ENDOCYTOSIS OF CATIONIZED FERRITIN

BY SOYBEAN (GLYCINE MAX L.) PROTOPLASTS

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy
in the
Department of Biology
University of Saskatchewan
Saskatoon, Saskatchewan

by

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February 1987

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Abstract

Protoplasts derived from soybean SB-1 suspension cell cultures were incubated in buffered osmoticum containing cationized ferritin (CF) or native ferritin (NF) to demonstrate the process of endocytosis in plant protoplasts. Uptake of CF occurred rapidly; with as little as a 10 s incubation, CF was found in the cytoplasm in coated vesicles (CVs). After 30 s, CF was also found in small smooth (i.e. non-coated) vesicles. By 2 to 6 min, CF was occasionally detected in three additional organelles; the partially coated reticulum (PCR), dictyosomes and multivesicular bodies (MVBs). This is the first demonstration that the PCR is involved in the process of endocytosis in plants. The PCR consists of a network of interconnected tubular membranes with a coat over part of its cytoplasmic surface. Serial sectioning established that CF was distributed throughout this organelle except for membrane dilations which occasionally contained small internal vesicles. The PCR was frequently, but not always, associated with dictyosomes. Only one example of a direct membrane connection between these organelles was observed.

Longer CF incubations resulted in increased labelling of the PCR, dictyosomes, and MVBs. Incubations of 60 min or longer resulted in the labelling of membranous masses within vacuoles. The membranous masses resembled the contents of some CF-labelled MVBs and are likely derived from them.
Endocytosis in soybean protoplasts was charge dependent since even at high concentrations, very little NF was internalized.

Results from protoplasts post-fixed in zinc iodide-osmium tetroxide (ZIO) indicate that membrane connections exist between the endoplasmic reticulum and dictyosomes. The lack of ZIO reaction product in some PCR is consistent with the proposed independence of this organelle from dictyosomes.

In preliminary experiments, whole SB-1 cells were incubated in NF, CF and lanthanum chloride. Uptake of CF and lanthanum was observed but could not unambiguously be attributed to the occurrence of endocytosis.

A scheme for the endocytotic pathway in soybean protoplasts is presented. The results from protoplasts are compared with those for receptor-mediated endocytosis in animal cells. The implications of this pathway are considered with respect to membrane recycling, molecular repair, degradation of macromolecules and receptor-mediated endocytosis in plant protoplasts and cells.
Acknowledgements

I wish to thank first and foremost my supervisor, Dr. Larry C. Fowke, for the opportunity to work on this project and for allowing me a sufficient degree of freedom to develop the project as I saw fit. I am especially thankful for the high degree of patience, perseverance, and skill displayed by Dr. Fowke while editing the numerous drafts of this thesis and of papers published and in press. I am also thankful for his words of encouragement and sound advice.

I would also like to thank the other members past and present of my supervisory committee, Drs. V.K. Sawhney, G.H. Rank, G. Burkholder, L.R. Griffing and B.G. Mersey for their thoughtful comments and questions. I would especially like to thank Dr. Sawhney for the excellent job he did as acting supervisor during Dr. Fowke's sabbatical leave. I would also like to thank Dr. Griffing, with whom I shared office space, for the numerous stimulating discussions and for use of his technical expertise in some experiments.

As is always the case, the work presented in this thesis could not have been produced without the technical assistance of others. In particular, I would like to thank Ms. Patricia Rennie for her technical assistance, especially with the generation and analysis of serial sections from soybean protoplasts, and for her generous offers to maintain my cell cultures during my absences from the Biology Department. I must also thank Pat for words of
encouragement, sympathy and advice. Excellent technical assistance was also provided by Mr. Yukio Yano (electron microscopy) and Mr. Dennis Dyck (photography). I would also like to thank Dr. J. King and the members of his laboratory for the use of their laboratory facilities. Assistance from Dr. F. Constabel and Mr. K. Pahl of the Plant Biotechnology Institute is gratefully acknowledged.

To my bridge partners, golf partners, soccer and fastball teammates, and friends, who are too numerous to name individually, I thank you for making my stay in Saskatoon more pleasurable and interesting than it would have been otherwise. Special mention and thanks go out to Ms. Rozwitha Marx for coming to the aid of a friend in need.

Financial assistance in the form of a Postgraduate Scholarship (4 years) from the Natural Sciences and Engineering Research Council, Canada (NSERC) is gratefully acknowledged. Research expenses and additional (non-overlapping) financial assistance were provided from an NSERC Operating Grant to Dr. L.C. Fowke.
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List of Abbreviations

cER- cisternal endoplasmic reticulum
CF- cationized ferritin
CP/CPs- coated pit/coated pits
CPB- cell phosphate buffer
CURL- compartment of uncoupling of receptor and ligand
CV/CVs- coated vesicle/coated vesicles
ER- endoplasmic reticulum
GERL- Golgi-associated endoplasmic reticulum which produces lysosomes
MES- 2-(N-morpholino)ethanesulfonic acid
MVB/MVBs- multivesicular body/multivesicular bodies
NF- native ferritin
PCR- partially coated reticulum/partially coated reticula
PPB- protoplast phosphate buffer
tER- tubular endoplasmic reticulum
Tris- Tris(hydroxymethyl) aminomethane
ZIO- zinc iodide-osmium tetroxide
1 Introduction

1.1 General Concepts

Endocytosis is the process by which molecules, particularly macromolecules, and particles from the extracellular environment are internalized into the cell via plasma membrane-derived vesicles. The term, endocytosis, actually encompasses two different but related processes. The first is called phagocytosis and is the process by which large particles or molecular aggregates (> 500 nm in diameter) are internalized (Steinman et al. 1983). The cell actively surrounds and engulfs these particles. Beneath the region of the plasma membrane where the vesicle called a phagosome is forming, there is usually an organelle-free filamentous network that contains actin and probably other contractile proteins (Steinman et al. 1983). Phagocytosis is used extensively by animal cells. An example is the mammalian immune response where certain cell types such as macrophages engulf large molecular aggregates or particles such as whole bacterial cells. The second process, called pinocytosis, involves the internalization of macromolecules or small particles (Steinman et al. 1983). The vesicles involved in this process are very much smaller than phagosomes. Furthermore, the cell does not engulf the material per se but rather the plasma membrane invaginates resulting in the eventual formation of a tiny vesicle or pinosome.
Phagosomes are easily identified because the particles contained therein can be seen by both light and electron microscopy (Steinman et al. 1983). Pinocytotic vesicles can only be detected in the light microscope when their contents have been labelled in a very specific manner which enables detection in specialized optical systems such as the fluorescence light microscope. Even in these cases, the actual nature of the vesicle must be determined through electron microscopy.

A number of terms are commonly used to describe specific types of endocytosis. While the word, endocytosis, is used in these terms, the process involved is usually pinocytosis rather than phagocytosis. These specialized terms include fluid-phase endocytosis, adsorptive endocytosis and receptor-mediated endocytosis. In fluid-phase endocytosis substances in solution or suspension are internalized within plasma membrane-derived vesicles; the substances do not interact directly with the plasma membrane. Adsorptive endocytosis involves the binding of molecules or particles to the surface of the plasma membrane prior to their internalization. Receptor-mediated endocytosis is one form of adsorptive endocytosis. This term is used to describe selective internalization of a particular substance or ligand (usually protein) following its binding to a specific receptor on the plasma membrane (Hermo et al. 1985).
1.2 Endocytosis in Animal Cells

Fig. 1 presents a diagrammatic representation of receptor-mediated (and adsorptive) endocytosis in animal cells (Breitfeld et al. 1985; Wileman et al. 1985; Stahl and Schwartz 1986). It does not represent any one cellular system but is a composite showing the major organelles involved in receptor-mediated endocytosis. The following discussion describes the structure and function of these organelles.

In the last ten years or so, research on pinocytosis in animal cells, especially receptor-mediated endocytosis, has indicated that a morphologically distinct class of vesicle, the coated vesicle (CV), is frequently involved in these processes. The CV can be distinguished from other classes of vesicles by the presence of a protein layer or coat on its cytoplasmic surface. When CVs are isolated and negatively stained, the surface coat has an appearance, in the electron microscope, reminiscent of a soccer ball. The coat consists of a series of interlocking pentagons and hexagons (Kanaseki and Kadota 1969). Biochemical studies on CV-enriched gradient fractions reveal that the coat consists primarily of a protein which has been given the name, clathrin (Pearse 1975). Clathrin is composed of two different classes of polypeptides. The "heavy chain" has a relative molecular mass of 180 KDa (Pearse 1975; Fine and Ockleford 1984) while the "light chains" have a relative molecular mass of 33–38
Fig. 1. Diagrammatic representation of the pathway of receptor-mediated endocytosis in animal cells. Receptor-ligand complexes localized in a coated pit (CP) are internalized when the CP pinches off the plasma membrane to form a coated vesicle (CV). The CV sheds its coat to become a smooth vesicle (SV) which fuses with an endosome. The endosome shown here is known by the acronym, CURL. Receptors and ligands which are recycled become localized within the tubules of CURL. Vesicles derived from these tubules return recycling molecules to the cell surface. These vesicles may originally be coated and then become smooth as they shed their coats. Recycling receptors and ligands may also pass through the Golgi stacks prior to returning to the cell surface. Transport to and from the Golgi is thought to be mediated by vesicles. Receptors and ligands which are to be degraded become localized within a vesicular or dilated region of CURL. This region is thought to transform into a multivesicular body (MVB) and then into a secondary lysosome. In this lysosome, the receptors and ligands are degraded. Composite diagram based on Breitfeld and co-workers (1985), Wileman and co-workers (1985) and Stahl and Schwartz (1986).
KDa (Ungewickell and Branton 1981; Kirchhausen and Harrison 1981). It appears that there are at least two different types of light chains differing in relative molecular mass by 2 or 3 KDa (Ungewickell and Branton 1981; Kirchhausen and Harrison 1981).

Ligands bind to their specific receptors on the plasma membrane and are internalized via coated pits (CPs) and CVs. CPs are specialized regions of the plasma membrane which have a clathrin coat on their cytoplasmic surfaces. By the process of invagination, these pits are thought to "bud off" the plasma membrane to form free CVs (Kanaseki and Kadota 1969). Coated regions are also found on some internal cellular membranes. In particular, portions of the Golgi apparatus, especially trans reticular Golgi, bear such coats (Willingham and Pastan 1982; Willingham et al. 1984). Other organelles, such as the compartment of uncoupling of receptor and ligand (CURL, Geuze et al. 1984, 1985) and the Golgi-associated endoplasmic reticulum which produces lysosomes (GERL, Novikoff 1976), may also have coats on portions of their cytoplasmic surfaces.

After CVs are formed, they are thought to rapidly shed their clathrin coat and fuse with one another or with organelles known as endosomes (Wileman et al. 1985). Endosomes are heterogeneous in morphology consisting of membranous sacs and associated tubules and more than one type of endosome may exist in a particular cell. Receptors
and/or ligands which are to be degraded are delivered ultimately to lysosomes. Receptors and/or ligands which are recycled back to the cell surface may recycle directly from endosomes such as CURL (e.g. Geuze et al. 1984). In this way, these receptors and ligands avoid exposure to the hydrolytic enzymes present in lysosomes.

Receptor-ligand complexes which experience different fates appear to be internalized via the same CP (Geuze et al. 1984). Therefore sorting of these different complexes from one another must occur within endosomes. It is known that endosomes are acidified membrane compartments (Tycko and Maxfield 1982). This is due to the presence of an ATP-dependent proton pump located within the membrane of the endosome (reviewed by Wileman et al. 1985). Coated vesicles are also thought to have a similar proton pump (reviewed by Wileman et al. 1985). Under acidic pHs, a number of receptor-ligand complexes are known to dissociate (Wileman et al. 1985). Thus the acidic environment of the lumen of endosomes may result in the dissociation of receptor-ligand complexes and in the subsequent targeting of these components to their different sites within the cell. Based on immunocytochemical and cytochemical results, it has been proposed that CURL (e.g. Geuze et al. 1983a, 1984) and/or trans reticular Golgi (Willingham et al. 1984) are the endosomal compartments responsible for these sorting events.
It is conceivable that these two structures are in fact the same organelle which has been given different names by different research groups (Willingham and Pastan 1984a).

The uptake of asialoglycoproteins provides a good example of sorting of ligand and receptor in the endosomal compartment of the cell (Geuze et al. 1983a). In CURL which consists of tubular membranes connected to vesicular profiles, the receptors tend to be localized within the tubular regions while the ligands tend to be located in the vesicular portion. Since the asialoglycoprotein receptors are recycled back to the plasma membrane while their ligands are degraded by the lysosomes, it was suggested that the vesicles derived from the tubular region of CURL would deliver the receptors to the plasma membrane either directly or after passage through the Golgi apparatus. It was also suggested that the vesicular portion of CURL could mature into a second class of endosome called a multivesicular body (MVB). In some cases, MVBs have also been referred to as multivesicular endosomes to emphasize their involvement in endocytosis. The MVB containing the asialoglycoprotein ligands would then fuse with a (primary) lysosome to produce a secondary lysosome where degradation of the ligands would occur (Geuze et al. 1983a).

In hepatocytes, both asialoglycoprotein receptor-ligand complexes and IgA receptor-ligand complexes are internalized (Geuze et al. 1984). In contrast to the situation for
asialoglycoproteins and their receptors, IgA molecules and their receptors do not dissociate upon internalization and neither the receptors nor the ligands are recycled. Instead receptor-ligand complexes are formed at the sinusoidal membrane of rat hepatic parenchymal cells and are ultimately delivered to the bile canaliculi where the receptors are cleaved to release IgA molecules with attached secretory components. This process of transferring ligands from one side of a cell to another is known as transcytosis. During transcytosis, the IgA-IgA receptor complexes become localized within the tubular component of CURL. There is a dramatic microheterogeneity in the distribution of receptors within the tubules of CURL. Therefore the receptors for asialoglycoproteins and the IgA receptor-ligand complexes are located within different regions of the tubules. This result demonstrates that sorting of different receptor-ligand complexes can occur within CURL.

Multivesicular bodies consist of a membrane bounded vacuole containing several smaller internal vesicles. On the cytoplasmic surface of the outer membrane there is frequently an electron dense plaque which has a lamellar appearance (Willingham and Pastan 1984a). In some cases, these organelles can contain hydrolytic enzymes (e.g. Friend and Farquhar 1967; Morales et al. 1985). These organelles are usually thought of as being the last membrane
compartment in the endocytic pathway before lysosomes. It has been suggested that they may form directly from other endosomes such as CURL via a maturation process and that they themselves are transformed into lysosomes (Geuze et al. 1983a, 1985; Harding et al. 1985).

Another organelle which is frequently involved in membrane traffic associated with receptor-mediated endocytosis is the Golgi apparatus. The Golgi apparatus consists of Golgi stacks, the number of which varies considerably from cell type to cell type. Physical connections may exist between the various Golgi stacks in a cell (Farquhar and Palade 1981). A Golgi stack consists of a set of 3 to 8 stacked flattened membrane sacs referred to as cisternae (Farquhar and Palade 1981). The Golgi apparatus or rather its constituent Golgi stacks tend to be concentrated within one region of animal cells (Farquhar and Palade 1981). The cisternae are not of identical composition and thus a Golgi stack has a distinct polarity. One side of the Golgi stack, the cis side, is thought to receive vesicles containing newly synthesized products from the endoplasmic reticulum (ER). At the other side, the trans side, secretory vesicles and granules are formed from the cisternae (Farquhar and Palade 1981). The Golgi stacks of animal cells are known to contain a number of enzymes (reviewed by Farquhar 1985), many of which appear to function in the post-translational modification of proteins newly
synthesized on the ER. These enzymes are located within specific cisternae of the Golgi stacks. In general, the enzymes involved in early steps of the post-translational modification of proteins are found in the cis cisternae while enzymes involved in later steps tend to be found in trans cisternae. Transport of proteins between cisternae is thought to be mediated by vesicles which form from the edges of the cisternae (Farquhar 1985).

There is evidence that components of the Golgi stacks function in the endocytotic pathway. In particular, vesicles and tubules associated with the trans side of the Golgi stacks (i.e. GERL, trans reticular Golgi and CURL) frequently become labelled during endocytosis (e.g. Willingham et al. 1984). These components seem to be involved with sorting events resulting in the separation of receptors and ligands which are to be recycled to the plasma membrane from those which are to be degraded in lysosomes (Willingham et al. 1984). However, the cisternae are also sometimes labelled and thus seem to be involved in the endocytotic pathway as well. Recent evidence suggests that biosynthetic repair of ligands and membrane constituents may take place in the cisternae of the Golgi apparatus (reviewed by Breitfeld et al. 1985). There is also evidence in secretory cells that components of the secretory granule membrane are recycled to the trans Golgi cisternae
presumably to be reutilized in the packaging of secretory products (reviewed by Farquhar 1985).

Fig. 1 presents the (receptor-mediated) endocytotic pathway of animals cells as it is commonly thought to occur. However, an alternative pathway (Fig. 2) has been proposed (Willingham et al. 1984) which is radically different from that illustrated in Fig. 1. While this alternative is often widely cited in the literature, it does not appear to be widely accepted. In this pathway, CPs are fixed structures on the plasma membrane which accumulate receptor-ligand complexes. These complexes are then transferred to an adjacent region of the plasma membrane where a smooth surfaced vesicle is forming via the process of invagination (Willingham et al. 1981). This smooth surfaced vesicle is called a receptosome. The receptosomes are then transported towards the center of the cell and during this process they may fuse with one another to form a MVB. Eventually the incoming receptosomes including MVBs fuse with the trans reticular Golgi where sorting of receptors and ligands occur. It is proposed that receptors and ligands which are to be degraded are delivered to lysosomes from the coated regions of the trans reticular Golgi. These coated regions may mirror the function of the CPs of the plasma membrane except that rather than delivering receptors and ligands to receptosomes they deliver them to newly forming lysosomes. Receptors and ligands which are to be recycled to the plasma
Fig. 2. Alternative representation of the pathway of receptor-mediated endocytosis in animal cells. Receptor-ligand complexes localized in a CP are transferred to an adjacent, non-coated region of the plasma membrane. From this region, a smooth vesicle, called a receptosome pinches off the plasma membrane. Typically, the receptosome contains a single small internal vesicle. A receptosome may fuse directly with the trans reticular Golgi (TRG) or may first fuse with other receptosomes to form a MVB (a.k.a. a multivesicular receptosome). The MVB then fuses with the TRG. In the TRG, receptors and ligands to be degraded become localized in a coated tip of a TRG tubule. From this coated region, they are transferred to a newly forming lysosome, possibly in a manner analogous to receptosome formation. In the lysosome, the receptors and ligands are degraded. Receptors and ligands which are recycled become concentrated in a non-coated region of the TRG and are transported to the cell surface in a vesicle derived from this region. Diagram based on Willingham and co-workers (1981,1984), and Willingham and Pastan (1984a).
membrane are thought to do so in vesicles originating from the smooth surfaced regions of the trans reticular Golgi (Willingham et al. 1984; Willingham and Pastan 1984a).

1.3 Endocytosis in Plant Cells

While the occurrence of endocytosis is well documented for animal cell systems, the same is not true for plant cells. However, there is a small body of work which provides evidence for the occurrence of endocytosis in plant cells and protoplasts. Some studies of plant-pathogen interactions indicate that the pathogens can gain entry to the host cells via an endocytotic process involving the invagination of the plant cell plasma membrane (e.g. Newcomb 1976). Furthermore, when plant tissues are incubated in solutions of heavy metal salts, evidence of uptake via plasma membrane-derived vesicles has been obtained (e.g. Wheeler and Hanchey 1971; Wheeler et al. 1972; Robards and Robb 1972, 1974; Peterson et al. 1986). In these examples, uptake is thought to involve the formation of smooth surfaced vesicles from the plasma membrane. The intracellular location of the crystallized heavy metal precipitates usually includes vacuoles of varying sizes (e.g. Wheeler and Hanchey 1971; Wheeler et al. 1972; Robards and Robb 1972; Peterson et al. 1986).

In plant protoplasts, a range of molecules or particles have been internalized by protoplasts. These include ferritin (e.g. Cocking 1966a; Power and Cocking 1970), virus
particles (e.g. Cocking 1966a,b; Honda et al. 1975), polystyrene beads (e.g. Mayo and Cocking 1969; Suzuki et al. 1977), liposomes (e.g. Fukunaga et al. 1983), isolated organelles (e.g. Davey et al. 1976), bacteria, and yeast cells and protoplasts (e.g Davey and Power 1975). In all cases, the endocytotic process involved the formation of a smooth surfaced vesicle from the plasma membrane. No attempt was made to determine the full extent of the pathway in any of these cases. Effort was concentrated on determining that endocytosis had occurred. In many cases, the demonstration of endocytosis was of dubious physiological significance as agents such as polyethylene glycol or poly-L-ornithine were used to stimulate the binding of particles to the surface of the protoplast and to induce the occurrence of endocytosis via osmotic shock (e.g. Davey and Power 1975; Honda et al. 1975; Davey et al. 1976; Fukunaga et al. 1983). In these cases, the occurrence of endocytosis may solely be the result of these manipulations and therefore, may bear no relationship to the normal functioning of protoplasts. Furthermore, protoplasts are themselves an artificial system and represent a significant physiological deviation from normal turgid plant cells. Therefore, results using plant protoplasts may not be directly applicable to normal turgid plant cells.

The existence of CVs is well documented for plant cells
Coated vesicles are frequently found in regions where active cell wall synthesis is occurring, for example, at the developing cell plate of dividing cells (Franke and Herth 1974; Nakamura and Miki-Hiroshige 1982) and in the region of the enlarging infection thread during the infection of a plant host by a symbiotic Rhizobium bacterium (Robertson and Lyttleton 1982). As a result the most frequently suggested functions for coated membranes in plants has been exocytosis either of cell wall protein (Bonnett 1969; Robertson and Lyttleton 1982) or cell wall polysaccharide (Nakamura and Miki-Hiroshige 1982), and the transport of new membrane to the plasma membrane (Franke and Herth 1974; Nakamura and Miki-Hiroshige 1982). However, others (e.g. Lucas and Franceschi 1981) have suggested that CPs and CVs may be involved in endocytotic processes.

Coated vesicles have been isolated from suspension cultured tobacco cells (Mersey et al. 1982), soybean protoplasts (Mersey et al. 1985) and suspension cultures of carrot cells (Depta and Robinson 1986). In each case, SDS-polyacrylamide gel electrophoresis indicated that a 190 KDa protein co-purifies with CV-enriched fractions. It has been suggested that this protein is the heavy chain of plant clathrin. Polypeptides co-purifying with CV-enriched fractions were also found in the 30-40 KDa range and may constitute the light chains of plant clathrin. Coats are also found on CPs, some cisternae of the dictyosomes (see...
terminology below) and on an organelle which is known as the partially coated reticulum (PCR), a tubular membrane system with coats on part of its cytoplasmic surface (Pesacreta and Lucas 1985).

The Golgi apparatus of higher plant cells tends to be organized in a manner somewhat different from its animal cell counterpart. In higher plant cells, it consists of a large number of small stacks of cisternae. Each stack of cisternae is referred to as a dictyosome. The dictyosome is the plant cell equivalent of the Golgi stack found in animal cells. Dictyosomes are distributed throughout the plant cell as opposed to being concentrated in one region of the cell as is frequently the case with Golgi stacks in animal cells. However, like the animal cell Golgi stacks, dictyosomes of plant cells are polarized and have cis and trans sides (Mollenhauer and Morre 1980; Robinson and Kristen 1982).

1.4 Research Objective and Rationale

In this research project, the hypothesis that coated membranes as well as other organelles are involved in endocytosis in plant cells and protoplasts was tested. While there is evidence for the presence of plasma membrane-associated receptors for beta glucans (Yoshikawa et al. 1983), auxins (Jacobs and Gilberts 1983) and abscisic acid (Hornberg and Weiler 1984), no plant plasma membrane
receptor for a specific signal has been isolated and fully characterized (Ralton et al. 1986). Therefore, it was not possible to use a specific receptor-ligand system to demonstrate the occurrence of endocytosis in plant cells and to demonstrate the involvement of coated membranes in this process as has been done in animal cell systems. Instead unmodified or native ferritin (NF) and cationized ferritin (CF) were used as markers for fluid phase and adsorptive endocytosis, respectively. Ferritin is an iron storage protein; its iron-rich central core can be identified in the electron microscope and is quite distinctive in appearance. CF is a chemically modified derivative of NF which has a relatively high isoelectric point such that it has a net positive charge at neutral and acidic pHs. This property is beneficial for studies of adsorptive endocytosis as plant plasma membranes, particularly those of protoplasts, have a net negative surface charge under these conditions (Nagata and Melchers 1978; Griffing et al. 1985; Fowke 1986). The only problem associated with the use of the two types of ferritin as markers for endocytosis is that these molecules are large proteins and therefore do not readily pass through the cell wall. For this reason, protoplasts were used for most experiments. After establishing the existence of an endocytotic pathway in protoplasts, preliminary experiments involving incubations of cells with lanthanum chloride which is a heavy metal salt, NF, and CF were performed in the hope
of demonstrating the occurrence of endocytosis in whole cells.

SB-I soybean cell suspension cultures were the source of cells and protoplasts used in this research project. This cell culture is a long established, easily maintained cell line and was readily available. Furthermore, a satisfactory protocol for the production of protoplasts from this cell culture had already been developed (Mersey et al. 1985).

Some of the results reported in this thesis have been published (Tanchak et al. 1984).
2 Materials and Methods

2.1 Cell Culture

The soybean cell culture used in all experiments was the SB-1 cell line. (This cell line was originally obtained from Dr. F. Constabel of the Plant Biotechnology Institute, National Research Council, Saskatoon, Sk., Canada.) The SB-1 cell line was maintained in 1-B5 medium (Gamborg, 1982) and was subcultured twice weekly. Subculturing of this cell line entailed the inoculation of a 250 ml DeLong flask containing 50 ml of fresh 1-B5 medium with 10 ml of the previous 3.5 day old culture. The cultures were kept in a culture room on a gyratory shaker (150 rpm) at 28°C, in continuous light (10 umol photons/m²/s from "cool-white" fluorescent lamps; Westinghouse, Pittsburg, PA, USA).

2.2 Buffers

A number of buffer systems were used during the course of this project. The composition of some of these buffers and where relevant a brief description of how they were produced and used is provided here.

Sorb-MES osmoticum contained 0.7 M sorbitol, 3 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 6 mM CaCl₂ and 0.7 mM NaH₂PO₄, pH 5.8. This buffer system was used during the production and isolation of protoplasts (sections 2.3 and 2.4).

Buffered osmoticum consisted of 50 mM sodium phosphate buffer and 350 mM sorbitol, pH between 6.7 and 6.8. It was
made by mixing equal volumes of 100 mM \( \text{Na}_2\text{HPO}_4 \), \( \text{100 mM Na}_2\text{HPO}_4 \), \( \text{1.4 M sorbitol} \) and glass distilled water. This osmoticum was used to wash protoplasts prior to and, where appropriate, following cationized ferritin (CF; horse spleen ferritin coupled with \( \text{N,N-dimethyl-1,3-propanediamine} \), in \( \text{150 mM NaCl} \); Sigma Chemical Co., St. Louis MO, USA) or native ferritin (NF; Ferritin Type 1 from equine spleen, in \( \text{150 mM NaCl} \); Sigma) incubations. It was also the osmoticum in which CF or NF incubation of protoplasts took place and in which glutaraldehyde fixation of protoplasts was performed. The derivatives of this buffered osmoticum were made by substituting an appropriate volume of CF, NF or (50 \% \text{ v/v}) glutaraldehyde solutions for some of the distilled water used to make up the buffered osmoticum. For example, to make up 4 ml of 1 mg/ml CF in buffered osmoticum, the following volumes and solutions were used; 1.0 ml \( \text{Na}_2\text{HPO}_4 \), 1.0 ml \( \text{Na}_2\text{HPO}_4 \), 1.0 ml 1.4 M sorbitol, 0.6 ml distilled water and 0.4 ml of 10.4 mg/ml CF in \( \text{150 mM NaCl} \).

Protoplast phosphate buffer (PPB) contained 50 mM sodium phosphate buffer, pH 6.8, and was produced by mixing 1 volume of \( \text{100 mM Na}_2\text{HPO}_4 \) with 1 volume of \( \text{100 mM Na}_2\text{HPO}_4 \) and 2 volumes of glass distilled water. It was used to wash protoplasts following glutaraldehyde fixation, for osmium tetroxide post-fixation of protoplasts and some subsequent washes. The osmium tetroxide post-fixative was made by
substituting 1 volume of 4% (w/v) osmium tetroxide for one of the 2 volumes of glass distilled water used to make up PPB.

Cell phosphate buffer (CPB) consisted of 25 mM sodium phosphate buffer, pH 6.8, produced by mixing 1 volume of 100 mM NaH$_2$PO$_4$ with 1 volume of 100 mM Na$_2$HPO$_4$ and 6 volumes of glass distilled water. This buffer system and its derivatives were used in all stages prior to ethanol dehydration (section 2.9) of an experiment where whole SB-1 cells were incubated in CF or NF for a period of 1 h (section 2.7). The derivatives of CPB included CPB plus 1 mM CaCl$_2$ (Ca-CPB), Ca-CPB with 1 mg/ml CF (15 mM NaCl), Ca-CPB with 10 mg/ml NF (15 mM NaCl), Ca-CPB with 15 mM NaCl, Ca-CPB with 3.1% (v/v) glutaraldehyde, and CPB with 1% (w/v) osmium tetroxide. These derivatives were made by substituting the appropriate volume(s) of 10 mM CaCl$_2$, 10.4 mg/ml CF (150 mM NaCl), 100 mg/ml NF (150 mM NaCl), 150 mM NaCl, 50% (v/v) glutaraldehyde, and 4% (w/v) osmium tetroxide for some of the distilled water used to make CPB. The pH of Ca-CPB with 3.1% (v/v) glutaraldehyde had to be adjusted to between 6.7 and 6.8 by the addition of 1 M sodium hydroxide.

MES-Tris buffer contained a mixture of MES and Tris(hydroxymethyl) aminomethane (Trizma Base; Sigma) and was used in all stages prior to ethanol dehydration in an experiment where intact SB-1 cells were incubated in a
solution of lanthanum chloride (LaCl$_3$). This buffer system was prepared in the following manner. A solution of 20 mM Trizma Base was added dropwise to a volume of 20 mM MES until the pH of the resulting mixture was 5.8. This mixture will be referred to as 20 mM MES-Tris. To produce the final buffer that was used in the experiment (i.e. MES-Tris buffer), a volume of 20 mM MES-Tris was mixed with an equal volume of glass distilled water. At this last step, derivatives of MES-Tris buffer were made by using 40 mM LaCl$_3$, 50 % (v/v) glutaraldehyde, or 4 % (w/v) osmium tetroxide in place of all or a fraction of the glass distilled water to produce, respectively, 10 mM LaCl$_3$, 3.1 % (v/v) glutaraldehyde (pH corrected with 1 M sodium hydroxide) and 1 % (w/v) osmium tetroxide in MES-Tris.

2.3 Production of Protoplasts

For the production of protoplasts, a dense cell suspension or slurry was produced by pooling the contents of two or three 3.5 day old cultures into one culture flask. The cells were allowed to settle and most of the medium was decanted leaving only the minimum volume of medium necessary for the pipetting of the cell slurry. Fifteen ml of this slurry, about the equivalent of the contents of one 3.5 day old SB-1 culture, were added to each sterile plastic 100 x 15 mm petri dish containing a cell wall-degrading enzyme mixture (See section 2.4) in 10 ml of a Sorb-MES osmoticum
(section 2.2). The petri dishes were then sealed with Parafilm M (American Can Company, Greenwich, CT, USA), covered with aluminum foil and incubated at room temperature, for 12 to 15 h on a gyratory shaker (50 rpm). The resulting protoplast preparation was filtered through glass wool and a nylon screen with an 80 or 85 μm mesh size. The protoplasts were collected by centrifugation (200X g, 4-8 min) and were washed by centrifugation (200X g, 4-6 min) in a wash medium, a 1:1 mixture of culture medium (1-B5) and Sorb-MES osmoticum, pH 5.8.

2.4 Composition of Cell Wall-degrading Enzyme Mixture

The cell wall-degrading enzyme mixture consisted of 1.2 % (w/v) Onozuka R-10 cellulase (Kanematsu-Gosha, Los Angeles, CA, USA), 0.5 % (w/v) Rhozyme HP-150 hemicellulase (Genecor Inc., Corning, NY, USA) and 0.5 % (w/v) pectinase in Sorb-MES osmoticum (section 2.2). Four different pectinases were used in this enzyme mixture. They were: pectinase (fungal; Sigma), pectinase (from Rhizopus sp.; Sigma), Serva pectinase (Feinbiochemica, Heidelberg, FRG) and pectinase (fungal origin; Fluka AG, Chem. Fabrik, Buchs, Switzerland). Onozuka R-10 cellulase was used in the form provided by the supplier. In contrast, the Rhozyme HP-150 hemicellulase and the various pectinases were usually desalted on a Sephadex G-25 column (Pharmacia (Canada) Inc., Dorval, P.Q. Canada) and freeze-dried for storage prior to use. On occasion, crude or non-desalted preparations of
these enzymes were used for protoplast experiments. All enzymes (except the pectinase from \textit{Rhizopus}) were generous gifts from Dr. F. Constabel and Mr. K. Pahl of the Plant Biotechnology Institute, National Research Council, Saskatoon, Sk., Canada.

Immediately prior to use, the cell wall-degrading enzyme mixture was filter-sterilized using a 0.45 μm sterile disposable Millipore filter (Millipore Corp., Bedford, MA, USA).

2.5 Cationized Ferritin Incubation and Fixation of Protoplasts

Prior to CF treatment, protoplasts were washed 2 or 3 times by centrifugation (200X g, 1-4 min) in the buffered osmoticum (section 2.2). Pellets containing approximately 0.15 ml of packed protoplasts were resuspended in 0.4 ml of buffered osmoticum. To commence CF treatment, 0.4 ml of buffered osmoticum containing 1 mg/ml CF (15 mM NaCl) were added to the resuspended protoplasts. For short and intermediate term CF treatments which lasted from 10 s to 30 min, the CF treatment was terminated by the addition of 0.4 ml of ice-chilled buffered osmoticum, pH 6.7-6.8, containing 3.1 % (v/v) glutaraldehyde. For long term CF treatments which lasted from 60 to 180 min, the CF treatment was terminated in the following manner. The protoplasts were washed 3 times by centrifugation (200X g, 1 min) with
buffered osmoticum, over approximately 30 min, and were resuspended in 0.8 ml of buffered osmoticum (In some cases, as indicated in the figure legends, this washing step prior to the addition of glutaraldehyde fixative was omitted). Finally, 0.4 ml of ice-chilled buffered osmoticum containing 3.1 % (v/v) glutaraldehyde were added to the protoplast preparations. All samples were then incubated on ice, with occasional resuspensions, for 1 to 2 h. The samples were pelleted by centrifugation (200X g, 1 min), resuspended in 1.2 ml of buffered osmoticum containing 3.1 % (v/v) glutaraldehyde and incubated at room temperature for 2 to 3 h. Finally, the protoplasts were pelleted by centrifugation (200X g, 1 min) and washed once by centrifugation (200X g, 1 min) in buffered osmoticum. The samples were then postfix fixed either with osmium tetroxide (See section 2.9) or with a zinc iodide-osmium tetroxide post-fixative (See section 2.10).

Control samples (without CF) were processed in an identical manner to the treated samples. In the controls, the buffered osmoticum containing CF was replaced by either 0.4 ml of buffered osmoticum or 0.4 ml of buffered osmoticum containing 15 mM NaCl.

For specific experiments where significant deviations from the basic protocol occur, the full extent of these deviations will be described in the appropriate figure legends.
2.6 Native Ferritin Incubation of Protoplasts

Incubation of soybean protoplasts with NF was carried out in the same manner as the CF incubations (section 2.5). Two NF solutions were used, 1 mg/ml NF (1.5 mM NaCl) and 20 mg/ml NF (30 mM NaCl) in buffered osmoticum.

The protoplasts were incubated in NF for 30 min (1 mg/ml NF) or 70 min (20 mg/ml NF). The incubations were terminated in different manners. When the 1 mg/ml NF solution was used, the incubation was terminated by the direct addition of 0.4 ml of ice-chilled 3.1 % (v/v) glutaraldehyde in buffered osmoticum in the manner described for the short and intermediate term CF incubations (see section 2.5). When the 20 mg/ml NF solution was used, the protoplasts were washed three times and then fixed with glutaraldehyde in the manner described for the long term CF incubations (see section 2.5). Subsequent manipulations were performed as described for the CF incubations (see section 2.5).

For comparison purposes, CF incubations were performed in parallel to the NF incubations. The standard CF solution was used (see section 2.5) and the resulting protoplast preparations were handled in an identical manner to those treated with NF.

As a control, in place of a ferritin solution, some protoplast preparations received 0.4 ml of buffered osmoticum containing either 1.5 mM NaCl or 30 mM NaCl and
then were handled in an identical manner to the preparations that had received the NF solutions.

2.7 Incubation of Whole SB-1 Cells with Cationized and Native Ferritin

Cells from a 1 d old SB-1 cell culture were pelleted (200X g, 2 min) and were washed three times by centrifugation (200X g, 1 min) in Ca-CPB (section 2.2). Cell pellets, approximately 0.15 ml packed cell volume, were resuspended in 0.4 ml of Ca-CPB.

For the ferritin incubations, 0.4 ml of Ca-CPB containing either 1 mg/ml CF (15 mM NaCl) or 10 mg/ml NF (15 mM NaCl) were added to the resuspended cells. As a control, 0.4 ml of Ca-CPB containing 15 mM NaCl were added to a resuspended cell preparation. After an 1 h incubation period, the cells were washed three times with a 1.0 ml volume of Ca-CPB over a period of approximately 40 min. Each cell preparation was resuspended in 0.8 ml of Ca-CPB and 0.4 ml of ice-chilled 3.1 % (v/v) glutaraldehyde in Ca-CPB. After 80 min on ice, the cell samples were transferred to 1.2 ml of 3.1 % (v/v) glutaraldehyde in Ca-CPB at room temperature. After a period of 140 min, each cell preparation was washed briefly with Ca-CPB and then was washed five times on ice over a period of 2 h with CPB. Each cell preparation was post-fixed with osmium tetroxide, dehydrated in ethanol and embedded in Araldite resin according to the protocols described in sections 2.9 and
Additional, longer term incubations of cells were performed using CF. For these experiments, LB5 culture medium was used in place of Ca-CPB.

In one experiment, cells from 2 ml of a 1 d old SB-1 culture were resuspended in 2.0 ml of LB5 medium containing 2.5 mg/ml of CF and 37.5 mM NaCl, pH 5.3. As a control, a similar cell sample was resuspended in LB5 medium containing 37.5 mM NaCl. These samples were incubated for 16 h in the dark on a gyratory shaker (50 rpm). (Sterile conditions were maintained during this period of time.) The cell preparations were then washed three times by centrifugation (150X g, 1 min) in LB5 medium and were fixed by resuspending the cells in 2.0 ml of LB5 medium plus 1.0 ml of LB5 medium containing 3.1 % (v/v) glutaraldehyde, pH 5.5. The cell preparations were left on ice for 75 min, transferred to 3.5 ml of 3.1 % (v/v) glutaraldehyde in LB5 medium and were incubated at room temperature for approximately 2 h. The cells were washed briefly in LB5 medium at room temperature and then five times over a period of 2 h on ice with CPB. The cell preparations were then post-fixed, dehydrated and embedded according to the protocols described in sections 2.9 and 2.12.

Another experiment was performed on cells in a manner very similar to the protocol of the preceding experiment.
However, cells for this experiment were obtained from a newly initiated culture and were incubated for 3.5 d in the LB5 medium containing 2.5 mg/ml CF.

2.8 Incubation of Whole SB-1 Cells with Lanthanum Chloride

Cells from a 1 d old SB-1 culture were washed three times in MES-Tris buffer (see section 2.2), pH 5.8. Cell pellets, approximately 0.15 ml packed cell volume, were resuspended in 0.4 ml of MES-Tris buffer. For the lanthanum chloride (LaCl$_3$) incubation, 0.4 ml of 20 mg/ml LaCl$_3$ in MES-Tris buffer was added to the resuspended cells for 1 h. As a control, 0.4 ml of MES-Tris buffer without LaCl$_3$ were added to a second cell preparation. The cells were washed three times with MES-Tris buffer, and were resuspended and fixed in 0.8 ml of MES-Tris buffer and 0.4 ml of ice-chilled 3.1 % (v/v) glutaraldehyde in MES-Tris buffer for 1 h on ice. The cells were then transferred to 1.2 ml of 3.1 % (v/v) glutaraldehyde in MES-Tris buffer at room temperature. After a period of 2 h, the cells were washed once in MES-Tris buffer at room temperature followed by five additional changes of this buffer on ice over a period of 2 h. The cells were post-fixed with 1 % (w/v) osmium tetroxide in MES-Tris buffer for 14 h on ice. The cells were then dehydrated in ethanol according to the protocol in section 2.9 and were embedded in Araldite resin according to the protocol in section 2.12.
2.9 Standard Post-fixation and Dehydration Protocol

Prior to post-fixation in osmium tetroxide, the protoplast preparations (see sections 2.5 and 2.6) were given 5 washes on ice, with PPB, over a period of 2 to 3 h. The samples were then resuspended in 1.2 ml of PPB containing 1 % (w/v) osmium tetroxide and left on ice for 11 to 17 h. Samples were given 2 washes on ice, with glass distilled water, and were dehydrated in ethanol. Ethanol dehydration involved the gradual transfer of the samples from distilled water to absolute ethanol by 10 % (v/v) increments. The samples were then given 2 or 3 additional changes with 100 % (v/v) ethanol and were stored in a -60°C freezer until they could be embedded. Dehydration was performed on ice and each change of solution was facilitated by centrifugation (200X g, 1 min).

For experiments using intact soybean cells, osmium tetroxide post-fixation was performed as described for protoplast preparations except that CPB rather than PPB was used for the washes preceding the post-fixation step and as the buffer system for the 1 % osmium tetroxide solution.

The dehydration protocol for the cell preparations was identical to the protocol described above for protoplast preparations.

2.10 Zinc Iodide-Osmium Tetroxide Post-fixation

Some protoplast preparations were post-fixed in a zinc iodide-osmium tetroxide (ZIO) post-fixative rather than in 1
% osmium tetroxide. The ZIO post-fixative (Harris and Chrispeels 1980) consisted of a mixture of equal volumes of 2 % (w/v) osmium tetroxide and a freshly prepared zinc iodide solution. The zinc iodide solution was made in the following manner. 1.5 g of zinc metal powder (BDH Chemicals Company Ltd., Saskatoon, Sk, Canada) was added to 10 ml of glass distilled water. This mixture was sonicated for 30 s and then 0.5 g of resublimed iodine were added to it. After 5 min of stirring with a magnetic stir bar, the resulting mixture was filtered by gravity through a disk of #2 Whatman filter paper (W & R Balston Ltd., England) and was used immediately.

The ZIO post-fixation was performed in the following manner. Glutaraldehyde-fixed protoplast preparations (see below) were given 5 washes on ice, with glass distilled water, over a period of approximately 2 h. Samples were resuspended in 1.2 ml of ZIO post-fixative. The samples were left in the dark either on ice for 16 h or at room temperature for 1 h. Samples on ice were transferred to a fresh ZIO post-fixative after 8 h of incubation. After ZIO post-fixation, the samples were given 2 or 3 washes on ice, with glass distilled water, dehydrated in ethanol according to the protocol in section 2.9 and embedded according to the protocol in section 2.12.

Some of the protoplast preparations which were post-
fixed with ZIO had previously been treated with CF and fixed with glutaraldehyde in buffered osmoticum (section 2.5). However, in many cases the protoplasts were freshly isolated but otherwise unmanipulated (section 2.3). Pellets containing approximately 0.15 ml (packed cell volume) of these protoplasts were suspended in 0.8 ml of wash medium, a 1:1 mixture of 1-B5 culture medium and Sorb-MES osmoticum, pH 5.8, and 0.4 ml of wash medium containing 3.1 % (v/v) glutaraldehyde. The protoplasts were left on ice for 1 to 2 h and then transferred to 1.2 ml of 3.1 % (v/v) glutaraldehyde in wash medium at room temperature. After 2 to 3 h, the protoplasts were washed briefly with wash medium and then post-fixed with ZIO as described above.

2.11 Uranyl Acetate En Bloc Staining of Protoplasts

In some experiments, protoplast preparations were en bloc stained by the following protocol. Following post-fixation in 1 % (w/v) osmium tetroxide (section 2.9), samples were given 2 washes with distilled water on ice and an additional 1 or 2 washes with distilled water at room temperature. The samples were resuspended in 1.2 ml of freshly prepared 2 % (w/v) uranyl acetate and were placed in a 60°C oven for 3 or 16 h. The preparations were then washed several times with distilled water at room temperature followed by one final distilled water wash on ice. Finally, the samples were dehydrated in ethanol according to the protocol in section 2.9.
2.12 Embedding Protocol

The embedding procedure used in all experiments was the procedure described by Fowke (1982).

Samples were removed from storage at -60°C where they had been placed following dehydration in ethanol (section 2.9) and were given 2 washes in ice-chilled 100% ethanol. Most of the ethanol was decanted and propylene oxide was added dropwise (approximately 5-10 drops per hour) over 6 h, on ice. When the ratio of propylene oxide to ethanol reached approximately 2 to 1, samples were transferred to pure propylene oxide and were left overnight in closed 1.5 ml Eppendorf tubes on ice. The next morning, the samples were brought to room temperature and were given 2 changes of propylene oxide. The samples were transferred to small glass vials. Most of the propylene oxide was decanted and a 1:1 (v/v) mixture of propylene oxide and Araldite resin was added dropwise over approximately 6 h until enough resin was present to ensure that the samples would be covered by resin when the propylene oxide was removed. The vials were covered with aluminum foil lids which had been punched with numerous holes, and were placed in a fume hood to allow the propylene oxide to evaporate overnight. The next morning, samples were transferred to fresh Araldite resin in Beem capsules (J.B. EM Services Inc., Pointe Claire - Dorval, P.Q., Canada). The samples were pelleted within the Beem capsules by
centrifugation (1500X g, 15-30 min) and were allowed to soak in the resin for 24-48 h and then were polymerized by placing the Beem capsules in a 60°C oven for 36-60 h.

The standard mixture of Araldite resin consisted of 12.5 g of Araldite 502 epoxy resin, 9.5 g of dodecenyl succinic anhydride, and 0.35 g of DMP-30 ([2,4,6-tri(dimethylamino)methyl]phenol). All components of the Araldite resin were obtained from Polysciences Inc., Warrington, PA, USA.

2.13 Sectioning and Staining

Samples embedded in Araldite resin were sectioned with a diamond knife on a Reichert-Jung Ultracut Microtome (C. Reichert Optische Werke AG, Wien, Austria). Thin sections displaying silver or grey interference colours and thick sections which were greater than 200 nm in thickness were picked up on flamed, uncoated, copper 300 or 300 x 75 grids (J.B. EM Services Inc., Pointe Claire, Dorval, P.Q., Canada).

Sections were usually unstained or stained for 7-12 min in Reynolds' lead citrate (Reynolds, 1963) prior to use. On occasion, sections were stained with a saturated uranyl acetate solution in 70 % (v/v) ethanol for 20-30 min, followed by post-staining in Reynolds' lead citrate for 5 min. Sections of protoplasts which were en bloc stained with uranyl acetate (section 2.11) did not receive any additional staining.

Serial sections were collected with the use of a
specially designed "third hand" device (Rostgaard 1973) on Notchdot 1 x 2 slot grids (J.B. EM Services Inc., Pointe Claire, Dorval, P.Q., Canada) coated with a formvar and carbon support film. Ribbons containing 10 to 25 consecutive thin sections with silver interference colours were commonly obtained. For the study of coated vesicles, ribbons containing 10 to 15 consecutive thin sections with grey interference colours were collected.

The presence of an electron-dense region on the cytoplasmic surface of an organelle, where some evidence of membrane structure could be distinguished, was the criterion by which presumptive clathrin coats were identified.

2.14 Electron Microscopy and Photography

Routine viewing and photography of thin and thick sections was done using a Philips 300 transmission electron microscope. Where tilting of the specimen was required, sections were viewed in a Philips 410 transmission electron microscope equipped with a variable +/- 60° specimen stage goniometer. For photomicrography, Kodak electron microscope film (Eastman Kodak Co., Rochester, NY, USA) was used. The film was developed using Kodak D-19 developer and Rapid Fixer according to the manufacturer's recommended development procedure.
3 Results

3.1 Location of Coated Membranes

SB-1 soybean suspension culture cells (Fig. 3) and protoplasts (Fig. 4 and 5) derived from them contained a number of organelles which bore a coat on at least a part of their surface. These organelles included coated pits (CPs), coated vesicles (CVs), dictyosomes and the partially coated reticulum (PCR).

Coated pits (CPs) were small invaginations of the plasma membrane (Fig. 5 to 8) which had a layer of material associated with their cytoplasmic surface. They appeared to be distributed in a non-random manner on the plasma membrane. In cross-sections through the plasma membrane, profiles of CPs sometimes appeared clustered into small regions of the plasma membrane (Fig. 6) which were scattered among larger tracts of non-coated membrane. When soybean material was unstained (Fig. 7) or stained only with lead citrate (not shown), these coats were often difficult to see in the electron microscope. In these instances, the coats appeared to be fuzzy regions, apparently lacking substructure, underlying the invaginations of the plasma membrane. In sections of protoplasts stained en bloc with uranyl acetate (Fig. 8) or sections double stained with uranyl acetate and lead citrate (Fig. 6), the coat was heavily stained and in some cases substructure could be seen in the form of striations running perpendicular to the
plasma membrane. In other cases, the coat still appeared to be a fuzzy region which was now quite electron dense. In rare profiles of glancing sections through CPs, the coat was seen to consist of a network of interconnected polygons reminiscent of a honeycomb or soccer ball (Fig. 6). The substructure of the coat was very similar to that typical of the protein coats composed of clathrin and associated proteins that have been found in animal cells.

Coated vesicular profiles or coated vesicles (CVs) were frequently seen in the cytoplasm of soybean cells and protoplasts (Fig. 5, 9, 10). These organelles were about 65 to 100 nm in diameter and consisted of a membrane bound vesicle about 30 to 50 nm in diameter with a coat 15 to 30 nm thick. This coat was identical in appearance to the coat observed on CPs. Again, in unstained sections (not shown) or sections stained only with lead citrate (Fig. 9), the coat had a fuzzy appearance and could be difficult to see. In sections of protoplasts stained en bloc with uranyl acetate (Fig. 10) or in sections double stained with uranyl acetate and lead citrate, this coat was heavily stained and was more distinct in appearance. Under ideal conditions the coat appeared to have a characteristic substructure consisting of striations radiating, at right angles, from the surface of the vesicle (Fig. 10).

The PCR consisted of a set of interconnecting tubular
membranes (Fig. 11) which appeared quite distinct from the cisternal membranes of the dictyosome. The PCR frequently bore a coat at the ends or tips of the tubules and coated vesicular profiles were frequently seen in the vicinity of this organelle (Fig. 11). Coats could also be found in more central locations on PCR tubules where the coated membrane appeared to be either fusing with or budding from the surface of the PCR. The coat was located on the cytoplasmic surface of the PCR and appeared to be identical in appearance to the coat on CPs and CVs. The PCR will be described in greater detail in section 3.6.2.

Dictyosomes also had a coat on part of their surface. The coat was found on the cytoplasmic surface, at the tip or dilated end, of a dictyosomal cisterna (Fig. 12). When the dictyosome was sectioned in face view, coats could sometimes be seen on the ends of tubules which projected from the periphery of the dictyosome cisterna (not shown). The coat seen on the dictyosome tended to be somewhat less distinct in appearance than that seen on CPs, CVs or the PCR (compare Fig. 12 to Fig. 8, 10, 11).

An additional organelle which merits a brief description at this time is the multivesicular body (MVB). It consisted of a large vesicle which contained several smaller internal vesicles (Fig. 13). Associated with the cytoplasmic surface on part of the large vesicle was a plaque somewhat similar but not identical to the coat seen on the organelles
described previously in this section. A more detailed description of this structure can be found in section 3.6.4.

3.2 Selection of Staining Protocols

A number of different staining protocols were employed during the course of this research project. Unstained sections were frequently used. In such sections, it was comparatively easy to positively identify and visualize markers such as CF (Fig. 14). However, the presence of coats and plaques on organelles was more difficult to establish (Fig. 15). Furthermore, the structural details of these coats and plaques were more difficult if not impossible to visualize in unstained sections (Fig. 14 compared to Fig. 8). Unstained sections were used in situations where it was necessary to unambiguously identify the presence of the various markers; CF, NF and lanthanum. For this reason, unstained sections were used from protoplast preparations which were incubated with CF, or with NF and from cell preparations which had been incubated with CF, NF, or lanthanum chloride. Unstained sections were also used on some occasions for the serial sectioning analysis of organelles labelled with CF (section 3.6).

Some thin sections were stained with lead citrate for 7 to 12 min. This staining protocol resulted in a slight increase in specimen contrast in comparison with unstained sections thus making the detection of coats and plaques
somewhat easier (compare Fig. 16 to Fig. 15). However, this staining protocol sometimes resulted in the formation of small precipitates which could on occasion make the identification of ferritin particles more difficult. Sections stained in this manner were primarily used for protoplast preparations which had been incubated in CF for short periods of time.

Some protoplast preparations were stained en bloc with 2% (w/v) aqueous uranyl acetate (section 2.11) prior to dehydration in ethanol. This staining protocol greatly improved the visibility of coats and plaques on cytoplasmic organelles (Fig. 17), particularly when staining was for a duration of 3 h, and resulted in greatly improved specimen contrast. However, en bloc staining resulted in the formation of many small electron dense precipitates. These precipitates were found on or around most membranous organelles and were especially prominent on the tonoplast of the central vacuole of the protoplasts (Fig. 17). The precipitates tended to be more prominent in preparations stained for 16 h than in those stained for 3 h. These precipitates made the identification of ferritin particles more difficult (Fig. 17). Furthermore, ferritin particles, on average, were much less distinct in appearance suggesting that the ferritin particles, or the material to which they were bound, were stained by this protocol. This staining protocol was used on protoplast preparations which were
primarily used for the serial sectioning analysis of organelles labelled with CP. In these instances, the increased specimen contrast proved beneficial in the delineation of organelles found adjacent to one another in the cytoplasm.

Traditional uranyl acetate staining with lead citrate counterstaining was not used extensively. With this staining protocol, CF particles became less distinct in appearance, probably due to staining with uranyl ions, and the specimen contrast was increased so greatly that it was extremely difficult to visualize ferritin particles (Fig. 18). This staining protocol was not used during serial sectioning analysis as the extra handling that was involved with this protocol tended to result in the breaking of the formvar film on the slot grids. This resulted in the loss of several series of sections which were difficult to obtain. This staining protocol was used on some occasions with thin sections of ZIO post-fixed material (see section 3.9) in the hope of increasing specimen contrast to detect the presence of coats. It was also used on some thin sections of control preparations (i.e. protoplasts which were not exposed to CP or NF) in order to reveal the morphological details of certain organelles such as MVBs.
3.3 Short Term (10 s to 2 min) Incubation of SB-1 Protoplasts with Cationized Ferritin

Before describing the uptake of CF by soybean protoplasts, it should be noted that ferritin, also known as phytoferritin, does occur naturally in both SB-1 culture cells and protoplasts derived from these cells. However, this fact does not complicate the interpretation of CF (or NF) uptake results as phytoferritin was only detected within the plastids of the protoplasts (Fig. 19) and cells. These organelles are not likely to be directly involved in the process of endocytosis.

When freshly isolated protoplasts from SB-1 cell cultures were exposed to CF for 10 s prior to fixation with glutaraldehyde, the plasma membrane was evenly labelled with CF particles. All regions of the plasma membrane were labelled including CPs. Probable stages of coated membrane invagination leading to the formation of free CVs were observed. These stages included very shallow CPs (Fig. 20), more highly invaginated CPs (Fig. 21), CPs with a narrow neck connecting the structure to the protoplast surface (Fig. 22 and 23) and free CVs (Fig. 24). Some of the free CVs were found quite deep within the cytoplasm (Fig. 25). After the 10 s exposure with CF, none of the CF particles were observed in smooth or uncoated vesicles or in deep, uncoated invaginations of the plasma membrane. The gentle undulations typical of the plasma membrane were however
labelled. There was no evidence of the formation of smooth or uncoated vesicles (receptosomes) from the plasma membrane in the vicinity of CPs as observed in some instances in animal cell systems (Willingham et al. 1981).

If protoplasts were exposed to CF for 30 s prior to fixation by glutaraldehyde, labelled CVs were again observed and in some cases were found in the vicinity of other cytoplasmic organelles such as dictyosomes (Fig. 26) and the PCR. At this time point, labelled smooth vesicles (SVs) or rather vesicles lacking a distinct coat were also observed (Fig. 27). Very rarely, at this and subsequent time points (Fig. 28), examples were found of what appeared to be labelled vesicles which only had a partial coat.

After a 2 min incubation of protoplasts with CF, smooth vesicles containing CF particles were quite common and were in fact more numerous than labelled CVs. These smooth vesicles could be found in the vicinity of structures such as dictyosomes (Fig. 29) and the PCR (not shown). The diameter of the smooth vesicles was similar to that of the membrane-bounded compartment of the CVs (Fig. 30), i.e. the size of a CV that had shed its distinctive coat. The labelled smooth vesicles were observed quite distant from the plasma membrane and could be found in transvacuolar strands. The vesicles did not appear to be attached to any subcellular structure. The mode of movement of these
vesicles to different locations in the cytoplasm could not be identified.

With a 2 min incubation of protoplasts with CF, examples of labelling of structures other than CPs, CVs and smooth vesicles were generally not observed. In the single exception, the structure which was labelled appeared to be a PCR (Fig. 31).

3.4 Intermediate Term Incubation (4 to 30 min) of SB-1 Protoplasts with Cationized Ferritin

With longer incubations of protoplasts with CF, labelling of internal membrane organelles became more frequent. The structures which became labelled were the PCR, dictyosomes and multivesicular bodies (MVBs). For protoplasts incubated with CF for 4 min, labelled organelles other than CPs, CVs and SVs were still very rare. However, when they occurred they appeared, mainly, to be examples of PCR. In fact, for incubations lasting 4 to 16 min in length, the PCR was the organelle that was most frequently labelled with CF (Fig. 32-34). Dictyosomes (Fig. 35) were less frequently labelled during these time points. Label was first observed in a small number of MVBs after a CF incubation of 6 min. In these very rare examples, only one or two particles of CF were observed in the MVB. With longer incubation times, the frequency of labelled MVBs increased but the number of CF particles per organelle remained quite low (Fig. 36). Similar changes in labelling pattern were
observed for the PCR and dictyosomes.

In protoplasts incubated with CF for 30 min., CPs, CVs, SVs, the PCR, MVBs and dictyosomes were all labelled with CF as would be expected. Furthermore, the amount of CF in the PCR (Fig. 37 and 38), dictyosomes and especially MVBs (Fig. 39) was significantly higher than had been encountered at earlier time points.

3.5 Long Term (60 to 180 min) Incubation of SB-1 Protoplasts with Cationized Ferritin

Long term incubations with CF were performed with SB-1 protoplasts in order to determine whether organelles other than CPs, CVs, SVs, dictyosomes, the PCR and MVBs become labelled with CF.

With CF incubations of 60 min or more, the central vacuole of the protoplast was occasionally labelled with CF (Fig. 40 and 41). The CF particles in these vacuoles did not appear to be free within the lumen of the organelle but rather appeared to be bound to the surface of a mass of material. In some cases, this material appeared to be a mass of coalescing vesicles (Fig. 42). An identical distribution of label was also found in smaller vacuolar profiles (Fig. 43).

No other organelles became labelled with CF, even with incubations of 3 h in length. However, it should be noted that there was some variation in the degree of labelling of
the various organelles from one long term experiment to another. In particular, while the PCR and MVBs were usually heavily labelled with CF, the labelling of dictyosomes was quite variable. In some experiments, a few particles of CF were found only in the terminal buds of one or two cisternae while in other experiments the dictyosomes were heavily labelled, with CF in all or most cisternae of the dictyosomes.

3.6 Ultrastructural Analysis of Organelles Labelled with Cationized Ferritin (Including Serial Sectioning Data)

The 3-dimensional morphology of a number of organelles in protoplasts was analysed using serial thin sections. The results of this analysis are presented in the following five sub-sections.

3.6.1 Coated Vesicles

Coated vesicular profiles were found throughout the cytoplasm but were particularly frequent in the region just below the plasma membrane and in the vicinity of dictyosomes and the PCR. Looking at single sections, it was impossible to determine whether these structures were free vesicles or whether the vesicular profiles were actually physically connected to another organelle via a membranous neck or tubule. Serial sectioning was used to determine whether coated vesicular profiles seen in soybean protoplasts were free coated vesicles.

Using thin sections of grey interference colour, it
was usually possible to section completely through a CV in 4 to 6 sections. This range included one section on each side of the series where the CV was clearly not present. (Based on the size of CVs (section 3.1), the thin sections are approximately 25-40 nm in thickness.)

Some of the coated vesicular profiles seen near the plasma membrane (Fig. 44) were found in subsequent sections to be connected to the plasma membrane (Fig. 45) and thus actually constituted CPs. However, in other cases, membrane connections to other organelles were not evident (Fig. 46 to 52). In these instances, the coated vesicular profile likely constituted a true CV.

3.6.2 Partially Coated Reticulum

The PCR was frequently seen in the perinuclear region of the protoplast where a major portion of the cytoplasm was located. However, PCR were also seen in the thin peripheral, cortical cytoplasm and in transvacuolar cytoplasmic strands.

The PCR was frequently but not exclusively seen associated with the dictyosomes of the protoplast. However, there were numerous examples where the PCR did not appear to be associated with any organelles in the cytoplasm (Fig. 53). Frequently, the PCR had a ring configuration (Fig.54) or a semicircular structure.

Serial sections were cut to determine whether or not the PCR was connected to other membranous structures of the
cytoplasm and to get some idea of the overall size and 3-dimensional shape of the PCR.

As mentioned in section 3.1, the PCR consists of a set of interconnecting tubular membranes with distinct coated regions at the tips of the tubules, or in more central locations. Serial sectioning confirmed the accuracy of this interpretation and also revealed a feature of the PCR which was not always obvious from single sections. Many but not all PCR possessed a membrane dilation. This dilation was often present in only one or two consecutive sections (Fig. 55-57) but could be present in several consecutive sections. In some cases, this dilation appeared to be a large vesicle free within the cytoplasm and only by examining adjacent sections was it possible to determine that it was connected to the PCR. This dilation in the membrane of the PCR never had, on its cytoplasmic surface, a coat of the type seen on the tips of the PCR tubules, CPs or CVs nor a distinct plaque like those seen on MVBs. However, in some instances, one or more internal membrane bounded vesicles were observed within the dilation of the PCR (Fig. 58, also see Fig. 34).

Cationized ferritin particles were generally distributed throughout the tubules of the PCR including the coated tips of this structure (e.g. Fig. 59-66). However, in sharp contrast, the dilations in the PCR including their internal vesicles were never labelled with CF (e.g Fig. 55-58).

As a general indication of the size of this organelle,
it usually required anywhere from 12 to 20 thin (60 to 70 nm) sections in order to section through a PCR.

The analysis of serial sections was most interesting with respect to connections or associations of the PCR with dictyosomes. For this analysis, an association was considered to exist between these organelles if a PCR was present within 200 nm of a dictyosome and if the only organelles occupying the space between them were SVs, CVs and/or ribosomes. In some cases, there was no evidence of an association between the PCR and dictyosomes (Fig. 59-66). However, often, the PCR was associated with dictyosomes but direct membrane connections between the two organelles were clearly not present (Fig. 67-78). In other such cases, the possibility of a direct membrane connection between the PCR and the dictyosome existed but could not be determined with certainty. However, in a single case, the membrane connection between the PCR and dictyosome appeared to be genuine (Fig. 79-86).

Dictyosomes were not the only organelle with which the PCR was associated. Occasionally, the PCR was either tightly appressed against elements of the ER or may have been connected to this organelle (Fig. 87).

3.6.3 Dictyosomes

Dictyosomes consisted of a set of 4 to 8 stacked cisternae. The cisternae were thin, flattened, fenestrated
sacs which tended to have a slightly dilated rim from which tubules frequently projected into the surrounding cytoplasm. Large dilations (>100 nm in diameter) in the cisternae and associated tubules of the dictyosomes were encountered very infrequently.

As was the case for the PCR, dictyosomes were frequently seen in the perinuclear region of the protoplast, in the thin layer of cortical cytoplasm and in transvacuolar strands.

During intermediate term incubations with CF, dictyosomes became labelled. Only a few particles of CF were observed in the tip or dilated rims of one or two cisternae of a dictyosome (see Fig. 35 and 39). Serial sections of protoplasts which had undergone a 60 min CF incubation were examined in order to determine the full extent of dictyosome labelling. Labelling occurred in several cisternae including those on both sides of the same dictyosome (Fig. 88-91). Thus labelling did not occur exclusively on one side or the other of the dictyosome. Labelling frequently occurred in the slightly dilated ends or rims of the cisternae or in close proximity to these regions. This peripheral labelling was seen in dictyosomes sectioned in face view (Fig. 92). In these cases, CF was seen around the periphery of the cisternae and in the tubules emanating from the periphery of the cisternae but not in the central region of the cisternae. However in at least one situation, cisternae
appeared labelled throughout their entire length (Fig. 93). These cisternae appeared to be the outer-most cisternae on one (trans ?) side of the dictyosome.

The PCR (section 3.6.2) was not the only organelle which may have been connected directly to dictyosomes. The ER was frequently found in close proximity to dictyosomes and in some cases, appeared to be either appressed against a dictyosome or physically connected to it (Fig. 94, also see Fig. 125-129).

3.6.4 Multivesicular Bodies

Multivesicular bodies were recognized as distinct organelles within soybean protoplasts. They consisted of a large membrane bounded vesicle which contained several smaller, internal membrane bounded vesicles. In thin sections, the profiles of the large vesicles generally had a diameter of 250 to 500 nm, although deviations from this range were observed. MVBs usually appeared circular in section, however, oval and irregular profiles were also frequently encountered. The profiles of the smaller, internal vesicles generally had diameters in the range of 40 to 100 nm. Occasionally, profiles of internal vesicles were encountered with diameters greater than 150 nm. Profiles of the internal vesicles usually were circular or oval in shape, however, profiles with irregular shapes were also seen. The density of the internal vesicles was variable.
Some vesicles were very electron-dense while others were relatively electron-lucent. The large vesicles (> 150 nm) were generally electron-lucent. The 40 to 100 nm internal vesicles were usually quite electron-dense although this was not always the case.

Multivesicular bodies were found distributed throughout the cytoplasm including transvacuolar strands and were especially common in the perinuclear region.

Typically, 5 to 9 consecutive thin sections were required in order to section through this organelle (Fig. 95-100). When serial sections of protoplasts labelled for 60 min with CF were examined, CF particles were observed bound to the outer surface of the internal vesicles of the MVB and to the inner surface of the limiting membrane of the MVB (e.g. Fig. 97). The possibility that CF may occur free within the lumen of the MVB could not be totally discounted (e.g. Fig. 99). MVBs from this 60 min CF preparation were more heavily labelled with CF than the MVBs from the intermediate term incubations (section 3.4), suggesting that CF accumulates in this organelle.

Multivesicular bodies frequently bore a plaque on the cytoplasmic surface of their limiting membrane. This plaque was usually seen in 1 to 3 consecutive serial sections. The plaque was quite distinctive in appearance. In some thin sections which were double stained with uranyl acetate and lead citrate, the plaques appeared to have a lamellar
substructure (Fig. 101). This substructure could not be seen in unstained sections (Fig. 102) or in sections of protoplasts which had been en bloc stained with uranyl acetate (Fig. 103). Only rarely were clathrin-like coats observed on MVBs.

In addition to the presence of a plaque, small membranous buds or tubules sometimes protruded from the main body of the MVB (Fig. 104). Also, in some instances, profiles were obtained which appeared to represent the formation of the internal vesicles via an invagination process from the limiting membrane of the MVB (Fig. 105, also see Fig. 13). In some MVBs, the internal vesicles appeared to be poorly resolved as if they were coalescing or disintegrating.

Elements of the ER were often observed in the vicinity of MVBs. In some cases, the ER appeared to be closely appressed against the MVB (Fig. 106, also see Fig. 135). Less frequently, MVBs were seen associated with dictyosomes.

3.6.5 Vacuoles

Labelling of the central vacuole was not observed in protoplasts from short term incubations in CF but was only seen in protoplasts that had been incubated with CF for a minimum of 60 min. Even then, labelling was not seen in all profiles of protoplasts in these preparations but did occur at a readily detectable level, in approximately 5 to 10 % of
protoplast profiles in individual sections.

CF particles found in the central vacuole were not free within the lumen but rather were bound to a membranous mass which appeared to contain vesicles coalescing together (see Fig. 42). These membranous masses were usually seen in 3 to 6 consecutive sections and tended to be located at or near the tonoplast. Exactly how the CF particles reached the central vacuole is not clear. However, the size and appearance of the membranous masses were comparable to the contents of some MVBs in the cytoplasm of the same protoplast (Fig. 107-109). Therefore it is possible that the contents of MVBs were transferred to the central vacuole, perhaps by the fusion of a-MVB with the tonoplast. The absence of a membrane surrounding the membranous masses supports this idea but examples of such fusion events were not observed. It should be noted, however, that the tonoplast of the central vacuole frequently appeared to be damaged. Therefore the possibility that the presence of CF in the central vacuole may be an artifact could not be totally discounted.

Similar labelling patterns (Fig. 110-113) were observed for the small vacuolar profiles as mentioned in section 3.5. It was possible that some of these vacuoles were connected to the central vacuole.
3.7 Effect of Cationized Ferritin Treatment on Protoplast Morphology

Preparations of protoplasts treated with CF were not identical in appearance to control preparations. Protoplasts incubated in CF were more likely to be partially collapsed resulting in nonspherical profiles in thin sections. At the ultrastructural level, the tonoplast of the central vacuole of protoplasts incubated in CF was not as well preserved by glutaraldehyde fixation. The tonoplast was either vesiculated or had blistered and separated from the surrounding cytoplasm. Here, it is important to note that even in protoplasts from control preparations, some degree of damage to the tonoplast was observed. Frequently, in membranes to which CF particles were bound, the trilamellar appearance of the unit membrane could not be detected. In addition, the cytoplasm of protoplasts incubated in CF tended to be less dense than the cytoplasm of protoplasts from control preparations. The appearance of the protoplasts was suggestive of swelling or partial lysis. Again, this difference was not absolute but rather relative. It is important to note, however, that some protoplasts which had been treated with CF appeared to be comparable to protoplasts from control preparations. An attempt was made to restrict observations to CF-treated protoplasts which were as similar as possible to the protoplasts of the control samples. Despite these differences between the two
preparations, it was apparent that there were no differences between the two types of preparations with respect to the distribution and the appearance of the structures which became labelled with CF.

3.8 Effect of Charge on Endocytosis

The effect of charge on endocytosis in SB-1 protoplasts was determined by comparing the uptake of CF with that of native ferritin (NF), that is, ferritin which had not been chemically modified. NF is thought to be a marker for fluid phase endocytosis (Morales et al. 1985).

In one experiment, protoplasts were incubated with NF or CF, at a final concentration of approximately 0.5 mg/ml, for 30 min prior to the addition of glutaraldehyde (section 2.6). When examined in the electron microscope, NF particles were not readily detectable within organelles in the cytoplasm but a small number of NF particles were bound to the external surface of the plasma membrane. In contrast, uptake of CF was readily detectable with all the appropriate structures being labelled (section 3.4). Protoplasts treated with NF appeared similar to those in control samples. The cytoplasm of these protoplasts was quite electron dense and overall preservation of the material was good.

Because NF has traditionally been considered a marker for fluid phase endocytosis, it was somewhat surprising that NF appeared to be bound to the plasma membrane. Since the
protoplasts were not washed after the ferritin incubation and prior to fixation with glutaraldehyde, it is possible that free ferritin particles were bound to the plasma membrane by the glutaraldehyde. To test this possibility and to determine if NF uptake could be detected by using higher concentrations of NF, the experiment was repeated except with a final NF concentration of approximately 10 mg/ml. Protoplasts were incubated for 70 min followed by 3 washes in the appropriate buffer system prior to fixation with glutaraldehyde (see section 2.6). From this experiment, the following observations were made. First, uptake of NF was observed at low levels in cytoplasmic structures such as MVBs (Fig. 114) and dictyosomes (Fig. 115). Second, NF particles were again observed bound, in small quantities, to the external surface of the plasma membrane (Fig. 116). In comparison, CF uptake at this time was much more extensive and included the labelling of vacuoles as would be expected from the results of sections 3.5 and 3.6.5. Furthermore, the amount of CF bound to the plasma membrane was significantly higher than that observed for the NF treatment, and the preservation of protoplasts was poorer than that observed for protoplasts treated with NF or for control samples.

3.9 Results from Zinc Iodide-Osmium Tetroxide Post-fixed Protoplasts

Zinc iodide-osmium tetroxide (ZIO) post-fixation is a special method which heavily labels or stains specific
membranes within cells or protoplasts. ZIO treatment usually results in heavy labelling of the nuclear envelope, the endoplasmic reticulum, and elements of the Golgi apparatus (dictyosomes). The improved membrane contrast resulting from ZIO treatment enables one to use relatively thick sections (up to approximately 1 um) combined with tilting of the specimen in the electron microscope to determine whether or not direct membrane connections exist between different organelles. Furthermore, ZIO staining results have in the past been used as a criterion for considering the PCR as a structure distinct from the Golgi apparatus (dictyosomes) (Pesacreta and Lucas, 1984).

ZIO post-fixation was used in this project in the hope of demonstrating that dictyosomes and the PCR display different staining properties. Differential staining would support the serial sectioning results (section 3.6.2) which suggested that the PCR was an organelle distinct, in at least some cases, from dictyosomes. It was also hoped that the ZIO post-fixation procedure would allow for the easy visualization of direct membrane connections between different organelles in the cytoplasm.

When ZIO post-fixation was performed for 1 h at room temperature or for 16 h on ice, similar results were obtained. In general, the nuclear envelope (Fig. 117), the tubular (tER) (Fig. 117) and cisternal ER (cER) (Fig. 118 and
119), and dictyosomes (Fig. 120 and 121) were labelled with the ZIO reaction product. However, the labelling was variable. In many instances, tER, cER and the nuclear envelope were not labelled or only very weakly labelled (not shown). Dictyosome labelling was also variable but not in a consistent manner relative to the labelling of other organelles. Also labelled were mitochondria (Fig. 117) and the internal membranes of the plastids (Fig. 117). In general, the plasma membrane, central vacuole and tonoplast were not labelled by this treatment except for the occurrence of rather large electron dense precipitates (not shown) which were likely a nonspecific overreaction product. These rather large precipitates on the plasma membrane and in the central vacuole tended to result in tearing of thin or thick sections in the electron microscope. Smaller precipitates were also found in small vacuoles and in the cytoplasm (Fig. 120). Some of the cytoplasmic precipitates could have been in vesicles but a bounding membrane could not be detected.

In thick (200-400 nm) sections, the ER appeared to be a very complicated network consisting of tER and cER. Tubular ER consisted of long, often branched tubules of variable width (Fig. 117). The tubules frequently connected with cER which consisted of sheets of membrane (Fig. 118 and 119). The labelling of cER by ZIO treatment was often uneven resulting in a "flaky" appearance (Fig. 118). In many cases,
there appeared to be fenestrations or holes in the membranes of the cER (Fig. 119). In some instances, the tER also appeared to have fenestrations (Fig. 122-124). In many thick sections, the tER appeared to be connected to the nuclear envelope (Fig. 117) and dictyosomes (Fig. 122-127). Tilting of the specimen through + or - 30° clarified the association of ER and dictyosome. In many instances, apparent connections between tER and dictyosomes disappeared when the specimen was tilted (Fig. 122-124). The appearance of a tER-dictyosome connection resulted from the overlapping of the images of two structures which were actually located in different planes in the thick section. In other instances, tilting of the specimen through + or - 30° did not result in the separation of the tER from the dictyosome (Fig. 125-127) and it is possible that there was a real, direct membrane connection between the tER and the dictyosome. Using thin sections, which is not a common practice with ZIO post-fixed material, it was possible to find examples where the ER, presumably tER, appeared to be connected to a dictyosome (Fig. 128 and 129). These results support the interpretation based on the thick sections.

In thick and more noticeably in thin sections of protoplasts post-fixed with ZIO, the cisternae of dictyosomes displayed considerable heterogeneity with respect to labelling. In some cases there was a clear
polarity across the dictyosome with the cisternae on one side being heavily labelled while cisternae on the other side of the dictyosome were less intensely labelled (Fig. 130). In other cases, it was one or more cisternae in the middle of the dictyosome which were most intensely labelled (not shown) or, alternatively, all cisternae were labelled with equal intensity. It was reasonably common for one or more cisternae of a dictyosome to be unlabelled by the ZIO post-fixative (not shown). When dictyosomes were seen in face view (ie. sectioned parallel to stack of cisternae), the increased contrast resulting from ZIO treatment was most beneficial in the visualization of fenestrations (Fig. 131) in the cisternae of the dictyosome. Coats-on dictyosomes were difficult to see even when thin sections were stained with uranyl acetate and counterstained with lead citrate. However, for dictyosomes sectioned in face view, coats frequently appeared to be present on some of the membranous buds or tubules (Fig. 132) which extended from the periphery of the cisternae. In addition to the apparent direct membrane connections between some dictyosomes and the tER described above, connections between adjacent dictyosomes were also seen in both thick (not shown) and thin sections (Fig. 131 and 133).

In thick sections of ZIO post-fixed material, it was not possible to identify the PCR. It was not clear whether this problem was caused by lack of staining of the PCR or by the
presence of other ZIO labelled organelles which masked or obscured the presence of the PCR in thick sections. In thin sections, as noted for dictyosomes, the presence of coats was difficult to detect even when the sections were stained with uranyl acetate and counterstained with lead citrate. However, examples of membranes which were probably PCR were found to be unlabelled by the ZIO treatment (Fig. 134). This was the case despite the fact that all dictyosomes in the same protoplast were labelled with ZIO reaction product. However, it was also possible to find examples of membranes which resembled PCR and contained ZIO reaction product. These may have been glancing sections through the periphery of stained dictyosomes.

As with the PCR, it was not possible to identify MVBs in thick sections of material post-fixed with ZIO. However, in thin sections, MVBs were readily identified. Labelling of the MVBs was variable. In some cases, some of the internal vesicles appeared to be labelled with ZIO (Fig. 135 and 136), while in other cases the MVBs appeared to be completely unlabelled (not shown). In protoplasts which had been labelled with CF prior to glutaraldehyde fixation and ZIO post-fixation, it was possible to identify, in thin sections, MVBs which contained both CF particles and ZIO reaction product (Fig. 137).
3.10 Uptake of Markers by Whole SB-1 Cells

Because of the positive results with CF (and to a lesser extent NF) demonstrating the occurrence of endocytosis in protoplasts derived from SB-1 cells, preliminary experiments were performed on whole turgid SB-1 cells in the hope of demonstrating the occurrence of endocytosis in higher plant cells. NF, CF and lanthanum chloride were used as potential markers of endocytosis.

3.10.1 Uptake of Lanthanum Chloride

Lanthanum chloride dissociates into Cl⁻ ions and electron-dense, membrane-impermeant La³⁺ ions. It was hoped that the La³⁺ ion would diffuse through the cell wall, bind electrostatically to and be deposited on negatively-charged sites on the plasma membrane. The labelled plasma membrane would then be internalized via CVs and this process could be followed by observing the distribution of La deposits.

When SB-1 cells were incubated in a final concentration of LaCl₃ of approximately 10 mg/ml for 1 h followed by washing with buffer and fixation with glutaraldehyde (see section 2.8), the following observations were made. The majority of La deposits were located on the external surface of the cell wall where the cell did not abut another cell in the culture and appeared as large numbers of densely packed fine needles (Fig. 138). These needles were also observed deeper within the cell wall although at much lower densities (Fig. 138). Furthermore, some of these needles were seen in
the vicinity of the plasma membrane and may have been in
physical contact with it. These needles were also seen in
the cell walls separating adjacent cells in the cell clumps
found in the SB-1 culture but at a lower concentration than
in walls of individual cells (Fig. 139). La deposits were
also observed within the cells. These deposits frequently
appeared to be within elements of the ER (Fig. 140).
Sometimes the ER appeared to be slightly dilated in the
region where the La deposit was found (Fig. 140). In some
cases, the La deposits appeared to be present within
vesicles (Fig. 138 and 139) which may be elements of the ER
seen in cross-section. The labelling within the cytoplasm
tended to be located around the periphery of the cell
although on occasion could be seen deep within the cytoplasm
in the perinuclear region of the cell. The La deposits
within the cell were quite distinct and could frequently be
found simply by scanning the cell using a low (3,300 to
5,400 X) magnification on the electron microscope. Cells
from the control sample which were not exposed to LaCl₃ did
not contain deposits comparable to the La deposits seen
within the cell wall and cytoplasm of cells treated with
LaCl₃. In fact, this was the basis on which lanthanum
deposits were identified. (Elemental analysis of the
deposits was not performed.)
3.10.2 Incubations with Native and Cationized Ferritin

In the hope of demonstrating the occurrence of endocytosis in whole SB-1 cells, cells from 1 day old SB-1 cultures were incubated in buffers containing NF and CF for 1 h (see section 2.7). For cells incubated in NF, few ferritin particles were distributed along the outer surface of the cell wall. There was no evidence of any uptake by the cell or of any penetration of NF particles through the cell wall. For cells incubated with CF, ferritin particles were present in large numbers along the outer surface of the cell wall (Fig. 141). The increased binding of CF to the cell wall occurred despite the fact that NF was used at 10 times the concentration of CF. CF particles penetrated the cell wall to some degree (Fig. 141). However, CF particles were not observed in the cross walls which separate adjacent cells. In some instances, CF particles even seemed to be bound to the outer surface of the plasma membrane (Fig. 142). Unambiguous labelling of cytoplasmic structures with CF was not observed. However, an interesting ER profile (Fig. 143) was obtained which indicated that direct membrane connections may exist between the ER and the plasma membrane. Combined with the results of the longer term CF incubations of cells, such connections may be of great significance (see Discussion, section 4.21).

When cells from a 1 day old SB-1 culture were incubated in LB5 medium containing 2.5 mg/ml CF for a period of 14
hours, CF particles were seen predominately along the outside surface of the cell wall and examples of CF particles penetrating through the cell wall were observed (Fig. 144-146). Most importantly, CF particles were bound to the plasma membrane and in several instances appeared to be localized within the cytoplasm (Fig. 144-146). Frequently, these examples of cytoplasmic labelling were located adjacent to a region of the cell wall containing CF particles (Fig. 144-146). The labelling of the cytoplasm was not strong and frequently the CF particles appeared to be in elements of the ER (Fig. 145), in large vesicles which likely were elements of the ER (Fig. 146) or free in the cytoplasm (Fig. 144 and 146). The cytoplasm of SB-1 cells was quite dense even in unstained sections. Therefore it is conceivable, in at least some cases, that the CF particles which appeared to be free in the cytoplasm were in fact in vesicles which were obscured by the dense cytoplasm. En bloc staining of the cell preparations did not help to resolve this question as the uranyl precipitates formed using this protocol prevented the unambiguous detection of CF particles within the cytoplasm.

When SB-1 cells from a newly initiated culture were incubated in 1B5 medium containing 2.5 mg/ml CF for a period of 3.5 days, cell growth was inhibited. These cells appeared to be either dead or badly stressed when examined in the
electron microscope.

In this experiment, most of the CF was observed bound to the outer surface of the cell wall. CF was again found part way through the cell wall, bound to the plasma membrane and within the cytoplasm. Much of the cytoplasmic label appeared to be free within the cytoplasm and in some instances appeared to be either bound to the tonoplast or free within the central vacuole.

One final observation regarding SB-1 culture cells should be made. In lanthanum-treated (not shown), ferritin-treated (not shown) and control cells (Fig. 147), invaginations of the plasma membrane were observed. Frequently, a number of membrane bounded vesicles (Fig. 147) were found in the resulting enlarged periplasmic space. It is conceivable that these profiles represent the results of membrane fusion events between MVBs and the plasma membrane.
Fig. 3. Cells from a SB-1 soybean suspension cell culture. Nomarski differential interference optics. Magnification as Fig. 4.

Fig. 4. Protoplasts derived from a SB-1 soybean suspension cell culture. Nomarski differential interference optics. Bar equals 50 μm.

Fig. 5. Electron micrograph of soybean protoplast. Note the presence of dictyosomes (D), coated vesicles (arrows), mitochondria (M), a partially coated reticulum (PCR) and lipid bodies (L) in the cytoplasm and coated pits (arrowheads) on the plasma membrane (PM). Stained with uranyl acetate and counterstained with lead citrate. Bar equals 500 nm. Micrograph reprinted from Mersey et al. (1985), Planta 163: 317-327.
Fig. 6-12. Coated membranes in soybean protoplasts.

Fig. 6. A number of CPs (arrowheads, double arrowhead, arrow) are clustered on one region of the plasma membrane (PM). Note the striated appearance of the coat on some of these CPs (arrows) and the honeycomb appearance of one coat (double arrowhead) which consists of a set of interconnected polygons. Stained with uranyl acetate and counterstained with lead citrate. Bar equals 300 nm. Fig. 7. Note the CP (arrowhead) with an indistinct coat lacking any apparent substructure. Unstained. Bar equals 300 nm. Fig. 8. A CP (arrowhead) bearing a striated coat. Stained en bloc with uranyl acetate. Magnification as Fig. 12. Fig. 9. Note the CV (arrowhead) with an amorphous coat. Stained with lead citrate. Magnification as Fig. 12. Fig. 10. A CV (arrowhead) with a very distinct coat. Note the alternating electron-dense and electron-lucent regions in the coat giving it a striated appearance. Stained en bloc with uranyl acetate. Magnification as Fig. 12. Fig. 11. A section through a PCR. Note the PCR consists of tubular membranes part of which are coated (arrowheads). CVs (arrows) are seen in close proximity to the PCR. Stained en bloc with uranyl acetate. Magnification as Fig. 12. Fig. 12. Dictyosome (D) consisting of a set of 8 stacked cisternae. Note that some of the tips of the cisternae (arrowheads) bear an indistinct, electron-dense coat. Stained en bloc with uranyl acetate. Bar equals 300 nm.
Fig. 13. A MVB. Note the small vesicle (arrow) apparently forming by invagination of the limiting membrane of the MVB. Also note a small profile of a plaque (arrowhead) on the cytoplasmic surface of the limiting membrane of the MVB. Stained en bloc with uranyl acetate. Magnification as Fig. 18.

Fig. 14-18. Comparison of staining protocols. All at the same magnification. Bar in Fig. 18 equals 300 nm. Fig. 14. CP (arrowhead) with an indistinct coat can be seen on the plasma membrane (PM). Note the small, distinct, electron-dense CF particles which line the outer surface of the plasma membrane. 6 min CF incubation, unstained. Fig. 15. Two vesicles (arrowhead, arrow) are labelled with CF. One vesicle (arrowhead) appears to lack a coat. The second vesicle (arrow) may be a CV although due to lack of contrast, it is not possible to clearly distinguish the coat. 6 min CF incubation, unstained. Fig. 16. CV (arrowhead) labelled with CF. Unlike in Fig. 15, the coat on this CV can be clearly distinguished. Also note that the overall contrast of the cytoplasm is increased. (Compare this figure to Fig. 14 and 15.) D-dictyosome. 10 min CF incubation, stained with lead citrate. Fig. 17. CF particles (arrowheads) on the plasma membrane (PM), in smooth vesicles and in a tubule are less distinct in appearance than in unstained sections (e.g. Fig. 14). Also note the presence of electron-dense precipitates (arrows) caused by the staining and the presence of a CV (double arrowhead) with a distinct coat and vesicle. Finally, note that the overall contrast of the cytoplasm is greatly increased especially in comparison with unstained sections (Fig. 14 and 15). T-tonoplast. 60 min CF incubation, stained en bloc with uranyl acetate. Fig. 18. CP (arrow) and CF particles bound to it and other regions (arrowhead) of the plasma membrane are so heavily stained by this staining protocol that they have become quite amorphous in appearance and difficult to distinguish. Also note the greatly increased contrast of the specimen. 60 min CF incubation, stained with uranyl acetate and lead citrate.

Fig. 19. Phytoferritin. Unlabelled soybean protoplast showing a plastid which contains a number of particles of phytoferritin (arrowheads) and a starch granule (SG). Unstained. Bar equals 300 nm.
Fig. 20-30. Results of short term incubations of protoplasts with CF. All stained with lead citrate and at the same magnification. Bar in Fig. 29 equals 300 nm. Fig. 20. CF particles in a shallow CP (arrowhead). 10 s CF incubation. Fig. 21. CF in a deep CP (arrowhead). 10 s CF incubation. Fig. 22. CF in a highly invaginated CP (arrowhead) which is connected to the rest of the plasma membrane by a narrow neck. 10 s CF incubation. Fig. 23. CF in a CV (arrowhead) which is possibly still attached to the plasma membrane. 10 s CF incubation. Fig. 24. A CF-labelled CV (arrowhead). 10 s CF incubation. Fig. 25. A CF-labelled CV (arrowhead) deep within the cytoplasm. Note the presence of the endoplasmic reticulum (ER) between the plasma membrane (PM) and the CV. 10 s CF incubation. Fig. 26. A CF-labelled CV (arrowhead) near a dictyosome (D). 30 s CF incubation. Fig. 27. A CF-labelled SV. 30 s CF incubation. Fig. 28. A CF-labelled vesicle (arrowhead) which appears to only have a partial coat. 2 min CF incubation. Fig. 29. A CF-labelled SV (arrow) near a dictyosome (D). Note the CV (arrowhead) which may be labelled with CF. 2 min CF incubation. Fig. 30. A CF-labelled SV (arrow) and a CF-labelled CV (arrowhead). Note that the SV is approximately the same size as the vesicle proper of the CV. 2 min CF incubation. Fig. 20-26, 28, and 29 are reprinted from Tanchak et al. (1984), Planta 162:481-486.
Fig. 31. Results of short term incubations of protoplasts with CF (continued). A PCR labelled with particles of CF (arrows). Note the coated membranes (arrowheads) which contain CF. 2 min CF incubation. Stained with lead citrate. Magnification as Fig. 36.

Fig. 32-36. Results of intermediate term incubations of protoplasts with CF. Fig. 32. Presumptive PCR tubule (arrow) labelled with CF. Note vesicles (arrowhead, double arrowhead), each appearing to contain a single particle of CF. One vesicle (arrowhead) appears to be coated. 4 min CF incubation, unstained. Magnification as Fig. 36. Fig. 33. Presumptive PCR tubules labelled with CF. One region of the tubules (arrowhead) may be coated. 16 min CF incubation, unstained. Magnification as Fig. 36. Fig. 34. Two PCR labelled with CF. Note the regions of the PCR (arrowheads) which appear to be coated and a CV (double arrowhead) which is labelled with CF. Also note the presence of a dilation (arrow) in one PCR which appears to have an internal vesicle. Incubated with CF for 3 min on ice, followed by 10 min at room temperature. Otherwise, as described in the Materials and Methods (section 2). Unstained. Magnification as Fig. 36. Fig. 35. Dictyosome labelled with CF (arrowhead). 10 min CF incubation, unstained. Bar equals 300 nm. Fig. 36. MVB which is labelled with CF (arrowhead). Note that CF appears to be bound to the outer surface of one or possibly two of the small internal vesicles of the MVB. 8 min CF incubation, unstained. Bar equals 300 nm.
Fig. 37-39. Results of intermediate term incubations of protoplasts with CF (continued). All are unstained and at the same magnification. Bar in Fig. 39 equals 300 nm. Fig. 37. PCR tubule heavily labelled with CF and bearing a coat (arrowhead) at one end. M—mitochondrion. Fig. 38. A branched tubular network of a PCR heavily labelled with CF. A coated membrane profile (arrowhead) may or may not be connected to the PCR. Fig. 39. MVB heavily labelled with CF. Note that one cisterna (arrowheads) of the accompanying dicytosome is also labelled with CF.
Fig. 40-42. Results of long term incubations of protoplasts with CF. Fig. 40. Low magnification of protoplast showing a CF-labelled membranous mass within the central vacuole (V). N— nucleus. Area outlined in black is shown below in Fig. 41. 60 min CF incubation, pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. Bar equals 1 μm. Fig. 41. An enlargement from Fig. 40 showing a membranous mass within the central vacuole. 60 min CF incubation, pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. Magnification as Fig. 42. Fig. 42. A membranous mass within the central vacuole which is labelled with CF particles. Note that the mass seems to consist of vesicles (arrowheads). 90 min CF incubation, post-fixation with ZIO rather than osmium tetroxide and no additional staining. Bar equals 300 nm.
Fig. 43. Results of long term incubations of protoplasts with CF (continued). A small vacuole contains one or possibly two fusing CF-labelled membranous mass(es) (arrowheads). 60 min CF incubation, stained en bloc with uranyl acetate. Bar equals 300 nm.

Fig. 44 and 45. Part of a series of sections through a coated vesicular profile. 30 min CF incubation, stained en bloc with uranyl acetate. Both at the same magnification. Bar in Fig. 45 equals 150 nm. Fig. 44. Section through a CF-labelled coated vesicular profile (arrowhead). PM—plasma membrane. Fig. 45. Serial section adjacent to that of Fig. 44. The CF-labelled profile (arrowhead) from Fig. 44 is clearly connected to the plasma membrane and thus is a CP.

Fig. 46-52. Serial sections through a CF-labelled coated vesicle. 30 min CF incubation, stained en bloc with uranyl acetate. All at the same magnification. Magnification as Fig. 45. An arrowhead indicates the position of a distinct ER profile which is first seen in Fig. 46 and last seen in Fig. 51. Double arrowheads indicate the position of a second ER profile which is first seen in Fig. 49 and last seen in Fig. 51. An arrow indicates the position of the CV of interest. The edges of the CV can be seen in Fig. 48 and 51 as patterning in the cytoplasm. A clear profile of the CV is seen in Fig. 49. In Fig. 50, the section is thinner than the others in the series. As a result while a profile of the CV can be seen, a distinct coat is not visible. There is no evidence of the CV in Fig. 46, 47 and 52.
Fig. 53-58. Ultrastructural details of the PCR. All at the same magnification. Fig. 55-57 are adjacent serial sections. Bar in Fig. 58 equals 300 nm. Fig. 53. PCR which does not appear to be associated with other organelles in the cytoplasm. Note that one end of the PCR (arrow) is coated and that other regions (arrowheads) may also be coated. Also note that CF labelling is seen throughout the tubule of the PCR. 60 min CF incubation, stained en bloc with uranyl acetate. Fig. 54. PCR in which the tubules have a ring configuration. Note the coated region (arrow) and another region (arrowhead) which may be coated. Also note that the CF appears to be distributed at random throughout the PCR. 60 min CF incubation with pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. Fig. 55. PCR with CF distributed throughout the tubules of the organelle. Note the coated vesicular profile (arrowhead) adjacent to the PCR. Also note the edge of a membrane dilation (see Fig. 56). 60 min CF incubation, stained en bloc with uranyl acetate. Fig. 56. Section serial to Fig. 55. The membrane dilation (arrow) in the PCR noted in Fig. 55 is clearly visible. Note that the dilation in the PCR is not labelled with CF. 60 min CF incubation, stained en bloc with uranyl acetate. Fig. 57. Section serial to Fig. 56. Note that CF is located throughout the PCR except for the portion of the tubules (arrow) which corresponds to the dilation in Fig. 56. 60 min CF incubation, stained en bloc with uranyl acetate. Fig. 58. A selected section from a series of sections through a PCR. The dilation in the PCR contains internal vesicles (arrowheads). Note that while a tubule (arrow) connected to the dilation is labelled with CF, the dilation itself and its internal vesicles are not labelled. 30 min CF incubation with pre-fixation washes normally used only for long term CF incubations (see section 2.5 of Materials and Methods), en bloc uranyl acetate staining (3h).
Fig. 59-62. Serial sections through a PCR. Every second section used. Series continued on next plate. 60 min CF incubation, stained en bloc with uranyl acetate. All at the same magnification. Bar in Fig. 62 equals 300 nm. Fig. 59. PCR is not present. Fig. 60. CF-labelled vesicles (arrowheads) and the edge of a PCR tubule (arrow) can be seen. Fig. 61. CF-labelled vesicular (arrowheads) and tubular (arrows) profiles are present. Some vesicular profiles may be coated. Fig. 62. Network of interconnected tubules is present in the cytoplasm. A portion (arrowhead) of this network may be coated. CF appears throughout the network. Note the endoplasmic reticulum (ER) between the PCR and a dictyosome (D).
Fig. 63-66. Serial sections through a PCR (continued from previous plate). All at the same magnification. Bar in Fig. 66 equals 300 nm. Fig. 63. The PCR consists of a set of CF-labelled tubules. A portion (arrow) of these tubules is coated and other membrane profiles (arrowheads) may also be coated. ER- endoplasmic reticulum, D- dictyosome. Fig. 64. All that remains of the PCR are two or three small CF-labelled tubular profiles (arrowheads). Fig. 65. A single small CF-labelled profile (arrowhead) is the only structure in this section which might be related to the PCR. Fig. 66. The PCR can no longer be identified. The only CF labelled structures in this section are two SVs (arrowheads). Throughout this series of micrographs (Fig. 59-66) there was no evidence of an association between the PCR and dictyosomes. The closest dictyosome to the PCR was always separated from the PCR by an element of the ER (Fig. 62-64).
Fig. 67-70. Selected serial sections of a PCR and dictyosomes. 60 min CF incubation, stained en bloc with uranyl acetate. All at the same magnification. Bar in Fig. 70 equals 300 nm. Fig. 67. PCR (arrows) well separated from two dictyosomes (D). Fig. 68. The closest approach by the PCR to the dictyosomes. While the PCR is in close proximity (arrowheads) to one of the dictyosomes, these organelles clearly remain separate and distinct. Fig. 69. The PCR is well separated from the dictyosomes. Fig. 70. In this section, only the PCR remains as a clearly identifiable organelle.
Fig. 71-74. Selected serial sections of a PCR and dictyosome. Series continued on next plate. 60 min CF incubation, stained en bloc with uranyl acetate. All at the same magnification. Bar in Fig. 74 equals 300 nm. Fig. 71 and 72. Tubular membranes of a PCR. Note the coated membrane profiles (arrowheads). Also note that CF is distributed throughout the PCR. Fig. 73. Part of a dictyosome (D) is now visible. While the PCR is near the dictyosome there is no evidence of a membrane connection occurring between them. Note the coated membrane profiles (arrowheads). Also note the possible connection (arrow) between a PCR tubule and a dilation in the PCR (see Fig. 74). Fig. 74. The PCR and dictyosome are not connected. Note the dilation (arrow) in the PCR and the coated membrane profiles (arrowheads).
Fig. 75-78. Selected serial sections of a PCR and dictyosome (continued from previous plate). All at the same magnification. Bar in Fig. 78 equals 300 nm. Fig. 75. The PCR consists only of short tubules and vesicular profiles (arrowheads) and has diminished in size and morphological complexity. There is still no evidence of a membrane connection between the PCR and dictyosome (D). Fig. 76. All that remains of the PCR is a few vesicular profiles (arrowheads), some of which may be coated. Fig. 77 and 78. The PCR is no longer present.
Fig. 79-82. Selected serial sections showing a possible membrane connection between a PCR and dictyosome. Series continued on next plate. 60 min CF incubation, stained en bloc with uranyl acetate. All at the same magnification. Bar in Fig. 82 equals 300 nm. Fig. 79. Tubule (arrow) and associated vesicular profiles of a possible PCR. Coated vesicular profile (arrowhead). Fig. 80. A more extensive set of tubular membranes weakly labelled with CF. Possible coated membrane (arrowhead). Fig. 81. Tubular membrane weakly labelled with CF. Possible coated membranes (arrowheads). Fig. 82. Note the presence of coated membranes (arrowheads) and the start of a dilation (arrow) in a tubule. Also note the edge of a dictyosome (D).
Fig. 83-86. Selected serial sections showing a possible membrane connection between a PCR and dictyosome (continued from previous plate). All at the same magnification. Bar in Fig. 86 equals 300 nm. Fig. 83. A tubule with a dilation (arrow) near the dictyosome (D). Fig. 84. A CF-labelled tubule with a dilation (arrow) appears to be connected to the dictyosome. This section marks the closest approach to the dictyosome by the PCR. Fig. 85. A short CF-labelled tubule (arrowhead) is present near the membrane dilation (arrow). Fig. 86. A glancing section through the dilation to which a short CF-labelled tubule is attached.
Fig. 87. Association of a PCR with the endoplasmic reticulum. A selected section from a series through a PCR. The PCR consists of an unlabelled membrane dilation (arrow) connected to CF-labelled tubules. The ends of the tubules (arrowheads) are coated. The PCR is appressed against, and possibly but less likely connected to an element of rough endoplasmic reticulum (ER). 60 min CF incubation, stained en bloc with uranyl acetate. Bar equals 300 nm.

Fig. 88-91. Selected serial sections through a CF-labelled dictyosome. 60 min CF incubation with pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. All at the same magnification. Bar in Fig. 91 equals 300 nm. Fig. 88. One cisterna (arrowhead) of the dictyosome is labelled with CF. Fig. 89. CF is seen in two cisternae (arrowheads) of the dictyosome. Fig. 90 and 91. CF appears to be in two cisternae (arrowheads) on opposite sides of the dictyosome. One section omitted between Fig. 90 and 91. For Fig. 88-91, note that the CF appears to be located at or near the ends of profiles of the labelled cisternae.

Fig. 92. Peripheral labelling of a dictyosome shown in face view. Note that CF (arrows) is distributed exclusively around the periphery of the dictyosome. 60 min CF incubation, pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. Bar equals 300 nm.
Fig. 93. CF-labelling throughout the length of the outermost cisterna (arrowhead) of a dictyosome. 60 min CF incubation, pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. Bar equals 300 nm.

Fig. 94. Membrane connection between a dictyosome and the endoplasmic reticulum. A dictyosome seen in face view appears to be connected (arrows) to an element of the rough endoplasmic reticulum (ER). Note the fenestrations (arrowheads) around the periphery of the dictyosome. Control (i.e. no CF) protoplast preparation, stained en bloc with uranyl acetate. Bar equals 300 nm.
Fig. 95-100. Series of sections through a MVB. 60 min CF incubation, pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. All at the same magnification. Bar in Fig. 100 equals 300 nm. Fig. 95. Note the vesicle (arrow) containing CF. Additional CF (arrowhead) may represent the edge of the MVB. Fig. 96. Glancing section through the MVB. Fig. 97. Section through the MVB showing CF on the inner surface of the limiting membrane and the outer surface of the internal vesicles (arrowheads). Fig. 98. Section through the MVB. Note that CF particles appear to be concentrated within two regions of the MVB perhaps obscuring the presence of internal vesicles. Fig. 99. Section through the MVB showing CF (arrowhead) apparently free within the lumen of the MVB. Fig. 100. Note the vesicle (arrow) containing CF. Additional CF (arrowheads) may represent the edge of the MVB.
Fig. 101-106. Detailed ultrastructure of MVBs. All at the same magnification. Bar in Fig. 106 equals 300 nm. Fig. 101. MVB with a lamellar plaque (arrow) on the cytoplasmic surface of the limiting membrane. Note the periodicity seen within the outer layer of the plaque. Also note the numerous small internal vesicles within the MVB. Control (i.e. no CF) protoplast preparation, double stained with uranyl acetate and lead citrate. Fig. 102. MVB bearing a plaque (arrow) on the cytoplasmic surface of its limiting membrane. Note that it is not possible to see substructure within the plaque. Control protoplast preparation, unstained. Fig. 103. MVB with a plaque (arrow) on the cytoplasmic surface of its limiting membrane. It is not possible to discern any substructure within the plaque. Control protoplast, en bloc uranyl acetate staining. Fig. 104. MVB with tubular extensions (arrows) protruding from the main body of the MVB. Note the CF bound to the outer surface of the small internal vesicles. 30 min CF incubation with pre-fixation washes normally used only for long term CF incubations (see section 2.5 of Materials and Methods), en bloc uranyl acetate staining (3h). Fig. 105. MVB containing a large number of internal vesicles. Note what appears to be a small vesicle (arrow) forming by invagination of the limiting membrane. Control protoplast preparation, double stained with uranyl acetate and lead citrate. Fig. 106. Rough endoplasmic reticulum (ER) appressed against a MVB (arrows). 60 min CF incubation, stained en bloc uranyl acetate.
Fig. 107-109. Selected serial sections showing a comparison of a CF-labelled vacuolar membranous mass with the contents of MVBs in the cytoplasm. 60 min CF incubation, stained en bloc with uranyl acetate. All at the same magnification. Bar in Fig. 107 equals 300 nm. The section numbers are indicated on each micrograph (i.e. S1, S3, S5). Fig. 108 and 109 show areas equivalent to those present in Fig. 107. Fig. 107. Two MVBs (MVB1 and MVB2) and the edge of a CF-labelled membranous mass (M) in the central vacuole are present. The membranous mass is labelled not only with CF (arrows) but also with electron-dense precipitates (arrowheads) resulting from en bloc uranyl acetate staining. Similar precipitates are also found along the tonoplast (T). Fig. 108. Profiles of MVB1 and MVB2 are again seen. MVB1 is strongly labelled with CF. Fig. 109. This figure shows another profile of the vacuolar membranous mass (M). Note the similarity in the appearance and size of this profile with that of the contents of MVB1 in Fig. 108.
Fig. 110-113. Selected serial sections of a small CF-labelled vacuole. 60 min CF incubation with pre-fixation washes omitted (see section 2.5 of Materials and Methods), unstained. All at the same magnification. Bar in Fig. 113 equals 300 nm. Fig. 110. A small vacuole (SVa) is near the central vacuole (V). Due to fixation damage (arrowheads), it is not possible to determine if the small vacuole is truly independent of the central vacuole. Fig. 111. The small vacuole contains a CF-labelled membranous mass (arrow). Note the presence of a vesicle (arrowhead) on the plasma membrane. Fig. 112. A CF-labelled membranous mass (arrow) is again seen in the small vacuole. One section with a CF-labelled membranous mass in the small vacuole was omitted between Fig. 111 and 112. Fig. 113. The membranous mass is no longer visible in the small vacuole. Again, note the presence of a vesicle (arrowhead) on the plasma membrane.
Fig. 114-116. NF uptake results. 70 min NF incubation, unstained. All at the same magnification. Bar in Fig. 116 equals 200 nm. Fig. 114. A MVB is labelled with NF (arrowheads). Also note the NF bound to the plasma membrane (arrows). Fig. 115. A single NF particle (arrowhead) in a dictyosome. Fig. 116. NF bound to the plasma membrane (arrowhead) and a CP (arrow).

Fig. 117. Low magnification of protoplast post-fixed with ZIO. The nuclear envelope (NE) is strongly labelled and nuclear pores (arrows) are easily visualized. The endoplasmic reticulum, in this case specifically the tubular endoplasmic reticulum (tER), is also heavily labelled and appears to be connected to the nuclear envelope. Note that mitochondria (M) are heavily labelled while only the internal membranes of plastids (P) are labelled. SG- starch granule, N- nucleus. Unstained, thick section (200-300 nm). Bar equals 500 nm.
Fig. 118 and 119. ZIO post-fixation results for cisternal endoplasmic reticulum. Unstained, thick sections (200-300 nm). Fig. 118. Uneven labelling of cisternal endoplasmic reticulum (cER) results in a "flaky" appearance. Note that the tER appears to be connected to the cER. D- dictyosome, M- mitochondrion. Magnification as Fig. 121. Fig. 119. Another example of cER which has fenestrations (arrowheads). Note that the cER again appears to be connected to tER (arrow). V- central vacuole. Bar equals 500 nm.

Fig. 120-121. ZIO post-fixation results for dictyosomes. Unstained, thick sections (200-300 nm). Both at the same magnification. Bar in Fig. 121 equals 300 nm. Fig. 120. Two dictyosomes (D) are seen in cross-section and are heavily labelled with ZIO reaction product. Also note the occurrence of small ZIO precipitates (arrowheads) in the general cytoplasm which may or may not occur in vesicles. Fig. 121. A dictyosome (D) seen in face view. Note the tubules which emanate from the periphery of the dictyosome (arrowheads). Also note the presence of tER (arrow).
Fig. 122-127. Results of tilting experiments with thick sections of ZIP post-fixed protoplasts. Unstained, thick sections (200-300 nm).

Fig. 122-124. A series of micrographs taken from the same section tilted at various angles (Fig. 122: -30°, Fig. 123: untilted, Fig. 124: +30°). All at the same magnification. Bar in Fig. 124 equals 300 nm. The approximate direction in which tilting occurred is indicated by the "two-headed" arrow in Fig. 122. D- dictyosome. Note the fenestration (arrow) in the ER which enlarges as the section is tilted from -30° (Fig. 122) to +30° (Fig. 124). Arrowheads and double arrowheads indicate the identical segments of tER in this series of micrographs. One ER tubule (double arrowhead) is apparently not attached to the dictyosome in Fig. 122 but appears to be connected to the dictyosome in Fig. 123 and 124. A second ER tubule (arrowhead) appears to be connected to the dictyosome in Fig. 122 and 123 but not in Fig. 124. Thus neither segment of tER remains attached to the dictyosome through the entire tilt series and therefore they are not physically connected to it.

Fig. 125-127. Series of micrographs from the same section tilted at various angles (Fig. 125: -30°, Fig. 126: untilted, Fig. 127: +30°). All at the same magnification. Bar in Fig. 127 equals 300 nm. The approximate direction of tilting is indicated by the "double-headed" arrow in Fig. 125. Arrowheads and double arrowheads are used to indicate the identical segment of ER throughout the tilt series. D- dictyosome. Two segments of ER (arrowhead, double arrowhead) appear to remain in contact with the dictyosome throughout the series and therefore may represent true membrane connections between the ER and the dictyosome.
Fig. 128-133. ZIO post-fixation results for dictyosomes using thin (60-80 nm) sections. Fig. 128. A dictyosome (D) which appears to be connected (arrowhead) directly to the endoplasmic reticulum (ER). Unstained, magnification as Fig. 133. Fig. 129. A dictyosome (D) which appears to be connected (arrowhead) to the endoplasmic reticulum (ER). Note that regions of a mitochondrion (M) probably corresponding to cristae are not labelled with ZIO reaction product. Unstained, magnification as Fig. 133. Fig. 130. A dictyosome (D) in which a clear polarity of ZIO labelling is seen between the cisternae on one side of the dictyosome relative to the cisternae on the other side. Unstained, bar equals 300 nm. Fig. 131. A dictyosome (D) in face view in which peripheral fenestrations (black arrowheads) are readily seen. A membrane connection (white arrowheads) between this dictyosome and a second dictyosome (D2) is also visible. Unstained, magnification as Fig. 133. Fig. 132. Two dictyosomes (D) are present. On these dictyosomes, membrane buds (arrowheads) which may be coated are visible. Stained with uranyl acetate and lead citrate. Magnification as Fig. 133. Fig. 133. Two dictyosomes, one in cross-section (D) and the other (D2) in face view, are present. Note the apparent membrane connection (arrowheads) between them. Unstained, bar equals 300 nm.
Fig. 134. ZIO post-fixation results with thin sections showing an unlabelled PCR. Note the coated ends (arrowheads) of the tubules of the PCR. Also note the presence of a dictyosome (D) which is heavily labelled with ZIO reaction product. Stained with uranyl acetate and lead citrate. Bar equals 300 nm.

Fig. 135-137. ZIO post-fixation results using thin sections for MVBs. All at the same magnification. Bar in Fig. 137 equals 300 nm. Fig. 135. Three MVBs (arrowheads) with internal vesicles containing very fine ZIO deposits. Note the ER which is appressed against the MVBs and is heavily labelled with ZIO. Unstained. Fig. 136. A MVB (arrow) with internal vesicles which are heavily labelled with ZIO. Unstained. Fig. 137. MVB (arrow) containing CF and internal vesicles heavily labelled with ZIO. Note that portions of the plastid (P) and the ER (arrowhead) are also labelled with ZIO. 90 min CF incubation, unstained.
Fig. 138-140. Results of lanthanum chloride uptake by whole SB-1 cells. 60 min lanthanum chloride incubation, unstained. All at the same magnification. Magnification as Fig. 143. Fig. 138. The majority of the lanthanum deposits (arrowheads) are found on the outer surface of the cell wall (CW) although they are also seen at lower frequency within the cell wall. Note the presence within the cytoplasm of a vesicular profile (arrow) containing lanthanum. Fig. 139. The cross-walls (CW) between adjacent SB-1 cells contain a few lanthanum deposits (arrowheads) indicating that lanthanum reaches these regions at least to some small degree. Note the lanthanum-containing vesicular profile (arrow) within the cytoplasm of one cell. Fig. 140. A lanthanum deposit (arrow) can be seen within an element of the ER. The ER appears to be slightly dilated at the region where the deposit occurs. CW- cell wall.

Fig. 141-143. Results of incubating whole SB-1 cells with CF for 60 min. All at the same magnification. Bar in Fig. 143 equals 300 nm. Fig. 141. Most CF appears to be bound to the outer surface of the cell wall (CW). Very little CF (arrowheads) appears to penetrate part way into the cell wall. Unstained. Fig. 142. A particle of CF (arrow) appears to be bound to the outer surface of the plasma membrane. Stained with lead citrate. Fig. 143. ER apparently connected (arrow) directly to the plasma membrane. Unstained.
Fig. 144-146. Results of 14 h incubation of whole SB-1 cells with CF. Unstained, all at the same magnification. Bar in Fig. 146 equals 300 nm. Fig. 144. Most CF is bound to the outer surface of the cell wall (CW). However, some CF (arrowheads) has penetrated into the cell wall. Note that there also appears to be some cytoplasmic labelling with CF (arrows). This CF appears to be free in the cytoplasm. Fig. 145. CF is seen within an element of the ER (arrows). Also note the presence of CF (arrowheads) part way through the cell wall. Fig. 146. Note the "trail" of CF (arrowheads) running through the cell wall (CW) to the plasma membrane. Also note the presence of CF (arrows) within the cytoplasm. Some of this CF appears to be free in the cytoplasm. Some is in the cytoplasm but possibly associated with a vesicular profile. Some is in a vesicular profile. This profile may be an element of the ER seen in cross-section.

Fig. 147. Additional observation of whole SB-1 cells. Note the invagination of the plasma membrane (arrow) where many small membrane-bounded vesicles are present in the periplasmic space. CW- cell wall, PM- plasma membrane. Lanthanum control (i.e. no lanthanum) cell preparation, unstained. Bar equals 300 nm.
4 Discussion

4.1 Introductory Remarks

Endocytotic processes are well known and well
demonstrated in animal cell systems (Steinman et al. 1983).
In sharp contrast, little is known about these processes in
plant cells or protoplasts. Uptake of a variety of markers,
such as native ferritin (Power and Cocking 1970), hemocyanin
(Williamson et al. 1976), latex beads (Suzuki et al. 1977),
viruses (Takebe 1979) and liposomes (Fununaga et al. 1983),
have been demonstrated in protoplasts. However, prior to the
start of this research project, there was no evidence for
the involvement of coated membranes in the endocytotic
process. Therefore, the results of this research project are
significant as they clearly implicate a number of coated
membranous organelles including; CPs, CVs, the PCR and
dictyosomes, in the process of endocytosis in soybean
protoplasts. Also, SVs, MVBs, small vacuoles and the central
vacuole appear to be involved in this process. All of the
organelles which become labelled with CF in soybean
protoplasts bear some similarity morphologically and/or
functionally to organelles involved in receptor-mediated
endocytosis in animal cells (see discussion below). An
endocytotic pathway for soybean protoplasts which is based
in part on an analogy with the receptor-mediated endocytotic
pathway in animal cells will be presented later in this
section.
A problem encountered constantly throughout this project was the question of specimen contrast. A number of staining protocols were used in an attempt to achieve sufficient specimen contrast such that coats and membranes could clearly be resolved while not seriously impeding one's ability to identify CF. None of these staining protocols proved to be adequate for general use. Unstained sections permitted clear resolution of CF as small electron-dense particles but the resolution of membranes and protein coats was poor. Staining thin sections with lead citrate improved the resolution of membranes and coats somewhat but still was not ideal. Staining thin sections with uranyl acetate followed by lead citrate provided good specimen contrast. However, this protocol also stained CF heavily, making it difficult or impossible to identify with any degree of confidence. Therefore, to unambiguously identify CF, unstained sections or sections stained with lead citrate alone were used. Sections were stained with uranyl acetate en bloc to resolve ultrastructural details of organelles (e.g. serial sectioning analysis) as well as to visualize CF particles.

4.2 Effect of Molecular Charge on Endocytosis

The uptake of ferritin particles via the endocytotic pathway in soybean protoplasts appeared to be very sensitive to the charge of the marker in use (section 3.8). Uptake of
CF particles could be easily detected at much lower protein concentrations than for NF. Furthermore, much greater amounts of CF were internalized even when the relative concentration of CF was considerably lower than the concentration of NF. In addition, the plasma membrane was more heavily labelled when CF was used rather than NF. These results are hardly surprising. When NF and CF have been used with animal cells, similar if not identical results were found. For example, with chick embryos, substantial uptake was observed using CF but uptake of NF was not detected even when NF was used at a concentration 40 times greater than that for CF (MacLean and Sanders 1983). Furthermore, in human fibroblasts, binding of NF to the plasma membrane and uptake were very low in comparison with CF. This was the case even when NF was used at 50 times the concentration of CF (Van Deurs et al. 1982). For animal cell studies, NF has been considered to be a marker for fluid phase endocytosis, i.e. the marker is not bound to the plasma membrane prior to uptake (e.g. Morales et al. 1985). Strictly speaking, for soybean protoplasts and at least some types of animal cells, NF is not a fluid phase marker as some NF particles were bound to the plasma membrane (Fig. 114 and 116, and Van Deurs et al. 1982). In the case of CF, it is considered to be a marker for adsorptive endocytosis, i.e. the marker is bound to the plasma membrane but not necessarily to a specific receptor prior to uptake (e.g. Van Deurs et al.)
1982; Morales et al. 1985). Adsorptive endocytosis is more efficient than fluid phase endocytosis as the marker is concentrated on the plasma membrane where the initial events of endocytosis are occurring. Therefore, much lower concentrations of markers are needed to detect uptake into the cell or protoplast when adsorptive endocytosis is being studied.

The plasma membrane of plant protoplasts is known to have a strong net negative surface charge (Nagata and Melchers 1978; Fowke 1986). In soybean, when isoelectric focusing was used to measure the surface charge of protoplasts derived from SB-1 cell cultures, the isoelectric point of these protoplasts was found to be 3.7 or 5.1, depending on the nature of the pH gradient (Griffing et al. 1985). Therefore at pHs near neutrality (e.g. pH 6.8), these protoplasts would have a strong net negative surface charge. CF particles would therefore bind electrostatically to the negatively charged sites on the plasma membrane resulting in the observed high levels of CF binding and uptake. It should be noted that electrostatic interactions are weak bonds (Van Deurs and Christensen 1984). Thus, it is possible that a CF particle can dissociate from the group to which it was originally bound (Van Deurs and Christensen 1984 and references cited therein) and may form another bond with another group or may remain free within the lumen of an
organelle. Therefore, it is possible that internalized CF particles may no longer be associated with membrane constituents originating from the plasma membrane. While acknowledging that the cytoplasmic location of any particular CF particle may not correspond to the location of plasma membrane constituent(s), it has been assumed, for the sake of discussion, that the overall cytoplasmic distribution of CF particles is representative of the overall distribution of membrane constituents and/or luminal contents from endocytotic, plasma membrane-derived vesicles.

4.3 Adverse Effects of CF on the Morphology of Protoplasts and Cells

While adverse effects of CF treatment on both SB-1 protoplasts (section 3.7) and cells (section 3.10.2) were detected, the morphological differences between control and CF-treated preparations were relative rather than absolute. That is to say, some protoplasts (and cells) incubated in CF were comparable to their controls. Every attempt was made to make observations from CF-treated protoplasts and cells which were similar to the controls.

Protoplasts treated with CF tended to collapse more than controls, contained extracted cytoplasm and exhibited more frequent damage to the tonoplast. Preliminary results indicated that cells from newly initiated cultures were also susceptible to damage by CF. During the study of ferritin uptake by the roots of Phaseolus vulgaris seedlings (Barton
1964), the ferritin solutions used in the experiments were found to be slightly toxic to root cells. Unfortunately, the specific symptoms of the toxic effect were not described and therefore a direct comparison to the present situation cannot be made. However, Barton did speculate that contaminants in the form of cadmium salts used in the preparation of ferritin may have been responsible for these toxic effects. This explanation is not likely to apply to the present situation as NF preparations which would also be contaminated with cadmium salts did not produce the effects associated with CF treatment. It is more likely that the problems associated with the CF incubations are related to the charge of this macromolecule. Other basic macromolecules, including histones and poly-L-lysine, are known to inhibit growth for instance in barley roots (Drew and McLaren 1970; Drew et al. 1970). This inhibition of growth was correlated with leakage of cell contents suggesting that basic macromolecules act primarily at the plasma membrane to alter the normal cell permeability (Drew and McLaren 1970). The difficulty in observing the typical unit membrane structure in CF treated protoplasts is consistent with the occurrence of damage to the plasma membrane or an alteration in its properties. The extracted appearance of some protoplasts and cells is consistent with the possible occurrence of increased membrane permeability.
Variations in the degree of damage seen in different protoplast and cell preparations may reflect interexperimental differences in the physiological state of these preparations, in the culturing procedures or in the procedures used for protoplast production and isolation. Drew and co-workers (1970) found that the inclusion of as little as $1 \text{ mM} \ \text{CaCl}_2$ resulted in the protection of plant tissue from the adverse effects of basic macromolecules. It was thought that the presence of $\text{Ca}^{2+}$ ions was responsible for this effect. It would be interesting to determine whether $\text{Ca}^{2+}$ ions would have the same effect during all CF incubations but especially with protoplasts.

4.4 Significance of Short Term CF Uptake by Protoplasts

The results from the short term CF uptake studies demonstrate the capacity of soybean protoplasts to internalize surface-labelled plasma membrane by endocytosis via their coated-membrane system. After 10 s of exposure to CF, this label was present on the entire plasma membrane surface, in CPs and CVs. The absence of labelled, large, smooth vesicles at this and other early time points (30 s, 2 min) indicates that phagocytosis was not responsible for the uptake of the plasma membrane. The evidence from Fig. 25 (section 3.3) favors the existence of free CVs. Furthermore, there was no evidence for the formation of uncoated or smooth vesicles either in the vicinity of CPs (i.e. the formation of receptosomes) or from elsewhere on the plasma
membrane. Therefore, the experimental evidence strongly suggests that CF uptake was mediated by the formation of CVs via the invagination of CPs on the plasma membrane.

The serial sectioning results from section 3.6.1 are consistent with the existence of free coated vesicles. However, there is some controversy in the literature on this matter. Some groups working on animal cell systems have used serial sectioning to provide evidence for the existence of free CVs (Fan et al, 1982; Petersen and Van Deurs 1983; Van Deurs et al. 1984). Depending upon the cell type and the section thickness used, 4 to 47 % of coated vesicular profiles in the vicinity of the plasma membrane were found to be unattached to other membranous organelles (Fan et al. 1982; Petersen and Van Deurs 1983; Van Deurs et al. 1984). In marked contrast, one research group (Willingham and Pastan 1983,1984b), using serial sections 70 to 100 nm in thickness and a special fixation procedure, found that all coated vesicular profiles in the vicinity of the plasma membrane were connected to it by a narrow membrane neck (Willingham and Pastan 1983). Furthermore, they found that uncoated or smooth membrane pits were sometimes found adjacent to CPs on the plasma membrane. They proposed that CPs were involved in the clustering of receptors and their bound ligands to specific regions on the plasma membrane, that these clusters of receptors and ligands were then
transferred to the adjacent plasma membrane from which a smooth or non-coated vesicle called a receptosome was formed by the process of invagination (Fig. 2 and Willingham et al. 1981). This controversy has not been resolved. For a more complete analysis of the debate see the papers by Van Deurs and co-workers (1984), and Willingham and Pastan (1984a,b). Current reviews by authors (Fine and Ockleford 1984; Breitfeld et al. 1985; Wileman et al. 1985; Stahl and Schwartz 1986) not directly involved in the debate indicate that the general consensus favours the existence of free CVs.

Occasionally vesicular profiles having the appearance of partially coated vesicles were seen in samples from the 30 s and subsequent time points, Fig. 28 (section 3.3). This observation suggests that the smooth vesicles seen during 30 s CF incubations and in all subsequent time points may have been formed by the uncoating of coated vesicles and that this uncoating process occurred rapidly. This would be consistent with results obtained from animal systems (Pearse and Bretscher, 1981). Partially coated or incompletely coated vesicles have also been seen in other plant cells (Ryser, 1979; Lucas and Franceschi, 1981). In one case, the existence of partially coated vesicles was taken as being consistent with an endocytotic mechanism (Lucas and Franceschi, 1981).

Overall, the results of short term uptake of CF by
soybean protoplasts are consistent with an endocytotic pathway which is similar to the conventional view of receptor-mediated endocytosis in animal cells (Fig. 1 in section 1.2) where CVs form from CPs, then shed their coats and fuse with other organelles. The results are not consistent with the formation of receptosomes as proposed by Willingham and co-workers (1981).

4.5 Significance of Intermediate Term CF Uptake by Protoplasts

Incubations of 4 to 16 min in CF were used on protoplasts to determine which organelles in addition to CPs, CVs and SVs become labelled and in what order. Unfortunately, the results were not as clear cut as one would have liked. Label rapidly appeared in three additional organelles; the PCR, dictyosomes, and MVBs. Label was seen in examples of the PCR as early as 2 min (Fig. 31), in dictyosomes as early as 4 min and in MVBs as early as 6 min. Thus a large separation in time of the labelling of these organelles did not occur. However, the PCR was more consistently labelled at the early time points and is thus thought to be labelled prior to the dictyosomes and MVBs. Using the same rationale, the results suggest that dictyosomes label prior to MVBs. In order, to rigorously demonstrate that one organelle was labelled prior to the others would require a detailed quantitative and statistical
analysis of the labelling pattern. Such an analysis was not attempted for two major reasons. First, the number of organelles which were labelled at these time points was low. Thus a prohibitively large number of organelles would have to be sampled before a statistically significant difference would be obtained. This problem is compounded by the fact that the organelles frequently contained one or a few particles of ferritin which could easily be missed when viewing the specimen in the electron microscope. Second, it was not always possible to unambiguously identify an organelle from a single section. For example, some profiles through a dictyosome can appear similar to the PCR and not all vesicles containing internal vesicles are MVBs. Therefore, the misidentification of unlabelled and labelled organelles would have been a problem.

The results from 30 min CF incubations were interesting as labelling of the PCR, dictyosomes and MVBs was readily detected. The labelling of the MVBs was particularly strong suggesting that CF particles accumulate in these organelles over time.

4.6 The Morphology of the Partially Coated Reticulum

The partially coated reticulum is an organelle in plant cells which has only recently been described in detail for the alga, Chara (Pesacreta and Lucas 1984) and some selected angiosperms (Pesacreta and Lucas 1985). For this reason, its presence and appearance in soybean protoplasts is of special
interest. The PCR in soybean protoplasts appeared very similar to the PCR found in cells from young *Phaseolus vulgaris* leaves and in *Zea mays* cortical root cells (Pesacreta and Lucas 1985). It consisted of a set of interconnected tubular membranes in which coated membrane regions could be found. Furthermore, in all cases, coated vesicles were seen in the cytoplasm around these organelles. One feature of the PCR of soybean protoplasts which is apparently unique was the occurrence of membrane dilations. Pesacreta and Lucas (1985) did not mention the occurrence of such dilations, but did note that in *Z. mays* the PCR could be found associated with small, dense zones of membranes that appeared to be either greatly anastomosed or vesiculated. However, these zones of membranes at least as illustrated by Pesacreta and Lucas (1985) bear little resemblance to the dilations of the PCR seen in soybean protoplasts.

The PCR of soybean protoplasts is also similar to those of *P. vulgaris* and *Z. mays* with respect to their distribution within the cytoplasm. Pesacreta and Lucas (1985) noted that the PCR in *P. vulgaris* and *Z. mays* was frequently but not always associated with Golgi stacks (dictyosomes). Only approximately 10% of the 100 PCRs that were examined in these two species were not closely associated with Golgi. Comparable data are not available for
the distribution of the PCR in soybean protoplasts and even if it were, a direct comparison would be difficult to make as Pesacreta and Lucas did not define what they meant by an association between the PCR and Golgi. However, the overall pattern of PCR distribution was the same in soybean protoplasts. The PCR was frequently seen in the vicinity of dictyosomes but in a number of cases was clearly not associated with dictyosomes.

Pesacreta and Lucas (1985) also noted that physical continuities between the PCR and Golgi stack membranes (cisternae of dictyosomes) were not evident in most cases or were "problematic" (i.e. ambiguous). Only occasionally did they find the PCR to be directly connected to the Golgi stack. Again, the results for soybean protoplasts are similar. In a number of cases, when the PCR was in the vicinity of a dictyosome there was clearly no evidence of a physical connection between the two organelles. In other cases, the organelles were closely associated but clear connections could not be demonstrated. In only one case was an unambiguous connection seen between a PCR and a dictyosome. A number of factors contributed to the uncertainty in establishing connections between the PCR and dictyosomes. First, sufficiently long series of sections were difficult to obtain. Second, the dictyosomes and the PCR were both, very complex organelles. The cisternae of dictyosomes frequently were curved and the tubules of the
PCR and of the dictyosomes could be curved or branched making glancing sections through these organelles difficult to interpret. In addition, both of these organelles had a tubular component which had a coat on part of its cytoplasmic surface and it was difficult to differentiate between these tubules. Thus when two or more of these organelles were found in close proximity to one another, it was frequently difficult to determine, with any degree of certainty, where one organelle began and another ended or whether the two organelles were in fact connected. Finally, it is possible that physical continuities between the PCR and dictyosomes or other organelles such as the ER (see below) may be transitory in nature. This possibility in combination with the technical problems discussed above may mean that a great deal of good luck would be required to unambiguously demonstrate membrane connections between these organelles.

Overall, the serial sectioning results for the PCR provided evidence that this organelle was at least in some cases distinct from the dictyosomes of the Golgi apparatus. Zinc iodide–osmium tetroxide postfixation was used in the hope of providing further evidence to support these results. In a study of the alga, Chara corallina, Pesacreta and Lucas (1984) were able to differentiate between the PCR which was lightly labelled with ZIO and Golgi stacks which were
generally heavily labelled. They reasoned that such results offered little support for the existence of either membranous connections or membrane flow from the Golgi to the PCR.

Unfortunately, the results from soybean protoplasts postfixed with ZIO were not as clear cut as with Chara. The labelling of dictyosomes was more variable than had been encountered in Chara thus making a comparison between the PCR and the dictyosomes more difficult. Furthermore, coats were more difficult to visualize in ZIO postfixed material thus making the identification of the PCR more difficult. Despite these problems, it was possible to identify probable PCR profiles which were unlabelled or only weakly labelled with ZIO reaction product while all profiles of the dictyosomes in the same protoplast were heavily labelled. These results are consistent with those for Chara discussed above and provide further evidence that the PCR can at least in some instances be distinct from the dictyosomes.

Pesacreta and Lucas (1985) considered the PCR to be a structural entity. But when faced with the range of morphology of this organelle, especially near the elements of the Golgi apparatus, they felt that it might be useful to consider the various structures as parts of an ultrastructural series. In the simplest case in such a series the PCR would consist of only a few, small coated membranes grouped together. The next case would involve a
larger but still relatively distinct membrane system. The final case would be the one in which physical continuities would exist between the PCR and the Golgi (dictyosomes). This type of series would reflect physiological differences in the rate, type and extent of interactions between the PCR and the Golgi (Pesacreta and Lucas 1985). Based on the similarities (discussed above) which exist between the PCRs of *P. vulgaris*, *Zea mays* and soybean protoplasts, it seems likely that such an ultrastructural series also exists in soybean.

The concept of an ultrastructural series is helpful in reconciling the existence of the PCR with previous observations of coated membranes located in the vicinity of dictyosomes, for instance the peripheral tubules of dictyosomes seen in protoplasts from *Vicia faba* (Joachim and Robinson 1984) and the trans pole complex in maize root cap cells (Hübner et al. 1985). These latter structures may be analogous to the PCR or may represent distinct forms of the PCR. The novelty of the results reported by Pesacreta and Lucas (1985) and of this project is not so much the discovery of a partially coated membrane network but rather the observation that some of these organelles are not associated with the Golgi (Pesacreta and Lucas 1985).

Pesacreta and Lucas (1985) also noted that organelles other than Golgi were often located near the PCR. Elements
of the ER were frequently seen adjacent to the PCR but there was no mention of possible connections between these organelles. In soybean protoplasts, not only was the PCR seen adjacent to the ER but in some cases the PCR appeared to be appressed against the ER or perhaps physically connected to it. This observation is quite important and may be of great significance. It has been noted (Harris 1986) that the PCR appears similar to the organelle which has been given the acronym, GERL (for Golgi-associated endoplasmic reticulum which gives rise to lysosomes). This organelle appears to be present in both animal and some plant cells (Novikoff 1976; Marty 1978). GERL is thought to be a partially coated tubular element of the ER which is located in close proximity to Golgi stacks, usually on the trans side of the stack (Novikoff 1976). While there is some debate about the structure of GERL, there can be no denying that the general description of the PCR is quite similar.

4.7 The PCR and Animal Cell Endosomes

The PCR bears considerable resemblance to a class of endosomes in animal cells which are known by a number of names including, trans reticular Golgi (Willingham and Pastan 1984a) and CURL, an acronym for compartment of uncoupling of receptor and ligand (Geuze et al. 1983a). Trans reticular Golgi is located, as its name indicates, on the trans side of the Golgi and consists of tubular elements which have coated buds (Willingham and Pastan 1984a;
Willingham et al. 1984). CURL is found in the peripheral cytoplasm and deeper within the cytoplasm in the vicinity of the Golgi apparatus (Breitfeld et al. 1985). It consists of interconnected tubular and vesicular profiles (i.e. dilations, Geuze et al. 1983a; Breitfeld et al. 1985). Small regions of the tubular component bear a clathrin coat on their cytoplasmic surface (Geuze et al. 1984, 1985; Breitfeld et al. 1985). Furthermore, internal vesicles have been observed within some of the vesiculated regions of this organelle (Geuze et al. 1985; Harding et al. 1985). It seems possible that these endosomes are perhaps the same organelles given different names by different research groups (Willingham and Pastan 1984a; Bretscher and Thomson, 1985). These structures also have some similarity to GERL (Willingham and Pastan 1984a; Geuze et al. 1985). In fact, the similarities are so strong that Willingham and Pastan (1984a) have suggested that CURL, trans reticular Golgi and GERL are the same organelle.

Studies of the function of CURL in receptor-mediated endocytosis have produced some most interesting results. It is thought that CURL is the endosome in which some receptor-ligand complexes dissociate, presumably due to a reduced pH within CURL, and where sorting of the receptors from the ligands occurs (Geuze et al. 1983a, 1984; Breitfeld et al. 1985). The sorting event may result simply through the
mechanism of mass action (Harding et al. 1985; Rome 1985). It has been estimated that roughly 90% of the membrane surface area in CURL is accounted for by the tubular portion of the organelle with the remaining 10% accounted for by the membrane surrounding the vesicular portion of the organelle. With respect to volume distribution, the values are reversed with the lumen of the vesicular portion of CURL accounting for the majority of the volume. If one assumes that receptors are free to move within the plane of the lipid bilayer of the membrane and that likewise free ligands are able to move freely within the lumen of CURL then by mass action the majority of the receptors (i.e. 90%) should be located within the tubular portion of the organelle while most of the ligand will be located within the vesicular portion. Thus quite efficient sorting of the receptor from the ligand could occur without proposing any special structural modifications to CURL (Rome 1985). It is thought that receptors could recycle to the plasma membrane in vesicles derived from the tubular portion. Recycling could occur directly from CURL or the receptors may first pass through the Golgi apparatus (Breitfeld et al. 1985). In contrast, for the transit of ligands, a very different mechanism has been proposed. There is some morphological evidence which suggests that multivesicular bodies in animal cells form as a result of a maturation process operating on the vesicular portion of CURL. It is proposed that the
tubular portions disappear as they produce outgoing receptor-rich vesicles while the vesicular portion produces internal vesicles via a simple invagination process. The resulting multivesicular bodies are then thought to fuse with (primary) lysosomes and thereby are transformed into (secondary) lysosomes (Geuze et al. 1983b; Harding et al. 1985).

It should be noted that the trans reticular Golgi has also been implicated with respect to the sorting of receptor-ligand complexes (Willingham et al. 1984). Epidermal growth factor and its receptor appear to be concentrated in the coated buds of the trans reticular Golgi. It is thought that this receptor-ligand complex is concentrated in the coated buds prior to its subsequent transfer to lysosomes. In contrast, transferrin and its receptor are generally recycled to the plasma membrane without being delivered to the lysosome. The transferrin-transferrin receptor complex is not concentrated in the coated buds of trans reticular Golgi but is generally found in the tubular portion of the organelle. It is thought that transferrin and its receptor are recycled back to the plasma membrane from this location (Willingham et al. 1984).

The observation in this study that the PCR becomes labelled with CF is a highly significant result as it provides the first direct evidence for the involvement of
the PCR in endocytosis. Based on an analogy with CURL or trans reticular Golgi, it is possible to envision the PCR playing a role in membrane sorting events and possibly in the dissociation of as yet unidentified receptor-ligand complexes. In this way, the PCR may be responsible for determining what material is directed into the degradative part of the pathway represented by MVBs and the central vacuole (see sections 4.9 and 4.11) and what material is recycled to the plasma membrane either directly from the PCR or indirectly via dictyosomes.

4.8 Significance of the Dilation in the PCR

The fact that the dilation is not seen in all of the PCR of soybean protoplasts and that the dilation sometimes contains internal vesicles creates a situation which lends itself to some interesting speculation. If one accepts the proposition that the MVB becomes labelled after the PCR and receives its label from the PCR then it is tempting to speculate that the MVB may form from the PCR via a maturation process analogous to the one described above for animal cell MVBs. However, a structure resembling the plaque of MVBs was not observed on the dilations of the PCR. Furthermore, the dilation and its internal vesicles, when they occurred, were never labelled with CF. Therefore, it seems unlikely that the PCR labelled with CF would generate an unlabelled MVB which only later would become labelled.

A second possible explanation for the occurrence of the
dilation in the PCR involves membrane influx as opposed to efflux. It is possible that the dilation represents a large vesicle that has fused with the tubular portion of the PCR thereby adding membrane to this organelle. As membrane recently incorporated into the PCR it would therefore be reasonable to expect that the dilation would not be fully integrated into the PCR and thus could be unlabelled. The influx of membrane resulting from the proposed fusion event could in theory compensate in part or in total for any loss of membrane that might occur as a result of the formation of vesicles from the coated regions of the PCR. The ER and dictyosomes would seem to be the most likely candidates as the source for the incoming vesicle. The occurrence of smaller vesicles within some dilations remains unexplained by this hypothesis. However, it is possible that these vesicles are simply fixation artifacts (Willingham and Pastan 1984a).

The clear segregation of CF label in the PCR could be readily explained if there is an obstruction or barrier within the lumen of the PCR. This obstruction could prevent the redistribution of CF particles from the tubular portion of the PCR to the dilation. This idea has limited appeal. While it would explain the lack of CF particles within the dilation, it reveals nothing about the nature or function of the dilation and would not provide an explanation for the
occurrence of small vesicles within the dilation. Furthermore, there is no ultrastructural evidence to support this idea.

Finally, it is possible that both the dilations and their internal vesicles are artifacts of glutaraldehyde (i.e. chemical) fixation. To discount this explanation would require the use of some procedure other than chemical fixation, such as freeze substitution, to provide truly convincing evidence that the dilations and/or their internal vesicles are "real" structures rather than fixation artifacts. However, if the dilation is a fixation artifact, it is a very special one. The fixation process would have to simultaneously cause the swelling of the PCR, vesiculation of the membrane of the PCR where internal vesicles are observed and the redistribution of CF particles such that they are excluded from the forming dilation.

Clearly from the discussion above, further work is required to determine if the dilation is a "real" feature of the PCR and to determine its significance.

4.9 The MVB of Soybean Protoplasts

The multivesicular body (MVB) of soybean protoplasts is a distinct organelle which is very similar in morphology to MVBs of animal cells. In particular, MVBs contain internal vesicles, bear tubular protuberances, have a plaque on the cytoplasmic surface of their limiting membrane and are involved in the process of endocytosis (see section 3.5.4;
The plaque on MVBs from animal cells sometimes has substructure which gives the plaque a lamellar appearance (Willingham and Pastan 1984a). Similar substructure in the plaque of MVBs from soybean protoplasts was only observed in conventionally double-stained preparations. The inability to detect substructure in other preparations is likely due to poor specimen contrast (in unstained sections) or overstaining of the specimen (in en bloc stained uranyl acetate stained preparations). The composition of the plaque is unknown. However, from immunocytochemistry experiments with animal cells, it is known that the plaque reacts poorly to antibodies against actin, clathrin, myosin, tubulin and fodrin (Willingham and Pastan, 1980, 1984a). The possibility that the plaque is composed of a modified or relatively inaccessible form of clathrin which would explain the plaque's poor reactivity to anti-clathrin antibodies can not be eliminated (Willingham and Pastan 1984a). The periodicity seen within the plaque (Fig. 101 and Willingham and Pastan 1984a) is certainly suggestive of a clathrin coat. Plaques appear to be present on some MVBs in cells of other plant species such as *Acer pseudoplatanus* (Nougarède and Lescure 1970; Fig. 4, 7 and possibly 10). In this case, their existence was not mentioned by the authors.
The MVBs of animal cells are generally considered to be a prelysosomal compartment but in some cases can contain acid hydrolases (e.g. Friend and Farquhar 1967, Morales et al. 1985) and thus are technically lysosomes. Some of the MVBs of soybean protoplasts contain peroxidase (Griffing and Fowke 1985) and acid phosphatase (L.R. Griffing and L.C. Fowke, unpublished results) and therefore constitute part of the lytic compartment in these protoplasts.

4.10 Formation of MVBs

Exactly how MVBs are formed is not clear. For animal cells, it has recently been proposed that MVBs may form by the fusion of plasma membrane-derived receptosomes (see Fig. 2 and section 1.2 of the Introduction, Willingham and Pastan 1984a) or that they may form from the vesicular portion of CURL via a maturation process (see section 4.7, Geuze et al. 1983a, b, 1985). In plants, dictyosomes have frequently been proposed as the site from which MVBs originate (e.g. Halperin and Jensen 1967, Nougarède and Lescure 1970, Dexheimer et al. 1975). This idea, however, is based entirely on circumstantial evidence. Exactly how the MVBs of soybean protoplasts are formed is not clear from the work reported here. It is possible that they could form from the dilations of the PCR via a maturation process. However, the morphological evidence from this research project is not totally consistent with this hypothesis. Specifically, while the MVBs are heavily labelled, the PCR dilations and their
internal vesicles are not. Alternatively, it is possible that MVBs might form via a two step process from dilations which sometimes occur in the cisternae of dictyosomes or in the ER. These dilations could pinch off dictyosomes or the ER to form the large vesicle of the MVB. The internal vesicles could then be produced by invaginations of the limiting membrane. Indeed, it was possible to find many profiles of vesicles which appeared to be budding off the limiting membrane into the lumen of the MVB (Fig. 13 and 105). CF might reach the developing MVB as a result of the fusion of labelled SVs (and/or possibly CVs) with the limiting membrane. Such vesicles might originate from the PCR or possibly dictyosomes. If a vesicle containing CF bound to its inner membrane surface fuses with a MVB, the CF would end up on the inner surface of the MVB limiting membrane, as observed. If the same region or an overlapping region of the limiting membrane then invaginated to form an internal vesicle, the CF would be present on the outer surface of the vesicle, as observed. Therefore the presence of CF on the inner surface of the limiting membrane and on the outer surface of the internal vesicles is consistent with the hypothesis that the internal vesicles form via an invagination process.

It should be noted that the idea that internal vesicles of MVBs form via invagination of the limiting membrane is
not new. Based on observations of MVBs in other plant cell systems, Halperin and Jensen (1967) and Dexheimer and co-workers (1975) have also proposed the same mechanism. Furthermore, it seems likely that the internal vesicles of animal cell MVBs are produced in the same manner (e.g. Smith and Farquhar 1966, Harding et al. 1984).

The fact that the internal vesicles of at least some MVBs of soybean protoplasts become labelled during post-fixation with ZIO is very interesting. If the preceding discussion concerning the formation of the internal vesicles is correct then the contents of these vesicles originate from the cytosol. The fact that some internal vesicles are labelled while the cytosol in general is not labelled suggests that selective uptake of cytosolic components into the MVB may be occurring. The labelling pattern of the MVBs is also significant because the internal vesicles of MVBs from animal cells are sometimes labelled by ZIO post-fixation (e.g. Loesch 1981, Weakley et al. 1981, Squier 1982). Thus ZIO labelling provides another criterion by which MVBs of soybean protoplasts are similar to MVBs of animal cells.

It was noted that elements of the ER were frequently observed in the vicinity of or appressed against MVBs in soybean protoplasts. These results may simply reflect chance associations between the two organelles in the cytoplasm or may represent a functional relationship between them in
which the transfer of material may be facilitated. Similar observations were made by Halperin and Jensen (1967) in embryogenic cells of carrot cell cultures.

4.11 MVBs and the Vacuoles of Soybean Protoplasts

Once labelled, the MVBs of animal cells apparently fuse with primary lysosomes producing secondary lysosomes where their contents are degraded by hydrolytic enzymes (Geuze et al. 1983b; Harding et al. 1985). In fact, degradation may begin within organelles which appear morphologically to be MVBs as in some cases the internal vesicles of MVBs become less distinct in appearance with increasing organellar age (Harding et al., 1985). In this way, the MVBs and lysosomes represent the degradative part of the generalized animal cell endocytic pathway. For soybean MVBs, it appears likely that they fuse with the central vacuole and smaller vacuolar profiles which may or may not be connected to the central vacuole. The presence of labelled membranous masses within vacuoles after prolonged CF incubation is consistent with this interpretation. These masses are similar in appearance and size to the contents of some MVBs from the same protoplasts (Fig. 107-109). They resemble coalescing vesicles with individual vesicles either being less distinct in appearance or not being recognizable at all. The fact that the actual fusion event between MVBs and vacuoles was not recorded does not invalidate this hypothesis. Such
fusion events may be rare and may occur quite rapidly therefore making it difficult or impossible to record. Since MVBs of soybean protoplasts contain hydrolytic enzymes (see section 4.9) and the central vacuole and smaller vacuoles are considered to be components of the lytic compartment of plant cells (Matile 1975), it seems likely that these organelles are functionally analogous to the MVBs and lysosomes, respectively, of the generalized animal cell endocytic pathway.

The successive labelling of MVBs and the membranous masses within vacuoles with CF may have important implications. This observation, in combination with the short term and intermediate term CF uptake results (sections 3.3, 3.4, 4.4 and 4.5), provides convincing evidence for a pathway including an intermediate set of membrane bounded organelles which results in the delivery of substances from the extracellular environment to the major lysosomal component of plant cells, the vacuole. The importance of this pathway is unclear but likely involves the degradation of macromolecules.

4.12 Possible Alternative Fate for MVBs

It should be noted that in at least one specialized type of animal cell, the reticulocyte, MVBs fuse with the plasma membrane releasing their contents including their internal vesicles to the extracellular medium (Harding et al. 1983, 1984). The internal vesicles of these MVBs appear to contain
transferrin receptors and therefore the exocytosis of MVBs provides a mechanism for the developmental loss of the receptors from these cells (Harding et al. 1984). The possibility that some MVBs from soybean protoplasts may behave in a similar manner can not be ruled out. However, it would be very difficult to record such a fusion event using protoplasts. Fusion of MVBs with the plasma membrane might release some or all of the internal vesicles into the surrounding medium. Small vesicles similar to those of MVBs were observed on the plasma membrane but they could represent remnants of protoplasts broken during protoplast isolation and labelling steps (Fig. 111 and 113). The presence of plasma membrane invaginations containing small vesicles in intact soybean cells (Fig. 147) is consistent with the idea that MVBs could fuse with the cell surface. Plant cells might utilize such a mechanism to remove materials from the cytoplasm. Further work is required to evaluate this possibility. It is of interest to note that Halperin and Jensen (1967), Nougarède and Lescure (1970) and Dexheimer and co-workers (1975) proposed, on the basis of ultrastructural evidence, that MVBs originate from the cisternae of dictyosomes and fuse with the plasma membrane releasing their contents including the internal vesicles.

4.13 Labelling of the Dictyosomes and Membrane Recycling

The dictyosomes of soybean protoplasts also become
labelled with CF. The amount of labelling seemed quite variable from one experiment to another. CF particles within the dictyosome could be found distributed within several cisternae and had a general peripheral distribution. The occurrence of dictyosomal labelling is a highly significant result as it has important implications concerning the function of the Golgi apparatus of plant cells. The dictyosomes of plant cells have been shown to be involved in exocytotic events (Brown et al. 1970; Brown and Romanovicz 1975; Rougier 1976). Vesicles derived from the dictyosomes fuse with the plasma membrane thereby delivering their membrane constituents to the plasma membrane and releasing their lumenal contents (e.g. cell wall matrix polysaccharides—reviewed by Willison 1981) to the periplasmic space. In this manner, a significant amount of membrane is added to the plasma membrane. There have been a number of estimates of this rate of flow of membrane (reviewed in Robinson and Kristen 1982). For instance, in the alga Pleurochrysis, scale exocytosis results in the addition of approximately 1% of the plasma membrane surface area per minute to the plasma membrane (Brown et al. 1970). Other estimates include 3% per minute for the gland cells of Mimulus tilingii (Schnepf and Busch 1976) and 9.4% per minute in the ovary gland cells of Atenis cordifolia (Kristen and Lockhausen 1982). In none of these cases does the cell under study enlarge at a rate which would require
this rate of increase in plasma membrane surface area. Therefore, the extra membrane which is continually being added to the plasma membrane must somehow be recycled back into the cell (Robinson and Kristen 1982). One way in which membrane recycling could be achieved is by endocytosis. Plasma membrane derived vesicles could deliver their membrane and lumenal contents or at least a fraction of them to the dictyosomes either directly by fusing with the cisternae of the dictyosomes or indirectly by fusion with some organelle such as the PCR followed by subsequent membrane transfer to dictyosomes. The labelling of dictyosomes with CF is consistent with this hypothesis and therefore provides excellent evidence for the occurrence of direct or indirect membrane recycling, involving coated membranes from the plasma membrane to the dictyosomes of soybean protoplasts.

4.14 Dictyosomes and Molecular Repair

The Golgi apparatuses of both animal and plant cells have been shown to contain a number of glycosyltransferases (reviewed by Farquhar 1986; Robinson and Kristen 1982) which are involved in the post-translational modification of newly synthesized proteins (reviewed by Farquhar 1986; Chrispeels 1984). It is possible that these enzymes may also function to repair damaged oligosaccharide chains on recycling glycoproteins. For example, partial resialylation of
asialotransferrin type 3 occurred during diacytosis by rat hepatocytes (Regoeczi et al. 1982). In addition, it has been demonstrated in K 562 (animal) cells that cell surface transferrin receptors which have been asialyated by a neuraminidase treatment are resialyated following internalization (Snider and Rogers 1984). It is thought that these repair processes might be mediated by the Golgi apparatus (Regoeczi et al. 1982; Snider and Rogers 1984; Breitfeld et al. 1985). It is conceivable that the dictyosomes of the plant cell Golgi apparatus could function in a similar manner. Therefore, the labelling of the dictyosomes with CF may indicate the occurrence of a pathway for the reparation of damaged membrane constituents in addition to a possible membrane recycling pathway.

4.15 Membrane Recycling and Membrane Sorting

While direct membrane recycling from the plasma membrane to the dictyosomes is certainly possible, there are reasons for arguing that the plasma membrane derived vesicles would fuse with another organelle, likely the PCR, followed by the subsequent transfer of membrane to the dictyosomes. It seems likely that the occurrence of endocytosis in soybean protoplasts involves more than mere membrane recycling since MVBs and vacuoles, lytic compartments of the protoplasts, also become labelled. Thus a significant portion of the membrane material and/or lumenal contents internalized via endocytosis is destined to be degraded rather than to be
recycled. This point is important in view of results which indicate that the initial endocytotic events in the protoplasts appear to be mediated exclusively by CPs. It seems unlikely that distinct populations of CPs exist on the plasma membrane such that one population is involved in the recycling of plasma membrane to the dictyosomes while a second population of CPs is involved in the delivery of material to the lytic compartment. At least, this does not appear to be the case in animal cells where different types of receptors with different fates following internalization via endocytosis occur randomly at the cell surface and in the CPs and CVs, i.e. the different receptors could be found within the same CP or CV and the distribution of the various types of receptors were independent of one another (Geuze et al. 1984). Furthermore, the sorting of different receptor-ligand complexes from one another and the sorting of the components of dissociated receptor-ligand complexes occurs intracellularly probably within CURL or some analogous endosome (Geuze et al. 1984; Willingham et al. 1984). Therefore, it seems more reasonable that membrane constituents destined to be recycled are co-localized within the same CP with constituents destined to be degraded and that sorting of these constituents occurs sometime after internalization. Based on its similarity to CURL and trans reticular Golgi, the PCR would seem to be the most likely
candidate as the organelle where sorting events occur within soybean protoplasts. Constituents to be recycled could then be transferred from the PCR to dictyosomes either via PCR-derived vesicles or via transitory direct membrane connections between the two organelles. It is also conceivable that some of the recycling membrane constituents might move directly from the PCR to the plasma membrane via PCR-derived vesicles. Constituents to be degraded by the lytic compartment may be transferred to MVBs via PCR-derived vesicles or perhaps possibly by the direct maturation of PCR dilations into MVBs. The PCR-derived vesicles might initially start out as CVs which could form from the coated tips of the PCR tubules. These vesicles could then shed their coat.

4.16 Dictyosome Heterogeneity and Membrane Flow

The labelling patterns seen for dictyosomes in protoplasts post-fixed with ZIO were quite interesting. The variation in the distribution and amount of label was considerable between dictyosomes, even dictyosomes within the same protoplast. In some cases, a clear gradient or polarity of labelling intensity was seen across a dictyosome but this was not true in other cases. Although the exact basis for ZIO labelling is not known (e.g. Pellegrino de Iraldi 1977; Rodriguez and Giménez 1981 and references cited therein), these results suggest that dictyosomes are not of identical composition and/or physiological state. It is
conceivable that different dictyosomes within a protoplast may be performing different functions or may be performing the same functions but at different rates. This would help explain the observed variability in CF labelling. Those dictyosomes most actively involved in exocytosis/endocytosis would be more heavily labelled with CF while those dictyosomes less actively involved or uninvolved in these processes would be poorly labelled or unlabelled.

The ZIO labelling of dictyosomes was also interesting because evidence from both thick and thin sections suggested that direct membrane connections existed between the ER and dictyosomes. These results are consistent with other recent work (e.g. Juniper et al. 1982), including ZIO labelling results (e.g. Harris and Oparka 1983), and indicate that the transport of material, in particular protein that is newly synthesized on the ER, may occur via these connections as opposed to via transition vesicles. The demonstration of two types of ER, cisternal and tubular, and the labelling of the nuclear envelope are also consistent with other recent work making use of a similar ZIO post-fixation procedure (e.g. Harris and Oparka 1983).

4.17 Nature of Internalized Material

The preceding discussion has suggested that the endocytotic pathway of soybean protoplasts is involved in the recycling, degradation and perhaps reparation of
membrane constituents and lumenal contents of plasma membrane-derived endocytotic vesicles. Perhaps at this point it would be a good idea to briefly consider the possible nature of the internalized material. As indicated earlier in this discussion, CF is a non-specific marker for adsorptive endocytosis. Furthermore, it is not a substance with which plant cells would normally come in contact. Therefore, the fact that CF particles are internalized via endocytosis does not reveal anything about the nature of the substances which would normally be internalized via this process or of the biological significance of this pathway.

Membranes consist of a bimolecular leaflet composed of lipids and associated integral and peripheral proteins. The lipid composition of the plasma membrane is thought to effect the physical properties of the membrane such as fluidity. Therefore, the ability of a protoplast or cell to recycle or degrade the lipid components of the plasma membrane would enable it to regulate and/or alter the composition of the membrane perhaps in response to changes in its physiological function or to changes in its environment. The demonstrated functions of the protein component of membranes include the catalysis of chemical reactions (by enzymes) and the exchange or transport of ions across the membrane (Leonard and Hodges 1980; Leonard and Rayner 1986). The ability to recycle, degrade or repair these proteins via endocytosis would enable a protoplast or
cell to regulate processes such as cell wall synthesis in response to changes in the environment or could provide a means to produce developmental changes in the protoplast or cell. It is conceivable that some of the proteins in the plasma membrane are receptors for specific molecules or classes of molecules. For example, there is at least some evidence that soybean membranes may contain a specific receptor for mycolaminaran, the phytoalexin elicitor from Phytophthora, which elicits the production of the phytoalexin, glyceollin, in soybean tissues (Yoshikawa et al., 1983). Therefore, it is possible that receptor-mediated endocytosis could occur in plant protoplasts or cells. To demonstrate the occurrence of this process would require the use of a specific ligand in uptake experiments for which a well characterized receptor exists. (Currently these conditions can not be met in any higher plant cell system.)

4.18 Overview of the Endocytotic Pathway in Soybean Protoplasts

The various components of the endocytotic pathway in soybean protoplasts are shown in Fig. 148. The position of the components in this figure is based upon the preceding discussion of the CF uptake results. Therefore, while Fig. 148 depicts a representation of the endocytotic pathway which is compatible with the experimental results with soybean protoplasts, other interpretations of these results
Fig. 148. Diagrammatic representation of the endocytotic pathway in soybean protoplasts. Material (e.g. CF) is internalized by a CP on the plasma membrane which pinches off the plasma membrane to form a CV. The CV sheds its coat to become a SV which in turn fuses with a PCR. From the PCR, the internalized material and membrane may be recycled directly to the protoplast surface (broken lines) within a vesicle although experimental evidence for this pathway was not obtained. The vesicle may originate from a coated tip of a PCR tubule and may shed its coat before reaching the plasma membrane. Second, the material and membrane may be transferred from the PCR to the dictyosomes of the Golgi apparatus. Transport to the dictyosome may occur via a vesicle in a manner analogous to transport from the PCR to the plasma membrane or may occur via transitory direct membrane connections between the two organelles. From the dictyosome, the material and membrane may be returned to the protoplast surface within a vesicle derived from this organelle. Third, the material and membrane may be transferred from the PCR to a MVB. Transfer may be mediated by vesicles or may result by the transformation of a PCR dilation into a MVB. The MVB may then fuse with a vacuole where the material and membrane may be degraded. Note that this pathway is very similar to that for receptor-mediated endocytosis in animal cells, Fig. 1.
are possible and may be equally valid. In this pathway, CF binds to the surface of the plasma membrane including CPs. The CPs pinch off the plasma membrane via the process of invagination to produce CVs. The CVs rapidly shed their coat and become SVs. The SVs fuse with the PCR delivering CF to this organelle. From the PCR, CF particles face three possible fates. First, these particles may be delivered directly back to the plasma membrane via vesicles derived from the PCR. This would represent direct membrane recycling from the PCR to the plasma membrane. Experimental evidence was not obtained for this pathway. Second, these particles may be delivered to the dictyosomes either via vesicles derived from the PCR or through transitory direct membrane connections between the PCR and dictyosome cisterna(e). CF could be redistributed to additional cisternae via vesicles originating from the periphery of the cisternae. This part of the pathway would result in the replenishment of the dictyosomal cisternae via the recycling of plasma membrane constituents and would complete a cycle of membrane flow originating with the dictyosomes. In theory at least, this part of the pathway could also result in the reparation of damaged plasma membrane constituents, particularly glycoproteins, prior to their return to the plasma membrane. From the dictyosome, membrane constituents may be recycled to the plasma membrane via exocytotic vesicles. Third, some CF particles could be delivered from the PCR to MVBs. This
transfer could be mediated by vesicles derived from the PCR or could result from the maturation of the PCR into a MVB. This would represent the degradative part of the pathway in which some plasma membrane constituents and the lumenal contents of plasma membrane-derived vesicles are delivered to the lytic compartment of the protoplast. In the MVB some initial degradation of material may occur, however, ultimately the contents of the MVBs are delivered to the vacuoles as a result of MVB-tonoplast membrane fusion events. In the vacuoles, further degradation takes place completing the degradative part of the endocytotic pathway.

4.19 Recent Demonstrations of Endocytosis in Protoplasts

Recently, Joachim and Robinson (1984) have demonstrated the occurrence of endocytosis in protoplasts from Vicia faba leaf cells. Their results provide at least partial confirmation of the endocytotic pathway in soybean protoplasts. In Vicia protoplasts, internalization of CF occurred via CPs and CVs although Joachim and Robinson did not rule out the possible involvement of uncoated pits. Smooth or uncoated vesicles also became labelled as did "peripheral tubules" (see below) on the trans side of the dictyosome. Finally, small vacuoles became labelled. Some micrographs suggested that these vacuoles might be formed directly from the plasma membrane. It is not clear whether these vacuoles are equivalent to the small vacuolar profiles which are labelled in soybean protoplasts. Joachim and
Robinson described structures which in their micrographs appear to be PCR. They refer to these structures as "peripheral tubules" of dictyosomes. The "peripheral tubules" of the dictyosome which were labelled with CF were not the PCR type tubules but rather appeared to be the dilated or vesiculated tip of a dictyosomal cisterna, at least in the example illustrated in their paper. Because Joachim and Robinson do not make a distinction between these two types of tubules, it is unclear whether the PCR type tubules are labelled in their protoplast preparations. Another difference between the results of Joachim and Robinson and the results reported here for soybean protoplasts is that they make no reference to labelling of MVBs or the central vacuole. Overall, the results of Joachim and Robinson confirm the occurrence of endocytosis in higher plant (angiosperm) protoplasts and the involvement of CPs and CVs in this process. However, some of the organelles involved in endocytosis in soybean protoplasts may not be involved in the same process in Vicia protoplasts.

More recently, Hillmer and co-workers (1986) have demonstrated the occurrence of endocytosis in bean (Vicia faba L.) leaf protoplasts and protoplasts from carrot suspension culture cells using a number of lectin-colloidal gold conjugates. Structures labelled during endocytosis included CPs, CVs, the Golgi apparatus (dictyosomes), MVBs, small vacuoles and the PCR. Interestingly, label was never
observed in the central vacuole. Label was seen only once in the ER of a bean leaf protoplast and never in carrot protoplasts. These results are similar to those reported here for soybean protoplasts, the major difference being the lack of labelling in the central vacuoles. This difference may be a result of the incubation times used by Hillmer and co-workers. The maximum incubation time used by this group was 1h. While an incubation of this length is sufficient for the detection of central vacuole labelling in soybean protoplasts, it is possible that longer incubations might be needed in other plant systems. It is also interesting to note, in particular, that for bean leaf protoplasts MVBs are labelled by lectins (Hillmer et al. 1986) but apparently not by CF (Joachim and Robinson 1984). These results suggest that either different relatively non-specific markers have different intracellular fates or that the CF uptake results were incomplete.

4.20 Coated Membrane Structures and Endocytosis in Plant Cells

Now that it is apparent that coated membrane organelles are involved in the process of endocytosis in protoplasts, the question arises as to whether these organelles are involved in the same process in normal turgid cells? This question is not as trivial as it may seem. Evidence for the occurrence of endocytosis in turgid cells is as scarce as
for protoplasts. In fact, it has been argued on theoretical grounds that endocytosis (pinocytosis) in turgid plant cells could not be a major uptake route of small-molecular-weight solutes (Cram 1980). It was thought that the energy required in vesicle formation to overcome turgor would be too excessive for endocytosis to be the mechanism for large scale uptake of material. Furthermore, results from studies on protoplasts may not be directly applicable to normal turgid plant cells. The physiological state of protoplasts and cells tends to be very different. In fact, at least for some characteristics, protoplasts appear to mimic the response of stressed plant cells (e.g. Mieth et al. 1986; Rao et al. 1984; Cress 1982; Fleck et al. 1982). Therefore, it is possible that endocytosis may not occur in normal turgid plant cells, or may occur only in certain specialized cell types or in cells that have been exposed to some sort of physiologically stressful situation or environment. With the previous statement in mind, the best evidence for the occurrence of endocytosis comes from studies of plant pathogens (e.g. Newcomb 1976) and plant-microbe symbioses. In these instances, microorganisms are internalized via plasma membrane-derived vesicles. This process does not appear to involve coated membrane organelles. The use of heavy metal salt solutions on various plant tissues also provided some evidence for the occurrence of endocytosis (e.g. Wheeler and Hanchey 1971; Wheeler et al. 1972; Robards
and Robb 1972; Robards and Robb 1974; Peterson et al. 1986). However, until very recently (see below), this process appeared to be mediated by smooth vesicles derived from the plasma membrane as opposed to CPs and CVs. Since heavy metals tend to be toxic to plant tissues, it is also unclear whether the endocytotic events revealed by exposure to these elements occur under normal conditions or whether the heavy metals stimulate the occurrence of these events (e.g. Wheeler and Hanchey 1971).

Very recently, Hübner and co-workers (1985) have provided evidence for the occurrence of endocytosis in maize (Zea mays) root cap cells using incubations with lanthanum and lead salt solutions. Lanthanum deposits were found in CPs, CVs, SVs and large vesicles present at the trans-pole of dictyosomes. Lead deposits were observed in CPs, smooth pits, CVs, SVs, large trans-pole dictyosome vesicles, the cisternal stack of the dictyosomes, coated portions of the PCR, small vacuoles and occasionally in the lumen of the ER. These results were interpreted to be evidence for coated pit/coated vesicle-mediated endocytosis and for the direct recycling of plasma membrane to the Golgi apparatus. Although it was conceivable that the presence of high concentrations of heavy metal salts might induce or stimulate endocytosis, it was thought that the presence of CPs on the plasma membrane and on the Golgi apparatus as
well as the presence of coated vesicles in control cells suggested that it was reasonable to assume that endocytosis could occur in undisturbed in vivo conditions.

4.2.1 Lanthanum and CF Uptake by SB-1 Cells

In this project, whole SB-1 soybean cells were incubated in solutions containing lanthanum chloride or CF particles in the hope of demonstrating the occurrence of endocytosis in cells. Unambiguous evidence of endocytosis was not obtained in these preliminary experiments. Lanthanum deposits and CF particles were seen on and throughout the cell wall, on the plasma membrane and within the cytoplasm. Lanthanum deposits and CF particles within the cytoplasm appeared to be located primarily in elements of the ER or within vesicular profiles which likely were cross-sections through the ER. Particles of CF were also found free within the cytoplasm. In some instances, these particles may have been within vesicles that were difficult to see against the electron-dense background of the ground substance. It was not apparent how the ER became labelled with these markers. It is possible that plasma membrane-derived vesicles may have carried these markers to the ER and that these vesicles were difficult to detect. If this was the case then the endocytotic pathway in SB-1 cells is quite different from the pathway in SB-1 protoplasts. Profiles in SB-1 protoplasts which were readily identifiable as elements of the ER were never labelled with CF. Alternatively, the ER in
intact SB-1 cells may be connected to the plasma membrane (see Fig. 143 for evidence). If this is the case then lanthanum ions and CF particles which had diffused through the cell wall may enter the lumen of the ER through the connections between the plasma membrane and the ER. In the case of lanthanum ions, these ions would be precipitated out wherever the ionic environment of the ER would favour this process. It is conceivable that the proposed plasma membrane-ER connections might be broken or sealed off as a result of the stress incurred during protoplast formation thereby explaining why ER labelling was observed in SB-1 cells and not in protoplasts from SB-1 cells.

The inability to detect CF particles in other cytoplasmic organelles may be due to the low level at which CF passed through the cell wall. Since very little CF was found bound to the plasma membrane (relative to the amount observed for protoplasts), it would be logical to expect that the amount of internalized CF would also be reduced by an equivalent degree. Therefore, it is conceivable that only a very few organelles would be labelled and that these organelles would only contain one or a few particles of CF. It would be quite easy to miss these organelles when scanning unstained thin sections of cells in the electron microscope.

With respect to lanthanum uptake, it is important to
note that one can only directly visualize, in the electron microscope, lanthanum which has precipitated out to form an electron-dense deposit. (It is a good practise, if one's electron microscope has the capability, to verify that these deposits contain lanthanum by using X-ray microanalysis.) Therefore, it is conceivable that other locations in the cell contain lanthanum which has not been precipitated. Furthermore, it is possible that some precipitates which have formed during the lanthanum chloride incubation could have been redissolved during the washes and other processing steps which followed the incubation. In fact, some authors (Revel and Karnovsky 1967) have recommended that lanthanum be included in every step of the processing sequence. This policy was not adopted here as it would not have been possible to argue convincingly that the location of any of the lanthanum deposits was the result of endocytosis.

In view of the above discussion, it is important to emphasize at this point that the lanthanum and CF uptake results are of a very preliminary nature and therefore do not eliminate the possibility that endocytosis can occur in whole SB-1 soybean cells. A wide range of parameters need to be explored before one can confidently rule out the occurrence of endocytosis in these cells. In fact, in view of Hübner's and co-workers's (1985) results it seems more likely that the occurrence of endocytosis will eventually be demonstrated.
The mere fact that CF (M.W. approx. 750,000) was able to pass through the cell wall of SB-1 cells is in itself very interesting. The cell wall of higher plant cells has generally been considered to be a significant barrier to high molecular weight compounds as these walls contain very small pores. The size of pores in the cell wall have been estimated by Carpita and co-workers (1979) to be between 3.7 and 5.2 nm in diameter. It is generally thought that the penetration through the cell wall of globular proteins with molecular weights greater than 17,000 to 60,000 is greatly impeded (Carpita et al. 1979; Tepfer and Taylor 1981). The low level at which CF particles pass through the cell wall of SB-1 cells bears testimony to this belief and yet at the same time demonstrates that given enough time these compounds do make it through the cell wall. The observation that NF particles do not appear to penetrate the cell wall during an 1 h incubation is also very interesting. In view of the CF results, it seems likely that the explanation for this lack of penetration by NF is related to net molecular charge. The cell walls of higher plant cells contain a large number of residues of carboxylated derivatives of monosaccharides. The negative charges on these residues may serve to repel the negatively charged NF particles so that these particles do not penetrate the cell wall under the conditions in which the experiment was performed. In
contrast, CF particles with their net positive charge would be electrostatically attracted to the acidic residues in the cell wall and could then pass through the cell wall as a result of mass action.

4.22 Other Functions for CVs

It is important to note that the results of this project and those of Robinson's laboratory (Joachim and Robinson 1984; Hübner et al. 1985; Hillmer et al. 1986) which demonstrate the involvement of CVs in endocytosis do not rule out the possibility that CVs may be involved in other membrane transport processes. It is possible as suggested by the work of others (e.g. Bonnett 1969; Robertson and Lyttledon 1982; Nakamura and Miki-Hiroshige 1982) that CVs may play some role in exocytotic processes such as the transport of cell-wall precursors or enzymes to the cell surface. It is conceivable that subpopulations of CVs which are functionally and perhaps morphologically different could exist within plant cells. With respect to this possibility, it should be noted that it has been reported that Golgi-associated coated membranes seemed to be of smaller diameter than those associated with the plasma membrane of protoplasts from cultured tobacco cells (Van der Valk and Powke, 1981).

4.23 Future Prospects

Further progress in demonstrating the occurrence of endocytosis in higher plant protoplasts and cells will
depend on the development of better membrane markers which are not toxic to the plant material and which can be used under normal culture or growth conditions. Ideally, these markers would bind to a specific molecule on the plasma membrane such as a receptor and would be small enough to pass unimpeded through the cell wall. It would also be beneficial if the marker was electron-dense so that it could be easily visualized in the electron microscope. However, this last property is not essential as one could employ autoradiographic or immunocytochemical protocols to determine the distribution of the marker in cells and protoplasts.

Further characterization of the organelles in the endocytotic pathway of soybean protoplasts would also be useful. At the ultrastructural level, a comparison of soybean protoplasts which have been freeze substituted with those which have undergone chemical (e.g. glutaraldehyde) fixation would be useful in determining whether or not the dilations and their internal vesicles are "real" features of the PCR. Furthermore, the use of cytochemical techniques, such as enzyme and 3-(2,4-dinitroanilino)-3'-amino-N-methylidipropyamine (DAMP) cytochemistry, could provide interesting information concerning the composition and the contents of the organelles involved in endocytosis in soybean protoplasts. For example, DAMP cytochemistry, used
for the identification of acidic organelles in animal cells (Anderson et al. 1984), could possibly be used to identify acidic organelles in soybean protoplasts. Such results could provide important clues to the function(s) of organelles in this endocytotic pathway as the dissociation of some endocytosed receptor-ligand complexes is thought to be mediated by acidic pHs and is thought to occur in the acidic organelles of animal cells.

Biochemical techniques could also be used to further characterize the organelles of the endocytotic pathway in soybean protoplasts. As has been done for animal cell systems (Hornick et al. 1985), cell fractionation protocols could be used to isolate fractions enriched for specific organelles such as MVBs. Furthermore, density shift protocols could possibly be used to separate organelles active in endocytosis from morphologically similar but endocytotically inactive organelles (Courtoy et al. 1984; Quintart et al. 1984). Enzyme and other assays on these cell fractions could be used to support results concerning the composition of various organelles obtained using cytochemical procedures (see above).

4.2.4 Summary

In summary, using CF, it was possible to demonstrate the occurrence of endocytosis in protoplasts from SB-1 soybean suspension culture cells. The endocytotic pathway included CPs, CVs, SVs, the PCR, dictyosomes, MVBs, small vacuoles
and the central vacuole. This is the first demonstration of the involvement of the PCR in endocytosis. The PCR appears to be a distinct organelle although in many cases it is associated with and sometimes possibly physically connected to the ER and the dictyosomes of the Golgi apparatus. The ultimate fate of some of the internalized CF label appears to be delivery, via MVBs, to the major lytic or lysosomal compartment of the plant cell, namely, the vacuole. It is proposed that the endocytotic pathway is involved in the recycling, degradation and possibly the repair of membrane constituents and lumenal contents of plasma membrane-derived vesicles. Unambiguous demonstration of endocytosis in whole SB-1 cells was not achieved using lanthanum chloride, NF or CF as markers of endocytosis. However, recent developments in this field of research (Hübner et al. 1985) suggest that an endocytotic pathway similar to the one described here for soybean protoplasts may function in at least some types of higher plant cells.
Literature Cited


