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Activity of Pectin Esterase and Cellulase in the Abscission Zone of Citrus Leaf Explants

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Abstract. **The activity of pectin esterase and cellulase in abscission of citrus explants was studied. No relation was established between pectin esterase and abscission, while cellulase activity was markedly increased before abscission and for a certain period after excision. IAA and cycloheximide delay abscission and cellulase activity, while ethylene and, to a lesser extent, GA_3 accelerate them. Application of cycloheximide during the lag period and before cellulase activity can be measured, inhibits to a certain extent the formation of cellulase. An escape from the inhibitory effect of cycloheximide is detected when inhibitor is supplied at the end of the lag period.**

Most of the studies on abscission were carried out on leaf explants of annual plants, such as bean, cotton, coleus, *etc.* Recently, Lewis (16, 17) studied the effects of GA_3 and IAA on the abscission of citrus leaf explants, and showed that the acceleration of abscission by GA_3 is probably due to stimulation of protein synthesis, while IAA maintains the rate of protein synthesis in the cells of the abscission zone. These findings are compatible with the results reported by Abeles (4), who showed that the abscission process is characterized by *"de novo"* synthesis of RNA and protein. Both authors believe that the increase in protein synthesis is due to formation of new enzymes which are responsible for the abscission process.

Osborne (21) claimed that pectin esterase (PE) participates in the abscission process of bean leaves. In a later report Horton and Osborne (12) mention that cellulase is also active in the abscission zone of bean leaves. While studying the role of PE in the abscission zone of citrus explants we found that cellulase rather than PE is responsible for the separation process.

Very recently, Abeles (5) published a parallel study, showing the role of cellulase in the abscission zone of bean, cotton, and coleus explants which were aged for 20 hr and then transferred into ethylene environment. He concludes that the mechanism of ethylene action during cell separation induces protein synthesis which is essential for cell separation by the production of essential enzymes for the physiological processes of abscission. The present work was carried out independently although simultaneously with that of Abeles (5). The objective was to define the time relationship which exists between the abscission mechanism and cellulase activity in citrus leaves.

Materials and Methods

Plant Material. Four to 10 months old leaves from 36 years old Shamouti orange trees [*Citrus sinensis* (L.) Osbeck], in the coastal area of Israel, were picked in the morning and processed immediately afterward. Twenty mm long explants (10 mm from the petiole and 10 mm from the midrib of the leaf blade) were excised and washed under running tap water. When not otherwise stated, explants were dipped for 10 min in the treatment solution immediately after preparation.

Application of ethylene was made by injecting into and mixing of the proper amount of the hormone in a humid and closed system (4.1 l volume), where 2 open Petri dishes (20 explants in each) were previously placed. CO_2 was absorbed by 20 % KOH in the presence of filter paper. The concentration of ethylene in the containers was determined according to Riov *et al.* (24) through the analysis of an air sample by means of gas-chromatography analyzer (Packard).

Petioles of 10 explants were tied to a glass-slide and kept for the desired period in a humidified Petri dish, 9 cm in diameter, in the dark and at 25°. At the end of each experiment the percent of abscission was determined by counting the distal sections which had already abscised and those which abscised due to a gentle touch administered by forceps (7).

Pectin Esterase (PE) Assay. The method employed was that of Kertez (14) with the modification of Rouse and Atkins (25). Two sections of 2 mm were cut from 20 explants, from each side of the abscission-layer over a 8 mm wide section from the proximal to the distal end (21). Twenty similar sections, representing the same portion with respect to the abscission line, were pooled, weighed, and homogenized in 20 ml of 0.2 N NaCl in a mortar. The homogenate was transferred into 50 ml of 1 %

Citrus pectin (Yakhin, Canning Company, Ltd., Israel) in 0.2 N NaCl. The pH was adjusted to 7.6 by 0.2 N NaCl and maintained at this level for 10 min under pH-meter check, by dropping 0.01 N NaOH at 30°. PE activity was expressed as (PE.u.) g concentrate =

$$\frac{\text{ml 0.01 N NaOH}}{\text{weight of sample (gF.W.)} \times \text{time (min)}} \times 100$$

Cellulase Assay. Cellulase assay was performed according to Maclachlan and Perrault (18), modified by Horton and Osborne (12). Sections from both sides of the abscission line were sampled from 40 explants and pooled as described above. The sections were homogenized in 15 ml of 0.05 % cysteine in phosphate buffer (0.1 M, pH 7.0) by means of Ultra turrax homogenizer (24,000 rpm) in an ice bath. The homogenate was centrifuged at 20,000*g* for 10 min at 4°. One ml of the supernatant (= enzyme solution) was transferred into 20 ml of 1.1 % carboxymethylcellulose (CMC) sodium salt (BDH, Ltd., England) in phosphate buffer (0.02 M, pH 6.0), and stirred immediately. For calculation purposes 100 units were arbitrarily defined as that amount of enzyme in 1 ml of enzyme solution which causes a decrease of 21 % in the flow-time of the enzyme-substrate mixture after 18 hr of incubation at 37°.

Attention was made that the recorded rate of decrease in flow-time during the assay period should be in the linear portion of the hyperbolic function. The enzyme-substrate relationship was also linear in the range of 0.2 to 2.0 % CMC. Optimal pH was at the range of 5.5 to 6.6. Each experiment was repeated 2 to 5 times; standard errors and significance levels (F values) were calculated.

Results

Studies on Abscission and PE Activity. It is a well established fact that the application of high concentrations of IAA to the distal zone delays abscission (8). Recently, this was also shown to be true in citrus explants (16, 20). The activity of PE in explants was therefore studied in the presence of 0.112 μg IAA applied to the distal end of the explants, as compared with control without IAA. Results are presented in table I.

It is evident that although abscission in the control was 45 %, no significant change in PE activity was found during the incubation period up to 72 hr. IAA almost completely inhibited abscission at the same period, but had little effect on PE activity. The activity of PE was studied at various intervals following the application of cycloheximide (CHI), a protein synthesis inhibitor (26) which delays abscission (3); and after an application of ethrel, which releases ethylene when it is in contact with plant tissues (29). Results (not presented) showed that CHI and ethrel delayed and accelerated abscission in citrus explants, respectively, while no changes in PE activity could be detected due to the above mentioned treatments.

Studies on Cellulase Activity. Anatomical and chemical changes occur mostly at the distal end of the separation-layer (8). Chaudhri (9) reported the same phenomenon in citrus fruit explants. Fig. 1 shows that the maximal cellulase activity is located at a 0.2 mm wide zone proximal to the separation-line, while only 37 % of maximum enzymatic activity could be found in the 0.2 to 0.4 mm wide zone of the proximal part of the explant. As little as 14 % of maximum cellulase activity was found in the first 0.2 mm zone of the distal end, and only 10 % were found in the next 0.2 to 0.4 mm zone. All further results on cellulase activity will therefore be based on a 2 mm section sampled proximal to the separation-line of leaf-explants.

Fig. 2 shows the relationships between abscission and cellulase activity after the application of 0.112 μg of IAA to the distal end of the explants, as compared with untreated control. It is clearly demonstrated that only little cellulase activity can be detected during the first 24 hr after excision of explants, but as abscission advances an increased activity of this enzyme occurs. The delay in abscission caused by IAA is accompanied by a lower cellulase activity. On the other hand results not presented indicate that an acceleration of abscission caused by 1 μg GA_3 per explant and which was significant after 72 hr is related to an increase in cellulase activity.

We further show that a delay in abscission which is caused by inhibition of protein synthesis also

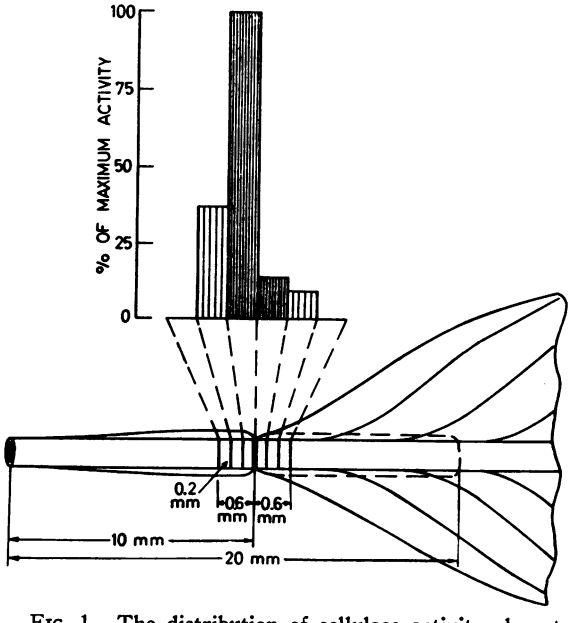

FIG. 1. The distribution of cellulase activity close to the abscission line of citrus leaf explants aged for 68 hr.

Table I. *PE.u./g in Various Sections of Citrus Explants With and Without 0.112 μg IAA*

Section No[1]		Hours after excision						
		0	24		48		72	
			—IAA	+IAA	—IAA	+IAA	—IAA	+IAA
Proximal	1	5.19	5.42	5.19	6.43	5.45	5.58	4.90
	2	5.33	5.35	5.41	5.70	5.27	5.30	5.00
Distal	3	5.52	5.56	5.24	5.58	5.08	4.97	4.50
	4	6.95	6.31	6.07	6.50	5.74	5.93	5.07
4:2 ratio		1.30	1.18	1.12	1.15	1.09	1.12	1.01
S. E.		±0.33	±0.19	±0.19	±0.26	±0.26	±0.48	±0.48
F		6.05*	5.43**	5.43**	3.83**	3.83**	N.S.	N.S.
% abscission		...	0	0	0	0	45	2.5

[1] Number of sections refer to 2 mm wide sections on both sides of the abscission line. Sections No. 2 and 3 are on the proximal and on the distal sides, respectively. * = significance at 0.05% level, ** = significance at 0.01% level. N.S. = not significant.

affects the activity of cellulase. The application of CHI inhibits (Fig. 3) the increase in cellulase activity, and the level of cellulase remains low although abscission is rapidly increasing later. Activity increases slowly and does not reach the level of control even 144 hr after excision. The effects of increasing concentrations of CHI on abscission and on cellulase activity are presented in Fig. 4. Up to 0.01 μg/ml (1 μg/ml = 3.5×10^{-6} M) only slight inhibitory effects of CHI on both parameters could be seen. When using higher concentrations of CHI, the inhibitory effect on abscission is more marked than its effect on cellulase activity.

Cellulase activity is detected well before abscission (Fig. 5). Control explants do not abscise even 48 hr after excision. On the other hand, significant amounts of cellulase can be detected as early as 28 hr after excision. In other cases (Figs. 2 and 3) cellulase activity was detected even after 24 hr. Ten ppm of ethylene (Fig. 5) markedly accelerated abscission, to 100 % 48 hr after excision, while the main increase in cellulase activity is found after 18 to 24 hr.

Fig. 6 depicts the effects of increasing concentrations of ethylene of both abscission and cellulase activity as measured 48 hr after excision. At the end of the experiment explants produced 0.1 ppm ethylene, and results were similar to those obtained with 0.1 ppm of exogenous ethylene. Explants which were left in Petri dishes under the same conditions, but were not confined in a closed container, did not show any abscission after 48 hr, while cellulase activity increased to 43.6 units. Abscission percentage after 48 hr increased from 10 % at 0.1 ppm to a maximum value at 10.0 ppm, while cellulase

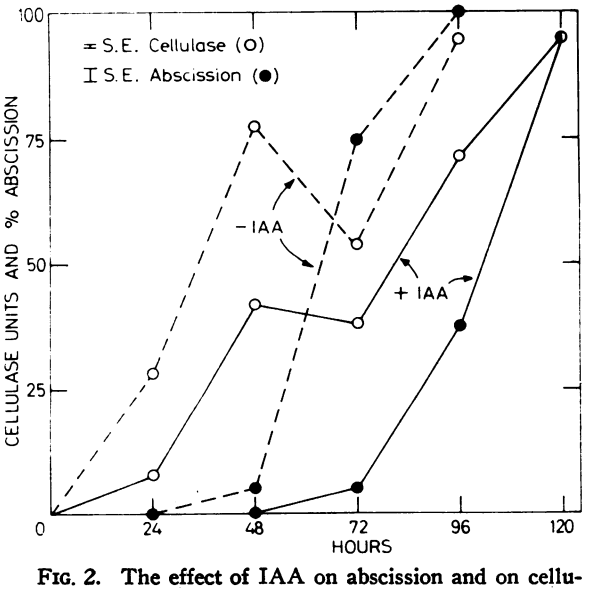

FIG. 2. The effect of IAA on abscission and on cellulase activity: 0.112 μg IAA in 2 μl were applied to the distal end of each explant immediately after excision.

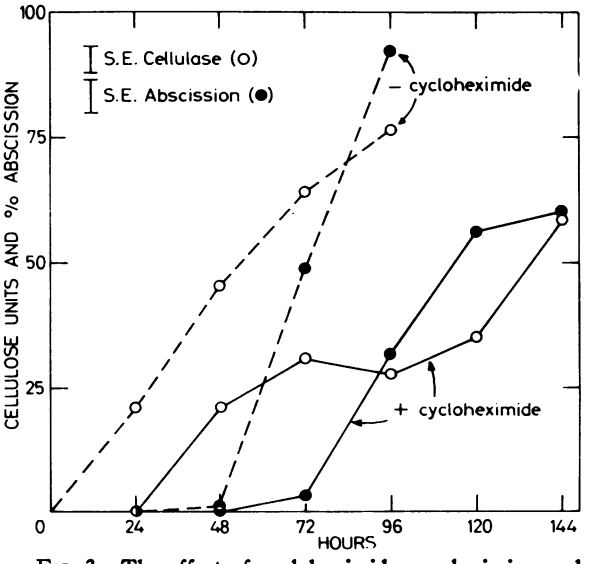

FIG. 3. The effect of cycloheximide on abscission and on cellulase activity; after excision explants were dipped for 10 min in 1.0 μg/ml of cycloheximide.

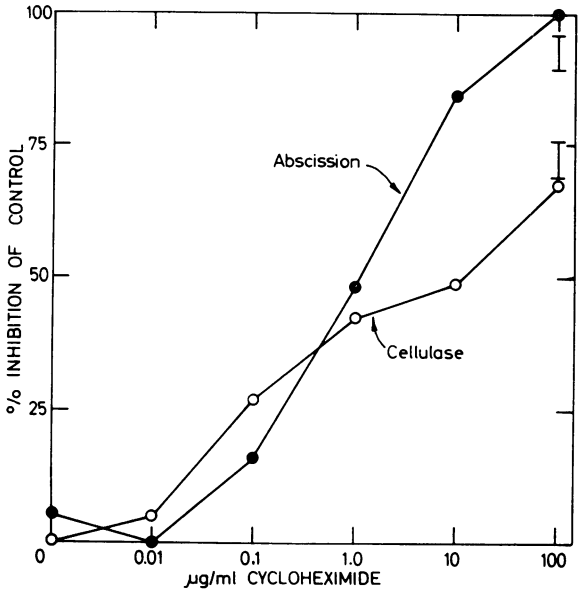

FIG. 4. The inhibitory effect of increasing concentrations of cycloheximide on abscission and on cellulase activity: treatments as explained in legend of Fig. 3; measurements applied 72 hr after excision.

activity showed a continuous increase from 1.0 to 100 ppm ethylene.

Earlier studies showed that the response of explants to exogenous treatment of ethylene takes place between 10 to 20 hr after excision (2, 11). In citrus, abscission starts between 24 to 28 hr after excision in the presence of 10 ppm of ethylene

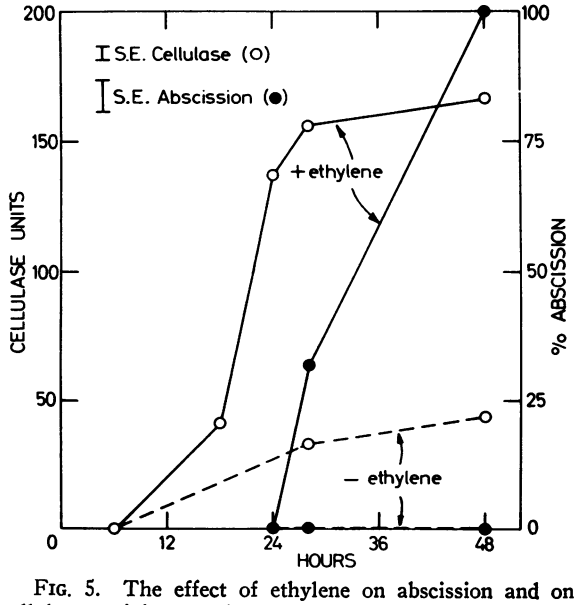

FIG. 5. The effect of ethylene on abscission and on cellulase activity; explants were stored in a humid and closed system containing 10 ppm ethylene.

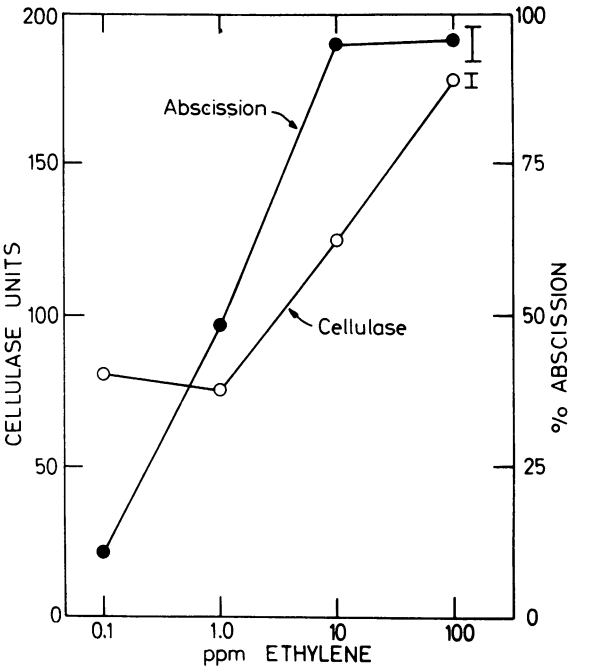

FIG. 6. The effect of increasing concentrations of ethylene on abscission and on cellulase activity. Explants were stored in ethylene in a humid and closed system containing various concentrations of ethylene. Measurements were taken 48 hr after excision.

(Fig. 5). It was deemed necessary to further study the timing effects of ethylene and CHI on cellulase activity. In Fig. 5 we prove that a lag period of at least 6 hr exists between the application of ethylene (10 ppm) and the appearance of detectable cellulase activity. If ethylene does not induce cellulase formation during that time, a treatment with CHI during that period should have inhibited cellulase activity since it will act prior to the induction stage. CHI was applied 0, 10, 18, and 24 hr after excision in the presence of 10 ppm of ethylene. Cellulase activity was measured after 28 hr, and compared with explants which were incubated with ethylene only (Fig. 7). CHI, which was applied during the first 10 hr after excision, inhibited ethylene-induced cellulase activity by nearly 70 %. When CHI was applied 18 hr after excision, a certain escape from its inhibitory effect could be detected, and became even more evident when the application of CHI treatment was delayed to 24 hr after aging in ethylene. Some inhibition of cellulase activity was still detectable at this treatment also.

Another approach to the study of the period required for ethylene to induce cellulase activity is by applying ethylene at different intervals after excision, as was done in previous studies which deal with the effect of ethylene on abscission. Explants were treated with 10 ppm of ethylene at various intervals after excision. Results show (Fig. 8) that

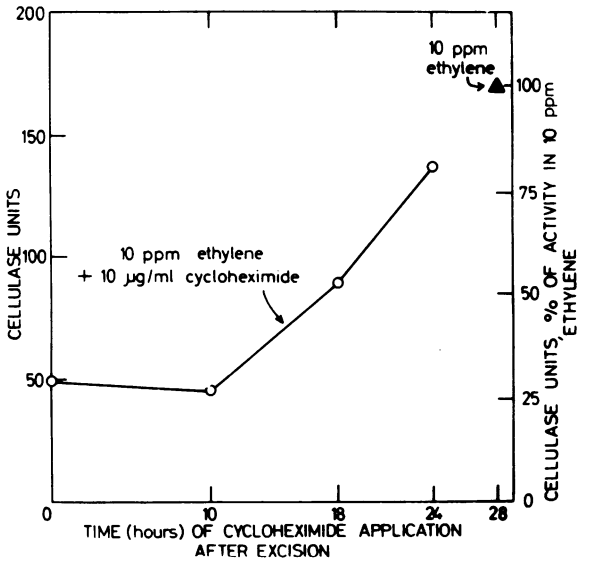

FIG. 7. The escape of cellulase formation from cycloheximide inhibition: explants were dipped in 10 µg/ml at different intervals after excision. 10 ppm ethylene was maintained throughout the experiment. Measurements were taken 28 hr after excision.

incubation in ethylene during the first 10 hr causes only a slight increase in cellulase activity as measured 28 hr after excision. When incubation time is prolonged to 18 hr, cellulase activity at 28 hr is higher, but the highest activity is obtained when a continuous incubation in ethylene is maintained during 28 hr. The same period of incubation which starts 18 hr after excision produces much more stimulatory effects on cellulase induction, which is even stronger than 18 hr of continuous incubation from excision.

The fact that CHI was found to inhibit cellulase activity in the presence of ethylene or in the absence of the hormone is not surprising, since abscission is known to be related to RNA and to protein synthesis (4, 14, 17, 22, 30). Results presented in table II show that the RNA analogue 2-thio-uracil (2-TU), significantly inhibited abscission and cellulase activity. 5-fluorodeoxyuridine (FUdR), a specific DNA synthesis inhibitor (15) behaved very much like 2-TU, but the most dramatic inhibition of both phenomena was obtained by CHI.

Table II. *The Effect of Different Metabolic Inhibitors on Cellulase Activity and on Percent of Abscission*

Treatment	Concn.	Cellulase units	Abscission
	µg/ml		%
Control		107.8	90
5-Fluorodeoxyuridine	12.5	52.1	28
2-Thiouracil	300.0	46.9	36
Cycloheximide	1.0	28.0	27

Discussion

Until recently, it was believed that the mechanism of abscission involves the pectic enzymes (6) which causes significant changes in PE activity during the abscission processes (21, 23, 31). We could not find any relationship between PE activity and abscission in citrus-leaf explants (table I). Cellulase, however, seems to be highly related to this process. It was already shown that during fruit ripening cellulose degradation is related to an increase in cellulase activity (10, 13, 19, 28). Moreover, results obtained by Horton and Osborne (12) might indicate that cellulose degradation in the abscission zone (30) could be attributed to cellulase activity.

We found (Fig. 1) that the locus of cellulase activity is very limited, and occurs close to the proximal border of the separation line. This is in accordance with anatomical data reported by Chaudhri for citrus explants (9) and by others for annual plants (7).

Figs. 2, 3, and 5 show clearly that whenever abscission occurs, it is preceded by an increase in cellulase activity. It can, therefore, be concluded that the time ratio interval between the increase in enzyme activity and the abscission process is constant. Any delay or acceleration of abscission by either hormonal (IAA and ethylene) or metabolic inhibitors, affects the cellulase activity.

Abeles (5, Fig. 2) showed that the main increase in cellulase activity starts to be evident 3 hr after the transfer of 20 hr aged explants into ethylene.

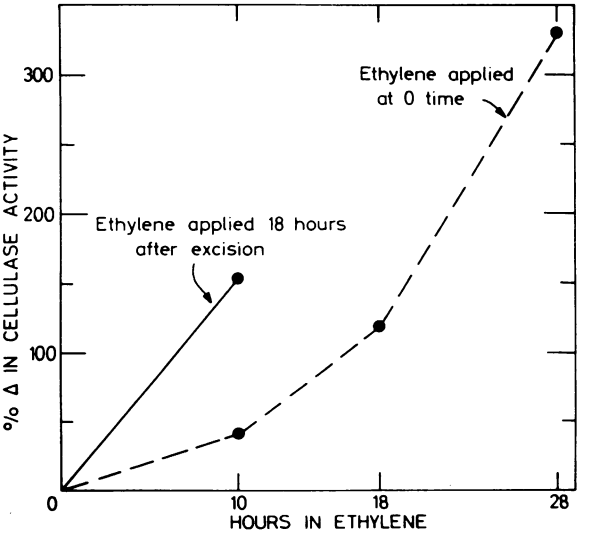

FIG. 8. Increase in cellulase activity due to various periods of incubation in 10 ppm of ethylene either from excision or from 18 hr after excision. Measurements were taken 28 hr after excision.

In his system, explants were found to be insensitive to ethylene during the first hours (1) and abscission is therefore not accelerated when explants are treated with ethylene during the first hours after excision. In citrus, the increase in cellulase activity in the presence of ethylene after a lag period of 6 to 10 hr is mostly evident between 18 and 24 hr.

It was interesting to find out whether ethylene induces the increase in cellulase activity during the lag period. The fact that CHI inhibits most cellulase activity at 0 and 10 hr after excision and fails to act in the same way after 18 hr (Fig. 7), indicates that in the presence of ethylene the increases in cellulase activity starts 10 hr after excision.

Riov *et al.* (24) showed recently in a similar way that exogenous ethylene stimulates the activity of phenylalanine ammonia-lyase (PAL) in external peel layers of intact grapefruits, and that CHI (1.4×10^{-5} M) inhibits both ethylene production and PAL activity in mature excised grapefruit-peel discs. Another enzyme whose synthesis is influenced by ethylene is peroxidase (27).

By applying exogenous ethylene to citrus leaf explants we could detect 3 stages of abscission as follows: A) a lag period (6 hr) before increased cellulase activity is detectable; B) increased cellulase activity (6–24 hr); and C) the separation processes (24–48 hr). Since citrus explants produce ethylene (Fig. 6), further work is needed to show whether ethylene is the endogenous inducer of cellulase formation (5) or whether it activates an inactive form of cellulase.

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