Plant Physiol. (1975) 55, 1043-1047

Regulation of Cell Wall Synthesis in *Avena* Stem Segments by Gibberellic Acid[1]

Received for publication October 17, 1974 and in revised form February 13, 1975

MICHAEL J. MONTAGUE[2] AND HIROSHI IKUMA
Department of Botany, University of Michigan, Ann Arbor, Michigan 48104

ABSTRACT

Gibberellic acid induces (*a*) increased elongation of *Avena sativa* stem segments, (*b*) increased formation of cell wall material, measured on the basis of dry weight, and (*c*) increased incorporation of ^{14}C-glucose into all fractions of the cell wall material. This increased incorporation of radioactivity correlates well with increased formation of cell wall material and shows a time-course pattern similar to the time course of the elongation response. Approximately one hour after the application of gibberellic acid, the rates both of growth and of incorporation of radioactivity accelerate to about 2-fold over the control rate. Gibberellic acid does not stimulate the incorporation of labeled glucose into the cell wall material simply by increasing the rate of uptake of glucose by internodal cells. The stimulation of the incorporation of ^{14}C-glucose into cell wall material, which reflects the stimulation of cell wall synthesis, seems to be an important and relatively early effect of gibberellic acid in this system and probably contributes significantly to the elongation response elicited by the hormone.

Using gravimetric analyses, Adams (2) showed that the content of cell wall material in the internode of *Avena* stem segments increased in response to the application of GA_3 and that this increase was correlated with internodal elongation. A similar correlation between elongation and formation of the cell wall has been observed for auxin-stimulated growth of *Avena* coleoptiles (4) and pea stem segments (1). Since the cell wall must increase in area in order for any plant cell to expand, these observations suggest that hormonal action in these cases involves increased biosynthesis of cell wall material, either directly or indirectly. In this study, the relationship between GA_3 stimulation of growth and biosynthesis of cell wall material was carefully examined by feeding ^{14}C-glucose to isolated *Avena* internodes and following the accumulation and distribution of radioactivity in the cell wall fractions.

MATERIALS AND METHODS

Tissue System and Growth Conditions. About 150 oat (*Avena sativa* cv. 'Victory') plants were routinely grown in flats (43.2 × 37.9 × 7.6 cm) in the greenhouse, or in some cases, in a growth chamber (18 hr light/6 hr dark; 22 C/16 C) for 40 to 45 days. Shoots containing the internodes immediately below the peduncular node (p-1 internodes) with lengths of 1 to 3 cm were carefully selected. As indicated in each experiment, 1-cm or 0.5-cm segments were prepared from the shoots with a razor blade cutting device. The p-1 internodal tissue, containing the intercalary meristem, was dissected from the node and leaf sheath. Segments thus prepared are designated "isolated internodes" and are highly suitable for studies involving uptake of isotopes because the effects of the node and sheath (2, 9) are avoided. The segments were routinely placed in Plexiglas frames on filter paper in 6-cm Petri dishes. They were allowed to grow in an upright position, since segments placed on their sides in the liquid medium (*a*) did not elongate as much, presumably because of the effects of anaerobiosis, and (*b*) became curled and were therefore difficult to measure. Each Petri dish contained from 1 to 3 ml of treatment solution. Segments were routinely allowed to grow at 30 C in the dark to avoid the effects of photosynthesis (2). At appropriate times, the lengths of the segments were measured with a millimeter ruler.

Isolation of Cell Wall Material. In general, cell wall material was isolated using the method of Baker and Ray (4). Briefly, the segments were first washed in distilled H_2O and H_2O was also forced through the central lacuna of each segment using a hypodermic syringe in order to remove residual treatment solution. Then, they were placed in 2 to 5 ml (depending on the number of segments) of 80% ethyl alcohol for at least 24 hr, the ethyl alcohol was collected, and the segments were again extracted with fresh 80% ethyl alcohol for at least 1 hr. The two ethyl alcohol extracts were combined, and an aliquot was spotted on a filter paper disc, dried, and counted. Next, the segments were crushed betwen two glass plates, and were placed in 2 ml of a pepsin solution containing 2 mg/ml of pepsin in 0.03 M potassium phosphate buffer, pH 2, for at least 24 hr. An aliquot of the pepsin solution was also counted in some experiments. The resultant cell wall material was rinsed in water, and allowed to dry before being counted or further fractionated. An alternative method for the isolation of cell wall material was used for the experiment in Table I. Cell wall material was prepared by grinding the internodes in cold distilled H_2O, filtering the slurry onto a Whatman GF/C glass fiber filter, and washing it thoroughly with ethyl alcohol.

Cell Wall Fractionation. Further fractionation was carried out on isolated cell wall material using methods based on the differential solubility of cell wall components in acids and bases (5). The cell wall material was first extracted with 0.05 N H_2SO_4 at 100 C for 2 hr to remove pectic and some hemicellulosic substances. Then, it was successively extracted with 4% (2 hr) and 17.5% (overnight) NaOH at room temperature to remove the remaining hemicellulose. The residue was pri-

[1] Supported by a National Science Foundation Predoctoral Fellowship to M.J.M. Material from a dissertation submitted by M.J.M. in partial fulfillment of the requirements for the Ph.D. at The University of Michigan.

[2] Present address: Department of Biological Sciences, Stanford University, Stanford, Calif. 94305.

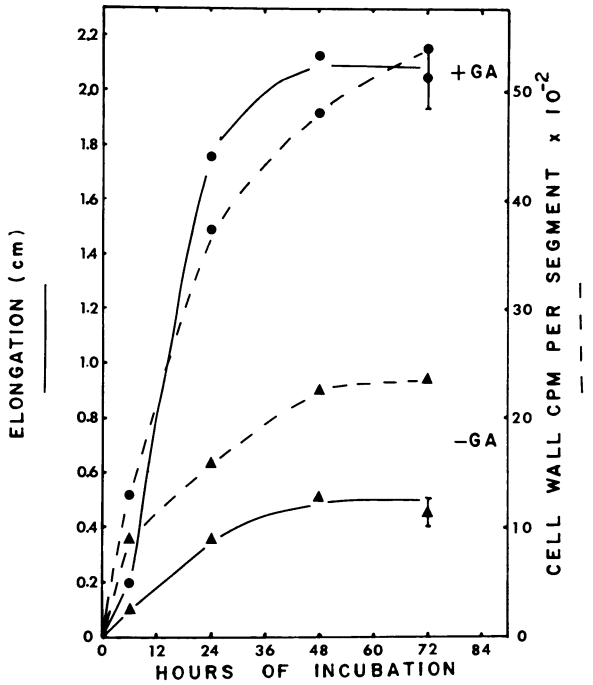

FIG. 1. Time course of elongation and incorporation of ^{14}C-glucose into cell wall material. Samples of 10 isolated internodes (1 cm) were incubated on 0.1 M ^{14}C-glucose (5098 cpm/μmole) with or without 10 μM GA_3.

marily cellulose since almost all of it was soluble in 72% (w/v) H_2SO_4.

Determination of Radioactivity. Radioactivity in extracts or cell walls was determined with a toluene scintillator containing 5 g/l of PPO and 0.3 g/l of POPOP. The channels ratio method (7) was used for quench correction.

Chemicals. PPO was purchased from Research Products International Corporation, Elk Grove Village, Ill. POPOP was a product of Packard Instrument Company, Inc. The ^{14}C-glucose was obtained from New England Nuclear and was prepurified by means of descending paper chromatography on Whatman No. 1 using *n*-butyl alcohol-ethyl alcohol-water (50:32:18, v/v) as solvent. Gibberellic acid was generously supplied by Mr. Douglas Broadbent of Imperial Chemical Industries, Ltd., Cheshire, England.

RESULTS

Correlation between Elongation and Cell Wall Biosynthesis. Figure 1 shows the relationship between growth of the isolated internode and ^{14}C-glucose incorporation into cell wall material of internodes incubated on 0.1 M glucose with and without GA_3. Treatment with hormone resulted in about a 5-fold increase in net elongation over the control at 72 hr. In terms of the total length, the internodes in GA_3 grew to about 2-fold the length of the control internodes. Total radioactivity detected in the wall material of GA_3-treated internodes was about 2-fold higher than that of the control internodes at the end of treatment. Thus, the radioactivity incorporated per unit length of the internodes was comparable for the two treatments. From these results, it can be concluded that the time-course patterns of elongation and of incorporation of labeled glucose into the cell wall are similar, although not identical. This conclusion holds quite well throughout the time course of Figure 1, except for the first 6 hr of treatment immediately after excision, when incorporation of radioactivity proceeded rapidly although elongation did not.

In order to determine the relationship between incorporation of radioactivity and cell wall synthesis, growth, measured as an increase in the dry weight of the cell wall material, was compared with the incorporation of radioactivity into the wall. For this purpose, isolated internodes were incubated for 12 hr on unlabeled glucose before transfer to ^{14}C-glucose with or without hormone. This pretreatment was introduced to reduce the effects of initial wounding responses. The results in Table I show that GA_3 causes about a 2-fold increase in both (*a*) dry weight of the cell wall material, and (*b*) radioactivity incorporated into the cell wall material during the 24-hr incubation. It should be added that the stimulation of the incorporation of labeled glucose into the cell wall usually ranged from about 1.5- to 2.5-fold in the experiments in this study. If the percentages of new cell wall derived from exogenous glucose are computed (last column of Table I), it is apparent that only about 20% of this new wall was derived from exogenous substrate. The percentage values for GA_3-treated and control internodes are very similar, strongly suggesting that GA_3 does not stimulate the preferential incorporation of exogenous or endogenous sugar into the new cell wall. Therefore, it can be concluded that these results show a strong correlation between the gravimetric increase in cell wall material and the incorporation of radioactive glucose into the cell wall.

Because not all of the cells along the length of the *Avena* internode elongate to the same extent (8), the distribution of labeled glucose along the internode was determined in the presence or absence of GA_3 (Table II). For this purpose, the internodal segments were placed either with their morphological base ends down in the medium (experiment 1), or with their morphological topmost ends down in the medium (experiment 2). After 24 hr, the internodes were cut manually into four equal pieces (referred to as basal, mid-basal, mid-topmost, and topmost) and were subjected to the usual preparation of cell wall material. It is clear in both experiments that those cells closest to the medium incorporated the most radioactivity into their walls and a decreasing amount was incorporated into the walls of cells further away from the medium. Gibberellic acid had little or no effect on the pattern of distribution of label, but it acted to increase by approximately

Table I. *Effect of GA_3 on Incorporation of ^{14}C-glucose into Cell Walls from Avena Stem Segments*

Samples of 15 isolated internodes (5 mm) were incubated for 12 hr on unlabeled 0.1 M glucose and then were transferred to 0.1 M ^{14}C-glucose (4847 cpm/μmole) with or without 500 μM GA_3 for 24 hr. At the start of the 24-hr incubation with labeled glucose, the dry weight of cell wall material from 15 representative internodes was 13.59 mg. Cell wall material was prepared by grinding the internodes in cold distilled H_2O, filtering the slurry onto a Whatman GF/C glass fiber filter, and washing with ethanol. Exogenous glucose equivalents incorporated into cell wall were calculated from the specific activity of the exogenous labeled glucose and the counts per minute incorporated into the wall.

Treatment	Gravimetric Increase in Cell Wall	Radioactivity Incorporated into Cell Wall	Exogenous Glucose Equivalents Incorporated into Cell Wall	New Cell Wall Derived from Labeled Glucose
	mg	*cpm*	*mg*	*%*
−GA	6.41	39,896	1.48	23.1
+GA	13.8	67,269	2.50	18.1

Table II. *Effect of GA_3 on the Distribution of ^{14}C-glucose in Cell Wall along Avena Stem Segments*

Samples of 10 isolated internodes (1 cm) were incubated for 24 hr on 0.1 M ^{14}C-glucose (5100 cpm/μmole) with or without 10 μM GA_3. Then, each internode was cut into four equal sections and cell wall material was prepared from each group of sections.

Section	Experiment 1		Experiment 2	
	Basal end in medium		Topmost end in medium	
	−GA	+GA	−GA	+GA
	cpm			
Basal	13,221	22,370	4,695	6,811
Mid-basal	7,235	15,580	3,545	9,350
Mid-topmost	3,508	11,271	5,041	11,685
Topmost	2,705	4,716	12,945	21,206
Total	26,669	53,937	26,226	49,052

2-fold the amount of incorporation in sections along the segment length. Because the cells furthest from the medium received only a small fraction of the total label taken up by the segment, they must have relied on endogenous carbohydrate for the synthesis of cell wall material. This may well account for at least some of the dilution of labeled substrate by endogenous carbohydrate noted in Table I.

From these results, it is clear that increased incorporation of ^{14}C-glucose into the cell wall correlates well with GA_3-induced increase in the dry weight of the cell wall, and shows a time-course pattern similar to the time course of the elongation response.

Early Time Course of Elongation and Synthesis of Cell Wall. In order to establish the early time-course pattern of growth and of incorporation of labeled glucose, isolated internodes were incubated on 0.1 M unlabeled glucose for 12 hr before they were transferred for 1, 3, or 6 hr to 0.1 M ^{14}C-glucose with or without GA_3. The results (Fig. 2, A and B) show that (*a*) during the first hour after application of GA_3, there was no difference between GA_3-treated segments and the control in either elongation or incorporation of label; and (*b*) after approximately one hour, the rates both of elongation and of incorporation of radioactivity accelerated to about 2-fold in GA_3-treated internodes over the control rates. The lag time for the beginning of the hormone effect varied somewhat from batch to batch of segments, but it was usually fairly close to one to two hours. This is in good agreement with recent growth measurements by Adams *et al.* (3; unpublished results) for *Avena* stem segments and by Rose (10) for gamma-irradiated wheat coleoptiles. These results again indicate a close correlation between GA_3-induced growth and increased incorporation of radioactivity. Furthermore, it is apparent that increased incorporation of radioactivity into the cell wall is a relatively early effect of GA_3 in these internodes.

Uptake of Glucose and Synthesis of the Cell Wall. In order to determine whether gibberellic acid was acting simply to increase the rate of uptake of labeled glucose into the internodes, isolated internodes were incubated for 12 hr on unlabeled 0.1 M glucose and then were transferred to 0.1 M labeled glucose with or without GA_3 and were incubated for 6 hr in a stoppered vessel. Radioactive CO_2 was collected in 2.5 N NaOH. After the incubation period, the segments were extracted with 80% ethyl alcohol and treated with pepsin, as usual. The results (Table III) show that (*a*) GA_3 caused only a slight increase in the total uptake of labeled glucose by the segment; (*b*) GA_3 caused an appreciable increase in the incorporation by cell wall material and by the pepsin-soluble fraction (which may include some cell wall carbohydrate); and (*c*) GA_3 caused a decrease in the amount of radioactivity in the ethyl alcohol-soluble fraction. From these results, it can be concluded that GA_3 does not stimulate the incorporation of ^{14}C-glucose into the cell wall material simply by increasing the rate of uptake of glucose by internodal cells.

Synthesis of Cell Wall Components as Affected by GA_3. The

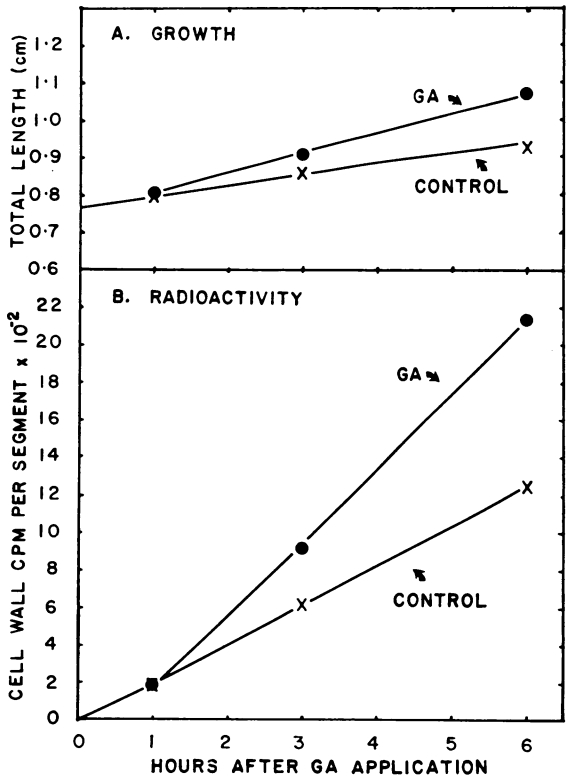

FIG. 2. Early time course of elongation (A) and incorporation of ^{14}C-glucose into cell wall material (B). Samples of eight to nine isolated internodes (5 mm) were incubated on unlabeled 0.1 M glucose for 12 hr and then were transferred to 0.1 M ^{14}C-glucose (16,745 cpm/μmole) with or without 500 μM GA_3.

Table III. *Metabolic Balance Sheet for Incorporation of ^{14}C-glucose into Avena Stem Segments*

Eleven isolated internodes (5 mm) were incubated for 12 hr on unlabeled 0.1 M glucose and then were transferred to 0.1 M ^{14}C-glucose (14,043 cpm/μmole) with or without 500 μM GA_3 for 6 hr in a closed vessel. Net growth over this 6-hr period was 0.12 cm without hormone and 0.29 cm with hormone.

Fraction	Radioactivity	
	−GA	+GA
	cpm	
80% Ethyl alcohol soluble	45,714	40,926
Pepsin soluble	2,316	3,317
Cell wall material	13,424	20,139
CO_2	9,382	11,671
Total	70,881	76,053

Table IV. *Effect of GA_3 on the Composition of the Cell Wall from Avena Stem Segments*

Fourteen isolated internodes (5 mm) were incubated for 12 hr on unlabeled 0.1 M glucose and then were transferred to 0.1 M ^{14}C-glucose (10,370 cpm/μmole) with or without 100 μM GA_3 for 6 additional hr. Cell wall material was fractionated using the solvents listed.

Fraction	Radioactivity			
	−GA		+GA	
	cpm	*% of total*	*cpm*	*% of total*
Hot 25 mM H_2SO_4	10,163	63.6	18,192	58.8
4% NaOH	2,599	16.3	5,770	18.7
17.5% NaOH	1,043	6.5	2,316	7.5
Residue	2,165	13.6	4,644	15.0
Total	15,970	100.0	30,920	100.0

question of whether GA_3 affects the synthesis of specific cell wall components was examined by fractionation of the cell wall into four components. Cell wall material was obtained from isolated internodes incubated on labeled glucose for 6 hr with or without GA_3, after they had been incubated on unlabeled 0.1 M glucose for 12 hr. The results (Table IV) show that GA_3 stimulates incorporation of radioactivity into all fractions by approximately 2-fold over the control. Within the limits of resolution inherent to this type of fractionation, it seems that the relative compositions of cell walls formed in the presence and absence of hormone are very similar. Gibberellic acid seems not to promote preferential synthesis of any specific wall component.

Effect of GA_3 on Incorporation of Labeled Endogenous Substrate into the Wall. All experiments to this point had been carried out by supplying labeled glucose at the same time that GA_3 was applied. In order to determine whether GA_3 also stimulated the incorporation of endogenous labeled substrate into cell wall material, isolated internodes were incubated on 0.1 M ^{14}C-glucose for 12 hr before they were placed on water or 0.1 M unlabeled glucose with or without GA_3 and allowed to incubate for an additional 24 hr. The results (Table V) show that a considerable amount of radioactivity was lost from the ethyl alcohol-soluble fractions in all treatments, presumably attributable to respiration (see Table III) since little radioactivity was found in the incubation media at the end of the experiment. However, radioactivity present in the cell wall material at the time that the internodes were removed from exogenous labeled glucose did not seem to be lost from the wall (treatment 1), even in the presence of large concentrations of unlabeled exogenous glucose (treatment 2). This strongly suggests that most cell wall material did not turn over rapidly. The application of GA_3 resulted in increased utilization of internal substrate to form cell wall material (treatments 3 and 4). This effect was especially apparent where GA_3 was given without exogenous unlabeled glucose (treatment 3 as compared to treatment 1). Thus, it can be concluded that GA_3 stimulates the incorporation of both endogenous and exogenous substrate into the cell wall, in good agreement with the findings presented in Table I.

The Effect of $CaCl_2$ on GA_3-induced Elongation. From the results presented thus far, it is clear that an increase in the incorporation of labeled glucose into the cell wall is an important, early effect of treatment with GA_3. However, it is possible that increased wall biosynthesis is merely the result of GA_3-induced elongation. Ray and co-workers (1, 4), studying auxin-sensitive systems, found that low concentrations of $CaCl_2$ (10 mM) effectively inhibited elongation but not wall synthesis. We examined the effects of $CaCl_2$ both on elongation and on the incorporation of radioactive glucose into the cell wall of *Avena* stem segments (Table VI). After previously titrating over a range of concentrations, 90 mM $CaCl_2$ was chosen for this experiment because it caused considerable inhibition of elongation with little osmotic effect. Isolated internodes (5 mm) were incubated for 12 hr on 0.1 M unlabeled glucose without $CaCl_2$ and then were transferred to 0.1 M ^{14}C-glucose with or without hormone and with or without $CaCl_2$. Although $CaCl_2$ severely inhibited elongation both with GA_3 (treatment 4 compared with treatment 2) and without GA_3 (treatment 3 compared with treatment 1), it did not abolish the stimulation of elongation by GA_3 (treatment 4 compared with treatment 3). It should be noted that $CaCl_2$ also failed to prevent GA_3-stimulated incorporation of labeled glucose into the cell wall (treatment 4 compared with treatment 3). Even so, because of the lack of complete inhibition of elongation, it is not possible, under these conditions, to show a direct effect of GA_3 on synthesis of the cell wall. In this respect, the *Avena* internode system differs from auxin-sensitive *Avena* coleoptiles and pea stem sections.

Table V. *Effect of GA_3 on the Incorporation of Endogenous Substrate into Cell Wall Material from Avena Stem Segments*

Isolated internodes (5 mm) were incubated on 0.1 M ^{14}C-glucose (6816 cpm/μmole) for 12 hr. After being washed with H_2O, they were transferred to H_2O or to 0.1 M unlabeled glucose, with or without 500 μM GA_3, and were allowed to grow for an additional 24 hr. Results are given as the mean of duplicate experiments.

Treatment	Radioactivity			
	Ethyl alcohol + Pepsin-soluble Fraction	Net Loss in Ethyl alcohol + Pepsin-soluble Fraction	Cell Wall Fraction	Net Gain in Cell Wall Fraction
	cpm/internode			
T_0 12 hr	4,178		1,952	
1. Water	2,224	1,954	2,102	150
2. 0.1 M Glucose	2,619	1,559	2,152	200
3. 500 μM GA_3	2,036	2,142	2,633	681
4. 500 μM GA_3 + 0.1 M Glucose	2,282	1,896	2,258	306

Table VI. *Effect of $CaCl_2$ on Avena Stem Segments*

Isolated internodes (5 mm) were incubated on 0.1 M unlabeled glucose for 12 hr and then were transferred to 0.1 M ^{14}C-glucose (5867 cpm/μmole) with or without 250 μM GA_3 in the presence or absence of 90 mM $CaCl_2$ and allowed to incubate for an additional 24 hr. Net growth over this 24 hr period is given as the mean of eight or nine internodes with standard errors.

Treatment	Net Growth	Cell Wall Radioactivity
	cm	*cpm/internode*
1. Control	0.42 ± .04	1101
2. GA_3	1.20 ± .07	2516
3. $CaCl_2$	0.13 ± .01	638
4. $CaCl_2$ + GA	0.25 ± .04	1129

DISCUSSION

Cell wall biosynthesis has been studied by many workers in order to investigate the hormonal control of plant growth (6). Plant cell expansion must result in an increased area of the wall which surrounds the cell, and in several cases, it has been shown that this increased area is accompanied by increased dry weight of the wall, and hence, by increased biosynthesis. From the results presented here (Table I) and elsewhere (2), it is clear that application of GA_3 causes increased synthesis of cell wall material in *Avena* stem segments. Because a strong correlation exists between the gravimetrically determined increase in cell wall material and incorporation of labeled glucose into the wall (Table I), incorporation of radioactivity can be used effectively to monitor the rate of cell wall biosynthesis, especially at short times after application of GA_3, when determination of extremely small gravimetric changes is not possible.

Baker and Ray (4) found that auxin caused increased biosynthesis of cell wall material in *Avena* coleoptiles and that this was reflected by the incorporation of externally supplied labeled glucose. In their system, endogenous substrate did not contribute substantially to wall synthesis when 50 mM labeled glucose was supplied exogenously. In oat stem segments, however, endogenous supplies of substrate contribute greatly to wall synthesis, even when 100 mM glucose is supplied externally (Tables I and V). The oat stem segments are in this way similar to pea stem segments, which also show substantial dilution of radioactivity by endogenous substrate (1). Unequal distribution of exogenously supplied glucose in the growing oat stem segment probably accounts for much of the dilution (Table II), although it seems that endogenous substrate can be used effectively in metabolism (Table V).

Our use of ^{14}C-glucose incorporation into the wall as a measure of wall synthesis revealed that GA_3 stimulates the synthesis of cell wall material in oat stem segments beginning about 1 hr after application, which is about the same time that the rate of elongation begins to accelerate (Fig. 2). The time lag for auxin stimulation of radioactive glucose incorporation into cell wall from *Avena* coleoptiles and pea stem segments is approximately 1 to 2 hr, and in this respect, pea stem segments, oat stem segments, and oat coleoptiles are similar (1, 4). Furthermore, GA_3 seems to stimulate the synthesis of all components of the wall, *i.e.*, cellulose, hemicellulose, and pectic substances (Table IV), as does auxin in peas and in *Avena* coleoptiles (1, 4). Using a gravimetric method, alternative extraction procedures, and a longer time of incubation, Adams (2) also showed that GA_3 stimulates the synthesis of all three components of oat stem segment cell wall. From the present study, it seems that this stimulation is not accomplished merely by increased rates of entry of glucose into the segment cells, but probably involves increased utilization of glucose (Tables III and V).

Auxin stimulates the incorporation of labeled glucose into cell wall material from both pea stem segments and *Avena* coleoptiles even when elongation is inhibited by $CaCl_2$ (1, 4). This indicates that auxin stimulates wall synthesis directly (1, 4). The determination of such a direct effect is dependent upon the application of an appropriate inhibitor of elongation. Although we investigated the effects of several inhibitors (cycloheximide, cold temperature, heavy metal cations, hyperosmotic media), these were found to be much less suitable than Ca for the determination of direct hormonal effects because of their inhibitory action on general metabolism. Unfortunately, however, in *Avena* stem segments, even a relatively high concentration of $CaCl_2$ (90 mM) failed to inhibit GA_3-induced elongation completely.

In conclusion, from the results presented in this study, stimulation of wall synthesis seems to be an important and relatively early effect of gibberellic acid in *Avena* stem segments and probably contributes significantly to the elongation response elicited by the hormone.

Acknowledgment—We gratefully acknowledge the gift of ^{14}C-glucose from Dr. Jane Taylor.

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