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Probing a Membrane Matrix Regulating Hormone Action

II. THE KINETICS OF LIPID-INDUCED GROWTH AND ETHYLENE PRODUCTION[1]

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ABSTRACT

Lipids which are active oleanimins, *i.e.*, those which stimulate respiration and auxin-induced cell elongation of pea stem sections, also initiate a period of ethylene formation in them after a lag period of at least 1 hour. Production of ethylene requires auxin, is inhibited by cycloheximide and dinitrophenol applied during or before the lag period, and is greatly stimulated by lipids longer than 20 Ångstroms in length such as heptadecylbenzene, chloro- or iodohexadecane, triolein, and vitamins E and K_1, but not by the shorter chloro- and iododecane. β-Stigmasterol at 10 to 40 μM concentrations depresses both oleanimin-induced growth and ethylene formation.

The effect of oleanimins on the growth rate steadily declines and disappears after 6 hours, whereas oleanimin induction of ethylene stays at a high level until it rapidly disappears after 6 hours. Nongrowing second internode sections also produce ethylene on oleanimin treatment, so ethylene formation is not dependent on cell elongation even though it requires auxin. Preincubation with heptadecylbenzene or auxin does not change the delay of an hour or more before ethylene is produced, whereas increases in growth are noted at the earliest measurements. Oleanimins stimulate growth at less than optimal auxin concentration, even as low as 20 nM, where a proportional ethylene formation is not noted. It is concluded that ethylene formation is not causally related to growth in these tissues.

The decline in oleanimin-induced ethylene formation is not changed by renewal of the incubation medium, and sucrose which is required to maintain growth for 20 hours does not influence growth or ethylene formation up to 6 hours. L-Methionine increases ethylene formed after heptadecylbenzene treatment, but unexpectedly, malonate is much more effective.

Auxin concentrations supraoptimal for growth cause no growth rate reductions for the first 10 hours, but they greatly enhance oleanimin-induced ethylene formation even when heptadecylbenzene is added after 6 hours. Applied ethylene even at concentrations much above those produced by the tissue itself fails to stimulate or inhibit short term pea stem section growth. It is concluded that the effect of oleanmins on growth is not mediated by ethylene. The similarities in concentration and molecular dimensions of these structurally diverse lipids which simultaneously stimulate respiration, growth, and ethylene formation, suggest a single site of action located in a regulatory membrane.

Trace quantities of various lipids and their derivatives can enhance respiration and the growth-promoting effect of auxin on pea stem sections. Stowe and Dotts (45) proposed the term "oleanimin" for these physiologically active oily substances and have demonstrated that the only consistent, common properties of oleanimins are that they possess molecular lengths longer than 20 Å and have a low polarity.

Preliminary experiments showed us that marked ethylene production is associated with the oleanimin effect, whereas no significant ethylene is produced by auxin application alone at the optimal concentration for growth of pea stem sections. The relationship of ethylene production to growth has been investigated by many workers with various organs of plants as noted in the review by Pratt and Goeschl (38), but some inconsistencies remain. Burg and Burg (5, 7) and Chadwick and Burg (9) showed that the inhibitory effect of supraoptimal concentration of IAA on the growth of pea stem and root was attributable to the auxin-induced ethylene, while Andreae *et al.* (2) postulated that it was unlikely that ethylene can play more than a minor role in mediating inhibition of pea root growth by IAA. According to Holm and Abeles (15) ethylene was partially responsible for the inhibition of elongation that occurred at supraoptimal concentrations of 2,4-D applied to excised soybean stems.

Penny and Stowe (37), having demonstrated the stimulating effect of oleanimins on respiration, noted that the similar concentrations and chemistry of effective molecules, along with other facts, suggested that there is only one site at which oleanimins act to increase both growth and respiration. Since a high rate of ethylene production is often accompanied by raised respiration in various phenomena such as fruit ripening (4), wounding (31, 32), physiological disorder (20), etc., it could be that the effect of oleanimins on growth is a result of respiratorily enhanced ethylene synthesis followed by ethylene-regulated growth.

In this study, the kinetics of growth and ethylene production of pea stem sections were examined, concurrently with the requirements of oleanimin-induced ethylene production, and the effects of ethylene on growth, in order to establish if oleanimin action can be attributed to the effects of ethylene.

MATERIALS AND METHODS

Bioassay. Progress No. 9 dwarf peas (*Pisum sativum* L.) were soaked in a thin layer of water for about 6 hr and grown on vermiculite under weak continuous red light at 25 C. On the 7th day after soaking, 10-mm sections were cut from the third internode 2.5 mm below apical hooks which had just reached a 90° angle. Ten randomized sections were placed in 20 ml of solution in a 10-cm Petri dish for each test. The basal medium used contained 1.5% sucrose, 5 mM KH_2PO_4 (pH 5.5), 50 μM

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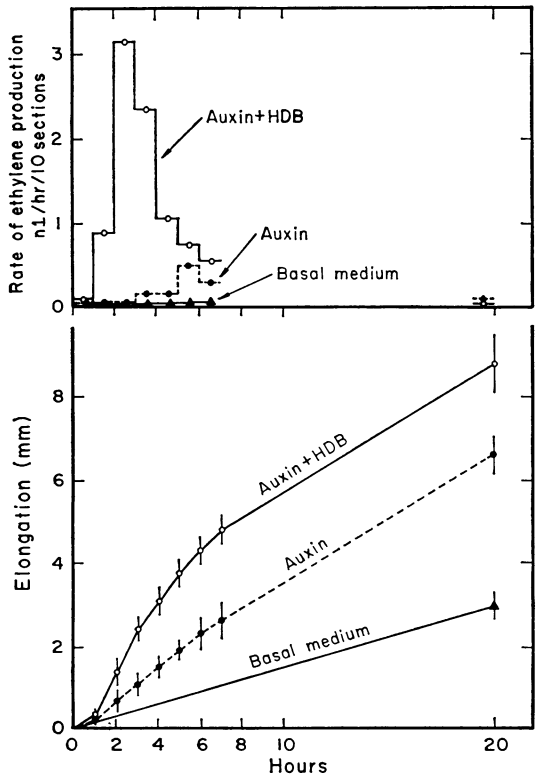

FIG. 1. The effect of HDB on growth and ethylene production of pea stem sections. Vertical lines show standard deviations. Auxin: 1.8 μM IAA + 0.3 μM GA_3; HDB: 20 μM.

$CoCl_2$, and 0.004% of the emulsion stabilizer Pluronic F-68 (Wyandotte Chemical Corp., Wyandotte, Mich.). Concentrations of IAA and GA_3 were 1.8 and 0.3 μM, respectively, unless stated otherwise, and are referred to as "standard auxin." These levels of hormones and cofactors have previously been shown to be optimal for growth responses in this bioassay (46); except for auxin their effect on ethylene formation was not investigated.

Incubation was conducted on a shaker in the dark room at 25 C, and the elongation of sections was measured. To get stable aqueous emulsions, oleanimins were dissolved in acetonitrile containing Pluronic F-68, and injected using a syringe with a relatively large bore needle into water in which Pluronic F-68 had been previously dissolved. The resulting emulsion was exposed to ultrasound for 1 min. The ultrasound treatment brought about a considerable ethylene formation, probably from impurities contained in acetonitrile; therefore, the sonicated emulsion was flushed with bubbles of nitrogen for 30 min before use. The concentration of oleanimin used was 20 μM, unless otherwise stated. These methods and the sources of chemicals used were described in detail in the previous paper (45). Since trace quantities of lipids are frequent environmental contaminants, and some commercial detergents are active in our assay (44), stringent precautions were taken to avoid inadvertent transfer of them to any part of the media or equipment used. All glassware was passed through a hot acid bath before final rinsing in distilled water.

Ethylene Determination. In preliminary tests, it was shown that confining bioassay materials to sealed containers for several hours brought about a reduction or complete disappearance of the growth-promoting effect of oleanimins on pea stem sections (see Table IV below). This consequence of sealing was not prevented by application of carbon dioxide or ethylene absorbents. Thus, in order to establish ethylene production, 10 sections in 20 ml of medium were transferred from a Petri dish to a 50-ml Erlenmeyer flask and sealed, using a silicone rubber stopper, for 2 hr or less time for each collection. Then, 4 ml of head space gas at atmospheric pressure were withdrawn by syringe and analyzed by gas chromatography. The sections which had once been sealed were not used for further determinations. Confinement for up to 2 hr did not influence the response of the sections. The 1/8-inch × 6-ft gas chromatographic column was packed with 60 to 80 mesh Alcoa type F-1 activated alumina and operated with nitrogen at 30 C, using a Perkin Elmer F-11 gas chromatograph with H_2 flame ionization detection. Identification of ethylene was by comparison of retention time and by absorption in $Hg(ClO_4)_2$ solution followed by release with LiCl or HCl (48). The lowest level of ethylene production detectable by these techniques was approximately 0.1 nl/hr·10 sections. As 10 sections initially weighed 0.35 ± 0.05 g, conversion to the more usual nl/g·hr may be approximated by multiplying by 3. Since these pea stem sections can double their fresh weight during an experiment, while their dry weight declines, it is preferable to report ethylene production on a per 10 section basis.

RESULTS

Time Course of Growth and Ethylene Production. Since Stowe and Dotts (45) showed that HDB[3] gave a typical oleanimin effect in enhancing the auxin-induced elongation of pea sections, and since it might be expected to be relatively resistant to metabolic change, this alkyl benzene was used for most of the present study. Figure 1 shows the effect of HDB on growth and ethylene production of sections which were measured at 1-hr intervals.

The growth-promoting effect of HDB was obvious; the effect became conspicuous in the 2nd hr after initiation of incubation and lasted for several hours, after which the rate of growth did not differ from the sections treated with auxin alone. In the absence of an auxin, HDB did not show any promoting effect. Ethylene production also showed a sharp increase in auxin + HDB-treated sections during the 2nd hr and reached a peak between 2 and 3 hr, followed by a steady decline. In the sections treated with auxin alone ethylene production remained at a low level throughout the incubation period rising to its maximal value between 5 and 6 hr.

To see if other, molecularly diverse, oleanimins effective on growth induced a similar ethylene output, isoprenoids and alkyl halides were tested. For convenience, their effect was assayed at 2-hr intervals. Vitamins E and K_1 were reported as effective isoprenoids by Stowe and Obreiter (46) and have lengths of 28 and 29 Å, near the optimum for the growth effect (45). Their marked growth-enhancing effect was confirmed in the present experiment (Fig. 2). Promotion of the rate of elongation was ended within 6 hr, the greatest increment of growth being attained during the 2 to 4 hr after initiation of incubation. Ethylene production also showed a sharp increase during the same 2- to 4-hr period in the auxin + vitamins-treated sections, then fell markedly. When auxin was not applied, these vitamins were ineffective on elongation during the earlier part of the experiment and by its end brought about an inhibition. Neither did they cause any increase of ethylene by themselves.

Stowe and Dotts (45) demonstrated that the major molecular requirements for oleanimin activity is suitable length of the lipid molecules. Effective substances have a molecular length

[3] Abbreviation used: HDB: heptadecylbenzene.

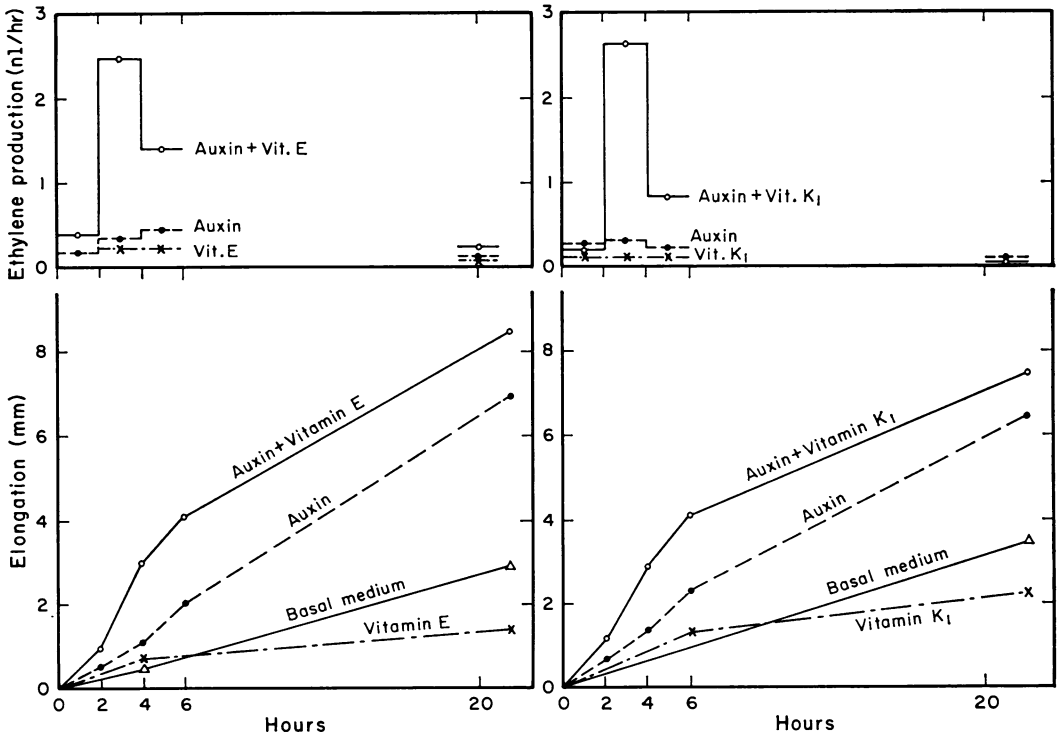

FIG. 2. The effect of 20 μM vitamines E and K_1 on growth and ethylene production (per 10 sections) of pea sections.

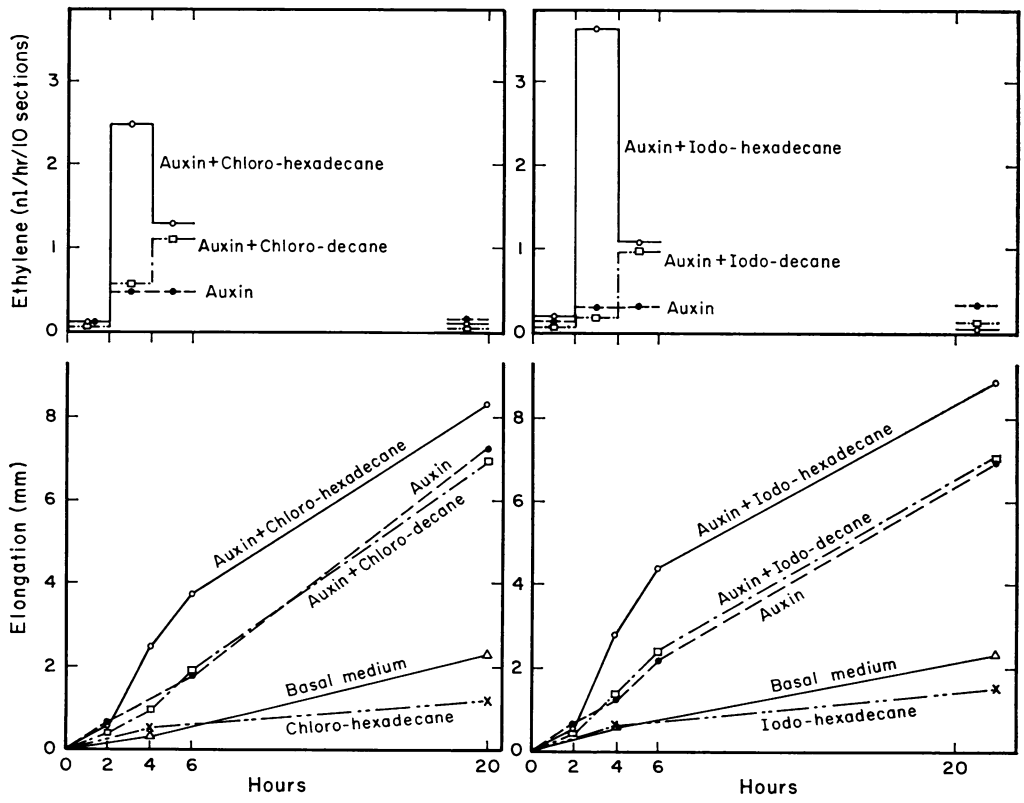

FIG. 3. Comparison of the effect of long and short alkyl halides at 20 μM on the growth and ethylene production of pea stem sections.

exceeding 20 Å. Figure 3 shows the comparison of the effect of short and long molecules of alkyl halides. 1-Chlorohexadecane (24.2 Å) and 1-iodohexadecane (24.8 Å) added to auxin induced rapid growth, especially during the 2- to 4-hr period, while 1-chlorodecane (16.7 Å) and 1-iododecane (17.3 Å) did not show any effect on growth of sections at any time. The rate of ethylene production also spurted during the 2- to 4-hr interval when sections were treated with the longer alkyl ha-

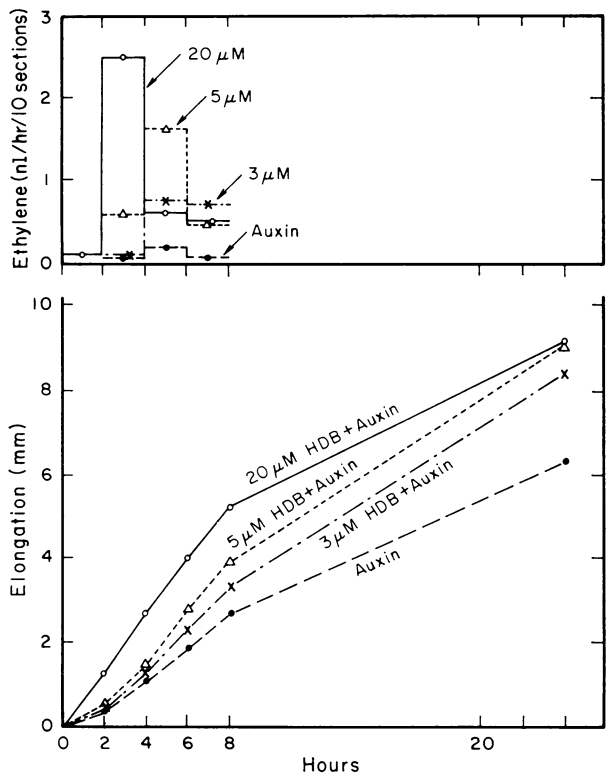

FIG. 4. The effect of the concentration of HDB on growth promotion and ethylene production by pea stem sections.

lides, while those sections treated with shorter molecules did not differ from those receiving only auxin, until the 4- to 6-hr period when a smaller rise occurred. Although not shown on the figure, no effect was noted when any alkyl halide was applied alone. Triolein, a typical oleanimin (36, 37, 46), also showed the same behavior as the effective substances mentioned above, with respect to both growth and ethylene production.

Effect of Concentration of Heptadecylbenzene. A range of concentrations of HDB were tested. No effect was found with 1 μM on both growth and ethylene production, and the effect of 10 μM was the same as 20 μM, similar to the concentration dependence of growth induced by vitamins E and K_1 (46). Figure 4 summarizes the effect of intermediate and optimal concentrations. When 5 μM was applied, a longer lag period resulted, and rapid growth began about 1 hr later than with 20 μM (see also Fig. 1). However, the growth rate in the last part of the incubation continued to be higher than with 20 μM, so that a similar length was reached in 24 hr. Ethylene production was delayed with 5 μM, too, and the peak which appeared during 4 to 6 hr was lower than that with 20 μM. The sections responded to treatment with 3 μM, but the growth was inferior to that with 5 μM, and the increase in ethylene production was less than that at higher oleanimin concentrations.

Effect of β-Stigmasterol. Stowe and Dotts (45) have postulated that sterols may act as space-filling materials within cavities formed in a lecithin monolayer which comprises half of a regulatory membrane's lipid bilayer, and predicted that sterols would antagonize the oleanimin effect. β-Stigmasterol, which is one of the major free sterols in peas (11, 45), was used in the present study (Fig. 5). Addition of 10 or 40 μM sterol to auxin + HDB solution decreased the growth-promoting effect of HDB, and the rates of ethylene production during 2 to 4 hr were reduced considerably depending on the concentration applied. No effect was found when β-stigmasterol was added to auxin solution without HDB.

Nongrowing Tissue and Ethylene Production. As shown above, the rapid increase of ethylene production in oleanimin-treated sections closely correlates with the growth rate increase of the sections. In many plant species the rapidly growing tissues are sites of ethylene production. The spurt in ethylene production, thus, could be a by-product of the enhanced elongation caused by oleanimins. To elucidate this, the first and second internodes of pea stems were measured with the usual third internode (Table I). The first internodes did not grow at all, and the ethylene production was at a very low level. The second internode sections treated with auxin + HDB, however, produced a considerable amount of ethylene and showed a peak during 2 to 4 hr in the same manner as the third internode sections. The growth was so little that all the second internode sections were significantly shorter than those from the third internode in basal medium. This indicates that the rapid increase of ethylene production does not result from the growth of sections and suggests that an ethylene-producing system was induced by treatment with an oleanimin and that the ability of sections to produce such a system diminishes with maturation and has disappeared in the first internodes.

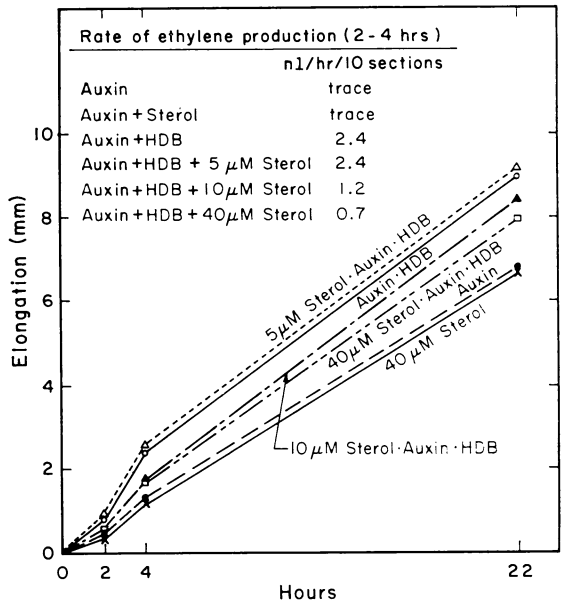

FIG. 5. The effect of the addition of β-stigmasterol on 20 μM HDB-induced elongation and ethylene production by pea stem sections.

Table I. *Comparison of Effect of Heptadecylbenzene on Growing and Nongrowing Pea Stem Sections*

Medium also contained 0.3 μM GA_3, 50 μM $CoCl_2$, 5 mM KH_2PO_4 (pH 5.5), 1.5% sucrose, and 0.004% Pluronic F-68.

Medium	Part of Stem (Internode)	Elongation		Rate of Ethylene Production	
		6 hr	20 hr	2-4 hr	4-6 hr
		mm		*nl/hr/10 sections*	
1.8 μM auxin	3rd	2.06	6.46	0.34	0.45
1.8 μM auxin + 20 μM HDB	1st	0	0	Trace	0.25
	2nd	0.45	1.03	1.43	1.02
	3rd	4.27	8.82	2.66	1.04

Effect of Preincubation in Heptadecylbenzene or Auxin Solutions. Sections, pretreated with auxin medium for various times, were then transferred into auxin + HDB solutions. The elongation and the ethylene production of the sections after transfer are presented in Figure 6. In comparison with the sections which were incubated in auxin + HDB medium from the beginning, the growth rate during the first 2 hr after transfer was almost the same in the sections pretreated for 2 hr, became slightly higher in those pretreated for 4 hr, and was reduced in those pretreated for 6 hr. Subsequent growth enhancement was markedly reduced with increasing hours of pretreatment, the elongation during 0 to 4 hr being 73 and 56% compared to nonpretreated sections for the 4 hr and 6 hr pretreated sections, respectively. On the other hand, ethylene production, although diminished during the hours of pretreatment, built up during the first 2 hr after transfer to amounts which were significantly higher, even in the sections pretreated up to 6 hr. The peak rate, however, still came during 2 to 4 hr after transfer. When the pretreatment exceeded 12 hr, an HDB effect was no longer found on either elongation or ethylene production.

When pretreatment was with HDB before auxin addition, the results in Figure 7 were obtained. Initial growth rates for as long as 4 hours after auxin addition were not changed by any pretreatment with HDB for periods up to 6 hr, and lengths at the end of the experiment were significantly greater in all but those given 6 hr of pretreatment. Ethylene production was considerably increased by 2 hr of pretreatment, and it was less in the 4 and 6 hr HDB-pretreated sections but still far above the HDB plus basal medium control. In the sections pretreated with HDB for 12 hr, a smaller but still marked oleanimin effect was found in growth rates although final lengths were less than in standard auxin, while no ethylene production occurred. After 20 hr, the sections pretreated with HDB in the absence of auxin had almost ceased growing and did not respond to auxin. When sections were subjected to pretreatment in basal medium alone, remarkably similar results were obtained; that is, the growth-promoting effect of auxin + HDB was decreased by pretreatment in basal medium for 6 hr or more, while the ethylene-producing effect was enhanced after pretreatment for 2 hr, then decreased after 4 hr or 6 hr of pretreatment, and the oleanimin effect on both growth and ethylene disappeared when pretreatment exceeded 12 hr.

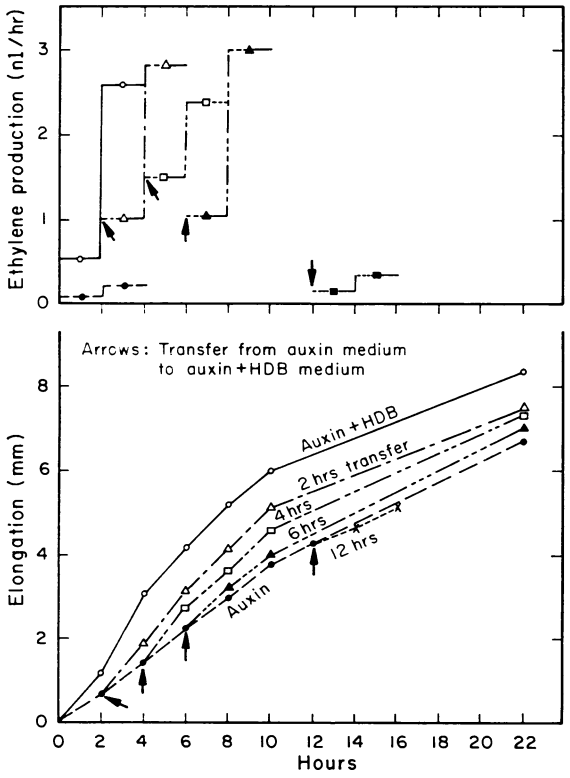

FIG. 6. The effect of preincubation in 1.8 μM IAA medium on growth and ethylene output of pea stem sections. Sections were transferred into auxin + 20 μM HDB medium after the hours of pretreatment noted (arrows). Ethylene production is per 10 sections.

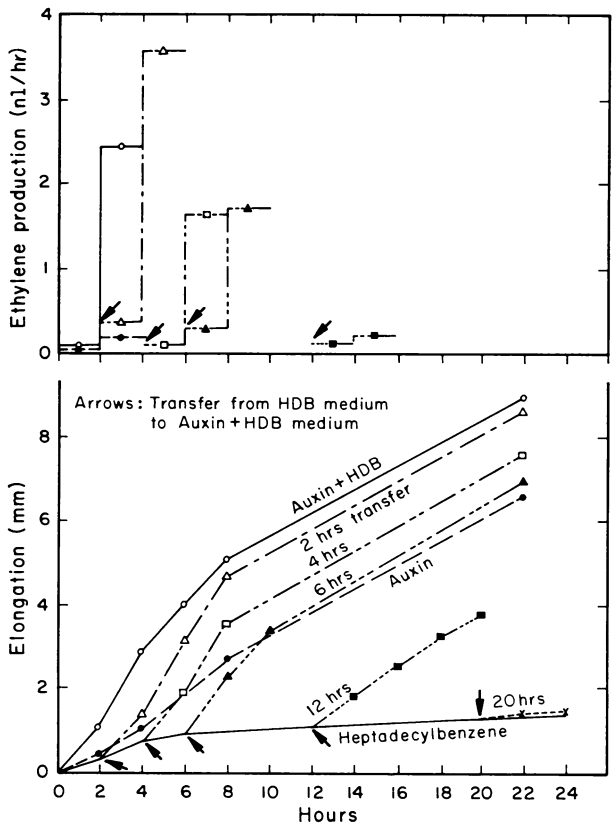

FIG. 7. The effect of preincubation in 20 μM HDB medium before transfer to 1.8 μM auxin medium on growth and ethylene output of pea stem sections. Ethylene production is per 10 sections.

Thus, the oleanimin effect was induced when both auxin and oleanimin were presented together. Pretreatment with either one of them was ineffective in accelerating the oleanimin effect. The changes of growth rate or ethylene production rate caused by pretreatment may be mainly a result of the changes in physiological age or maturation of sections. Pretreatment with auxin may accelerate the maturation, and matured pea segments lose the ability to grow further or to produce ethylene. Responsiveness to HDB decays more rapidly than responsiveness to auxin.

Effect of Metabolic Inhibitors. To examine the nature of ethylene production by oleanimin treatment, the effects of some metabolic inhibitors were examined. As shown in Table II, cycloheximide progressively inhibited elongation as its concentration increased and the degree of inhibition was almost the same in auxin alone as in the auxin + HDB medium. Ethylene production was also greatly suppressed by this protein synthesis inhibitor, suggesting rapid synthesis of ethylene-producing enzymes in auxin + HDB medium. This does not necessarily imply a close correlation to the inhibition of elongation. The peak rate of ethylene production in the sections treated with auxin plus HDB and 5 μM cycloheximide was as

Table II. *Effect of Metabolic Inhibitors and Heptadecylbenzene on Growth and Ethylene Production by Pea Stem Sections*

Medium also contained 0.3 μM GA_3, 50 μM $CoCl_2$, 5 mM KH_2PO_4 (pH 5.5), 1.5% sucrose, and 0.004% Pluronic F-68. Control data were obtained separately from 10 sections not treated with inhibitor; >20% growth inhibitions are significant.

Inhibitor	Medium	Concn of Inhibitor	Growth Inhibition		Ethylene Production	
			4 hr	20 hr	Rate (2-4 hr)	Percentage of control
		μM	%	%	*nl/hr·10 sections*	
Cycloheximide	1.8 μM auxin	0.5	8	16	0.23	43
		5	16	43	0.29	54
		50	65	80	trace	<30
	1.8 μM auxin + 20 μM HDB	0.5	10	36	2.09	65
		5	26	45	0.57	18
		50	61	77	0.44	14
2,4-Dinitrophenol	1.8 μM auxin	5	−10	8	0.42	221
		50	37	75	tr.	<50
		500	100	100	tr.	<50
	1.8 μM auxin + 20μM HDB	5	11	7	1.65	79
		50	51	73	0.76	36
		500	100	100	tr.	<8

low as 0.57 nl/hr·10 sections, which did not differ from the control sections in auxin medium without HDB and cycloheximide. Moreover, the elongation of these inhibitor-treated sections during the first 4 hr was still significantly higher than the control sections (2.42 ± 0.16 mm *versus* 1.54 ± 0.16 mm). The effect of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, was also determined. A slight inhibition was shown at 5 μM in both elongation and ethylene production of the sections treated with auxin + HDB, but a marked oleanimin effect on ethylene production still remained. When 50 μM DNP was applied, the elongation was extremely suppressed, and the ethylene production considerably decreased, too.

Effects of Methionine and Malonate Addition. Methionine and its derivatives have been shown to act as precursors of ethylene in several species of plants (26). Using etiolated pea stems, Burg and Clagett (8) demonstrated that the sections produce ethylene from methionine in the presence of a high concentration (1 mM) of IAA, and Ku *et al.* (24) extracted an enzyme system capable of ethylene production from methionine and methional. The effect of methionine on our oleanimin-induced ethylene-producing system was examined (Table III). Interestingly, methionine had no, or a slightly inhibitory, effect in auxin medium without HDB. In auxin + HDB medium, however, it brought about a pronounced increase of ethylene production at 10 mM, while no effect was found on elongation of sections. No significant effect was observed at 1 mM although this concentration has been reported as effective in other species (27).

Tests of malonate were initiated to examine its effect as a respiratory inhibitor acting on the tricarboxylic acid cycle, and that effect was obvious at the relatively high concentration of 50 mM where marked suppression of elongation in both auxin and auxin + HDB media was obtained (Table III). Ethylene production induced by HDB treatment was also reduced at this dose but still remained at a relatively high level. This led to the discovery that lower concentrations (5 mM) of malonate increased the ethylene production induced by auxin + HDB. While the figure (278% of control) in Table III was a conspicuous example, in part due to a low control value, increases of more than 30% were regularly observed. Shimokawa and Kasai (43), using banana fruit, have suggested a biosynthetic pathway of ethylene as follows: acetate→malonate→malonic semialdehyde→β-hydroxypropionate→ acrylic acid→ ethylene, thus involving malonate as an intermediate. This, or related ramifications of malonate metabolism, may explain these results with pea stem sections.

Effect of the Sucrose Concentration of the Medium. Stowe (44) demonstrated that sucrose is needed in the incubation medium if oleanimins are to increase growth when it is measured after 20 hr. The time course of elongation and ethylene production in relation to sucrose concentration was examined in the present study (Fig. 8). It was found that the sucrose requirement is time-dependent. That is, the HDB-induced rapid growth during the first 4 hr was not affected by sucrose, but the subsequent growth was greatly inhibited in the media in which no or insufficient sucrose was added. Ethylene was produced at a high level in all treatments, but its peak production was delayed in the sections lacking sucrose.

Effect of Renewal of Medium. Since the high rates of growth and ethylene production induced by oleanimin were shown above to decline rapidly after several hours (Figs. 1–4), in the experiment of Table IV, the sections were transferred into new media after 4 and 6 hr to confirm whether or not these decreases were caused by changes in the medium. Table IV demonstrates that renewal of medium did not affect the decline of ethylene productions, indicating that the drastic decrease was brought about by changes occurring inside the pea sections. Further, instead of stimulating the elongation, renewal of media resulted in a significant decrease of oleanimin-induced

Table III. *Effect of Methionine, Malonate, and Heptadecylbenzene on Growth and Ethylene Production of Pea Stem Sections*

Medium also contained 0.3 μM GA_3, 50 μM $CoCl_2$, 5 mM KH_2PO_4 (pH 5.5), 1.5% sucrose, and 0.004% Pluronic F-68.

Substrate	Medium	Substrate Concn	Elongation		Ethylene Production	
			4 hr	20 hr	Rate (2-4 hr)	Percentage of control
		mM	mm		*nl/hr·10 sections*	
L-Methionine	1.8 μM auxin	0	1.78 ± 0.36	5.99 ± 0.86	0.82	100
		10	1.59 ± 0.23	5.52 ± 0.88	0.67	82
	1.8 μM auxin + 20 μM HDB	0	3.02 ± 0.24	8.27 ± 0.78	2.22	100
		10	2.71 ± 0.19	8.11 ± 0.63	3.51	174
Malonate	1.8 μM auxin	0	1.30 ± 0.38	6.69 ± 0.53	0.51	100
		5	1.36 ± 0.35	6.30 ± 0.98	0.51	100
		50	0.44 ± 0.10	1.13 ± 0.11	0.51	100
	1.8 μM auxin + 20 μM HDB	0	2.35 ± 0.54	8.22 ± 0.93	1.71	100
		5	2.65 ± 0.29	7.44 ± 0.92	4.75	278
		50	0.97 ± 0.21	1.97 ± 0.18	1.10	64

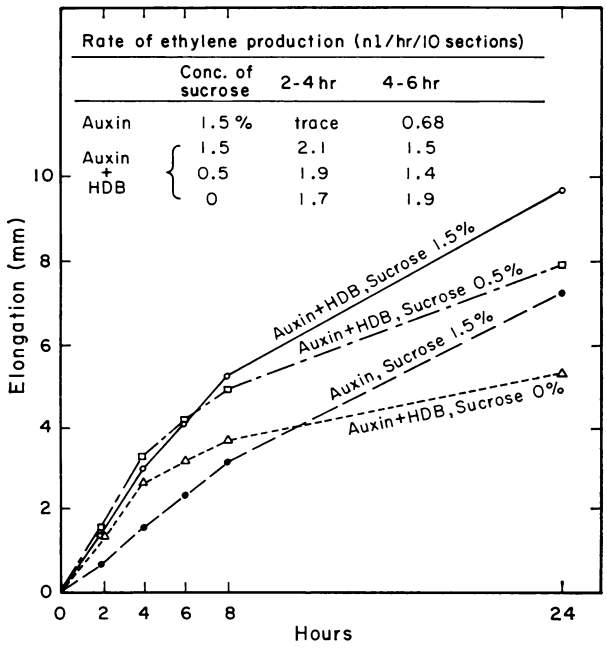

FIG. 8. The effect of sucrose concentration of incubation medium on 20 μM HDB-induced elongation and ethylene production by pea stem sections.

Table IV. *Effect of Renewal of Medium during Incubation of Pea Stem Sections*

Medium also contained 0.3 μM GA_3, 50 μM $CoCl_2$, 5 mM KH_2PO_4 (pH 5.5), 1.5% sucrose, and 0.004% Pluronic F-68.

Medium	Renewal	Elongation		Rate of Ethylene Production		
		8 hr	22 hr	2-4 hr	4-6 hr	6-8 hr
		mm		*nl/hr·10 sections*		
1.8 μM auxin	None	3.43 ± 0.36	7.04 ± 0.50	Trace	0.57	0.28
1.8 μM auxin + 20 μM heptadecylbenzene	None	5.23 ± 0.28	9.88 ± 0.50	2.85	0.63	0.34
	After 4 hr	5.24 ± 0.28	8.75 ± 0.59	...	0.63	Trace
	After 6 hr	4.90 ± 0.30	8.16 ± 0.79	...	...	Trace

growth by 22 hr. A possible explanation of this decrease may be that there is a favorable concentration of CO_2 in medium for elongation, as discussed previously by Penny and Stowe (36).

Effect of IAA Concentration. In the present experiments, 1.8 μM IAA was used as the standard auxin because this concentration had been found by Stowe (44) to provide the largest growth increments. On the other hand, it has been well known that high concentrations of auxins produce ethylene in various organs of plants, and auxin-induced inhibition of growth has been attributed to the ethylene produced (5, 7, 9), but this depends on the system studied (2, 38). For these reasons, lower and higher concentrations of IAA were applied, and the effects on oleanimin-induced growth and ethylene production were determined. The high concentration of IAA was neutralized to pH 5.5 with sodium hydroxide prior to addition.

Figure 9 shows the effect of low concentration of IAA. With 0.2 μM IAA, HDB-enhanced growth was statistically significant above any concentration of auxin alone, but the ethylene production was not sufficient to have a physiological role even though a slight increase was observed during 2 to 4 hr. Even at the very low concentration of 20 nM, a significant growth-promoting effect of HDB was found during the early hours of incubation, while it was not significant later. In this case, no stimulation of ethylene production was noted at all. These results suggest that the oleanimin effect in promoting elongation is not necessarily associated in these tissues with ethylene production.

The effect of a high concentration of IAA on HDB-induced growth and ethylene formation was determined at 1-hr intervals (Fig. 10). After a 1-hr lag period a striking increase in ethylene production occurred with 200 μM IAA, reaching a value 6 times higher than that of sections treated with the standard 1.8 μM concentration of IAA at its peak. The peak of the latter was reached during 2 to 3 hr as in the earlier experiments, but the maximum of the high auxin treatment came during 3 to 5 hr, and a high rate of ethylene production continued for some hours. However, the growth of sections was almost the same with standard or high concentrations of IAA for at least 8 hr of incubation, although the final elongation was significantly lower in the high concentration of IAA.

Table V shows the effect of various high concentrations of IAA with and without HDB. In the media without HDB, only the highest concentration of IAA (1 mM) significantly inhibited the final growth attained, but the inhibition was not obvious until after 8 hr. Ethylene production was enhanced with all high concentrations of IAA and the peak were reached during 4 to 6 hr. The peak rates were almost comparable to that treated with HDB and the standard 1.8 μM concentration of IAA.

In the media containing HDB, growth was inhibited by all concentrations of IAA higher than the standard, but the effect was not apparent within 8 hr and the characteristic rapid growth in the early period was similar in all media. Ethylene production was greatly enhanced, and peak rates were found

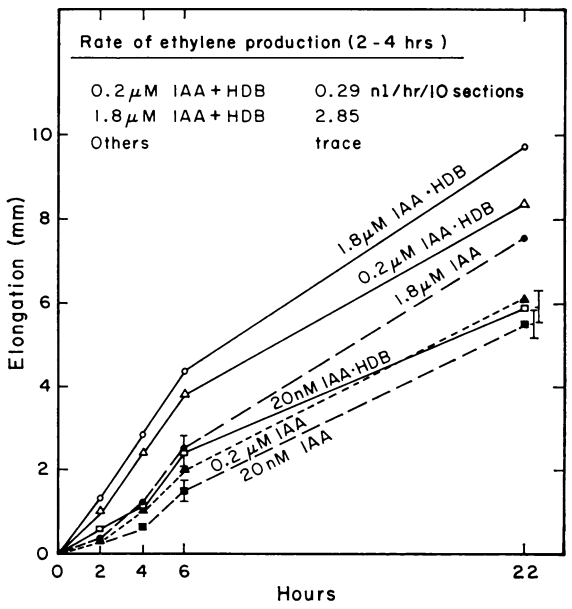

FIG. 9. The effect of 20 μM HDB at low concentrations of IAA on growth and ethylene production of pea stem sections. Representative significance limits indicated by vertical bars at 6 and 22 hr.

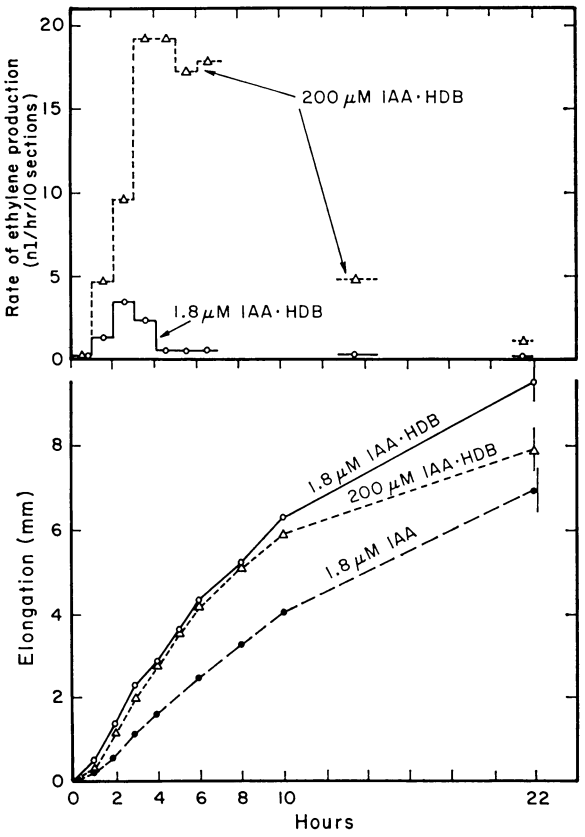

FIG. 10. The effect of 20 μM HDB at a high concentration of IAA on growth and ethylene production of pea stem sections measured at 1-hr intervals. Standard deviations at end of experiment indicated by vertical bars.

Table V. *Effect of High Concentrations of IAA with Heptadecylbenzene on Growth and Ethylene Production of Pea Stem Sections*

Medium also contained 0.3 μM GA_3, 50 μM $CoCl_2$, 5 mM KH_2PO_4 (pH 5.5), 1.5% sucrose, and 0.004% Pluronic F-68.

Medium	IAA Concn	Elongation			Ethylene Production		
		4 hr	8 hr	23 hr	2-4 hr	4-6 hr	12-14 hr
	μM	mm			nl/hr·10 sections		
IAA	1.8	1.41	2.97	6.66	Trace	0.43	Trace
	20	1.38	2.67	6.13	0.72	1.52	...
	200	1.43	2.81	6.19	1.29	2.47	Trace
	1000	1.39	2.62	4.41	1.18	2.28	Trace
IAA + 20 μM HDB	1.8	2.65	4.86	9.28	2.09	0.91	Trace
	20	2.51	4.83	7.88	7.56	8.89	...
	200	2.69	4.50	7.84	12.4	15.4	6.65
	1000	2.47	4.25	6.79	...	14.6	12.2

during 4 to 6 hr. There was no difference between 0.2 mM and 1 mM treatments in the peak reached, but the decline following was slower in the sections treated with 1 mM, which maintained a high rate of ethylene production even after 12 hr.

The effect on ethylene production of the time of application of a high concentration of IAA was investigated (Fig. 11). The peak rate of ethylene production in the sections treated from the beginning with a high concentration (0.2 mM) of IAA plus HDB was about 20 nl/hr. Increasing the IAA level to 0.2 mM caused the sections which had been incubated in standard media (1.8 μM IAA) with HDB for 4 hr to increase ethylene production. A peak was reached 2 to 4 hr after addition, but the peak rate was less than 70% of that reached when high auxin was applied from the start of the experiment. When the auxin concentration was stepped up at 6 hr, a rate was attained which was only 30% of the high auxin throughout controls. Thus, the amount of ethylene the pea sections can synthesize after treatment with high levels of auxin declines in the standard medium containing HDB, a result similar to the data of Figure 7 when optimal auxin was added to sections incubated in HDB alone.

The effect of methionine and malonate addition to the media containing a high concentration (20 μM) of IAA was also studied. Ethylene production of the sections treated with HDB was increased 40% when 10 mM L-methionine was added at its peak 4 to 6 hr after the start of the experiment and by 30% with 5 mM malonate. The addition of these compounds did not affect elongation at all. These results are comparable to those in Table III, when the sections were incubated in the standard IAA concentration.

Effect of Ethylene Application. A growth-inhibiting effect of ethylene has been reported by several workers in various experimental systems, including etiolated pea stem sections. In our investigations considerable amounts of ethylene were produced when pea sections were growing rapidly. Thus, the effect of exogenous ethylene was investigated (Table VI). Various concentrations of ethylene were applied from 1 to 3 hr after initiation of incubation, corresponding to the period when the highest rate of ethylene production occurs after oleanimin treatment. These applications did not produce any significant stimulation or inhibition of growth of sections in either auxin or auxin + HDB media. Even a 4-hr treatment, from 1 to 5 hr after the start of the experiments, at doses as high as 10 μl/liter did not show a significant effect in the auxin alone medium. Constant application for 20 hr did result in a statistically significant growth inhibition. These are largely negative results, but they contradict any suggestion that the ethylene produced after auxin treatments is itself mainly responsible for short term growth responses of these red light-treated tissues.

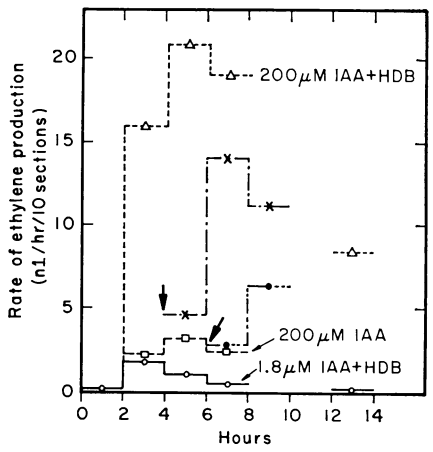

FIG. 11. The effect of the time of addition of a high concentration of IAA on the ethylene production of pea stem sections. Arrows indicate when 200 μM IAA was added to sections which had been incubated in standard concentration (1.8 μM) IAA + 20 μM HDB medium for 4 and 6 hr, respectively.

DISCUSSION

Time Course of Growth and Ethylene Production. An observation common to all our active substances (Figs. 1–3) was that enhancement of auxin-induced growth of pea stem sections occurred for several hours following a lag period of about 1 hr. Later, the growth rate subsided to almost the same as in auxin treatments alone. Measuring the growth attained during 2 to 4 hr after initiation of incubation with all six effective substances tested, the average growth rate was 2.8 times higher in the sections treated with oleanimins + auxin than that treated with auxin only. The ethylene production maxima were less proportional to the growth rates obtained with different oleanimins.

Nonetheless, ethylene production increased when growth was increased by oleanimins, after a lag period of about 1 hr, corresponding to that of growth promotion. The peak of ethylene production came between 2 and 4 hr after treatment with the optimal concentration of oleanimin, then rapidly decreased, reaching a level corresponding to auxin alone treatments several hours later. Consequently, the period of raised ethylene production corresponded to the rapid growth period.

When HDB, which is a representative active substance, was applied at concentrations lower than the optimal dose (Fig. 4), a delay of growth promotion was noted, and ethylene too showed a delayed increase and lower rate of peak production. The inhibition caused by β-stigmasterol (Fig. 5) also resulted in a close correlation of the reduction of growth and decreased ethylene production.

Role of Ethylene in Growth of Sections. Burg and Burg (5, 7) have studied ethylene formation and its relation to the inhibition of pea bud growth caused by IAA and postulated that IAA-induced ethylene formation accounts for the entire inhibition of bud growth. Chadwick and Burg (9) also stated that a large portion of IAA-induced inhibition of excised pea root tips and virtually all such inhibition of intact roots are the result of IAA-dependent ethylene production.

Even in our auxin medium without oleanimin, concentrations of IAA as high as 20 or 100 μM did not exhibit a significant inhibitory effect, in apparent contrast to some of Burg and Burg's experiments (5, 7), but in agreement with theirs when red light-treated peas were used (6). The rate of ethylene production by our pea sections was also considerably lower than their etiolated pea sections, whose values were two times, or perhaps even higher, than ours, but again red light treatment reduces the discrepancy (6). While the response of pea stem sections to auxin (39) and the rate of ethylene production (7, 41) are known to vary greatly with the location of the section on a stem, small inconsistencies in cutting sections are unlikely to explain the large differences from the rates they observed. Our basal medium contained 0.3 μM GA, but it would probably have a minor effect on ethylene production (10). Some of these differences could also result from the differences in cultivar (Alaska *versus* Progress No. 9 dwarf), but the light regime during seedling growth (complete darkness *versus* continuous weak red light) is more likely to have been decisive. Red light treatment is necessary for the oleanimin effect (46). Another difference results from the time intervals involved. Usually, to bring about an effect of ethylene application, a lag period of some hours is required. For example, according to Imaseki's data (18), at least 1 day was required to enhance peroxidase activity, and the growth-promoting effects with rice coleoptiles or intact oat and rice mesocotyls were not marked until 2 days or longer after ethylene treatment (23, 47). For the stimulation of respiration rate in potato tuber, Reid and Pratt (40) showed a lag of 8 hr. In our experiments, however, the duration of ethylene production having a physiologically effective rate may be estimated as less than 4 hr in the sections treated with the standard 1.8 μM concentration of IAA + oleanimins. Since exogenous ethylene applications to auxin-treated sections for periods as long as 4 or 6 hr did not give any effect even with a dose as high as 10 μl/liter (Table VI), it seems unlikely that ethylene could be the cause of the decline in growth rate after 6 hr in the standard 1.8 μM concentration of IAA. Only when a high concentration of IAA was applied along with HDB did a high rate of ethylene production last for a considerable period of time and a significant inhibition of growth occur (Table V and Fig. 10). In these cases an inhibitory effect of ethylene is more likely.

Table VI. *Effect of Ethylene Application on the Growth of Pea Stem Sections*

Medium also contained 0.3 μM GA_3, 50 mM KH_2PO_4 (pH 5.5), 1.5% sucrose, and 0.004% Pluronic F-68; 500 ml stoppered Erlenmeyer flasks were used for ethylene application.

Medium	Ethylene Concn	Period of Confinement[1]	Elongation	
			6 hr	20 hr
	μl/liter	*hr*	*% of control*	
1.8 μM auxin	0	...	100	100
	0.5	1–3	102	112
	1	1–3	101	112
	5	1–3	91	111
	10	1–3	95	99
	10	1–5[2]	94	97
	10	0–6	...	97
	10	0–20	...	90
1.8 μM auxin + 20 μM heptadecylbenzene	0	...	100	100
	0	1–3	104	102
	1	1–3	107	95
	5	1–3	102	96
	0	1–5[2]	90	64
	10	1–5[2]	85	60

[1] Hours after initiation of incubation.
[2] Atmosphere was renewed at 1-hr intervals.

Quantitatively, using Burg and Burg's data (6, 7) concerning ethylene formation in pea seedlings, Chadwick and Burg (9) estimated that a production rate of 6 nl/g·hr is equivalent to applying 1 μl/liter ethylene. Goeschl and co-workers (12) have reached a similar conclusion. Our pea sections produced ethylene approximately ranging from 2 to 4 nl/hr·10 sections at peak values when both oleanimin and auxin were added, and after 4 hr of growth the fresh weight of 10 sections ranged from 0.4 to 0.5 g. Applying these estimations to our data, a level of 0.7 to 1.7 μl/liter of ethylene would be reached within our pea sections during 2 to 4 hr. The minimal concentration of ethylene for inducing physiological effects varies to some extent depending whether fruit ripening, abscission, growth, or other effects are involved (4, 38), but half-maximal values of about 0.1 to 0.2 μl/liter are common. Thus, our tissues may have received physiologically effective concentrations even though no inhibitions were observed.

On the other hand, an effect of ethylene in stimulating growth has been reported on some species of plants. Ku *et al.* (23) found that rice coleoptiles grown with 10 μl/liter ethylene elongated rapidly and continued to grow for about 9 days whereas coleoptiles grown without ethylene elongated at a considerably lower rate and ceased to grow 3 days afterwards.

Imaseki and Pjon (19) also observed, using apical coleoptile segments of etiolated rice seedlings, that elongation induced by exogenous auxin was promoted by ethylene, while growth promotion did not occur unless exogenous auxin was added. Suge (47) found that a low concentration of ethylene stimulated growth of intact oat and rice mesocotyls. Hirai *et al.* (14), Maxie and Crane (30), and recently Marei and Crane (29) showed that ethylene markedly stimulated the enlargement and maturing of immature fig fruits. These are particularly germane observations, since fig ripening is the only other well substantiated situation in which lipids induce ethylene formation (13, 14), and the fig fruit is anatomically derived from a stem. It is well known, too, that ethylene activates the formation of some enzymes (17, 18) and stimulates respiration (17, 40). Again, however, all these stimulatory responses require relatively long exposures and have relatively long lag times. Thus, the failure of our experiments with applied ethylene to show any effect on short term growth is not contrary to previous results and seems substantiated by the failure of oleanimin-induced ethylene to inhibit growth either. In our case, the relatively rapid growth responses to oleanimins do not appear to be mediated by ethylene.

Relation of Ethylene to Metabolism of Sections. Penny and Stowe (36) have shown that the increase of respiration associated with oleanimin stimulation of growth of pea stem sections occurs by 3 hr or before. Such a respiratory increase seems unlikely to be due to oleanimin-induced ethylene because of this short time interval and the fact that respiration of nongrowing first internode sections was also stimulated by oleanimin (36), while these sections showed no increase of ethylene production in our experiments (Table I).

In many experiments above, a good correlation was found between growth promotion and rapid ethylene production. But discrepancies were observed in some cases, such as with cycloheximide inhibition (Table II), or when low concentrations of IAA were tested (Fig. 9). All in all, from such data too, we can conclude that oleanimin-induced ethylene neither enhances nor inhibits the growth of pea stem sections which are treated with 1.8 μM IAA, which is optimal for growth.

Nonetheless, growth and ethylene production could still be both secondary results of oleanimin action. Short term studies of respiration have not yet been made, but it is also stimulated by oleanimins and its products are effective in enhancing both growth and ethylene formation, respiratory stimulation could be the first step in a chain of events. We were not able to obtain growth stimulation without respiration stimulation in earlier work (36).

Thus, the site of action of oleanimins still seems most likely to be in a respiration-modulating membrane. Stowe and Dotts (45) postulated that oleanimins are active in the pea assay by forcing apart lecithin molecules, changing the charge distribution or chelating properties of such a regulatory membrane. The results with sterols (Fig. 5) and with the shorter alkyl halides (Fig. 3) reported in this paper are in accord with this hypothesis. In a study of light-scattering response and photosynthetic activities of spinach chloroplasts, Mukohata *et al.* (34) found a difference in the effect of *n*-alkane treatment between shorter and longer molecules and suggested that structural alterations in chloroplasts aged in the dark may be enhanced by longer alkanes. Ours could be a similar phenomenon, although different membranes would be involved. Any changes in properties of regulatory membranes could produce energy and enhance the ability of IAA to synthesize or modulate an ethylene-producing system as well as a cell elongation system.

Nature of Ethylene Production by Oleanimins. The effective lipids never produced significant quantities of ethylene unless auxin was applied with them, and the parameters of ethylene production such as lag period, peak rates, and duration were very similar at the same characteristically low lipid concentrations, even though the compounds would be expected to be metabolized by quite different pathways (45).

It seems unlikely that such diverse substances as iodohexadecane, triolein, vitamin E, and HDB could be equally effective substrates for the lipoperoxidase type of ethylene synthesis (27, 28), while even the metabolically available fatty acid ester oleanimins were shown earlier (37) not to enter rapidly into the general metabolism of these tissues. However, Hulme *et al.* (16) have noted that increases in lipid turnover precede increases in ethylene production by apple peel.

It is well known that high concentrations of IAA produce ethylene in various kinds of plants. Our experiments, too, showed a considerable ethylene production with 20 to 1 mM IAA giving peak rates comparable to those treated with 1.8 μM IAA and oleanimin (Table V). A delay of 1 hr was found for both high concentration IAA- and oleanimin-stimulated ethylene production (Fig. 10). When oleanimin was applied together with a high concentration of IAA, a very striking production of ethylene occurred, but the lag time did not alter. Burg and Burg (5) and Sakai and Imaseki (41) using pea and mung bean stem sections, respectively, also found a 1-hr lag time of ethylene production which was induced by high concentrations of IAA. Furthermore, the latter workers reported that the lag time did not change when auxin concentration was varied, while the rate of ethylene production increased as the auxin concentration was raised. Penny *et al.* (35) found auxin to increase growth after a lag time of only a few minutes.

These results suggest that the ethylene produced by oleanimin treatment is not derived from oleanimin itself, but rather that oleanimins enhance the ability of auxin to induce an ethylene-producing system. This conclusion is further supported by experiments which showed that the effect of oleanimins on growth declines more rapidly than does the enhancement of ethylene production (Fig. 6). Precursors of ethylene were not utilized until an oleanimin was supplied (Table III). This suggests a relationship of protein synthesis to auxin-induced ethylene production as has been reported (21), and this is supported by the effect of metabolic inhibitors on the oleanimin-induced production in our experiments (Table II).

After reaching a peak, a rapid decrease of ethylene production followed in oleanimin-treated sections. A quite similar pattern is well known in the ethylene production induced with a high concentration of IAA. Kang and co-workers (21), using etiolated pea shoots, suggested that the ethylene-producing system requires continued synthesis of protein, and that IAA induces the formation of a short-lived RNA required for the synthesis of a highly labile protein which controls the rate of ethylene production. They demonstrated a high rate of IAA conjugation and decarboxylation and stated that ethylene production and growth parallel the free IAA level to the tissue. In our experiments, the peak of ethylene production was always found during the 2- to 4-hr period in the sections treated with the standard 1.8 μM concentration of IAA + oleanimin, while that of high concentration IAA− or high concentration IAA+ oleanimin-treated sections came during the 4- to 6-hr interval. This may be due to a more rapid decline in endogenous free IAA concentration in the 1.8 μM IAA medium, below some threshold level. However, the ineffectiveness of renewal of incubation medium (Table IV) and the marked decrease in effect of adding high concentrations of IAA during incubation (Fig. 11) seem to indicate the development of a system to degrade or inhibit the ethylene-producing system rather than a system to lower free IAA levels. A rapid turnover

of the ethylene-producing system has been suggested by Sakai and Imaseki (41) and Kang *et al.* (21) in high concentration IAA-treated sections.

CONCLUSIONS

The goal of this study was to delineate the relationship between ethylene effects and the growth-promoting lipids we have termed oleanimins. We conclude (*a*) that since growth, respiration, and ethylene production can all be stimulated by molecularly diverse, yet dimensionally similar, oleanimins at the same low concentrations and with comparable kinetics, oleanimin action at a single locus in a membrane is probably regulating all three enhancement phenomena; (*b*) that ethylene is neither stimulating nor inhibitory to growth at auxin levels optimal in our material and during time intervals prevailing under our conditions and is therefore not regulating growth; (*c*) that although ethylene is frequently, but not always a result of oleanimin application and ethylene appearance is protein synthesis-dependent, therefore, oleanimins induce a system producing ethylene, for which auxin is also required; (*d*) that the above conclusions and the metabolic diversity existing among oleanimin molecules rule out ethylene production from them as substrates; and (*e*) thus, that ethylene or its production in no way explains the oleanimin effect.

In the next paper of this series we shall return to a study of oleanimin molecules and what their dimensions reveal concerning the neighborhood of the active site.

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