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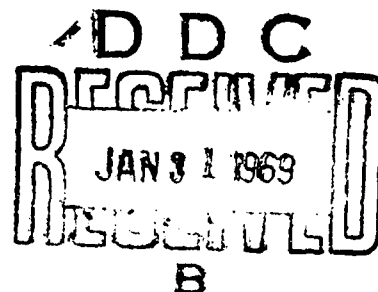
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TECHNICAL MANUSCRIPT 488

ABSCISSION: ROLE OF CELLULASE

Frederick B. Abeles



NOVEMBER 1968

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TECHNICAL MANUSCRIPT 488

ABSCISSION: ROLE OF CELLULASE

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Plant Physiology Division
PLANT SCIENCES LABORATORIES

Project 1B562602A061

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ABSTRACT

Cellulase (β -1,4-glucan-glucanohydrolase EC 3.2.1.4) activity increased during abscission and was localized in the cell separation layer of Phaseolus vulgaris L. var. Red Kidney (bean), Gossypium hirsutum L. var. Acala 4-42 (cotton), and Coleus blumei Benth. Princeton strain (coleus) abscission zone explants. Cellulase activity was optimum at pH 7, was reduced by one-half after heating to 55 C for 10 minutes, and was associated with the soluble components of the cell. Explants treated with aging retardants (indoleacetic acid, N⁶-benzyladenine, and coumarin), CO₂, actinomycin D, or cycloheximide had less cellulase activity when compared with untreated controls. Ethylene increased cellulase activity of aged explants after a 3-hour lag period. It was concluded that one of the roles of ethylene in abscission is to regulate the production of cellulase, which in turn is required for cell separation.

I. INTRODUCTION

A number of investigators have explored the role of cell wall - degrading enzymes in abscission. Osborne¹ reported that high levels of pectin methylesterase were associated with the separation layers of Phaseolus vulgaris L. (bean) abscission zone explants in which abscission was delayed by treatment with 2,4-dichlorophenoxyacetic acid. Acceleration of abscission by ethylene resulted in reduced levels of pectin methylesterase. Yager² reported similar results with Nicotiana tabacum L. (tobacco) explants. In his experiments indoleacetic acid was used as the abscission retardant and methionine as the accelerant. However, it is likely that the stimulatory effect of methionine was due to a stimulation of ethylene production.³ Rasmussen⁴ also reported that decreasing levels of pectin methylesterase were associated with aging bean explants. Rasmussen also found that polygalacturonase activity decreased during abscission.

Using the loss of cellular material from cucumber slices as an assay for pectinase, Morré⁵ reported that increasing enzyme activity was associated with abscission of bean petiole explants. A second enzyme known to increase during abscission is cellulase. Horton and Osborne⁶ reported that cellulase activity was localized in the separation layer of bean explants and that 2,4,5-trichlorophenoxyacetic acid inhibited cellulase activity but ethylene increased it.

Ethylene action during abscission is thought to be hormonal, that is, regulating the production of enzymes required for cell separation by regulating RNA and protein synthesis.⁷ This report presents experiments designed to support the above hypothesis, by showing that the regulation of cellulase activity by ethylene corresponds to data obtained earlier on the regulation of RNA and protein synthesis by ethylene.

II. MATERIALS AND METHODS

Cellulase (β -1,4-glucan-glucanohydrolase EC 3.2.1.4) was assayed by measuring the loss of viscosity of a sodium carboxymethyl cellulose (CMC) solution with a model LVT Wells-Brookfield microviscometer.* This cone plate viscometer was especially useful for measuring small samples (1 ml) over a range of 0 to 2,000 centipoises and has been described earlier.⁸

A 1.5% CMC solution was prepared by slowly adding powdered CMC** to 0.05 M potassium phosphate, pH 7, and 0.05 M NaF in a Waring Blendor and then autoclaving the mixture for 15 minutes. After the solution cooled, toluene to give a 0.5% solution was added as a preservative.

Cellulase activity was measured by adding 1 ml of enzyme solution to 1 ml of 1.5% CMC held at 40 C. Enzyme preparations with high activity were analyzed by placing 1 ml of the enzyme substrate mixture in the viscometer and determining the viscosity 10 minutes after mixing. The viscosities of preparations with lower activity were determined after a 4- to 6-hour incubation period at 40 C. The enzyme substrate mixture was equilibrated in the viscometer for 10 minutes before determining viscosity. While methods for expressing cellulase activity in absolute terms have been proposed,^{9,10} it is simpler and just as meaningful to present data as the per cent change in viscosity of the CMC plus enzyme solution compared with a blank without enzyme ($\Delta\eta$). The viscosity of a 0.75% CMC solution was about 50 centipoises.

Methods for growing, preparing, and storing Phaseolus vulgaris L. var. Red Kidney (bean), Gossypium hirsutum L. var. Acala 4-42 (cotton), and Coleus blumei Benth. Princeton strain (coleus) explants were described earlier.¹¹ For most of the experiments described in the present paper, bean explants were placed petiole-end down and cotton and coleus explants stem-end down in a 3-mm deep layer of 1.5% agar in petri plates. Bean explants treated with indoleacetic acid (IAA), coumarin, or the cytokinin N⁶-benzyladenine were placed pulvinal-end down in the agar. Actinomycin D (1 μ g) and cycloheximide (0.25 μ g) were injected as 1 μ liter solutions into bean explants with a microliter syringe by sticking 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. A water injection was used as a control in these experiments and had no effect on abscission.

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** Nutritional Biochemicals Corporation, Cleveland, Ohio.

Explants were treated with ethylene and CO₂ by placing the petri plates in 10-liter desiccators. The contents of the desiccators were first subjected to a partial vacuum, and then ethylene and CO₂ were added to the gas phase by a syringe inserted through the rubber vaccine cap covering the desiccator outlet. The vaccine cap was then removed to equilibrate the contents to atmospheric pressure.

For convenience, the methodology for each experiment is described with the presentation of results because of the variation of specific details among experiments.

III. RESULTS

Preliminary experiments consisted of assaying for cellulase activity in various parts of abscission zone explants before and after abscission. In the case of bean explants, the pulvinus represents the top 3 mm, the separation layer the middle 2.5 mm, and the petiole the remaining 4.5 mm. The petiole of cotton explants represents the top 2.5 mm of cotyledonary petiole, the separation layer the remaining petiole tissue flush with the stem, and the nodal tissue the stem tissue between the two petiole bases. Node number 4 coleus explants were subdivided in a similar manner. To insure that cellulase activity did not represent bacterial contamination, bean and cotton explants were surface sterilized with a 30-second wash of 2% NaOCl followed by two rinses of sterile distilled water. The explants were then stored in sterile agar. Sterile homogenates of abscising explants were free of bacterial contamination when plated out on nutrient agar. Because essentially similar data on cellulase production were obtained from explants isolated under nonsterile conditions, the NaOCl treatment was not used in subsequent experiments.

In Table 1 initial cellulase activity was determined on freshly excised explants (100 bean, 65 cotton, and 12 coleus) subdivided into separation layer and surrounding tissue and frozen. An equal number of explants were aged 24 hours in air, 24 hours in 1 ppm ethylene, separated into separation layer and surrounding tissue, and frozen until subsequent analysis.

TABLE 1. LOCALIZATION OF CELLULASE ACTIVITY IN ABSCISSION ZONE EXPLANTS

Plant	Explant Section	ΔA_{710} 10 Minutes	
		Initial Activity	1 ppm C ₂ H ₄ Treatment
Bean ^{a/}	Pulvinus	+4	-20
	Separation layer	+7	-46
	Petiole	+1	+6
Cotton ^{a/}	Petiole	-8	-2
	Separation layer	-6	-20
	Node	+2	-13
Coleus	Petiole	+4	-35
	Separation layer	-2	-61
	Node	+7	-41

a. Surface sterilized with 2% NaOCl.

Cellulase was extracted from bean and cotton explants by homogenization in 10 ml of 0.05 M potassium phosphate, pH 7, in a VirTis homogenizer. Coleus sections were ground in a Ten Broeck homogenizer with 4 ml of buffer. Polyvinylpyrrolidone (2%) was added to the buffer for cotton and coleus explants to protect the enzyme from any possible detrimental effect of gossypol and other phenolic substances present in these tissues. The homogenates were filtered through Miracloth (CalBiochem Corp.) and centrifuged at 10,000 x g for 10 minutes. Cellulase activity of the supernatant fluid is indicated in Table 1. Freshly excised explants were free of cellulase activity, but significant amounts of the enzyme were found in the abscising explants. In bean explants, cellulase activity was greatest in the separation layer, less in the pulvinus, and absent in the petiole. In cotton and coleus explants, cellulase was also localized in the separation layer with less amounts in the surrounding tissues.

The data in Table 2 indicate that cellulase is a soluble enzyme and is not associated with the particulate material of the cell. In this experiment, 250 bean explants were aged 24 hours in air, 24 hours in 10 ppm ethylene, homogenized with 10 ml 0.05 M potassium phosphate, pH 7 buffer, and then filtered through Miracloth. A sample of the filtered homogenate was assayed directly in the viscometer. The remaining homogenate was centrifuged at 2,000 x g for 10 minutes and the resulting pellet was resuspended in buffer so that the volumes of resuspended precipitate and of supernatant were equal. Samples of both the precipitate and supernatant

were assayed for cellulase activity. This procedure was repeated at 10,000 x g for 10 minutes and 100,000 x g for 1 hour using the subsequent supernatant solutions. The data in Table 2 indicate that the cellulase activity remained primarily in the supernatant fractions.

TABLE 2. FRACTIONATION OF ABSCISSION ZONE CELLULASE BY CENTRIFUGATION

Fraction	$\Sigma \Delta \eta$ 10 Minutes	
	Precipitate	Supernatant
Original homogenate		46
2,000 x g for 10 minutes	13	46
10,000 x g for 10 minutes	10	36
100,000 x g for 1 hour	6	33

The data in Table 3 indicate that it was possible to precipitate cellulase with $(\text{NH}_4)_2\text{SO}_4$ and acetone. Supernatant (10,000 x g for 10 minutes) from aged ethylene treated explants was brought to 20% $(\text{NH}_4)_2\text{SO}_4$ saturation, held at 0 C for 15 minutes, and centrifuged at 10,000 x g for 10 minutes. The supernatant was then brought to 80% saturation, held at 0 C for 30 minutes, and centrifuged at 10,000 x g for 10 minutes. The pellet was then taken up in a volume of buffer equal to the original solution of crude cellulase. The cellulase was also precipitated by adding 2 ml of 0 C acetone for each ml of supernatant and centrifuging at 3,000 x g for 10 minutes. After washing the precipitate twice with 70% acetone (v/v with water), it was taken up in a volume of phosphate buffer equal to the original crude preparation. The data in Table 3 indicate that $(\text{NH}_4)_2\text{SO}_4$ and acetone were able to precipitate most of the cellulase.

TABLE 3. PRECIPITATION OF ABSCISSION ZONE CELLULASE BY ACETONE AND $(\text{NH}_4)_2\text{SO}_4$

Treatment	$\Sigma \Delta \eta$ 10 Minutes
Control	64
20 to 80% $(\text{NH}_4)_2\text{SO}_4$	63
70% acetone	56

The effect of pH on cellulase activity was measured by homogenizing ethylene-treated explants in water and mixing portions of supernatant centrifuged at $10,000 \times g$ for 10 minutes with equal volumes of MacIlvaine's buffer ($0.1 \text{ M K}_2\text{HPO}_4 + 0.05 \text{ M citric acid}$). The cellulase activity of these samples was measured on CMC dissolved in water. As shown in Figure 1, cellulase activity was greatest at pH 7.0, with another peak at pH 3.4. Subsequently, all extractions and analyses were performed with 0.05 M potassium phosphate, pH 7, buffer.

As shown in Figure 2, cellulase activity increased with increasing temperatures up to 50°C . It was not possible to obtain higher temperatures with the water bath used to supply water to the Wells-Brookfield viscometer.

The heat stability of cellulase was tested by exposing samples of the enzyme to different temperatures for 10 minutes and then measuring subsequent cellulase activity at 40°C . As shown in Figure 3, a 10-minute treatment at 55°C resulted in a 50% loss in activity.

A comparison between bean and commercial cellulase (CalBiochem Corp.) concentration and the reduction of CMC viscosity is shown in Figure 4. This figure indicates that the viscometric technique described here measured as little as $1 \mu\text{g}$ of cellulase after 10 minutes.

The time course for cellulase formation in explants that received a 20-hour aging period is shown in Figure 5. Aged explants were used in this and subsequent experiments because earlier work showed that freshly excised explants are insensitive to ethylene.¹² Samples of 25 explants were placed either in air or stored in desiccators with 10 ppm ethylene. Samples were withdrawn and frozen every 3 hours for subsequent cellulase assays. As shown in Figure 5, a 6-hour ethylene treatment caused an increase in cellulase activity compared with air-treated controls.

IAA, coumarin, and N^6 -benzyladenine block the ability of ethylene to stimulate abscission.¹³ The data in Table 4 show that explants treated with these compounds produced less cellulase when exposed to 1 ppm ethylene. In these experiments, as well as those presented in Tables 5 and 6, one set of 25 separation layers from freshly excised explants represents initial activity. The remaining sets of 25 explants were placed pulvinal-end down in plain agar or agar containing the abscission retardants. After 24 hours, the explants were placed in a desiccator containing 1 ppm ethylene for an additional 24 hours before separation layers were excised and frozen. The 25 separation layers were homogenized in 4 ml of buffer in a Ten Broeck homogenizer, centrifuged at $10,000 \times g$ for 10 minutes, and then assayed for cellulase.

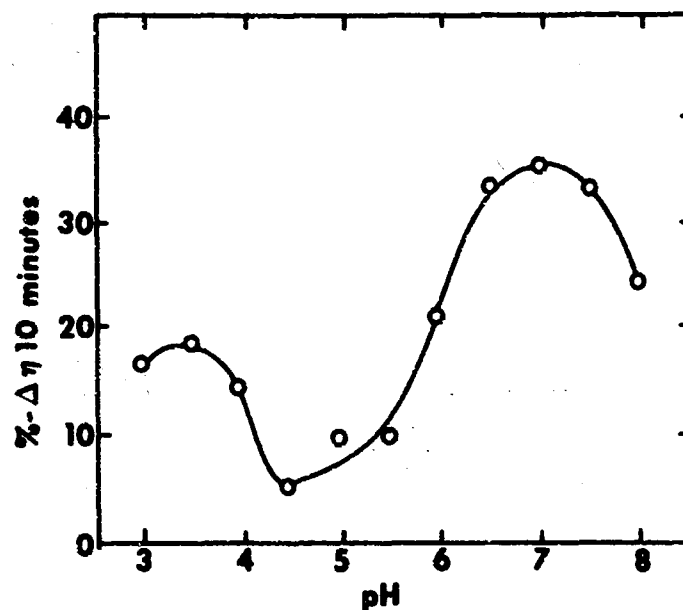


FIGURE 1. Effect of pH on Abscission-Zone Cellulase Activity. MacIlvaine's buffer (0.1 M K_2HPO_4 and 0.05 M citric acid) was used to establish pH values.

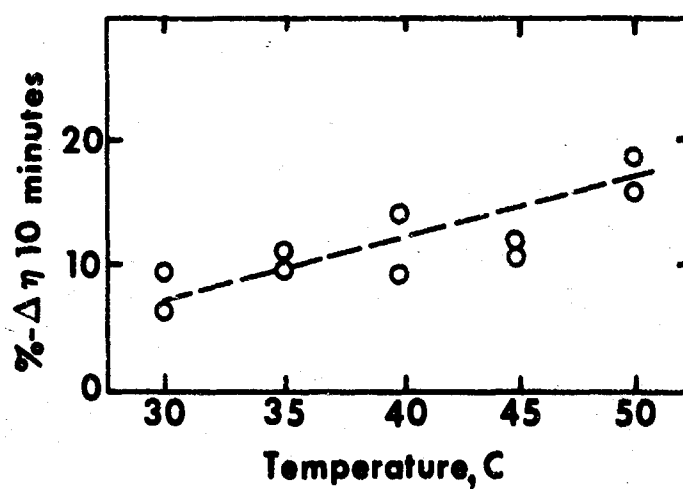


FIGURE 2. Effect of Temperature on Abscission-Zone Cellulase Activity.

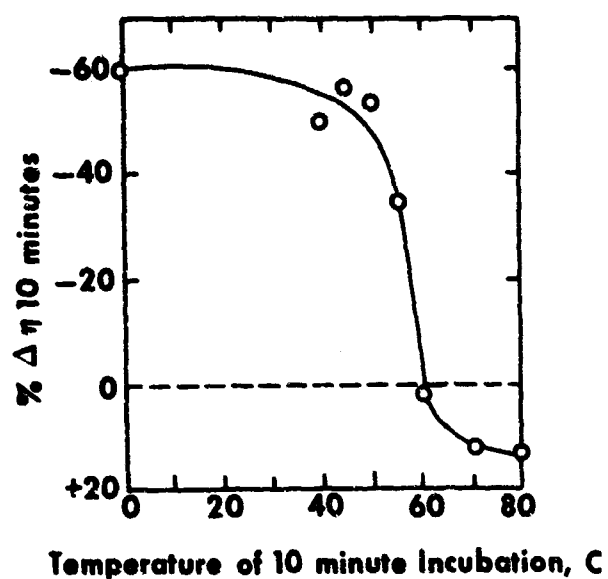


FIGURE 3. Effect of Temperature on Stability of Abscission-Zone Cellulase.

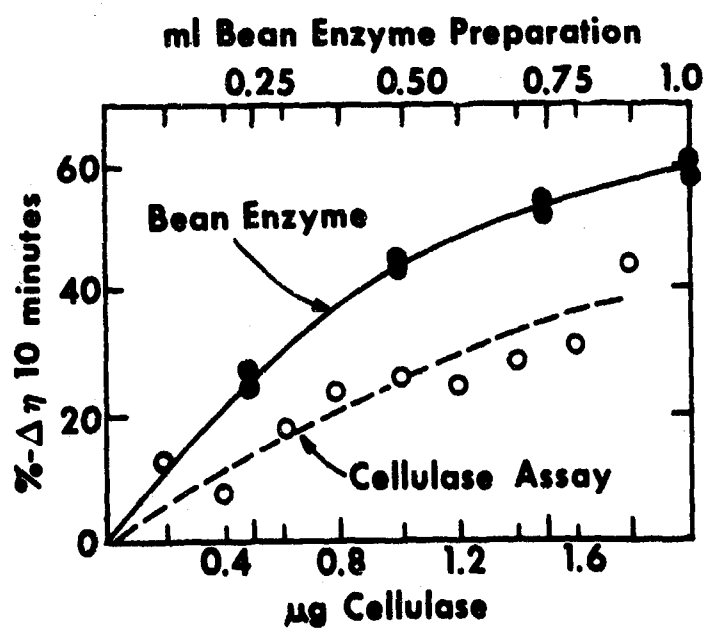


FIGURE 4. Enzyme Concentration Curve. Effect of increasing concentrations of abscission-zone cellulase and commercial cellulase preparations on the viscosity of CMC.

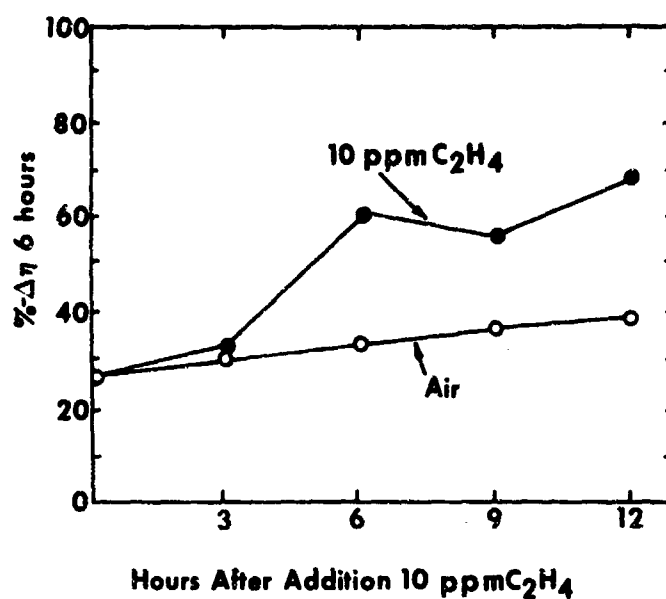


FIGURE 5. Time Course for Induction of Separation-Layer Cellulase in Ethylene-Treated and Control Explants.

TABLE 4. EFFECT OF IAA, COUMARIN, AND N⁶-BENZYLADENINE IN THE PRESENCE OF ETHYLENE ON CELLULASE INDUCTION IN BEAN EXPLANT SEPARATION LAYER^a

Treatment	%-Δη 4 Hours
Initial	5
Control	77
C ₂ H ₄	91
C ₂ H ₄ + IAA	18
C ₂ H ₄ + coumarin	6
C ₂ H ₄ + N ⁶ -benzyladenine	32

a. 5×10^{-5} M IAA; 10^{-3} M coumarin; 10^{-3} M N⁶-benzyladenine; 1 ppm ethylene.

Carbon dioxide competitively inhibits the acceleration of abscission by ethylene.¹⁴ The data in Table 5 indicate that carbon dioxide also inhibits the action of ethylene in accelerating cellulase formation. Sets of 25 explants were stored in air for 24 hours before being placed in desiccators filled with the gases indicated in Table 5. After a 24-hour exposure to the gases, the separation layers were excised and cellulase was extracted as in the experiment described in Table 4.

TABLE 5. EFFECT OF C₂H₄ AND CO₂ ON CELLULASE FORMATION IN BEAN EXPLANT SEPARATION LAYER^a

Treatment	%-Δη 4 Hours
0-Hour Initial	16
Control	42
C ₂ H ₄	67
CO ₂	37
C ₂ H ₄ + CO ₂	61

a. 1 ppm C₂H₄; 10% CO₂.

Actinomycin D and cycloheximide have been used to demonstrate a requirement for RNA and protein synthesis in abscission.¹¹ If cellulase is one of the proteins synthesized during abscission, it should be possible to reduce the amount of this enzyme by treating explants with actinomycin D and cycloheximide. After a 24-hour aeration period, groups of 25 explants were injected with actinomycin D and cycloheximide as described in Section II. The explants were then given an 8-hour 1-ppm ethylene treatment before excising and freezing the separation layer for subsequent cellulase determinations. The data in Table 6 indicate that actinomycin D and cycloheximide inhibited the increase in cellulase activity by ethylene.

TABLE 6. EFFECT OF ACTINOMYCIN D AND CYCLOHEXIMIDE
IN THE PRESENCE OF ETHYLENE
ON CELLULASE FORMATION^a

Treatment	%-Δ 5 Hours
0-Hour Initial	7
C ₂ H ₄	43
C ₂ H ₄ + actinomycin D 1 μg	11
C ₂ H ₄ + cycloheximide 0.25 μg	7

a. 1 μg actinomycin D; 0.25 μg cycloheximide;
1 ppm ethylene.

IV. DISCUSSION

The experiments described in Table 1 confirm and extend to cotton and coleus the findings of Horton and Osborne⁶ that cellulase activity was associated with the separation layer of abscission zone explants undergoing abscission.

Separation layer cellulase is a soluble enzyme (Table 2) with maximum activity at pH 7.0 (Fig. 1) and with stability up to 40 C (Fig. 3). Fungal cellulases usually show optimum activity at pH 4.0 to 5.5,¹⁵ tomato cellulase at 5.0,¹⁶ snail cellulase at 5.6,¹⁷ bacterial cellulase around 6.0,¹⁸ and nematode cellulase from 5.5 to 8.0.¹⁹ Abscission zone cellulase was not as stable as crude preparations of Irpex lacteus and of Trichoderma viride which retain 16 to 30% of their original activity after 30 minutes at 99 C.²⁰ Sison, Schubert, and Nord²¹ reported a 44% loss in activity of cellulase from Poria vaillantii after a 70 C 10-minute treatment, while Myers and Northcote¹⁷ found that snail cellulase was rapidly inactivated at 30 C.

Ethylene increased cellulase activity of separation layer cells after a 3-hour lag period (Fig. 5). These findings agree with the earlier observations that ethylene increased RNA synthesis after an hour lag period and protein synthesis after a 2-hour lag period.²² Data obtained with cycloheximide also suggested that proteins essential to abscission were synthesized after a lag period.

Results of experiments measuring the influence of aging retardants, CO_2 , and the inhibitors actinomycin D and cycloheximide on cellulase formation in the separation layer of bean explants agree with the known action of these compounds on abscission. The aging retardants are thought to slow down or prevent the onset of the ethylene-sensitive stage of abscission.¹³ These compounds also prevented ethylene from inducing cellulase activity (Table 4). The gas CO_2 , on the other hand, acts as a competitive inhibitor of ethylene in abscission⁷ and, as shown in Table 5, was able to overcome some of the effect of ethylene in increasing cellulase formation. Finally, actinomycin D and cycloheximide are known to inhibit abscission and presumably act by blocking RNA and protein synthesis required for the formation of degradative enzymes.¹¹ As shown in Table 6, actinomycin D and cycloheximide blocked the induction of cellulase activity.

Control of cellulase activity by inhibitors of low molecular weight or activators could explain some of the results presented here. However, experiments designed to observe such control mechanisms have given negative results. For example, an inhibitor of cellulase activity might be present in unaged tissue and these materials could decrease with age. However, protein-free extracts of juvenile explants had no effect on the cellulase produced by aged explants. Conversely, dialysis or $(\text{NH}_4)_2\text{SO}_4$ precipitation of proteins from juvenile explants did not result in increases in cellulase activity. In other experiments the possibility that activators may play a role in cellulase activity was examined. In these experiments dialysis or $(\text{NH}_4)_2\text{SO}_4$ precipitation of aged explants did not reduce activity nor did protein-free extracts from aged explants increase cellulase activity from unaged explants.

As discussed in greater detail in a recent review,⁷ abscission can be described by an aging-ethylene hypothesis. The essential features of this hypothesis as applied to explants is that excision of explants cuts off the supply of juvenility factors, such as auxin, normally supplied by the leaf. In the absence of these juvenility factors, the explants age and the separation layer becomes increasingly sensitive to ethylene. From work with RNA and protein metabolism, it was concluded that the mechanism of ethylene action during cell separation was to induce protein essential for the cell separation process. Both the work by Horton and Osborne⁸ on cellulase induction during abscission and this report confirm this interpretation and indicate that the mechanism of ethylene action, like other hormones, is to regulate the production of enzymes that are essential for physiological processes.

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13. ABSTRACT		
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