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Ethylene Effects in Pea Stem Tissue[1]

EVIDENCE OF MICROTUBULE MEDIATION

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DAVID A. STEEN[2] AND ARTHUR V. CHADWICK
Department of Biology, Loma Linda University, Loma Linda, California 92354

ABSTRACT

The marked effects of ethylene on pea stem growth have been investigated. Low temperature and colchicine, both known microtubule depolymerization agents, reverse the effects of ethylene in straight growth tests. Low temperature (6 C) also profoundly reduces the effects of gas in terms of swelling, hook curvature, and horizontal nutation. Deuterium oxide, an agent capable of rigidifying microtubular structure, mimics the effects of ethylene. Electron microscopy shows that microtubule orientation is strikingly altered by ethylene. These findings indicate that some of the ethylene responses may be due to a stabilizing effect on microtubules in plant cells.

The multinet hypothesis suggests that isodiametric expansion is prevented in normally elongating cells by radially oriented microfibrils in the cell wall (25). The finding that the orientation of microfibrils usually parallels that of microtubules (9, 21) has led to the suggestion that microtubules may be responsible for determining the orientation of newly deposited cellulose microfibrils (21, 23). Colchicine, which is known to alter microfibrillar deposition (12) and produce a mottled birefringence pattern (10), is also known to depolymerize microtubules (3, 12). On the other hand, D_2O produces a banded birefringence pattern similar to that exhibited by C_2H_4-treated tissue (8) and is known to have a "stabilizing" effect on microtubules (13).

The effects of C_2H_4 on microtubules have not been investigated in detail and, in light of the apparent link between microfibrils and microtubules, it seemed imperative to do so. Here, we present evidence suggesting that microtubules are indeed altered as a consequence of C_2H_4 treatment and that some of the pronounced effects caused by the gas can be reversed by low temperature or colchicine. A model is proposed in which C_2H_4 stabilized microtubular structure, which may cause the observed change in the orientation of microtubules and microfibrils leading to radial cellular expansion.

MATERIALS AND METHODS

Hook Curvature Studies. Seeds of *Pisum sativum* L. (cv. Alaska) were soaked for 6 h in running water and planted in moist vermiculite in wide-mouth glass jars. After growing 3 to 4 days in darkness at 24 C, the jars were sealed with air-tight covers and C_2H_4 was added to make appropriate final concentrations. Some jars were kept at 6 C for 48 h in either light or dark and others were incubated for various times at 24 C in the light or dark. After the predetermined period of time, epicotyls were cut from the seed and shadow-graphed. Hook angles were measured with a protractor (16).

Straight Growth Tests. Seeds were soaked as previously described, germinated in plastic bins containing moist vermiculite and grown in darkness at 24 C for 7 days. Under dim green light, 10-mm subhook sections were excised from the third internode of selected seedlings (plants whose third internode was less than 30 mm). Ten sections were floated in 10 ml standard growth media [2% sucrose, (w/v), 5 mM $CoCl_2$, 5 mM phosphate buffer (pH 6.8), 1 mM IAA, and appropriate concentrations of colchicine and C_2H_4] in 125-ml Erlenmeyer flasks which were sealed with vaccine caps and gently shaken in the dark at 24 C for 12 h. In some experiments, some of the flasks were incubated for 48 h at 6 C. After incubation, C_2H_4 levels were determined by GC, stem sections were weighed on an analytical balance, and lengths were measured to the nearest 0.1 mm.

Long Term Low Temperature Experiments. Pea seeds were surface-sterilized with a 5% Clorox solution, rinsed and soaked in sterile water for 6 h, and planted in moist vermiculite in autoclaved 1-liter glass jars. The jars were sealed with air-tight lids and appropriate concentrations of C_2H_4 were introduced. They were incubated at 6 C for up to 60 days in the dark. At 3-day intervals, the jars were ventilated and fresh C_2H_4 was introduced. Observations were made and pictures were taken periodically.

D_2O Growth Studies. Peas were treated as above except that

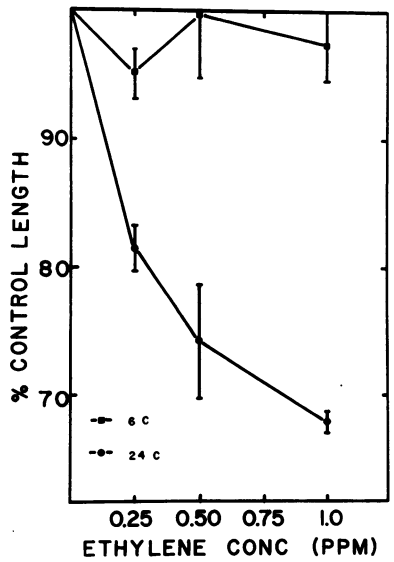

FIG. 1. Effects of C_2H_4 on elongation of 10-mm subapical sections when incubated at 24 C for 12 h (●) and at 6 C for 48 h (■). Vertical lines represent ±1 SE. Data are means of at least 30 replicates.

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[2] Present address: Southern Missionary College, Collegedale, TN 37315.

Table I. *Hook Curvatures of Etiolated Seedlings Treated with and without Added C_2H_4 at 24 and 6 C*

Treatment				Hook Angle				
Temperature	Light conditions	Incubation time	C_2H_4 (when added)	Control		C_2H_4		*P* Value[c]
				$\bar{X}$[a] ± SE	N[b]	$\bar{X}$ ± SE	N	
C		*h*	*ppm*	*degrees*				
24	Dark	24	10	125 ± 5	42	164 ± 5	48	<0.001
	Dark	12	1	120 ± 8	31	166 ± 5	41	<0.001
	Light	24	10	85 ± 5	29	178 ± 8	22	<0.001
6	Dark	168	100	125 ± 5	27	120 ± 6	19	>0.5
	Dark	48	10	104 ± 4	62	108 ± 4	73	>0.5
	Light	48	10	45 ± 4	24	94 ± 6	22	>0.001

[a] $\bar{X}$, mean.
[b] Number of replicates.
[c] *P* derived from *t* test.

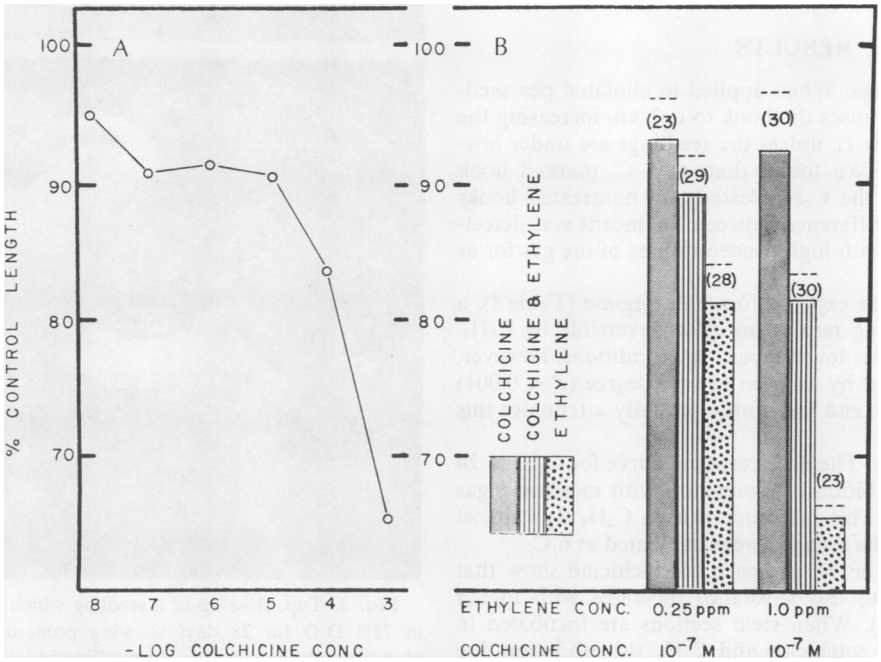

FIG. 2. A, effects of various concentrations of colchicine on elongation of 10-mm subhook sections; B, effects of various combinations of C_2H_4 and colchicine concentrations on elongation of 10-mm subhook sections. (- - -), ±1 SE. Numbers in parentheses are numbers of replicates.

Table II. *Effects of Low Temperature, C_2H_4, and D_2O on Germination and Morphological Development*

These data represent the general trend of at least six experiments (three for D_2O). After 9 days, all observed effects are ±3 days. E, epicotyls; R, roots.

Days from Planting	24 C						6 C			
	Control		C_2H_4		D_2O		Control		C_2H_4	
	E	R	E	R	E	R	E	R	E	R
	cm									
3	<1[a]	3–4[a]				<1				
6	20–30[a]	>15[a]		<0.1		1				
9				0.5		1.5		<1		<1
12				1.2	<1[b]	2[c]		1–4		1–2
15			1[b]	3[c]	1[b]	3[c]		3–4		2–3
24			2[b]	4[c]	2[b]	4[c]	1–2[a, d]		0.5[a, d]	
50							2–3[a, d]		1–2[a, e]	

[a] Geotropic.
[b] Swollen.
[c] Ageotropic.
[d] No swelling.
[e] Moderate swelling.

D_2O was substituted for various amounts of H_2O and growth was at 24 C. Samples of air were withdrawn periodically to check for D_2O-induced C_2H_4 production. Observations were made and pictures were taken periodically.

Tissue Preparation for Electron Microscopy. Presoaked seeds were planted in moist vermiculite in 1-liter wide-mouth jars. After 4 days in the dark, the jars were sealed and C_2H_4 was added to some for 12 h. Five-mm subhook sections then were excised from normal looking plants with terminal internode lengths of at least 15 mm. The sections were halved longitudinally and placed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 24 C for 24 h. Sections then were rinsed with buffer, dehydrated in an ethanol/propylene oxide series, and infiltrated with Epon. Sections were made with glass knives. The grids were poststained with uranyl acetate and lead citrate. Cell wall regions were photographed at various magnifications with a Siemens 1A transmission electron microscope. Three separate tissue batches were prepared and photographed in this manner.

RESULTS

Hook Curvature Studies. When applied to etiolated pea seedlings in the dark, C_2H_4 causes the hook to tighten, increasing the angle of curvature (Table I), unless the seedlings are under low-temperature conditions. We found that, at 6 C, marked hook opening occurs in both the C_2H_4-treated and nontreated hooks and that no significant difference between the means was detectable even when treated with high concentrations of the gas for as long as 7 days.

When pea seedlings are exposed to a light regime (Table I), a pronounced hook opening results, an effect reversible by C_2H_4. Our data show that, under low temperature conditions, however, the pea seedlings respond by an even greater degree ($P < 0.001$) of hook opening in light and C_2H_4 only partially attenuates this response.

Straight Growth Tests. The dose-response curve for C_2H_4 at 24 C shows increasing inhibition of elongation with increasing gas concentrations (Fig. 1). This inhibition due to C_2H_4 was almost entirely reversed when the sections were incubated at 6 C.

Straight growth tests in the presence of colchicine show that growth inhibition due to this substance increases with higher concentrations (Fig. 2A). When stem sections are incubated in various combinations of colchicine and C_2H_4, the inhibition due to C_2H_4 is significantly ($P < 0.01$) prevented (Fig. 2B). Instead of a synergistic effect of the two elongation inhibitors, there appears to be an antagonistic effect, with colchicine lessening the observed effect of C_2H_4.

Long Term Growth at 6 C. Alaska variety seeds germinate and grow much more slowly at low temperatures than they do at 24 C (Table II). Concentrations of C_2H_4 which have a characteristic and marked effect at 24 C show little or no effect on cold-grown seedlings (29). Horizontal nutation of stems is absent, and subhook swelling and growth inhibition due to C_2H_4 is drastically reduced. C_2H_4-treated roots are geotropic in the cold and show practically no swelling, in contrast to the striking effects caused by similar treatment at 24 C.

D_2O Growth Studies. Deuterated water causes delayed germination (Table II), swelling, ageotropic roots (Fig. 3), and, in 75% D_2O-treated seedlings, a curious release of axillary buds (29).

Electron Microscopy. Longitudinal and transverse sections were examined to determine microtubule orientation in elongating pea-stem parenchyma cells. The normal orientation of microtubules is radial, *i.e.* they are seen to run circumferentially just inside the plasmalemma (21). This orientation was confirmed in most cases by both longitudinal (Fig. 4) and transverse (Fig. 5) sections of control tissue. In tissue treated with C_2H_4, however, the microtubule orientation was found to be altered so that the patterns in transverse and longitudinal sections were reversed with respect to control tissue (Figs. 6 and 7). This condition was observed in 70% of the fields photographed in which microtubules were discernible (37 fields had microtubules). Microtubules in both orientations were seen in 11% of the fields and the remaining 19% (most of which were fields of C_2H_4-treated tissue) showed microtubules with orientations opposite to the stated conditions.

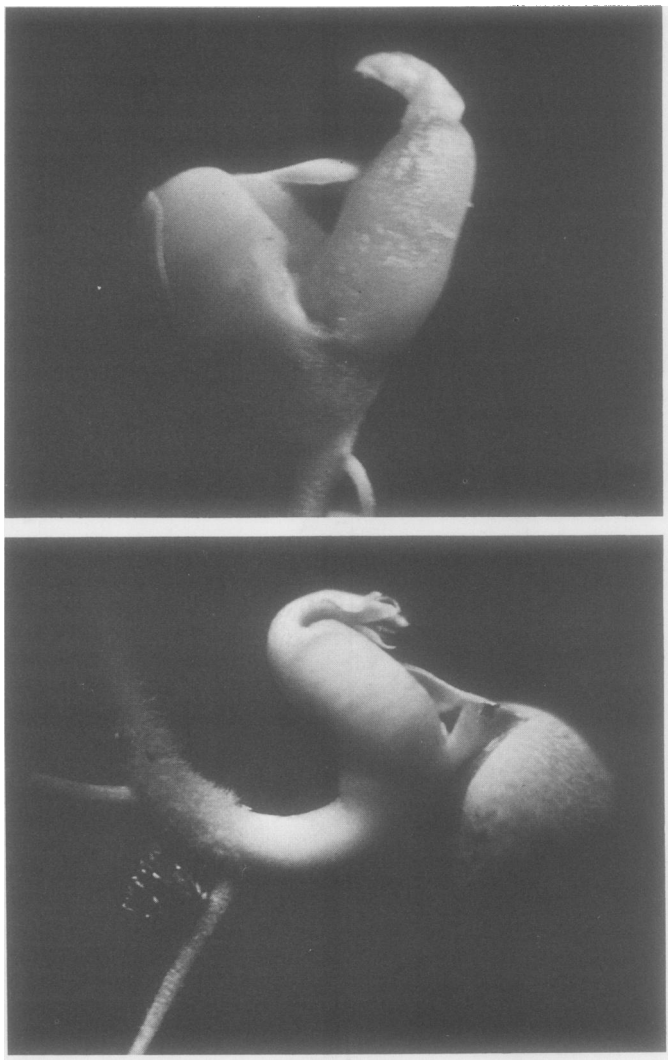

FIG. 3. Top, close-up of a seedling which was germinated and grown in 75% D_2O for 28 days showing pronounced swelling and reduced plumule; bottom, close-up of a seedling which was germinated and grown in a 5 ppm C_2H_4 atmosphere for 7 days at 24 C. Swelling is pronounced and the plumular hook is tight. The root is ageotropic and with prolific root hairs.

DISCUSSION

Radial cellular expansion is prevented by circumferentially oriented microfibrils in the cell wall of many plant tissues (25). Agents which cause swelling in etiolated pea-stem sections, such as benzimidazole (5, 10, 24), BA (10), kinetin and other cytokinins (11), colchicine and vinblastine sulfate (4), supraoptimal auxin concentrations, and C_2H_4 (5, 10), all reorient microfibrils, as evidenced by changes in the optical birefringence patterns. The characteristic banded pattern produced by C_2H_4 is indistinguishable from that observed in cells treated with benzimidazole, BA, kinetin, or supraoptimal auxin concentrations. Microfibril orientation is altered by a different mechanism, however, when cells are treated with colchicine and vinblastine sulfate. In this case, the

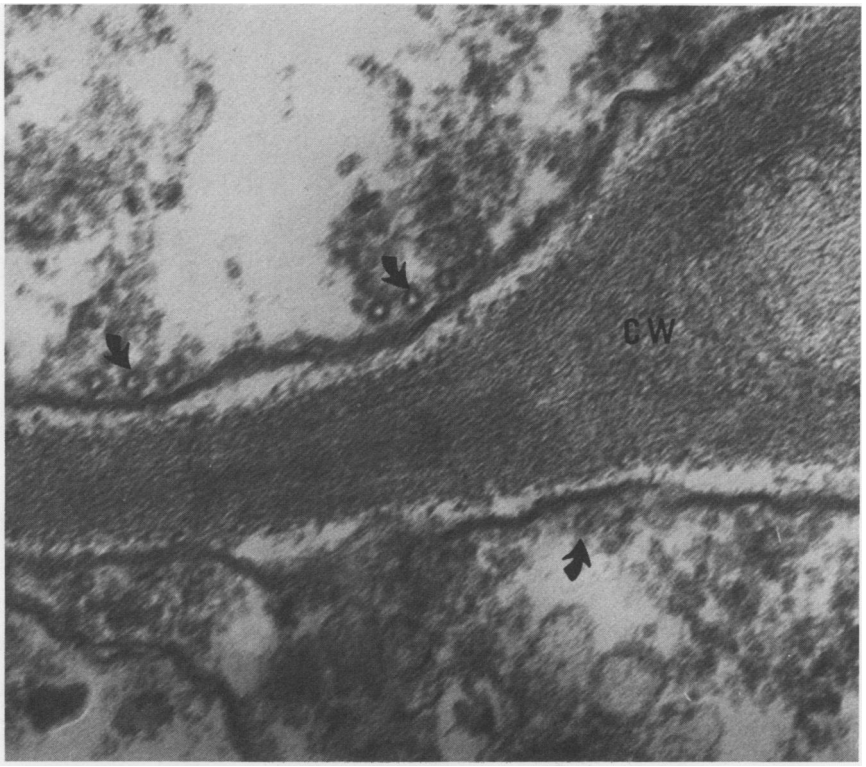

FIG. 4. Longitudinal section through the subapical zone of a pea stem from a control plant showing the cell w ' (CW) region of parenchyma cells. Note the microtubules (arrowed) commonly found in groups of three just beneath the plasmalemma. × 100,000.

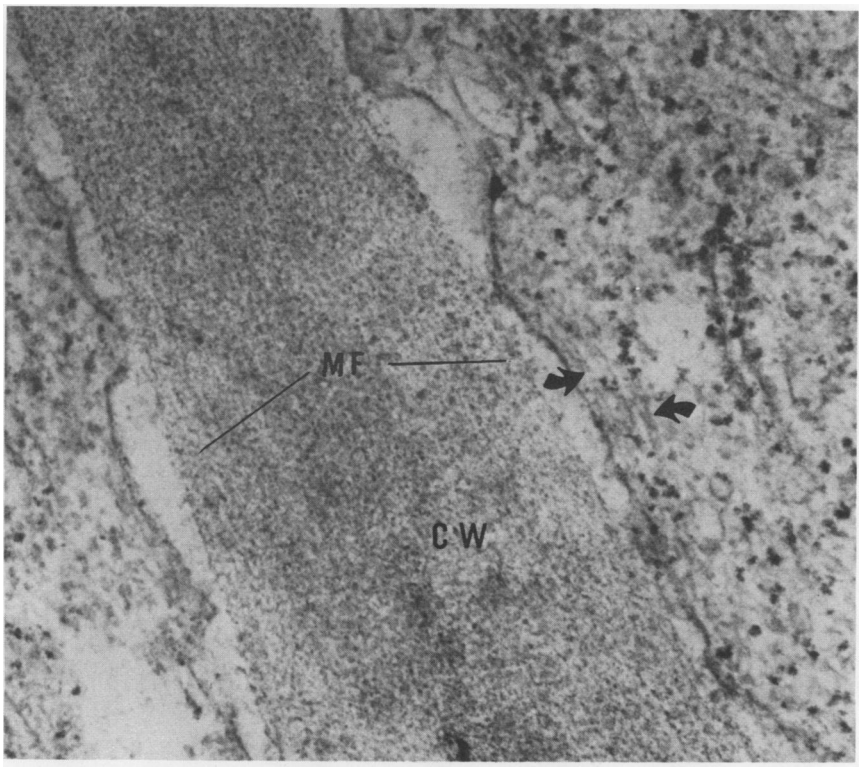

FIG. 5. Transverse section through the subapical zone of a pea stem from a control plant showing the cell wall (CW) region of parenchyma cells. Microtubules (arrowed) are found running circumferentially just beneath the plasmalemma. Note the orientation of newly deposited microfibrils (MF) which mirror the orientation of the microtubules. × 100,000.

optical birefringence pattern appears diffuse or mottled (10, 23), apparently due to the depolymerization of microtubules (21, 23). All these agents which cause swelling do so by altering cellulose microfibrillar deposition; the auxins, C_2H_4, BA, and benzimidazole alter the microfibrils to a longitudinal direction by an orderly redirecting of cellulose deposition (1, 24, 31) and the others do so

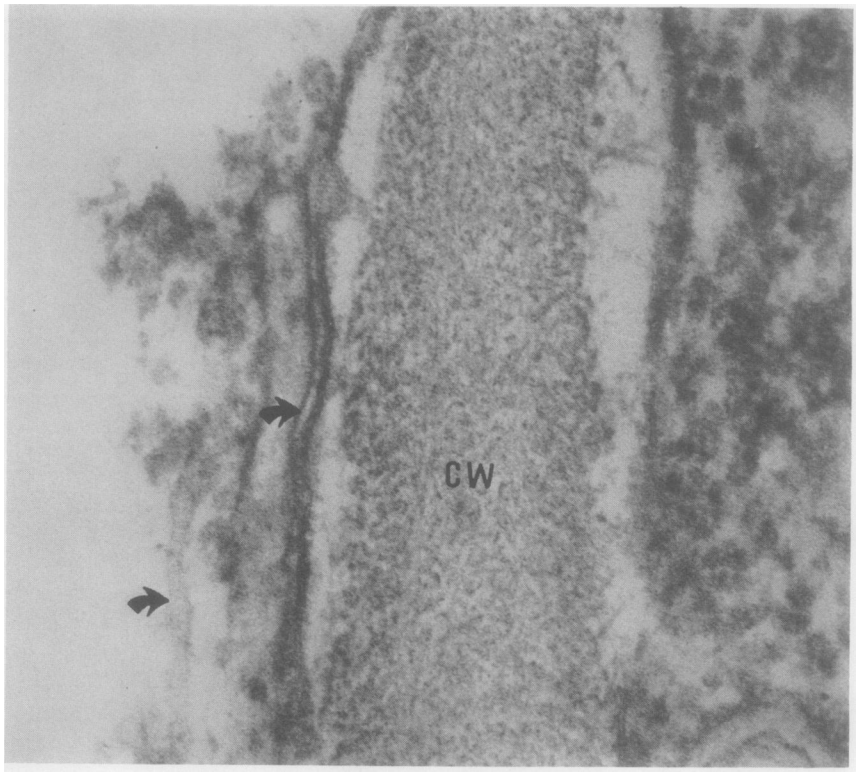

FIG. 6. Longitudinal section through the subapical zone of a pea stem from an C_2H_4-treated plant (1.0 ppm for 6 h) showing the cell wall (CW) region of parenchyma cells. Microtubules (arrowed) appear in a longitudinal orientation paralleling the long axis of the cell. × 130,000.

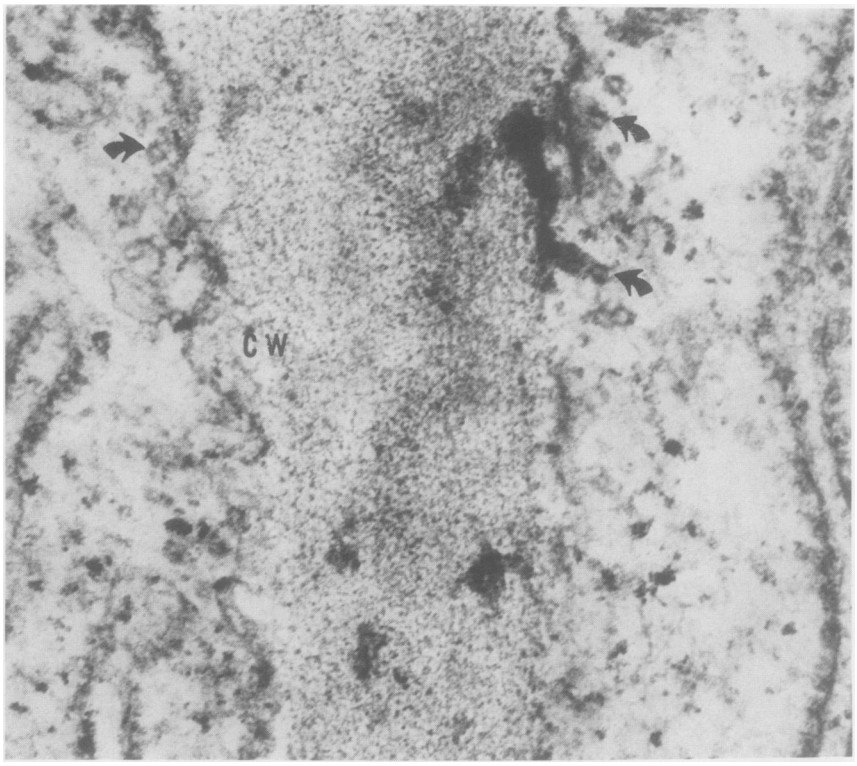

FIG. 7. Transverse section through the subapical zone of a pea stem from an C_2H_4-treated (1.0 ppm for 6 h) showing the cell wall (CW) region of parenchyma cells. Microtobules (arrowed) are shown in cross-section parallel to the orientation of newly deposited cellulose microfibrils in the cell wall. × 130,000.

by random deposition (4, 10). These findings clearly suggest that swelling is mediated by microfibrillar orientation.

Inasmuch as microtubule orientation usually (9, 21), but perhaps not always (28), parallels that of newly deposited cellulose microfibrils, it has been suggested that microtubules may be responsible for microfibrillar deposition (21, 23). Further support

for this suggestion is evidence presented here that C_2H_4-treated tissue had microtubules which, like the microfibrils, are reoriented to a predominantly longitudinal direction. Therefore, a key to microfibrillar orientation and radial swelling in cells may lie in the structure and orientation of microtubules.

Microtubules are protein polymers in which the spiraling monomers form hollow, unbranched, cylindrical structures about 240 Å in diameter (21). Inoué and Sato (15) have proposed a dynamic state of equilibrium between pools of monomers and the microtubule polymers which undergo cyclic breakdown and reformation. Under certain conditions, such as low temperature (14, 30), high hydrostatic pressure (14, 22), and treatment with colchicine and certain other compounds, the dynamic equilibrium is shifted towards the monomer state, resulting in a breakdown of microtubules. Conversely, elevated temperature (19, 30), low hydrostatic pressure (19), and D_2O (7, 19) result in stabilization of microtubular structure due to a shift towards increased polymerization. Thus, conditions which alter the birefringence patterns and microfibrillar deposition also alter the stability of microtubules.

The similarity of effects of C_2H_4 and D_2O (Table II) suggest that C_2H_4 may be affecting microtubules in a manner similar to that of heavy water, that is, by stabilization of the microtubular structure. This does not immediately suggest a reason why the microtubular orientation should be altered, but little is known about organization and movement of microtubules. The same type of alteration has been reported in response to applied kinetin in experiments where C_2H_4 levels were not monitored (26). One may infer some relationship at the microtubular level between the observed effect of C_2H_4 on microtubules and the ability of the gas to inhibit mitosis (2).

Gross and Spindle (13) have suggested hydrogen bonding to be the force responsible for stabilization of microtubules composing the mitotic apparatus. This suggestion was based largely upon the rapid and reversible arrest of mitosis following application of D_2O, apparently as a result of excessive stabilization of microtubules and the evidence that D_2O forms stronger intermolecular D—O bonds.

A consideration of temperature effects upon the stability of microtubules leads one to suspect that a major component of microtubular bonding forces may be other than hydrogen bonds (19, 30). If hydrogen bonding supplied the major impetus, the bonds should be weaker at higher temperatures and stronger at lower temperatures. One of the most consistent observations in connection with microtubules is that, in fact, the opposite is true. This latter condition is precisely what one would expect were hydrophobic bonds the major source of interaction between subunits (20). The observed effect of D_2O would be explainable on the basis of an increased strength of hydrophobic bonding resulting from the reduction of entropy imposed by slightly stronger D—O attractive forces. Hydrophobic bonds are being recognized, with increasing frequency, to have a significant role in interactions at the cellular level (32).

If hydrophobic bonds constitute the major stabilizing force in microtubule polymerization, one possible mechanism for C_2H_4 action suggests itself. Although speculative, that mechanism is based on the well-accepted presence of binding sites on the tubulin molecule for Mg^{2+} (18) and Ca^{2+} (27) and the suggestion that the latter may be capable of binding C_2H_4 (6). Our model suggests that, in the absence of C_2H_4, a dynamic equilibrium is maintained between the polymer and monomers by the presence of hydrophilic sites (divalent cations) on the individual subunits. When C_2H_4 is bound to this site, the equilibrium is shifted in favor of the polymer by the resulting enhancement of hydrophobic bonds. This mechanism would explain the observed competitive inhibition of D_2H_4 action by CO_2 [which is thought to bind to the C_2H_4 site (6, 17)] inasmuch as CO_2 would create a hydrophilic center due to the polarity of the molecule and its affinity for water. Binding of CO_2 on a microtubule polymer in place of C_2H_4 would shift the equilibrium toward a depolymerized or "loose" state by the reduction of hydrophobic bonding. It is significant in this regard that Weisenberg (33) found it necessary to remove Ca^{2+} in order to facilitate polymerization of microtubules *in vitro*. Also of interest is the finding that colchicine binds to a hydrophobic site (3).

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