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Regulation by Auxin of Carbohydrate Metabolism Involved in Cell Wall Synthesis by Pea Stem Tissue[1]

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ABSTRACT

Promotion of cell wall synthesis (from glucose) in pea (*Pisum sativum*) stem segments by indoleacetic acid (IAA) develops over a period of 1 to 2 hours and is comprised of a promotion of glucose uptake plus a promotion of the utilization of absorbed glucose. The effect of IAA resembles, in these and other respects, its effect on cell wall synthesis in oat coleoptile segments, but the pea system differs in not being inhibited by galactose or mannose, in involving considerably more isotope dilution by endogenous substrates, and in certain other respects.

Effector influences upon and total activities of the following enzymes obtained from etiolated pea stem segments pretreated with or without IAA were examined: phosphoglucomutase, uridine diphosphate glucose (UDP-glucose) pyrophosphorylase, nucleoside diphosphokinase, UDP-glucose dehydrogenase, inorganic pyrophosphatase, hexokinase (particulate and soluble), and UDP-glucose-β-1,4-glucan-glucosyl transferase (β-glucan synthetase). The first three enzymes mentioned exhibit high activity relative to the flux *in vivo*, do not appear to show physiologically significant effector responses, and are concluded not to be control points. UDP-glucose dehydrogenase activity is regulated by UDP-xylose. Hexokinase is a potential control point but does not exhibit regulatory effects related to the IAA response. β-Glucan synthetase is the only one of these enzymes with activity which is increased by treatment of tissue with IAA, and this may be responsible for the effect of IAA on wall synthesis.

Assays of metabolite pools support the conclusion that stimulation of polysaccharide synthesis by IAA is due partly to changes in hexokinase reaction rate resulting from an increase in metabolic glucose pool size caused by increased glucose uptake, and partly to increased activity at the polysaccharide synthetase level.

A promotive effect of auxin on synthesis of cell wall materials is known in a number of tissues that are capable of a cell enlargement response to auxin (2, 7–10, 24, 37, 39). The effect has been studied most extensively with oat coleoptiles. This report describes characteristics of the wall synthesis response to auxin in pea stem tissue and presents evidence regarding the biochemical sites of regulation *in vivo* of wall polysaccharide synthesis.

MATERIALS AND METHODS

Plant Material. Pea seedlings (*Pisum sativum* L., cv. Alaska) were grown in vermiculite for 7 days in the dark at 25 C and 70% relative humidity, with occasional exposure to dim red light (60-w ruby red lamp). Under similar light, segments 8 mm long were cut from the third internode beginning 3 mm below the top of the apical hook. After pretreatment in water with or without IAA and other additions the tissue was incubated in uniformly labeled ^{14}C-glucose normally containing 0.05 M unlabeled glucose and other components as indicated in the individual experiments. After incubation, the tissue was washed, frozen, homogenized, and assayed for soluble and cell wall-bound radioactivity as previously described (38).

Further fractionation of the cell wall material (Table I) was performed directly on stainless steel planchets by the same procedure as used in Table III of Reference 39.

Percentage incorporation into the cell wall is calculated on the basis of total uptake estimated as the sum of the cell wall incorporation and water-soluble uptake, *i.e.*, not including activity released as CO_2, which is a small fraction of the total uptake, as shown by data in Table VI.

Enzyme Assays. The methods used for extraction of each enzyme were devised in preliminary experiments, beginning with methods described in the literature, in which we sought to obtain reproducible and maximal yields of activity and to optimize the conditions of assay. Chilled tissue was ground in a mortar at 0 C in 10 to 20 mM potassium phosphate buffer, pH 7.0 to 7.5 except as noted below. Cell wall debris was removed by low speed centrifugation. Total activity of soluble enzymes was obtained by assaying the supernatant solution after centrifugation at 10,000*g* for 20 min (phosphoglucomutase [11], UDP-glucose pyrophosphorylase [30; cf. 18], nucleoside diphosphokinase [4], UDP-glucose dehydrogenase [43], inorganic pyrophosphatase, soluble hexokinase). Except for the last two, the activities were assayed spectrophotometrically at 23 C by following the reduction either of NADP (first three enzymes mentioned) by coupling to glucose-6-P dehydrogenase through addition of intermediary enzymes as required, or the reduction of NAD in case of UDP-glucose dehydrogenase. Nucleoside diphosphokinase was also assayed in the reverse direction by coupling to lactic dehydrogenase and NADH via pyruvate kinase (1). Inorganic pyrophosphatase (the homogenization was in 0.1 M tris, pH 7.5) was assayed by the determination of P_i produced from pyrophosphate (21).

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Particulate and soluble hexokinases were assayed radioisotopically (33) at 25 C. The homogenization medium for hexokinase extraction contained 0.15 M KCl and 1 mM EDTA in addition to phosphate, and the particles precipitated by centrifugation at 10,000*g* (20 min) were resuspended in the same medium and similarly reprecipitated prior to suspension in 1 mM EDTA-0.04 M tris, pH 8.0, for assay. The soluble hexokinase was dialyzed against repeated changes of the latter medium prior to assay.

Tests of effector influences were performed upon enzyme preparations fractionated from crude homogenates as follows. Phosphoglucomutase was precipitated by 70% saturated $(NH_4)_2SO_4$. UDP-glucose pyrophosphorylase was precipitated between 40 and 80% saturated $(NH_4)_2SO_4$ (16), then fractionated through Sephadex G-150 with 0.1 M tris, pH 7.0, to give about a 70-fold increase in specific activity. UDP-glucose dehydrogenase was prepared and purified through the second $(NH_4)_2SO_4$ precipitation of the procedure of Strominger and Mapson (44), giving about 15-fold increase in specific activity. Nucleoside diphosphokinase was purified by treatment of the homogenate for 1 min at 55 C followed by precipitation between 35 and 55% saturated $(NH_4)_2SO_4$ (25), and fractionation through Sephadex G-150 with 0.1 M tris-acetate buffer, pH 7.5.

Glucan synthetase was extracted by grinding with 0.1 M tris buffer, pH 7.5 (3) or, in later experiments, in the homogenizing medium given in Reference 40. The homogenate was centrifuged at 1,000*g* for 2 min, and the precipitate was resuspended in homogenizing medium, ground a second time in the mortar, and centrifuged as before. The supernatants were combined and centrifuged for 45 min at 38,000*g*. The resulting precipitate was suspended in homogenizing medium and assayed with ^{14}C-UDP-glucose as described (40). In contrast to earlier experiments (38) in which tissue was ground only once in tris buffer, not more than 10% of the total detectable glucan synthetase activity was retained in the 1,000*g* precipitate, an amount of activity too small to assay accurately because of the bulkiness of this material. The glucan synthetase particles from this tissue have been isolated and characterized as reported elsewhere (40).

Extraction and Assay of Metabolites (Table IV). Frozen tissue was ground in 1.5 ml of ice-cold 6% (w/v) perchloric acid, centrifuged 2 min at 1,000*g*, ground with another 1.5 ml of perchloric acid, and centrifuged, the two supernatants being combined. After bringing the pH to 6.8 with KOH or K_2CO_3, the sample was kept in ice for 30 min and centrifuged to remove $KClO_4$.

Metabolites were determined enzymatically by the glucose-6-P dehydrogenase method (6), with successive addition of phosphoglucomutase and UDP-glucose pyrophosphorylase plus pyrophosphate, to determine glucose-1-P and UDP-glucose, or of hexokinase to determine ATP. The UDP-glucose pyrophosphorylase used in these assays was purified from pea tissue as described in the preceding section; the enzyme was inactive with GDP-glucose. The remaining enzymes used in metabolite assays and as auxiliaries in the enzyme assays of the preceding section were obtained from Sigma Chemical Co.[2] and were the highest purity grade available.

Metabolite Fractionation (Table VI). Frozen tissue was ground in a cold mortar in ice-cold water and centrifuged at 3,000*g* for 10 min. The pellet (cell wall material) was washed twice with cold water, and the combined supernatants were kept in boiling water for 10 min to precipitate protein, then cooled and centrifuged. The precipitated protein and the cell wall material were washed again with cold water and finally with absolute ethanol. The boiled supernatant was made 80% (v/v) in ethanol, kept at 0 C for 1.5 hr, and then centrifuged. The precipitate (cold water-soluble polysaccharides) was washed twice in 95% ethanol, and the washings were combined with the original supernatant. The ethanol-soluble fraction was evaporated at reduced pressure, then passed through Dowex 50 (H^+). The effluent was passed through Dowex 1 (acetate). The resulting neutral fraction was freeze-dried. The Dowex 50 and Dowex 1 were each eluted with 45 ml of 1 N HCl, yielding the basic (amino acid) fraction and acidic fraction, respectively. These were evaporated at reduced pressure and finally freeze-dried. The neutral fraction was chromatographed on Whatman No. 1 paper with 2-butanone-acetone-water-pyridine, 150:30:20:1 (v/v) to separate fructose, glucose, and sucrose, which were located by scanning the paper for radioactivity and comparing with known markers, and eluted.

RESULTS

Figure 1 illustrates the wall synthesis response of pea stem segments to IAA. The response is completed in about 2 hr and is reversible, over a period of several hours, upon removal from auxin. After a 2-hr pretreatment with IAA the cell wall incorporation obtained during a 1-hr ^{14}C-glucose incubation was not dependent upon the presence of IAA in the incubation medium.

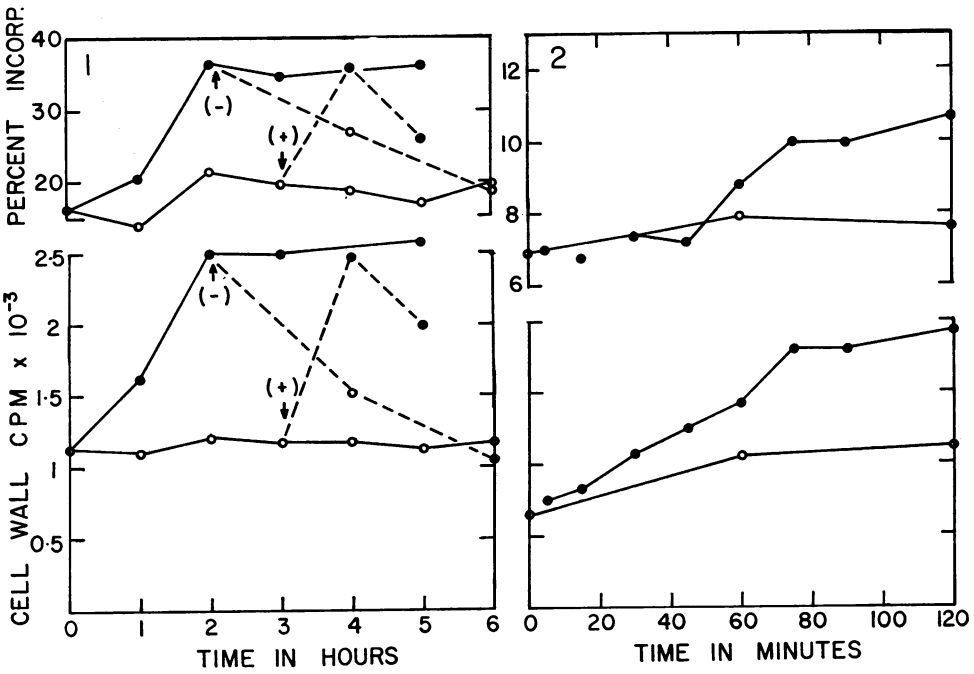
Figure 2 shows a more detailed time course for the response, obtained by incubating for only 20 min in ^{14}C-glucose after increasingly long treatments with auxin. Because this incubation is well within the isotope transient period for incorporation into the cell wall (*cf.* Fig. 3), the percentage incorporation into the cell wall was small, but the data indicate that the promotive effect of auxin appears about 1 hr after exposure to auxin.

The promotion of wall synthesis by IAA occurs in the presence of 0.02 M $CaCl_2$, 0.1 M mannitol, or 0.084 M galactose, all of which substantially inhibit elongation. Therefore, the auxin effect on wall synthesis is not due merely to elongation. However, IAA did not promote wall synthesis when given in the presence of 0.2 M mannitol.

Table I shows that IAA promotes incorporation into all cell wall fractions, whether given in the absence or presence of Ca^{2+}. In the absence of Ca^{2+}, IAA promotes uptake of labeled glucose, whereas in the presence of 0.02 M $CaCl_2$ IAA does not increase the rate of glucose uptake (Table I). Although the absolute effect of IAA on cell wall incorporation was larger in the absence than in the presence of Ca^{2+}, the effect of IAA upon percentage incorporation (incorporation as percentage of total uptake) was similar in the absence and presence of Ca^{2+}. Experiments showed that when the rate of glucose uptake was increased either by increasing the external concentration (Table II) or by cutting the stem segments in half lengthwise, cell wall incorporation increased similarly and the percentage incorporation remained unchanged. Therefore, the promotion of cell wall incorporation by IAA in the absence of Ca^{2+} is compounded of an intrinsic promotion (measurable as an increase in percentage incorporation) and a promotion of glucose uptake.

The gravimetric experiments shown in Table II were performed to relate radioisotope incorporation to actual wall synthesis. Supplying the tissue with glucose at substrate levels (25–50 mM) markedly increases wall synthesis above that which is due to endogenous substrates when micromolar lev-

[2] Mention of specific instruments or trade names is made for identification purposes only and does not imply any endorsement by the United States Government.



FIGS. 1 AND 2. Time courses of response of cell wall synthesis to supply and withdrawal of IAA. Segments were kept in water (open points) or in 3 μg/ml IAA (solid points) for times indicated on abscissa, then incubated in 0.02 M $CaCl_2$-0.05 M ^{14}C-glucose, for 1 hr in Figure 1 (left), or for 20 min in Figure 2 (right). Points on dashed lines in Figure 1 show segments which were transferred from water to auxin (+) or vice versa (−) at times shown by arrows. Upper part of each figure shows cell wall incorporation as percentage of total uptake. Each point is mean of three replicate samples of 10 segments.

els of glucose are fed. Wall synthesis from endogenous substrates is only slightly promoted by IAA, and there is no appreciable effect of IAA on isotope incorporation into the cell wall when labeled glucose is supplied at micromolar levels.

Even in 50 mM glucose the tissue utilizes substantial amounts of endogenous substrate for wall synthesis as shown by the difference between the absolute increase of wall material and the increase due to incorporation of labeled glucose.

Characteristics of the response of cell wall synthesis to auxin in pea stem segments, just described, resemble for the most part the comparable response observed in oat coleoptiles (2). Differences are (*a*) peas utilize much more endogenous substrate for wall synthesis than do oats; (*b*) wall synthesis in peas is insensitive to inhibition by exogenous galactose or mannose; (*c*) auxin promotes incorporation into α-cellulose in peas even when elongation is inhibited by Ca^{2+}, but not in oats (39); and (*d*) the effect of auxin on wall synthesis in peas is less sensitive to inhibitors of RNA and protein synthesis than in oats (38).

Enzyme Studies. The enzymes listed in Table III were investigated because of their probable involvement in formation of UDP-sugars, and thence polysaccharides, from glucose (*cf.* Fig 4).

Effects of hexose phosphates, nucleotide phosphates, and sugar nucleotides on activities of these enzymes were examined in a search for regulatory behavior that might be significant in the control of polysaccharide synthesis. The effects found are indicated in Table III. Certain of these effects are in agreement with previous reports (15, 31, 34). Inhibition of UDP-glucose pyrophosphorylase by high concentrations of UDP-glucose may be related to the much more sensitive inhibition of this enzyme by UDP-glucose reported by Tsuboi *et al.* (48). In their assays the reaction was run in the direction opposite to that used in the present assay, and it is possible that formation of UDP-glucose from UTP and glucose-6-P by pea UDP-glucose pyrophosphorylase is more sensitive to inhibition by UDP-glucose than is the pyrophosphorolysis of UDP-glucose shown in Table III.

Table I. *Effect of IAA on Incorporation into Pea Stem Tissue*

Stem segments were kept for 3 hr in water, then pretreated for 3 hr in water, 3 μg/ml IAA, 0.02 M $CaCl_2$, or IAA + $CaCl_2$, and finally incubated for 2 hr in 0.02 M $CaCl_2$/0.05 M glucose containing 2.6 μc/ml ^{14}C-glucose. Each figure is the mean of three replicate samples of 10 segments each.

Pretreatment	Final Elongation	Water-soluble Uptake	Water-soluble Polysaccharide[1]	Cell Wall Fractions				
				Hot water	Dimethyl sulfoxide	Ammonium oxalate	KOH (4N)	Cellulose residue
	%	*cpm*	*cpm*	*cpm*				
Water	10.0	93,600	2,930	1,170	410	960	1,840	6,480
IAA	27.3	108,300	3,750	2,540	810	1,680	3,760	15,200
$CaCl_2$	6.6	83,000	1,180	820	360	690	1,440	4,110
$CaCl_2$ + IAA	8.3	84,100	2,630	1,440	740	1,600	3,380	8,320

[1] Polysaccharides soluble during homogenization of tissue and subsequently precipitated by 80% ethanol.

No activation effects, comparable to those that have been reported for certain other synthetase systems (35, 46), were found for the pea synthetase system with cellobiose, fructose, sucrose, glucose, or glycerol.

Table III shows total activity of enzymes from tissue segments that had been pretreated with or without IAA. The data quoted are individual experiments from among two or three similar experiments of this type that were carried out on each enzyme listed. Among the enzymes investigated, UDP-glucose-dependent glucan synthetase was the only one with activity affected by an IAA pretreatment; this effect has been confirmed in numerous additional experiments. The effect re-

Table II. *Comparison of Wall Synthesis Measured Isotopically and Gravimetrically*

Samples of 50 segments were incubated 12 hr in labeled glucose (0.29 μc/ml in experiment 1, 0.047 μc/ml in experiment 2), either undiluted or with unlabeled glucose added to give concentrations of 0.025 or 0.05 M as indicated, and with or without 3 μg/ml IAA. Each figure is the mean of three (experiment 1) or four (experiment 2) replicate samples. The gravimetric increase in wall material (column 1, ± standard deviation) was calculated by subtracting the mean cell wall weight of initial samples, which was 14.26 mg in experiment 1 and 15.39 mg in experiment 2. The uptake and incorporation of labeled carbohydrate was calculated in gravimetric terms (third and fifth columns), on an anhydroglucose basis, from the known specific radioactivity of the glucose supplied.

Treatment	Increase in Cell Wall	Incorporation into Cell Wall		Glucose Uptake		Incorporation
	mg	*dpm* × 10^{-3}	*mg or* μg[1]	*dpm* × 10^{-3}	*mg or* μg[1]	%
Experiment 1						
1.3×10^{-6} M glucose	1.84 ± 0.11	618	0.19*	1638	0.50*	38
Same + IAA	2.18 ± 0.47	606	0.18*	1750	0.53*	34
0.05 M glucose	4.06 ± 0.16	133	1.67	502	6.26	26
Same + IAA	5.93 ± 0.14	236	2.95	640	7.96	37
Experiment 2						
4.7×10^{-6} M glucose	2.74 ± 0.11	89.3	0.66*	169	1.24*	53
Same + IAA	3.15 ± 0.42	89.4	0.66*	145	1.07*	61
0.025 M glucose + IAA	5.65 ± 0.15	45.0	1.77	112	4.43	40
0.05 M glucose + IAA	7.11 ± 0.25	36.1	2.86	97	7.56	37

[1] Figures marked with (*) are in micrograms, remaining figures in milligrams.

sembles that reported for oat coleoptile tissue by Hall and Ordin (19). In our early experiments (38) the IAA-induced increase in synthetase activity was relatively small, but in experiments under optimal conditions, to be described elsewhere, a 2- to 3-fold effect is obtained.

In repeated trials no effect was obtained by addition of IAA, over a wide concentration range, to synthetase preparations using either total particles, obtained as described in "Materials and Methods," or isolated synthetase particles, obtained as in Reference 40, whether the IAA was added before or during assay. No improvement in the effect of an IAA pretreatment was obtained by including IAA in the media used for tissue homogenization and resuspension of synthetase particles.

Metabolite Assays. A series of experiments was performed to determine how incubation with and without IAA affects the *in vivo* levels of metabolites that are important in the conversion of glucose to polysaccharides. Aggregate results of a number of experiments are given in Table IV. The levels of glucose phosphates and UDP-glucose declined markedly during incubation without glucose; this decline was largely prevented by feeding glucose. Treatment with IAA in the absence of glucose caused a consistent increase in ATP level relative to the control, but IAA did *not* cause an appreciable increase in the level of ATP when the incubation medium contained glucose. In short term (10–30 min) experiments no effects of IAA on the ATP pool comparable to those reported in the literature (27, 42, 47) were detected.

IAA caused a depletion of UDP-glucose and glucose phosphates, especially under treatment without exogenous sugar. The effect on hexose phosphate pools resembles that inferred for *Avena* coleoptiles, from ^{32}P labeling experiments, by Trewavas *et al.* (47), who found, however, an increase in the apparent UDP-glucose pool, contrary to our results.

Metabolic Glucose Pool Size. We previously suggested (38) that a compartmentation effect influencing the size of the metabolically active glucose pool could be the basis for regulation of carbon flow from glucose to polysaccharides. This idea was tested by determining the size of the internal isotope-trapping pool between glucose and wall polysaccharides in terms of the kinetics of the isotope-transient period.

As shown in Figure 3, the isotope-transient period following a transfer from 0.05 M unlabeled glucose to the same concentration of ^{14}C-labeled glucose lasts about 45 min and leads into a steady state of constant incorporation rate. By extrapolation of this rate back to zero time (dotted lines in Fig. 3) the amount of radioactivity that is trapped, at isotope equilibrium, in all pools between the fed glucose and the wall polymers can be determined. From this radioactivity the quantity of material in these pools can be estimated by dividing by the specific radioactivity of the glucose supplied and multiplying by the isotope dilution factor found, from data in Table II, for cell wall incorporation from external glucose under these conditions. We expected that any major effect on the size of the metabolic glucose pool would be detectable as an effect on the measured trapping pool size.

Results of several representative isotope-transient experiments of this type are listed in Table V. The calculated size of the isotope-trapping pool is about 2 μmoles/100 segments, substantially larger than the sum of glucose phosphates and UDP-glucose (1.3–1.4 μmoles/100 segments) determined for such tissue (Table IV). Therefore, the metabolic glucose pool size may be about 0.5 μmole/100 segments. Data in Table V indicate an increase by IAA in the size of the isotope-trapping pool in the case of incubation in glucose without Ca^{2+}, as is to be expected since under these conditions IAA treatment increases glucose uptake. In the presence of 0.02 M $CaCl_2$, under which conditions glucose uptake is not increased by IAA, there was only a small effect of IAA on the calculated trapping pool size.

Metabolic Balance Sheet. Table VI presents data on conversion of glucose into major classes of metabolic products. When the ^{14}C-glucose incubation was performed in presence of 0.02 M $CaCl_2$ (experiment 1), so that treatment with IAA would cause no increase in glucose uptake to complicate the interpretation, the increase in incorporation from ^{14}C-glucose into cell wall and soluble polymers caused by IAA was accompanied by a decreased incorporation into all classes of soluble metabolites, both amino acids, organic acids, and sugars other than glucose. Only a minor proportion of the increased cell wall incorporation could be attributed to a decrease in uptake into the glucose storage pool (step b in Fig. 4).

When Ca^{2+} was not added to the ^{14}C-glucose incubation medium (experiment 2, Table VI), incorporation into soluble cell constituents other than glucose was almost as great in IAA-treated tissue as in the control, and the total production of metabolites from glucose was increased over the control. IAA increased the accumulation of radioactivity as free glucose within the tissue, as was to be expected in view of the increased uptake of glucose that IAA induces in the absence of Ca^{2+}.

DISCUSSION

The problem of how cell wall polymer synthesis from glucose is regulated in response to auxin and to exogenous sugar

Table III. *Enzyme Activities from Pea Stem Tissue*

Enzyme[1]	Total Activity in Tissue[2]		K_m (substrate)	Negative Effectors (C_{50} values)[3]
	Control	IAA-treated		
	μmoles/min × 100 segments		*mM*	*mM*
Hexokinase[c]				
Particulate	0.410 ± 0.006	0.381 ± 0.031	3.5 (ATP), 0.2 (glucose)	ADP (1.1), GDP (2.5), UDP (1.9)
Soluble	0.104 ± 0.003	0.102 ± 0.003	...	...
Phosphoglucomutase[d]	2.47 ± 0.14	2.52 ± 0.20	0.7 (glucose-1-P)	Galactose-1-P (5.5)
UDP-glucose pyrophosphorylase[e]	33.0 ± 1.6	34.8 ± 2.0	0.25 (UDP-glucose)	UDP-glucose,[4] UDP-galactose (10), UDP-xylose (8), galactose-1-P (10)
UDP-glucose dehydrogenase[f]	0.065 ± 0.001	0.063 ± 0.005	0.05 (UDP-glucose)	UDP-xylose (0.03), UDP-glucuronic acid (1.0), UDP-galactose (4)
Nucleoside diphosphokinase[g]	10.2 ± 0.2	9.5 ± 0.05	0.7 (UTP)	ADP (0.25), UDP (0.25)
Inorganic pyrophosphatase[h]	0.25 ± 0.004	0.27 ± 0.10	...	...
β-Glucan synthetase[i]	0.181[5] ± 0.003	0.29[5] ± 0.012	0.04 (UDP-glucose)[6]	UDP (2.3), UTP (4.0), GTP (3)

[1] Superscript letters refer to the designated reactions in Figure 4.

[2] Assays were performed at substrate saturation except in the case of hexokinase and glucan synthetase. Hexokinase was assayed at 1 mM glucose and the activity at 50 mM glucose (substrate saturation) calculated by multiplying by the factor 1.6 as estimated from separate kinetic experiments. Tissue initial fresh weight (prior to 2-hr pretreatment in water or 3 μg/liter IAA) was 1.9 g/100 segments. Each assay is the mean for triplicate samples of tissue and is the result obtained from one of two or more comparable experiments.

[3] C_{50}, in parentheses, is concentration of effector giving 50% inhibition. Tests were made using substrate concentration(s) equal or close to Km, excepting glucan synthetase (2 μM UDP-glucose) and hexokinase (1 mM glucose, 5 mM Mg^{2+} ATP).

[4] 20 mM UDP-glucose gave 33% inhibition relative to V_{max} (5.0 mM UDP-glucose).

[5] β-Glucan synthetase activity, assayed with 2 μM UDP-glucose as described in "Materials and Methods," is given in nanomoles per min per 100 tissue segments.

[6] In the 1 to 200 μM range, glucan synthetase exhibits an apparent Km of about 40 μM for UDP-glucose, but incorporation continues to increase with concentration of UDP-glucose, with no indication of true substrate saturation, at least to 1 mM UDP-glucose at which concentration the apparent reaction rate is about 100 times that assayed in the standard assay with about 2 μM UDP-glucose.

Table IV. *Levels of Metabolites in Pea Stem Segments*

Each figure represents the mean and standard error of the mean of three to seven experiments in each of which three to four replicate samples of tissue were extracted and assayed. Each experiment included minus and plus IAA (3 μg/ml) treatments run simultaneously. ATP was assayed in separate experiments from those in which glucose-1-P and UDP-glucose were assayed; glucose-6-P was assayed in both types of experiments and the results of all of these have been averaged in the figures quoted. Initial fresh weight was 1.9 g/100 segments.

Compound	Initial	After 2-hr Pretreatment			
		Without glucose		With 0.05 M glucose	
		Minus IAA	Plus IAA	Minus IAA	Plus IAA
	nmoles/100 segments				
Glucose-6-P	1081 ± 21	657 ± 20	465 ± 21	910 ± 21	866 ± 20
Glucose-1-P	38 ± 3	29 ± 2	22 ± 2	29 ± 2	21 ± 3
UDP-glucose	544 ± 10	376 ± 9	277 ± 4	502 ± 13	432 ± 15
ATP	111 ± 7	56 ± 5	76 ± 5	69 ± 4	65 ± 4

supply will be considered with reference to the metabolic diagram in Figure 4. The primary question is whether synthesis of wall polysaccharides from glucose is regulated at the level of precursor formation (steps c–g), as is believed regarding the control of starch synthesis in plants (13, 14, 29, 49, 50) or is regulated at the level of polysaccharide synthetase reactions (steps i–k), as in glycogen synthesis (12, 17, 23, 28, 36, 41). It should be noted that carbon may reach nonhexose polysaccharides via the inositol-glucuronic acid pathway (26), which bypasses reactions d–f; this pathway was not investigated in the present work. Also outside the scope of the present study is the regulation of polysaccharide synthesis from sucrose.

Except for the inhibition of UDP-glucose dehydrogenase by UDP-glucuronic acid and UDP-xylose, we do not think that effects of metabolites on enzyme activities (Table III) are likely to be significant in the regulation of carbon flow from glucose to polysaccharides, because (*a*) they occur mostly at what are probably unreasonably high concentrations physiologically, and (*b*) the inhibitory effects of nucleoside diphosphates would tend to oppose rather than to facilitate carbon flow toward UDP-glucose from glucose, because concentrations of nucleoside diphosphates would tend to be raised by sugar nucleotide utilization and resultant shifts in equilibria between nucleoside di- and triphosphates shown in Figure 4. Some significance might be imagined for the inhibition of hexokinase by ADP, which would tend to couple carbon flow in Figure 4 positively to the "energy charge" and thus could bring about an acceleration of polysaccharide synthesis as a result of promotion of respiration by IAA. However, for reasons to be given below it is clear that, apart from consequences of the IAA effect on glucose uptake, promotion of polysaccharide synthesis by IAA does not result primarily from a change in the flux at the hexokinase step.

The activities of all the enzymes that participate in conversion of glucose-6-P to UDP-glucose are high compared with that of hexokinase (Table III), as has been observed in several other systems (5, 45, 51). The intermediary activities are also very high compared with the flux from glucose to polysaccharides in this tissue, which may readily be calculated as 0.1 to 0.12 μmole of glucose per min per 100 tissue segments from data given in Table II. This is further reason for doubting that the reactions between glucose-6-P and UDP-glucose can constitute points of control for polysaccharide synthesis. Because of these high activities it appears probable that virtual equilib-

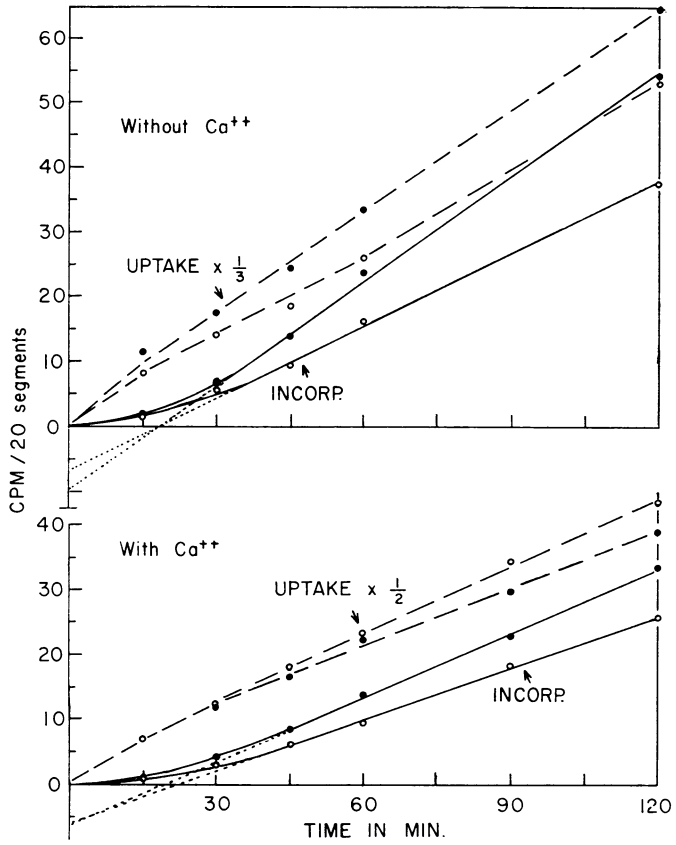


FIG. 3. Time courses for uptake of ^{14}C-glucose and incorporation into cell wall when pea segments pretreated with 0.05 M glucose minus or plus 3 μg/ml IAA and/or 0.02 M $CaCl_2$ are transferred to the same medium containing 1.02 μc/ml ^{14}C-glucose. Dashed lines show method of estimating radioactivity, at isotope equilibrium, in trapping pools between fed glucose and cell wall polymers.

rium should actually prevail *in vivo* at the phosphoglucomutase, nucleoside diphosphokinase, and UDP-glucose pyrophosphorylase steps. The existence of such equilibria is made additionally probable by the low activity found for inorganic pyrophosphatase (Table III) and by the fact that the ratio of glucose-1-P to glucose-6-P is practically the equilibrium value. On the other hand, the modest activity found for UDP-glucose dehydrogenase (step f) relative to the flux *in vivo* is compatible with the possibility that this step is a control point for formation of nonhexose polysaccharide precursors and that the feedback control of this enzyme by nonhexose sugar nucleotides is of physiological significance in the regulation of wall synthesis. The low activity of UDP-glucose dehydrogenase suggests further that a significant fraction of the carbon flow into nonhexose polysaccharides could be occurring via the inositol pathway (26).

The flux *in vivo* through the polysaccharide precursor pathway in Figure 4 is of the same magnitude as the particulate and soluble hexokinase activities, which is biologically reasonable since step c should be a control point owing to its irreversibility. That it is a control point is indicated further by the strong dependence of wall polymer synthesis, *in vivo*, upon the concentration of glucose supplied to the tissue. However, it is clear that hexokinase activity does not increase in response to IAA, and the data in Tables V and VI show that the hexokinase step is not being stimulated *in vivo* by treatment with IAA in presence of Ca^{2+} since, if it were, the metabolic glucose pool size must be reduced by IAA and the total yield of glucose metabolites must be increased by IAA, neither of which is observed. In presence of Ca^{2+} the increase in cell wall incorporation caused by IAA appears actually to be largely at the expense of the flux into storage pool metabolites, via steps m–r. An increased yield of glucose metabolites due to

Table V. *Estimation of Isotope-trapping Pool in Conversion of Glucose to Cell Wall Polymers*

Segments were pretreated for 2 hr in 0.025 M glucose with or without 3 μg/ml IAA and, in experiments 3 and 4, 0.02 M $CaCl_2$. They were then transferred to the same medium plus 1.02 μc/ml ^{14}C-glucose, and triplicate samples of 10 segments were taken after 45, 60, 75, and 90 min. Radioactivity in the isotope-trapping pool was estimated, and pool size was calculated, as described in text. The isotope dilution factor used was 2.25 (mean for plus IAA samples in Table II), corrected for differences in glucose uptake by multiplying by u_1/u_2 where u_1 is mean 60-min total uptake for minus Ca, plus IAA samples and u_2 is uptake for the sample in question.

Experiment	Ca^{2+}	IAA	Radioactivity after 60-min Incubation		Trapping Pool at Equilibrium	Pool Size
			Cell wall	Total uptake		
			cpm × 10^{-3}			*nmoles/100 segments*
1	–	–	5.93	28.2	2.7	2060
		+	9.50	38.9	4.9	2700
2	–	–	6.80	32.3	3.4	2260
		+	9.28	39.0	5.6	3080
3	+	–	3.57	20.1	2.15	2300
		+	5.23	19.9	2.5	2700
4	+	–	3.74	21.2	2.0	2020
		+	5.68	20.9	2.3	2360

Table VI. *Metabolic Balance Sheet for Pea Stem Segments in ^{14}C-Glucose*

Samples of 20 segments were pretreated 2 hr in 0.05 M glucose with or without 3 μg/ml IAA and/or 0.02 M $CaCl_2$, then incubated for 3 hr (experiment 1) or 2 hr (experiment 2) in the same medium plus 2 μc/ml ^{14}C-glucose. Figures in experiment 1 are the mean of duplicate samples.

Fraction	Experiment 1		Experiment 2	
	$CaCl_2$ in media		Without $CaCl_2$	
	– IAA	+ IAA	– IAA	+ IAA
	cpm × 10^{-3}			
Cell wall	158.0	213.2	74.4	108.6
Water-soluble polysaccharides	53.5	64.0	—[1]	—[1]
CO_2	69.8	68.9	37.0	42.3
Heat-precipitated protein	8.7	6.0	23.9	28.7
Bases (amino acids)	37.7	24.0	21.1	21.2
Organic acids (and phosphate esters)	62.6	49.0	32.7	30.1
Sucrose	9.9	5.5	16.8	16.0
Fructose	25.1	12.6	10.9	9.5
Glucose	62.5	41.8	41.2	46.6
Sum of nonpolysaccharide metabolites	213.8	166.0	142.4	147.8
Total	487.8	485.0	258.0	303.0

[1] Not determined.

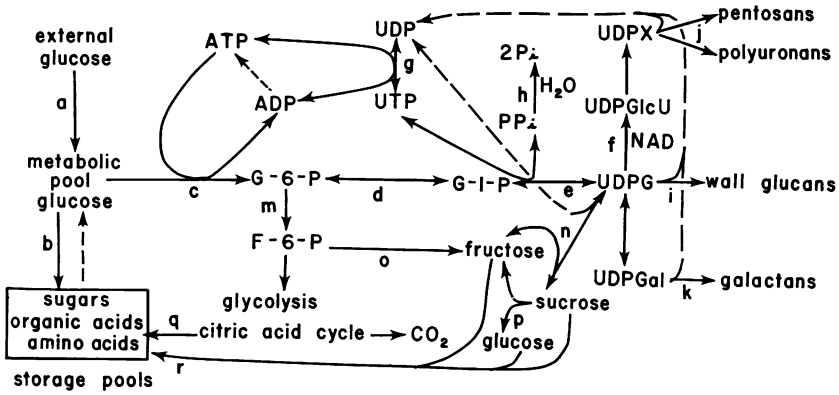


FIG. 4. Diagram of metabolic pathways related to cell wall synthesis that are considered in interpreting the results. Lower case letters identify enzymes listed in Table III or denote steps referred to in the text. UDPX: UDP-galacturonic acid and UDP-pentoses; UDPGlcU: UDP-glucuronic acid; UDPG: UDP-glucose; UDPGal: UDP-galactose; G-1-P and G-6-P: glucose-1-P and -6-P; F-6-P: fructose-6-P.

IAA is, however, observed in the absence of Ca^{2+}; this is attributable to the stimulated glucose uptake (step a) and resultant increase in size of the metabolic glucose pool, upon which the rate at step c depends *in vivo*.

The fact that IAA causes a depletion of UDP-glucose and glucose phosphates, especially in the absence of exogenous sugar (Table IV) suggests that utilization of sugar nucleotides in polysaccharide synthetase reactions (steps i–k) is being activated by auxin, leading to a drain-down in UDP-glucose and glucose phosphates when inflow via step c is restricted by a limited glucose supply. A similar observation has been made regarding hormone-induced glycogen synthesis in liver (22). The enzyme assays (Table III) agree with and support this inference, since polysaccharide synthetase is the only relevant enzyme with activity increased by IAA during the period in which the wall synthesis response takes place. It contrasts with the inferred regulation of wall synthesis at the pyrophosphorylase level (step e) in *Acetabularia* (52, 53) and *Dictyostelium* (32), although in the latter a dramatic regulation of polysaccharide synthetase, due apparently to enzyme formation and inactivation, also occurs and may be controlling.

A major weakness of the present glucan synthetase assays is that, apparently in common with other plant glucan synthetase systems that have been studied (20), they measure an incorporation rate that is only a minute fraction of the rate of wall glucan synthesis *in vivo*. Because of the peculiar kinetics of the system (Table III, footnote 6) it is not possible to estimate a substrate-saturated activity for comparison with the other enzymes in Table III, and the factor of about 100 by which the apparent incorporation of glucose from UDP-glucose may be increased over that under standard assay conditions by using high UDP-glucose concentrations in the range that may occur *in vivo* is still far from sufficient to raise glucan synthetase assay values such as those in Table III to a level comparable with the flux *in vivo*. Synthetase activities using other donors (GDP-glucose and UDP-galactose) are somewhat lower than with UDP-glucose (40) and thus do not help to close the gap. Future research must seek to account for wall synthesis *in vivo* by assayable polysaccharide synthetase activity and must supply an explanation of the regulation of polysaccharide synthetase activity by auxin.

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