Plant Physiol. (1973) 52, 171–173

Effects of Cycloheximide on Indoleacetic Acid-induced Ethylene Production in Pea Root Tips[1]

Received for publication October 13, 1972

DAVID A. STEEN AND ARTHUR V. CHADWICK
Department of Biology, Loma Linda University, Loma Linda, California 92354

ABSTRACT

Cycloheximide inhibited ethylene production in excised pea root tips treated with high levels of indoleacetic acid (100 μM and 10 μM). In contrast, cycloheximide did not inhibit ethylene production induced by a lower concentration (1 μM) of indoleacetic acid unless it was added 2 hours before the indoleacetic acid treatment. These observations suggest that indoleacetic acid has two effects on the enzyme system involved in ethylene synthesis. At low concentrations (1 μM) indoleacetic acid increases ethylene production without protein synthesis, whereas at the higher concentrations, the synthesis of new protein is associated with increased ethylene production.

Previous research has suggested that auxin may stimulate the induction of an enzyme system involved in ethylene production (1). Kang *et al.* (10) and Sakai and Imaseki (13) propose a short lived RNA involved with synthesizing an extremely labile protein controlling the rate of ethylene production. Such a system requires an induction period following application of auxin, in contrast to the earlier observation (5) that ethylene production is stimulated immediately in pea roots, and also under certain conditions in mung bean stem tissue (13).

Evidence is presented here that ethylene production in pea root tips responds to applied IAA in two phases, and that the first phase accounts for the immediate stimulation of ethylene in response to applied auxin. Since this ethylene production is not affected by cycloheximide, a potent and fast acting protein synthesis inhibitor (8, 9, 12), it apparently does not involve *de novo* protein synthesis. The second phase, which can be partially or completely blocked by CH,[2] seemingly requires new synthesis of protein.

MATERIALS AND METHODS

Seeds of *Pisum sativum* L. var. Alaska were soaked in running tap water for 6 hr, planted 1 cm deep in moist vermiculite, and grown in darkness for about 48 hr at 24 C. All subsequent manipulations were performed under dim green light to avoid photomorphic and phototrophic responses. Ten 5-mm apical tips were cut from roots 2 to 3 cm long, placed in 10-ml microfernbach flasks with 2 ml of media containing 2% sucrose (w/v), and 5.0 mM phosphate buffer (pH 6.8). An appropriate concentration (5) of IAA and CH (10 or 20 μM) were added in some experiments. The flasks were sealed with vaccine caps and gently shaken in the dark. At intervals of 1, 2, 4, 8, 16, and 20 hr, 5-ml samples were withdrawn using a 5-ml glass syringe. The vacuum was relieved by admitting room air through a second needle. Ethylene levels were determined by gas chromatography (3).

Experiments designed to determine the effect of pretreatment with CH on ethylene production were carried out by incubating the tissue in 1.8 ml of standard media with or without CH for 2 hr. After pretreatment, sufficient IAA was injected through the cap to produce the correct auxin concentration in 2-ml total volume. The flasks were subsequently sampled as described above to determine ethylene production.

Roots were weighed on an analytical balance and length measurements were made with an optical stage micrometer and a 10X stereomicroscope.

Experiments to determine the effect of 10 μM CH were done using a method adapted from Klein *et al.* (11). Ten 5-mm root apices were incubated in 10-ml microfernbach flasks with 2 ml of standard media, 1.2 μM ^{14}C-leucine and with or without 10 μM CH. At predetermined times the tissue was removed from the flask and rinsed with 5% trichloracetic acid which contained 100 μg/ml unlabeled leucine. The tissue was then homogenized at 0 C in 4 ml of this solution, heated to 90 C for 15 min, chilled, and then centrifuged for 5 min at 2,000*g*. An aliquot of supernatant was dried on a planchet and assayed for radioactivity. The pellet was resuspended three times in 4 ml of 5% trichloracetic acid (0 C), and the final precipitate was transferred to a planchet and dried. The radioactivity was measured using a Nuclear Chicago gas flow planchet counter.

RESULTS

Initial control experiments to determine ethylene evolution in response to applied IAA demonstrate (Fig. 1A) that ethylene production is a function of the applied IAA concentration (5). Ethylene was evolved to a similar extent at all concentrations of applied auxin during the first 2 hr. Between 2 and 4 hr, the rate of ethylene production in roots treated with 100 μM IAA increased sharply, then leveled off and returned nearly to the control rate after 16 to 20 hr. A marked increase in ethylene production also resulted after 4 hr treatment with 10 μM IAA. This enhanced production continued for several hours and returned to control rate after about 12 hr. No increase in rate after the first 4 hr was experienced by roots treated with 1 μM IAA. Instead, the roots returned to a rate not significantly different from that of the control, between 4 and 8 hr after auxin application.

[1] Computation assistance was received from the Loma Linda University Scientific Computation Facility supported in part by National Institutes of Health Grant RR-276-08.

[2] Abbreviation: CH: cycloheximide.

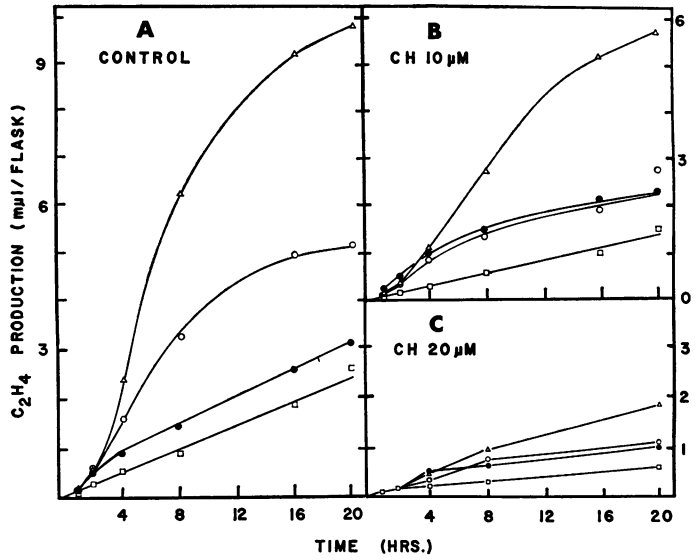

FIG. 1. Time course showing effects of various IAA concentrations on cumulative ethylene production, A: Control with IAA only; B: IAA and 10 μM CH; C: IAA and 20 μM CH. IAA concentrations are 100 μM (△); 10 μM (○); 1 μM (●); and control (□).

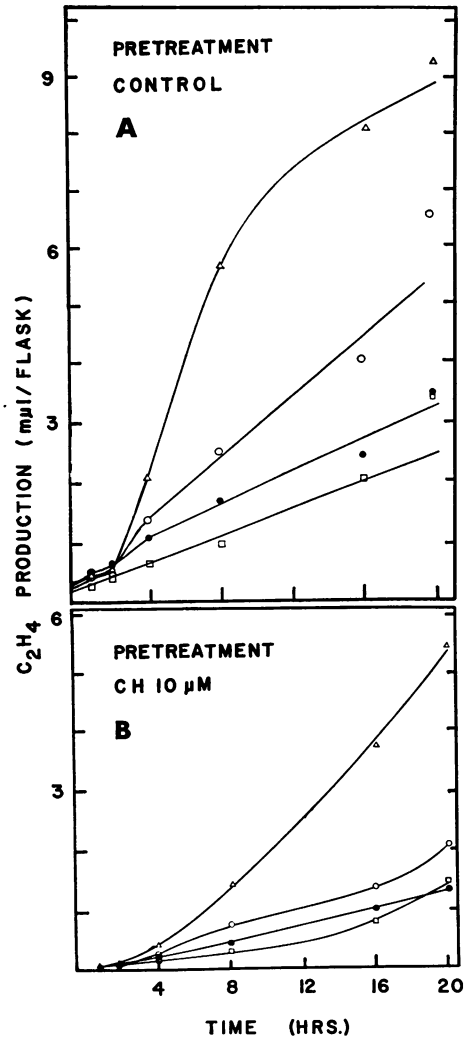

FIG. 2. Time course showing effects of 2 hr pretreatment. A: In standard media; B: in 10 μM CH, before addition of various IAA concentrations at 0 hrs. Final IAA concentrations are 100 μM (△); 10 μM (○); 1 μM (●); and control (□).

Table I. *The effects of IAA and CH on growth parameters of root tips measured after 20 hr of incubation*

Treatment	Weight of 10 Root Tips	Length of Root Tip	Ratio of Increase in Weight to Increase in Length
	mg	*mm*	*swelling ratio*
Fresh cut	43.7	5.0	
Control			
No IAA	99.4	12.5	1.00
IAA, 1 μM	90.0	10.2	1.20
IAA, 10 μM	80.3	8.3	1.47
IAA, 100 μM	63.3	6.4	1.94
Control			
CH, 10 μM	68.9	7.8	1.21
IAA, 1 μM + CH 10, μM	68.4	8.4	0.97
IAA 10, μM + CH 10, μM	63.2	7.8	0.94
IAA, 100 μM + CH, 10 μM	55.9	6.8	0.87

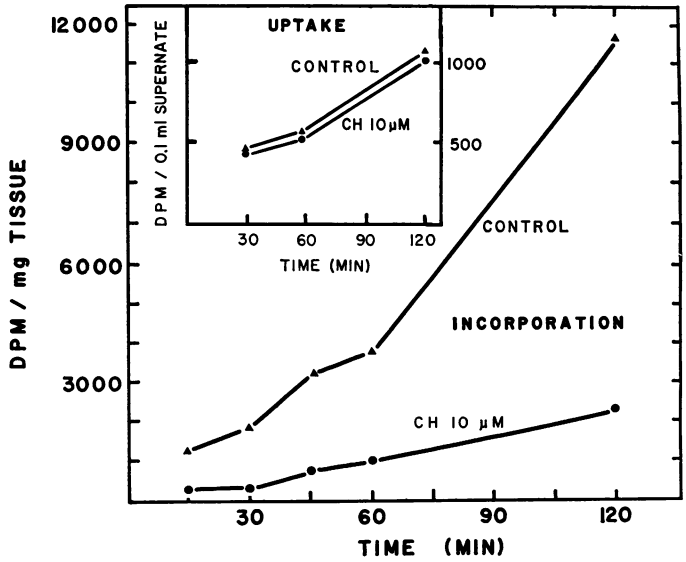

FIG. 3. Time course showing relative radioactivity of pea root tissue after incubation: (▲) in standard media with ^{14}C-leucine; (●) in standard media with ^{14}C-leucine and 10 μM CH. The inset shows the radioactivity of the supernatant of control (▲) and 10 μM CH (●), after homogenization in trichloracetic acid.

When excised roots were treated with 10 μM CH, the production of ethylene was reduced by 50% (Fig. 1, compare A with B), but the rate of production of the treated control remained almost linear throughout the experimental period. Production of ethylene stimulated by 1 μM IAA was not significantly changed by CH, whereas that induced by higher auxin levels was markedly ($P < 0.001$) altered in that the rate increase which normally occurred after 2 hr was virtually eliminated at 10 μM, and the rate was considerably reduced in roots treated with 100 μM IAA during this period.

Doubling the CH concentration resulted in a further depression of ethylene production by the control tissue and a general reduction in ethylene evolution by roots treated with 10 and 1 μM IAA (Fig. 1C). The ethylene production induced by IAA at 100 μM was almost entirely eliminated by this higher concentration of CH.

When the roots were pretreated with 10 μM CH for 2 hr before auxin was added (Fig. 2), a marked reduction occurred in the ability of auxin to stimulate ethylene production during the following few hours. There was no effect on the control,

whereas roots treated with 10 and 1 μM IAA evolved considerably less ethylene after CH pretreatment than roots treated with auxin and CH simultaneously ($P < .05$). Ethylene production by roots pretreated with CH, to which 100 μM IAA was subsequently added, were not markedly different from those of roots continuously in the presence of CH and 100 μM IAA.

The effects of IAA and CH on various growth parameters of pea root tips are described in Table I. The concentration of CH employed resulted in a considerable reduction in growth. This inhibition is increased by the presence of 100 μM IAA but is little influenced by lesser concentrations of IAA. The ratio of increase in weight to increase in length (swelling ratio) indicated in Table I is decreased by CH.

The incorporation of ^{14}C-leucine into pea root tips was reduced 80% by 10 μM CH (Fig. 3). Maximal inhibition was already present as early as 15 min and continued little changed throughout the two hour experiment. CH did not appreciably affect the uptake of ^{14}C-leucine into the tissue (Fig. 3, inset).

DISCUSSION

Ethylene production stimulated by the application of 1 μM IAA is of short duration and involves only a doubling of the control rate. This production is not affected by CH applied in conjunction with the IAA; furthermore, ethylene production induced by higher concentrations of IAA is reduced to this level, but not lower, by 10 μM CH. CH is known to block ribosomal synthesis of protein in pea tissue (6), but it does not inhibit mitochondrial respiration (7) or free IAA levels (10). However CH does affect other cellular processes (8). CH effectively reduced amino acid incorporation in pea root apices (Fig. 3), but it did not affect uptake of the amino acid. These facts suggest that 1 μM IAA is directly stimulating an extant ethylene forming system.

That IAA is also able to induce the formation of nascent protein, capable of enhancing the rate of ethylene production, is indicated by the susceptibility to CH of the large, delayed increases in ethylene production caused by 10 and 100 μM IAA. The stimulatory effect of 10 μM IAA is completely removed by CH at 3 μg/ml, whereas that caused by 100 μM IAA is only partially prevented. However, when a higher concentration of CH is applied, 100 μM IAA loses most of its stimulatory ability.

It is apparent that pea root tissue responds to relatively low levels of IAA (1 μM), increasing its ethylene production without the necessity for new protein synthesis. It has recently been suggested that the auxin-induced ethylene-producing system in stem tissue has a fairly short half-life (< 1 hr [10], [12]) and thus may require the continuous synthesis of protein in order to continue ethylene production.

Our data suggest the existence in pea roots of two systems producing ethylene—a stable basal system, insensitive to IAA and CH—and a labile auxin-induced system which normally accounts for about one-half the ethylene production. The latter responds immediately and directly to applied IAA, increasing several fold in rate, without a significant lag period, and at higher IAA concentrations is induced after a considerable lag. CH prevents the enzyme induction but not the immediate response. Pretreatment with CH allows the natural labile auxin-induced and auxin-sensitive component to decay so that it can no longer respond to IAA. This concept is supported by the work of Sakai and Imaseki (13), who found that ethylene production induced in mung bean stems a few hours after application of a moderate concentration of auxin was enhanced in rate without a lag when subsequently a higher auxin concentration was applied. Apparently, a substantial component of the naturally occurring ethylene production in pea roots is due to stimulation by endogenous auxin in the root tip, and hence is sensitive to IAA without a lag phase.

The duration of the stimulatory phase of ethylene production is not significantly altered by the presence of CH. Since it has previously been demonstrated that ethylene production in pea roots (5) and stems (10) correlates closely with endogenous auxin levels, this suggests that the conjugative and destructive mechanisms may not be affected by CH. Previous data support this, indicating that decarboxylation and conjugation do not exhibit a lag or inductive phase after IAA is applied to pea roots (5). Conflicting data have also been published (2) and in the case of pea stems, while CH is known to alter neither the internal free IAA concentration nor the rate of decarboxylation, it does decrease the rate of auxin conjugation (10).

Pea root tips continue to produce ethylene at a uniform rate throughout the experimental period. This linearity is not affected by CH when it reduces the rate of production. This suggests that a stable enzyme system is responsible.

It is clear from Table I that swelling is a normal response of roots to auxin or ethylene (5), but in the presence of a combination of auxin and CH, this swelling response is eliminated. Similar data concerning the necessity for protein synthesis in auxin and ethylene-induced swelling in pea stem tissue have been presented (4).

Excised pea roots respond to exogenous IAA in a complex manner. A simple response to IAA is exhibited in the presence of CH, and may be due to direct stimulation of a labile protein present under natural conditions in pea roots. A secondary response to IAA occurs in the absence of CH and involves a time delay for full activity. This response is exhibited only at IAA concentrations sufficiently high to assure the presence of free IAA in the roots for several hours (5). It apparently involves the *de novo* synthesis of protein.

A low level of ethylene is produced by the control which exhibits no change in rate for the duration of the experimental period, either in the presence or absence of CH. This suggests a basal system which is not labile.

Acknowledgment—The authors wish to express their appreciation to Dr. S. P. Burg for helpful discussions and for a critical reading of the manuscript.

LITERATURE CITED

1. Abeles, F. B. 1966. Auxin stimulation of ethylene evolution. Plant Physiol. 41: 585–588.
2. Andreae, W. A. 1963. Auxin metabolism and root growth inhibition. *In:* Regulateurs Naturels de la Croissance Vegetale, Intern. Colloq., Gif France, 1963 (C.N.R.S. Paris, 1964). pp. 558–573.
3. Burg, S. P. and E. A. Burg. 1966. The interaction between auxin and ethylene and its role in plant growth. Proc. Nat. Acad. Sci. U.S.A. 55:262–269.
4. Burg, S. P. and E. A. Burg. 1969. Interaction of ethylene, oxygen and carbon dioxide in the control of fruit ripening. Qual. Plant. Mater. Veg. 19: 185–200.
5. Chadwick, A. V. and S. P. Burg. 1970. Regulation of root growth by auxin-ethylene interaction. Plant Physiol. 45: 192–200.
6. Davies, E. and G. A. MacLachlan. 1969. Generation of cellulase activity during protein synthesis by pea microsomes *in vitro*. Archives. Biochem. Biophys. 129: 581–598.
7. Ellis, R. J. and I. R. MacDonald. 1968. Characterization of amino acid incorporation by subcellular fractions from sterile beet disks. Planta 83: 248–256.
8. Ellis, R. J. and I. R. MacDonald. 1970. Specificity of cycloheximide in higher plant systems. Plant Physiol. 46: 227–232.
9. Evans, M. L. and P. M. Ray. 1969. Timing of the auxin response in coleoptiles and its implications regarding auxin action. J. Gen. Physiol. 53: 1–20.
10. Kang, B. G., W. Newcomb, and S. P. Burg. 1971. Mechanism of auxin-induced ethylene production. Plant Physiol. 47: 504–509.
11. Klein, S., H. Barenholz, and A. Burdick. 1971. The initiation of growth in isolated lima bean axes. Physiological and fine structural effects of actinomycin D, cycloheximide and chloramphenicol. Plant Cell Physiol. 12: 41–60.
12. Penny, P. 1971. Growth-limiting proteins in relation to auxin-induced elongation in Lupin hypocotyls. Plant Physiol. 48: 720–723.
13. Sakai, S. and H. Imaseki. 1971. Auxin-induced ethylene production by mung bean hypocotyl sections. Plant Cell Physiol. 12: 349–359.