does not involve an external chemical agent as essential for any of its stages. The "inducers" of fusion studied were effective by decreasing the electrostatic forces of repulsion between the protoplasts. They may, however, facilitate the

fusion process but are not essential to it. The polycationic compounds may well have this type of effect, as they have been reported to bring about immediate membrane contractions when applied to various protozoa.[31]

Our model for fusion relies on the physical and electrical interactions of the constituent membrane molecules when in close proximity to bring about the necessary structural rearrangements.

The fusion model of Poste and Allison shows many similarities to our own, but ATP and calcium ions are implicated as essential to the mechanisms.[32] Whilst accepting the need for ATP, our experiments would not appear to confirm a need for calcium.

Lucy has proposed a model for fusion which necessitates a high proportion of membranous lipid to be in a micellar form.[33] Our experimental plan has not been comprehensive enough to enable us to tender any valid evidence for or against this proposal.

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