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Synthesis of Cellulase during Abscission of *Phaseolus vulgaris* Leaf Explants

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ABSTRACT

When abscission in leaf explants from *Phaseolus vulgaris*, cultivar Red Kidney, was allowed to proceed while the explants were in ^2H_2O, a 1.25% increase in the buoyant density of cellulase in a cesium chloride gradient was observed. These data indicate that the increase in cellulase activity during abscission is a result of the synthesis of new protein. Two differentially soluble forms of cellulase are present in the abscission zone. The form which is soluble only in a high salt buffer seems more closely related to the abscission process than the form which is soluble in dilute buffer. The correlation between changes in pull force and increase in cellulase activity and the effects of several hormones on cellulase activity are discussed.

The increase in activity of an enzyme can be the result of the synthesis *de novo* of the enzyme, or it can be due to the activation of an existing protein. This work was done primarily to determine how cellulase increases in activity during the abscission process (1, 10) and to examine the causal relationship of this hydrolase to abscission. To establish that an enzyme is being synthesized at a particular time can be shown if the tissue is incubated in a solution containing an amino acid precursor labeled with a heavy isotope (*e.g.*, $^{15}NO_3^-$, $^{15}NH_4^+$, ^2H_2O, *etc.*) during this time; the enzyme increases in buoyant density as measured by isopycnic equilibrium sedimentation. If in the presence of a heavy isotope, the cellulase increases in buoyant density when abscission is allowed to occur, the more dense cellulase would represent new synthesis. An increase in cellulase activity will depend upon the relative rates of synthesis and turnover of the enzyme; that is, an increase in activity could result from either an increase in the rate of synthesis with the same rate of turnover or a decrease in the rate of turnover with the same rate of synthesis. No increase in buoyant density means that all the protein was present prior to abscission, and that the increase in activity was due either to an activation of a cellulase proenzyme or to a decrease in the level of cellulase inhibitors which are known to exist in many plants (13).

Because it is difficult to establish cause and effect relationships between morphogenetic responses such as those involved in abscission and the activity of an enzyme, correlations between enzyme activity and the response must often suffice. Therefore, we have compared enzyme activity with changes in pull force (tension which is required to pull apart the abscission zone).

This report also shows that viscometric data for bean cellulase can be converted to units of relative and absolute activity, as demonstrated by Almin *et al.* (2) for a fungal cellulase, thus providing a convenient method for measuring the activity of cellulases from higher plants.

MATERIALS AND METHODS

Abscission zones were taken from the primary leaves of *Phaseolus vulgaris* L., cultivar Red Kidney. The plants were grown in a growth chamber for 10 to 12 days at 24 C with 8 hr of light from both incandescent and fluorescent lamps at an intensity of 2100 ft-c, followed by 2 hr of incandescent light of 100 ft-c to provide an exposure to far red light typical of sunset conditions, and 14 hr of dark. Plants were grown in a vermiculite-gravel medium and watered daily with a 0.5 Hoagland's solution.

Debladed seedlings consisted of the intact plant with only the leaf blades removed. Petiole explants consisted of 5 mm of pulvinus, the upper abscission zone, and about 17 to 18 mm of the petiole. Approximately 50 explants were placed with the proximal ends in 1.25% (w/v) agar in a deep Petri dish. In the density labeling experiment, agar was prepared with 80% ^2H_2O (v/v). Leucine-U-^{14}C and GA_3 were applied in 5-μl agar droplets to the distal end of the explants. Ethrel (2-chloroethyl phosphonic acid) and abscisic acid were applied in 5-μl drops of water. To minimize microbial contamination, the explants were soaked in 1% (v/v) Clorox (sodium hypochlorite) for 15 min before insertion into the agar, and the agar contained 50 mg/liter of chloramphenicol.

Pull force measurements were made with a Chatillion strain gauge calibrated in 10 g increments. The explant was clamped to the gauge and force was applied to the section by mechanically pulling the gauge at the rate of 2 mm/sec. The force at which the abscission zone separated was recorded as the pull force of abscission.

Soluble cellulase was extracted by grinding, with mortar and pestle, about 100 mg of tissue in 4 ml of 0.02 M sodium phosphate buffer, pH 6.1. After centrifuging for 10 min at 10,000*g*, the supernatant solution was analyzed for cellulase activity. The residue was washed three more times with dilute buffer, then extracted with 1 M NaCl in 0.02 M phosphate buffer, pH 6.1, to remove the residual cellulase.

Cellulase activity was assayed by measuring the reduction in viscosity of a solution of CM-cellulose,[1] types 7HF or 7HP (Hercules Powder Co., Wilmington, Del.). The assay mixture contained 2 parts of CMC-7HF, 1.33% (w/v) in 0.02 M sodium phosphate buffer, pH 6.1, and 1 part of the enzyme solution. CM-cellulose 7HP, 1.5% (w/v), was used in some experiments,

[1] Abbreviation: CM: carboxymethyl.

and results were similar to the results with CM-cellulose 7HF. Drainage time through a calibrated portion of a 100-μl pipette was used as a measure of viscosity. Viscosity was usually measured after a 20-hr reaction time. Enzyme reactions and viscosity measurements were done at a room temperature of about 23 C. Viscosity data were converted to relative units of activity ($B \cdot g^{-1} \cdot hr^{-1}$) as described in Almin *et al.* (2).

The technique of Filner and Varner (7) was used to test for synthesis *de novo* of cellulase 48 hr after preparing the explants. A heavy isotope, ^2H, was used to label newly synthesized protein, and its buoyant density was compared with the buoyant density of protein synthesized in the absence of ^2H. Isopycnic equilibrium centrifugation was done on the International B-60 ultracentrifuge with an SN-405 head at 60,000 rpm and on the Beckman L2-50 with an SW-50L head at 50,000 rpm. One milliliter of CsCl, density 1.56 g/cm^3, was added to each of two centrifuge tubes. One milliliter of cellulase from the ^2H_2O-treated tissue was added to one tube, and 1 ml of cellulase from the H_2O-treated tissue was added to the second tube. One milliliter of cellulase represented the enzyme from 25 bean abscission zones 1 mm wide, or about 100 mg of tissue, fresh weight. Pancreatic α-amylase was added to both tubes as an internal standard.

The tubes were filled with 2 ml of paraffin oil layered over the protein solution and centrifuged for 84 hr at about 2 C. After centrifugation, 3-drop fractions were collected by puncturing the bottom of the tubes. Five fractions were selected for refractive index readings to evaluate the established gradient. All other fractions were assayed for cellulase activity with the CM-cellulose substrate. After determining cellulase activity, the fractions were diluted with 1 ml of water and assayed for amylase (7). The fraction number corresponding to the mean of each band of enzymatic activity was calculated by plotting the data on Gaussian scale graph paper *versus* tube number on the second axis. Fraction number and buoyant density were correlated through the refractive index readings. Radioactivity due to ^{14}C-leucine incorporation into protein was measured by adding a sample from each tube after dilution for the amylase assay to 5 ml of Bray's solution (3) and counting in a scintillation counter. If the newly synthesized protein were synthesized from amino acids in which all of the hydrogen atoms were replaced by deuterium atoms, an increase in buoyant density of about 5% would be expected. The maximal possible increase would depend upon the amino acid composition of the enzyme; this is not known. The increase in the buoyant density of the ^{14}C band due to leucine-^{14}C incorporation into protein synthesized in the presence of deuterium is a measure of the average shift in buoyant density of the newly synthesized protein.

The conversion of viscometric values to cellulase activity in absolute terms has been demonstrated by Almin *et al.* (2) who measured the enzymatic activity of a cellulase from the fungus *Penicillium chrysogenum notatum*. Their cellulase was sufficiently active to measure initial rates of enzyme activity in minutes. In our experiments with cellulase from the abscission zone of Red Kidney beans, the activity had to be measured in hours. The results show, however, that the calculations are also valid with the less active higher plant cellulase and that the mathematical conversion provides a workable system for calculating intrinsic viscosity [η], relative activity (B), or absolute activity (A) from viscometric readings.

The adaptability of the equations and constants of Almin *et al.* (2) to our data is shown by Figures 1, 2, and 3. Figure 1 shows that the relation between $[\eta]^{-3.66}$ and time is linear for about 22 hr. Figure 2 demonstrates the linear relation between enzyme concentration and $[\eta]^{-3.66}$ for a given time. The linearity of the relative activity value (B) with an increasing concentration of cellulase enzyme is shown in Figure 3.

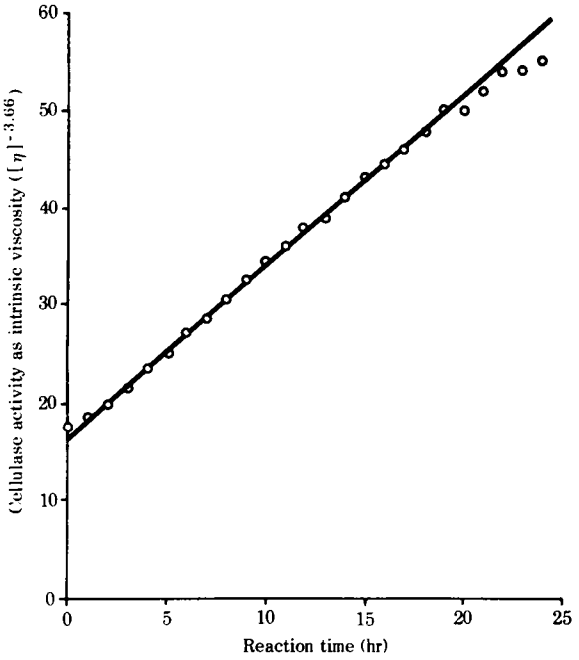

FIG. 1. Linear relation between time and cellulase activity plotted as intrinsic viscosity.

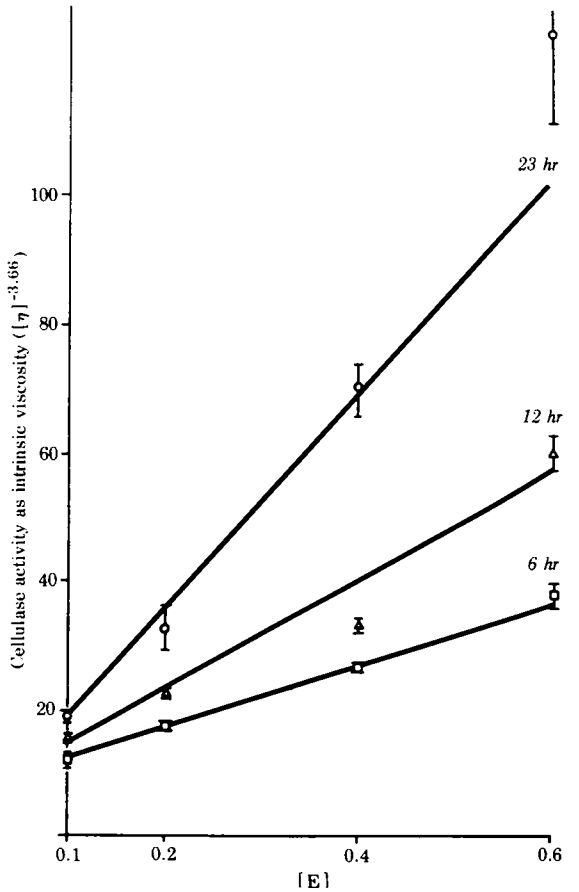

FIG. 2. Cellulase activity plotted as intrinsic viscosity of the -3.66 power is linear with enzyme concentration within limits. The number above each line is the length of the reaction time elapsed before determining viscosity. The relative enzyme concentration of 0.6 was too high for linearity when the reaction was allowed to run for 23 hr. The exponential nature of the calculations is such that very low viscosity readings translate into erroneously high cellulase activity values.

RESULTS

Cellulase Activity during Abscission. The cellulase activity extractable with 0.02 M phosphate buffer, pH 6.1, did not show any consistent changes during the abscission process in bean leaf explants or debladed bean seedlings. Nor was there any striking difference between this cellulase activity in the proximal, abscission, and distal zones of the explants. These results differ from those reported by Horton and Osborne (10) and by Abeles (1) who had found increases in a similarly extracted cellulase in the abscission zone during the abscission process. We found, however, a cellulase fraction which could be extracted with 1.0 M NaCl in 0.2 M phosphate buffer, pH 6.1 (Table I). This fraction, which we call residual cellulase, showed changes in activity during the abscission process similar to those reported previously. Although Triton X-100 seemed to increase the extraction of residual cellulase, the use of detergents was discarded because of their interference with subsequent purification and assay.

In addition to the fact that residual cellulase was extractable only with a high salt buffer, the particulate nature of the enzyme was apparent from the fact that enzymatic activity could be recovered in a 10,000*g* pellet after the tissue was ground in dilute phosphate buffer; this activity could be solubilized by treatment of the pellet with M NaCl.

Residual cellulase activity in the pulvinus, abscission zone, and proximal tissue of the intact petiole was about 10 relative units $\cdot g^{-1} \cdot hr^{-1}$ at the time the explant was prepared. Within 48 hr after deblading, there were 50 to 70 relative units $\cdot g^{-1} \cdot hr^{-1}$ in the pulvinus and in the abscission zone. The level in the proximal tissue remained very low. The level in the abscission zone was usually higher than in the pulvinus (Table II), although the difference was not very striking (10–20 units).

To determine whether a relationship could be found between cellulase and abscission, experiments were done to correlate the pull force required to break the abscission zone with the level of cellulase activity. An increase in cellulase activity preceding the decrease in pull force would be a convincing indication that cellulase is causal to abscission. It appears that cellulase activity may increase slightly prior to a decrease in pull force (Fig. 4). However, the variability in break strength at any given time was too great to refine these measurements, and so a different approach was used. Explants were grouped according to the pull force required to separate the abscission zone, and each category was analyzed for cellulase activity. These data (Fig. 5) showed a close correlation between the decrease in pull force and the increase in residual cellulase activity down to about 140*g* pull force From this point on, the decrease in pull force did not seem to be accompanied by a further increase in cellulase activity. Although the initial decrease in pull force correlated closely with the increase in residual cellulase activity, there was no observable increase in activity preceding the actual loosening of the abscission zone.

Auxins (2,4-D, 2,4,5-T, IAA, and napthaleneacetic acid were tested) inhibited the abscission process and the accompanying

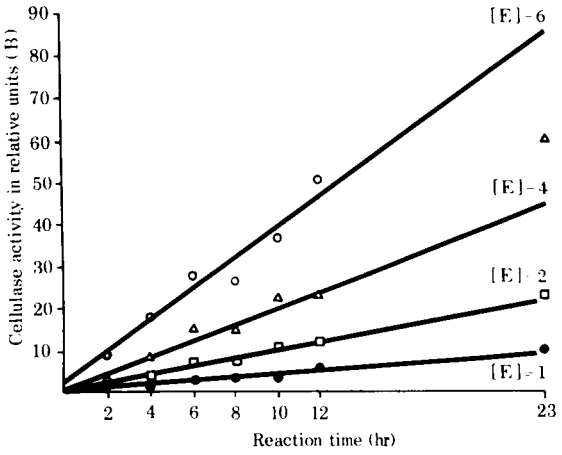

FIG. 3. A series of four enzyme concentrations exhibit increasing relative units of activity which were linear with time up to 12 hr. The 23-hr points were linear only with the lower concentrations of enzyme. Since these units have initial viscosity subtracted from the final values, the lines should intercept the ordinate axis at zero.

Table I. *Effect of Salt Concentration and Detergent on Recovery of Residual Cellulase from 0.02 M Phosphate Buffer Residue 48 hr after Excision*

Twenty relative units ($g^{-1} \cdot hr^{-1}$) of soluble cellulase activity were extracted from this tissue in 0.02 M phosphate prior to the recovery of residual cellulase.

Extraction Solution	Cellulase Activity	Relative Reducing[1] Groups of CM-Cellulose
	relative units $\cdot g^{-1} \cdot hr^{-1}$	
Buffer (0.02 M phosphate, pH 6.1)	0	0
Buffer + 0.05 M NaCl	16	0.115
Buffer + 1.0 M NaCl	100	0.735
Buffer + 0.5 M NaCl + 20% Triton X-100	36	[2]
Buffer + 1.0 M NaCl + 20% Triton X-100	212	[2]
Buffer + 0.5 M NaCl + 20% Tween 20	14	[2]
Buffer + 1.0 M NaCl + 20% Tween 20	117	[2]

[1] Reducing power determined by the Nelson-Somogyi arsenomolybdate method (14).

[2] Detergents interfered with the reducing sugar assay.

Table II. *Residual Cellulase Activity in the Proximal, Abscission, and Distal Zones of Bean Explants*

Time after Deblading	Cellulase Activity		
	Proximal	Abscission	Distal
hr	*relative units* $\cdot g^{-1} \cdot hr^{-1}$		
0	2	2	9
48	12	65	48

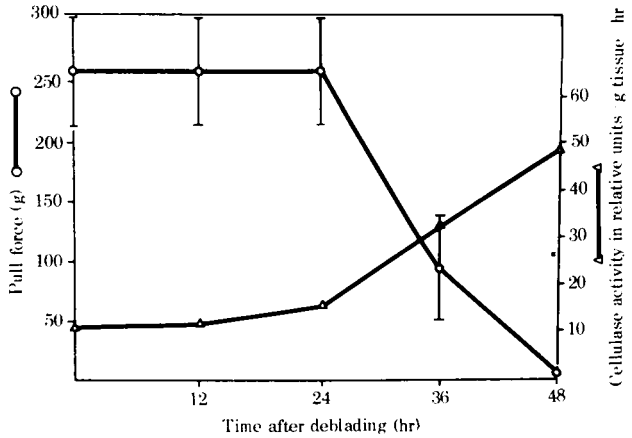

FIG. 4. Comparison of the changes in pull force and changes in cellulase activity with time after deblading. Brackets represent ±1 standard deviation.

increase in residual cellulase activity. One microliter per liter ethylene in the atmosphere accelerated abscission and stimulated an increase in total cellulase activity to about 300 relative units·g^{-1}·hr^{-1}. Application to the petiole of 5 to 100 mg of Ethrel accelerated abscission and increased cellulase activity similar to the ethylene treatment, except that the acceleration of abscission and the increase in cellulase activity due to Ethrel followed that due to ethylene by about 24 hr (Fig. 6). This is probably the time required for the Ethrel to move into the abscission zone and to be degraded to ethylene. It is well established that Ethrel is readily converted to ethylene in plant tissue; however, caution must be exercised in equating the effects of ethylene and Ethrel, because Ethrel delayed the loss of chlorophyll in citrus leaves while ethylene did not (8). The increase in cellulase activity which resulted from ethylene and Ethrel treatments was primarily in soluble cellulase, *i.e.*, the fraction extractable in dilute buffer without NaCl.

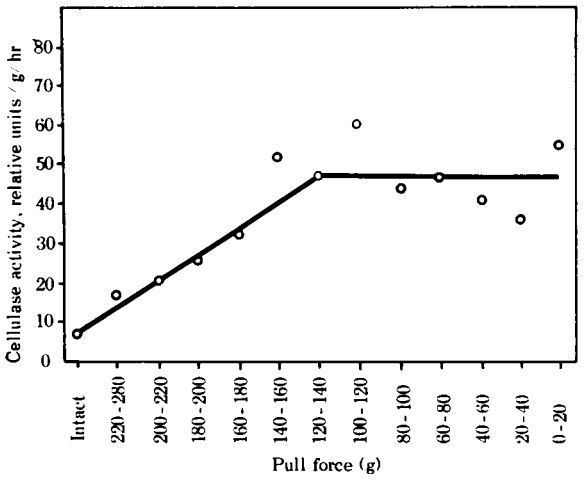

FIG. 5. Cellulase activity in the pulvinal tissue adjacent to the abscission zone. The abscission zones were separated with a Chatillion strain gauge and grouped according to the pull force required for the separation. Each point is mean of two experiments. One hundred milligrams (about 25 tissue slices) was extracted for each pull force category.

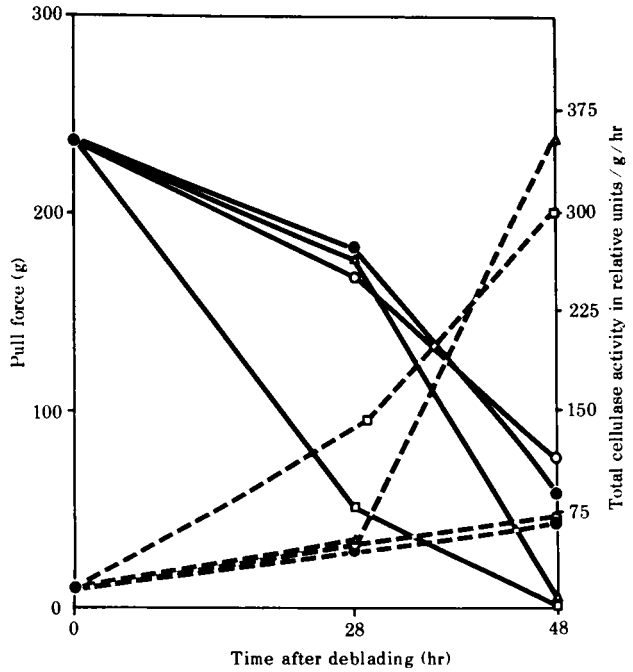

FIG. 6. Changes in pull force (solid lines) following deblading of bean seedlings and zero time application to the pulvinus 10-μl aqueous solutions of abscisic acid, 4×10^{-4} M (○); or 2-chloroethyl phosphonic acid (Ethrel), 3.5×10^{-3} M (△); or placing the seedlings in an atmosphere containing 1 μl/liter of ethylene (□); or control, which received plain water (●). Relative units of cellulase activity are represented by the dashed lines and the same point markers noted above.

Table III. *Effect of GA_3 on Cellulase Activity in the Abscission Zone of Intact and Debladed Bean Seedlings*

GA_3 Applied to Debladed Petiole (48 hr)			GA_3 Applied to Intact Plant		
				Soluble Cellulase Activity	
Concn	Soluble Cellulase Activity in Abscission Zone	Pull Force	Concn	Distal half of abscission zone	Proximal half of abscission zone
M	*relative units·g^{-1}·hr^{-1}*	*g*	M	*relative units·g^{-1}·hr^{-1}*	
Water	25	68.0	Water	11	7
6×10^{-5}	50	68.0	3×10^{-6}	15	16
6×10^{-4}	42	77.0	3×10^{-5}	14	16
6×10^{-3}	34	79.0	3×10^{-4}	13	15

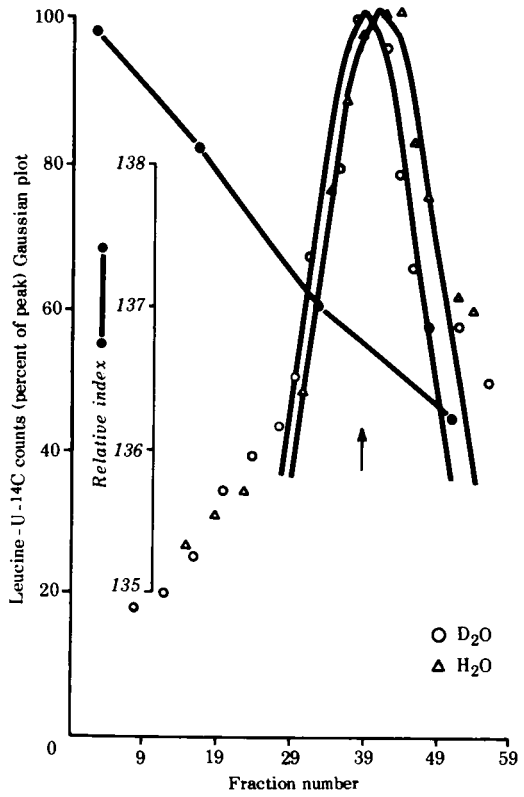

FIG. 7. Equilibrium distributions in a cesium chloride gradient of ^{14}C activity extracted with 1 M NaCl in 0.02 M phosphate buffer from the abscission zone of bean explants treated for 48 hr with leucine-U^{14}-C while supported in either an agar-D_2O or an agar-H_2O medium. The protein soluble in 0.02 M phosphate buffer was extracted prior to the high salt extraction. The curves are a Gaussian plot of the data, but the open circles and triangles represent the raw data plotted as a percentage of maximal peak height. The line drawn through the solid circles describes the cesium chloride gradient. Pancreatic α-amylase was included in all tubes as a buoyant density standard and the arrow indicates the mean of the amylase band.

Gibberellic acid applied to the debladed petiole increased the level of cellulase activity without altering the rate of abscission (Table III). Applying 5 μl of a 6×10^{-5} M solution of GA_3 to the debladed petiole doubled the level of soluble cellulase activity, compared to debladed controls, within 48 hr after removing the leaves, but the pull force was not changed. Higher concentrations of GA_3 also increased cellulase activity but to a lesser degree. Spray applications of 3×10^{-6}, 3×10^{-5}, 3×10^{-4} M GA_3 to intact plants also increased the level of soluble cellulase activity in the abscission zone tissue with no evidence of senescence or abscission. Residual cellulase was not altered by GA_3 treatment. Cellulase activity in the intact plant was much lower than in the debladed ones.

***De novo* Synthesis of Cellulase.** Following isopycnic equilibrium centrifugation, there was an increase of 1% in the buoyant density of newly synthesized protein, as measured by the band density of leucine-^{14}C-labeled protein synthesized in the presence of ^2H_2O, compared with that synthesized in the presence of H_2O (Fig. 7). This is the average shift in buoyant density of most of the newly synthesized protein and provides an indication of what should be expected for an individual enzyme being synthesized under these conditions. In the same experiment cellulase increased in buoyant density by 1.25% (Fig. 8). This means that the increase in buoyant density of the cellulase was the maximum that could be expected for our system. The shifts in buoyant density for total new protein and for cellulase were about the same when crude extracts were used as when the extracts were partially purified by polyethylene glycol precipitation.

Since the band shape of the cellulase produced by the explants in ^2H_2O is very similar to the band shape of cellulase produced in H_2O, and since the entire band of enzyme activity was displaced by about the same amount, it would appear all residual cellulase present in the abscission zone 48 hr after cutting the explants was a result of synthesis *de novo* of the enzyme, rather than of an activation of even a part of already existing enzyme molecules.

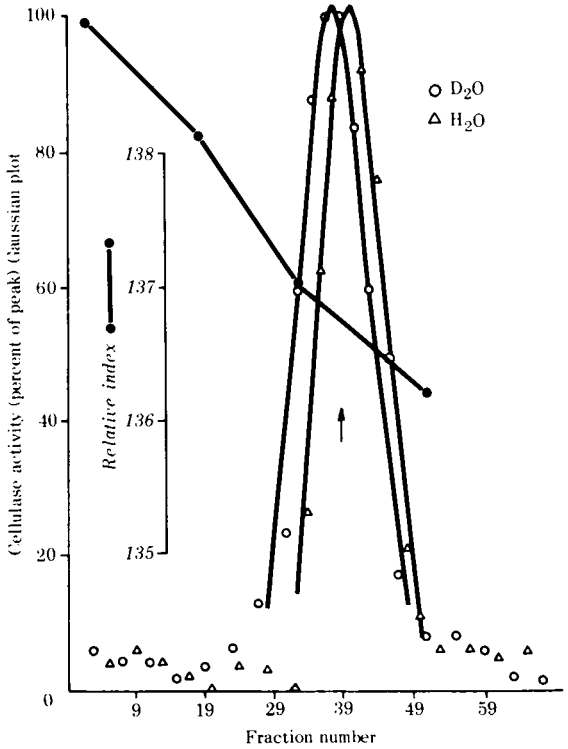

FIG. 8. Equilibrium distributions of cellulase from the same plant extracts as described in Figure 7.

A disconcerting point about the deuterium labeling was the fact that the deuterium oxide treatment stopped the senescence and abscission process. The pulvinus did not lose its green color and the sections did not abscise, but residual cellulase activity did increase to the same level as in sections placed in agar-H_2O.

There was no evidence of an increase in the buoyant density of any portion of the soluble cellulase band. This means that only the residual cellulase was being synthesized *de novo*. The failure to observe an increase in the enzymatic activity of the soluble cellulase would support this conclusion.

DISCUSSION

Our data support the conclusion of Horton and Osborne (10) and of Abeles (1) that there is an increase in cellulase activity accompanying the senescence and abscission process in debladed bean seedlings and in bean petiole explants. Contrary to the observations of these authors, we observed this increase in activity only in residual cellulase, a fraction soluble only in a buffer containing 1 M NaCl. We have no explanation of the apparent difference in the solubility of cellulase between our system and that of these investigators. Fan and Maclachlan (6) reported that they were unable to find a residual cellulase unless they used an extraction medium containing calcium, and then it appeared that the calcium was aiding in the binding of the enzyme to the cell residue. We could not observe any effect of Ca^{2+} or Mg^{2+} on the binding of the enzyme, but Fan and Maclachlan were not working with senescent tissue, and we observed almost no activity in the residual fraction until the tissue began to senesce following deblading. Cellulase also appears to be a bound enzyme in ripening tomato fruit, as Hobson (9) has reported the need for a a high salt buffer to extract cellulase from this tissue.

Although we do not know the nature of the cell material to which the cellulase was bound, it would be logical to expect that an enzyme which is acting on an insoluble and immobile substrate such as cellulose would be found associated with the cell wall, and that the residual cellulase would represent the fraction of the enzyme actively engaged in altering the cell wall. Lee *et al.* (11) demonstrated that the cell wall hydrolases of corn coleoptiles were bound to the isolated cell walls and were autolyzing the cell walls following isolation of the wall-enzyme complex. Soluble cellulase, on the other hand, might be that portion which is not in contact with the cell wall.

This explanation, however, does not explain the observed deuterium labeling of the residual cellulase and its lack in the soluble cellulase. In fact, if the residual cellulase is the fraction associated with the cell wall and the soluble cellulase is cytoplasmic cellulase, it might be expected that the soluble cellulase would be the most recently synthesized one, whereas the ^2H_2O labeling experiment suggests exactly the reverse. An alternative possibility which cannot be rigorously eliminated in our experiments is that the residual cellulase was absorbed to the particulate matter of the cell during the grinding and extraction procedure. However, the fact that only the residual cellulase became labeled with deuterium from ^2H_2O is a very strong indication that soluble and residual cellulase exist as separate entities within the intact cell, perhaps as two different molecular forms of the enzyme.

There can be no doubt that cellulase activity increases when abscission is allowed to proceed naturally following deblading, that it is increased further by abscission accelerants such as ethylene or Ethrel, and that it remains low when abscission is retarded by auxins. However, it is still difficult to decide whether the enzyme plays a causal role in abscission, for three reasons. (*a*) GA_3 increased cellulase activity without altering the rate of abscission. Perhaps the cellulase synthesized in response to

GA_3 was a different form of the enzyme or was localized in different cells than the cellulase associated with abscission. It was apparent from the GA_3 treatment of the intact seedlings that the larger increase was in the proximal portion of the abscission zone. Increases in activity typical of the abscission response are in the distal portion. (*b*) ^2H_2O stopped abscission but did not inhibit the increase in the activity of residual cellulase. (*c*) The increase in cellulase activity paralleled the decrease in pull force of the abscission zone. Had the increase preceded the drop in pull force, causal relationship would have appeared more convincing.

The density labeling experiment clearly shows that the residual cellulase activity present in the 48-hr explants was a result of synthesis *de novo* of the enzyme. Since the total amount of cellulase present at any given time depends on both synthesis and turnover, the observed increase in residual cellulase could have been due to an increased rate of synthesis, a decreased rate of turnover, or both. Further experiments will be needed to clarify this point. The fact that we were able to obtain only about 20% of the theoretically possible increase in buoyant density means that a considerable amount of the amino acids used in protein synthesis during the abscission process came primarily from the hydrolysis of existing protein, with no extensive changes in their carbon skeletons and hence affording little opportunity for ^2H replacement of their H. Transamination of these amino acids in ^2H_2O could account for the incorporation of one ^2H per amino acid molecule. Maximal incorporation of deuterium may be due either to the synthesis of all amino acids from their precursors, or to extensive reworking of the carbon skeletons of amino acids resulting from the hydrolysis of existing protein. It is also probable that the increase in buoyant density of the enzyme was limited by biological discrimination against ^2H_2O in favor of the 20% H_2O present in the agar. Longo (12) noted a doubling in deuterium incorporation between a 100 and 80% ^2H_2O system. These qualifications do not alter the fact that synthesis *de novo* of protein in general and cellulase in particular accompanies the abscission process.

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