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Isolation of plasma membrane from protoplasts of *Lolium multiflorum* (ryegrass) endosperm cells

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Plasma membranes have been isolated from protoplasts of suspension-cultured ryegrass (*Lolium multiflorum*) endosperm cells. The protoplast membrane is coated before cell disruption with murine myeloma protein J539, a galactose-binding immunoglobulin A. The plasma membrane is labelled with ^{125}I by using chemically or enzymically catalysed iodination techniques, or, more conveniently, by using ^{125}I-labelled myeloma protein J539, which enables the membrane to be simultaneously coated and labelled. Protoplast lysis is effected by gentle mechanical means after swelling in hypo-osmotic medium. The plasma-membrane fraction is recovered at low centrifugal forces by fractionation of cell lysates on a discontinuous sucrose/sorbitol gradient. The plasma-membrane fraction is enriched 96-fold on a protein basis with respect to the specific radioactivity of ^{125}I-labelled myeloma protein J539 in the homogenate. Electron microscopy showed long membrane profiles often associated with one another.

In order to study the location of polysaccharide synthases involved in plant cell-wall formation (Fincher & Stone, 1981), components of the endomembrane system must be fractionated, and we report here a method for the isolation of plasma membranes from ryegrass (*Lolium multiflorum*) endosperm cells.

The preparation of plant plasma membranes for biochemical studies is not straightforward. They comprise only a very small proportion of the total cellular membranes (Quail, 1979) and the severity of the treatments required to disrupt cell walls results in extensive vesiculation of the plasma membrane and other cell membranes, and damage to subcellular organelles. Isolation of the plasma membranes from the resulting mixtures is difficult and is made more so by the lack of universal or valid markers for their identification (Quail, 1979).

We have circumvented the problems imposed by the need to disrupt the cell wall by using, as starting material, protoplasts, which can be disrupted by gentle methods. To facilitate separation of plasma membrane from other cellular membranes we have adopted the strategy used by Scarborough (1975) in the isolation of fungal plasma membranes. The method involves attaching a carbohydrate-binding protein to the cell surface before disruption. The protein-coated protoplasts are lysed to form large, sheet-like structures that can be readily separated from membrane vesicles of intracellular origin at low centrifugal forces.

We have avoided basing the identification of plasma membrane on putative chemical or enzyme markers by using specific labelling methods, involving radioactive probes (Galbraith & Northcote, 1977; Perlin & Spanswick, 1980), or a labelled carbohydrate-binding protein. A preliminary report of this work has been presented (Schibeci *et al.*, 1981).

Materials and methods

Chemicals and radiochemicals

Immobilized lactoperoxidase and glucose oxidase (Enzymobeads), Bio-Gel P-2 and Bio-Gel A5m were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.) Evan's Blue, DEAE-dextran (mol.wt. approx. 500000), poly-DL-lysine (mol.wt. 30000–70000), bovine serum albumin, fluorescein diacetate, Neutral Red, concanavalin A and fluorescein isothiocyanate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Sephadex G-25 and Blue Dextran were from Pharmacia Fine Chemicals (Uppsala, Sweden), deoxyribonuclease was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), Calcofluor White ST was from American Cyanamid Co. (Bound Brook, NJ, U.S.A.), Driselase was from Kyowa Hakka Kogyo Co. (Tokyo, Japan) and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen) was from Pierce Chemical Co. (Rockford, IL, U.S.A.)

The radiolabelled sodium [^{125}I]iodide (17 Ci/mg) was obtained from New England Nuclear Corp.

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(Boston, MA, U.S.A.) and was stored in 0.1M-NaOH at a concentration of 10–20mCi/ml.

Axinellin was kindly provided by Dr. H. Bretting (Universität Hamburg, Hamburg, Germany) and tridacnin was generously donated by Dr. A. E. Clarke (University of Melbourne, Australia).

Ryegrass endosperm cell cultures

Liquid-suspension cultures of ryegrass endosperm cells were maintained in modified White's medium containing 4% (w/v) sucrose as carbon source (Smith & Stone, 1973).

Protoplast isolation

Protoplasts were prepared by incubating endosperm cells for 16h with a commercial cellulase preparation (Driselase) as described by Keller & Stone (1978). Protoplasts were typically 90–95% viable when tested with fluorescein diacetate (Heslop-Harrison & Heslop-Harrison, 1970), and no residual cell-wall material could be detected after staining with Calcofluor White ST (Nagata & Takebe, 1970).

Preparation of murine myeloma protein J539

Mouse myeloma protein J539 was purified from ascites fluid by the method of Eichmann *et al.* (1976), on Sepharose 2B pre-treated with 1M-propionic acid. Tumour-bearing mice were generously donated by Dr. M. Potter (National Institutes of Health, Bethesda, MD, U.S.A.). The protein was coupled to fluorescein isothiocyanate as described by Goldman (1968).

Agglutination of protoplasts

Protoplasts (approx. 4×10^4) in 20μl of 50mM-Tris/HCl buffer, pH7.5, containing 10% (w/v) sorbitol and 0.5mM-$CaCl_2$ (sorbitol/calcium buffer, pH7.5) were incubated with various proteins (0.2–1.0mg/ml in the same buffer) for 40min at 25°C with gentle shaking in a total volume of 100μl. Cell suspensions were examined by bright-field microscopy and the percentage agglutination calculated by comparing the number of free protoplasts 40min after addition of protein with the number of free protoplasts in a suspension containing no added protein.

Surface-labelling of protoplasts

(*a*) *Sodium [^{125}I]iodide.* (i) Chemical catalysis. Small glass vials were coated with 0.1mg of Iodogen/ml in methylene chloride (Fraker & Speck, 1978). Solvent was removed by evaporation and the coated reaction vessels stored over silica gel. Washed protoplasts [$(1–2) \times 10^7$] in 1.0ml of sorbitol/calcium buffer, pH7.5, were incubated in coated reaction vessels at 25°C with $Na^{125}I$ (50–500μCi) for 10min. Iodination was terminated by removing the suspension from the reaction vessel. Bound ^{125}I was determined by precipitating the protoplast suspension with 5% (w/v) trichloracetic acid containing 1mM-KI, washing the precipitate twice with the same solution and three times with 5% trichloracetic acid at 4°C. The ^{125}I in the precipitate was counted for radioactivity directly in a gamma counter.

Protoplasts labelled with ^{125}I were washed once with sorbitol/calcium buffer, pH7.5, containing 1mM-KI, and four times with sorbitol/calcium buffer, pH7.5, before disruption.

(ii) Enzymic catalysis. Washed protoplasts [$(1–2) \times 10^7$/ml] in sorbitol/calcium buffer, pH7.5, were incubated at 25°C with 0.2% (w/v) glucose, Enzymobeads (0.2mg/ml) and 50–500μCi of $Na^{125}I$ in a total volume of 1.0ml for 30min. Bound ^{125}I was measured after labelled protoplasts were precipitated and washed with trichloroacetic acid/KI solution as described above.

(*b*) *^{125}I-labelled myeloma protein J539.* Myeloma protein (5mg) in 1.0ml of 10mM-Tris/HCl, pH7.0, and 50μCi of $Na^{125}I$ were incubated in an Iodogen-coated reaction vessel for 10min at 25°C. The reaction was stopped by removing the solution, and unbound $Na^{125}I$ was separated by gel-filtration chromatography on a Bio-Gel P-2 column (bed dimensions 1.2cm × 6cm). The ^{125}I-labelled myeloma protein J539 (50×10^6d.p.m./mg) was used immediately or stored at −15°C. Protoplasts [$(1–2) \times 10^7$] in 1.0ml of sorbitol/calcium buffer, pH7.5, were incubated at 25°C for 20min with ^{125}I-labelled myeloma protein J539 (17.6×10^6d.p.m., specific radioactivity 31.4×10^6d.p.m./mg). The suspension was diluted 5-fold with buffer, and labelled protoplasts were collected by gentle centrifugation (60*g*, 90s).

Disruption of protoplasts

(*a*) *Mechanical disruption.* Protoplasts (10^7/ml), coated with myeloma protein J539, were suspended in 10mM-Tris/HCl buffer, pH7.5, containing 5mM-$MgSO_4$ and deoxyribonuclease I (1mg/ml), homogenized in a glass/Teflon tissue homogenizer (10–20 passes at a clearance of approx. 0.15mm) and the homogenate incubated at 4°C for 10min (Scarborough, 1975).

(*b*) *Polycation-induced lysis.* Protoplasts (10^7/ml) in 10mM-Tris/Mes (4-morpholine-ethanesulphonic acid) buffer, pH6.0 (containing 10% sorbitol and 0.5mM-$CaCl_2$), were incubated with DEAE-dextran (1mg/ml) or poly-DL-lysine for 1–2min at 4°C, followed by 15–20min at 30°C (Dürr *et al.*, 1975).

(*c*) *Osmotic lysis.* Protoplasts (10^7/ml) were allowed to swell in 10–20vol. of 50mM-potassium phosphate buffer, pH8.0 ('hypo-osmotic' buffer) containing 5mM-$MgSO_4$ and 0.2–1.0mg of deoxyribonuclease I/ml (Wagner & Siegelman, 1975).

After 5–10 min at 4°C, the suspension was blended for 2 min at low speed in a Sorvall Omnimixer (Sorvall, Newtown, CT, U.S.A.).

(d) *Assessment of lysis.* Lysis was assessed by optical microscopy and with Evan's Blue-exclusion test (Taylor & West, 1980) as an indicator of the degree of cell disruption. Intact vacuoles were stained with Neutral Red.

Optical and fluorescence microscopy

Specimens were examined with a Zeiss microscope equipped with epifluorescence optics (Carl Zeiss, Oberkochen, Germany). Illumination was provided by an HBO 50 W high-pressure mercury arc lamp. The filter combination for examining specimens stained with Calcofluor White ST was UG5 excitation/LP 418 barrier/Rf 420 reflector filters, and for fluorescein isothiocyanate, UG1-BG12 excitation/LP 520 barrier/Rf 460 reflector. For comparison, protoplasts were examined by using Nomarski optics. Specimens were photographed with Kodak Tri-X pan or Ektachrome film (ASA 400).

Electron microscopy

Membrane fractions were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4 (containing 0.5 mM-$CaCl_2$), for 2–4 h at 4°C and post-fixed with 1% OsO_4 for 2 h at 4°C. For fixation of protoplasts, solutions were supplemented with 0.5 mM-$CaCl_2$ and with sorbitol to a final osmolarity of 0.54. Protoplasts were also fixed and embedded in agarose by the method of Seed (1980). The specimens were dehydrated through a graded series of ethanol concentrations and propylene oxide and embedded in Spurr's resin. Thin sections were stained either with phosphotungstic acid/chromic acid (Roland *et al.*, 1972), or uranyl acetate and lead citrate, and examined in a Siemens 1 A transmission electron microscope.

Gel-filtration chromatography

Sephadex G-25 (1.2 cm × 6 cm) and Bio-Gel A5m (2.2 cm × 6 cm) columns were eluted with 50 mM-potassium phosphate buffer, pH 8.0 (containing 5 mM-$MgSO_4$), and fractions (0.5 and 1.0 ml respectively) were collected. Columns were calibrated with Blue Dextran (void volume, V_0) and tyrosine (bed volume, V_T).

Protein determination

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Selection of plasma-membrane-binding protein

In attempts to find a surface-binding protein suitable for stabilizing the ryegrass plasma membranes, the ability of a number of carbohydrate-binding proteins to agglutinate ryegrass endosperm protoplasts was compared (Table 1). Concanavalin A did not agglutinate the protoplasts, whereas axinellin, a 1,6-β-D-galatose-binding protein from the marine sponge *Axinella polypoides* (Bretting & Kabat, 1976) and the mouse myeloma protein J539, which is also specific for 1,6-β-D-galactose residues (Jolley *et al.*, 1974), agglutinate approx. 80% of the protoplasts under the conditions used. Tridacnin, an agglutinin from the giant clam *Tridacna maxima* that binds *N*-acetyl-D-galactosamine residues and, to a lesser extent, D-galactose residues (Baldo *et al.*, 1978), did not agglutinate the protoplasts.

Myeloma protein J539 can be readily purified (Eichmann *et al.*, 1976) and was used routinely in the isolation of plasma membranes. Agglutination by myeloma protein J539 occurred between pH 5.5 and 8.0, and was optimal at pH 6.0–7.5. Agglutination of ryegrass protoplasts with fluorescein-labelled myeloma protein J539 was examined by Nomarski interference and epifluorescence microscopy (Plate 1).

In some regions (Plate 1*d*) the fluorescence appears to be concentrated at the interface of agglutinated cells, whereas on other cells it is distributed more evenly. Agglutination was much reduced in the presence of 0.5 M-lactose (Plates 1*e* and 1*f*).

Protoplast disruption

Several procedures for disrupting protoplasts were investigated with a view to maximizing cell breakage, but minimizing damage to subcellular organelles (Table 2). The latter was assessed by measuring the

Table 1. *Agglutination of ryegrass protoplasts by carbohydrate-binding proteins*

Protoplasts in sorbitol/calcium buffer, pH 7.5, were incubated with the proteins for 40 min at 25°C with gentle shaking. The number of free protoplasts remaining in the presence of protein, compared with the number observed with no added protein, was used to calculate the percentage agglutination.

Protein	Concentration range (mg/ml)	Agglutination (%)
Control (no addition)	—	5
Protoplast lysate	0.5–1.0	5
Bovine serum albumin	0.5–1.0	5
Concanavalin A	0.5–1.0	5
Tridacnin	0.5–1.0	5–10
Axinellin	0.2–0.5	80–90
Mouse myeloma protein J539	0.2–0.5	70–80

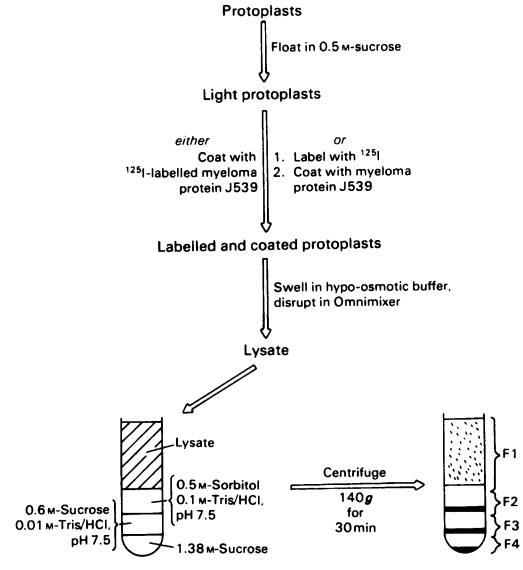
Table 2. *Comparison of methods for disruption of protoplasts from ryegrass endosperm*
Mechanical disruption was effected in a glass/Teflon homogenizer, by polycation-induced lysis with DEAE-dextran or poly-DL-lysine, and by osmotic breakage by gently blending protoplasts previously swollen in hypo-osmotic buffer at pH 8.0.

	Mechanical	Polycation-induced	Hypo-osmotic–Omnimixer
Protoplasts lysed (%)	>95	60–75	85–95
Vacuoles intact (%)	<10	30–40	20–40

percentage of intact vacuoles released by the disruption. Mechanical disruption in a glass/Teflon homogenizer resulted in breakage of most protoplasts, but more than 90% of vacuoles were also disrupted. Polycation-induced lysis (Dürr *et al.*, 1975) led to 60–75% protoplast breakage, with a relatively high percentage of the vacuoles released intact. The optimum concentration for protoplast lysis by either DEAE-dextran or poly-DL-lysine was 0.2 μg/protoplast, a concentration much higher than that used to disrupt yeast spheroplasts (Dürr *et al.*, 1975). Complete lysis could not be induced by increasing the polycation concentration. Buser & Matile (1977) observed a similar effect with protoplasts from *Bryophyllum* leaves. A modification of the hypo-osmotic-lysis procedure of Wagner & Siegelman (1975) resulted in disruption of a high proportion of the protoplasts, whereas vacuole preservation compared favourably with polycation-induced lysis (Table 2). This method had the added advantage that the extent of breakage could be relatively easily controlled by varying the intensity of the Omnimixer treatment (see the Materials and methods section). Hypo-osmotic swelling/mechanical disruption was therefore used for lysing protoplasts during plasma-membrane isolation.

^{125}I-labelling of protoplasts

When ryegrass protoplasts were labelled with $Na^{125}I$, up to 70% of the radioactivity was incorporated into the protoplasts after 30 min in the presence of a chemical catalyst (Iodogen), but less than 40% of the total radioactivity was incorporated in the same time when enzymic catalysis (Enzymobeads) was used. The chemical catalyst was used in subsequent radioiodination experiments.



Scheme 1. *Procedure for plasma-membrane isolation*

Plasma-membrane isolation

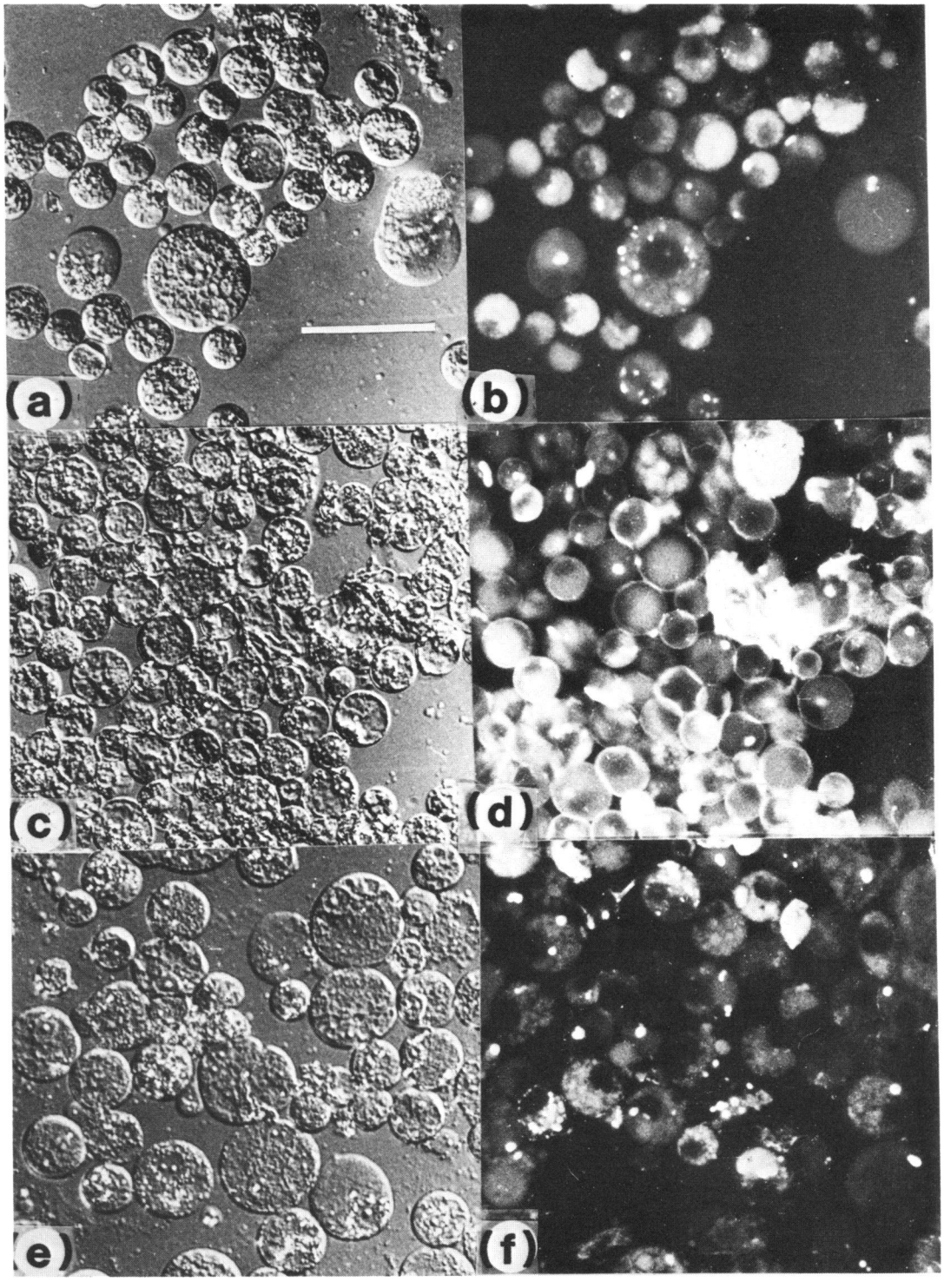
The method adopted for isolation of plasma membrane is summarized in Scheme 1. In order to avoid contamination of the plasma-membrane preparation with intact protoplasts that survive the disruption process, protoplasts were fractionated by centrifugation for 10 min at 400 *g* in 0.5 M-sucrose, and the floating ('light') protoplasts collected. Protoplasts sedimenting under these conditions were discarded.

The 'light'-protoplast fraction was labelled with

EXPLANATION OF PLATE 1

Binding of fluorescein-labelled murine myeloma protein J539 to protoplasts derived from suspension-cultured ryegrass (Lolium multiflorum) endosperm

All photomicrographs are of the same magnification. The bar on (a) corresponds to 100 μm. (a) Isolated protoplasts photographed by using Nomarski interference illumination. (b) Same field showing autofluorescence of the protoplasts. (c) Protoplasts agglutinated with fluorescein-labelled myeloma protein J539, photographed by using Nomarski interference illumination. (d) Same field as shown in (c), but photographed by using epifluorescence illumination. (e) Protoplasts incubated with fluorescein-labelled myeloma protein J539 in the presence of lactose, photographed by using Nomarski interference illumination. (f) A similar field to that shown in (e), but photographed by using epifluorescence illumination.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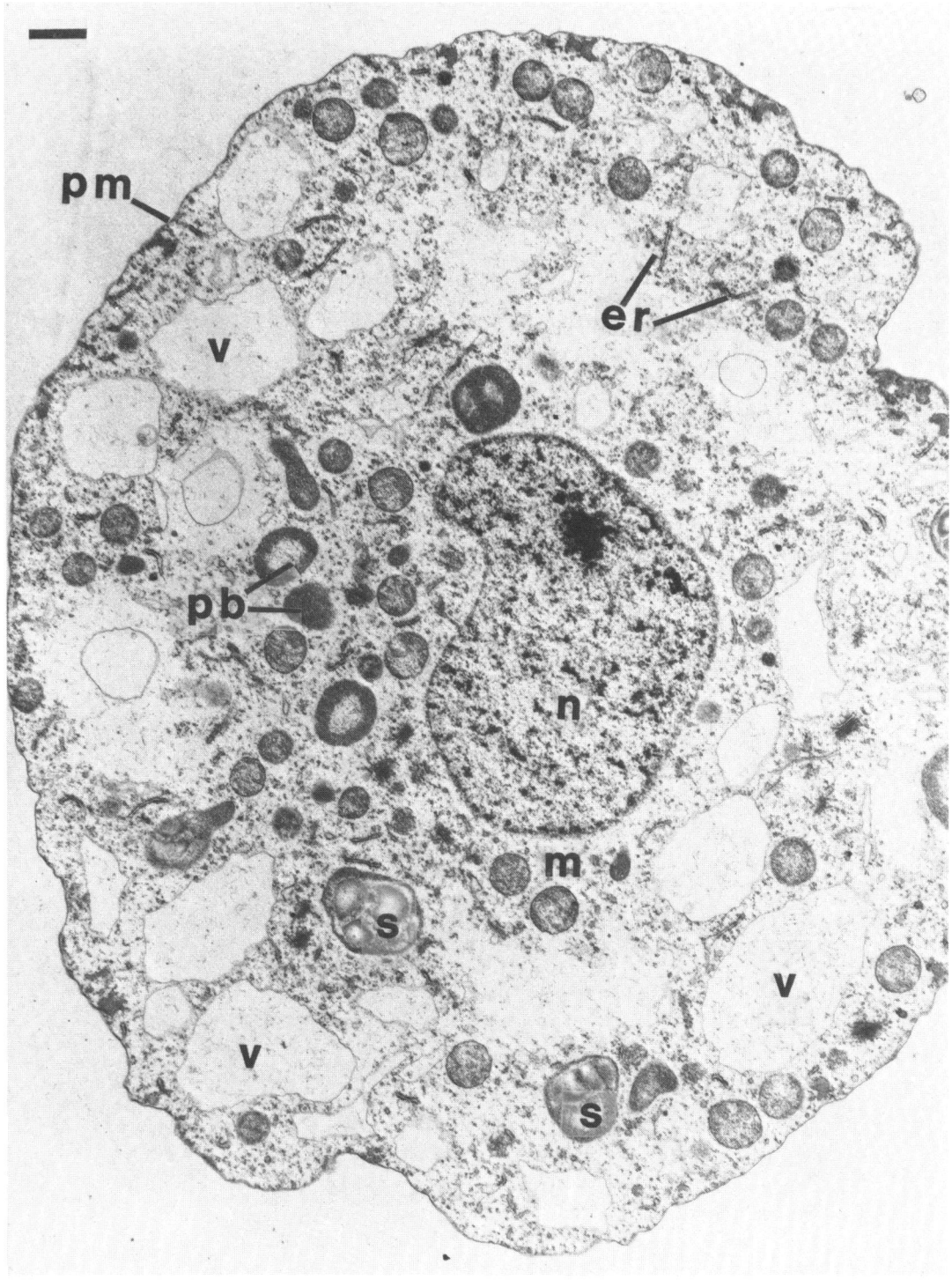
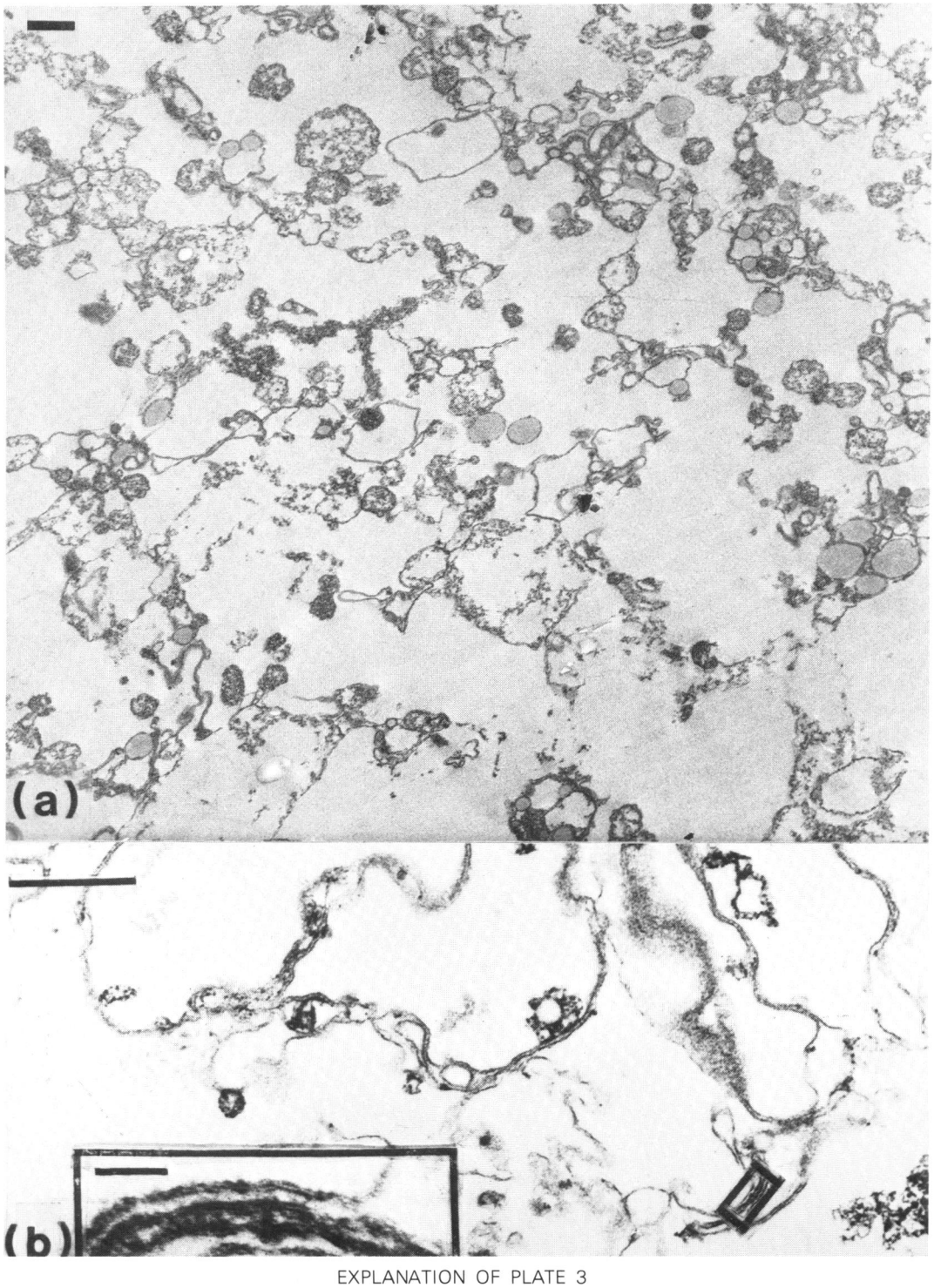


EXPLANATION OF PLATE 2

Electron micrograph of a ryegrass endosperm protoplast

Protoplasts were stained with phosphotungstic acid/chromic acid. The plasma membrane (pm) is intact and the nucleus (n), vacuoles (v), mitochondria (m), starch granules (s), endoplasmic reticulum (er) and protein bodies (pb) are visible. The bar represents 1 μm.



EXPLANATION OF PLATE 3

Electron micrographs of plasma-membrane fraction F3

Sections of washed fraction F3, embedded in Spurr's medium, were stained with uranyl acetate and lead citrate. (a) Low magnification (the bar represents 1 μm); (b) high magnification (the bar represents 1 μm). The inset depicts an enlargement of the enclosed area showing associated plasma membranes, each with a trilamellar structure (the bar represents 0.1 μm).

$Na^{125}I$ by using catalysts that are excluded from the cytoplasm, and the plasma membrane stabilized by coating with myeloma protein J539 (0.2mg/ml, 1 μg/10^4 protoplasts). Labelled protoplasts were at least 95% viable when assessed by the fluorescein diacetate method. Labelled and coated protoplasts were allowed to swell in hypo-osmotic buffer and subsequently lysed by gentle mechanical treatment. The lysate was loaded on to a discontinuous sucrose/sorbitol gradient (Scheme 1), consisting of the following steps: 0.5 M-sorbitol in 0.1 M-Tris/HCl buffer, pH 7.5; 0.6 M-sucrose in 0.01 M-Tris/HCl buffer, pH 7.5; and a cushion of 1.38 M-sucrose.

Membranes were fractionated on the gradient in a low centrifugal field (140 *g* for 30 min). Fractions F1–F4 as shown in Scheme 1 were collected with a J-shaped pipette, or by upward-flow displacement with an ISCO gradient fractionator equipped with a UA-2 u.v. analyser (Instrument Specialities Co., Lincoln, NE, U.S.A.).

The F1 fraction, which remained on the top of the gradient, was shown by electron microscopy to contain membrane fragments and vesicles, and constituted the bulk of the total cellular membranes and cytoplasmic components. Microscopic examination of the F2 fraction revealed the presence of intact protoplasts and vacuoles, and a small number of membrane sheets. The F3 fraction contained large, sheet-like structures similar in appearance to

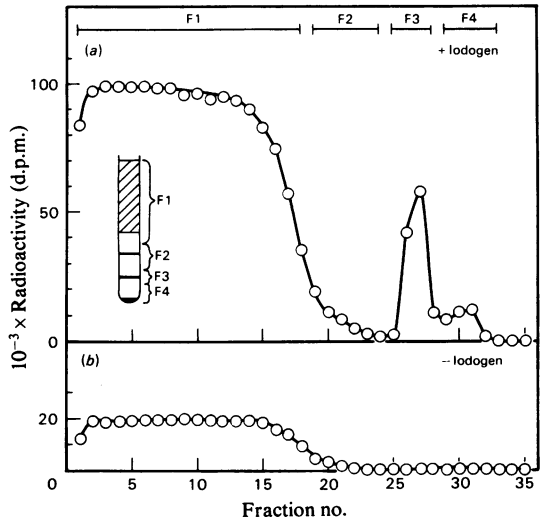


Fig. 1. *Fractionation of lysate obtained from protoplasts labelled with $Na^{125}I$*

After incubation with $Na^{125}I$ at 25°C for 10 min in (*a*) the presence or (*b*) the absence of Iodogen, protoplasts were washed and coated with myeloma protein J539 before disruption. The lysate was loaded on to a discontinuous sorbitol/sucrose gradient (Scheme 1) and centrifuged at 140 *g* for 30 min. Fractions were collected by upward-flow displacement and radioactivity determined.

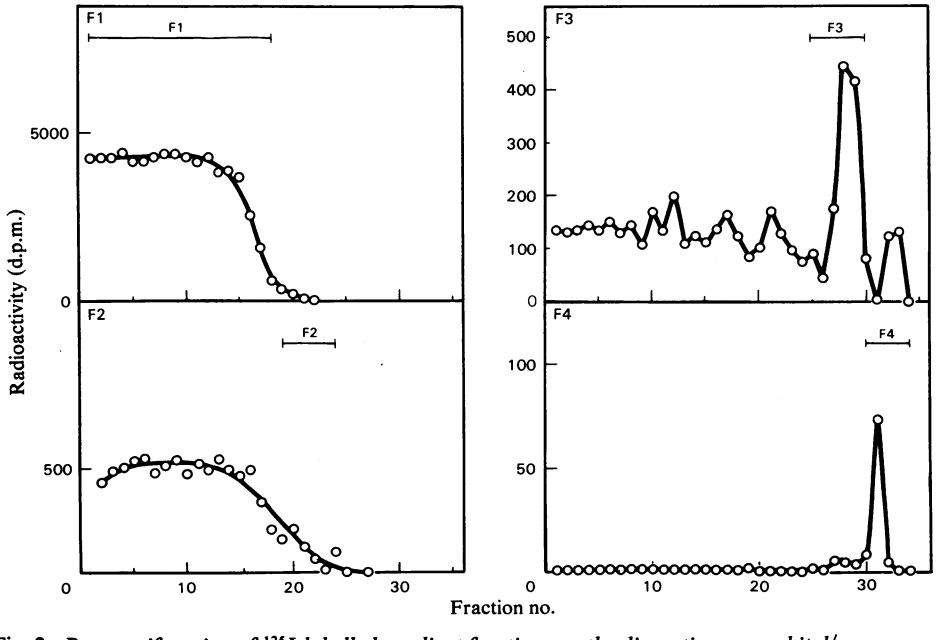


Fig. 2. *Re-centrifugation of ^{125}I-labelled gradient fractions on the discontinuous sorbitol/sucrose gradient*

Individual gradient fractions were pooled as indicated in Fig. 1. Portions of the resulting fractions, F1–F4, were re-centrifuged on the discontinuous sorbitol/sucrose gradient as shown in Scheme 1.

Table 3. *Distribution of radioactivity after centrifugation of a lysate obtained from protoplasts labelled with sodium [^{125}I]iodide in the presence of Iodogen*

Fractions were prepared as indicated in Scheme 1.

Fraction	Specific radioactivity (d.p.m./mg)	Relative specific radioactivity
Lysate	2562*	1.0
F1	1800*	0.7
F2	4555	1.8
F3	149400	58.3
F4	60166	23.4

* Specific radioactivity in lysate and fraction F1 are based on membrane-bound radioactivity determined by gel-filtration chromatography (3% of total radioactivity).

Table 4. *Distribution of radioactivity after centrifugation of a lysate obtained from protoplasts labelled with ^{125}I-labelled myeloma protein J539*

Fractions were prepared as indicated in Scheme 1. Percentage d.p.m. was calculated from the radioactivity profile shown in Fig. 3(*a*).

Fraction	D.p.m. (%)	Protein (%)	Relative specific radioactivity
Lysate	100	100	1.0
F1	25.0	91.9	0.3
F2	16.6	7.3	2.3
F3	57.7	0.6	96.0
F4	0.8	0.2	4.0

the plasma-membrane sheets isolated from fungi and yeast (Scarborough, 1975; Durán *et al.*, 1975). Some protein bodies and starch were also present in fraction F3. The F4 fraction consisted of nuclei, starch and protein bodies and a few adhering sheet-like membranes. The inclusion of the 1.38 M-sucrose cushion (Scheme 1) in the sucrose/sorbitol gradient was essential to minimize contamination of fraction F3 with these heavier subcellular organelles and to avoid pelleting of the membrane sheets.

^{125}I-labelling

When protoplasts were labelled with Na^{125}I by chemical (Iodogen) catalysis, and lysates were fractionated on the discontinuous gradients, radioactive material was detected not only in fraction F1, but also in F3 and to a lesser extent in F4 (Fig. 1*a*). In the absence of catalyst, no radioactivity was detected in fractions F3 or F4, and radioactivity associated with F1 was low (Fig. 1*b*). Similar results were obtained when iodination was catalysed enzymically (results not shown).

Reproducible sedimentation patterns were observed after re-centrifugation of the individual fractions (Fig. 2). Since most radioactivity was associated with F1, this fraction was examined by gel-filtration

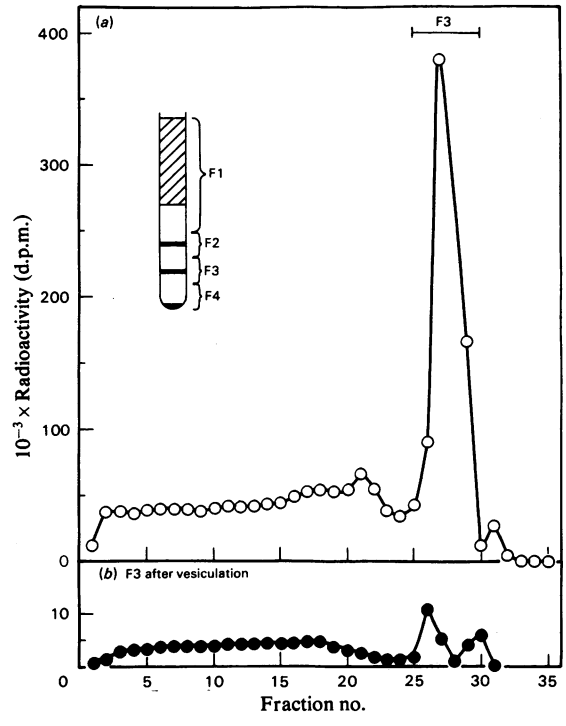


Fig. 3. *Fractionation of lysate obtained from protoplasts labelled with ^{125}I-labelled myeloma protein J539*

(*a*) Protoplasts were labelled with ^{125}I-labelled myeloma protein J539 at 25°C for 20 min and washed. After disruption, the lysate was fractionated on a discontinuous sorbitol/sucrose gradient as described in Scheme 1. (*b*) A portion of fraction F3 was treated with 0.5 M-lactose in 10 mM-Tris/HCl, pH 7.5, homogenized and re-centrifuged on the discontinuous sorbitol/sucrose gradient.

chromatography on Bio-Gel A5m and Sephadex G-25. The material excluded from the Bio-Gel A5m column contained only 3% of total radioactivity. Gel filtration of fraction F1 on Sephadex G-25 indicated that the ^{125}I-labelled material is of very low molecular weight.

After fraction F1 was centrifuged at 100000 *g* for 1 h or precipitated with trichloroacetic acid, only 3 and 4% respectively of the total radioactivity was recovered in the pellets, confirming that most of the radioactivity was associated with low-molecular-weight material.

The distribution of radioactivity in fractions from the discontinuous gradient is summarized in Table 3. The apparent enrichment factor of specific radioactivity in fraction F3 was approx. 58.

Labelling with ^{125}I-labelled myeloma protein J539

When protoplasts were labelled with radioiodinated myeloma protein J539, and lysate mem-

branes fractionated on discontinuous gradients, the pattern of label distribution (Fig. 3*a* and Table 4) was significantly different from that obtained after Iodogen-catalysed iodination (Fig. 1). Most radioactivity sedimented in the F3 fraction and a lower proportion was found in the F1 fraction. The apparent enrichment of specific radioactivity was highest in fraction F3 (96-fold, Table 4).

Gel-filtration chromatography of the F3 fraction on Bio-Gel A5m showed that most radioactivity was excluded from the gel and was therefore likely to be associated with membrane-bound proteins (results not shown). When fraction F3 was treated with 0.5 M-lactose, homogenized and a portion of the resultant vesicle suspension reapplied to the discontinuous gradient, radioactivity originally associated with fraction F3 was distributed mainly in the F1 fraction (Fig. 3*b*).

Of the total radioactivity in the original lysate, 25% was recovered in fraction F1 (Fig. 3*a*). Chromatography of this material on Bio-Gel A5m showed that 76% of the radioactivity was excluded (results not shown). Assuming that the excluded radioactivity is bound to plasma membrane, it can be calculated that fraction F1 contains a maximum of 20% of the plasma membrane.

Electron microscopy

The appearance of a ryegrass protoplast after staining with phosphotungstic acid/chromic acid is shown in Plate 2. Numerous mitochondria and vacuoles are apparent, as well as developing amyloplasts, some profiles of rough endoplasmic reticulum, a few dictyosomes and spherical bodies that resemble developing protein bodies (cf. Mares & Stone, 1973). The plasma membrane is intact.

Examination of the F3 fraction from discontinuous density gradients (Scheme 1) revealed expanses of sheet-like membranes admixed with spherical, membrane-limited structures that could not be positively identified (Plate 3*a*). Starch granules were sometimes seen. Contaminating components were partly removed by resuspending and diluting the F3 fraction in 0.01 M-Tris/HCl buffer, pH 7.5, and re-centrifugation in the discontinuous sorbitol/sucrose gradient. This 'washing' procedure resulted in a significant enrichment of the membrane sheets, although some membrane-limited structures remained.

At higher magnifications (Plate 3*b*), long plasma-membrane profiles were seen. These were often associated, presumably due to myeloma protein J539 binding, and had a typical trilamellate structure (Plate 3*b*, inset).

Discussion

Our method for the isolation of plasma membrane from ryegrass endosperm cells is based on that described by Scarborough (1975), in which concanavalin A was used to coat the plasma membrane of cell-wall-less mutants of *Neurospora crassa*. After cell disruption, the fungal plasma membrane formed large sheets that could be separated readily from other cellular membranes at low centrifugal forces (Scarborough, 1975). Parish & Müller (1976) have also used concanavalin A in the isolation of plasma membranes from *Dictyostelium discoideum* cells. In an attempt to apply this method to carrot (*Daucus carota*) protoplasts, Boss & Ruesink (1979) observed agglutination with concanavalin A, but were unable to isolate large plasma-membrane sheets. Ryegrass protoplasts, in contrast with fungal (Scarborough, 1975), yeast (Durán *et al.*, 1975) and carrot protoplasts (Boss & Ruesink, 1979), were not agglutinated by concanavalin A (Table 1). However, the 1,6-β-galactose-binding proteins axinellin (Bretting & Kabat, 1976) and murine myeloma protein J539 ((Jolley *et al.*, 1974) both agglutinated protoplasts derived from the ryegrass endosperm cells (Table 1). This is consistent with the observation that galactose-containing molecules are associated with cell membranes of ryegrass endosperm (Mascara & Fincher, 1982).

We have successfully used the myeloma protein J539 to coat protoplast surfaces in the isolation of ryegrass plasma membranes; the observation that galactose-binding proteins agglutinate protoplasts from several other plant species (Larkin, 1977; Raff *et al.*, 1980) suggests that these proteins may be generally useful as agents for coating plant plasma membranes. Furthermore, the myeloma protein J539 does not interfere with assays for β-glucan synthases (R. J. Henry, A. Schibeci & B. A. Stone, unpublished work).

The use of protoplasts as a starting material is important because removal of the cell wall allows direct access of coating agents to the plasma membrane, and allows gentle cell-disruption procedures to be used. Protoplasts were successfully lysed with polycations or by gentle mechanical means after swelling in hypo-osmotic medium. In both methods, up to 40% of the vacuoles were released intact (Table 2). The conditions of disruption are important determinants of the success of subsequent isolation of intact plasma membranes, since excessive damage leads to membrane vesiculation and attendant difficulties in resolving specific membrane components, and because vacuole disruption is likely to liberate degradative enzymes.

To enable identification of plasma membrane during fractionation of cell lysates, protoplasts were surface-labelled with a number of radioactive probes before disruption. Radioiodination with [^{125}I]iodide or labelling with ^{125}I-labelled myeloma protein J539 were the most reliable. The latter procedure has the

advantage that plasma membranes can be coated and labelled simultaneously. Diazotized sulphanilate derivatives have been used to label surface membranes in animal (Edwards *et al.*, 1979), microbial (Scarborough, 1975) and plant systems (Galbraith & Northcote, 1977; Perlin & Spanswick, 1980), but ryegrass protoplasts were permeable to these compounds and very little incorporation of radioactivity from either ^{35}S- or ^{125}I-labelled diazosulphanilate into ryegrass membranes was observed (results not shown).

We conclude that surface-labelling of the protoplasts with ^{125}I or with ^{125}I-labelled myeloma protein J539 provides a good means for plasma-membrane identification in cell homogenates. Although we cannot rule out that labelling of cytoplasmic contents of small numbers of ruptured or leaky cells occurs, this would produce labelled components that, under the centrifugation conditions used, would remain in the supernatant fraction (fraction F1, Scheme 1).

Galbraith & Northcote (1977) noted that suspension-cultured soya-bean (*Glycine max*) cells contained high levels of endogenous peroxidase; this effectively precluded the use of $Na^{125}I$ for specifically labelling soya-bean plasma membranes. However, it is apparent that although $Na^{125}I$ penetrates the ryegrass protoplasts and may label intracellular proteins, there is very little labelling of intracellular membranes. Approx. 60% of the ^{125}I in fraction F3 appears to be non-covalently bound, as judged by gel-filtration chromatography on Bio-Gel A5m (results not shown), and may be associated with membrane lipids or bound to the few starch granules that contaminate this fraction. Such associations probably account for the lower enrichment factor in fraction F3 with $Na^{125}I$ compared with ^{125}I-labelled myeloma protein J539.

The ^{125}I-labelled myeloma J539 gave a 96-fold enrichment factor, which is consistent with the expected yield of plasma membrane. Our conclusion that this fraction is predominantly plasma membrane is supported by the results of electron microscopy. The membrane profiles seen in the F3 fractions (Plate 3) can be identified as plasma-membrane sheets on the basis of their length and smooth appearance. Further, their thickness (approx. 8nm) is the same as the intact protoplast plasma membrane and within the range (6–10nm) normally observed for other higher-plant cells (Gunning & Steer, 1975). Long regions of laterally associated membranes are frequently observed and presumably arise from agglutinated protoplasts, which are bridged at their surfaces by the multivalent myelona protein J539. This interpretation is supported by the observation that, at high magnification (Plate 3*b*), each membrane can be seen to consist of three regions, staining dark–light–dark in a pattern of characteristic of unit membranes. The associated membranes seen in Plate 3(*b*) appear very similar to plasmalemma fractions isolated from rat liver by Benedetti & Emmelot (1968).

Just how concanavalin A and myeloma protein J539 achieve the stabilization of the plasma membranes that allows them to maintain their natural sheet-like conformation and largely prevents their vesiculation, is at present unknown. Possibly it relates to the 'freezing' of mobile membrane glycoproteins or glycolipids in the membrane matrix by the multivalent carbohydrate-binding proteins.

The method described for the preparation of highly enriched plasma membranes will permit chemical and enzymic analysis of their composition, in particular with respect to β-glucan synthases and the nature of ligands for the galactose-binding myeloma protein J539.

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References

Baldo, B. A., Neukom, H., Stone, B. A. & Uhlenbruck, G. (1978) *Aust. J. Biol. Sci.* **31**, 149–160

Benedetti, E. L. & Emmelot, P. (1968) in *The Membranes* (Dalton, A. J. & Haguenau, F., eds.), pp. 33–120, Academic Press, New York and London

Boss, W. F. & Ruesink, A. W. (1979) *Plant Physiol.* **64**, 1005–1011

Bretting, H. & Kabat, E. A. (1976) *Biochemistry* **15**, 3228–3236

Buser, C. & Matile, P. (1977) *Z. Pflanzenphysiol.* **82**, 462–466

Durán, A., Bowers, B. & Cabib, E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3952–3955

Dürr, M., Boller, T. & Wiemken, A. (1975) *Arch. Microbiol.* **105**, 319–327

Edwards, R. M., Kempson, S. A., Carlson, G. L. & Dousa, T. P. (1979) *Biochim. Biophys. Acta* **553**, 54–65

Eichmann, K., Uhlenbruck, G. & Baldo, B. A. (1976) *Immunochemistry* **13**, 1–6

Fincher, G. B. & Stone, B. A. (1981) in *Plant Carbohydrates II: Cell Walls of Higher Plants* (Tanner, W. & Loewus, F. A., eds.) (*Encyclopaedia of Plant Physiology, New Series*), vol. 13B, pp. 68–132, Springer-Verlag, Heidelberg

Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857

Galbraith, D. W. & Northcote, D. H. (1977) *J. Cell. Sci.* **24**, 295–310

Goldman, M. (1968) *Fluorescent Antibody Methods*, p. 101, Academic Press, New York and London

Gunning, B. E. S. & Steer, M. W. (1975) *Ultrastructure and the Biology of Plant Cells*, p. 312, Edward Arnold, London

Heslop-Harrison, J. & Heslop-Harrison, Y. (1970) *Stain Technol.* **45**, 115–120

Jolley, M. E., Glaudemans, C. P. J., Rudikoff, S. & Potter, M. (1974) *Biochemistry* **13**, 3179–3184
Keller, F. & Stone, B. A. (1978) *Z. Pflanzenphysiol.* **87**, 167–172
Larkin, P. J. (1977) *J. Cell Sci.* **26**, 31–46
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mares, D. J. & Stone, B. A. (1973) *Aust. J. Biol. Sci.* **26**, 135–150
Mascara, T. & Fincher, G. B. (1982) *Aust. J. Plant Physiol.* **9**, 31–45
Nagata, T. & Takebe, I. (1970) *Planta* **92**, 301–308
Parish, R. W. & Müller, U. (1976) *FEBS Lett.* **63**, 40–44
Perlin, D. S. & Spanswick, R. M. (1980) *Plant Physiol.* **65**, 1053–1057
Quail, P. H. (1979) *Annu. Rev. Plant Physiol.* **30**, 425–484
Raff, J., McKenzie, I. F. C. & Clarke, A. E. (1980) *Z. Pflanzenphysiol.* **98**, 225–234
Roland, J. C., Lembi, C. A. & Morré, D. J. (1972) *Stain Technol.* **47**, 195–200
Scarborough, G. A. (1975) *J. Biol. Chem.* **250**, 1106–1111
Schibeci, A., Fincher, G. B. & Stone, B. A. (1981) *Proc. Aust. Biochem. Soc.* **14**, 127
Seed, L. J. (1980) *J. Microsc.* **120**, 109–112
Smith, M. M. & Stone, B. A. (1973) *Aust. J. Biol. Sci.* **26**, 123–133
Taylor, J. A. & West, D. W. (1980) *J. Exp. Bot.* **31**, 571–576
Wagner, G. J. & Siegelman, H. W. (1975) *Science* **190**, 1298–1299