Bioenergetics (1971) **2**, 371–382

A Mechanism for the Pinocytosis of Latex Spheres by Tomato Fruit Protoplasts

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Received 11 *June* 1971

Abstract

Plant protoplasts isolated from tomato fruit locule tissue take up 0·12 μ polystyrene latex spheres by an endocytotic process. Freeze-etching enables the process to be clearly visualized in the electron microscope and provides important data from face view membrane fractures. When protoplasts are glutaraldehyde fixed prior to mixing with latex, the spheres adhere to the plasmalemma and cause some localized membrane distortion. This shows that adhesive forces are sufficient to initiate the invagination process. Freeze-etch membrane fracture faces carry numerous granules, the density of which is lower in the invaginating region. This reduction is in accordance with the expected value if only that part of the membrane which comes in intimate contact with the sphere expands to surround it. There is no change in the granule densities in the surrounding membrane. In fractured membrane surface views distinct phases can be delimited, and their relative occurrence indicates that the second half of the invagination process (i.e. after the diameter of the sphere has reached the membrane plane) is some thirty times quicker than the first half. It is proposed this indicates operation of surface tension forces during the fast phase, and it may be that cellular energy is required only for the initial slow phase of membrane expansion.

Introduction

Endocytosis is the entry of material into cells by means of invagination of the cell membrane. It exists in various forms and probably has various mechanisms. The process is initiated by adsorption of the material to be taken up to the cell membrane. It is usually supposed that this adsorption is charge dependent. Polystyrene latex particles are charged and the charge varies with pH and ionic conditions.[1] The adsorption of latex spheres to a plastic plate is effectively dependent on there being opposite charges on plate and spheres.[2] The mechanism of invagination following adsorption is, however, unknown.[3] With regard to the mechanism, the most studied aspect of endocytosis is pinocytosis in amoebae. It may be induced by salt solutions which produce a change in the electrical resistance of the plasma membrane.[4] Brandt[5] considers that once the membrane is activated in this way its "internal structure is weakened", and it may be pulled in by the normal gel contraction occurring immediately beneath the plasma membrane. However, no such gel contraction occurs in plant protoplasts or in many of the other cells which undergo endocytosis.

The vesicles formed by surface endocytosis in amoebae often produce small evaginations by micropinocytosis. Opposing views concerning these are held by Chapman-Andresen[6] and by Roth.[7] The first considers that there is no resulting change in the total area of membrane, but only that plasma membrane is transferred from outside the cell to the inside. Roth, on the other hand, holds that there is a huge increase in membrane area, proposing that this is by rapid membrane synthesis. It may be significant that the size of micropinocytotic vesicles are comparable with those described in this paper. An alternative to membrane synthesis is membrane stretch. The observation of Burton,[8] that in a stretched membrane pores stretch much in excess of the remainder of the membrane, may be significant in appreciating the physiological value of micropinocytosis in altering the permeability of a cell.[9]

Mayo and Cocking[10] showed that isolated tomato fruit protoplasts would take up latex spheres by pinocytosis (see note on nomenclature), but the use of water-miscible methacrylate embedding media precluded the possibility of obtaining extensive quantitative data. Thus, we re-examined the phenomenon using the freeze-etching technique in order to verify and expand these observations. We realized during this work that the freeze-etch replicas contained information of considerable importance to an understanding of the mechanism of the invagination phase of latex sphere pinocytosis. In freeze-etching fracture occurs in the plane of biological membranes. It is now generally accepted that this fracture lies along an internal plane.[11,12] Most membranes fractured in this way are covered, to a greater or lesser extent, with granules, *c.* 10 nm diameter, probably composed of protein. Regular variations in the distribution density of these granules have been used to indicate regions in which, it is suggested, membrane expansion is occurring by interpolation of lipo-protein material.[13] Equivalent changes occur in association with latex sphere pinocytosis. In addition, large areas of membrane may be exposed on which the relative proportions of the various stages of latex sphere pinocytosis are written more reliably than in thin sections prepared for electron microscopy.

Nomenclature

We have frequently used the term pinocytosis, rather than endocytosis, phagocytosis or micropinocytosis in this paper in order to maintain continuity with previously published work[10,14–16] and because no other term has been used with respect to plant cells or protoplasts.[17,18] However, we recognize that the multitude of terms which have been used in the past[19] tend to lead to confusion in the mind of the reader, by implying differences or similarities which may not be real, and that it is better to use the "blanket" term endocytosis.[20] Furthermore there is at this stage no reason to believe that there is an intrinsic difference requiring separate terminology between the processes which occur in higher plants and those which occur in animal or protistan cells; however, there may be such a difference between uptake into large vesicles and uptake into small vesicles.[21]

Materials and Methods

Protoplasts were isolated from tomato fruit locule tissue obtained from tomato plants grown under controlled conditions.[22] The diced tissue was incubated with 0·5% Macero-

zyme (All Japan Biochemicals Co. Ltd) and 5% cellulase ("Onozuka" P 1500) for 3 hours and the protoplasts released were then washed with 20% sucrose[23] before transfer to fresh 20% sucrose. 0·12 micron diameter latex spheres (Dow Chemical Co., obtained from Serva, Heidelberg, W. Germany) were ultrasonically treated using an ultrasonic probe (M.S.E.) to disperse any aggregates and the suspension made up to 20% sucrose. These were then added in equal proportion to the protoplast suspension and the mixture was stirred. Samples were taken at ½, 1, 3 and 6 hour intervals and fixed in 6% glutaraldehyde in 0·05 M phosphate buffer pH 7 with 20% sucrose for 3 to 6 hours. The protoplasts were then washed and resuspended in 0·05 M phosphate buffer with 20% glycerol by volume and mounted onto copper discs for freeze-etching[24] using a Balzers BA 360m freeze-etcher. Replicas were shadowed with platinum/carbon and cleaned with bleach ("Chloros", ICI), 70% sulphuric acid and tetrahydrofuran consecutively. Grids were examined in an AEI, EM6B electron microscope. The number of granules per unit area (granule density) was measured directly from the plate using a graticule inscribed with circles of various radii attached to a magnifying glass (Lumex Limited, England). Any granule touching the circle was counted as being within the circle. All micrographs are presented in order to show shadows in white.

Results

The pinocytosis of latex spheres was observed at all times during the experimental period. Regions in which the plasmalemma was cross-fractured showed clearly, but qualitatively, that the process was occurring (Figs. 1 and 2). Such invaginations were not found in control material in which protoplasts were mixed with latex spheres after fixation, but in both cases latex spheres were to be found bound closely to the plasmalemma (Figs. 3 and 4). Indeed some indentation of the plasmalemma did occur in the prefixed control (Fig. 5) which suggestes that adhesive forces alone are sufficient to distort the membrane. Like most membranes the plasmalemma of these protoplasts fractures during freeze-etching to produce a convex densely granular face and a concave sparsely granular face.[11, 12] The densely granular face faces outward from the protoplast and provides the most valuable freeze-etch images with regard to pinocytosis. Latex spheres were taken up singly and were closely bound by the membrane. The distribution of pinocytosing sites was random (Fig. 6) as was the distribution of the various stages. Thus, there do not appear to be specific active sites, or to be regions which become active for a limited period of time. We were not able to distinguish the exact extent of development of each individual invagination but three stages were clearly distinguishable, namely: invaginations from which latex spheres had been removed by fracture; invaginations which still contained latex spheres; and small, granule free, depressions which presumably represent the surface view of the "pinched off" invagination before it is released as a separate vesicle (Fig. 7). The diameter of the granule-free depressions varied between 50 and 100 nm; these two extremes being of different appearance as shown in Fig. 8. Their appearance on the sparsely granular concave face is shown in Fig. 9.

The relative proportions of the three strictly definable stages, 3 hours after mixing latex with protoplasts, is presented in Table I. All stages were found at each sampling time but insufficient data is available for statistically valid results to be presented for

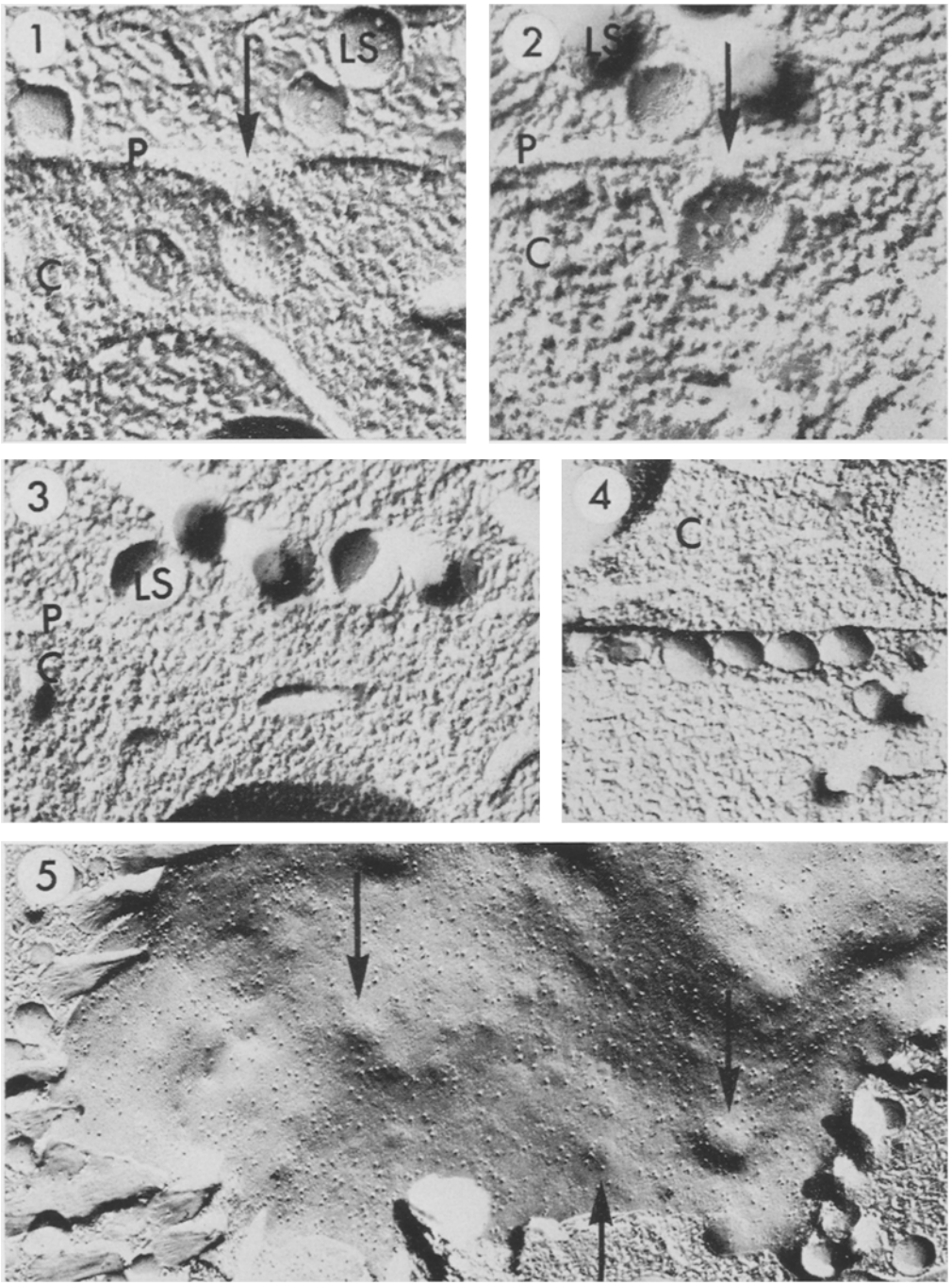


Figure 1. An indentation of the plasmalemma (arrow) from which the latex sphere has been removed in the fracture process. Plasmalemma P, Cytoplasm C, latex sphere LS. (×100,000).

Figure 2. As Fig. 1, but with a narrowed neck. (×114,000).

Figure 3. Part of a protoplast to which latex spheres have adhered. Plasmalemma P, Cytoplasm C, latex sphere LS. (×81,000).

Figure 4. Part of a protoplast, glutaraldehyde fixed before mixing with latex showing latex sphere adhesion similar to Fig. 3. (×60,000).

Figure 5. Protoplast glutaraldehyde fixed before mixing with latex. The plasmalemma has fractured in sparsely granular face view showing that indentations are produced by adhesion (arrows). (×39,000).

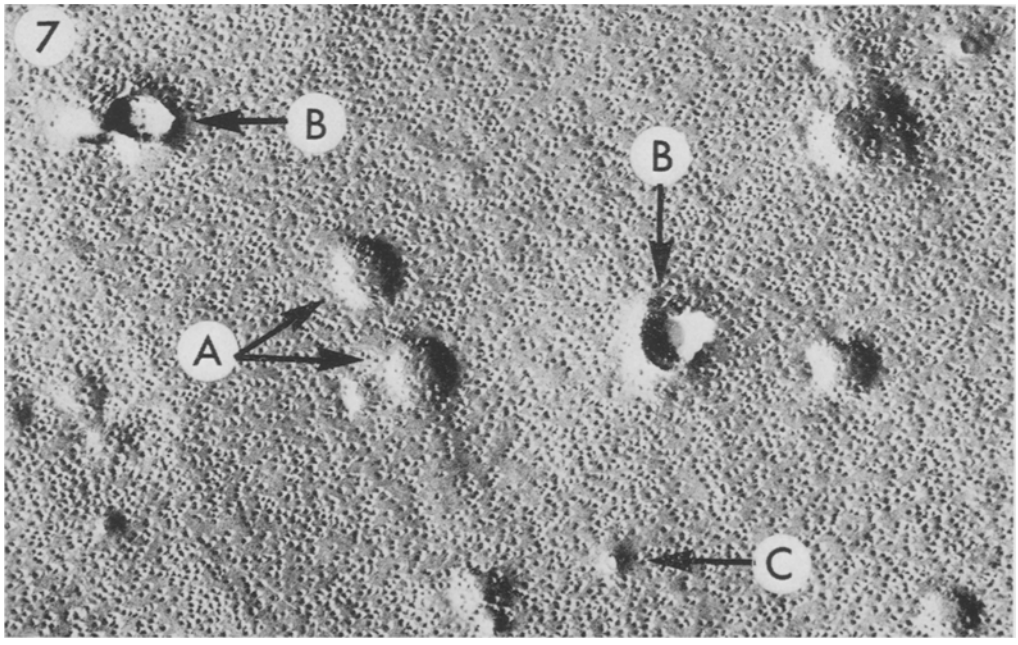


Figure 6. Plasmalemma of a pinocytosing protoplast 3 hours after mixing latex with protoplasts. The different stages of the pinocytotic process are randomly arranged. (×32,000).

Figure 7. Higher magnification of part of Fig. 6. A—hollows from which latex spheres have been removed by fracture. B—latex spheres embedded in the plasmalemma. C—"granule-free depressions", the surface view of pinched-off invaginations. (×72,000).

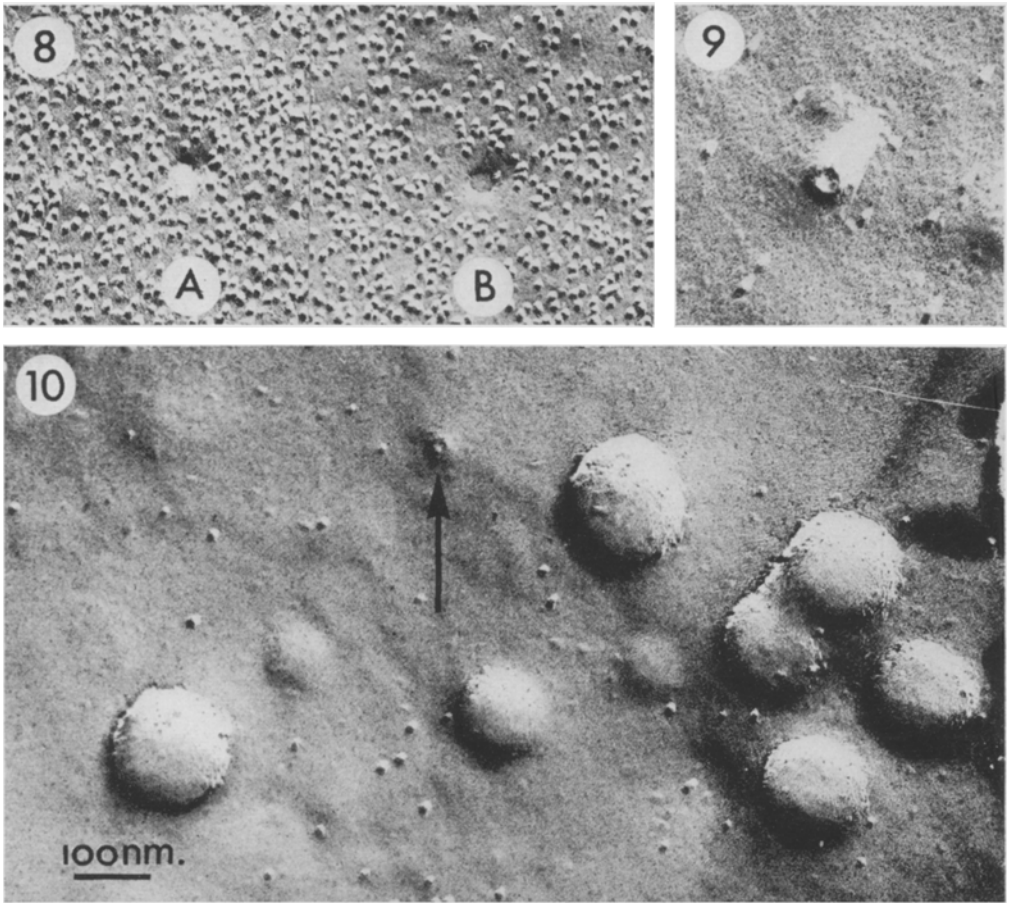


Figure 8. The small granule free depressions which appear at the densely granular face (C, Fig. 7) have an appearance varying between closed (A) and open (B) forms. (×105,000).

Figure 9. The pinched-off neck of the invagination as seen from the sparsely granular face—the attached vesicle has probably been severed by fracture. (×105,000).

Figure 10. The sparsely granular face of the protoplast plasmalemma. There is no increase in granule density corresponding with the decrease, on the opposing face, associated with the invaginations. A pinched-off neck (arrow, cf. Fig. 9) is also shown which has almost completely closed. (×105,000).

all sampling times. There did however, seem to be an increase in the proportion of later stages relative to earlier stages as the experiment progressed.

Analyses of the number of granules per unit area (granule density) were hampered by the small size of latex spheres used. In this, especially, there was considerable variation and the statistical data (Tables I and II) provided applies only to variation in one region chosen for close study, that is, a 900 μm^2 region of plasmalemma from a single protoplast of which Fig. 6 is typical. There is a reduction in the granule density in the base of the hollows relative to the background. There is however, no change in the granule density between the edge of the hollows and the background, neither is there a gradual reduction in granule density in the region surrounding the granule-free depressions (Table IIB). The complementary concave face of the fractured plasmalemma does not show any

TABLE I. The relative proportions of the strictly definable stages of latex sphere pinocytosis as seen in membrane face view 3 hours after mixing latex spheres and protoplasts

Percentage invaginations from which latex has been removed	Percentage invaginations containing latex spheres	Percentage small granule free depressions	Total number counted
66·4	2·2	31·4	916

measurable change in granule density (Fig. 10) due to its sparse granularity. However, there is no reason to believe that there is an increase in the number of granules which could account for the reduction on the opposed face. There appears to be an unaccountable variation from protoplast to protoplast in the extent of the reduction in the granule density of the invaginated region (Fig. 11 shows the plasmalemma of a $\frac{1}{2}$ hour incubated

TABLE II. Granule densities (granules per unit area) found in face views of plasmalemma 3 hours after mixing latex spheres and protoplasts (see Plate 7)

(A)

In base of deepest hollows (selected visually)	Background	Unit measured (diameter of effective circle)*	Number of observations
4·3 ± 1·2	7·5 ± 1·4	80 nm	21

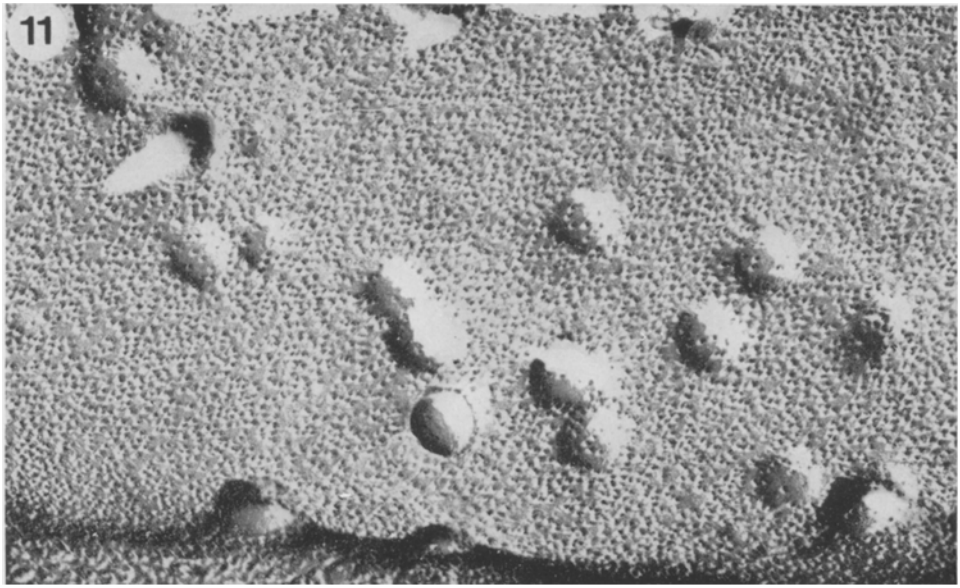
(B)

0–125 nm from edge of hollow	125–250 nm from edge of hollow	Background	Unit measured (diameter of effective circle)*	Number of observations
26·9 ± 3·2	26·8 ± 3·5	26·7 ± 3·0	145 nm	20

* Diameter of circle + twice average diameter of granules.

protoplast in which there appears to be a greater reduction than that shown in Fig. 7).

If the decrease in granule density is a product of membrane expansion, then the tabulated results represent a membrane expansion of 1·72 times. This is, at least, the expansion required to swallow only half the sphere.



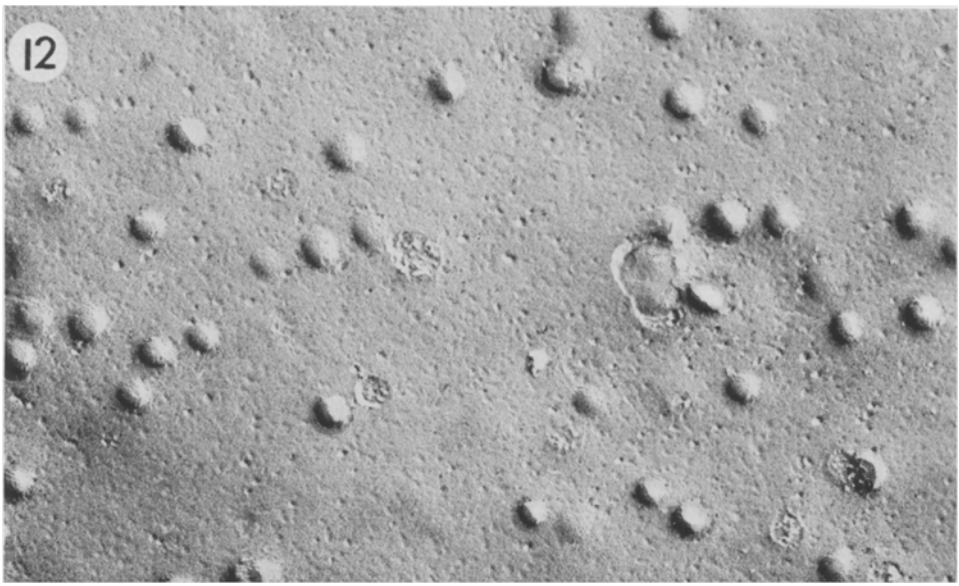


Figure 11. Protoplast incubated with latex for $\frac{1}{2}$ hour. The reduction in granule density in the pinocytosing region is more marked in this case. (×78,000).

Figure 12. Glutaraldehyde fixed endothelial cell plasma membrane from human placenta fractured in face view. Numerous pinocytosing sites can be seen with a possible associated reduction in granule density corresponding with that shown by plant protoplasts. (×90,000).

Attempts to find an equivalent change in granule densities in blood capillary plasma membranes proved difficult since the micropinocytotic invaginations are very small. However, the appearance of these invaginations by freeze-etching suggests that a similar phenomenon might be occurring (Fig. 12).

Discussion

It is reasonable to propose that adsorption initiates the pinocytosis of latex spheres by plant protoplasts and that the adsorbing surfaces of membrane and latex effectively carry opposite charges (see Introduction). This suggestion is supported by the observation that uptake is random, all regions of the membrane being potentially pinocytotic and requiring only the adsorption of a sphere for the process to occur.

The behaviour of the membrane in endocytosis is sometimes described as "infolding". There are clearly no creases in the membranes shown in this paper. Bennett[25] proposed that the mechanism by which the membrane entered the pinocytotic channel was "membrane flow" indicating diagrammatically that membrane was transferred from the top of the channel to the bottom. The changes in granule density which we have described suggest that only that membrane intimately involved with the spheres takes part in the process. The most straightforward explanation for our results is that the region of membrane in contact with the particle expands as a result of changes induced by contact. This expansion may be by membrane stretch, by synthesis or both, e.g. by interposition of lipoprotein. Certainly if any synthesis does occur, then the granules typically found in freeze-etched membranes[24,26] are not synthesized. If the expansion is by membrane stretch then, as stated in the results, an expansion factor of $3{\cdot}5 \pm 2{\cdot}2$ is required. This is much in excess of the expansion capacity of whole protoplasts when placed in hypotonic solutions.[27,28] However, in localized regions expansion capacities above these values might be expected. Interestingly, an expansion factor of 4 is required if a sphere is pushed through an elastic sheet in which the surrounding region does not contribute (surface area of a sphere $= 4\pi r^2$, its cross-sectional area $= \pi r^2$), and this is well within the limits we have obtained. Another explanation for the reduction in granule density in the pinocytosing region is that granules disappear, for instance, in association with the energy supply for conformational changes, perhaps by supplying ATP-ase. It seems unnecessary however to imply a functional significance for these granules when the observed changes can be explained without their mediation. In amoebae, in association with the change in electrical resistance induced by salt solutions, there is a change in the plasma membrane structure visualized in thin-section, the distance between the two dark staining lines of the "unit-membrane" becoming greater than usual.[4] A similar change has recently been reported in plant roots under the influence of uranyl acetate.[33] It is suggested that this change is indicative of the weakening of the membrane structure. The reduction in granule density which we observed might be part of the same phenomenon.

The relative proportions of the three definable stages found in plasmalemma face views are clearly proportional to the relative times for these stages to occur. Thus, if we assume that latex spheres remain embedded in the membrane once they have passed beyond half-way, then the second half of the uptake process is some thirty times faster than the first. Although the initial premise is probably not strictly correct, in that spheres might be ripped from the membrane when beyond half-way, the discrepancy cannot be accounted for solely in this way. Thus, we can say that the second half of the invagination is considerably quicker than the first. This is what might be expected according to the early, passive, surface-tension theories of endocytosis,[29] in which it was proposed that surface tension forces alone could account for the phenomenon. The internal pressure of the cell will, however, act to close off the vesicle once the mid-plane

of the sphere has passed beyond the plasmalemma and will thus facilitate this half of the invagination process. In closing off the neck of the invagination a small granule free area of membrane arises on the plasmalemma surface, presumably by the same means that reduces the granule-density in the base of the invagination. The values for granule densities presented in Table IIB suggest that, again, only a localized region is involved in this process.

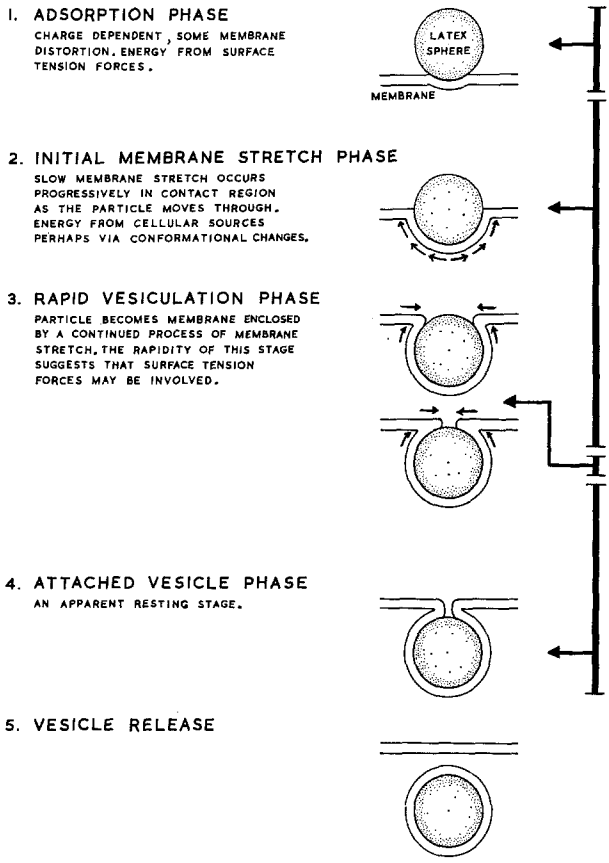


Figure 13. Diagram of the phases of latex sphere pinocytosis by tomato fruit protoplasts. The divided bar (right hand side) represents the relative times for the phases to occur. Arrows indicate regions of membrane stretch.

Variations in granule-density have been shown by Staehelin and Kiermayer[13] in a Golgi complex. At the peripheral region of a mature cisterna, where vesiculation is taking place, there is a marked reduction in the concentration of granules. They interpreted this as being by means of interposition of lipoprotein membrane material. Membrane stretch might also be implicated here.

It may be that a reduction in granule density is found associated with other types of endocytosis. The relatively low granule densities found on fractured membranes other than plant plasmalemma makes observation difficult; however, Fig. 12, and the micro-

graphs of Nickel and Grieshaber,[30] and Kouri, Finlay and Stark[31] do indicate a possible parallel.

The proposed mechanism of uptake of latex spheres by plant protoplasts is shown diagrammatically in Fig. 13. Some suggestions for the sources of energy are included. It seems possible that ATP mediated cellular energy acting directly through the membrane is required only during the slow first half of the invagination process. Casely-Smith[21] proposed that there are two, energetically distinct, types of endocytosis. Classical pinocytosis and phagocytosis being dependent upon cellular energy, but micropinocytosis being independent of cellular energy. This view is often criticized, but it might be that an energetic supply of the type envisaged by Penniston and Green[32] is involved which is built into the membrane as conformational energy and which might not be affected by normal inhibitors. The observations of Penniston and Green on erythrocyte ghosts however, do not relate to the more usual forms of endocytosis since they observed the transition of evaginations to invaginations in which there is no requirement for membrane expansion.

Acknowledgement

We would like to thank Prof. D. D. Eley and Dr. D. Cooper (Chemistry Department, University of Nottingham) for valuable discussion on the nature of latex spheres. The electron microscope used in this work was purchased with a grant from the S.R.C.

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