Plant Physiol. (1972) 50, 510-517

Ethylene-induced Pea Internode Swelling

ITS RELATION TO RIBONUCLEIC ACID METABOLISM, WALL PROTEIN SYNTHESIS, AND CELL WALL STRUCTURE[1]

Received for publication July 30, 1971

W. R. EISINGER[2] AND S. P. BURG
Department of Biology, University of Miami, and Fairchild Tropical Gardens, Coral Gables, Florida 33156

ABSTRACT

Exposure of etiolated pea (*Pisum sativum* L. var. Alaska) subapical sections to ethylene disrupts normal polar cell expansion, but fresh weight increase is little affected and the tissue expands radially, swelling. Ethylene has no effect on gross ribonucleic acid metabolism before or during the period when swelling occurs, but incorporation of ^{14}C-proline and leucine into wall-associated protein is markedly inhibited after an initial 3-hour lag period which precedes swelling. Ethylene affects the composition of this protein, altering the proline-hydroxyproline ratio. The gas also alters the optical birefringence pattern of the cell wall, indicating that the cellulose microfibrillar orientation has been changed.

The normal growth pattern of excised subapical sections from the third internode of etiolated pea seedlings is a highly polar expansion. When ethylene is applied, linear expansion is markedly inhibited, but fresh weight increase is hardly affected and the tissue expands radially. Since no cell divisions occur in this internodal tissue of either intact seedlings or excised sections in the presence or absence of ethylene, the ethylene-induced shift to nonpolar, radial expansion is due solely to cellular swelling (2). Ethylene induces subapical swelling in excised sections by a process resembling that which occurs in intact seedlings; for example, the same concentrations of ethylene are effective (6, 7). *In vivo*, however, ethylene reduces the rate, but greatly prolongs the duration of cellular expansion (3, 5), whereas excised sections are not so affected and invariably stop growing in 12 to 18 hr. When excised sections swell in response to ethylene, their total dry weight, weight of wall material, respiration, content of various sugars, and permeability are not altered (6, 7). Slight stimulation of RNA synthesis in response to ethylene has been noted in certain intact vegetative tissues (14, 16), but does not occur in isolated soybean hypocotyl (15) and has not previously been studied in pea internodal sections. During ethylene-induced swelling the optical birefringence pattern of the cell wall is altered (7) and newly deposited cellulose microfibrils are laid down in a longitudinal rather than radial direction (1). In a preliminary note we reported an inhibition by ethylene of ^{14}C-proline incorporation into a wall-bound protein (5) of excised sections and similar results have been obtained using tissue derived from ethylene-pretreated seedlings (5). However, Ridge and Osborne (22) found enhanced levels of hydroxyproline in wall protein derived from ethylene-treated pea stem tissue. In the present communication the effects of ethylene on RNA metabolism and cell wall composition and structure are comprehensively investigated in relation to the swelling response.

MATERIALS AND METHODS

Seeds of *Pisum sativum* L. (var. Alaska) were soaked in tap water for 6 hr, planted in vermiculite, and grown in darkness at 25 C. After 7 days, 1-cm sections were cut from the third internode of the epicotyl immediately below the hook and lots of 10 or 15 were incubated with or without 10 μl/liter of ethylene in sealed 125-ml flasks with 10 ml of medium containing 5 mM potassium phosphate buffer (pH 6.8), 2% sucrose, 5 μM cobalt chloride, and varying amounts of IAA and ^{3}H- or ^{14}C-labeled substrates (6). Following isotope incubations, tissue was rinsed with distilled water and floated in ice water for 15 min to clear residual isotope from the free space. Under these conditions, sections exposed to ^{14}C-proline (0.05 μc/ml) for about 1 sec retained less than twice background count.

Effects of Ethylene on Uptake. Short term uptake of ^{14}C-proline by pea internode tissue was studied after a 4-hr preincubation with or without ethylene in the presence of 1 μM IAA. The tissue was then exposed to U-^{14}C-proline (210 to 220 mc/mmole, 0.05 μc/ml) for 1 sec (0 time), 1, 5, 15, or 30 min. After sections were floated on ice water, they were rinsed, squashed on a planchet, dried, and counted using a thin window gas flow counter. Self-absorption was negligible under these conditions.

Effects of Ethylene on RNA Metabolism. RNA was extracted from internodal sections using the method of Key and Shannon (18) and total RNA content of tissue determined spectrophotometrically (A_{260}-A_{290}) using a hydrolyzed yeast RNA standard. Synthesis of new RNA was studied using 8-^{14}C-ATP (15 mc/mmole, 0.01 μc/ml).

Effects of Ethylene on Wall-associated Protein Synthesis. Tissue sections were incubated with ^{14}C-labeled proline, leucine, or glucose (usually 0.05 μc/ml) for 3 hr following a 4-hr preincubation, all in the presence of 1 μM IAA with or without 10 μl/liter of ethylene. Cell wall material was extracted and purified using two methods, one for homogenized tissue (9) and the other for whole sections (3). Wall protein purified by the method of Cleland (9) was extracted either with 1 N NaOH or with 200 μg/ml Pronase. Whole sections were extracted three

[1] This investigation was supported in part by a National Science Foundation Grant GB-27424 to S. P. Burg, and W. R. Eisinger was supported in part by a predoctoral trainee grant from the National Institutes of Health to the Department of Cellular and Molecular Biology, University of Miami.

[2] Present address: Department of Biological Sciences, Stanford University, Stanford, Calif. 94305.

times with 80% (v/v) methanol at 2 C, then twice for 24 hr with 200 μg/ml Pronase at 24 C. Tritium-labeled materials and ^{14}C radioactivity in base extracts were determined by liquid scintillation counting; all other samples were counted using a thin window gas flow counter.

Conversion of ^{14}C-proline to ^{14}C-hydroxyproline was determined using Pronase extracts from methanol-treated whole sections. After hydrolysis with 6 N HCl at 90 C for 18 hr in a nitrogen atmosphere, amino acid residues were separated using two-dimensional silica gel thin layer chromatography ($CHCl_3$-methyl alcohol-17% NH_4OH, 3:3:1; and phenol-water, 3:1).

Autoradiography and Optical Birefringence Patterns. For autoradiography studies, sections were incubated with either ^3H-proline or ^3H-glucose (50 μc/ml) for 3 hr following a 4-hr preincubation, all in the presence of 1 μM IAA with or without 10 μl/liter of ethylene. Sections were then extracted with 80% methanol at 2 C for 3 days and macerated with a 1:1 mixture of H_2O_2 and glacial acetic acid. Cells freed by this treatment were dried on microscope slides and coated with nuclear track emulsion (Kodak, NTB2) which was developed after 2 weeks using the method described by Kodak (19). Optical birefringence patterns of cell wall materials from macerated tissue were observed using a polarizing microscope.

RESULTS

The fresh weight of subapical sections increases linearly with time and is not affected by ethylene (Fig. 1A), but the gas specifically inhibits linear expansion after about 3 hr (Fig. 1B) reducing it to about one-fifth the normal rate. Coincident with the swelling response, changes in cell wall composition and structure can be detected.

Effects of Ethylene and IAA on Uptake. IAA (1 to 100 μM) stimulates uptake of labeled glucose, proline, leucine, and ATP by about 10 to 20% during a 3-hr period, and 10 to 40% when label is added for 3 hr after a 4-hr preincubation. No significant ethylene-induced change in uptake was observed within 3 hr with any of the labeled materials, but following a 4-hr preincubation with ethylene total uptake of proline and leucine is strongly inhibited during a subsequent 3-hr period. These effects may be related to incorporation of the amino acids into tissue components as discussed below, for ethylene has no effect on short term uptake of ^{14}C-proline by pea tissue. Tissue preincubated with ethylene for 4 hr and then exposed to ^{14}C-proline briefly, takes up radioactivity at a linear rate during a 30-min period and this uptake is not affected by ethylene pretreatment.

Effects of Ethylene and IAA on RNA Metabolism. Ethylene had no significant effect on the total RNA content of pea internode sections after 2, 4, 8, or 18 hr of treatment (Fig. 2 and Table I) in the presence or absence of 1 or 100 μM IAA. Similarly, IAA treatment had no effect on total RNA content between 0 and 8 hr, but slight stimulation was observed after 18 hr of incubation with 100 μM IAA. In general, the RNA content of excised sections declined during incubation.

Ethylene had no significant effect on the total incorporation of ^{14}C-ATP into RNA regardless of whether IAA was present or absent (Fig. 3 and Table I). Incorporation of ^{14}C-ATP into RNA occurred at a linear rate for 4 hr and was not affected

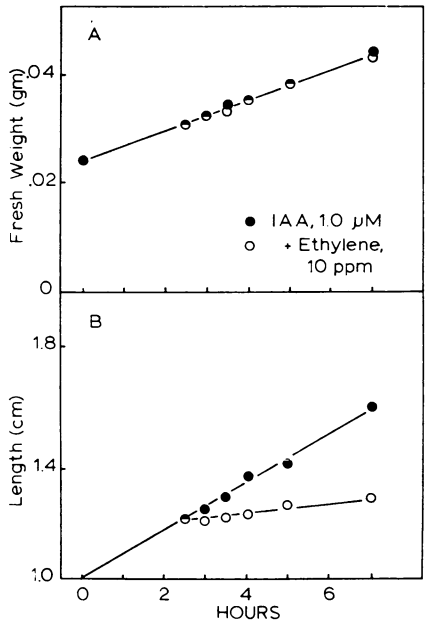

FIG. 1. A and B: Growth of etiolated pea internode sections treated with IAA or IAA plus ethylene.

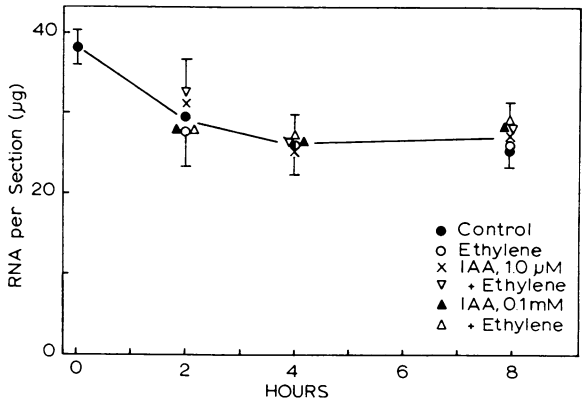

FIG. 2. Effects of IAA and ethylene on RNA content of etiolated pea internode sections.

Table I. *Effects of Ethylene and IAA on RNA Metabolism in Pea Internode Sections After an 18-hr Incubation*

	Total ^{14}C-ATP Uptake	^{14}C-ATP Incorporation into RNA	^{14}C-ATP Incorporation per g Fresh Wt	Total RNA	RNA per g Fresh Wt	RNA Specific Radioactivity
	dpm × 10 μM	*dpm × 0.1 mM*		*μg*	*μg/g*	*dpm/μg*
Control	3.54	2.42	4.39	324 ± 30[1]	580	76 ± 7[1]
Ethylene, 10 μl/liter	3.43	2.46	4.23	334 ± 20	600	71 ± 10
IAA, 1.0 μM	6.42	4.06	5.31	350 ± 30	450	118 ± 9
IAA, 1.0 μM + ethylene	6.18	3.60	4.80	348 ± 30	450	106 ± 10
IAA, 100 μM	5.08	3.89	5.25	387 ± 20	530	106 ± 12
IAA, 100 μM + ethylene	5.17	4.01	5.49	388 ± 30	530	102 ± 13

[1] Standard deviation of four replicate experiments.

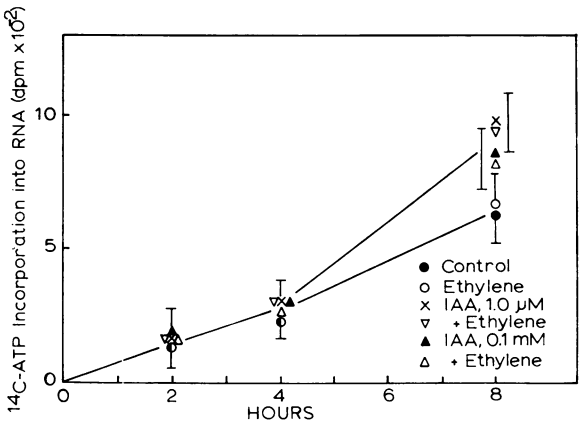

FIG. 3. Effects of IAA and ethylene on incorporation of ^{14}C-ATP into RNA of etiolated pea internode sections.

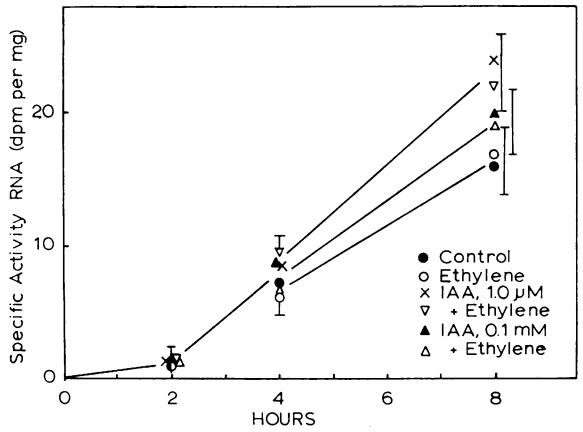

FIG. 4. Effects of IAA and ethylene on the specific activity of RNA in etiolated pea internode sections.

by either IAA or ethylene during this period (Fig. 3). During 8 hr, however, IAA stimulated total incorporation by about 30% (Fig. 3) and in 18 hr by about 60% (Table I). Addition of ethylene had no effect on total incorporation in any case and the specific radioactivity of RNA extracted from tissue sections incubated with ^{14}C-ATP was not significantly changed by the gas (Fig. 4 and Table I). Between the 2nd and 8th hr of incubation the specific activity of RNA increased approximately at a linear rate and IAA caused a progressive stimulation, whereas ethylene was without effect both in the presence or absence of IAA (Fig. 4). After 18 hr (Table I) the stimulation of specific radioactivity caused by IAA was similar to that at 8 hr; addition of ethylene again had no effect.

The effects of applied ethylene and IAA on the RNA content of intact pea seedlings were also investigated. Ethylene had no effect on the RNA content of the internode region immediately below the hook of intact seedlings during 18-hr exposure, although this region swelled markedly in response to the gas (Table II). If seedlings were decapitated immediately below the hook, ethylene treatment had no effect on the RNA content of the internode (Table II). Under these conditions so little new tissue formed in the absence of IAA that it was not possible to determine whether the cells swelled in response to the gas (Table II). When 0.5% IAA was applied in lanolin paste to the cut surface of decapitated seedlings, no significant change was observed in the RNA content of the internode after 24 hr, but the tissue swelled greatly. Ethylene supplied to the IAA-treated tissue caused no significant change in the RNA content and caused no further swelling of the internode since during this time IAA induces cellular swelling by stimulating ethylene formation (6), whereas later IAA-induced cellular divisions may contribute to the swelling response (10).

Effects of Ethylene on Wall Protein Composition. Ethylene treatment with or without IAA resulted in a 30 to 40% reduction in ^{14}C-proline (200 mc/mmole) incorporation into a Pronase- or base-extractable wall fraction when the isotope was added at a concentration of 0.25 μM after a 4-hr preincubation with the gas (Table III). The effect was observed with tissue sections which were homogenized, purified, and extracted, and also with whole sections extracted with methanol and then treated with Pronase. However, extraction of whole sections with Pronase was far more efficient in removing counts from the wall than extraction of homogenized tissue with base or Pronase (80 *versus* 30 to 40%). Ethylene had no significant effect on the amount of label found in the supernatant of homogenized tissue (cytoplasmic fraction) or in counts extracted by methanol from whole sections (Table III). IAA stimulated incorporation of ^{14}C-label from proline into the wall fraction as well as into the "cytoplasmic" fraction, and ethylene inhibited incorporation into the wall in the presence of IAA to the same extent as in the absence of IAA.

The kinetics of the ethylene-induced inhibition of ^{14}C-proline incorporation into the wall fraction are closely similar to those of the swelling response. Ethylene does not affect ^{14}C-proline incorporation into the wall fraction during the 3-hr period before swelling is induced. No inhibition of label incorporation was observed with tissue preincubated with ethylene for 1.5 hr and then exposed to isotope for 1.5 hr, but if the tissue was preincubated for 2 hr then exposed to isotope for 2 hr, about a half-maximal inhibition occurred compared to that after 4-hr preincubation with the gas. These data indicate that ethylene-induced swelling and inhibition of proline incorporation both begin only after sections have been exposed to ethylene for about 3 hr. Ethylene also inhibits incorporation of ^{14}C-leucine (25 mc/mmole; 10 μM) but not ^{14}C-glucose (5 μc/μmole; 2 μM) into the wall fraction (Table IV). Inhibition of leucine incorporation occurs only after a 3-hr lag period, and in these studies was slightly less striking than that with proline (about 30 *versus* 40%). Ethylene has no significant effect on incorporation of ^{14}C-glucose into any of the cellular fractions investigated during the 7-hr period studied (Table IV). Sucrose was omitted from the incubation medium for the

Table II. *Effect of IAA and Ethylene on Growth and RNA Content of Subapical Zone of Etiolated Pea Seedlings During an 18-hr Treatment*

	Fresh Wt	Length	ΔWeight-ΔLength	Total RNA
	mg	*cm*	*ratio*	*μg*
Initial	18.8	1.00		26 ± 4[1]
	Intact Seedlings			
Control	74.6	2.55	36	25 ± 3
Ethylene, 10 μl/liter	45.0	1.21	125	25 ± 4
	Decapitated Seedlings			
Control	32.8	1.26	54	28 ± 7
Ethylene, 10 μl/liter	29.7	1.19	58	32 ± 7
IAA, 0.5%	57.6	1.25	155	34 ± 6
IAA + ethylene	63.9	1.28	161	36 ± 7

[1] Standard deviation of four replicate experiments. Data presented on a per section basis.

Table III. *Effects of Ethylene and IAA on Distribution of ^{14}C-Label Following ^{14}C-Proline Incubation of Etiolated Pea Internode Sections*

^{14}C-Proline (0.05 μc/ml; 0.25 μM) was present between the 4th and 7th hr of incubation.

	Radioactivity in Fraction of Homogenized Tissue				
	NaOH treatment			Pronase treatment	
	Supernatant after homogenization	Extracted	Residue	Extracted	Residue
	dpm × 10⁻³/section				
Control	22.4	12.6 ± 1.0[1]	23.3 ± 2.8	11.2 ± 0.9	22.0 ± 3.1
Ethylene, 10 μl/liter	18.5	9.2 ± 0.6	20.9 ± 2.6	8.4 ± 0.5	22.4 ± 3.4
IAA, 1.0 μM	30.5	22.6 ± 4.0	32.8 ± 4.6	16.2 ± 2.2	32.3 ± 5.5
IAA + ethylene	27.6	16.0 ± 1.6	25.4 ± 1.5	11.9 ± 0.7	29.1 ± 2.9
	Radioactivity in Fractions of Whole Sections				
	Methanol extract	Pronase extract	Cell wall residue		
	dpm × 10⁻³/section				
Control	15.2 ± 1.1	43.5 ± 3.6	12.2 ± 1.2		
Ethylene, 10 μl/liter	14.3 ± 0.9	27.5 ± 1.6	9.6 ± 1.8		
IAA, 1.0 μM	16.7 ± 2.9	60.0 ± 6.3	17.1 ± 4.1		
IAA + ethylene	16.0 ± 3.6	41.9 ± 5.4	13.6 ± 3.1		

[1] Standard deviation of four replicate experiments.

^{14}C-glucose studies to prevent isotope dilution through hydrolysis of sucrose in the medium.

Subapical sections treated with an optimal concentration of benzimidazole (2 mM), colchicine (1 mM), benzyladenine (10 μM), supraoptimal IAA (100 μM), or applied ethylene (10 μl/liter) all swell to approximately the same extent and are inhibited similarly in incorporation of ^{14}C-proline into the wall fraction. None of the above substances induce swelling during the initial 3-hr incubation period or have a substantial effect on proline incorporation during that period. None of the above substances at any time significantly affected fresh weight increase, counts in methanol extracts or ethylene production by the tissue (1), except supraoptimal IAA which enhances ethylene production. However, after a 4-hr preincubation all the substances inhibit ^{14}C-proline incorporation into the wall fraction by about 40% and induce swelling.

Table IV. *Effects of Ethylene and IAA on Distribution of ^{14}C-Label from ^{14}C-Leucine and ^{14}C-Glucose Incubations of Etiolated Pea Internode Sections*

Isotope was present between the 4th and 7th hr of incubation.

	Radioactivity in Fractions after Extraction		
	Methanol extract	Pronase extract	Cell wall residue
	dpm × 10⁻³/section		
^{14}C-Leucine incubation (25 μc/μmole; 10 μM)			
Control	14.0 ± 1.3[1]	43.2 ± 4.3	3.5 ± 0.5
Ethylene, 10 μl/liter	12.6 ± 1.0	33.0 ± 2.0	2.8 ± 0.4
IAA, 1.0 μM	12.7 ± 1.0	61.0 ± 8.5	5.8 ± 0.7
IAA + ethylene	11.4 ± 2.3	47.2 ± 4.7	4.3 ± 0.8
^{14}C-Glucose incubation (5 μc/μmole; 10 μM)			
Control	106.0 ± 6.4	6.7 ± 0.5	13.2 ± 1.0
Ethylene, 10 μl/liter	105.0 ± 2.1	6.9 ± 0.3	12.9 ± 0.5
IAA, 1.0 μM	114.0 ± 9.1	8.0 ± 0.7	25.0 ± 2.2
IAA + ethylene	99.0 ± 6.9	8.1 ± 0.9	23.9 ± 2.4

[1] Standard deviation of four replicate experiments.

Table V. *Effects of Ethylene on Conversion of ^{14}C-Proline to Hydroxyproline in Wall Protein*

^{14}C-Proline (0.05 μc/ml; 0.25 μM) was present between the 4th and 7th hr of incubation.

	Radioactivity in Proline and Hydroxyproline				
	Methanol extract proline/hydroxyproline	Pronase digest			
		Proline	Hydroxyproline	Proline/hydroxyproline	Recovery
	ratio	*dpm × 10⁻³*		*ratio*	%
Control	2.4 ± 0.4[1]	28.0	17.6	1.6 ± 0.3	60
Ethylene, 10 μl/liter	1.8 ± 0.6	13.4	5.3	2.5 ± 0.5	50
IAA, 1.0 μM	1.8 ± 0.7	40.0	18.7	2.1 ± 0.2	50
IAA + ethylene	2.2 ± 0.6	28.0	9.3	3.0 ± 0.4	40

[1] Standard deviation of four replicate experiments.

Ethylene treatment may affect the labeled amino acid composition of the wall, and definitely alters the conversion of ^{14}C-proline to ^{14}C-hydroxyproline in wall fraction protein extracted by Pronase. Incorporation of label from 0.25 μM ^{14}C-proline is inhibited more strongly than that from 2 μM ^{14}C-leucine, and similar results were obtained in double label experiments with ^{14}C-proline and ^{3}H-leucine, so that ethylene-treated tissue appears to have a relatively higher labeled leucine ratio in the wall. However, this difference could be related to the fact that the concentration of leucine used in these studies was about one-tenth that of proline, as discussed below. IAA had little or no effect on the proline-leucine balance in the wall under the conditions of these experiments. Conversion of ^{14}C-proline to ^{14}C-hydroxyproline in wall fraction protein is suppressed more strongly by ethylene than is ^{14}C-proline incorporation (Table V). Therefore, the labeled proline to hydroxyproline balance in the wall is shifted in favor of proline con-

Table VI. *^{14}C-Proline Uptake and Incorporation into Wall-associated Protein after Application of 0.25 μM or 20 mM Uniformly Labeled Proline (1 μc/ml) in Presence of 0.2 μM IAA*

Isotope Present	Uptake of ^{14}C-Proline		Percentage Conversion[1]			
			Pronase extract		Wall residue	
	0.25 μM	20 mM	0.25 μM	20 mM	0.25 μM	20 mM
hr	*dpm × 10^{-3}/g fresh wt*		*%*			
0–1	91.6	68.4	21.1	20.4	2.2	1.8
0–3	314.3	47.6	61.4	21.6	14.4	2.3
4–7	1008	57.1	62.5	13.4	19.3	2.6
6–7	1548	95.7	71.5	12.7	14.8	1.5

[1] Values are for per cent of conversion of total radioactivity taken into tissue to the pronase-extractable or residual wall fraction.

tent. IAA had little or no effect on the labeled proline to hydroxyproline ratio in the wall, as previously reported (12), and neither IAA nor ethylene had any significant effect on the proline-hydroxyproline ratio in methanol-extractable counts.

No ethylene-induced inhibition or auxin-induced stimulation of ^{14}C-proline incorporation into wall fractions occurred when sections were exposed to a high concentration of applied 20 mM ^{14}C-proline (2 μc/mmole), although the growth of the sections and their response to ethylene and IAA was not changed by the proline. Under these conditions, using 1 μc/ml isotope but either 0.25 μM or 20 mM applied proline, total uptake of counts and relative conversion of ^{14}C-proline into Pronase-extractable or -nonextractable wall fractions was almost independent of proline concentration during the 1st hr of exposure to isotope (Table VI). With the low concentration of proline, uptake increased rapidly throughout a 7-hr period whereas it remained relatively constant with the higher concentration (Table VI). The per cent of conversion of ^{14}C-proline into the Pronase-extractable and -nonextractable fractions was independent of proline concentration during the 1st hr of incubation, and remained relatively constant or declined slightly thereafter when 20 mM proline was applied. However, with a low concentration of proline, the per cent of conversion to wall-bound protein increased abruptly during the first 3 hr and slightly thereafter. According to Christiansen and Thimann (8) during a 24-hr pea straight growth test the free amino acid content of sections decreases by about 86% without IAA and 96% with 50 μM IAA. If the free amino acid pool is representative of the proline pool available for isotope dilution, and if total isotope uptake depends upon the conversion of proline to wall-bound protein, the different results obtained with 20 mM and 0.25 μM proline could be interpreted to be a reflection of the proline pool size, or rate of turnover in the tissue, or both. When the concentration of isotope fed is so low that it does not influence endogenous proline availability, uptake and incorporation into wall-associated protein should increase progressively as the endogenous proline pool decreases; and the per cent incorporation of applied ^{14}C-proline would be expected to increase and approach some limiting value. This in fact is observed to be the case, and the magnitude of the changes in proline uptake and incorporation into wall protein are similar to the reported changes in free amino acid content. When a high concentration of proline is applied to sustain the endogenous free proline pool, none of these changes occur. These data suggest that the responses to ethylene and IAA noted in the presence of low applied concentrations of ^{14}C-amino acids may be related to hormone-induced changes in pool size or turnover rate instead of or in addition to changes in wall protein synthesis. This hypothesis is in accord with the observation that auxin stimulates loss of free amino acid from pea tissue at the same time that it enhances proline uptake and incorporation into wall-associated protein (8). To further evaluate this interpretation the effect of ethylene on proline incorporation was studied after exposing intact seedlings to the gas for 24 or 48 hr and then pulse labeling subapical tissue sections with ^{14}C-proline at high and low concentration for 1 or 3 hr. This approach should eliminate or at least minimize the complication of free amino acid depletion if this is mainly caused by incubating excised tissue in the absence of a nitrogen source. A 1-cm subapical zone was demarcated with two ink spots applied to 7-day-old etiolated pea seedlings. When 1-cm pieces of this zone were cut from control plants after 48 or 96 hr, it was found that the ability of the tissue to take up proline and incorporate it into wall-associated protein during a 3-hr pulse feeding increased as the tissue aged (Table VII). If plants had been exposed to ethylene for 24 or 48 hr (Table VII) the excised sections incorporate proline into wall-associated protein at a decreased rate during a 1- or 3-hr pulse feeding. When after 48 hr of exposure to ethylene, the plants were placed in a ventilated area for an additional 48 hr, the marked tissue recovered its ability to incorporate proline into wall-associated protein. These studies were carried out using a low concentration of applied proline; when 20 mM ^{14}C-proline was used instead, again no effect of ethylene on proline incorporation into wall-associated protein could be discerned.

Effects of Ethylene on Wall Structure. Ethylene and other substances which cause swelling in pea tissue alter the optical birefringence patterns of cell walls. Ethylene-induced dark and light banding patterns paralleling the long axis of the cell were observed clearly after a 7-hr exposure to ethylene (Fig. 5B); similar but less distinct patterns were observed after a 3-hr ethylene treatment. Cell walls from tissue treated with near-optimal IAA were similar in appearance to untreated cell

Table VII. *Effect of Ethylene Applied to Intact 7-Day-Old Etiolated Seedlings on Incorporation of ^{14}C-Proline by Marked 1-cm Subapical Zone*

	Radioactivity in Fractions after Treatment			
	Methanol extract	Pronase extract	Wall residue	Fresh wt
	dpm × 10^{-3}/g fresh wt			*mg/cm*
Initial, 0 hr[1]	70.2	19.2	2.0	25
Control, 24 hr	68.6	31.6	5.9	23
Ethylene, 24 hr	67.8	20.3	2.9	50
Initial, 0 hr[2]	28.0	42.0	11.0	35
Control, 48 hr	44.2	45.0	18.5	37
Ethylene, 48 hr	33.5	27.5	7.4	111
Control, 96 hr	51.0	74.0	26.0	39
Recovery, 96 hr[3]	40.0	64.0	29.5	95

[1] At indicated times, 1-cm sections were cut from the marked zone and incubated for 1 hr with 1 μc/ml ^{14}C-proline (2.5 μc/μmole) for 1 hr in the presence of 0.2 μM IAA.

[2] At the indicated times, 1-cm sections were cut from the marked zone and incubated for 3 hr with 0.05 μc/ml ^{14}C-proline in the absence of IAA.

[3] Recovery, 48-hr treatment with 10 μl/liter ethylene and then ventilated for 48 hr without ethylene. Standard deviation of four replicate experiments ranged from ±5 to ±10%.

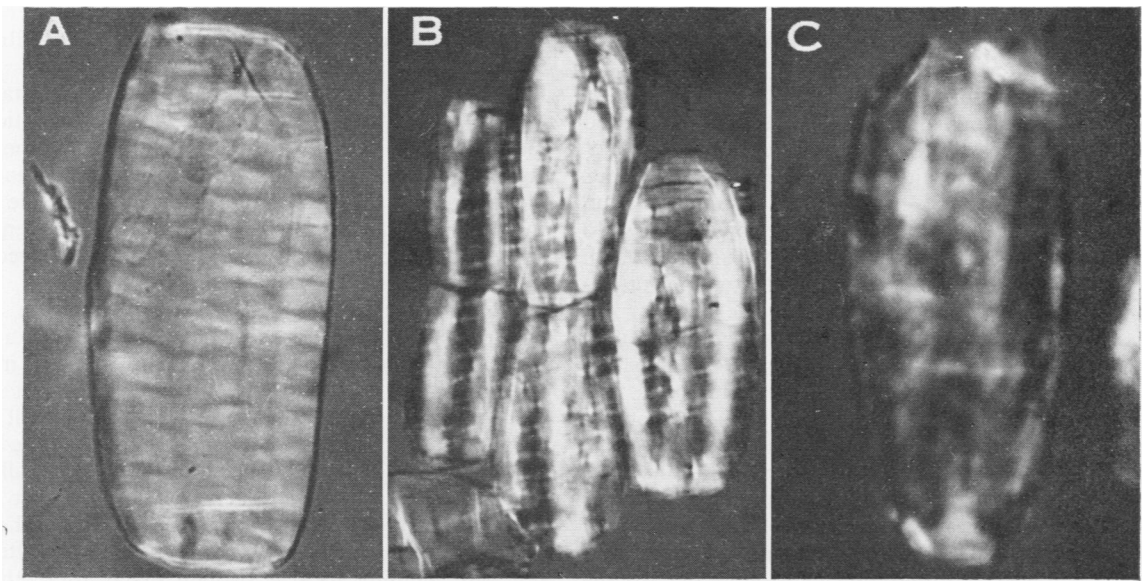

FIG. 5. Polarizing micrographs of parenchyma cells from macerated pea internode tissue. Internode sections were immediately macerated (A) or treated with ethylene (B) or colchicine (C) for 7 hr.

walls (Fig. 5A). However tissue treated with benzimidazole, benzyladenine, or supraoptimal IAA for 7 hr swelled and showed optical banding patterns similar to those observed with ethylene treatment. Colchicine induced a mottled optical pattern (Fig. 5C) distinct from either untreated or ethylene-like patterns. Microautoradiography revealed no relationship between ethylene-induced optical banding patterns in cell walls of tissue incubated with ^{3}H-proline or ^{3}H-glucose for 7 hr, and the distribution of silver grains from ^{3}H-label. In both ethylene and control cell walls ^{3}H-label from either proline or glucose was evenly distributed over the cell wall. Similar microautoradiographs were obtained with tissue incubated with ^{3}H-glucose for 18 hr in the presence of 2 mM benzimidazole.

DISCUSSION

Effects of Ethylene on Solute Uptake. Ethylene inhibition of proline incorporation does not appear to be a result of inhibition of amino acid uptake. This is indicated by the fact that inhibition by ethylene of total incorporation of ^{14}C-proline is not related to the rate of uptake into cytoplasmic fractions. Moreover, in whole sections ethylene had no effect on methanol-extractable counts (Table III) or on short term uptake of ^{14}C-proline, although total uptake and incorporation of ^{14}C-proline following a 4-hr preincubation (Table III) is inhibited by ethylene along with incorporation into the wall fraction. During short term exposures to label (less than 30 min) following preincubation with ethylene for as long as 7 hr, cytoplasmic incorporation appears to overshadow the effect of ethylene on wall fraction incorporation and no ethylene effect on ^{14}C-proline content of the tissue is observed. However, using isolated segments, within 3 hr after ethylene is applied, the total incorporation into the wall fraction becomes the dominant factor in total incorporation by the tissue. Therefore, ethylene-induced inhibition of wall-associated protein synthesis must be the cause rather than an effect of the altered uptake patterns observed after ethylene treatment of excised pea internode tissue.

Effects of Ethylene and IAA on RNA Metabolism. Since no effect of ethylene on RNA metabolism could be detected during 18 hr with excised sections, and as IAA stimulated RNA synthesis with equal efficiency in the presence or absence of ethylene, ethylene treatment must induce swelling with no change in gross RNA metabolism. Similarily, swelling in intact pea seedlings during an 18-hr period was not accompanied by any change in total RNA, in contrast to reports of stimulation of RNA synthesis in other ethylene-treated tissues (14, 16). However, when data of these studies are expressed as RNA/g fresh weight, the ethylene effect becomes insignificant, as shown with isotope experiments in the same tissue (15). Possibly the change in RNA synthesis induced by ethylene in these long term studies may be due to a prolongation of the growth phase (2). Auxin stimulation of RNA synthesis has been reported in excised soybean tissue (14, 16, 17) and Fan and Maclachlan (10) observed "massive" synthesis of RNA in decapitated, etiolated pea seedlings in response to an extremely high auxin treatment which induced cellular swelling and proliferation. However, our attempts to reproduce this effect using similar conditions, but for only 18 hr, failed to yield any significant increase in RNA content of the internodal tissue at that time (Table II).

Ethylene Effects on Cell Wall Composition and Structure: Their Relation to Swelling. The kinetics of the ethylene-induced swelling response and inhibition of ^{14}C-proline incorporation into wall protein are closely related in excised sections exposed to the gas, indicating a possible direct correlation between these ethylene-induced responses. Inhibition of both elongation and proline incorporation begin after 3-hr exposure to ethylene and both set in at a near-maximal rate shortly thereafter. The Pronase-extractable wall fraction from methanol-extracted whole sections shows the same hormone response pattern as wall protein obtained from homogenized, purified wall material using a method described by Cleland (9), suggesting that the Pronase-extractable fraction is identical to extensin (20). Inhibition of ^{14}C-proline incorporation into this wall fraction occurs without a lag in tissue cut from the growing zone of plants pretreated with ethylene.

In the presence of 20 mM ^{14}C-proline, no ethylene inhibition of label incorporation into the wall fraction was observed even in tissue excised from plants exposed to the gas for 24 hr, indicating that ethylene may be affecting the effective proline pool size, rather than, or in addition to, incorporation into wall protein. Christiansen and Thimann (8) have reported that the free amino acid pool size in pea internode sections rapidly declines

with time and that this decline is stimulated by IAA. The inhibition of incorporation seen with ethylene pretreatment would occur if ethylene treatment sustained the proline pool or increased the proline turnover rate relative to control or IAA-treated tissue, resulting in a greater dilution of supplied ^{14}C-proline and therefore less label in the wall fraction. Incubation with 20 mM proline would greatly swell the endogenous pool and mask any differences induced during preincubation so that no effect of ethylene treatment on incorporation into the wall fraction would be seen. The mechanism by which ethylene treatment might affect proline pool sizes is not apparent, and there is no indication that extension is labile.

Incorporation of labeled leucine into the wall fraction may be inhibited by ethylene to a somewhat lesser extent than that of proline, but this result can be complicated by pool size. Ethylene treatment definitely results in a larger proline-hydroxyproline ratio in wall protein, indicating that the gross composition or relative proportions of proteins in the wall fraction is altered. Such a change in wall protein might be related to the altered growth pattern, swelling, of ethylene-treated cells. Studies using ^{14}C-glucose further demonstrate the preferential nature of the ethylene inhibition of proline and leucine incorporation into wall material. However, long term exposure of intact seedlings to ethylene gas can result in tissue which continues to grow and incorporate glucose into wall material after control tissue has stopped elongating and synthesizing large amounts of wall material (3).

Ridge and Osborne (22), measuring total hydroxyproline content of etiolated pea seedling stem tissue, found an increased wall-associated hydroxyproline content when measurements were made after ethylene treatment lasting 1 or more days. Their results, showing a positive (stimulatory) effect of ethylene on hydroxyproline content contrasts sharply with the negative (inhibitory) effect of ethylene using a low concentration of applied ^{14}C-proline, or lack of an effect at high concentrations, on proline and hydroxyproline incorporation and synthesis presented here. The apparent incompatibility of these results is not readily obvious, but might be related to the differences in methods used and parameters studied, Ridge and Osborne measured total wall-associated hydroxylproline whereas we have measured the incorporation of ^{14}C-label from proline into wall protein. Evidence has been presented to indicate that our results might be complicated by ethylene-induced changes in the effective proline pool size. Furthermore, Ridge and Osborne measured long term effects of ethylene on intact seedlings, and these are difficult to interpret in terms of the initiation of the swelling response. Ethylene treatment of pea seedlings stops cell divisions (2) and alters the long term growth characteristics of this tissue (3). Comparisons of control and ethylene-treated tissue after 1 or more days of treatment are complicated by the fact that in the apex of ethylene-treated seedlings cell divisions slow within 2 hr whereas new cells are constantly being produced in untreated plants. Ethylene-treated subapical cells continue to expand for many days whereas control cells stop growing in about 24 hr (3), and ethylene treatment also delays lignification and other aspects of differentiation. The increases in hydroxyproline reported by Ridge and Osborne may be related to some of these other actions of ethylene, but it is noteworthy that the discrepency in results still is apparent even when whole plants are treated with ethylene for several days.

Inhibition of ^{14}C-proline incorporation into wall protein seems to be an integral part of the swelling response since it can be induced by a variety of chemical substances, but only during the period when swelling occurs. The swelling induced by these substances (supraoptimal IAA, benzyladenine, benzimidazole, and colchicine) is of a similar character and magnitude to that induced by ethylene. Possibly the swelling response operates by a common mechanism induced by any of the above substances. None of the above listed substances except supraoptimal IAA induce any significant stimulation of ethylene production by the tissue during the experimental period. Fuchs and Lieberman (11) have reported that kinetin slightly stimulates ethylene production in pea internode tissue, but only after a 6 hr or longer lag period, and the magnitude of the stimulation is considerably less than that required to induce swelling (1).

Ethylene and other substances which induce swelling all produce altered optical birefringence patterns during the period when swelling occurs, suggesting a change in wall microfibrillar orientation. A loss of directional orientation of growth has been correlated with random organization of wall microfibrils of *Nitella* (13) treated with colchicine and Probine (21) has described altered birefringence patterns in cell walls from pea tissue induced to swell with benzimidazole. Under a polarizing microscope evenly lighted areas indicate ordered microfibrils; such a pattern is typical of control tissue. The patterns of dark and light banding seen in swollen tissue after ethylene treatment indicate a different microfibrillar orientation, as does the amorphous pattern after colchicine treatment. Veen (23, 24) using pea internode tissue induced to swell with supraoptimal IAA (*i.e.*, via induced ethylene production), reported that the orientation of walls microfibril synthesis changed from initially transverse to a longitudinal orientation even before growth had begun. Veen concluded that the newly synthesized, longitudinal microfibrils encourage lateral expansion. These studies with supraoptimal IAA suggest that ethylene must cause cells to swell by facilitating radial expansion with newly synthesized, longitudinal microfibrils, and such a change in ethylene-treated pea cells has recently been observed (1). However, the work of Veen (24) indicated that radial expansion can occur independently of inhibited elongation under certain conditions, and hence is not necessarily a direct consequence of restraining elongation. Rather it would appear that cells swell because they are directed to do so, and that elongation may suffer when this occurs because there is a limit to the rate of cellular expansion.

Probine (21) reported that benzimidazole treatment (3 mM, 18 hr) of etiolated pea internode tissue sections induced swelling and resulted in distinct areas of concentration of label following ^{14}C-sucrose incubation, in zones corresponding to the optical banding patterns seen with polarizing optics. However, attempts to duplicate these results using nearly identical conditions (^{3}H-glucose substituted for ^{14}C-sucrose) failed to show any pattern or area of concentration of silver grains in autoradiographs of walls from tissue induced to swell with benzimidazole. These data, in contrast to Probine's results, suggest that ethylene or benzimidazole treatment of pea internode tissue causes swelling without affecting the normal, even distribution of proline or glucose incorporation into the cell wall.

Acknowledgments—The authors would like to acknowledge the assistance of Doctors Bin G. Kang and Akiva Apelbaum in studies on the incorporation of ^{14}C-proline into sections excised from plants pretreated with ethylene, and also in studies utilizing a high concentration of applied ^{14}C-proline.

LITERATURE CITED

1. Apelbaum, A. and S. P. Burg. 1971. Altered cell microfibrillar orientation in ethylene-treated *Pisum sativum* stems. Plant Physiol. 48: 649-652.
2. Apelbaum, A. and S. P. Burg. 1972. Effect of ethylene on cell division and DNA synthesis in *Pisum sativum*. Plant Physiol. In press.
3. Apelbaum, A. and S. P. Burg. 1972. Effects of ethylene and 2,4-D on cell expansion in *Pisum sativum*. Plant Physiol. In press.

4. Baker, D. B. and P. M. Ray. 1964. Direct and indirect effects of auxin on cell wall synthesis in oat coleoptile tissue. Plant Physiol. 40: 345–352.
5. Burg, S. P., A. Apelbaum, W. Eisinger, and B. G. Kang. 1971. Physiology and mode of action of ethylene. Hort. Sci. 6: 359–364.
6. 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: 262–296.
7. Burg, S. P. and E. A. Burg. 1968. Auxin stimulated ethylene formation: its relation to auxin inhibited growth, root geotropism and other plant responses. *In:* F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. Runge Press Ltd., Ottawa, Canada. pp. 1275–1294.
8. Christiansen, G. S. and K. V. Thimann. 1950. The metabolism of stem tissue during growth and its inhibition. III. Nitrogen metabolism. Arch. Biochem. Biophys. 28: 117–129.
9. Cleland, R. 1968. Distribution and metabolism of protein-bound hydroxyproline in an elongating tissue, the *Avena* coleoptile. Plant Physiol. 43: 865–870.
10. Fan, D. and G. A. Maclachlan. 1967. Massive synthesis of RNA and cellulase in the pea epicotyl in response to indoleacetic acid with and without concurrent cell division. Plant Physiol. 42: 1114–1123.
11. Fuchs, Y. and M. Lieberman. 1968. Effects of kinetin, IAA and gibberellin on ethylene production, and their interactions in growth of seedlings. Plant Physiol. 43: 2029–2036.
12. Giesen, M. and D. Klämbt. 1969. Untersuchungen zur Beeienflussung des Prolin-Einbaues in Proteine durch Auxin und Hydroxyprolin in Weizenkoleoptilcylinder. Planta 85: 73–84.
13. Green, P. B. 1963. On mechanisms of elongation. *In:* M. Locke, ed., Cytodifferentation and Macromolecular Synthesis. Academic Press, Inc., New York. pp. 203–234.
14. Holm, R. E. and F. B. Abeles. 1968. The role of ethylene in 2,4-D-induced growth inhibition. Planta 78: 293–304.
15. Holm, R. E. and J. L. Key. 1971. Inhibition of auxin-induced deoxyribonucleic acid synthesis and chromatin activity by 5-fluorodeoxyuridine in soybean hypocotyl. Plant Physiol. 47: 606–608.
16. Holm, R. E., T. J. O'Brien, J. L. Key, and J. H. Cherry. 1970. The influence of auxin and ethylene on chromatin-directed ribonucleic acid synthesis in soybean hypocotyl. Plant Physiol. 45: 41–45.
17. Key, J. L. 1964. Ribonucleic acid and protein synthesis as essential processes for elongation. Plant Physiol. 39: 365–370.
18. Key, J. L. and J. C. Shannon. 1964. Enhancement by auxin of RNA synthesis in excised soybean hypocotyl tissue. Plant Physiol. 39: 360–364.
19. Kodak Pamphlet No. P-64. 1966. Kodak materials for nuclear physics and autoradiography. Eastman Kodak Company, Rochester, N. Y. p. 10.
20. Lamport, D. T. A. 1963. Oxygen fixation into hydroxyproline of plant cell wall protein. J. Biol. Chem. 238: 1438–1440.
21. Probine, M. C. 1964. Chemical control of plant wall structure and cell shape. Proc. Royal Acad. 161: 526–527.
22. Ridge, I. and D. J. Osborne. 1970. Hydroxyproline and peroxidases in cell walls of *Pisum sativum:* regulation by ethylene. J. Exp. Bot. 21: 843–856.
23. Veen, B. W. 1970. Orientation of microfibrils in parenchyma cells of pea stem before and after longitudinal growth. Kon. Ned. Akad. Wetensch. 73: 113–117.
24. Veen, B. W. 1970. Control of plant cell shape by cell wall structure. Kon. Ned. Akad. Wetensch. 73: 118–121.