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Bound Auxin Formation in Growing Stems

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ABSTRACT

The term "bound auxin" is herein used to describe auxin conjugates insoluble in organic solvents which dissolve indoleacetic acid (IAA) and its derivatives, but hydrolyzable by NaOH to release IAA. Bound auxin from pea stems was fractionated into water-soluble, water-insoluble/NaOH-hydrolyzable, and insoluble components. Formation of bound auxin commenced with 15 minutes of applying exogenous labeled IAA, and progressively increased in amount, relative to IAA uptake, over 6 hours. Formation was not restricted to any particular zone of the stem and occurred in both light- and dark-grown stems. A greater quantity of bound auxin was formed in light-grown stems, reaching 4.2 and 7.7%, of the IAA taken up, in the water-soluble and water-insoluble/NaOH-hydrolyzable fractions after 6 hours. The presence of sucrose, during either the IAA treatment or an aging pretreatment had no effect, though 6 hours aging did cause a subsequent increase in the water-insoluble fraction of the bound auxin. Bound auxin formation in light-grown stems was dependent on respiratory metabolism, being reduced by KCN. It was also reduced, compared to total uptake, by inhibitors of RNA, and protein synthesis (6-methylpurine and cycloheximide) but only when the inhibitors preceded auxin addition and were present for a 4-hour period. Addition of inhibitors following auxin had no effect, suggesting an early inductive effect of auxin on bound auxin formation. Inhibitors of cell elongation had no effect. Deoxyglucose, an inhibitor of glucan synthesis, had only a small effect on the water-soluble fraction. Bound auxin is an important auxin product in growing plants. Its function is unknown, but some possibilities are discussed.

The terms "bound auxin" has been used to describe various substances in which auxin is unavailable as a result of chemical combination into some inactive form. The presence of auxin activity in a bound form was originally described by Berger and Avery (5), who found that if a water extract of corn kernels was tested for auxin activity, the activity of the aqueous extract was considerably enhanced by alkaline hydrolysis of the extracts. They termed the alkali labile, auxin-containing compound "auxin precursor" because of the liberation of auxin on hydrolysis. Their precursor was insoluble in most organic solvents, but soluble in aqueous alcohol or acetone, and in their continued characterization they employed 50% aqueous acetone as an extraction solvent. Their work was continued by Hamilton *et al.* (10) who used the term "bound auxin" to describe the fraction. Subsequent work by Bandurski and co-workers (1, 2, 11, 13, 14, 19–21) has shown that their bound auxin from corn kernels consists of numerous IAA-*myo*-inositol-glycosyl and IAA-glucan esters which have now been characterized in detail.

A nontransportable bound auxin was found during auxin transport experiments by Winter and Thimann (24). Some auxin remained in the tissue even after a 2-hr export period, at the end of which further diffusion of IAA from the tissue had largely ceased. This auxin could not be extracted by grinding in water but could be extracted with diethyl ether as free IAA, and they suggested that the IAA might be associated with a protein.

There is a clear difference between the bound auxin referred to in the two above instances, the former being IAA in a covalent chemical compound, the latter representing a much weaker complex. In this paper I will use the term bound auxin to represent alkali-hydrolyzable IAA-containing compounds in the same sense as used by Hamilton *et al.* (10).

An additional type of alkali-hydrolyzable IAA compound has been described by Bendaña *et al.* (3) and Davies and Galston (9). This is a compound which is water-soluble, yet insoluble in cold 70% ethyl alcohol, and, as in the auxin complexes of Berger and Avery (5) and Bandurski (2), releases IAA on alkaline hydrolysis. A distinct difference, however, is that the 70% ethyl alcohol-soluble bound auxin is found in immature corn kernels, whereas the ethyl alcohol-insoluble compound is formed following the application of exogenous radiolabeled IAA to growing pea stem sections. An IAA-glucan from corn kernels, insoluble in cold 70% ethyl alcohol, was described by Piskornik and Bandurski (14), though this is again a different compound, as it is insoluble in water.

It has been hypothesized (20) that the IAA-inositol compounds of Bandurski and co-workers might be a storage form of IAA which would protect the IAA during the desiccation of the corn kernel. Bendaña *et al.* (3) suggested originally that their compound was an IAA-RNA complex involved in the growth-promoting action of IAA. It has since been shown that while IAA is bound in the manner described by Bendaña *et al.* (3) the binding is not to RNA (6, 9). Preliminary separations (unpublished) have suggested that the compound might actually be an IAA-glycoside compound of similar nature to Bandurski's IAA-inositol-glycoside compounds.

One of the actions of auxin in growth promotion is the promotion of cell wall synthesis. In view of the formation of bound auxin in growing stems and its possible connection with glycoside metabolism, the physiology of bound auxin formation in both light- and dark-grown stems of peas has been investigated.

MATERIALS AND METHODS

One-cm sections were cut from the apical or subapical internode of 14- to 15-day-old light-grown peas or from 6-day-old dark-grown peas, 3 mm below the apical hook. The sections were washed and then incubated in 4 μM IAA-2-^{14}C (60 mCi/mmole) in 0.02 M phosphate buffer, pH 6.2, containing 30 mM sucrose, and other additives in some experiments, for 3 hr unless otherwise stated. Prior or subsequent treatments were in buffer and sucrose as above. The volume of incubation solution varied between experiments but was held at 1 ml for approximately every 15 stem sections. Mean fresh weight of the sections was 20 mg/section for light-grown sections and 30 mg/section for dark-grown sections unless otherwise stated.

The dark-grown sections were incubated in the dark, and light-grown sections were incubated in an illuminated growth cabinet with a light intensity of 3400 $\mu w/cm^2$ provided by fluorescent and a few incandescent lamps, both at 22 C. All manipulations and subsequent extractions were performed in dim laboratory light at 21 C. Following incubation the sections were removed from the incubating solution and washed for 5 sec in running distilled H_2O to remove surface radioactivity. Sections were then placed in 8-ml polyethylene scintillation vials containing 2 ml of 95% ethyl alcohol, with two sections/vial and six replicate vials/treatment, for 24 hr at 21 C with occasional agitation, and then into vials containing additional 2 ml of ethyl alcohol for 24 hr. The alcohol extracts were bulked and evaporated down to 0.5 ml prior to counting. Thereafter the pairs of sections were transferred in sequence to vials containing 1.4 ml of distilled H_2O, 0.5 ml of 1 M NaOH, and finally 0.1 ml of Soluene 100 (a tissue-dissolving solution, Packard Instrument Co.) with the sections remaining 24 hr in each vial. The final vial with Soluene was held at 55 C overnight to solubilize the residue. Five ml of toluene-Triton X-100 (2:1) scintillation solution, containing PPO and dimethyl-POPOP, were added to each vial of alcohol, water, and NaOH solution (with the latter diluted with 1 ml of water per vial to prevent precipitation) while 5 ml of toluene scintillation fluid including 0.5% acetic acid were added to the vials containing solubilized tissue. To check hormone uptake in some experiments, six replicate pairs of sections from each treatment were placed directly into vials and 0.3 ml of Soluene 100 was added, followed by overnight digestion at 55 C and addition of scintillation solution as above. The vials were counted in a Packard Model 3375 scintillation spectrometer with counting efficiency (70–80% depending on solvent) recorded using the automatic external standard channels ratio. All counts were then individually computed into dpm, and the mean and its standard error were calculated for each extract. Because two sections weighed about 60 mg, carryover of radioactivity from one solvent to the next was approximately 1/1000 from the ethyl alcohol to the water (a maximum of about 40 dpm) and 1/30 from water to NaOH. Significant differences were well in excess of these levels.

RESULTS AND DISCUSSION

Nature of Bound Auxin. In these experiments bound auxin is defined as those auxin compounds insoluble in 95% ethyl alcohol, but soluble in water. It is clearly necessary to determine the relationship between this bound auxin fraction and the IAA-inositol-glycosides of Bandurski and co-workers (see above). Accordingly, sections were placed separately in vials of either 95% ethyl alcohol, 70% ethyl alcohol, 50% acetone (as used for the IAA-inositol esters) or boiling water for 5 min followed by 95% ethyl alcohol. The results (Table I) show clearly that 95% ethyl alcohol extracts less radioactivity than either 70% ethyl alcohol or 50% acetone, in that the following water extractions extract more radioactivity from sections previously treated with 95% ethyl alcohol than those treated with 70% ethyl alcohol or 50% acetone. There is no difference in the amount the three solvents leave in the water-insoluble but NaOH-hydrolyzable or residue fractions. Boiling water prior to ethyl alcohol did not remove all the water-soluble material and generally left more radioactivity in the residue fraction, possibly due to the precipitation of protein associated IAA which would not be removed by alkali (1). Ninety-five percent ethyl alcohol has been shown (7) to remove only IAA and its immediate derivatives. The differences in water-extractable radioactivity following the different solvents clearly show that there are compounds present with solubility properties similar to IAA inositols in that they are soluble in 50% acetone but not in 95% ethyl alcohol. The material insoluble in 70% ethyl alcohol or 50% acetone is IAA-containing material as when stem sections are extracted with phenol-water and the water fraction made 70% in ethyl alcohol an NaOH-hydrolyzable IAA-containing fraction is precipitated (6, 9). This is clearly a fraction which falls under the definition of "bound auxin" yet one which is probably lost in an extraction in 50% acetone used for IAA-inositols or IAA-glucan (14). Its greater polarity than the IAA-inositol glycosides suggest that it could possibly be a similar compound with a greater proportion of glycoside residues in the molecule.

The material which is insoluble in ethyl alcohol, acetone, or water but which releases radioactivity on hydrolysis represents a more extremely bound auxin which would be included in bound auxin if auxin activity was measured simply following NaOH hydrolysis of the tissue as done by Bandurski and Schulze (1). Its insolubility eliminates its being any compound like the extractable IAA-inositols. This insoluble IAA-containing polymer is possibly located in the cell wall as radioactivity supplied as IAA has been localized in the cell wall (17), though no removal of this activity following NaOH treatment has been reported.

Rate of Formation of Bound Auxin. Bound auxin is clearly a quantitatively important product formed after the addition of

Table I. *Influence of Different Extraction Methods on Solvent Distribution of Bound Auxin*

The pea stem sections were incubated in 8.3 μM IAA-^{14}C for 4 hr. The sections were then extracted twice with different solvents as noted below. Following this initial extraction, they were treated with water and 1 M NaOH; the remaining material is the residue.

Initial Solvent	Soluble in Initial Solvent	Water-soluble	NaOH-soluble	Residue	Total
			dpm/2 sections		
Light-grown stems					
95% ethyl alcohol	39,690 ± 1,627	1,738 ± 100	2,567 ± 127	1,540 ± 93	45,535 ± 1,947
70% ethyl alcohol	40,460 ± 1,163	323 ± 10	2,392 ± 83	1,441 ± 36	44,616 ± 1,292
50% acetone	41,225 ± 1,082	236 ± 5	2,580 ± 92	1,499 ± 39	45,540 ± 1,218
Boiling water then	35,330 ± 679				
95% ethyl alcohol	1,765 ± 93	384 ± 114	2,937 ± 149	2,117 ± 146	42,533 ± 1,181
Mean uptake (by direct measurement) = 47,913 ± 189					
Dark-grown stems					
95% ethyl alcohol	58,053 ± 6,537	1,201 ± 118	2,191 ± 258	1,213 ± 197	62,658 ± 7,110
70% ethyl alcohol	66,231 ± 11,802	456 ± 42	2,684 ± 165	1,531 ± 70	70,902 ± 12,079
50% acetone	51,672 ± 9,371	401 ± 37	2,618 ± 208	1,311 ± 154	56,002 ± 9,770
Boiling water then	66,014 ± 5,551				
95% ethyl alcohol	2,264 ± 95	202 ± 11	3,294 ± 169	2,034 ± 185	73,808 ± 6,011
Mean uptake (by direct measurement) = 65,752 ± 11,728					

exogenous IAA. Its formation commences without a lag and it is present in significant quantities within 15 min after IAA application (Fig. 1) at about 1.4% and 2.6% of the total radioactivity in the light-grown stem sections for water-soluble and NaOH-soluble conjugates, respectively. Slightly less is present in dark-grown peas. The amount of bound auxin increases at a greater rate than does the total IAA uptake, so that after 6 hr the percentage of bound auxin in light-grown peas has increased to 4.2% and 7.7% for water- and NaOH-soluble conjugates, respectively. This indicates either a specific compartmentation for formation which auxin enters more slowly than the total level in the tissue, or a steady induction of the binding system. More label was found in the NaOH-soluble than in the water-soluble fraction. Previous experiments extracting the water- and NaOH-soluble fractions together have shown that the main labeled component after NaOH hydrolysis is IAA itself (7), and that after 24 hr most of the non-degraded IAA in light-grown stems is in the form of bound auxin (8). Bound auxin is not only formed after the exogenous addition of IAA but exists in growing tissue (330 μg/kg alkali-hydrolyzable IAA ester in *Zea* coleoptiles *versus* 24 μg/kg free IAA) (1).

Bound Auxin Formation in Relation to Position of Origin of Stem Segments. Segments were taken from various regions of light- and dark-grown stems, including regions of active elongation near the top of the stem and mature regions at the base of the stem. While auxin uptake was greatest in sections from parts of the stem where elongation was almost complete (Fig. 2, A and D), there was no enhancement of bound auxin formation in this region. All bound auxin fractions were formed throughout the stem (Fig. 2, B, C, E, and F) with the highest level in the recently mature locations and a slight decline in the lowermost section. This indicates that a role for bound auxin in cell elongation is unlikely. This distribution and speed of formation is somewhat similar to that noted for the stimulation of glucan synthesis in the upper parts of dark-grown pea stem segments (15), so that a role in wall synthesis is a possibility and will be considered below.

Light- *versus* Dark-grown Stems. Only experiments performed on light- and dark-grown stems at the same time with identical auxin solutions are directly comparable though even here differences in plant age may confuse any interpretations (Figs. 1 and 2). In all other experiments the light and dark

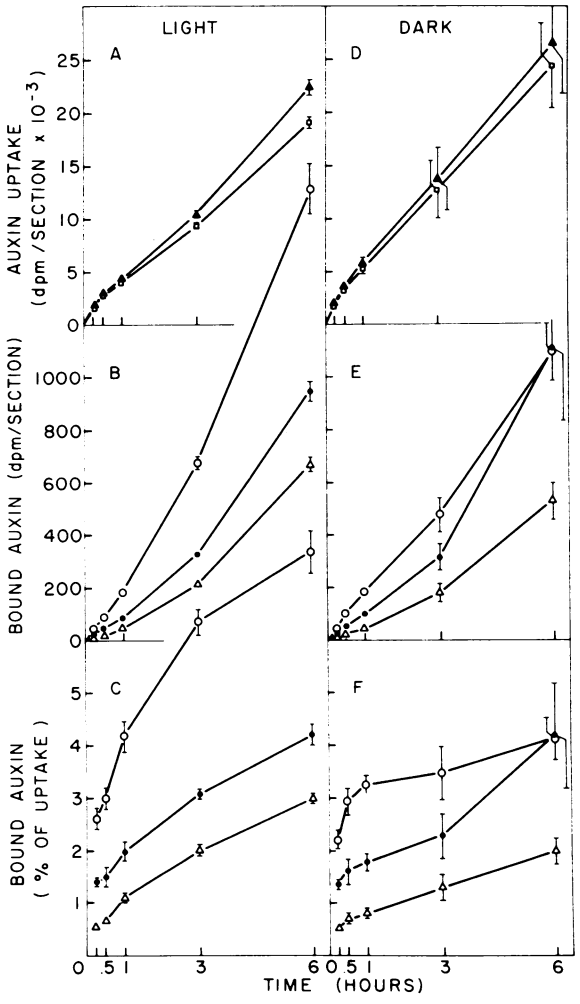

FIG. 1. Kinetics of bound auxin formation in sections from light- (A–C) and dark-grown (D–F) peas. A, D: Total uptake of labeled IAA (▲); amount of ethyl alcohol-extractable label (□); B, E: formation of bound auxin as radioactivity soluble in water (●); 1 M NaOH (○); and insoluble residue (△); C, F: bound auxin formation in relation to total uptake of label (symbols as above). Bars represent standard error. Where no bars are shown, the error was less than the area occupied by the point.

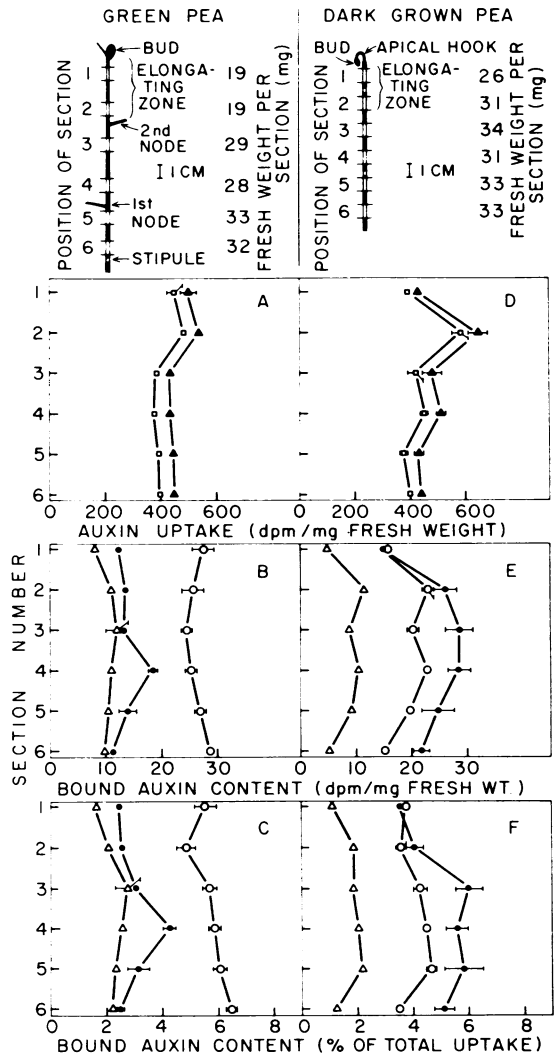

FIG. 2. Formation of bound auxin in sections from light- (A–C) and dark-grown (D–F) peas, in relation to the position in the stem. A, D: Total uptake of labeled IAA (▲); amount of ethyl alcohol-extractable label (□); B. E: bound auxin formation per unit fresh weight of the stem sections; C, F: bound auxin formation in relation to total uptake of label: water extractable (●); 1 M NaOH-extractable (○); nonextractable residue (△). Error as Fig. 1.

treatments were on different days with different auxin solutions. In general, dark-grown pea stem sections were found to take up more label from the treating solution, although light-grown stem sections converted more of that taken up into ethyl alcohol-insoluble compounds. The exact extent of bound auxin formation varied with many factors such as plant age, growing conditions, position of the stem section, and time of treatment, so that minor variations always occurred. In all cases, bound auxin formation was considerable, going as high as 17% in light-grown sections after a 3-hr treatment (Table II).

Effect of Sucrose. The inclusion of a low concentration of sucrose in the incubating medium for IAA-treated stem sections has previously been shown to enhance glucan synthesis (15). Light- and dark-grown sections were therefore incubated in labeled auxin with or without 30 mM sucrose, and the various extracts were measured. No effect of sucrose on the formation of bound auxin was found except for a very small increase in the water-soluble fraction in the light (3.79 ± 0.14% of total IAA uptake minus sucrose *versus* 4.44 ± 0.34% with sucrose).

Effect of Aging Prior to IAA Addition. In experiments by Ray (15) on the effect of auxin on glucan synthesis, it was found that glucan synthesis dropped if stem sections were aged but that this could be prevented by IAA or sucrose. To determine whether there is any connection between bound auxin and auxin-induced glucan synthesis, the influence of aging stem sections before treating with auxin was examined. Aging of light-grown sections in light was found to have no effect on the water-soluble fraction of bound auxin (Fig. 3) but, between about 3 and 6 hr of aging, the formation of the NaOH-soluble bound auxin fraction increased when recorded as a percentage of the total auxin uptake. A slight increase in the insoluble residue labeling was also recorded over the same time period. Uptake itself increased slightly with aging but was only significant in the minus sucrose treatment after 6 hr (about a 30% increase from the '0–1 hr aged' uptake). It is likely that the increase in bound auxin formation following aging resulted simply from a decrease in the endogenous auxin content giving an increase in the specific radioactivity of the auxin pool and thus more binding of the labeled molecule. The presence of sucrose during aging has no effect in light-grown sections.

Effect of Inhibitors on Bound Auxin Formation. The similarities between the kinetics and position of bound auxin formation and IAA-stimulated glucan synthesis (15) led to further testing of the conditions promoting the formation of bound auxin. Various inhibitors which affect (or fail to affect) glucan synthesis were applied to stem sections together with the radiolabeled auxin. The inhibitors used were those of protein synthesis (cycloheximide), RNA synthesis (6-methylpurine), glucan synthesis (2-deoxyglucose), cell expansion (high Ca^{2+} concentrations, which prevent cell expansion without affecting wall or glucan synthesis [16]) osmotic inhibitors of cell expansion (mannitol and sucrose), and respiratory metabolism (KCN). As the labeled auxin is added exogenously the uptake of auxin may also be affected by the inhibitors. The inhibitors were therefore added prior to, with, or following the auxin. Early addition served to ensure the action of the inhibitors before the addition of auxin, while addition of inhibitors following a treatment period with radioactive auxin was made to eliminate any effect on auxin uptake. In the latter case, the inhibitors could still affect transport or elution out of the sections. All results have also been presented in terms of the total uptake in the presence of the various inhibitors so that uptake can be taken into account.

Uptake of auxin in light-grown sections (Table II) was slightly reduced by cycloheximide, 6-methylpurine, and 2-deoxyglucose, particularly following pretreatment with the inhibitors, and greatly reduced by KCN. Ca^{2+} tended to increase uptake slightly. Little difference in total radioactive content was seen

Table II. *Influence of Various Inhibitors on Formation of Bound Auxin in Sections from Light-grown Pea Stems*

The sections were treated in 3.5 μM IAA-^{14}C together with inhibitors for 3 hr (A); or following 1 hr of preincubation in inhibitors without IAA (B); or treated with IAA-^{14}C (7 μM) alone for 1 hr followed by inhibitors without IAA for 3 hr (C).

Treatment and Inhibitor	Ethyl alcohol-soluble		Water-soluble		NaOH-soluble		Residue		Total	
	dpm/2 sections	*% of uptake*	*dpm/2 sections*	*% of uptake*	*dpm/2 sections*	*% of uptake*	*dpm/2 sections*	*% of uptake*	*dpm/2 sections*	*% of control*
A. 3 hr IAA-^{14}C + inhibitors										
None (control)	16.984 ± 637	82.52 ± 3.09	1.078 ± 61	5.24 ± 0.29	1.324 ± 43	6.43 ± 0.21	1.196 ± 43	5.81 ± 0.21	20.582 ± 784	100 ± 3.81
20 μg/ml cycloheximide	14.983 ± 971	82.25 ± 5.33	748 ± 83	4.11 ± 0.46	1.336 ± 117	7.33 ± 0.64	1.150 ± 92	6.31 ± 0.51	18.217 ± 1.263	88.51 ± 6.14
1 mM 6-methylpurine	15.476 ± 862	82.22 ± 4.80	837 ± 46	4.45 ± 0.24	1.377 ± 75	7.32 ± 0.40	1.133 ± 48	6.02 ± 0.26	18.823 ± 1.031	91.45 ± 5.01
50 mM 2-deoxyglucose	16.741 ± 813	84.59 ± 4.11	770 ± 54	3.89 ± 0.27	1.271 ± 62	6.42 ± 0.31	1.009 ± 51	5.10 ± 0.26	19.791 ± 980	96.16 ± 4.76
20 mM $CaCl_2$	19.702 ± 945	82.70 ± 3.97	1.440 ± 79	4.19 ± 0.33	1.531 ± 53	6.43 ± 0.22	1.150 ± 54	4.83 ± 0.23	23.823 ± 1.131	115.74 ± 5.50
0.25 M mannitol	18.031 ± 439	83.73 ± 2.04	1.242 ± 102	5.77 ± 0.47	1.309 ± 47	6.08 ± 0.22	952 ± 53	4.42 ± 0.25	21.534 ± 641	104.63 ± 3.11
0.33 M sucrose	18.224 ± 554	83.95 ± 2.55	1.227 ± 59	5.65 ± 0.27	1.309 ± 56	6.03 ± 0.25	948 ± 44	4.37 ± 0.20	21.708 ± 713	105.47 ± 3.46
1 mM KCN	10.363 ± 205	90.00 ± 1.78	240 ± 11	2.08 ± 0.09	615 ± 18	5.34 ± 0.16	296 ± 13	2.57 ± 0.11	11.514 ± 247	55.94 ± 1.20
B. 1 hr inhibitors → 3 hr IAA-^{14}C + inhibitors										
None (control)	21.024 ± 924	81.01 ± 3.56	1.574 ± 122	6.06 ± 0.47	1.942 ± 56	7.48 ± 0.22	1.413 ± 65	5.44 ± 0.25	25.953 ± 1.167	100 ± 4.50
20 μg/ml cycloheximide	18.436 ± 603	85.73 ± 2.80	644 ± 28	2.99 ± 0.13	1.310 ± 45	6.09 ± 0.21	1.114 ± 38	5.18 ± 0.18	21.504 ± 714	82.86 ± 2.75
1 mM 6-methylpurine	18.164 ± 677	83.45 ± 3.11	752 ± 43	3.45 ± 0.20	1.585 ± 124	7.28 ± 0.57	1.265 ± 55	5.81 ± 0.25	21.766 ± 899	83.87 ± 3.46
50 mM 2-deoxyglucose	17.528 ± 658	82.65 ± 3.10	891 ± 76	4.20 ± 0.35	1.664 ± 111	7.84 ± 0.52	1.125 ± 68	5.30 ± 0.32	21.208 ± 913	81.72 ± 3.52
20 mM $CaCl_2$	22.951 ± 1.847	82.94 ± 6.67	1.641 ± 66	5.93 ± 0.23	1.782 ± 99	6.43 ± 0.36	1.298 ± 61	4.69 ± 0.22	27.672 ± 2.073	106.62 ± 7.99
0.25 M mannitol	22.226 ± 885	83.42 ± 3.32	1.442 ± 114	5.41 ± 0.43	1.741 ± 99	6.53 ± 0.37	1.233 ± 50	4.63 ± 0.19	26.642 ± 1.148	102.65 ± 4.42
0.33 M sucrose	21.559 ± 679	83.95 ± 2.64	1.400 ± 111	5.45 ± 0.43	1.599 ± 85	6.22 ± 0.33	1.123 ± 39	4.37 ± 0.15	25.681 ± 914	98.95 ± 3.52
1 mM KCN	10.890 ± 356	89.70 ± 2.93	267 ± 15	2.19 ± 0.12	670 ± 27	5.52 ± 0.22	313 ± 15	2.58 ± 0.12	12.140 ± 413	46.78 ± 1.59
C. 1 hr IAA-^{14}C → 3 hr inhibitors										
End of IAA-^{14}C pretreatment	15.903 ± 319	85.96 ± 1.72	578 ± 41	3.12 ± 0.22	1.079 ± 48	5.83 ± 0.26	941 ± 58	5.41 ± 0.31	18.501 ± 466	100 ± 2.52
None (control)	9.528 ± 379	75.08 ± 2.99	1.103 ± 73	8.69 ± 0.58	1.127 ± 50	8.88 ± 0.39	933 ± 47	7.35 ± 0.37	12.691 ± 549	100 ± 4.33
20 μg/ml cycloheximide	9.112 ± 345	74.69 ± 2.83	956 ± 41	7.84 ± 0.34	1.189 ± 83	9.75 ± 0.68	943 ± 50	7.73 ± 0.41	12.200 ± 519	96.13 ± 4.10
1 mM 6-methylpurine	8.497 ± 400	74.26 ± 0.35	1.031 ± 91	9.01 ± 0.80	1.036 ± 47	9.05 ± 0.41	878 ± 37	7.67 ± 0.32	11.442 ± 575	90.16 ± 4.53
50 mM 2-deoxyglucose	9.274 ± 343	75.64 ± 2.80	857 ± 70	6.99 ± 0.57	1.156 ± 48	9.43 ± 0.39	973 ± 47	7.94 ± 0.38	12.260 ± 508	96.60 ± 4.00
20 mM $CaCl_2$	9.238 ± 570	73.76 ± 4.55	1.148 ± 55	9.17 ± 0.43	1.145 ± 67	9.14 ± 0.53	992 ± 41	7.92 ± 0.33	12.523 ± 733	98.68 ± 5.78
0.25 M mannitol	10.184 ± 659	77.02 ± 4.98	1.072 ± 130	8.11 ± 0.98	1.035 ± 75	7.83 ± 0.57	932 ± 73	7.05 ± 0.55	13.223 ± 937	104.19 ± 7.38
0.33 M sucrose	9.258 ± 228	75.52 ± 1.86	1.053 ± 77	8.59 ± 0.63	1.034 ± 62	8.43 ± 0.51	914 ± 47	7.46 ± 0.38	12.259 ± 414	96.60 ± 3.26
1 mM KCN	9.746 ± 445	81.62 ± 3.73	501 ± 45	4.20 ± 0.37	932 ± 79	7.80 ± 0.66	762 ± 49	6.38 ± 0.41	11.941 ± 618	94.09 ± 4.87

with the different inhibitors in the pulse chase experiment though a loss of about 30% of the total radioactivity in the section occurred during the 3-hr chase period.

In light-grown sections, the formation of bound auxin was shown to require metabolism, although the reduction in formation brought about by KCN was larger in the water soluble complex (64% reduction in sections pretreated for 1 hr in KCN then 3 hr in IAA-^{14}C+KCN) or residue (52%) than in the NaOH hydrolyzed conjugate (30%). The formation of bound auxin depends on protein synthesis but only over a prolonged period. No effects were found if cycloheximide followed the IAA (Table IIC), while a small effect on the water-soluble conjugate only was noted when the auxin and inhibitor were added together and a 50% reduction when the cycloheximide was added prior to the auxin. A slight reduction of the NaOH-hydrolyzed conjugate was also noted in this instance. The effects of 6-methylpurine on the water-soluble conjugate were similar to those of cycloheximide, but less pronounced (43% reduction with prior addition of inhibitor), and no effects was found on the other fractions. Ca^{2+} and the osmotic inhibitors produced no consistent effects, though the latter did reduce the label in the residue if added with or prior to the auxin. Deoxyglucose reduced the formation of only the water-soluble fraction irrespective of the time of addition though the effect was maximal with prior addition of the inhibitor (30% reduction). Similar experiments on dark-grown stem sections showed no effect of most inhibitors, due either to different metabolic conditions or to the masking of differences due to the much higher variation noted in dark- as compared to light-grown stem sections. Though KCN reduced bound IAA in dark-grown sections no further reduction other than that caused by the decreased IAA content in the sections was noted.

The addition of inhibitors after auxin uptake had least effect, suggesting that the auxin binding system was already promoted by the prior addition of auxin. Addition of inhibitors prior to auxin produced the greatest effects (though never complete inhibitions). The timing of the effects would indicate that either the auxin binding system in existence in response to native auxin lasts for at least 3, but is dropping by 4 hr or that when auxin and inhibitors are added simultaneously the promotion of the binding system by auxin precedes its inhibition by the inhibitors, so that no effect of the inhibitors is noted. When addition of the inhibitors precedes auxin, the binding system can be seen to be partially sensitive to cycloheximide indicating a requirement for protein synthesis. There is also a requirement, though less pronounced, for RNA synthesis. Osmotic solutes or Ca^{2+} outside the stem had no effect showing that cell expansion is not a requisite for auxin binding. 2-Deoxyglucose, which inhibits the incorporation of sugars into wall polymers (16), only had an effect on the water-soluble fraction. These results are at variance with those found by Ray (16) for auxin-induced glucan synthesis which did not require protein or RNA synthesis, though glucan synthesis was assayed after only 2 hr of inhibitor treatment so the glucan synthetase enzymes relying on IAA stimulation might simply have a life in excess of 2 hr. Glucan synthesis was inhibited by osmotic inhibitors, but not Ca and by 2-deoxyglucose so the agreement with auxin binding is only with the slight effect of 2-deoxyglucose.

Because sucrose, both prior to and during auxin treatment, has no effect on bound auxin formation, and the pattern of inhibition by added inhibitors is different between bound auxin formation and IAA-induced glucan synthesis, it seems that these two processes have little in common. This does not, however, rule out the possibility of some involvement of bound auxin with wall synthesis. The fact that a considerable proportion of the bound auxin could, on the basis of solubility, be IAA-inositol-glycosides (2, 19, 21), and that much of the remainder of the NaOH-hydrolyzed material is insoluble in 70 to 95%

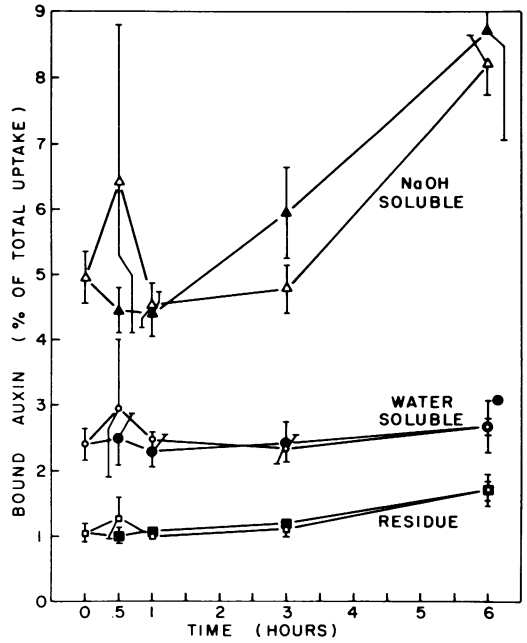

FIG. 3. Influence of aging light-grown stem sections with or without sucrose on the formation of bound auxin. Sections were aged for up to 6 hr then transferred to 5 μM IAA-^{14}C for 1 hr. Without sucrose (△, ○, □); with sucrose (▲, ●, ■). Error as Fig. 1.

ethyl alcohol, 50% acetone, cold and boiling water, would indicate that bound auxin may be involved in polysaccharide and wall synthesis and possibly actually part of the wall itself. This is backed up by the histoautoradiographic studies mentioned earlier (17). A further finding is that IAA increases the content of cold water-soluble xyloglucans, possibly through the modification of existing xyloglucans rather than their *de novo* synthesis (12). This might occur by means of direct auxin binding.

One other possible role for bound auxin is in auxin metabolism. Berger and Avery (5) termed their alkali hydrolyzable bound auxin "auxin precursor" because it liberated IAA on hydrolysis. There is indeed some evidence that such compounds in caryopses of Gramineae may, in fact, be actual precursors of auxin. Early work by numerous investigators (reviewed by Went and Thimann [23]) indicated that auxin was absent from dry caryopses but appeared as soon as the caryopsis took up water. Went and Thimann (23) suggested that an ester precursor of auxin moves from the seed to the coleoptile tip where it is converted into auxin, while van Overbeek (22) showed that auxin production from a precursor occurred in coleoptile tips. Further recent reinvestigation of this problem by Sheldrake (18) has shown that the auxin precursors move to the tip of the coleoptile and are possibly activated in the tip. IAA was found to be released by hydrolysis of guttation fluid of decapitated coleoptiles. Thus in coleoptiles bound auxin appears to be a physiological auxin precursor. In these experiments in pea stems, since the auxin was supplied as IAA, the bound auxin cannot be an absolute precursor. Bentley (4) noted that the ease with which bound auxin releases free auxin suggests that it is in a dynamic state in the plant, controlling the level of auxin and thus influencing the effect of auxin. Should this be so, the formation of bound auxin from exogenously supplied auxin could represent a temporary sequesting of the auxin rather than a complete breakdown of the molecule, so that either transport (as noted in coleoptile xylem by Sheldrake [18]), or storage against future utilization could occur.

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