

Enhancement of RNA Synthesis, Protein Synthesis, and Abscission by Ethylene

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Summary. Ethylene stimulated RNA and protein synthesis in bean (*Phaseolus vulgaris* L. var. Red Kidney) abscission zone explants prior to abscission. The effect of ethylene on RNA synthesis and abscission was blocked by actinomycin D. Carbon dioxide, which inhibits the effect of ethylene on abscission, also inhibited the influence of ethylene on protein synthesis. An aging period appears to be essential before bean explants respond to ethylene. Stimulation of protein synthesis by ethylene occurred only in receptive or senescent explants. Treatment of juvenile explants with ethylene, which has no effect on abscission also has no effect on protein synthesis. Evidence in favor of a hormonal role for ethylene during abscission is discussed.

In an earlier paper (2) we showed that the protein synthesis inhibitors actinomycin D and cycloheximide would block abscission, and that during the course of ethylene-enhanced abscission L-leucine-¹⁴C was incorporated into a trichloroacetic acid-precipitable fraction of tissue homogenates from the separation zone of bean (*Phaseolus vulgaris* var. Red Kidney) and cotton (*Gossypium hirsutum* var. Acala 4-42) explants. Protein synthesis in the stem and in the nodal tissue surrounding the separation zone was not influenced by the ethylene treatment. These results supported the view that ethylene, like other hormones (4) was capable of acting as an effector or regulatory metabolite.

In this paper we will show that the stimulation of RNA synthesis and protein synthesis is analogous to that reported for other hormone systems.

Materials and Methods

Methods used to grow and prepare explants and to measure ethylene in the surrounding gas phase have been described earlier (3, 17).

Preparation of RNA. The perchloric acid method of RNA extraction was modified after Key and Shannon (13). One and one-half grams of tissue (10-15 explants) were homogenized in 2 ml 0.01 M Tris buffer, pH 7.5, in a ground glass homogenizing flask with a ground glass pestle for 1 to 2 minutes. The sides of the homogenizing flask were washed down with 3 ml of 0.01 M Tris buffer and the resultant suspension was filtered through glass wool. Samples (3.5 or 4.0 ml) were taken immediately after filtration through glass wool, made to 0.2 N with respect to HClO₄, thoroughly mixed, and centrifuged at 1000 × g for

10 minutes. The pellets were then suspended and washed twice with 0.2 N HClO₄ and centrifuged at 1000 × g for 10 minutes. The resulting pellets were then suspended and washed twice in methanol containing 0.05 M formic acid and centrifuged at 2000 × g for 10 minutes. All above steps, including centrifugation, were carried out at 2° to 4°. The washed pellets were twice extracted at 37° for 30 minutes in a 2:2:1 mixture of ethanol: ether:chloroform to remove lipids and centrifuged at 3000 × g for 10 minutes. RNA was hydrolyzed in 0.3 N KOH for 18 hours at 37°. After chilling, HClO₄ was added to a final concentration of about 0.3 N, followed by centrifugation at 2000 × g for 10 minutes to remove the KClO₄ precipitate, protein, and DNA. Absorbance of samples of RNA was measured at 260 and 290 mμ with a spectrophotometer. After determining the RNA, each sample was neutralized with KOH to pH 4.5 to 5.5. After chilling, the KClO₄ precipitate was removed by centrifugation and samples were plated, dried, and counted for determination of ³²P incorporation into RNA.

Protein Extraction Methods. A modified Peterson and Greenberg (16) method was used. Tissue was homogenized in ground glass homogenizers with 1 ml 0.01 M Tris buffer, pH 7.5, containing 0.2 mg L-leucine ¹⁴C per ml. After 2 minutes of homogenization, 3 ml of Tris buffer were added and the resultant suspension was poured into centrifuge tubes. A small sample of the homogenate was taken for extraction in hot 80% (v/v) ethanol to determine the amount of ethanol-soluble L-leucine-¹⁴C. The amount of ethanol-soluble of L-leucine-¹⁴C was essentially the same (± 10%) from ethylene-treated and control explants. The remaining homogenate was centrifuged at 775 × g for 10 minutes. The sediment (cell wall

material) was washed with distilled water and 0.01 *N* NaOH. The supernatant fractions were combined with the original supernatant. The proteins were precipitated by adding 2 volumes of 20% (w/v) trichloroacetic acid and purified by successive washings with 5% trichloroacetic acid (4 times), ethanol (once), 3:1 ethanol-ether, 3 minutes at 60° (3 times), and ether (once). During the second 5% trichloroacetic acid wash, the suspension was heated at 90° for 15 minutes. The final protein pellet was suspended in 1 *N* NaOH and a sample was taken and diluted to 0.1 *N* NaOH for protein analysis by the Lowry method (14). The protein associated with the cell wall was extracted in the same manner as above. A less exhaustive but simpler method of protein extraction that gave identical results is based on a method described by Key (12) and has been published earlier (2).

Incubation of Explants. Except for part A of figure 5, only senescent explants were used in the experiments reported in this paper. After the explants were cut from the primary bean leaf, they were stored by sticking them in 3 mm of 1.5% agar for 22±2 hours and placing the petri dishes in the incubation chamber described below. Six ml of 1.5% agar were poured into 43±2-ml gas-collection bottles (5 cm in diameter and 2.5 cm high) and 10 senescent explants were inserted petiole end down in the agar so that 3 mm of the explant were submerged. The bottles were fitted with a neck in which a 25-mm-diameter rubber vaccine cap could be placed. Where required, ethylene was injected into the bottles through the vaccine cap to give the specified ethylene concentration. The gas-collection bottles were cut horizontally 1.25 cm from the bottom to facilitate manipulation of the agar containing radioactive compounds and explants. To make the bottles gastight, silicone grease was applied to the ground surfaces and the bottle halves were held together with adhesive tape. Control explants were aerated by opening and resealing the gas-collection bottles. The ethylene concentration in the gas phase was monitored by gas chromatography, and in the aerated bottles the concentration was not allowed to exceed 0.1 nl ethylene per ml (0.1 ppm) during the experiment. Previous experiments by Rubinstein and Abeles (17) indicate that this amount of ethylene does not accelerate abscission over that of aerated controls. The explants were incubated at 25° under 400 ft-c of continuous fluorescent light (General Electric type 30T8-ww).

Application of ³²P and L-leucine-¹⁴C to Abscission-Zone Explants. Radioactive orthophosphate in neutralized solution, carrier-free, was incorporated in 1.5% agar disks (1.4 mm × 3 mm diameter) that contained approximately 3 μc of ³²P. The amount of L-leucine-¹⁴C incorporated in these agar blocks (0.01 ml of 2.8 × 10⁻⁵ M) was not high enough to act as an abscission stimulant (17). Concentrations greater than 1 mM are required for this effect. The radioactive agar blocks

were placed on the pulvinal end of the 10 mm-explants.

The disks of L-leucine-¹⁴C and ³²P were saved at the end of an experiment, placed in planchets, and melted by adding 1 ml of water and heat. This treatment spread the disks (usually 10 per replicate treatment) evenly over the bottom of the planchet. The planchets were then counted to check whether ethylene had any effect on isotope uptake into the tissue. No effect of ethylene on L-leucine-¹⁴C or ³²P uptake has been observed.

All tissue samples were immediately frozen after harvesting and thawed just before homogenization.

Results

Inhibition of Abscission by Actinomycin D and Cycloheximide. Since actinomycin D inhibits at an earlier point than cycloheximide in the sequence of events leading to protein synthesis, a time course curve showing the effect of these inhibitors on ethylene-treated explants should show that the tissues will be sensitive to cycloheximide longer than actinomycin D.

Actinomycin D and cycloheximide were injected into the explant in 1 μl of solution with a micro-liter 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. After the needle was withdrawn, all of the solution remained within the cavity of the petiole tissue. The first injection was made immediately prior to the addition of the ethylene. Two, 4, and 6 hours later, a set of bottles was opened, inhibitor was injected

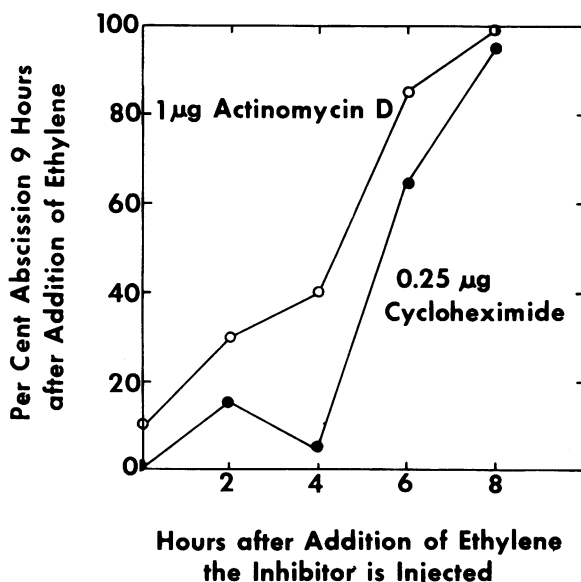


FIG. 1. Effect of actinomycin D and cycloheximide on abscission of senescent explants.

into the explants and the bottles were resealed, and the ethylene reinjected. Abscission was measured 9 hours after the start of the experiment. As shown by the representative experiment in figure 1, the ability of the actinomycin D to delay abscission decreases when actinomycin is added after the ethylene, but cycloheximide retains its effectiveness for about 4 hours.

Table I. *Effect of 1 µg Actinomycin D on ³²P Incorporation into Bean Explant RNA*

Explants were aged 22 hours at 25° and then injected through the petiole with either 1 µl of water or 1 µl of actinomycin D. Two hours later the agar blocks containing ³²P were put on the pulvinus and the gas collection bottles were sealed. The explants were harvested 5 hours later. RNA was extracted and was purified by the perchloric acid method.

Treatment	% Abscission after 29 hrs	CPM/mg RNA ± SD
Control	23	44,000 ± 7000
Ethylene, 2 ppm	68	102,000 ± 7000
Actinomycin D	8	27,000 ± 5000
Ethylene, 2 ppm + actinomycin D	8	34,000 ± 6000

Data in table I show that the action of actinomycin D in explant tissue is due to an inhibition of RNA synthesis. Explants were injected with actinomycin D (described above) before exposure to ethylene. The agar blocks containing ³²P were placed on top of the explants, and the RNA was extracted and measured 5 hours later. In order to enhance the difference between ethylene treated and control explants the ³²P was placed on pulvini which had the top 2 mm removed. Earlier experiments (2), indicated that ethylene had only a negligible effect on RNA synthesis in the upper part of the pulvinus. Data in table I show that actinomycin D inhibits RNA synthesis of both control and ethylene-treated explants.

Effect of X-Irradiation. Another way of implicating an interaction between ethylene and the nucleic acids of the cell in abscission is to alter the priming activity of the DNA with X-irradiation.

If an intact nucleus is required for ethylene action, then X-irradiation prior to addition of ethylene to susceptible explants should block or retard the abscission-stimulating effect of ethylene. If the nucleus is exposed to X-rays after ethylene has had a chance to initiate polypeptide formation, the X-rays should have little or no effect on abscission. Senescent explants were exposed to a General Electric Maxitron 1000 for 30 minutes at a distance of 10 cm, giving a total dose of 260,000 r. Explants were treated immediately prior to the addition of ethylene and then 2.5 and 5 hours later. Abscission was measured 8 hours after the start of the experiment. As shown in figure 2, an exposure of 260,000 r prior to an inductive ethylene treatment resulted in an inhibition of abscission. The same

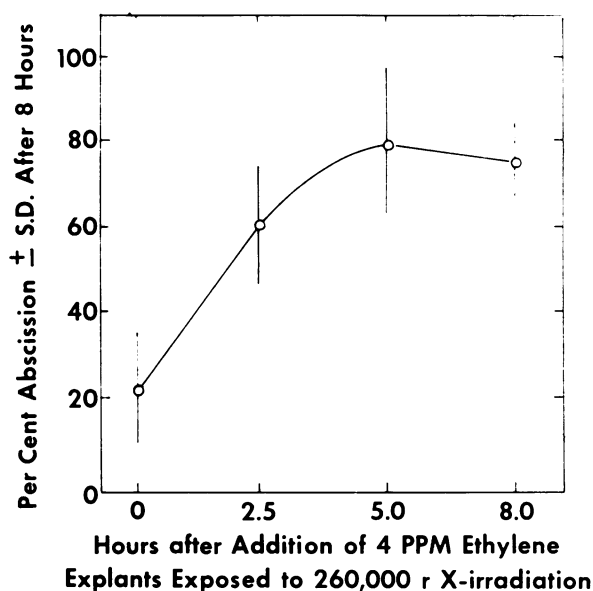


FIG. 2. Effect of x-irradiation on abscission of senescent explants exposed to 4 ppm ethylene.

treatment 2.5 hours later had only a residual effect, and a 5-hour treatment gave results similar to that of untreated controls.

Enhancement of RNA and Protein Synthesis by Ethylene. The use of inhibitors and X-irradiation can only give indirect support to the idea that ethylene is capable of acting as an inducer in the abscission process. A more direct approach is to show that ethylene is capable of stimulating RNA synthesis in receptive tissue, followed by a stimulation in protein synthesis.

In this experiment, agar blocks of ³²P and L-leucine-¹⁴C were placed on top of senescent explants, and the explants were incubated in either air or an atmosphere containing 4 ppm ethylene. The RNA and protein was extracted at 2, 4, 6, and 8 hours. Figure 3 shows the percentage difference in incorporation of tracer into RNA and protein in ethylene-treated versus control explants. Enhancement of RNA synthesis occurs after an hour lag; the enhancement in protein synthesis occurs after a 2-hour lag. These results indicate that stimulation of protein synthesis occurs only after a stimulation in RNA synthesis.

Another possible explanation for the enhancement of protein synthesis is that ethylene caused a solubilization of wall proteins which resulted in a larger amount of tagged material in the supernatant. No evidence for this explanation was obtained. We found that ethylene also stimulated incorporation of L-leucine-¹⁴C into wall protein, that similar amounts of protein were extracted from control and ethylene treated explants, and that the enhancement of protein synthesis was actinomycin D sensitive.

Effect of Ethylene on Juvenile and Senescent

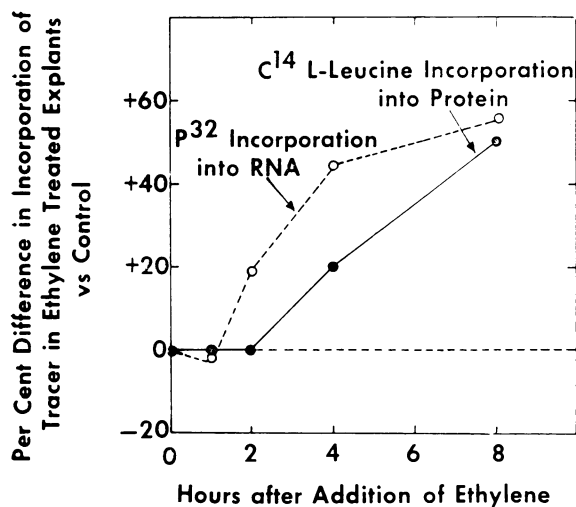


FIG. 3. Time course of enhancement of ^{32}P and L-leucine- ^{14}C incorporation into RNA and proteins of ethylene-treated vs. control bean explants.

Explants. In an earlier paper (2) we presented evidence that the stimulation of protein formation occurred only in the separation layer of cotton and bean explants and not in the surrounding petiole or nodal tissue. Another way of demonstrating that ethylene action is specifically directed toward ethylene-sensitive tissue is to treat juvenile explants with the gas. Yamaguchi (19) and Abeles and Rubinstein (3) have shown that ethylene was without effect on abscission when juvenile explants were treated with gas. It follows that if the gas has no effect on abscission at this time, it should also have no effect on protein synthesis. Figure 4 shows that this is true: an ethylene-dependent enhancement of protein synthesis is absent in juvenile explants but readily observable in senescent explants.

Effect of Carbon Dioxide on Abscission and Protein Synthesis. CO_2 is known to block the effect of ethylene in accelerating abscission (19), and fruit ripening (6). Burg and Burg (6) have shown with a Lineweaver and Burk kinetic analysis that CO_2 acts as a competitive inhibitor in the inhibition of pea stem growth by ethylene. Since CO_2 inhibits abscission, it also should inhibit the

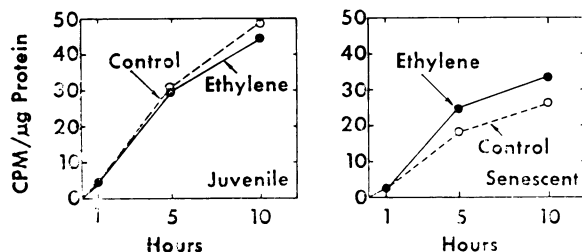


FIG. 4. Effect of ethylene on L-leucine- ^{14}C incorporation into abscission-zone proteins of juvenile and senescent explants.

acceleration of protein synthesis by ethylene A) if the assumption that the carbon dioxide occupies the same site as ethylene can be extrapolated from data on pea stem growth, and B) if the stimulation of protein synthesis is an integral part of abscission.

Data presented in table II support this interpretation. This table shows that 15% CO_2 blocks abscission of bean explants and that the inhibiting effect of the CO_2 is partially relieved by the addition of 0.75 ppm ethylene. Carbon dioxide inhibited the incorporation of leucine into protein by 7%, which might reflect the interaction of CO_2 with endogenous ethylene production. As usual, 0.75 ppm ethylene stimulated protein synthesis (by 30%) and this stimulation was almost completely overcome by 15% CO_2 . Addition of ethylene and CO_2 simultaneously gave a rate of protein synthesis and abscission, somewhat greater than that of the controls.

Table II. *Inhibition of Explant Abscission and Protein Synthesis by Carbon Dioxide*

Explants were aged 22 hours at 25° before L-leucine- ^{14}C in agar blocks was applied to the pulvinal stump, incubated in the below atmospheres for 5 hours, and then the protein was extracted by the trichloroacetic acid method.

Treatment	% Abscission	CPM/mg protein ± SD
Control	70	21,100 ± 400
15% CO_2	0	19,600 ± 700
0.75 ppm ethylene	100	27,200 ± 100
15% CO_2 ± 0.75 ppm ethylene	80	21,600 ± 300

Discussion

Ethylene appears to stimulate abscission of senescent explants only. Some of the factors that maintain juvenility of explants, measured as an insensitivity toward ethylene, include storage at low temperatures (19) and treatment with auxins (3), kinins, and carbon dioxide. After the tissue has aged, carbon dioxide can still block ethylene action although auxins and kinins are without effect (unpublished results). This second effect of carbon dioxide may be similar to the competitive inhibitor effect known for the inhibition of pea stem growth by ethylene (6). This 2-fold action of carbon dioxide on abscission, first retarding senescence and second blocking ethylene action, was previously recognized by Yamaguchi (19).

Once the abscission zone becomes receptive, the action of ethylene appears to be similar to that of other plant and animal hormones. Although the idea of a gaseous hormone is not new (20), it has never achieved the popularity accorded the less ephemeral ones such as auxin, gibberellins, and kinins. Nevertheless, ethylene is known to be

evolved from fruits, flowers, leaves, stems, roots, tubers, and seeds from a large variety of plants (5), and characteristic of hormones is active in small amounts (ca. 1 ppm) for most of its effects. Since the gas is produced in all parts of the plant, translocation from the site of production to the site of action does not seem to be a problem.

The observation that ethylene action is blocked by actinomycin D and cycloheximide is evidence in favor of an effector role for ethylene. The fact that the inhibiting effect of cycloheximide persisted longer than that of actinomycin D after the addition of ethylene is evidence that the mode of action of these compounds in explants is similar to that ascribed to them from studies of other systems, that is, the inhibiting effect of cycloheximide lasts longer than that of actinomycin D because it acts at a later point in protein synthesis.

The effect of ethylene was blocked by 250 krad of X-irradiation. Although no direct evidence is offered, we assumed that the effect of the radiation was preferentially on the nucleic acid-dependent part of protein synthesis and that proteins were more resistant to the action of this high-energy radiation. This assumption was supported by the fact that X-irradiation was most effective prior to the addition of ethylene and that after polypeptide synthesis had taken place, its effectiveness was lost. A similar observation on the effect of gamma radiation on ripening pears has been reported by Maxie et al. (15). They found that 300 krad of gamma radiation would prevent normal ripening of preclimateric pears but did not inhibit ripening when the pears were one-half way through the ascending portion of the climacteric.

When we examined the effect of ethylene on RNA and protein synthesis, we found that results with explant tissue agree with what is well known for polypeptide synthesis in other organisms; that is, a short lag period occurs after the addition of ethylene before an enhancement of RNA synthesis begins, followed by another lag period and the stimulation of protein synthesis. As anticipated, actinomycin D blocked the stimulation of RNA synthesis by ethylene. Additional support for the idea that ethylene-dependent protein synthesis is an integral part of abscission comes from the experiments that show that carbon dioxide blocked both abscission and protein synthesis. When the inhibiting effect of the carbon dioxide was overcome by additional amounts of ethylene, protein synthesis also proceeded.

In this paper we offer evidence in favor of the hypothesis that ethylene is a plant hormone by showing that ethylene is capable of acting as an effector substance. Other hormonal attributes of ethylene such as its effectiveness in small amounts, its catalytical characteristics, and its ubiquity has been described by Burg (6).

Other possible interpretations of these data include the idea that ethylene treatment results in an alteration of membrane permeability or causes an-

other substance to be an effector, either through synthesis or release by alteration of cellular compartmentalization. These ideas are not advanced to avoid the risk of speculation but represent a real problem in studies with plant hormones. Sacher has presented evidence that changes in compartmentalization by an alteration of membrane permeability account for some of the phenomena associated with ripening of bananas (18) and senescence of bean pods (10). Earlier workers have found that ethylene may increase (11) or have no effect (9) on membrane permeability. We have found that ethylene had no effect on the rate of sodium azide uptake into senescent abscission zone explants measured as an inhibition of respiration or on the rate of ion leakage measured as a change in the conductivity of bathing solutions.

It is well known that IAA (13) can stimulate RNA synthesis. It is also known that IAA can stimulate ethylene synthesis, and interestingly enough that this IAA effect can be blocked by actinomycin D and puromycin (1). Since we show here that ethylene is capable of stimulating RNA synthesis under certain circumstances, how can we be sure that some auxin effects are not ethylene effects?

Burg and Burg have shown that the inhibiting effect of high concentrations of IAA on pea stem growth (7) and stimulation of floral initiation in the pineapple by NAA (8) are probably due to enhanced ethylene production. Other effects of growth regulators such as epinasty, guttation, release of dormancy, root initiation, fruit ripening, and intumescence formation that are known to be similarly influenced by ethylene could be profitably re-examined, [see Abeles and Rubinstein (3) for appropriate references].

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