

THE SITE OF CELLULOSE SYNTHESIS

Hormone Treatment Alters the Intracellular Location of Alkali-Insoluble β -1,4 -Glucan (Cellulose) Synthetase Activities

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ABSTRACT

Membrane preparations from growing regions of 8-day old *Pisum sativum* epicotyls contain multiple β -1,4-glucan (cellulose) synthetase activities (UDP- or GDP-glucose: β -1,4-glucan-glucosyl transferase), and the levels of some of these are influenced by treatments with the growth hormone, indoleacetic acid (IAA). When membranes from control epicotyl segments (zero time) are fractionated by isopycnic sedimentation in sucrose density gradients, all of the synthetase activities are associated mainly with Golgi membrane (density 1.15 g/cm³). After decapitation and treatment of epicotyls with IAA, synthetases also appear in a smooth vesicle fraction (density 1.11 g/cm³) which is rich in endoplasmic reticulum (ER) marker enzyme. Major fractions of these synthetases are not recovered in association with plasma membrane or washed cell walls.

When [¹⁴C]sucrose is supplied in vivo to segments \pm IAA, radioactive cellulose is deposited only in the wall. Cellulose or cellodextrin precursors do *not* accumulate in those membranes in which synthetase activities are recovered in vitro. In experiments where tissue slices containing intact cells are supplied with [¹⁴C]sugar nucleotide in vitro, alkali-insoluble β -1,4-glucan is synthesized (presumably outside the protoplast) at rates which greatly exceeded (20–30 times) those obtained using isolated membrane preparations. Progressive disruption of cell structure results in increasing losses of this high activity.

These results are consistent with the interpretation that Golgi and ER-associated synthetases are not themselves loci for cellulose synthesis in vivo, but represent enzymes in transit to sites of action at the wall:protoplast interface. There they operate only if integrity of cellular organization is maintained.

In higher plants, cellulose biosynthesis is generally considered (5, 24–26, 28–30, 40, 54) to occur at the plasma membrane:cell wall interface, i.e., at the site of microfibril deposition. The most convincing evidence for this is derived from autoradiographic studies in vivo (54, see also 5, 25, 26), which indicate synthesis of cellulose only outside the protoplast. In biochemical studies using particulate enzyme preparations, alkali-insoluble β -1,4-glucan (cellulose) synthetase activity has been

identified primarily in association with Golgi membrane (35, 50). There is only one clear demonstration (10) of this enzyme active in fractions containing plasma membrane, albeit at lower activity levels than Golgi-bound synthetase. It may be that cell-surface synthetase activity is particularly labile and lost during extraction. Certainly, the maximum rates of cellulose synthesis attained *in vitro* using membrane-bound preparations have never reproduced the rate of cellulose deposition commonly observed *in vivo* (1, 4, 13).

It has been shown repeatedly (1, 12, 32, 33, 45, 46, 47) that treatments with the growth hormone indoleacetic acid (IAA) can regulate net levels of certain β -1,4-glucan synthetase activities and, at the same time, affect rates of cellulose deposition (31, 34, 45, 46, 53). The existence of multiple β -1,4-glucan synthetase activities was demonstrated recently (45) in growing tissues from *Pisum sativum* epicotyls, and some of these are subject to rapid turnover and hormonal control. It was suggested that the IAA-regulated activities could account for a major part of total cellulose deposited in this tissue, with nonregulated activity responsible for a basal rate of cellulose deposition which proceeds even in the absence of added hormone. The present study utilizes this same plant system and describes effects of IAA on the intracellular distribution of β -1,4-glucan synthetases. Attention is confined to membrane-bound activity leading to formation of alkali-insoluble glucan which is not lipid soluble. It is concluded that such synthetases are found in association with Golgi membrane and endoplasmic reticulum (ER) and are not active *in vivo*; but rather they represent enzymes in transit to extracellular site(s) of action which are readily inactivated during the usual methods of extraction.

MATERIALS AND METHODS

Plant Material

Seeds (*P. sativum* L. var. Alaska) were surface-sterilized in 0.5% NaOCl (20 min), soaked in tap water (8 h), and grown on vermiculite in darkness until third internodes reached 3–5 cm (7–8 days). Epicotyls were decapitated by removing the plumule and hook (the natural source of IAA), and the apical 10-mm region was marked to delineate a "segment" of tissue. Segments were analyzed either immediately (zero time) or after 2 days in darkness during which time the apex was covered with lanolin paste (40% H₂O, wt/wt) \pm IAA (approximately 10 μ g/apex). Manipulations were performed under dim green light. In the intact epicotyl, the apical 10-mm

segment encompasses the region of rapid elongation growth. Decapitation prevents elongation, and addition of IAA causes swelling (11).

Tissue Extraction Procedures

Routinely, 75–200 segments (4–7 g) were homogenized at 2°C in approximately 0.3 vol of an extraction medium containing 0.1 M Tris, pH 8.0, 0.4 M sucrose, and 5 mM dithiothreitol (DTT) in a Plexiglass tray using a motor-driven razor-blade chopper (22). After squeezing the homogenate through nylon, a filtrate was obtained from which components sedimenting between 500 g (20 min) and 130,000 g (20 min) were collected. This "particulate fraction" contained the membrane-bound material minus nuclei and cell wall debris. It was resuspended in 0.1 M Tris, pH 8.0, 5 mM DTT, and aliquots were used for estimating synthetase activities as described below.

For experiments requiring an isolated cell wall fraction (see Table III), the residue retained by nylon was further extracted at 2°C by grinding vigorously using a mortar and pestle and washing extensively (40–60 vol) in extraction medium to remove cytoplasmic contamination. The washed "wall fraction" was collected by centrifuging the brei for 20 min at 500 g.

Cellulose Estimations

Washed wall fraction was extracted three times with hot (85°C) H₂O followed by 2.5 N NaOH (3–4 h, 25°C) and hot (85°C) 1 N NaOH (two 1-h periods). The final alkali-insoluble residue was neutralized, dissolved in 72% (wt/wt) H₂SO₄ (25°C), and cellulose content was estimated with anthrone (15).

Sucrose Density Gradient Analysis

A 500-g supernate was prepared from tissue segments as above except that the extraction medium contained 0.1 mM MgCl₂. Aliquots of this supernate (2.0 ml, containing 2–9 mg particulate protein from 50 to 160 tissue segments) were layered on 10 ml of 25–55% (wt/vol) sucrose gradients (containing 0.1 M Tris, pH 8.0, and 5 mM DTT) and centrifuged at 2°C in an IEC SB 283 rotor at 20,000 rpm. Tests indicated that isopycnic sedimentation of β -1,4-glucan synthetase activity was obtained by 2 h. A total of 20 fractions (0.5 ml each) was collected and analyzed for various enzyme activities, turbidity (absorbance at 600 nm), protein content (Lowry method [20]), and refractive index. Sucrose density distribution in gradients did not change from experiment to experiment, and is therefore shown only in Figs. 1 and 3.

β -1,4-Glucan (Cellulose) Synthetase Assays

Assay mixtures (45–47) contained in a total volume of 275 μ l: 100 μ l resuspended particulate fraction (up to 0.6 mg protein), 100 μ l 0.1 M Tris, pH 8.0, 11 mM MgCl₂, and 5 mM cellobiose as activators, and either

UDP-[¹⁴C]glucose or GDP-[¹⁴C]glucose as substrates (600 μM, 1.5–2.5 μCi/μM or 6 μM, 150–275 μCi/μM). Incubations were carried out for 7 min at 35°C. Concentrations were such that the amounts of particulate protein present were rate limiting for the individual synthetase activities examined. Reactions were terminated by boiling (5–10 min), and 20 mg Whatman cellulose powder were added as carrier for radioactive products. The mixture was washed with hot (85°C) H₂O for three 10-min periods (15 ml total) followed by two 10-min washes with hot (85°C) 1 N NaOH (10 ml total). Radioactivity present in alkali-insoluble products was measured by suspending the neutralized residue in Aquasol (New England Nuclear, Boston, Mass.) and counting in a Beckman CPM 100 liquid scintillation system (90% efficiency).

Enzymatic hydrolysis of alkali-insoluble products obtained in these assays demonstrated (45) that most of the radioactivity derived from either high or low substrate concentrations of GDP-glucose or UDP-glucose was present in β-1,4-linked glucan. Further extraction with hot alkali (up to 2.5 N) did not change the yields of glucan in these mixtures. None of this alkali-insoluble product was lipid soluble and <10% was degraded to alkali-soluble material after extensive (50 h) incubation with purified β-1,3-endoglucanase. Consequently, activity is referred to in present tests as β-1,4-glucan (cellulose) synthetase, recognizing that it is due to several enzyme activities with different substrate specificities, and physical properties such as crystallinity and degree of polymerization have yet to be determined. One unit of activity is equivalent to 1 pmol [¹⁴C]glucose incorporated into alkali-insoluble glucan/7 min.

Marker Enzyme Assays

Association of IDPase with Golgi membrane of plant cells has been demonstrated by both cytochemical (7) and biochemical (35) techniques. In present tests, latent IDPase activity was measured in gradient fractions which had been stored at 2°C for 3 days (35). Aliquots (0.1 ml, containing up to 120 mg protein) were incubated at 35°C for 1 h in a reaction medium (final volume, 200 μl) containing 75 mM Tris, pH 7.8, 2.5 mM inosine diphosphate (IDP), and 1.0 mM MgCl₂. Activity is expressed as micromoles P_i released per hour per fraction. Inorganic phosphate was measured with the ferrous sulfate-molybdate reagent (48).

NADH cytochrome *c* reductase activity is present in the ER of animal (10, 27) and higher plant cells (18). It was measured by following the increase in absorbancy at 550 nm at 25°C when 50-μl aliquots from gradient fractions (containing up to 50 μg protein) were added to 2.5 ml of a solution containing 50 mM sodium phosphate, pH 7.5, 0.2 mM NADH, 1.7 mM KCN, and 30 μM cytochrome *c* (Sigma Chemical Co., St. Louis, Mo., type III). The rate of cytochrome *c* reduction was calculated using an extinction coefficient of 18.5 mM⁻¹ cm⁻¹ (14).

In Vivo Radioisotope Incorporation

Freshly excised segments (20–75) were floated for periods of up to 1 h at 25°C on 4.0 ml of a solution containing 0.1 mCi [U-¹⁴C]sucrose (680 cpm/pmol), 0.1 M sodium phosphate, pH 7.0, and 1 drop 10% Tween 80. After rinsing with a solution of unlabeled sucrose, segments were homogenized and radioactivity was measured in the particulate (membrane) fraction and the washed wall fraction. Chromatography showed that the extraction procedures removed all traces of labeled free sugars from these fractions.

Hydrolysis of Radioactive Glucans

Samples of radioactive products formed by excised segments of tissue supplied with [¹⁴C]sucrose, or by tissue slices, homogenates or membrane fractions supplied with GDP-[¹⁴C]glucose or UDP-[¹⁴C]glucose, were subjected to enzymic hydrolysis by adding purified preparations of fungal cellulase or β-1,3-glucanase. Reaction mixtures (0.1–0.3 ml) contained *Streptomyces* (QM B814) β-1,4-endoglucanase (cellulase) (1–5 mg/ml in 50 mM sodium acetate, pH 5.5, containing 3 mM NaN₃) or *Rhizopus arrhizus* β-1,3-glucanase (3 mg/ml in 50 mM citric-Na₂HPO₄, pH 4.5, containing 3 mM NaN₃), and incubations were carried out for up to 5 days at 50°C. Aliquots were chromatographed (descending, Whatman no. 1 paper) in *n*-propanol:ethylacetate:water (7:1:2) for 40 h and radioactivity was measured by cutting chromatographs into 1-cm sections and counting in scintillation fluid. Cellodextrins are hydrolyzed by cellulase to products including primarily cellobiose and glucose under these conditions (33, 36, 45). Almost complete degradation (>95%) of alkali-insoluble products to these two sugars was achieved in present tests by hydrolyzing for 5 days with high concentrations (5 mg/ml) of cellulase.

Electron Microscopy

Cross sections of tissue (approximately 1 mm thick) were taken from freshly decapitated epicotyls at a point 3–5 mm below the apex. They were fixed in 2.5% glutaraldehyde buffered with 0.1 M potassium phosphate (pH 7.3) for 2 h at 4°C. Sections were washed three times with buffer over a period of 1 h and were postfixed in buffered 1% OsO₄ (pH 7.3) for 3 h at room temperature. After washing thoroughly in H₂O, sections were dehydrated in acetone and embedded in Epon (21).

Gradient fractions were diluted with 0.1 M Tris, pH 8.0, containing 5 mM DTT until sucrose concentrations reached 0.4 M. Material pelleting from these fractions at 130,000 *g* (30 min) was fixed overnight (4°C) in a solution containing 2.0% glutaraldehyde, 0.4 M sucrose, and 0.1 M potassium phosphate, pH 7.3. It was postfixed for 1 h at room temperature in buffered 1% OsO₄. After dehydration in acetone, pellets, were embedded in Epon.

Membranes in thin sections were stained routinely using alkaline lead citrate (37). Alternatively, thin sec-

tions were subjected to a staining procedure (16, 41) specific for plant cell plasma membrane (39, 41) as follows: sections mounted on gold grids were floated on 1% periodic acid (30 min), washed for five 10-min periods in H₂O, and stained (5 min) with a solution containing 1% phosphotungstic acid (PTA) and 10% chromic acid. Excess stain was removed by a brief rinse in H₂O. Specimens were observed and photographed with a Philips EM 200.

Quantitative estimations of plasma membrane content of gradient fractions were made by measuring the amount of membrane material which stained with PTA as a percent of total membrane per fraction (which stained with lead citrate), using alternate groups of three to five sections cut from the same block. Using the method of Loud (19), photographs (8 × 10 inches, magnification approximately × 80,000) were overlaid with a transparency having a grid of parallel lines spaced 1 cm apart. All clearly stained membrane edges intersecting grid lines were scored. Estimations for each determination involved 2,000–5,000 intersections with lead citrate-positive material and 500–1,500 intersections with PTA-positive material. Sample variation between photographs was calculated for both staining procedures and yielded standard deviations for plasma membrane estimations which represented approximately 10% of values recorded (see Fig. 7).

Chemicals

GDP-[¹⁴C]glucose was purchased from ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif. and UDP-[¹⁴C]glucose from New England Nuclear. Unlabeled substrates were obtained from Sigma Chemical Co. Purified glucanases (samples S199A and S176L) were kindly supplied by Dr. E. T. Reese (U. S. Army Natick Laboratories).

RESULTS

Cellulose Deposition and β-1,4-Glucan (Cellulose) Synthetases

Table I records certain changes which occur in apical segments of pea epicotyls after removal of the plumule and hook and treatment of the cut apex ± IAA (see also 45). In the absence of hormone (control), segments do not grow, total particulate protein declines (–30% in 2 days), and net cellulose levels increase (+40% over zero-time value). Treatment with IAA causes lateral swelling of segments, a net increase in particulate protein (+30%), and a very substantial enhancement of cellulose deposition (eight times control increment). The amount of cellulose laid down in the decapitated epicotyl in response to IAA treatment is comparable to that formed by this same sector of tissue when it is left intact in the epicotyl.

Measurements of particulate β-1,4-glucan synthetase activities present in decapitated epicotyls were made using either high (600 μM) or low (6 μM) substrate concentrations. It was observed (Table I) either that activity declines drastically after decapitation but is maintained in the presence of IAA (using GDP-glucose or 6 μM UDP-glucose) or, that activity is stable (600 μM UDP-glucose) and not greatly affected by either aging after decapitation or treatment with hormone.

Sucrose Gradient Profiles

At zero time, isopycnic sedimentation profiles (Fig. 1) of the multiple β-1,4-glucan synthetase

TABLE I
IAA Stimulates Cellulose Deposition and Maintains Certain β-1,4-Glucan (Cellulose) Synthetase Activities in Decapitated Pea Epicotyls*

Treatment	Swelling	Protein	Cellulose	Synthetase activity			
				6 μM		600 μM	
				GDPG	UDPG	GDPG	UDPG
	<i>mg/mm</i>	<i>μg/seg</i>	<i>μg/seg</i>	<i>units/segment</i>			
Zero time	2.5	62	83	10.1	7.2	51.8	127
2 days, control	3.1	43	118	0.5	0.6	0.9	121
2 days, + IAA	6.7	81	374	12.8	7.9	55.7	124

* Epicotyls were decapitated at zero time and the apex was treated with lanolin ± IAA. The apical 10 mm (segment) did not elongate appreciably (10–20% in 2 days). Segments were chopped with razor blades at 2°C, the brei was squeezed through nylon, and cellulose was estimated as alkali-insoluble anthrone-positive material in the residue. The particulate (membrane) fraction which sedimented between 500 and 130,000 g was collected from the filtrate, and aliquots were tested for protein (20) and synthetase activity by assaying in a standard reaction medium containing [¹⁴C]glucose-labeled nucleoside diphosphates at concentrations indicated (see Materials and Methods). Activity units are picomoles [¹⁴C]glucose transferred to alkali-insoluble glucan/7 min/segment.

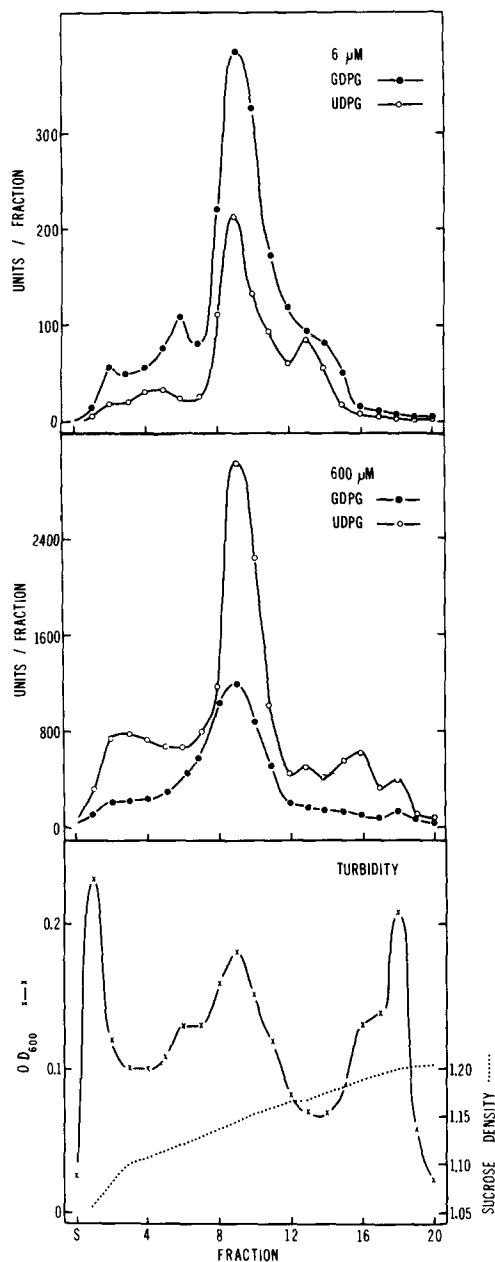


FIGURE 1 Isopycnic sedimentation of particulate β -1,4-glucan (cellulose) synthetase activities derived from growing regions of the pea epicotyl (apical segments at zero time). Approximately 4-g tissue segments (see Table I) were chopped with razor blades at 2°C in 0.3 vol of medium, the brei was squeezed through nylon, the filtrate centrifuged (500 g, 20 min), and the supernate (2.0 ml from 160 segments containing 8.8 mg particulate protein) was layered on a sucrose gradient and centrifuged (see Materials and Methods). Fractions (0.5 ml) were collected and aliquots (0.1 ml) were assayed in a standard

activities which use either UDP-glucose or GDP-glucose at 6 or 600 μ M show that all cosediment in a major peak (fractions 8–10) at an approximate density of 1.15 g/cm³. This corresponds to a peak of high turbidity in the gradient. Ray et al. (35) identified it as IDPase-rich Golgi membrane by electron microscope observation of purified samples.

In control (untreated) epicotyls after 2 days, the one synthetase (using 600 μ M UDP-glucose) which remains active after decapitation displays (Fig. 2) a gradient profile which now includes a major peak of activity (fractions 14–16) at approximately 1.18 g/cm³, in addition to the peak at 1.15 g/cm³ (fractions 8–10).

In profiles obtained from IAA-treated epicotyls (Fig. 3), all of the synthetase activities, which were also present in zero-time segments, continue to cosediment. A new major peak of activity appears (fractions 2–4), however, which sediments at approximately 1.11 g/cm³.

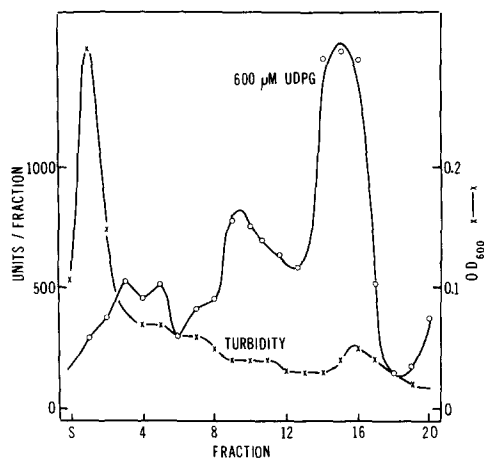


FIGURE 2 Sucrose gradient profile of the β -1,4-glucan (cellulose) synthetase which remains in epicotyls after decapitation (2-day controls, see Table I). A 500-g supernate (2.0 ml containing 3.6 mg particulate protein) extracted from 90 segments was overlaid on a gradient and centrifuged for 2 h at 20,000 rpm. Methods and activity units are described in Fig. 1. The major peaks of activity (fractions 9–10 and 14–16) are at densities of 1.15 and 1.18 g/cm³.

reaction mixture. All synthetase activity was present in the gradient, i.e., none remained in the supernate (S) or pelleted to the bottom of the tube. The major peak of all activities (fractions 8–10) occurred at a density of 1.15 g/cm³ (cf. IDPase distribution, Fig. 3).

Identification of Membrane Fractions

In profiles from IAA-treated epicotyls (Fig. 3), the synthetase activities which band at 1.15 g/cm^3 (fractions 8–10) cosediment with the major peaks of IDPase activity (Golgi marker) and those at 1.11 g/cm^3 (fractions 2–4) with the major component of NADH cytochrome *c* reductase activity (ER marker). The ER-rich region (fractions 2–4) is not clearly associated with a peak of turbidity. Nevertheless, these fractions contain a relatively homogeneous population of smooth membrane vesicles (Fig. 4). The major peak of turbidity nearby in fraction 1 (Figs. 1 and 3) contains most of the ribosomes in the gradient.

The heavy synthetase fraction (1.18 g/cm^3) in profiles from control tissue (Fig. 2) could hardly be expected to reside in mitochondria which are the main components responsible for turbidity in this region of the gradient. No effort was made to identify which of the minor components in this fraction contained this synthetase.

Plasma membrane in sectioned pea epicotyl cells stains specifically with PTA-chromic acid (Fig. 5, see also 39, 41). Specificity for this reagent is retained by isolated membrane fractions (Fig. 6, see also 41). This stain was used to determine the distribution of plasma membrane in pooled gradient fractions in relation to synthetase profiles (Fig. 7). At zero time, plasma membrane was concentrated at a density of approximately 1.13 g/cm^3 (fractions 5–7) which was distinct from the main location of synthetase activity (fraction 8–10, see also Fig. 1). Plasma membrane did not change density after IAA treatment, i.e., it remained at an intermediate level between the two main loci of synthetase activities appearing under these conditions (cf. Fig. 3). In the ER-rich fraction (density 1.11 g/cm^3) from IAA-treated tissue, plasma membrane accounted for less than 2% of the total membranous material.

Fig. 8 illustrates the effect of Mg^{++} concentration on the distribution of NADH cytochrome *c* reductase and β -1,4-glucan synthetase activities in membrane profiles from IAA-treated epicotyls. Extraction and sedimentation of particulate (membrane) material in the presence of relatively high Mg^{++} concentration (5 mM) results in a shift of about half of total reductase activity from fractions 2–5 to fractions 12–16. Analysis of RNA distribution in these experiments (legend Fig. 8, see also 18) indicates that the redistributed reductase peak is accompanied by redistributed ribosomes. High Mg^{++} concentration evidently conserves the

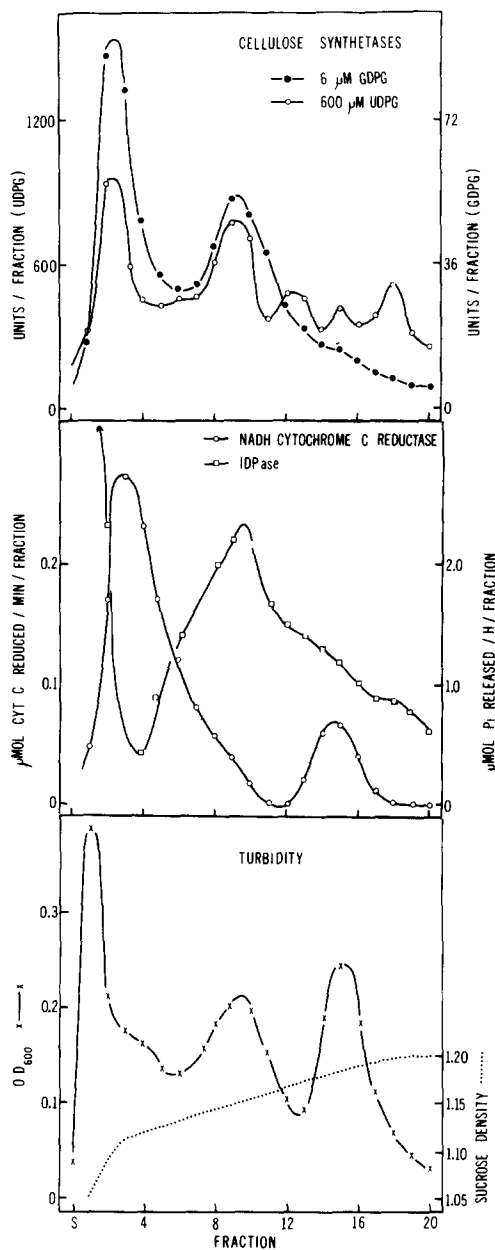


FIGURE 3 Sucrose gradient profiles of β -1,4-glucan (cellulose) synthetase and marker enzyme activities derived from IAA-treated epicotyl segments. A 500-g supernate (2.0 ml containing 4.6 mg particulate protein) extracted from 50 segments was overlaid on a gradient as described in Fig. 1. Marker enzymes were assayed as described in Materials and Methods. The major peaks of synthetase activities (fractions 2–4 and 8–10) are at densities of 1.11 and 1.15 g/cm^3 .

integrity of rough ER and leads to its sedimentation at a relatively high density. High Mg^{++}

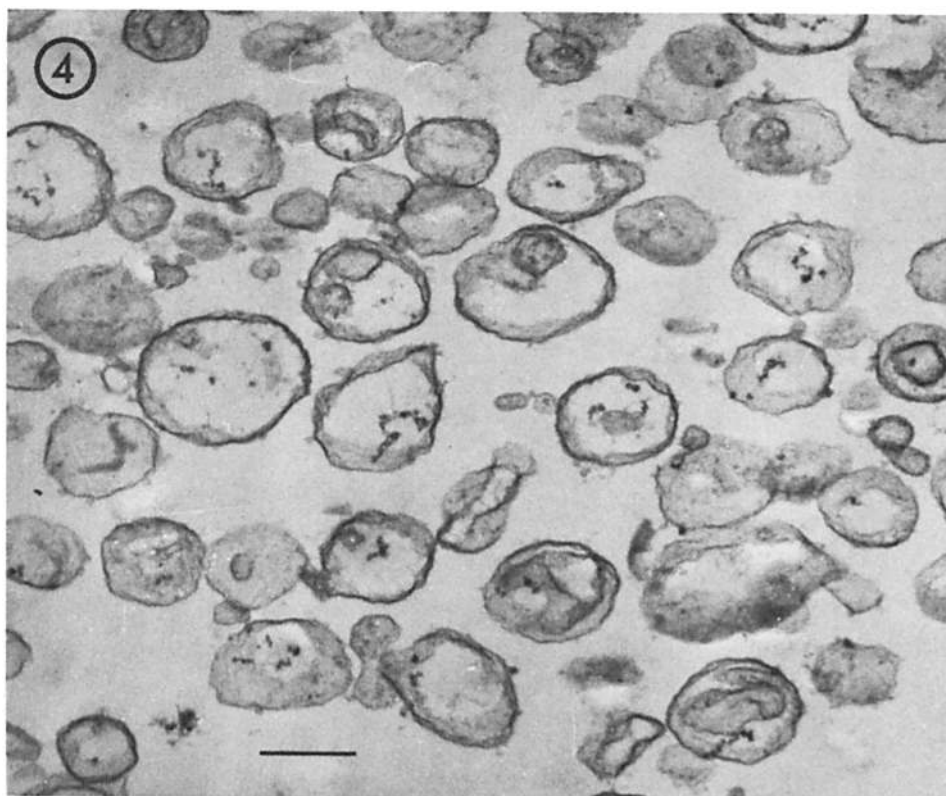


FIGURE 4 Membrane vesicles present in the ER-rich region of sucrose density gradients (fractions 2-4, Figs. 1 and 3). Membranes were stained using lead citrate (see Materials and Methods). $\times 65,000$. Bar: 0.2 μm .

concentration has *no* effect, however, on the distribution of synthetase activity or on about half of total reductase activity which remains at the lighter density. The β -1,4-glucan synthetase activities in the light fraction may be associated with an ER component which bears no ribosomes but retains part of total NADH cytochrome *c* reductase. Electron microscopy of cross sections through the epicotyl \pm IAA indicates that all *bilamellar* ER configurations *in situ* bear ribosomes (see Fig. 5). *Smooth* ER, therefore, may exist in a different configuration, presumably as smooth vesicles. Indeed, rough ER often terminates in what appears to be potential vesicles which do not bear ribosomes (see arrows Figs. 5, 3, and references 42, 52).

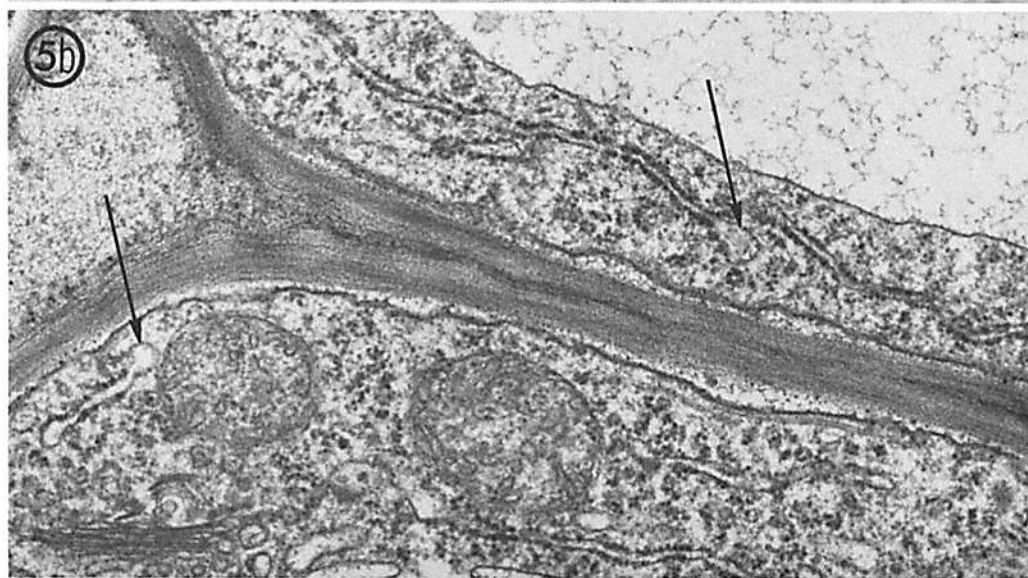
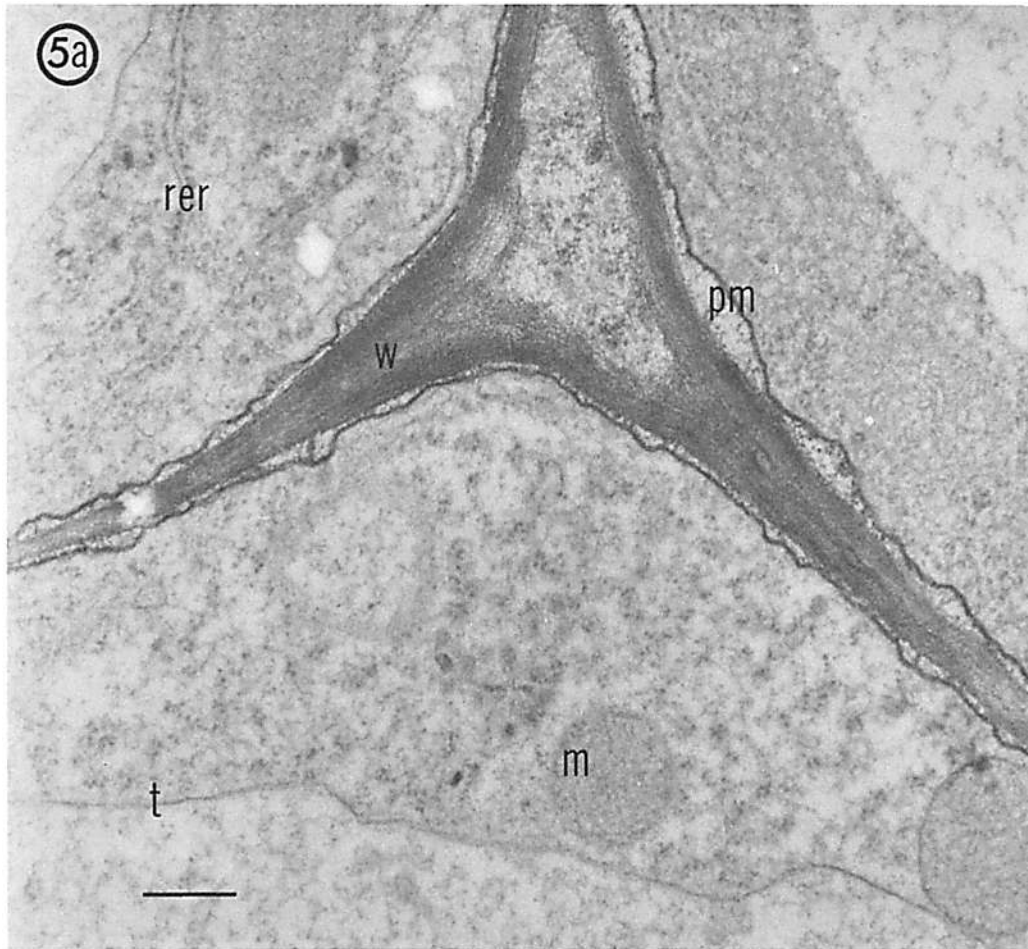
Formation of Cellulose In Vivo from [¹⁴C]Sucrose

Tests in which [¹⁴C]sucrose was supplied *in vivo* to freshly excised segments (Table II) revealed that no newly formed alkali-insoluble glucan (cellulose)

is detectable in the particulate (membrane) fractions which bear β -1,4-glucan (cellulose) synthetase activities. High molecular weight cellulose is found associated only with the wall fraction.

It is unlikely that the β -1,4-glucan synthetase activities recovered in membrane fractions were derived during extraction from the wall as a result of physical dislodging. The recovered activities were clearly associated only with certain intracellular organelles from which solubilization required treatment by detergent (unpublished data, 17, 49), *i.e.*, they were not merely adsorbed. It is also unlikely that alkali-insoluble glucan products were formed by intracellular organelles *in vivo*, released free in suspension, and then lost to the wall during extraction. For example, it can be shown that when membrane fractions are allowed to synthesize [¹⁴C]alkali-insoluble glucan *in vitro* and then are mixed with homogenized, unlabeled wall fractions and refractionated by standard procedures, most label (>85%) is retained by the membrane fraction, not the wall.

The only products formed from [¹⁴C]sucrose by



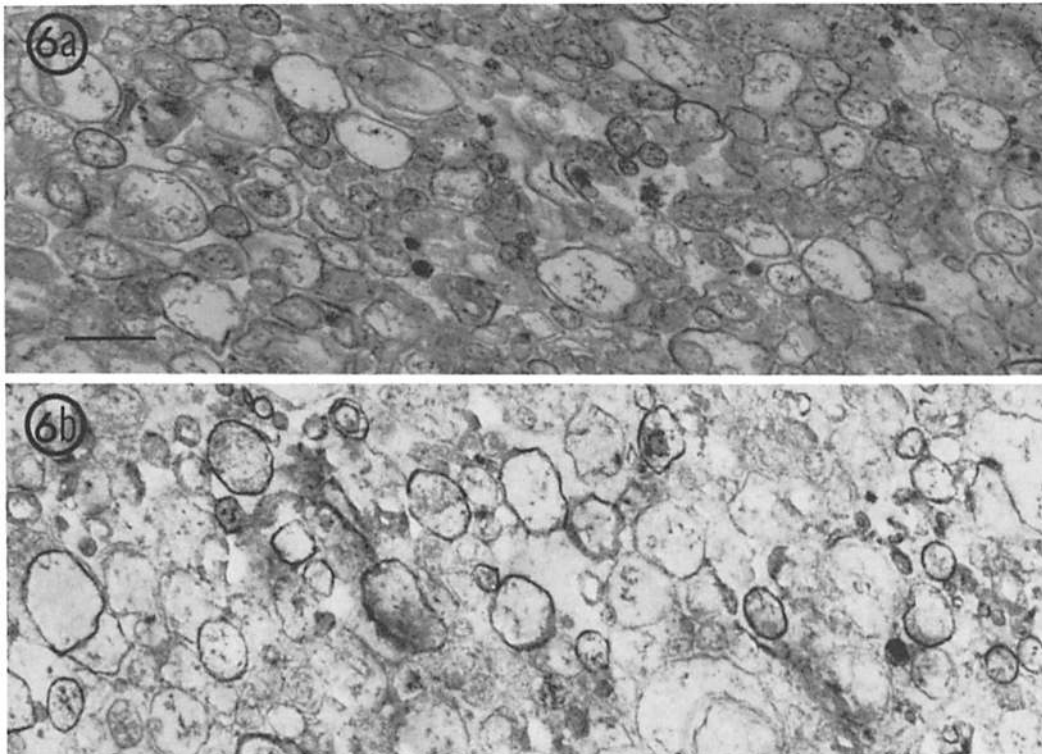


FIGURE 6 Identification of plasma membrane present in sucrose density gradients using PTA-chromic acid. Membrane collected from gradient fractions 5 to 7 in the experiment described in Fig. 7 was prepared for electron microscopy (see Materials and Methods) and thin sections were stained using either lead citrate (Fig. 6 a) or PTA-chromic acid (Fig. 6 b). $\times 41,000$. Bar: $0.3 \mu\text{m}$.

excised tissue segments *in vivo* and found after homogenization associated with membrane fractions were those soluble in hot water (Table II). These products cosedimented in gradients with the major membrane components bearing β -1,4-glucan synthetase activity (Fig. 9, see also 33). They were incubated with *Streptomyces* cellulase under conditions which are effective in degrading cellulose (36, 45); nevertheless, they failed to migrate in standard chromatographic solvents and none co-chromatographed with cellobiose. These products were evidently not low molecular weight celloextrin precursors of cellulose.

Formation of Alkali-Insoluble β -1,4-Glucan from UDP-Glucose by Semi-Intact Tissue

If alkali-insoluble glucan is not formed *in vivo* by intracellular membrane-bound synthetases, but rather at the cell surface, it may be reasoned (see also 8) that surface enzyme activity might accept sugar nucleotides as substrates supplied directly to intact cells.

As shown by data in Table III, relatively very high rates of formation of alkali-insoluble β -1,4-glucan can be obtained by supplying $600 \mu\text{M}$ UDP-glucose directly to pea tissue sections or

FIGURE 5 Plasma membrane of intact cells is the only membrane component which stains with PTA-chromic acid. Cross sections from zero-time segments taken 3–5 mm below the apex were prepared for electron microscopy as described in Materials and Methods. Thin sections were stained using either PTA-chromic acid (Fig. 5 a) or lead citrate (Fig. 5 b). Similar results were obtained using hormone-treated tissue. *m*, mitochondrion; *pm*, plasma membrane; *rer*, rough ER; *t*, tonoplast; *w*, wall; arrows point to blebbing of ER. $\times 42,000$. Bar: $0.3 \mu\text{m}$.

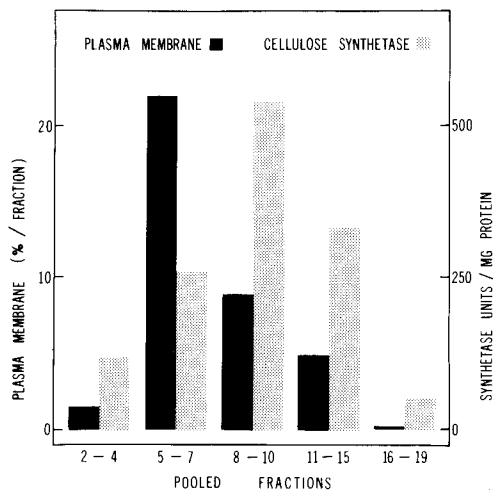


FIGURE 7 β -1,4-Glucan (cellulose) synthetase activity and plasma membrane are concentrated in different membrane fractions. A 500-g supernate derived from 150 zero-time segments was fractionated in a sucrose density gradient as described in Fig. 1. Selected 0.5-ml fractions were pooled and aliquots were assayed for synthetase specific activity using 6 μ M GDP-glucose as substrate. Protein levels of pooled fractions were as follows: fractions 2-4 (2.2 mg), 5-7 (1.5 mg), 8-10 (1.6 mg), 11-15 (2.2 mg), and 16-19 (1.7 mg). Membrane present in pooled fractions was identified in electron micrographs using either lead citrate or PTA stains (see Fig. 6). The relative amount of plasma membrane was estimated as described in Materials and Methods (expressed as percent of the total membrane present in each fraction). The plasma membrane profile in preparations from IAA-treated epicotyls was similar to the above (peak in fractions 5-7, with <2% in fractions 2-4). It did not correspond to the two peaks of cellulose synthetase activity (in fractions 2-4 and 8-10, Fig. 3).

fragments prepared by slicing, i.e., systems in which cellular organization remains comparatively intact. GDP-glucose was not utilized as effectively under these conditions. Progressive disruption of cell structure by grinding (\pm sand as an abrasive) resulted (Table III) in extensive loss in the amount of total synthetase activity measured per segment. When wall material (containing broken cells and trapped membrane) was separated from the total homogenate obtained by grinding, further losses in synthetase activity were observed, i.e., the sum of activities in the crude wall pellet plus cytoplasmic membranes (500-130,000-g pellet) represented about 50% of activity in the original homogenate. When the separated wall and membrane fractions were recombined and then assayed, a partial

recovery of the activity in the initial homogenate was observed.

The best rates of utilization of UDP-glucose for alkali-insoluble glucan synthesis by tissue fragments were at least 20 times the highest rates observed in experiments using isolated membrane

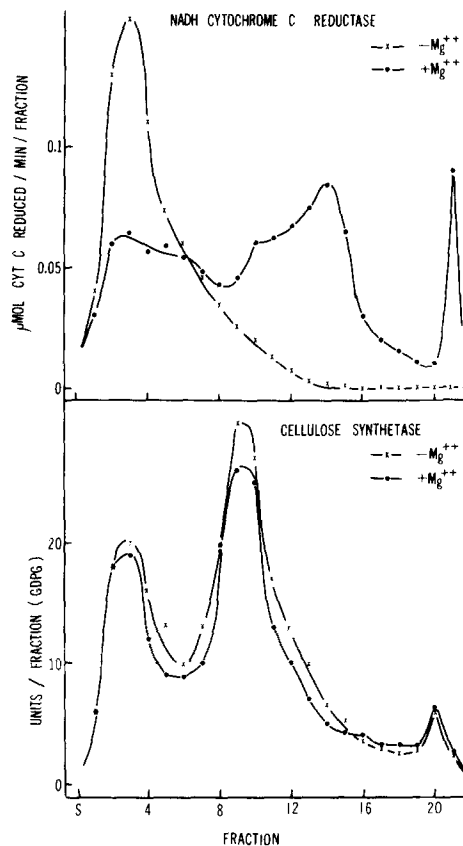


FIGURE 8 Effect of magnesium ions on marker enzyme and synthetase profiles. Segments from IAA-treated tissue were chopped in a standard medium (Fig. 1) or in a medium containing 5 mM $MgCl_2$ (2 vol). Particulate protein (2.5 mg from 45 segments) in the 500-g supernates (concentrated to 2.0 ml) from the two extracts was sedimented in corresponding sucrose gradients (formed on 70% wt/vol sucrose cushions) which either contained no $MgCl_2$ or 5 mM $MgCl_2$ (designated - or + Mg^{++} above). Fractions were assayed for GDP-glucose-dependent (6 μ M) β -1,4-glucan (cellulose) synthetase and NADH cytochrome *c* reductase as described in Materials and Methods. The sucrose density distributions in the two gradients (\pm Mg^{++}) were identical. The addition of Mg^{++} resulted in a shift in the sedimentation of RNA to the region of the gradient which acquired NADH cytochrome *c* reductase activity (fractions 12-16). This region acquired approximately 15% of total RNA compared to <1% in the absence of Mg^{++} .

TABLE II
*High Molecular Weight Cellulose Does Not Accumulate In Vivo in Association with Fractions Bearing β -1,4-Glucan (Cellulose) Synthetase Activity In Vitro**

Solubility of radioactive products	In vivo synthesis from [14 C]sucrose		In vitro synthesis from GDP-[14 C]glucose	
	Wall	Membrane	Wall	Membrane
	% total		% total	
H ₂ O (85°C)	15	31	1	1
CCl ₄ :CH ₃ OH:H ₂ O (50°C)	1	1	0	2
1 N NaOH (85°C)	17	1	1	49
NaOH insoluble (cellulose)	34	0	2	45

* For the experiments in vivo, epicotyl segments (2 day + IAA) were floated on radioactive sucrose for 1 h, homogenized, and separated into wall (extensively washed, see Materials and Methods) and membrane (particulate) fractions. Cold (2°C) water-insoluble material was extracted sequentially with hot water; warm chloroform:methanol:water (1:2:0.8); and hot alkali as in assays for synthetase activity, and the distribution of labeled products was determined (to the nearest 1%, 7,500 cpm total/segment). For tests in vitro, washed wall and membrane fractions were assayed for synthetase activity using 6 μ M GDP-glucose as substrate. Control (zero time) segments yielded similar results.

TABLE III
*Intact Cells Synthesize β -1,4-Glucan from UDP-glucose at Much Higher Rates Than Particulate Membrane Fractions**

Treatment of 10-mm apical epicotyl segments	Cellulose synthetase activity per segment \ddagger	
Cut into 1-mm sections	1,304	
Sliced into small fragments	1,848	
Homogenized by grinding	Minus abrasive	Plus abrasive
Total homogenate	912	665
0-500-g pellet	334	280
500-130,000-g pellet	68	85

* Epicotyl segments (zero time) were cut or sliced into relatively small pieces of tissue to facilitate uptake of substrate into intercellular spaces. The preparations were either used directly as source of synthetase, or they were homogenized by grinding in a mortar \pm sand (15 mg/g tissue). Tissue sections, fragments, or homogenates were transferred to conical graduated tubes and extraction medium was added until tissue plus medium reached a volume of 100 μ l. Assay components (including 600 μ M UDP-[14 C]glucose, 1.5 mCi/mM) were then added (275 μ l total, see Materials and Methods), and a thin glass rod was inserted to keep tissue fragments suspended during incubation and shaking. Formation of alkali-insoluble glucan proceeded at a constant rate for 12 min with no detectable lag period. The 0-500-g pellet (assayed as above) contained wall material, broken cells, and trapped membrane. The 500-130,000-g pellet contained the remaining particulate membrane material normally employed in assays for membrane-bound synthetase activity (see Materials and Methods and Table I). Negligible synthetase activity was recovered in the 130,000-g supernate.

\ddagger Synthetase activity is expressed as picomoles glucose incorporated into alkali-insoluble glucan/12 min/segment. Maximum rates of cellulose deposition in intact pea epicotyls (apical 10 mm) used in these experiments ranged from 3,000 to 6,000 pmol glucose incorporated/12 min/segment (data taken from increments measured in marked segments at 6-h intervals over a 48-h period of normal elongation, footnote 1).

fractions (see also 1, 4, 13), and approached the rates of cellulose deposition in the intact epicotyl (see legend Table III). That the product formed by tissue slices from UDP-glucose was almost entirely β -1,4-glucan (cellulose) was indicated by observations of the progress of hydrolysis by high concen-

trations (5 mg/ml) of *Streptomyces* cellulase. In early stages of the incubation (1–2 days), about half of the product degraded to glucose, cellobiose, and higher cellodextrins. By 5 days, most (>95%) was converted to glucose and cellobiose, with minor components cochromatographing with celotriose and cellotetraose. No trace of such hydrolysis products were observed after treatment with *Rhizopus* β -1,3-endoglucanase.

In assays using slices or fragments of tissue as source of synthetase (Table III), UDP-glucose or its immediate breakdown product glucose-1-phosphate would not be expected to diffuse readily across plasma membrane of intact cells. Chromatography of total reaction mixture from these experiments revealed that some [14 C]sucrose had been formed from UDP-glucose. However, in control tests where [14 C]sucrose or [14 C]glucose was supplied to tissue slices at the same concentration and specific activity (approximately 3 cpm/pmol) as UDP-[14 C]glucose, negligible radioactivity was incorporated into alkali-insoluble glucan. It appears, therefore, that incorporation in these experiments must have proceeded extracellularly directly from UDP-glucose rather than indirectly from a degradation or conversion product.

DISCUSSION

This study demonstrates that hormone treatment changes the intracellular distribution of β -1,4-glucan (cellulose) synthetases between membrane fractions. The problem is to assign a function to these synthetases, since the intracellular membranes do not appear to be sites of cellulose synthesis (Table II, Fig. 9). It is suggested that such loci in both elongating and swelling pea tissue represent synthetases in transit to sites of action at the cell wall:protoplast interface (i.e., the cell surface).

In elongating pea epicotyl tissue (zero-time segments), β -1,4-glucan synthetase activities recovered in membrane profiles after relatively gentle extraction procedures (chopping) are mainly associated with Golgi membrane (35 and Fig. 1). After IAA treatment, these activities are associated with Golgi plus a membrane fraction rich in smooth ER vesicles (Figs. 3 and 8). Plasma membrane in this latter fraction accounted for <2% of total membranous material (Fig. 7). Absolute identification of the ER as the new site bearing β -1,4-glucan synthetase activity after hormone treatment is very difficult, however, owing to

the possible presence in this gradient region of other smooth membranes (e.g., vacuolar). Nevertheless, in view of the evidence (Table II, Fig. 9, and references 5, 54) that alkali-insoluble cellulosic glucan is not *formed* by endocyttoplasmic membranes *in vivo*, it seems highly unlikely that β -1,4-glucan (cellulose) synthetase would accumulate in membranes or organelles other than those responsible (Golgi and ER) for exporting protein to the cell surface, and those at the surface itself.

Little synthetase activity was present in plasma membrane-rich regions of gradient profiles at any time (Figs. 1, 3, 7), in contrast to the situation in onion stem preparations (50) where this was a major locus (in addition to Golgi membrane) for such activity. Of course, plasma membrane, due to its intimate association with the cell wall, may be selectively entrapped by the wall residue or inactivated during normal homogenization and not entirely recovered in membrane profiles. Certainly, in peas, cell wall residues require extensive

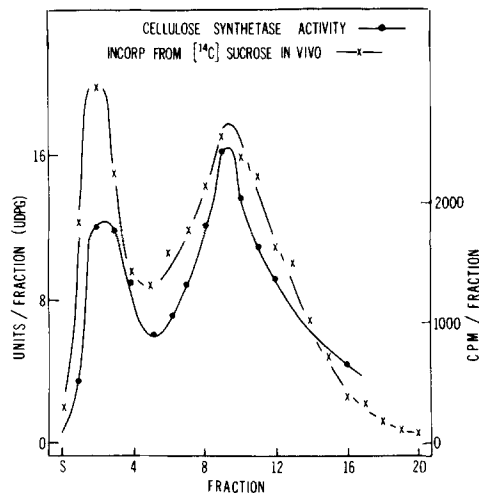


FIGURE 9 Hot water-soluble products formed from [14 C]sucrose *in vivo* and recovered in membrane fractions cosediment with β -1,4-glucan (cellulose) synthetase activity. IAA-treated segments were provided with [14 C]sucrose for 20 min and after homogenization, the particulate (membrane) fraction (500–130,000-g pellet) was resuspended in extraction medium (2.0 ml) and centrifuged in a sucrose gradient (see Materials and Methods and Fig. 3). Aliquots of gradient fractions were assayed for radioactivity incorporated from [14 C]sucrose (hot water soluble) (see also Table II), and for synthetase activity using 6 μ M UDP-glucose. Radioactivity derived from [14 C]sucrose which was alkali insoluble was too small (see Table II) to interfere with calculations of values for the synthetase assay.

grinding and washing to remove all membrane-bound synthetase activity (see Table I and Materials and Methods), and activity in homogenates is very easily lost when walls and membranes are separated (Table III).

The β -1,4-glucan synthetase activities which were recovered in membrane profiles in these experiments accounted for only about 5% of the rates of cellulose deposition commonly encountered in the intact epicotyl (see legend Table III). This relatively low activity in vitro has been noted before (1, 4, 13), and improvements in isolation techniques, assay conditions, etc. have failed to increase the activities to values which approach those in vivo. The rates did approach in vivo rates, however, when relatively intact tissue slices or fragments (containing intact cells) were used in the assay medium (Table III). The intent of such tests was to assay for extracellular synthesis directly from sugar nucleotides under conditions where wall:protoplast associations were minimally disturbed. It appears that cellulose synthetases at this site can operate effectively only insofar as the integrity of cellular organization is retained. Normal methods of homogenization evidently disrupted surface integrity to the point where essential components of the active cellulose synthetase systems were either separated (e.g., enzyme from microfibril primer), denatured, or selectively inactivated.

The Golgi complex is generally viewed as a transport organelle (see 23) and, in certain instances (42), ER has been shown to give rise to transport vesicles which operate independently of the Golgi complex (see also 3, 23, 40, 52). Neither organelle accumulated any trace of cellulose during synthesis in vivo from [14 C]sucrose (Table II and Fig. 9). Presumably, cellulose synthetases in these organelles are active in vitro only because all of the various components necessary for activity are provided in the assay medium (see 47). In view of the evidence for active cellulose synthesis only outside the protoplast (Table III and references 5, 54), the simplest interpretation of these observations is that cellulose synthetases recovered from gradients in association with Golgi and ER-rich fractions represent enzymes being transported to extracellular sites of action.

IAA greatly enhanced the deposition of cellulose in vivo (Table I), and this correlates well with effects of IAA on maintenance and/or generation of high activity levels of certain of the membrane-bound synthetases (45). The question now arises of

whether there is a special role for the cellulose synthetases which cosediment with ER membrane (Figs. 3, 8) after IAA treatment. Further tests¹ have indicated that such ER-associated synthetases are also found after other treatments (e.g., ethylene) which prevent normal elongation growth and result in tissue swelling, but not after treatments (e.g., gibberellic acid) which maintain the ability for elongation. They also appear during the normal completion of cell elongation in the intact epicotyl. There is strong evidence (2, 6, 9, 38, 43, 44, 51) that the orientation of newly formed cellulose microfibrils in lateral cell walls changes during transition from cell elongation (mainly transverse fibrils) to either maturation and/or swelling (mainly longitudinal fibrils). It may be that the Golgi-associated synthetases are destined to function in deposition of transversely oriented microfibrils (which do not impede elongation growth), whereas the ER-associated synthetases contribute to longitudinally oriented microfibrils (which prevent elongation but not lateral swelling). This possibility is currently under investigation.

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