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Characterization and Quantitation of Concanavalin A Binding by Plasma Membrane Enriched Fractions from Soybean Root

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ABSTRACT

The binding of concanavalin A (Con A) to soybean root membranes in plasma membrane enriched fractions (recovered from the 34/45% interface of simplified discontinuous sucrose density gradients) was studied using a radiochemical assay employing tritiated (^{3}H)-Con A. The effect of lectin concentration, time, and membrane protein concentration on the specific binding of ^{3}H-Con A by the membranes was evaluated. Kinetic analyses showed that Con A will react with membranes in that fraction in a characteristic and predictable manner. The parameters for an optimal and standard binding assay were established. Maximal binding occurred with Con A concentrations in the range of 8 to 16% of the total membrane protein with incubation times greater than 40 min at 22 C. Approximately 10^{15} molecules of ^{3}H-Con A were bound per microgram of membrane protein at saturation. Binding was reversible. Greater than 92% of the total Con A bound at saturation was released by addition of α-methyl mannoside.

A major peak of ^{3}H-Con A binding was also observed in fractions recovered from the 25/30% interface of a complex discontinuous sucrose density gradient when membranes were isolated in the absence of Mg^{2+}. When high Mg^{2+} was present in the isolation and gradient media, the peak was shifted to a fraction recovered from the 34/38% sucrose interface. These results suggest that Con A binding sites are also present on membranes of the endoplasmic reticulum. The amount of Con A bound by endoplasmic reticulum membranes was at least twice the amount bound by membranes in plasma membrane enriched fractions when binding was compared on a per unit membrane protein basis. In contrast, mitochondrial inner membranes, which equilibrate at the same density as plasma membranes, had little ability to bind the lectin.

Protoplasts from several plant sources (3, 4, 25, 26) have the ability to bind Con A.[1] Although binding is apparently specific for mannosyl and glucosyl residues associated with the cell surface, the capacity for cell wall regeneration by isolated protoplasts has made Con A binding to the plasma membrane difficult to distinguish from its binding to nascent cell wall material. However, the temperature-dependent mobility of Con A receptor sites, indicative of their residence in a fluid matrix, has provided strong evidence for their presence on the membrane surface itself (4). That conclusion was also supported by the report that isolated protoplasts were extensively labeled by Con A-F even though their cell wall regenerative capacity had been eliminated by fixation of the protoplasts immediately following cell wall removal (25). The ability of isolated plasma membranes from soybean roots to bind the lectin has also been reported (23). Because isolated membranes are incapable of cell wall resynthesis, that result further supports the contention that there are Con A receptors specifically associated with the plant plasma membrane. It also suggests that subcellular plasma membrane vesicle preparations may prove useful in studies concerned with the elucidation of the structure and function of lectin binding sites at the plant plasma membrane surface. However, ultimate attainment of that objective will require a kinetic analysis of Con A binding to isolated vesicles. That analysis is reported in this paper and the quantity of receptor sites associated with those membranes is determined.

In previous reports (1, 23) we noted that enriched plasma membrane fractions prepared from soybean roots contain about 20 to 25% contaminating membranes. In order to arrive at a relatively definitive interpretation of the binding data as it specifically relates to the plasma membrane complement of those fractions it is also essential to evaluate the extent to which the presence of contaminating membranes may affect the kinetic analyses. Evidence obtained by Hendrix and Kennedy (5), from marker enzyme studies, suggest that mitochondrial and ER membranes, and possibly Golgi membranes, are the major contaminants in the enriched fraction. However, all membranous components probably exist to some extent in that fraction. Available evidence indicates that intact proplastids and chloroplasts isolated by techniques which do not include the use of cell wall macerating enzymes (20, 21), inner and outer chloroplast envelopes (21), and the tonoplast (23) do not bind Con A. However, some Con A binding by thylakoid membranes was noted (21). Con A binding by plant cell membranes of the ER, Golgi apparatus, mitochondrion and nuclear envelope have not been studied directly. With membrane isolates from rat liver tissue (6) those components bound significant amounts of Con A only when subjected to procedures which reduced membrane integrity. Those results indicate that receptor sites were localized on the inner vesicular surface and were not accessible to the lectin under standard conditions. Since membrane isolation from plant tissue requires somewhat harsher conditions than do similar isolations from animal tissue, the potential for disruption of membrane integrity is enhanced. Hence the capacity for Con A binding by mitochondrion and ER membranes was also assessed. The extent to which binding or lack of binding of Con A by those fractions might affect the kinetic analyses is evaluated. Analysis of Con A binding by Golgi and nuclear membranes was not evaluated in this study.

MATERIALS AND METHODS

Plant Tissue. Soybean seedlings (*Glycine max* (L.) Merr. cult. Wells) were germinated in darkness in plastic dishpans containing moist vermiculite at 30 C. Four-day-old roots were excised below the region of lateral root development into ice-cold aerated deionized H_2O.

Preparation of Membrane Fractions Used in Con A Binding

[1] Abbreviations: Con A, Concanavalin A; Con A-F, Con A-ferritin conjugate; ^{3}H-Con A, tritiated Con A prepared by acetylation of Con A with tritium-labeled acetic anhydride; α-MM, α-methyl mannoside; PACP, phosphotungstic acid-chromic acid procedure.

Studies. Plasma membrane enriched fractions were prepared by homogenization in a medium containing 25 mM Tris-Mes (pH 7.2), 3 mM EDTA, 2.5 mM DTT, and 250 mM sucrose. No Mg^{2+} was present. Homogenization and sucrose density gradient centrifugation were as previously described (24). Plasma membrane enriched fractions were recovered from the 34/45% sucrose interface.

In studies aimed at assessing the level of Con A binding by SER and RER membrane isolates, membrane fractions were isolated and fractionated under two sets of conditions. In experiments involving "zero" Mg^{2+}, the homogenization medium was identical to that used to prepare plasma membrane enriched fractions as described above. Differential and isopycnic sucrose density gradient centrifugation were also as previously described except that the 80,000*g* microsomal pellet was fractionated on complex discontinuous sucrose density gradients (7) consisting of 8 ml of 45% sucrose and 7 ml each of 38, 34, 30, and 25% sucrose (w/w). All sucrose solutions were in 1 mM Tris-Mes (pH 7.2). In experiments in which high Mg^{2+} was included, the procedures and conditions used were identical to those used in experiments with "zero" Mg^{2+} except that homogenization media contained 4 mM $MgSO_4$ (EDTA not present). All other media in the density gradient procedure contained 3 mM $MgSO_4$.

In each case plasma membrane fractions and membrane fractions rerecovered from complex gradients were diluted with incubation buffer containing 5 mM Hepes (pH 7.2), 19 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgSO_4$. Membranes were pelleted 35 min at 80,000*g* (Spinco T65 rotor) to remove sucrose, washed, and resuspended in incubation buffer, repelleted 35 min at 80,000*g* to remove residual sucrose and resuspended in incubation buffer prior to their use in Con A binding studies.

Mitochondrial membranes were isolated by a modification of the procedure of Bonner (2). Approximately 25 g of root tissue were homogenized for 3 to 4 min with a mortar and pestle, without added abrasive, in four volumes of homogenizing medium (25 mM Tris-Mes [pH 7.2], 3 mM EDTA, 2.5 mM DTT, 250 mM sucrose) and filtered through Miracloth. The crude homogenate was centrifuged 15 min at 13,000*g*. The pellet was resuspended in homogenizing medium and centrifuged 15 min at 1,000*g*. The resulting supernatant was centrifuged 15 min at 10,000*g*. The 10,000*g* pellet was resuspended in homogenizing medium and centrifuged 15 min at 6,000*g*. The pellet was resuspended in resuspension buffer (1 mM Tris-Mes [pH 7.2], 20% sucrose [w/w] and 1 mM $MgSO_4$), layered on a simplified sucrose density gradient, consisting of 8 ml of 34% sucrose layered over a 28 ml cushion of 45% sucrose, and centrifuged for 135 min at 80,000*g* (Spinco SW27 rotor). The particulate fraction recovered from the 34/45% sucrose interface was pelleted, washed free of sucrose, and separated into two fractions. One fraction was resuspended in ^{3}H-Con A incubation buffer (see below) and used in ^{3}H-Con A binding assays. The other was resuspended in 0.25 M sorbitol in 1 mM Tris-Hepes (pH 7.2), and used on Con A-F binding studies.

Preparation of ^{3}H-Con A. Con A (Calbiochem) was acetylated (14) with ^{3}H-labeled acetic anhydride (50 mCi/mmol) to a specific radioactivity of 7.47×10^5 cpm/mg. The acetylated derivative was lyophilized and stored at −20 C. Stock solutions of 1.0 mg/ml in 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 19 mM NaCl, and 5 mM Hepes (pH 7.2) were stored for 3 weeks at 4 C with no loss of activity.

Assay for ^{3}H-Con A Binding to Isolated Membranes. The reaction between ^{3}H-Con A and isolated membranes was quantitated using a modification of the procedure of Keenan *et al.* (6). The reaction mixture contained 5 mM Hepes (pH 7.2), 19 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 500 μg of membrane protein and the desired amount of ^{3}H-Con A in a final volume of 1.0 ml. Reaction vessels were incubated with agitation at 22 C for the desired period of time. After incubation the assay mixture was transferred to a Whatman GF/C glass fiber filter disc (2.2 cm diameter) under vacuum. The reaction tube was washed with duplicate 5-ml aliquots of incubation buffer and the wash was transferred to the filter. Filters were washed with duplicate 25-ml aliquots of incubation buffer and counted in 10 ml of Bray's solution. Parallel incubation mixtures contained 0.1 M α-MM in addition to the above constituents. Specific binding was determined from the difference in counts bound in the absence and presence of α-MM.

Con A-F Binding to Vesicles in Fractions Enriched For Mitochondrial Membranes. Fractions enriched for mitochondrial membranes were incubated for 80 min at 22 C in a reaction mixture containing 0.25 M sorbitol in 1 mM Tris-Hepes (pH 7.2) in the presence of Con A-F at a concentration of approximately 16% of the total membrane protein present. The reaction was stopped by centrifuging the reaction mixture for 35 min at 80,000*g* (Spinco, T65 Rotor). For complete removal of unreacted Con A-F, the pellet was resuspended in buffered sorbitol and centrifuged a second time. The final pellet was fixed in 2% glutaraldehyde in 0.25 M phosphate buffer for 12 h at 0 to 2 C, postfixed in 1% OsO_4 for 1.5 h, dehydrated in a graded acetone series, and embedded in Epon. Thin sections were stained with either uranyl acetate-lead citrate (17) or PACP (19). Sections were viewed with a JEOL-100S electron microscope.

RNA Analysis. Plasma membrane fractions recovered from the 34/45% sucrose interface of discontinuous sucrose density gradients were diluted with incubation buffer and centrifuged for 35 min at 80,000*g* (Spinco T65 rotor). Pellets were resuspended in 3 ml of extraction buffer (6 M urea, 50 mM NaOAC [pH 5.5], 200 mM NaCl, 10 mM EDTA, 0.75% SDS). The RNA was extracted by shaking in a vortex with 1 volume of phenol:chloroform: isoamyl alcohol (25:24:1; v/v/v) for 10 min. The phases were separated by centrifuging at 10,000*g* for 20 min and the water phase was reextracted with 1 volume of the chloroform-isoamyl alcohol (24:1; v/v). The water phases were pooled and the RNA was precipitated by adding 2.5 volumes of ethanol, 200 mg of maize tRNA and stored overnight at −20 C. The RNA precipitate was recovered by centrifugation at 10,000*g* for 20 min. To distinguish between rRNA and potential contamination by soluble RNA, the pellets were dissolved in water and subjected to electrophoresis.

Gel electrophoresis of RNA was on 9 cm 2.4% polyacrylamide gels containing 0.5% agarose in 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA (pH 7.2) (18). Gels were prerun for 90 min at 5 mamp/gel, then overlaid with 50 μl samples containing sucrose. Electrophoresis was performed at room temperature for 2.5 h at 6 mamp/gel. Gels were soaked in distilled H_2O and scanned at 260 nm with a Gilford Model 2410 gel scanner.

Protein Determinations. Proteins were determined by the method of Lowry *et al.* (8).

RESULTS

Analysis of ^{3}H-Con A Binding by Membranes in Plasma Membrane Enriched Fractions. Plasma membrane enriched fractions isolated from soybean roots were recovered from the 34/45% sucrose gradient interface. The level of enrichment for the plasma membrane was approximately 75% (23). The binding of Con A by membrane vesicles in those fractions was measured using radioactively labeled Con A prepared by acetylation with (^{3}H)-acetic anhydride. The kinetics of ^{3}H-Con A binding are shown in Figures 1 and 2. At a constant membrane protein concentration of 500 μg/1-ml assay mixture and constant temperature of 22 C, binding increased nearly linearly as the concentration of ^{3}H-Con A increased up to about 40 μg/ml (Fig. 1). There was little further increase in binding at higher concentrations of Con A, indicating that binding sites were saturated. Results from the time course assay (Fig. 2) indicated that nearly maximal amounts of Con A were bound within the first 40 min. At longer incubation times a

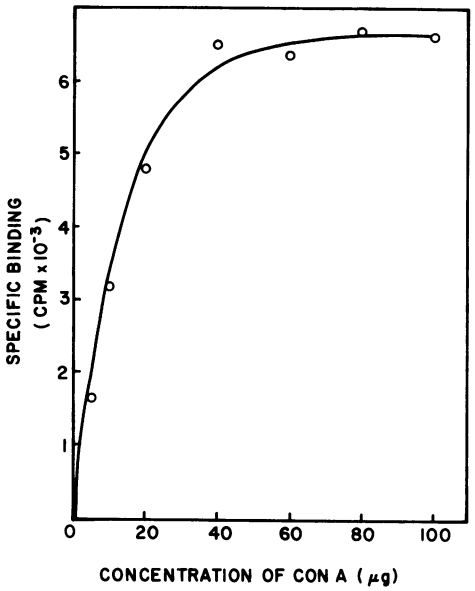

FIG. 1. Effect of ^{3}H-Con A concentration on binding of the lectin by plasma membrane enriched fractions isolated from soybean roots. The concentration of membrane protein was 500 μg/assay. Binding was measured after 80 min at 22 C. Specific binding was measured as the difference between binding in the presence and absence of α-methyl mannoside.

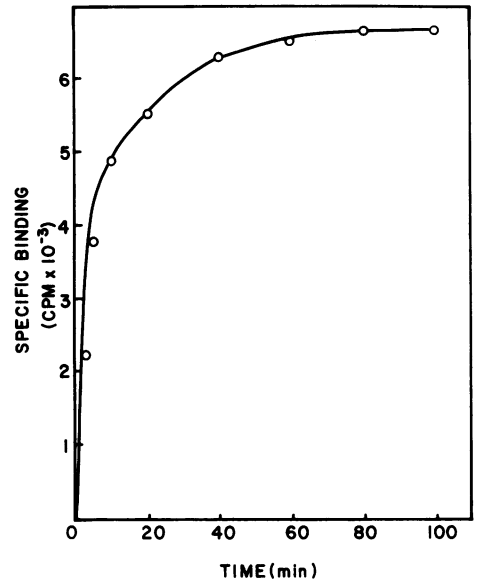

FIG. 2. Effect of incubation time on ^{3}H-Con A binding by plasma membrane-enriched fractions isolated from soybean roots. Binding was measured at 22 C. The concentration of membrane protein and Con A was 500 and 60 μg/assay, respectively. Specific binding was measured as the difference between binding in the presence and absence of α-methyl mannoside.

slight increase in binding was observed up to 80 min. Thereafter, no additional increase in the amount of Con A bound was observed. The amount of Con A bound was linear over the range of membrane protein concentrations from 0 to 500 μg/ml (Fig. 3). From the slope of this line it was calculated that approximately 13 cpm of Con A were bound/μg of membrane protein at saturation.

Binding of Con A to the membranes was reversible (Table I). Membrane fractions incubated with ^{3}H-Con A in the absence of the hapten inhibitor α-MM bound approximately 8,000 cpm. Preincubation of Con A with the inhibitor reduced bound counts to 3,200. The addition of α-MM to the membrane fraction after 1 h incubation with ^{3}H-Con A released bound Con A; approximately 3,000 cpm remained bound. The GF/C filters alone bound 2,800 cpm. Thus, nonreversible, nonspecific binding of Con A to the membrane fraction was on the order of 200 to 400 cpm.

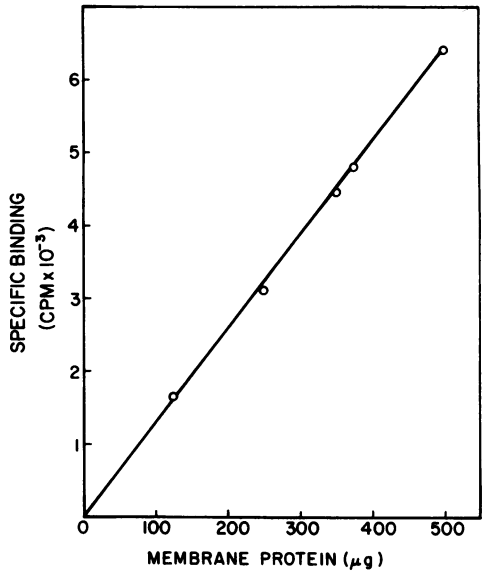

FIG. 3. Effect of membrane protein concentration on the specific binding of Con A. Binding was measured after 80 min at 22 C. The concentration of Con A was 60 μg/assay. Specific binding was measured as the difference between binding in the presence and absence of α-methyl mannoside.

Table I. *Release of ^{3}H-Con A from Enriched Plasma Membrane Fractions by α-Methyl Mannoside*

A plasma membrane-enriched fraction was recovered from the 34/45% interface of a simplified discontinuous sucrose density gradient, repelleted 35 min at 80,000g, washed free of sucrose, and resuspended in incubation buffer. Membrane protein (375 μg) was incubated in the presence of 40 μg of ^{3}H-Con A at 22 C in a final volume of 1.0 ml. The concentration of α-methyl mannoside, when present, was 0.1 M. Vesicles were recovered on 2.2 cm Whatman GF/C filters.

Treatment	Radioactivity Bound
	cpm
Control: Membranes + ^{3}H-Con A, 2 h	8,000
^{3}H-Con A preincubated with α-methyl mannoside, 45 min, + membranes, 2 h	3,200
^{3}H-Con A + membranes 1 h, followed by α-methyl mannoside, 1 h	3,000
Background: ^{3}H-Con A bound to GF/C filters in absence of membranes	2,800

Evidence for Con A Binding by the ER. The capacity for ^{3}H-Con A binding by membranes of the ER, potential contaminants in plasma membrane preparations (5), was analyzed by comparing the relative distribution and the level of counts bound on sucrose density gradients by membrane fractions isolated and fractionated under conditions which either favor dissociation of ribosomes from the ER (zero Mg^{2+}; 3 mM EDTA) or which facilitate their retention (3–4 mM Mg^{2+}; no EDTA). This experiment was predicated upon the finding that the major fraction of SER characteristically bands on sucrose density gradients at a buoyant density of about 1.10 g/cc, whereas RER equilibrates at approximately 1.16 g/cc (5, 10–12, 16). The distribution of ^{3}H-Con A binding is presented in Table II. When the membranes were isolated in the absence of Mg^{2+}, the peak activity (cpm ^{3}H-Con A bound/μg protein) was recovered from the 25/30% (1.10-1.13 g/cc) sucrose

Table II. *^{3}H-Con A Binding to Subcellular Membranes from Soybean Roots Isolated in the Absence and Presence of Mg^{2+}*

Membrane fractions were recovered from sucrose density gradients, pelleted 35 min at 80,000g, washed free of sucrose, and resuspended in incubation buffer. Membrane protein (500 μg) was incubated for 80 min at 22 C in the presence of 60 μg of ^{3}H-Con A in a final volume of 1.0 ml.

Gradient Interface	Specific Radioactivity[a]	
	$-Mg^{2+}$ [b]	$+Mg^{2+}$
20/25	9.6	5.3
25/30	23.6	12.7
30/34	17.8	15.0
34/38	15.5	26.3
38/45	12.4	13.2

[a] Values given represent difference between cpm/μg protein in the presence and absence of α-methyl mannoside.

[b] 3 mM EDTA also present.

interface. Conversely, when vesicles were isolated in the presence of 3 to 4 mM Mg^{2+} peak activity equilibrated at the 34/38% (1.15-1.17 g/cc) interface. The results suggest that the shift in the level and distribution of Con A binding/μg membrane protein was associated with a concomitant shift, depending upon the presence or absence of Mg^{2+}, in the position of the major ER fraction on the gradient. The results further show that the specific radioactivity (cpm bound/μg protein) of both SER and RER was greater than either peak value. This is evident from the additive effect on counts bound/μg protein obtained by shifting the position of the major ER fraction on the gradient. (*e.g.* An increase in counts bound/μg protein from 15.5 [$-Mg^{2+}$, 34/38% interface] to 26.3 [$+Mg^{2+}$, 34/38% interface] would require the addition of a fraction with a higher specific radioactivity than the resulting peak value.)

Estimation of Level of ER Contamination in Plasma Membrane-Enriched Preparations. The level of rRNA contamination was evaluated to estimate the extent of ER contamination in plasma membrane fractions used in this study. The concentration of rRNA was found to be approximately 40 μg/mg protein (4%). Assuming an rRNA to protein ratio of 0.45 for RER-enriched fractions (reported by Philipp *et al.*, for onion root), it was estimated that ER contamination may be on the order of 8%.

Analysis of ^{3}H-Con A Binding by Mitochondrial Membranes. Mitochondrial membranes, known contaminants in the plasma membrane fractions used in this study (5), were also tested for their ability to bind the lectin. A mitochondrial membrane enriched fraction, recovered from the 34/45% sucrose interface, was assayed for ^{3}H-Con A binding. Approximately 2.5 cpm/μg protein were bound under optimum conditions. The level of binding was considerably lower than that associated with plasma membrane enriched fractions (13 cpm/μg protein as derived from data in Fig. 3). To determine whether the low level of binding was actually associated with the mitochondrial membranes, the remaining portion of the fraction was treated with Con A-F and prepared for electron microscope analysis. Morphometric analyses of the fractions showed that approximately 80 to 90% (as determined from two different preparations) of the membranes present were in the form of large membrane vesicles (average 0.8 μm in diameter, Fig. 4A) which appeared to be derived from inner mitochondrial membranes. Smaller vesicles were often contained within the intravesicular spaces. Few intact mitochondria were present. Also present, in lower proportion, were small membrane vesicles (average 0.2 μm in diameter) and some membrane fragments which were not associated with the large mitochondrial vesicles. Electron micrographs of PACP stained sections (Fig. 4B) showed that ferritin particles were associated only with the independent PACP positively stained small vesicles and membrane fragments. The large mitochondrial vesicles were not stained by this procedure. The association of ferritin particles with PACP positively stained vesicles and the absence of ferritin associated with the unstained mitochondrial membranes of similar buoyant density strongly suggests that the low level of ^{3}H-Con A binding by membranes in that fraction can be accounted for by the presence of contaminating plasma membrane.

DISCUSSION

In a previous study (23) we established that Con A is extensively bound by vesicles in plasma membrane-enriched fractions isolated from soybean root. Detailed kinetic analyses were not performed at that time nor was the quantity of Con A bound by membrane vesicles in those fractions determined. To provide that information, and since electron microscope techniques used in that study were found to be unsuitable for that purpose, an assay using the tritiated-acetylated form of Con A was developed. Optimal assay conditions were established by evaluating the effect of lectin concentration, time, and membrane protein concentration on the specific binding of ^{3}H-Con A by membrane vesicles. With that assay we demonstrated that Con A will react with vesicles in plasma membrane-enriched fractions in a characteristic and predictable manner. Maximal binding occurred with Con A concentrations in the range of 8 to 16% of the total membrane protein concentration. The number of lectin receptor sites per μg of membrane vesicle protein can be estimated from the amount of lectin specifically bound at saturation and its specific radioactivity. Assuming a mol wt of 110,000 (13) for the Con A tetramer, approximately 9.67×10^{14} molecules of Con A were bound/μg of membrane protein. The calculated value reflects the concentration of binding sites associated with the membrane fraction taken as a whole, and, without further information with regard to the lectin binding ability of contaminating membranes, may only be taken as a rough estimate of the concentration of binding sites specifically associated with the plasma membrane (approximately 75% of the total vesicle population [1,23]). However, an analysis of the binding potential associated with two major contaminants in that fraction (discussed below), suggests that as much as 80% of the total Con A bound may be specifically associated with plasma membranes.

When membrane fractions which had been isolated in the absence of Mg^{2+} but in the presence of EDTA (conditions which cause dissociation of ribosomes from the ER) were recovered from complex discontinuous gradients and assayed for their ability to bind Con A (Table II), it was found that significant levels of Con A binding occurred in all fractions. This result by itself does not preclude Con A specificity for the plasma membrane since data presented by Hendrix and Kennedy (5) showed that significant levels of K^+-stimulated ATPase activity (plasma membrane marker) were also present in all gradient fractions when membranes were isolated from soybean roots under similar conditions. However, in that study, peak enzyme activities were found to be associated with membranes recovered from the 30/34% and 34/38% sucrose interface. In contrast, here, peak activity for Con A binding occurred in a fraction recovered from the 25/30% sucrose interface. A shift in the position of that peak to a position of greater buoyant density at 34/38% sucrose, which occurred when Mg^{2+} was present in the isolation media (conditions which favor ribosomal binding to the ER), indicated that fraction to contain a high proportion of ER with a significantly higher specific radioactivity than the value associated with plasma membrane preparations, greater than 26 cpm (Table II) and 13 cpm (Fig. 3) per μg of membrane protein, respectively.

Since *in vitro* assays have indicated that RER is a major site of GDP-mannosyl transferase activity in plant cells (10) and *in vivo* incorporation of [^{14}C]mannose into lipid and protein components of those membranes has been demonstrated (11), it is not surprising that they may contain receptor sites with an affinity for Con

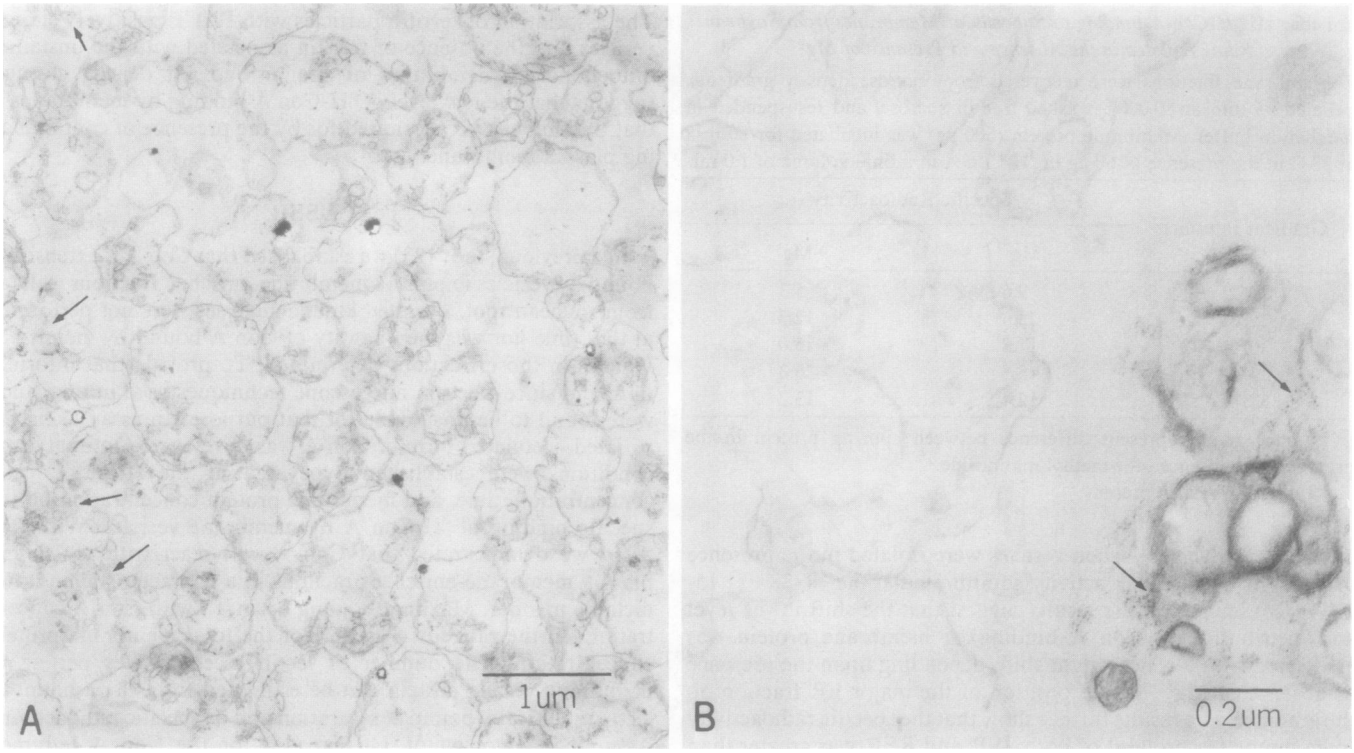

FIG. 4. Electron micrographs of sections from pellet of Con A-F-treated mitochondrial membrane vesicle preparations from soybean root. (A) Section stained with uranyl acetate-lead citrate showing large membrane vesicles derived from inner mitochondrial membranes. Also present, in lower proportion, are relatively small membrane vesicles (arrows) (×16,750). (B) Section stained by PACP. Ferritin particles are present on oblique portions of small PACP positively stained vesicles (arrows), but not on unstained membranes of larger vesicles (×56,000).

A. What is surprising is that those sites, which in the intact cell are believed to be asymetrically localized on the intracisternal membrane face, are localized in such a manner on isolated vesicles as to be accessible to the ^{3}H-Con A molecule. When membranes were isolated from rat liver cells (6), the vesicles were shown to retain an orientation similar to their intracellular counterparts. ^{3}H-Con A binding to vesicles which were not plasma membrane derived was only observed after the vesicles were subjected to procedures which disrupted membrane integrity. A possible reason for the binding of Con A reported here may relate to the more vigorous homogenization procedures used to disrupt the plant cell wall. Those procedures may lead to the production of a large number of inverted vesicles or it may be that vesicles are either perforated or partly ruptured during homogenization, thereby facilitating the passage of Con A to the inner vesicular space. The latter alternative is supported by two lines of evidence. In the first case, electron micrographs of isolated RER from plant tissue (10, 16) show that in most cases ribosomes are present on the outer surface of those vesicles as would be expected from their localization *in vivo*, indicating that most vesicles are probably right side out. Second, it has been suggested by Sze and Hodges (22) that their inability to demonstrate an ATP-driven ion flux with plasma membrane vesicles obtained from oat roots isolated by similar techniques to those employed here, was related to the presence of a large number of partially sealed and completely unsealed vesicles in their preparation. In the present study, when membrane fractions containing ER, and presumably other endomembrane components, were subjected to several cycles of freezing and thawing (data not shown) no additional increase in Con A binding was observed. That suggests that either all binding sites on the inner and outer surface of the vesicles were accessible prior to the freeze-thaw treatment or that those procedures were insufficient to disrupt the membranes. The latter interpretation is unlikely in light of the results reported by Sze and Hodges (22). In either case, the high specific radioactivity associated with the ER indicates that its presence as a major contaminant in plasma membrane fractions would significantly affect the ability of those fractions to bind the lectin.

An mRNA to protein ratio of approximately 0.04 was calculated for plasma membrane preparations used in this study as compared to a value of 0.45 which was reported by Philipp *et al.* (15) for RER-enriched preparations (approximately 83% RER) from onion root. A comparison of the two ratios suggests that as much as 8% of the total protein in plasma membrane preparations may be ER and ribosomal derived. Inasmuch as plasma membrane fractions were isolated using conditions which cause dissociation of ribosomes from the ER, it is not yet clear what proportion of the total rRNA content may be directly related to the presence of ER in these preparations. Freely associated ribosomal aggregates and smooth-surfaced ribosome enclosing vesicles may also be present (15). Inasmuch as the specific radioactivity associated with ER is at least twice that for the plasma membrane fraction taken as a whole, RER contamination levels on the order of 8% of the total protein present may account for more than 16% the total amount of Con A bound by all membranes present in that fraction.

In contrast to the results obtained with ER membrane isolates, "purified" mitochondrial membranes which equilibrated at the same buoyant density as plasma membranes showed little ability to bind the lectin. Thus, it can be concluded that their presence in plasma membrane fractions can only serve to dilute the specific radioactivity associated with those fractions by a factor proportional to the amount of mitochondrial membrane protein present. Therefore, the concentration of receptor sites associated with Con A binding membranes in plasma membrane preparations may be somewhat higher than the value of approximately 10^{15} sites/μg of membrane protein calculated above.

Still to be resolved is the question of Con A binding by isolated Golgi membranes which may also be present as contaminants in

the plasma membrane-enriched fractions. If plasma membranes and membranes of the ER contain a large number of Con A binding sites and if one accepts the endomembrane hypothesis (9), then it is quite possible that a significant number of Con A binding sites will also be present on Golgi membranes. A direct resolution of this question by the techniques employed in the present study will depend on developing a method to obtain relatively pure fractions of Golgi membrane isolates while eliminating significant contamination by vesicles of the plasma membrane and the ER.

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