

UNCLASSIFIED

AD NUMBER
AD860421
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; SEP 1969. Other requests shall be referred to Department of the Army, Fort Detrick, MD.
AUTHORITY
BRDL D/A ltr, 29 Sep 1971

THIS PAGE IS UNCLASSIFIED

AD 860421

AD

TECHNICAL MANUSCRIPT 562

TEMPORAL AND HORMONAL CONTROL  
OF  $\beta$ -1,3 GLUCANASE  
IN PHASEOLUS VULGARIS L.

D D C  
RECEIVED  
OCT 7 2 1969  
REGIMENTAL  
B

Frederick B. Abeles  
Leonard E. Forrence

STATEMENT #2 UNCLASSIFIED

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of Dept. of Army, Fort Detrick, ATTN: Technical Release Branch/TID, Frederick, Maryland 21701

SEPTEMBER 1969

DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland

Approved by the  
CLEARINGHOUSE  
for General Scientific & Technical  
Information (Standard No. 2, 1961)

26

Reproduction of this publication in whole or in part is prohibited except with permission of the Commanding Officer, Fort Detrick, ATTN: Technical Releases Branch, Technical Information Division, Fort Detrick, Frederick, Maryland, 21701. However, DDC is authorized to reproduce the publication for United States Government purposes.

DDC AVAILABILITY NOTICES

Qualified requesters may obtain copies of this publication from DDC.

Foreign announcement and dissemination of this publication by DDC is not authorized.

Release or announcement to the public is not authorized.

DISPOSITION INSTRUCTIONS

Destroy this publication when it is no longer needed. Do not return it to the originator.

The findings in this publication are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

ACCESSION for	
CPSTI	WHITE SECTION <input type="checkbox"/>
DDC	BUFF SECTION <input checked="" type="checkbox"/>
UNANNOUNCED	<input type="checkbox"/>
JUSTIFICATION	
BY	
DISTRIBUTION/AVAILABILITY CODES	
DIST.	AVAIL. and/or SPECIAL
2	

DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 562

TEMPORAL AND HORMONAL CONTROL OF  $\beta$ -1,3 GLUCANASE  
IN PHASEOLUS VULGARIS L.

Frederick B. Abeles

Leonard E. Forrence

Plant Physiology Division  
PLANT SCIENCES LABORATORIES

Project 1B562602AD04

September 1969

ABSTRACT

The endo- $\beta$ -1,3 glucanase ( $\beta$ -1,3-glucan 3-glucanhydrolase, EC 3.2.1.6) extracted from Phaseolus vulgaris L. cv. Red Kidney had a pH optimum of 5 and a temperature optimum of 50 C. Excision of plant tissue resulted in an increase in  $\beta$ -1,3 glucanase activity after a 6-hour lag period. The increase could be prevented by indole-3-acetic acid, gibberellic acid, and cytokinins. Ethylene (half-maximal concentration = 0.1 ppm) promoted the synthesis of  $\beta$ -1,3 glucanase, and 10% CO<sub>2</sub> overcame some of the ethylene effect. Cycloheximide prevented the induction of  $\beta$ -1,3 glucanase but actinomycin D and chromomycin A<sub>3</sub> had only a partial effect.

The amount of callose in sieve tube cells correlated with levels of  $\beta$ -1,3 glucanase, suggesting that this enzyme played a role in the degradation of  $\beta$ -1,3 glucans.

CONTENTS

Abstract . . . . .	2
I. INTRODUCTION . . . . .	5
II. MATERIALS AND METHODS . . . . .	5
A. Plant Material . . . . .	5
B. Reagents . . . . .	6
III. RESULTS . . . . .	9
A. Localization of $\beta$ -1,3 Glucanase Activity in the Bean Plant . . . . .	9
B. Effect of Ethylene . . . . .	9
C. Effect of Other Hormones . . . . .	13
D. Effect of RNA and Protein Synthesis Inhibitors . . . . .	13
IV. DISCUSSION . . . . .	20
Literature Cited . . . . .	23
Distribution List . . . . .	25
DD Form 1473 . . . . .	27

FIGURES

1. Comparison Between Colorimetric and Viscometric Assays for $\beta$ -1,3 Glucanase . . . . .	8
2. Increase in $\beta$ -1,3 Glucanase Activity in Abscission Zone Explants Measured Colorimetrically and Viscometrically . . . . .	10
3. Time Course for the Induction of $\beta$ -1,3 Glucanase Activity in Abscission Zone Explants in the Presence of 1 ppm Ethylene and $10^{-4}$ M IAA . . . . .	11
4. Increase in $\beta$ -1,3 Glucanase Activity by Ethylene . . . . .	12
5. Inhibition of $\beta$ -1,3 Glucanase Activity by Plant Hormones . . . . .	14
6. Time Course for the Inhibition of $\beta$ -1,3 Glucanase by $10^{-4}$ M IAA . . . . .	16
7. Time Course for the Inhibition of $\beta$ -1,3 Glucanase by 0.25 $\mu$ g Cycloheximide . . . . .	18

TABLES

1. Increase of $\beta$ -1,3 Glucanase Activity by 1 ppm Ethylene and Reversal by 10% CO <sub>2</sub> . . . . .	13
2. Inhibition of $\beta$ -1,3 Glucanase Activity by the Cytokinins, N <sup>6</sup> Benzyladenine, SD 8339, Zestatin, and Kinetin . . . . .	15

3. Inhibition of Induction of  $\beta$ -1,3 Glucanase Activity by IAA, GA, and Cytokinin SD 8339 . . . . . 15
4. Inhibition of Ethylene-Induced  $\beta$ -1,3 Glucanase Activity by Cycloheximide and Actinomycin D . . . . . 17
5. Frequency of Sieve Tube Callose Deposits in the Pulvinus of Bean Leaf Abscission Zone Explants after Aging in the Presence of Various Chemicals . . . . . 19
6. Effect of 10 ppm Ethylene on Callose and  $\beta$ -1,3 Glucanase from Bean Leaves . . . . . 19

## I. INTRODUCTION\*

Regulation of enzymatic activity is frequently incorporated into explanations of hormonal action on plants. Two examples are the induction of  $\alpha$ -amylase during gibberellic acid - stimulated barley germination<sup>1</sup> and cellulase induction in ethylene-regulated abscission.<sup>2</sup> This paper presents data in favor of the view that regulation of another carbohydrase,  $\beta$ -1,3 glucanase, is important in the degradation of sieve tube callose and that a variety of plant hormones control the production of this enzyme.

## II. MATERIALS AND METHODS

### A. PLANT MATERIAL

Bean (Phaseolus vulgaris L. cv. Red Kidney) plants were grown at  $24 \pm 2$  C, 64% relative humidity, and a 12-hour photoperiod supplied by fluorescent and incandescent lamps that yielded 1,200 ft-c at plant level. Petiole abscission-zone explants (4.5 mm pulvinus, 5.5 mm petiole) from the primary leaf were excised from 2-week-old plants and inserted into a 3-mm layer of 1.5% agar contained in either 15-cm petri plates or  $43 \pm 2$  ml gas collection bottles. The gas collection bottles were 5 cm in diameter and 2.5 cm high and had a neck that accommodated a 2.5-cm-diameter rubber vaccine cap. Except for experiments with RNA and protein synthesis inhibitors, the explants were treated by incorporating various chemicals in the agar. During the experiments, the explants were exposed to 25 C and 400 ft-c of continuous fluorescent light. The light, however, had no effect on induction of  $\beta$ -1,3 glucanase; results similar to those reported here were obtained with explants stored in the dark. The treatments were terminated by freezing the explants at -16 C and storing them at that temperature until subsequent extraction of enzyme. When whole plants were treated with ethylene, they were placed in plastic boxes (60 by 60 by 60 cm) in a 25 C growth chamber with a 400 ft-c 16-hour photoperiod supplied by fluorescent lamps. Experimental data shown in the figures and tables are the means of two or three replicates and are representative of three experiments performed on separate occasions.

---

\* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.



## B. REAGENTS

The following chemicals were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio: indole-3-acetic acid (IAA), 75% K salt gibberellic acid (GA), N-6-benzyladenine, actinomycin D, and laminarin. Zeatin was purchased from Calbiochem Corp., Los Angeles, California. Cytokinin SD 8339 6-benzylamino-9,2-(tetrahydropyran-9 H purine) was a gift of the Shell Development Co., Modesto, California. The R.J. Reynolds Tobacco Co., Winston-Salem, North Carolina, kindly supplied the abscisic acid (47.3% cis,trans). The pachyman used to make carboxymethylpachyman (CMP) was a gift of Dr. B.A. Stone, University of Melbourne, Parkville, Victoria, Australia. The CMP was made according to the method described by Clarke and Stone.<sup>3</sup> The procedure was modified by adding 3 liters of 2-propanol to the original reaction mixture of pachyman and NaOH. Chromomycin A<sub>3</sub> was purchased from the Mann Research Laboratories, New York, New York.

Explants were treated with ethylene and CO<sub>2</sub> by adding appropriate dilutions of these gases to the gas phase inside the gas collection bottles with a syringe inserted through the rubber vaccine cap. A flame ionization gas chromatograph fitted with a  $\frac{1}{4}$ -inch, 60-cm activated alumina column run at 100 C was used to measure ethylene.

Actinomycin D (1  $\mu$ g), cycloheximide (0.25  $\mu$ g), and chromomycin A<sub>3</sub> (5  $\mu$ g) were injected as 1- $\mu$ liter solutions into bean explants with a microliter syringe by inserting the needle up through the center of the petiole tissue to a depth of about 5 mm, at which point the firmer pulvinal tissue resists further movement of the needle.

$\beta$ -1,3 Glucanase was extracted from plant tissue  $\beta$ -1,3 glucan 3-glucanohydrolase, EC 3.2.1.6, by homogenizing 10 explants, or an equivalent amount of other tissue, in 4 ml of 0.05 M, pH 5, potassium acetate buffer in a Ten Broeck homogenizer. The homogenates were filtered through Miracloth (Calbiochem Corp.) premoistened with acetate buffer centrifuged at 10,000 x g for 10 minutes and the resulting supernatant liquid was retained for subsequent assay of activity. The  $\beta$ -1,3 glucanase is a soluble enzyme; 100,000 x g for 1 hour removed only 16% of the original activity from the crude homogenate and no activity could be recovered from the precipitate.

Both CMP and laminarin were used as substrates for the  $\beta$ -1,3 glucanase. The reaction of  $\beta$ -1,3-glucanase with CMP was measured in a Wells-Brookfield microviscometer (Brookfield Engineering Laboratories, Stoughton, Massachusetts). Equal volumes of enzyme and 2% CMP were mixed together and a 1-ml sample was placed in the microviscometer. The resulting viscosity after 10 minutes' incubation at 40 C (timed from the moment of mixing) indicated the activity of the enzyme. The viscometric technique was a sensitive method of measuring  $\beta$ -1,3 glucanase, but it was time-consuming and not suited to measuring large numbers of samples. For routine experiments, a colorimetric assay utilizing laminarin as a substrate and the dinitrosalicylic reagent (prepared by adding 300 ml of 4.5% NaOH to 880 ml of a

solution containing 8.8 g dinitrosalicylic acid and 255 g potassium sodium tartrate·6H<sub>2</sub>O) was used to measure the reducing sugars produced. After initial experiments revealed that optimum conditions for assay were pH 5 and 50 C, 0.5 ml enzyme in 0.05 M, pH 5, potassium acetate buffer was routinely added to 0.5 ml of 2% (w/v) laminarin in water and incubated at 50 C for 1 or 2 hours. The laminarin was dissolved by heating the 2% solution briefly in a boiling water bath before use. The reaction was stopped by adding 3 ml of the dinitrosalicylic reagent and heating the tubes for 5 minutes at 100 C. The tubes were then cooled to 25 C, the contents were diluted 1:10 with water, and the optical density was read at 500 nm. Figure 1 presents data comparing the viscometric and colorimetric assays for  $\beta$ -1,3 glucanase. The Nelson reagent was also utilized in a number of experiments and gave similar results.<sup>4</sup> Because of the complex nature of the reaction, straight-line kinetics were not obtained with these assays. However, at lower enzyme concentrations, the change in optical density and viscosity gave a practical approximation of relative enzyme activity. Lack of linearity was due in part to the fact that the  $\beta$ -1,3 glucanase is an endo- as opposed to exo-enzyme. This was established by chromatographing the reaction products on a thin layer of cellulose with isopropanol:acetic acid:water (67:10:23). To reveal the sugars produced, the chromatographs were sprayed with benzidine (0.5 g benzidine, 20 ml glacial acetic acid, 80 ml absolute alcohol) and heated at 100 C for 5 minutes. The  $\beta$ -1,3 glucanase released no observable glucose from the laminarin, but rather a series of short fragments (presumably  $\beta$ -1,3 oligosaccharides) that chromatographed behind a glucose standard. The failure of the glucose oxidase reaction<sup>5</sup> to give a positive result with the products of a  $\beta$ -1,3 glucanase digestion supported chromatographic evidence.

Cellulase deposits were measured according to the methods of Scott et al.<sup>6</sup>

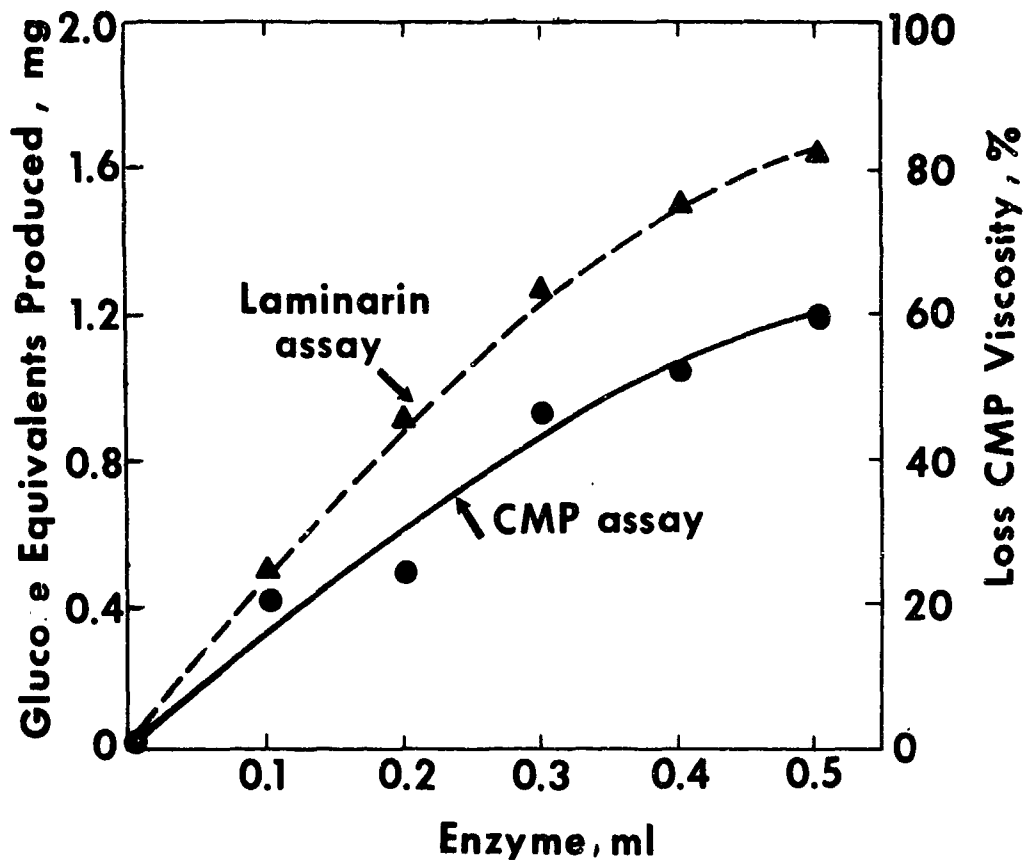


FIGURE 1. Comparison Between Colorimetric and Viscometric Assays for  $\beta$ -1,3 Glucanase. For the laminarin assay 0.5 ml of 2% laminarin was added to 0.5 ml of an enzyme buffer (0.05 M, pH 5, sodium acetate) mixture and incubated at 50 C for 2 hours. Reducing groups were measured by the dinitrosalicylic method. The viscometric assay utilized a 1-ml example containing 0.5 ml 2% CMP and 0.5 ml enzyme acetate buffer mixture. The activity of the enzyme was expressed as the per cent loss of viscosity of the mixture after a 10-minute 40 C incubation period compared with a substrate buffer control.

### III. RESULTS

#### A. LOCALIZATION OF $\beta$ -1,3 GLUCANASE ACTIVITY IN THE BEAN PLANT

All of the above-ground portions of the bean plant contained  $\beta$ -1,3 glucanase; the activity of the enzyme increased in aged excised tissues. Figure 2 shows representative data on the increase in  $\beta$ -1,3 glucanase activity in abscission zone explants with time as measured both viscometrically with CMP as the substrate and colorimetrically with laminarin as the substrate. The difference in time required for the increase in activity between the two curves is due in part to the fact that the experiments were performed on separate occasions. After these initial experiments, the viscometric assay, although more sensitive, was discontinued because more samples could be examined colorimetrically in an equivalent amount of time.

The increase in  $\beta$ -1,3 glucanase activity occurs for at least 3 days, as shown in Figure 3. When the experiments were originally planned, it was felt that  $\beta$ -1,3 glucanase might play some role in abscission, and so both abscission-accelerating (ethylene) and inhibitory (IAA) hormones were applied to the explants. The data indicate that ethylene increased  $\beta$ -1,3 glucanase activity but IAA delayed the increase. Transferring IAA-treated explants to fresh IAA after 1 day had no further effect on blocking the increase.

The idea that the increase in  $\beta$ -1,3 glucanase represented a wounding response was tested by subdividing ten 1-cm petiole sections into 2, 3, and 4 pieces and comparing their  $\beta$ -1,3 glucanase activity with that of intact sections after a 24-hour aging period. It was found that subdividing the petiole sections caused a loss rather than increase in  $\beta$ -1,3 glucanase activity. The wounding explanation was tested further by harvesting explants a day after they had been scratched with a needle and comparing them with untreated controls. No difference was found between explants from treated and control plants. It was also possible that the increase in activity was due to changes in inhibitors and activators in the tissue. To check this explanation, extracts from initial and aged explants were deproteinized by heating them to 100 C for 10 minutes and then adding samples back to enzyme solutions from initial or aged explants. The protein-free solutions had no effect on enzyme activity from initial or aged explants and had no activity themselves.

#### B. EFFECT OF ETHYLENE

A concentration curve is shown in Figure 4 and, characteristic of ethylene effects, 0.1 ppm was approximately half-maximal and 10 ppm was essentially saturating. The characteristic reversal of ethylene action by CO<sub>2</sub> is shown in Table 1. However, the effect was small and 10% CO<sub>2</sub> was unable to reverse completely the effect of 1 ppm ethylene or reduce the control value to the initial activity. The maximum rate of increase of  $\beta$ -1,3 glucanase activity depended on a continual supply of ethylene. Removal of ethylene after 14 hours caused the rate of increase to slow to that shown by controls.

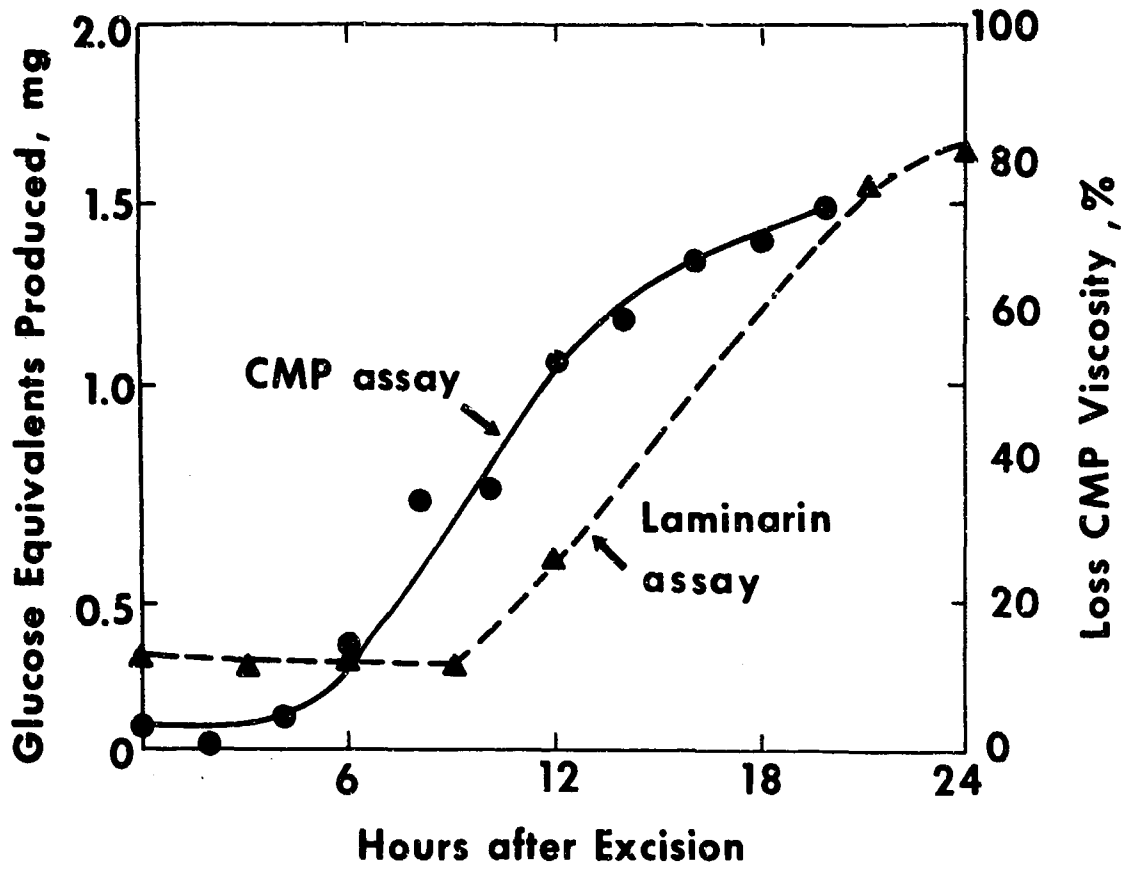


FIGURE 2. Increase in  $\beta$ -1,3 Glucanase Activity in Abscission Zone Explants Measured Colorimetrically and Viscometrically. Explants were excised and stored on plain agar in petri plates and frozen at the times indicated.

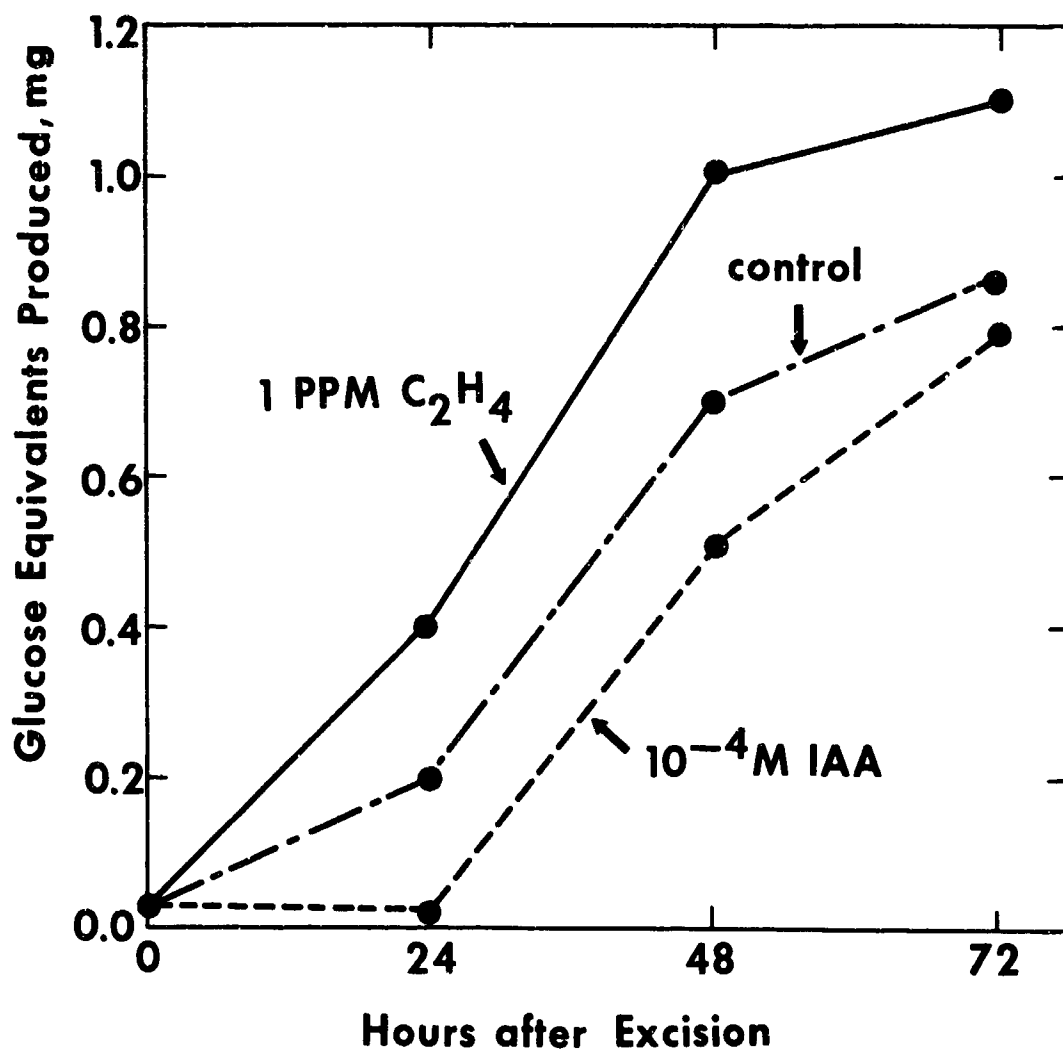


FIGURE 3. Time Course for the Induction of  $\beta$ -1,3 Glucanase Activity in Abscission Zone Explants in the Presence of 1 ppm Ethylene and  $10^{-4}$  M IAA. The explants were placed in gas collection bottles containing plain agar or  $10^{-4}$  M IAA. Explants were placed pulvinal-end down in the IAA to facilitate transport into the tissue. Explants were harvested at the time indicated and frozen until subsequent extraction of  $\beta$ -1,3 glucanase. Flasks were vented and resealed at 24 and 48 hours and ethylene was reinjected at those times. Because of the high activity after 3 days, only 0.1 ml enzyme was used in the laminarin assay.

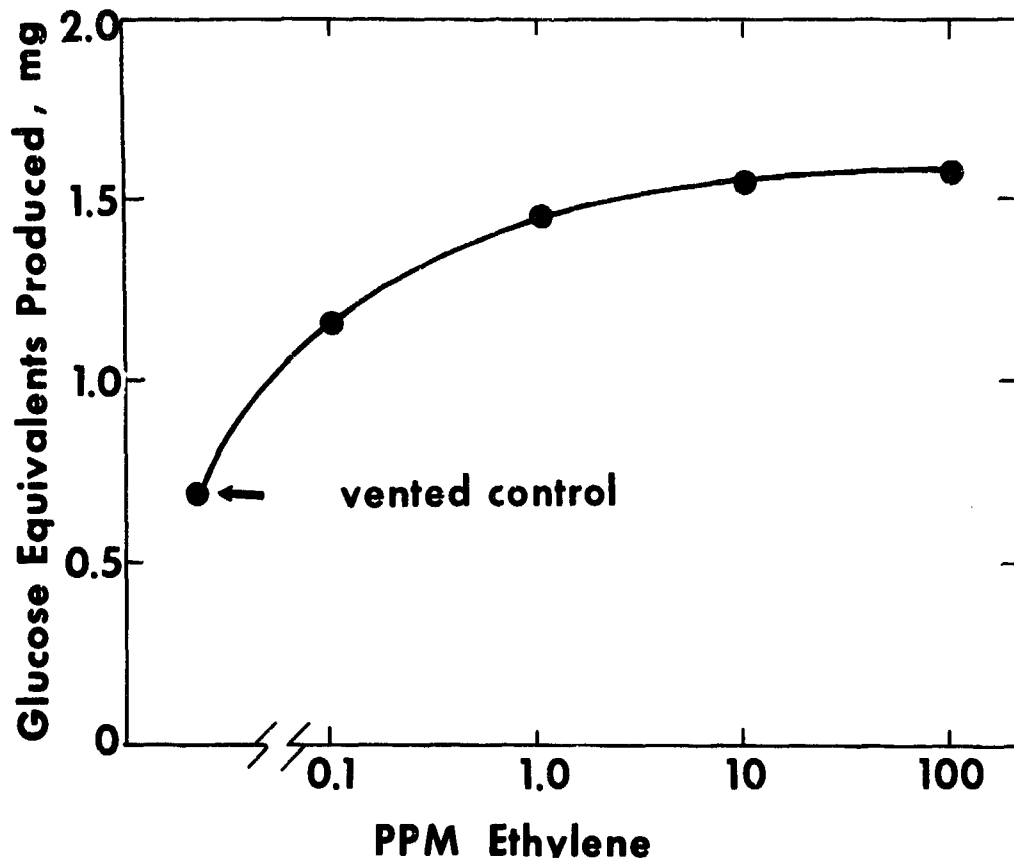


FIGURE 4. Increase in  $\beta$ -1,3 Glucanase Activity by Ethylene. Explants were treated with ethylene in gas collection bottles. The bottles were vented and resealed after an initial 7 hours to remove "wound" ethylene. Vented control bottles were covered with cheesecloth instead of the usual rubber vaccine stopper. Explants were frozen 24 hours after the start of the experiment and stored until subsequent extraction for  $\beta$ -1,3 glucanase activity.

TABLE 1. INCREASE OF  $\beta$ -1,3 GLUCANASE ACTIVITY  
BY 1 PPM ETHYLENE AND REVERSAL BY 10% CO<sub>2</sub><sup>a/</sup>

Treatment	Glucose Equivalents Produced, mg
0 hour initial	0.49
Control	1.20
Ethylene	1.73
CO <sub>2</sub>	1.11
Ethylene + CO <sub>2</sub>	1.51

a. Explants were placed petiole-end down in 1.5% agar in sealed bottles for 24 hours in the gas phases indicated. Bottles were vented and resealed, and ethylene and/or CO<sub>2</sub> was reinjected 6 hours after the start of the experiment.

#### C. EFFECT OF OTHER HORMONES

Figure 5 shows that IAA was more effective than GA or SD 8339 in inhibiting the increase of  $\beta$ -1,3 glucanase activity. Table 2 presents data that show that, of the cytokinins available for testing, SD 8339 was the most effective in blocking the increase in  $\beta$ -1,3 glucanase activity. The action of these hormones was complex. Table 3 presents data on the effect of low concentrations of these substances added singly or in combination to explants. When IAA, GA, and SD 8339 were added in combination, they had a synergistic effect compared with the action of these hormones alone. Abscisic acid was also tested for its effect on the increase in  $\beta$ -1,3 glucanase activity. However, concentrations up to 10<sup>-4</sup> M were inactive.

Figure 6 shows the effect of adding IAA at 2-hour intervals after excision of the explants. The data show that IAA was capable of blocking the increase in  $\beta$ -1,3 glucanase activity when added as much as 6 hours after excision of the explants.

#### D. EFFECT OF RNA AND PROTEIN SYNTHESIS INHIBITORS

Table 4 shows that cycloheximide blocked the increase in  $\beta$ -1,3 glucanase activity but actinomycin D had only a partial effect. Another RNA-synthesis inhibitor, chromomycin A<sub>3</sub>, which has an action similar to that of actinomycin D,<sup>7,8</sup> was also tested and had an effect similar to that of actinomycin D. The amounts of inhibitor used represent the minimal concentration that would completely block abscission of the explants. Since both RNA and protein synthesis are required for abscission,<sup>9</sup> this served as a check for the effectiveness of these compounds on RNA and protein synthesis.



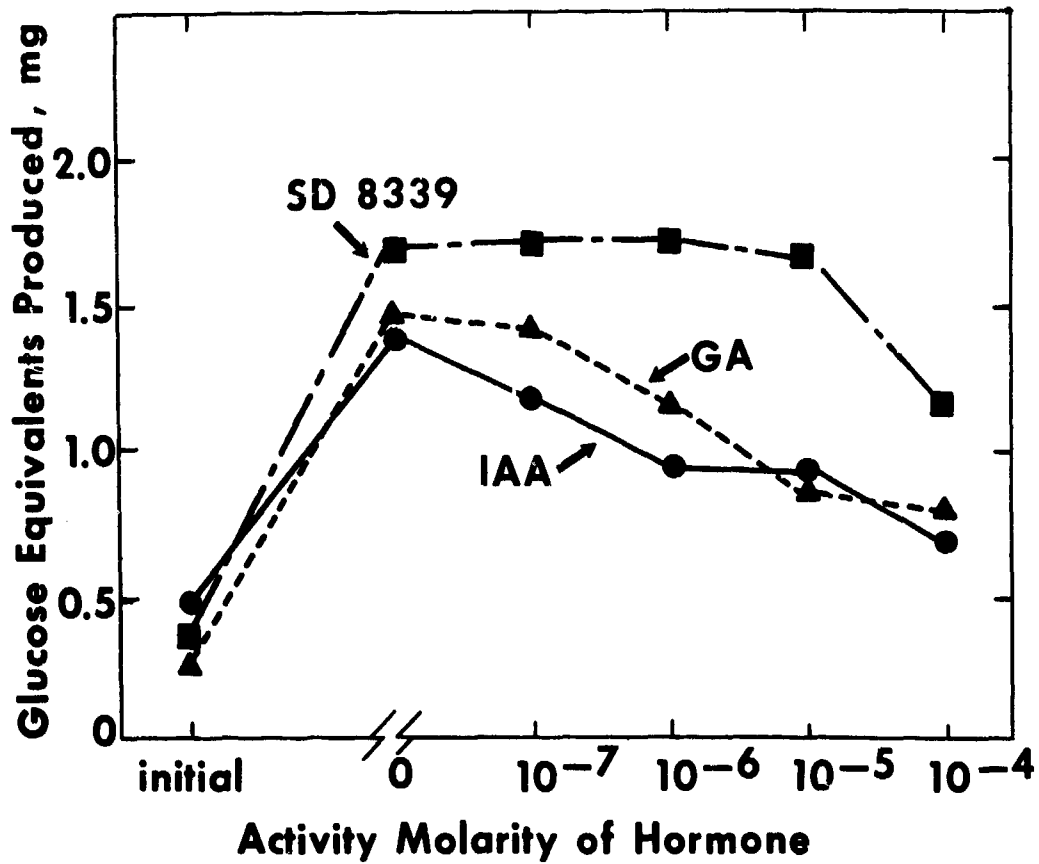


FIGURE 5. Inhibition of  $\beta$ -1,3 Glucanase Activity by Plant Hormones. Explants were inserted pulvinal-end down in petri plates containing the substances to be tested. Except for the freshly harvested explants (initial), explants were harvested after 24 hours of treatment and frozen until subsequent determination of  $\beta$ -1,3 glucanase activity.

TABLE 2. INHIBITION OF  $\beta$ -1,3 GLUCANASE ACTIVITY  
BY THE CYTOKININS, N<sup>6</sup> BENZYLADENINE,  
SD 8339, ZEATIN, AND KINETIN<sup>a/</sup>

Treatment	Glucose Equivalents Produced, mg
0 hour initial	0.34
Control	1.07
SD 8339	0.47
Kinetin	0.68
N <sup>6</sup> Benzyladenine	0.70
Zeatin	0.73

a. Explants were placed pulvinal-end down in 1.5% agar containing the cytokinins indicated at a concentration of  $10^{-4}$  M for 24 hours before determination of enzyme activity.

TABLE 3. INHIBITION OF INDUCTION OF  $\beta$ -1,3 GLUCANASE ACTIVITY  
BY IAA, GA, AND CYTOKININ SD 8339<sup>a/</sup>

Treatment	Glucose Equivalents Produced, mg
0 hour initial	0.55
Control	1.27
IAA	1.23
GA	1.00
SD 8339	0.68
IAA + GA	0.62
IAA + SD 8339	0.47
GA + SD 8339	0.62
IAA + GA + SD 8339	0.33

a. Explants were placed pulvinal-end down in 1.5% agar containing  $10^{-5}$  M IAA,  $10^{-5}$  M GA, or  $10^{-4}$  M SD 8339, singly or in combination as indicated, for 24 hours before determination of enzyme activity.

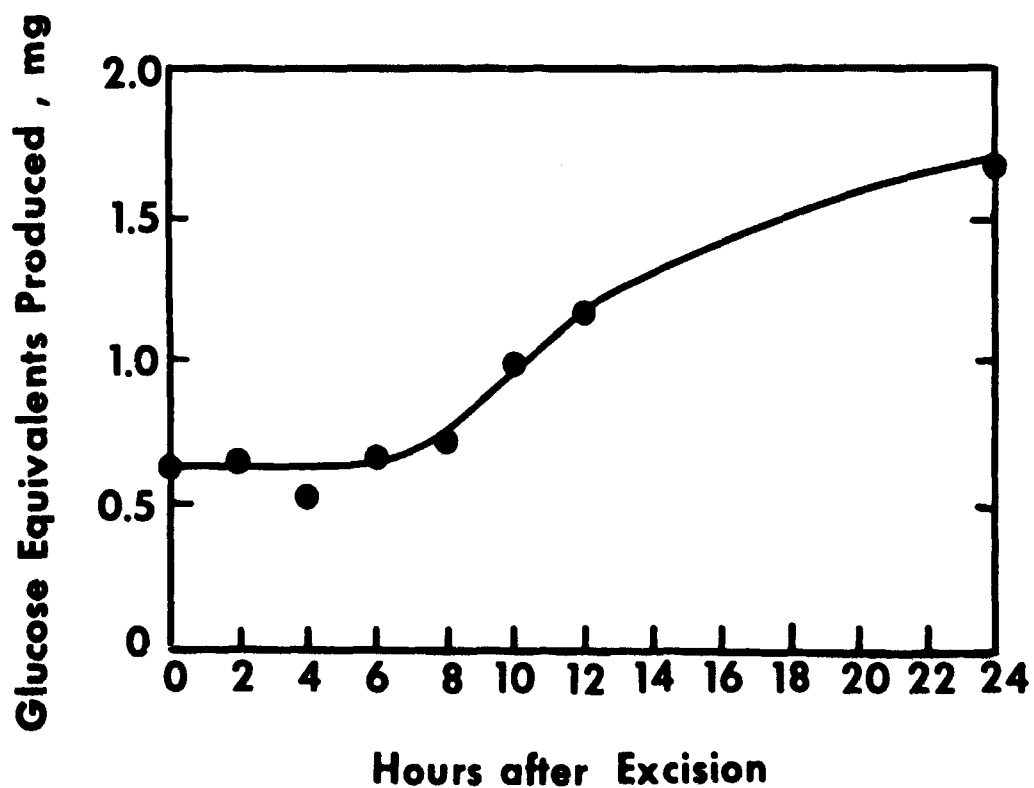


FIGURE 6. Time Course for the Inhibition of  $\beta$ -1,3 Glucanase by  $10^{-4}$  M IAA. Explants were excised and placed in petri plates containing plain agar. At the time indicated they were inserted pulvinal-end down in IAA agar. The explants were harvested 24 hours after the start of the experiment and frozen.

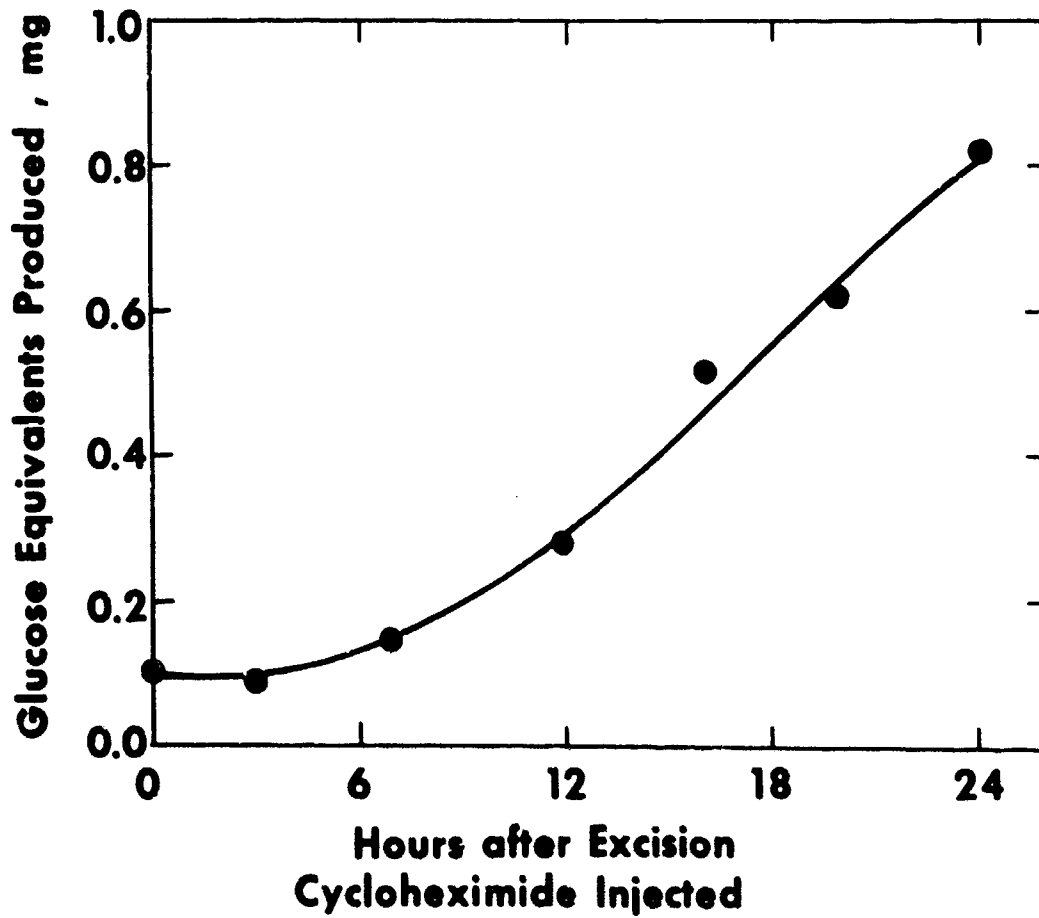
TABLE 4. INHIBITION OF ETHYLENE-INDUCED  $\beta$ -1,3 GLUCANASE ACTIVITY BY CYCLOHEXIMIDE AND ACTINOMYCIN D<sup>a</sup>/

Treatment	Glucose Equivalents Produced, mg		
	Cycloheximide	Actinomycin D	Chromomycin A <sub>3</sub>
0 hour initial	0.22	0.14	0.22
Control	1.22	1.59	0.92
Ethylene	2.01	1.67	1.77
Inhibitor	0.24	1.54	0.81
Ethylene + inhibitor	0.20	1.57	0.90

a. Explants were injected with 1  $\mu$ liter of 0.25  $\mu$ g cycloheximide per  $\mu$ liter or 1  $\mu$ g actinomycin D per  $\mu$ liter after excision and inserted petiole-end down in bottles containing 1.5% agar. Bottles were sealed and, as required, 1 ppm ethylene was added to the gas phase.

Figure 7 shows that cycloheximide can be injected into explants as much as 7 hours after they are excised from the plant and still block most of the subsequent increase in  $\beta$ -1,3 glucanase activity.

Sieve tube callose is a carbohydrate containing  $\beta$ -1,3 linkages and as such represents a logical substrate for increased levels of  $\beta$ -1,3 glucanase. Table 5 shows that  $\beta$ -1,3 glucanase activity in pulvinal tissue of explants correlated well with the number of callose deposits in the same tissue. In another experiment (Table 6) whole plants were gassed with 10 ppm ethylene and produced large quantities of  $\beta$ -1,3 glucanase in the leaves. Callose deposits in the pulvini of these leaves decreased during this same period. After 3 days of ethylene fumigation the plants were completely defoliated and the leaves wilted and chlorotic.



**FIGURE 7.** Time Course for the Inhibition of  $\beta$ -1,3 Glucanase by 0.25  $\mu$ g Cycloheximide. Explants were excised and stored on plain agar. At the times indicated they were injected with 1  $\mu$ liter of cycloheximide solution and returned to the agar. After 24 hours' incubation, the explants were harvested and frozen.

TABLE 5. FREQUENCY OF SIEVE TUBE CALLOSE DEPOSITS IN THE PULVINUS OF BEAN LEAF ABSCISSION ZONE EXPLANTS AFTER AGING IN THE PRESENCE OF VARIOUS CHEMICALS<sup>a/</sup>

Treatment	Number of Callose Deposits <sup>b/</sup>	Glucose Equivalents Produced, $\mu$ g
Initial	41 $\pm$ 4	0.54
24 hour control	11 $\pm$ 6	1.18
24 hour 10 <sup>-4</sup> M IAA	12 $\pm$ 3	0.81
24 hour 10 ppm ethylene	9 $\pm$ 5	1.89
24 hour 0.25 $\mu$ g cycloheximide	33 $\pm$ 6	0.59

- a. Longitudinal sections 32  $\mu$  thick were prepared from four pulvini and stained with 0.005 aniline blue in 0.15 M pH 8, potassium phosphate buffer. The number of fluorescent sieve tube end walls observed in ten 0.62-mm-diameter fields from each pulvinus was counted and averaged.  $\beta$ -1,3 Glucanase activity represents 0.25 ml enzyme preparation extracted from 10 pulvini.
- b.  $\pm$  = standard deviation.

TABLE 6. EFFECT OF 10 PPM ETHYLENE ON CALLOSE AND  $\beta$ -1,3 GLUCANASE FROM BEAN LEAVES<sup>a/</sup>

Day	Number Callose Deposits <sup>b/</sup>		Glucose Equivalents Produced, $\mu$ g	
	Control	Ethylene	Control	Ethylene
0	62 $\pm$ 12	-	0.0	-
1	58 $\pm$ 12	39 $\pm$ 20	0.02	0.23
2	60 $\pm$ 14	36 $\pm$ 13	0.03	0.46
3	64 $\pm$ 15	31 $\pm$ 15	0.02	0.62

- a. Longitudinal sections 32  $\mu$  thick were prepared from four pulvini and stained with 0.005% aniline blue in 0.15 M pH 8 phosphate buffer. The number of fluorescent sieve tube end walls observed in ten 0.62-mm-diameter fields from each pulvinus was counted and averaged.  $\beta$ -1,3 Glucanase was assayed by extracting leaf blade tissue with 0.05 M pH 5 acetate buffer (1 ml buffer per gram tissue) and incubating 0.1 ml of homogenate after 10 min centrifugation at 10,000  $\times$  g with 10  $\mu$ g laminarin in 0.9 ml 0.05 M, pH 5 acetate buffer at 50 C for 1 hour.
- b.  $\pm$  = standard deviation.

#### IV. DISCUSSION

The biochemical properties of bean  $\beta$ -1,3 glucanase reported here (pH optimum of 5, 50 C optimum, and non-particulate nature) are similar to those reported by others studying plant  $\beta$ -1,3 glucanase.<sup>3,10,11</sup> However, Clarke and Stone<sup>3</sup> reported that the  $\beta$ -1,3 glucanase preparation they isolated from grape vines produced glucose from laminarin, but the bean enzyme was an endo-enzyme producing only oligosaccharides from laminarin. The  $\beta$ -1,3 glucanases studied by Mandels et al.<sup>11</sup> and Esser<sup>10</sup> were also endo-enzymes.

The observations that 0.1 ppm ethylene was half-maximal in increasing  $\beta$ -1,3 glucanase (Fig. 4) and that CO<sub>2</sub> was partially able to overcome the ethylene effect (Table 1) are typical of ethylene action in plants. An earlier paper from this laboratory reported analogous results with the effect of ethylene on abscission and summarized similar examples from earlier literature on other ethylene-mediated responses.<sup>12</sup> Apparently the attachment site of ethylene in the cell that eventually gives rise to an increase in  $\beta$ -1,3 glucanase activity is the same or similar to the one that regulates a diverse array of processes such as abscission (RNA-dependent cellulase synthesis), ripening, inhibition of cell elongation, peroxidase synthesis, and flower fading. Cytokinins, auxin, and gibberellins delayed the appearance of  $\beta$ -1,3 glucanase activity in excised bean tissue (Fig. 5 and 6, and Tables 2 and 3). Why these basically dissimilar compounds have corresponding effects is puzzling. It should be noted, however, that the concentrations used to block  $\beta$ -1,3 glucanase synthesis are high compared with those for the normal growth regulator effects ascribed to these hormones in plants. It is possible, however, that the aging- or senescence-retarding capabilities these materials display at high concentrations may be a part of the explanation. In other words, excision of plant tissue sets off an aging phenomenon as the endogenous supply of aging retardants are used up in the tissue and no replenishment from the normal source of supply is possible. The effect is demonstrated in Figure 6. The data indicate that IAA can be added to the explant 6 hours after it has been excised and still block the subsequent rise of  $\beta$ -1,3 glucanase activity. This is analogous to what has been observed with the induction of cellulase (a  $\beta$ -1,4 glucanase) during abscission. IAA can be added to abscission zone explants up to 6 hours after excision and still block the formation of cellulase.<sup>13</sup> It appears likely that inhibition of  $\beta$ -1,3 glucanase by these plant hormones occurs in different ways. If IAA, GA, and SD 8339 were having exactly the same effect, then combinations of these hormones should have additive effects. However, the data in Table 3 suggest an interaction of these hormones because a synergistic effect was observed.

The effect of IAA on  $\beta$ -1,3 glucanase has been studied earlier by others. Unlike the results presented here, they found that auxin either promoted<sup>14</sup> or had no effect<sup>15</sup> on  $\beta$ -1,3 glucanase. The ability of cycloheximide to block the appearance of  $\beta$ -1,3 glucanase activity is taken as evidence in favor of the idea that the increased  $\beta$ -1,3 glucanase activity is due to

de novo protein synthesis (Table 4). However, positive proof will ultimately depend on the demonstration that radioactively labeled amino acids are incorporated into a purified enzyme. The  $\beta$ -1,3 glucanase induction curve shown in Figure 2 indicates that about 6 hours are required after excision before the enzyme is synthesized. This was demonstrated another way by showing that an injection of cycloheximide lost its full inhibitory effect by 6 hours following excision (Fig. 7).

On the other hand, inhibitors of RNA synthesis (actinomycin D, chromomycin A<sub>3</sub>) had only a partial effect on  $\beta$ -1,3 glucanase induction. Lack of effectiveness is not thought to be due to inactivity of these compounds because they completely blocked abscission in this tissue, which we had shown earlier to require RNA synthesis.<sup>16</sup> The data in Table 4 suggest either that the temporal effect does not need RNA synthesis and the ethylene effect does, or that both processes have some requirement blocked by these antibiotics.

A number of functions for  $\beta$ -1,3 glucanase in plant tissue have been suggested. These include degradation of seed glucans,<sup>17</sup> control of cell elongation,<sup>18</sup> regulation of pollen tube growth,<sup>19</sup> cell expansion of yeast,<sup>18</sup> fertilization,<sup>10</sup> and removal of callose.<sup>3</sup>

Data in Tables 5 and 6 present evidence in favor of the last idea. Earlier, Scott et al.<sup>6</sup> reported that ethylene-treated bean abscission zone explants had less callose than controls and that auxin-treated ones had more. Using their methods and measuring the fluorescence of phloem stained with aniline blue, we found that callose deposition correlated with  $\beta$ -1,3 glucanase activity. Such data are consistent with, but do not prove, the suggestion that one function of  $\beta$ -1,3 glucanase is to regulate the amount of  $\beta$ -1,3 glucans plugging sieve tube cell plates. On the other hand, it is unlikely that  $\beta$ -1,3 glucanase plays any role in abscission. Unlike cellulase,  $\beta$ -1,3 glucanase activity is not localized in the separation layer. Secondly, even though actinomycin D and chromomycin A<sub>3</sub> block abscission, they do not completely prevent the increase in  $\beta$ -1,3 glucanase. Finally, even though gibberellic acid was able to block the temporal increase in  $\beta$ -1,3 glucanase, it does not prevent abscission.



LITERATURE CITED

1. Varner, J.E.; Chandra, G.R. 1964. Hormonal control of enzyme synthesis in barley endosperm. *Proc. Nat. Acad. Sci.* 52:100-106.
2. Abeles, F.B. 1969. Abscission: Role of cellulase. *Plant Physiol.* 44:447-452.
3. Clarke, A.E.; Stone, B.A. 1962.  $\beta$ -1,3-Glucanase hydrolysis from the grape vine (*Vitis vinifera*) and other plants. *Phytochemistry* 1:175-188.
4. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153:375-380.
5. Bergmeyer, H.U. 1963. *Methods of enzymatic analysis*, p. 123-130. Academic Press, New York.
6. Scott, P.C.; Miller, L.W.; Webster, B.D.; Leopold, A.C. 1967. Structural changes during bean leaf abscission. *Amer. J. Bot.* 54:730-734.
7. Kaziro, Y.; Kamiyama, M. 1965. Inhibition of RNA polymerase reaction by chromomycin A<sub>3</sub>. *Biochem. Biophys. Res. Comm.* 19:433-437.
8. Miura, Y.; Moriyama, Y.; Hasegawa, S.; Fukui, N. 1965. Studies on the metabolism of rat-ascites-hepatoma with nitrogen mustard sensitive and resistant strains: VIII. Effect of chromomycin A<sub>3</sub> on the nuclear RNA metabolism. *J. Biochem. (Tokyo)* 57:440-444.
9. Abeles, F.B. 1968. Role of RNA and protein synthesis in abscission. *Plant Physiol.* 43:1577-1586.
10. Esser, K. 1963. Bildung und abbau von Callose in den Samenanlagen der *Petunia hybrida*. *Zeit. f. Bot.* 51:32-51.
11. Mandels, M.; Parrish, F.W.; Reese, E.T. 1967.  $\beta$ -(1,3) Glucanases from plant callus cultures. *Phytochemistry* 6:1097-1100.
12. Abeles, F.B.; Gahagan, H.E. 1968. Abscission: Role of ethylene, ethylene analogues, carbon dioxide, and oxygen. *Plant Physiol.* 43:1255-1258.
13. Craker, L.E.; Abeles, F.B. 1969. Abscission: Role of abscisic acid. *Plant Physiol.* 44:(in press).
14. Tanimoto, E.; Masuda, Y. 1968. Effect of auxin on cell wall degrading enzymes. *Physiol. Plant.* 21:820-826.

15. Datko, A.H.; MacLachlan, G.A. 1968. Indoleacetic acid and the synthesis of glucanases and pectic enzymes. *Plant Physiol.* 43:735-742.
16. Holm, R.E.; Abeles, F.B. 1967. Abscission: The role of RNA synthesis. *Plant Physiol.* 42:1094-1102.
17. Dillon, T.; O'Colla, P. 1951. Enzymatic hydrolysis of 1,3 linked polyglucosans. *Chem. Ind. London* 1951:111.
18. Shimoda, C.; Yanagishima, N. 1968. Strain dependence of the cell-expanding effect of  $\beta$ -1,3 glucanase in yeast. *Physiol. Plant.* 21:1163-1169.
19. Roggen, H.P.J.R.; Stanley, R.G. 1969. Cell-wall-hydrolysing enzymes in wall formation as measured by pollen-tube extension. *Planta* 84:295-303.

Unclassified  
Security Classification

DOCUMENT CONTROL DATA - R & D		
<i>(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)</i>		
1. ORIGINATING ACTIVITY (Corporate author)		2a. REPORT SECURITY CLASSIFICATION
Department of the Army Fort Detrick, Frederick, Maryland, 21701		Unclassified
		2b. GROUP
3. REPORT TITLE		
TEMPORAL AND HORMONAL CONTROL OF $\beta$ -1,3-GLUCANASE IN <u>PHASEOLUS VULGARIS L.</u>		
4. DESCRIPTIVE NOTES (Type of report and inclusiv dates)		
5. AUTHOR(S) (First name, middle initial, last name)		
Frederick B. Abeles Leonard E. Forrence		
6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
September 1969	27	19
8a. CONTRACT OR GRANT NO.		9a. ORIGINATOR'S REPORT NUMBER(S)
a. PROJECT NO. 1B562602ADO4		Technical Manuscript 562
c.		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.		CMS 6596
10. DISTRIBUTION STATEMENT		
Qualified requesters may obtain copies of this publication from DDC. Foreign announcement and dissemination of this publication by DDC is not authorized. Release or announcement to the public is not authorized.		
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY
		Department of the Army Fort Detrick, Frederick, Maryland, 21701
13. ABSTRACT		
<p>The endo-<math>\beta</math>-1,3 glucanase (<math>\beta</math>-1,3-glucan 3-glucanhydrolase, EC 3.2.1.6) extracted from <u>Phaseolus vulgaris L. cv. Red Kidney</u> had a pH optimum of 5 and a temperature optimum of 50 C. Excision of plant tissue resulted in an increase in <math>\beta</math>-1,3 glucanase activity after a 6-hour lag period. The increase could be prevented by indole-3-acetic acid, gibberellic acid, and cytokinins. Ethylene (half-maximal concentration = 0.1 ppm) promoted the synthesis of <math>\beta</math>-1,3 glucanase, and 10% CO<sub>2</sub> overcame some of the ethylene effect. Cycloheximide prevented the induction of <math>\beta</math>-1,3 glucanase but actinomycin D and chromomycin A<sub>3</sub> had only a partial effect.</p> <p>The amount of callose in sieve tube cells correlated with levels of <math>\beta</math>-1,3 glucanase, suggesting that this enzyme played a role in the degradation of <math>\beta</math>-1,3 glucans.</p>		
14. Key Words		
<p><math>\beta</math>-1,3 Glucanase Bean Ethylene Carbohydrases Callose Hormones (plant)</p>		

DD FORM 1473

REPLACES DD FORM 1473, 1 JAN 60, WHICH IS OBSOLETE FOR ARMY USE.

Unclassified  
Security Classification