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CHARACTERIZATION, ENZYMATIC AND LECTIN PROPERTIES OF ISOLATED MEMBRANES FROM *PHASEOLUS AUREUS*

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SUMMARY

Cellular membranes were prepared from the non-extending part of dark grown hypocotyls of *Phaseolus aureus*. The relative effectiveness of continuous and discontinuous sucrose gradient centrifugation for the separation of membranes was investigated. Characteristic densities of membranes were determined by the localization of enzyme activities on continuous sucrose gradients: NADH-cytochrome *c*-reductase for endoplasmic reticulum, β-1-3-glucan synthetase for plasma-membrane and IDPase for dictyosomes. The difficulties involved in the application of ATPase and IDPase as specific membrane markers are discussed. Negative staining of isolated fractions indicated that intact dictyosomes could be prepared from this tissue without the use of chemical fixatives in the homogenization medium.

Extraction of isolated membranes showed that carbohydrate-binding proteins (lectins) were present both in an easily removable and in a more strongly bound form. In vivo incorporation of D-[U-^{14}C]glucose and subsequent isolation and solubilization of the different membranes showed that sugar-containing polymers could be released without hydrolytic techniques and were present in the equivalent extracts that exhibited lectin activity. The possibility of lectin-polysaccharide complexes in endoplasmic reticulum and dictyosomes and their involvement in the synthesis and transport of secretory substances by the membranes is discussed.

INTRODUCTION

Hypocotyls of *Phaseolus aureus* have often been used as the biological system in investigations of polysaccharide synthesis in plant tissues [1]. The hypocotyl is composed of two very distinct growth regions: one zone, localized in the upper part of the hypocotyl exhibits very rapid extension growth, whereas the other zone, at the base of the hypocotyl, is composed of non-extending tissue. The composition of the cell wall is very different in the two growth regions, especially with respect to

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the proportion and type of glycoprotein(s) present [2]. In vitro synthesis of cell wall polysaccharides has involved the use of a total membrane fraction generally isolated from whole hypocotyls [3–5]. In a preliminary report, we used purified membranes of endoplasmic reticulum, dictyosomes, plasma membranes and mitochondria separated using continuous sucrose gradient centrifugation, to demonstrate the presence of carbohydrate-binding proteins in intracellular membranes [6]. The membranes were prepared from actively extending regions of the hypocotyl.

Due to the great diversity of techniques to prepare purified membranes from plant tissue (e.g. cf. refs. 7–11) and due to contradictory reports on the presence of marker enzymes for specific plant cell membranes (e.g. cf. refs. 12 and 13) we decided to investigate initially the various characteristics of the intracellular membrane system of *P. aureus* in order to provide a foundation for further work now in progress on in vitro synthesis of cell wall polymers by isolated fractions of endoplasmic reticulum dictyosomes and plasma membranes.

METHODS AND MATERIALS

General preparation of membrane fractions

Mung bean (*P. aureus*) seeds were germinated and grown in the dark under a constant spray of water at 22 °C. On the third day of growth, seedlings (with 4–5 cm hypocotyl length) were harvested, and 1 cm segments were cut from the lower portion of the hypocotyl. Fresh weight used for each experiment was 20 g. The material was homogenized at 4 °C in a pestle and mortar, in 20 ml of medium containing 50 mM Tris · HCl, 1 mM EDTA, 0.1 mM $MgCl_2$ and 3 % w/w sucrose at pH 8.0. All other sucrose solutions used in gradients were made up in 10 mM Tris · HCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM KCl at pH 7.0 and are given in w/w %.

The homogenate was squeezed through muslin and centrifuged at $1000 \times g$ for 10 min at 4 °C to remove cell walls. This first centrifugation step was carried out in a Sorvall RC-2-B centrifuge, all subsequent steps were carried out in a Beckman ultracentrifuge at 4 °C using an SW 27.1 rotor head.

In the determination of characteristic densities of the membranes from the hypocotyls, the total homogenate, after removal of cell walls, was layered onto a 2 step sucrose cushion (2 ml 45 %, 2 ml 18 %) and particulate material was sedimented onto the 18–45 % interface by centrifugation at $100\,000 \times g$ for 90 min. After dilution of the sucrose to 19 % by addition of gradient medium, the particulate material was layered onto a continuous sucrose gradient 20–45 % (2 ml membrane suspension per 15 ml gradient) and centrifuged at $100\,000 \times g$ for 2 h. Gradients were then fractionated and the sucrose density of the fractions and activity of marker enzymes determined.

A discontinuous gradient method was utilised in the preparation of bulk membrane fractions. The homogenate, after removal of cell walls, was layered onto a 3 step sucrose cushion (1 ml 45 %, 2.5 ml 33 %, 1 ml 18 %) and centrifuged at $100\,000 \times g$ for 90 min. Membrane at the 18–33 % interface was collected using a Pasteur pipette and loaded onto a discontinuous gradient: 3 ml 33 %, 6 ml 25 %, 4 ml 20 %; similarly membrane at the 33–45 % interface was loaded onto 3 ml 45 %, 6 ml 39 %, 4 ml 33 %. The gradients were centrifuged at $100\,000 \times g$ for 2 h and particulate material at each interface was collected.

Marker enzyme assays

NADPH-cytochrome-*c*-reductase assay contained: 100 µl of 200 mM sodium phosphate buffer pH 7.4, 50 µl cytochrome *c* (5 mg · ml^{-1}) 5 µl NaCN, 50 µl H_2O and 100 µl membrane fraction. The reaction was started by the addition of 50 µl NADPH (3 mg · ml^{-1}), followed at 550 nm using a Zeiss spectrophotometer, and rate of µM cytochrome *c* reduced per min was calculated using an extinction coefficient of $18.5 \cdot 10^6$ cm^2 per mol. NADH-cytochrome-*c*-reductase assay contained: 100 µl of 200 mM $NaPO_4$ buffer pH 7.4, 50 µl cytochrome *c* (5 mg · ml^{-1}), 5 µl NaCN, 140 µl H_2O and 10 µl membrane fraction. The reaction was started by addition of 50 µl NADH (3 mg · ml^{-1}) followed at 550 nm and the rate calculated as for NADPH-cytochrome-*c*-reductase. The effect of antimycin on enzyme activity was investigated by adding 5 µl antimycin (2 mg · ml^{-1} in ethanol).

ATPase activity was measured at pH 6.0 and pH 9.0. The assay contained 200 µl of 200 mM Tris/MES (pH 6.0 or pH 9.0), 100 µl 20 mM $MgCl_2$, 250 µl 200mM KCl and 300 µl of 10 mM ATP as the sodium salt. The reaction was started by the addition of 150 µl of membrane fraction, and incubated at 25 °C for 2 h. 1.5 ml of 12.5 % w/w trichloroacetic acid was added and the phosphate released was measured using the method of Taussky and Shorr [14]. IDPase activity was measured 48 h after isolation of the membranes. The assay contained: 250 µl 10 mM IDP as the sodium salt, 50 µl 20 mM $MgCl_2$, 600 µl 100 mM Tris · HCl pH 7.4. Reactions were started by the addition of 100 µl of membrane and after incubation at 25 °C for 1 h 1.5 ml of 12.5 % w/w trichloroacetic acid was added. Phosphate was determined as for ATPase. New plastic tubes and disposable plastic cuvettes were used for all phosphatase assays since the method of phosphate determination was found to be extremely sensitive and could give positive results with traces of phosphate present in glassware or cuvettes.

Glucan synthetase was carried out in conditions of high UDP-glucose concentration. The incubation mixture contained: 20 µl 250 mM Tris/acetate pH 8.0, 10 µl 10 mM UDPglucose, 10 µl UDP-[^{14}C]glucose (0.05 µCi, spec. act.: 280 Ci/mol, Radiochemical Centre Amersham). The reaction was started by addition of 50 µl of membrane suspension, incubated at room temperature for 30 min and stopped by addition of 1 ml of chloroform/methanol (3 : 2, v/v). The polymer fraction was extracted, washed and suspended in dioxane scintillation liquid for determination of radioactivity [15]. In assays using gradient fractions, it was necessary to add 50 µl of boiled crude membrane suspension, after chloroform/methanol, in order to provide sufficient protein to form a sizeable precipitate.

Extraction of isolated membrane fractions

The fractionation procedure was developed to investigate the possibility that sugar-binding proteins were carried within membrane vesicles in addition to their presence as membrane components. Membrane fractions at the 18–25 %, 25–33 % and 33–39 % interfaces of the discontinuous gradient were dialysed against 2×5 l of phosphate buffered saline (0.05 M sodium potassium phosphate buffer pH 7.4, 0.9 % NaCl) to produce conditions of osmotic shock, and the material remaining in the dialysis tubing was centrifuged at 100 000×*g* for 15 min at 4 °C in Ti 65 rotor. The supernatant (extract 1) was removed, and the membrane pellet was sequentially extracted further by sonication in phosphate buffered saline (extract 2), sonication

in 0.5 M potassium phosphate buffer pH 7.1 (extract 3), sonication in EDTA (100 mM)/Triton-X-100 (0.06 %) (extract 4). The membrane was pelleted between each extraction by centrifugation as above. Extracts 3 and 4 were dialysed against 4 × 5 l of phosphate buffered saline, and volumes of all fractions were made up to 12.0 ml. Protein was determined in each fraction using the method of Lowry et al. [16], with bovine serum albumin (Serva Heidelberg) as a standard. Agglutination activity and sugar specificity of the lectins were tested using the titer plate method and trypsinized rabbit red blood cells as previously described [6, 17]. Lectin activity is given as titer, which is defined as the reciprocal of the greatest dilution at which full agglutination occurred.

Incorporation of radioactive precursor

Hypocotyl segments were incubated in vitro in the dark on a shaker in 25 ml H_2O containing of 100 μCi D-[U-^{14}C]glucose (Radiochemical Centre Amersham, spec. act. 283 Ci/M) for 1 h. After incubation, the segments were washed with H_2O, and membrane fractions were obtained by discontinuous gradient centrifugation. Extracts 1–4 of endoplasmic reticulum, dictyosomes and plasma membranes were prepared as described above, and were dialysed against 15 l of H_2O at 4 °C. Samples were evaporated to dryness under reduced pressure. Hydrolysis, neutralization and analytical methods were all as described previously [11]. Since incorporation from glucose into proteins was found always to be very high [11, 18] radioactive amino acids were removed from the hydrolysate by paper electrophoresis, prior to analysis of the amount of radioactive incorporation into components of sugar-containing polymers [11].

Electron microscopy

Negative stain preparations of isolated membranes of the Golgi apparatus were performed. Membrane at 25–33 % interface of the discontinuous gradient was diluted 1 : 1 with water, and further diluted 1 : 1 with 12 % w/v glutaraldehyde (Serva Heidelberg), prior to addition of 2 % w/v sodium phosphotungstate (pH 6.8). Preparations were placed on Formvar coated copper grids (300 mesh) and viewed in a Zeiss electron microscope.

RESULTS AND DISCUSSION

Determination of marker enzyme activities

In order to determine characteristic densities of different membranes of *P. aureus* hypocotyls, a particulate fraction sedimenting onto a 45 % sucrose cushion was further centrifuged for 2 h on a continuous sucrose gradient. The distribution of certain enzyme activities in gradient fractions is shown in Fig. 1.

NADH-cytochrome *c* reductase has been shown to be localized in endoplasmic reticulum from both plant [19] and animal tissue sources [20]. Activity of the enzyme in *P. aureus* consistently occurred between 19–25 % glucose, (1.08 and 1.10 g · ml^{-1}) which is in agreement with that observed for endoplasmic reticulum in oat roots [21] maize roots (Bowles, D. J., unpublished) and extending hypocotyls from *P. aureus* [6]. In castor bean endosperm, peak activity of the enzyme occurred at a lower density of 1.12 g · ml^{-1} [19] and in pea epicotyl at 1.11 g · ml^{-1} [13]. It should be noted that

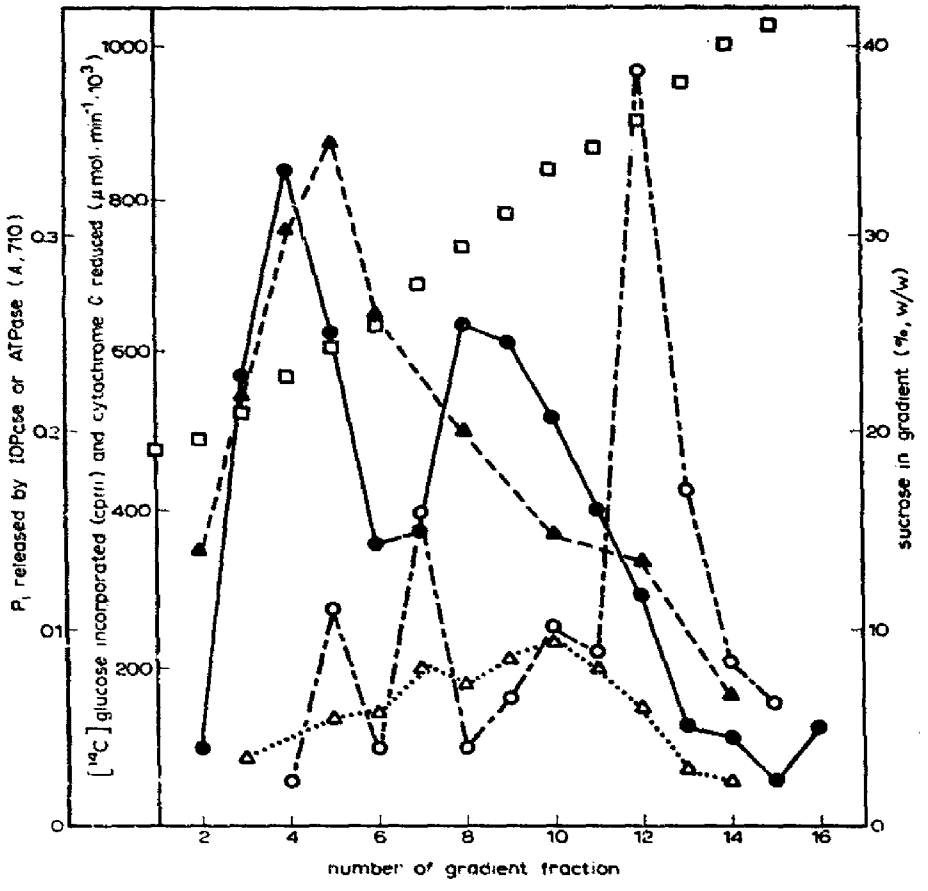

Fig. 1. Enzyme activities of membranes separated by continuous sucrose gradient centrifugation. A particulate fraction sedimenting at 18–45 % w/w sucrose interface, was centrifuged for 2 h at 4 °C and 100 000 × *g* on a continuous sucrose gradient 20–45 % w/w. The gradient was fractionated, and enzyme activities in different fractions determined. □□□, sucrose % w/w; ○– · –○, Glucan synthetase; △ . . . △, ATPase; ● — ●, IDPase; ▲– – –▲, NADH-cytochrome *c*-reductase.

the conditions of homogenization and gradient media used in our report cause dissociation of the ribosomes from "rough" endoplasmic reticulum, with resultant sedimentation of total endoplasmic reticulum membrane at low density.

IDPase has become known as a specific marker for dictyosomes of plant cells, due to the report of Ray and coworkers [12] on a coincidence of IDPase with glucan synthetase activity in pea epicotyls. In non-extending hypocotyls of *P. aureus*, it can be seen from Fig. 1 that there are 2 zones of IDPase activity, one very localized peak at 22% sucrose (1.09 g · ml^{-1}), and another broad peak which occurs between 28–33 % (1.12 and 1.15 g · ml^{-1}). Throughout a number of experiments the peak of enzyme activity occurring at 1.09 g · ml^{-1} remained consistently at that density, whereas the other peak of activity drifted considerably between extremes of 1.12 and 1.16 g · ml^{-1}. From continuous gradient data of pea epicotyls [12] and oat roots [21] two peaks of IDPase activity were also observed in those tissues; the enzyme activity at low

density was shown to be only slightly lower than that at high density. In comparison, in rapidly extending hypocotyls from *P. aureus* [6] and in maize roots (Bowles, D. J., unpublished) only one peak of IDPase was recovered on a continuous gradient and that occurred at 1.13 g · ml^{-1}. Similarly, in castor bean endosperm [19] only one peak of activity was found, but at 1.15 g · ml^{-1}.

Thus, although the IDPase may be located in dictyosome membranes, it is either not specific for such membranes in certain tissues or different parts of the dictyosomes e.g. vesicles as opposed to cisternal sacs, exhibit different equilibrium densities. It is now generally thought that the intracellular membrane system of endoplasmic reticulum, dictyosomes and plasma membrane is in a dynamic state, with either the turnover of membrane or specific membrane components occurring to produce a directional flow of membrane and secretory substances towards the cell surface. At the time of homogenization, certain parts of the membrane must therefore be in transition and have intermediate characteristics. It may be that the IDPase activity at 1.09 g · ml^{-1} is located in transition elements which exhibit enzyme activities typical of both the endoplasmic reticulum and Golgi apparatus.

ATPase activity, measured at pH 6.0, was not high, but showed a consistent broad peak of activity between 28–33 % sucrose (1.12 and 1.15 g · ml^{-1}). Using an isopycnic sucrose gradient for separation of membranes of pea epicotyl, ATPase activity (pH 7.5) was found to peak at 1.15 g · ml^{-1} [12]. This is in contrast to the activity in root tissues of maize and oats [21] where the enzyme was located at densities 1.17–1.22 g · ml^{-1} and has been suggested to be associated with plasma membrane. Work on Mg^{2+}-stimulated acidic ATPases in mung bean hypocotyls cannot be compared since activity was only measured in either a 20 000 × *g* or 100 000 × *g* membrane pellet which was not characterized enzymatically or by electron microscopy [22, 23]. It would therefore seem that the location of ATPase in plant membranes is dependent on the membrane source. In situations of very rapid growth, as in roots or rapidly extending zones of hypocotyls the ATPase activity is associated with a membrane of high buoyant density suggested to be plasma membranes, whereas in less actively growing tissues the ATPase may be associated with the dictyosome membranes.

Under conditions of high substrate concentration (in excess of 100 μM), it has been demonstrated that a total membrane fraction prepared from either oat coleoptile [24, 25] or *P. aureus* hypocotyls [26] produces a β-1-3-glucan from UDPglucose. It can be seen in Fig. 1 that using a UDPglucose concentrations of 1 mM, synthesis of glucan in *P. aureus* occurs preferentially in membranes of a density of 36–38 % sucrose (1.17 g · ml^{-1}). This density has been suggested to be characteristic for plasma membrane in other systems which have utilized continuous gradient centrifugation for membrane separation [7, 21]. In onion stem, at concentrations of 1 mM UDPglucose, β-1-3-glucans were shown to be synthesised by both dictyosomes and plasma membrane, but the membranes were fractionated using a discontinuous sucrose gradient prepared using coconut milk and characterized electron microscopically on the basis of distribution of material preferentially stained by phosphotungstic acid/chromic acid [9].

In this investigation, membranes were also fractionated using discontinuous sucrose gradient centrifugation, in an attempt to compare both the relative efficiency of the two types of gradient (continuous versus discontinuous) in the separation of

TABLE I

ENZYME ACTIVITIES DETERMINED IN ISOLATED MEMBRANE FRACTIONS OF *P. AUREUS* HYPOCOTYLS, PREPARED USING A DISCONTINUOUS SUCROSE GRADIENT

Membrane fraction tested	NADPH cytochrome *c*-reductase (cytochrome *c*-reduced: min^{-1} per μmol and mg protein)	NADH cytochrome *c*-reductase (cytochrome *c*-reduced: min^{-1} per μmol and mg protein)		ATPase (μmol P_i released per mg protein in 2.0 h)		IDPase (μmol P_i released per mg protein in 1.0 h)	Glucan synthetase (cpm in polymer per 50 μl membrane in 30 min)
		+antimycin	−antimycin	pH 6.0	pH 9.0		
Total homogenate	13.1	n.d.	2400	214.8	78.1	135	n.d.
18–25 % interface = endoplasmic reticulum	92.9	12387	13612	0	53.8	161	480
25–33 % interface = Golgi apparatus	40.7	6725	7450	383.3	75.0	325	765
33–39 % interface = plasma membrane	41.1	3828	6028	47.6	57.1	86	1150
39–45 % interface = mitochondria	18.1	1297	4162	0	297.3	0	490

n.d., not determined

plant membranes, and also to provide rapidly large amounts of the different membranes for further analysis. Results of enzyme activities in different fractions are shown in Table I. It can be seen that the particulate material sedimenting at each interface is enriched in specific membranes.

Activity of NADH-cytochrome-*c*-reductase was found to be approx. 150-times higher than that of NADPH-cytochrome-*c*-reductase. Antimycin A insensitive NADH-cytochrome-*c*-reductase has been associated with endoplasmic reticulum and outer membranes of mitochondria [19]. It was found that the NADH-cytochrome-*c*-reductase activity in the 18–25 % interface was inhibited 8.9 % by antimycin, whereas that located in the 39–45 % interface was inhibited by 68.8 %. This is consistent with previous data, and is also consistent with the localization of ATPase (optimum activity at pH 9.0) in mitochondria [21] which sediment at the 39–45 % interface. Relatively high IDPase activity was recovered in the 18–25 % interface, but since two peaks of activity were found on the continuous gradient, it is probable that the activity in the 18–25 % fraction is representative of endoplasmic reticulum membrane and not due to contamination by heavier particles. The activity of β-1-3-glucan synthetase in different discontinuous sucrose gradient fractions would suggest the enzyme is less specific for one type of membrane than that suggested by the data obtained with continuous gradient separation. It is probable that equilibrium centrifugation is necessary to ensure clear separation of plasma membrane from other intracellular membranes. The results also emphasise the fact that data obtained from plant membrane fractions isolated only by discontinuous gradient separation must be treated cautiously and cannot be used as the sole criteria for allocating a particular enzyme activity to a particular intracellular membrane.

Electron microscopy of dictyosomes

Membrane material sedimenting at the 25–33 % interface was negatively-stained using sodium phosphotungstate, and viewed using an electron microscope. The results are shown in Fig. 2. It has been considered that plant dictyosomes cannot remain intact and therefore morphologically distinct after the comparatively rigorous homogenization required to break the plant cell walls. Only one investigation, using enzymatic digestion of the cell wall prior to homogenization resulted in the isolation of intact dictyosomes [27]. However, the typical method of overcoming the problem has been to include glutaraldehyde in the homogenization medium [11, 28–30]. In such conditions, where proteins are cross-linked and stabilized, the isolated membranes cannot be used for in vitro biosynthesis studies. No glutaraldehyde was used either in the homogenization or the gradient media in the present report, but the fixative was used just prior to negative staining of the isolated material. If the fixative was not used, no characteristic cisternae could be observed in negatively stained preparations. Therefore, it would seem that the membrane structure is more labile to negative stain than to procedures involved in homogenization of the plant cells. This is consistent with the report that sodium phosphotungstate is an extremely effective solvent for membrane proteins of isolated dictyosomes from rat testes cells [31].

Presence of lectins and polysaccharides in isolated membrane fractions

The intracellular plant membrane system has been shown to synthesise polysaccharides and glycoproteins, and transport the polymers to the cell surface for

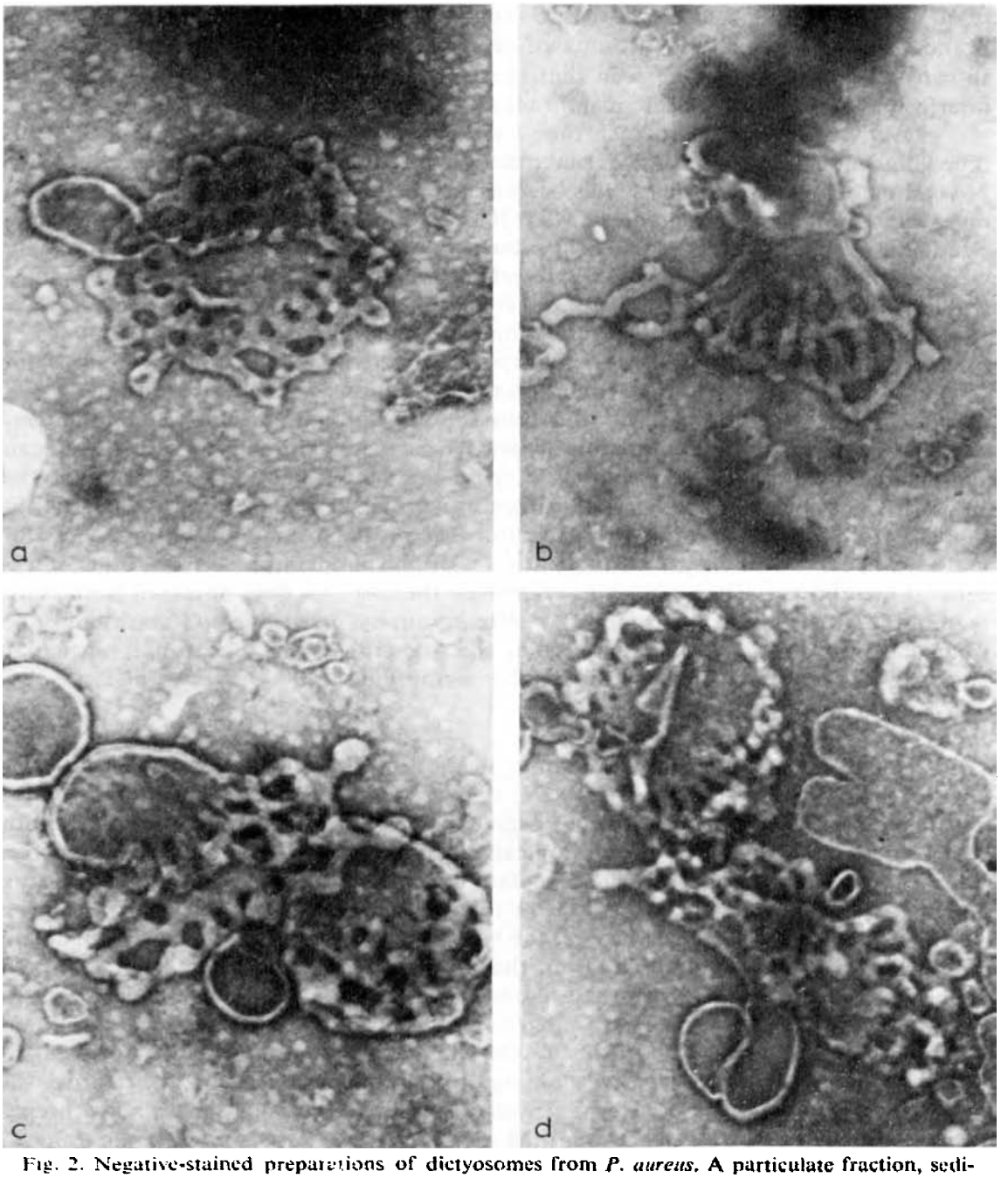

Fig. 2. Negative-stained preparations of dictyosomes from *P. aureus*. A particulate fraction, sedimenting at 25/33 % w/w sucrose interface of a discontinuous gradient was diluted with water, and glutaraldehyde was added to a final concentration of 6 % w/v. The membrane was then negatively-stained using sodium phosphotungstate. (a) and (b) (magnification × 40 500) show single cisternae of isolated dictyosomes, which exhibit central plate-like zones and fenestrated membrane. (a) is a surface view, whereas in (b) the cisternae is seen from the side. (c) and (d) (magnification × 32 400) show examples of joined cisternae and may represent parts of cisternal stacks.

TABLE II

AGGLUTINATION ACTIVITY OF FRACTIONS OBTAINED FROM ISOLATED MEMBRANES OF *P. AUREUS*

Membranes were isolated from hypocotyls using a discontinuous sucrose gradient, and fractionated sequentially to progressively solubilize contents of the membrane vesicles and membrane components. Agglutination activity in the fractions was determined using the titer plate method against trypsinized rabbit erythrocytes.

Solubilization procedure used on extract	Membrane type					
	Plasma membrane		Golgi apparatus		Endoplasmic reticulum	
	Protein $\mu g \cdot ml^{-1}$	Lectin activity	Protein $\mu g \cdot ml^{-1}$	Lectin activity	Protein $\mu g \cdot ml^{-1}$	Lectin activity
1. Osmotic shock	5	0	7	16/32	9.5	16/32
2. Sonication	trace	0	12	32/64	5.0	8/16
3. High molarity buffer	8.2	0	16.8	32/32	7.0	8/8
4. Chelating agent + detergent	23.5	32/64	23.5	128/128	18.0	16/16

TABLE III

EFFECT OF DIFFERENT SUGARS ON THE AGGLUTINATION ACTIVITY OF MEMBRANE EXTRACTS

Membranes were isolated from hypocotyls, and sequentially extracted (extract 1, osmotic shock; extract 2, sonication; extract 3, high molarity buffer; extract 4, chelating agent/detergent). The effect of different sugar derivatives on the agglutination activity of the lectins was tested using the titer plate method, with the sugars dissolved in phosphate buffered saline at an end concentration of $5 \cdot 10^{-3}$ M. + represents weak inhibition (less than 2 dilution steps); ++ represents moderate inhibition (more than 2) +++ represents strong inhibition (more than 3 dilution steps) L-arabinose, L-fucose, *N*-acetylgalactosamine and *N*-acetylglucosamine were found to have no effect.

	Golgi apparatus				Endoplasmic reticulum				Plasma membrane
	1	2	3	4	1	2	3	4	4
D-Galactose	++	+	++	O	O	O	O	O	+
pNP-α-D-galactoside	++	+++	+++	O	+++	++	++	O	++
pNP-β-D-galactoside	++	+	+	O	+	+	+	O	+
methyl-α-D-xyloside	+	+	+++	+++	++	+	++	+++	+++
methyl-β-D-xyloside	+	+	++	++	++	+	++	O	++
γ-D-galactonolactone	+++	+++	+++	O	+++	++	O	O	++
Mycinositol	O	O	++	O	O	O	O	O	+
Cellobiose	O	O	++	O	O	O	O	O	+++

incorporation into the cell wall [32, 33]. Previous work in our laboratory showed the presence of carbohydrate-binding proteins both in extracts of isolated membranes of endoplasmic reticulum, dictyosomes and plasma membranes from rapidly extending hypocotyls of *P. aureus* [6] and in cell wall extracts from the same tissue [17, 34]. It appeared possible, therefore, that the lectins recovered in isolated membranes could be both membrane components and secretory substances carried within the membrane vesicles for deposition at the cell surface. In order to try to determine the validity of these two alternatives, isolated membrane fractions were sequentially extracted. Results are shown in Table II.

Lectins could be solubilized from fractions of endoplasmic reticulum and dictyosomes by osmotic shock, sonication and high molarity buffer. These most probably represent proteins loosely-bound to the membranes or substances carried within vesicles. It could also be possible that soluble lectins present in the cytosol bind to sugar moieties of the glycoprotein and glycolipid components of cellular membranes during homogenization of the tissue. However, such unspecific adsorption is unlikely to have occurred, since equivalent extracts 1–3 of the plasma membrane do not exhibit lectin activity. In addition, it was found that the cytosol (equivalent to the $100\,000 \times g$ supernatant in our fractionation scheme) when dialysed against phosphate buffered saline and tested with trypsinized erythrocytes, did not exhibit any lectin activity.

The greatest amount of protein and lectin activity was released from both intracellular membranes and plasma membranes by the combined effect of chelating agent and detergent. These results suggest that lectins can be tightly-bound to the membranes and therefore may be classed as membrane components. Confirmatory evidence for this suggestion is to be presented using mitochondrial inner membranes from *Ricinus communis* [35].

The sugar specificity of the lectins in the different extracts was also tested and the results are shown in Table III. Lecin activity in extracts 1 and 2 of the endoplasmic reticulum and Golgi apparatus was inhibited strongly by γ-L-galactonolactone, and α-galactosides. It is of interest that the carbohydrate binding activity of cell wall extracts from the same tissue is also specifically inhibited by γ-D-galactonolactone

TABLE IV

INCORPORATION OF RADIOACTIVITY INTO SUGAR-CONTAINING POLYMERS AND GLYCOSIDES IN DIFFERENT MEMBRANES, AFTER IN VIVO INCUBATION OF *P. AUREUS* HYPOCOTYLS IN D-[U-^{14}C]GLUCOSE

Hypocotyls were incubated for 30 min in D-[U-^{14}C]glucose, and were used to prepare membrane fractions by discontinuous sucrose gradient centrifugation. After isolation, the membranes were progressively solubilized, and radioactivity in each fraction was determined.

Method of extraction	Total radioactivity in each membrane (cpm)		
	Plasma membrane	Golgi apparatus	Endoplasmic reticulum
1. Osmotic shock	460	1464	900
2. Sonication	360	4200	440
3. High molarity buffer	90	1915	317
4. Chelating agent + detergent	570	5620	956

TABLE V

PERCENTAGE INCORPORATION OF RADIOACTIVITY INTO POLYMERS CONTAINING SUGARS AND URONIC ACIDS OF DIFFERENT EXTRACTS OF ENDOPLASMIC RETICULUM AND GOLGI APPARATUS AFTER IN VIVO INCUBATION OF THE HYPOCOTYLS IN D-[U-^{14}C]GLUCOSE

Hypocotyls were incubated for 30 min and were used to prepare membrane fractions which were progressively solubilized by non-hydrolytic techniques. After hydrolysis of the sugar-containing polymers and determination of total radioactivity in each fraction as shown in Table IV, the neutral sugars were separated by paper chromatography (ethyl acetate/pyridine/water, 8 : 2 : 1) and radioactivity in each sugar determined.

	Radioactivity (%)							
	Fraction 1 (osmotic shock)		Fraction 2 (sonication)		Fraction 3 (high molarity buffer)		Fraction 4 (chelating agent/detergent)	
	Golgi apparatus	Endoplasmic reticulum	Golgi apparatus	Endoplasmic reticulum	Golgi apparatus	Endoplasmic reticulum	Golgi apparatus	Endoplasmic reticulum
Uronic acids	58	27	44	30.5	48	29.5	24	9.4
Galactose	35	35.8	32	45.6	5.2	47.0	6.5	53.7
Glucose	0	16.2	5	9.4	35	5.0	40.5	3.8
Mannose	0.9	6.5	2.5	12.6	3.0	14.7	9.3	21.0
Arabinose	5	7.3	4.6	1.7	4.7	4.2	12.5	12.0
Xylose	1.2	7.0	9.1	0	4.8	0	5.7	90
Rhamnose	0	0	2.7	0	0	0	1.8	0

[34]. The great variation shown by the sugar inhibition characteristics of the different extracts and membranes may indicate the existence of multiple lectin activities, but as yet the knowledge of the specific function of each intracellular membrane of plant cells is insufficient to allow the full significance of the results to be understood.

In order to determine the type of polysaccharide synthesised by membranes of *P. aureus*, segments from hypocotyls were incubated with D-[U-^{14}C]glucose in vivo, and membrane vesicles were isolated and sequentially solubilized to provide fractions equivalent to those which had been tested for lectin activity. Table IV shows the total radioactivity in neutral sugars and uronic acids recovered in hydrolysates of each membrane extract. Radioactivity was present in all fractions and extracts, although that recoverable in the fraction of dictyosomes is higher than that in either the plasma membrane or endoplasmic reticulum fractions. This is in contrast to results from maize roots [11, 28, 30], where the amount of radioactivity in an isolated endoplasmic reticulum fraction was found to be much greater than in a fraction of dictyosomes; however, the proportion of endoplasmic reticulum membrane observed in thin sections of hypocotyl tissue is always very much less than that observed in maize roots (Rougier, M., personal communication).

An analysis of the radioactive sugars and uronic acids in extracts 1–4 of the endoplasmic reticulum and dictyosome fractions is shown in Table V. Of interest is the considerable incorporation of radioactivity into glucose, only in extracts 3 and 4 of the dictyosome fraction. In a following report, we demonstrate that the glucosylation of an endogenous steroid acceptor in vitro occurs in membranes of isolated Golgi apparatus from *P. aureus* [36]. It may be possible that the relatively high proportion of glucose, only released by harsh treatment of the vesicles, represents not polysaccharides but steryl glucoside and acylated steryl glucoside present as components of dictyosome membranes.

The proportion of radioactivity in galactose and uronic acids is extremely high for extracts 1 and 2 of both the endoplasmic reticulum and the Golgi apparatus. These substances are characteristic components of hemicellulose and pectic substances [1], and their presence suggests that such polymers are solubilized from the membrane vesicles by mild treatment and are present in the extracts. This would be in accordance with results obtained using maize roots, which indicated that very high molecular weight sugar-containing polymers could be released from isolated membranes of endoplasmic reticulum and Golgi apparatus using non-hydrolytic techniques [30]. Such pectic substances would presumably contain suitable receptor groups for the carbohydrate-binding proteins/glycoproteins (lectins) present in the same extracts, which exhibit galactoside-specific binding sites. This report may therefore present the first indication that lectin-cell wall polysaccharide complexes do occur in the plant intracellular membrane system. The lectins may represent a device for binding the secretory substances to the membranes during synthesis and transport. Alternatively, the complexes may represent completed structural units of non-cellulosic components of the cell wall, preformed by polymerization in the membrane compartment before secretion and incorporation into the preexistent wall at the cell surface.

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