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Ethylene Production by Auxin-Deprived, Suspension-Cultured Pear Fruit Cells in Response to Auxins, Stress, or Precursor

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ABSTRACT

Auxin-deprived, mannitol-supplemented, suspension-cultured pear (*Pyrus communis* L. Passe Crassane) fruit cells produce large quantities (20–40 nanoliters ethylene per 10^6 cells per hour) of ethylene in response to auxins, $CuCl_2$ or 1-amino-cyclopropane-1-carboxylic acid (ACC). Maximum rates of production are achieved about 12 hours after the addition of optimal amounts of indoleacetic acid (IAA), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4 to 5 hours after the addition of $CuCl_2$ and 1 to 2 hours after the addition of ACC. Supraoptimal concentrations of IAA result in a lag phase followed by a normal response. High concentrations of NAA and 2,4-D result in an early (4–5 hours) stress response and injury.

Continuous protein and RNA synthesis are essential for elaboration of the full IAA response; only protein synthesis is necessary for the response to $CuCl_2$ and ACC. Based on polysomal states and rates of amino acid incorporation, $CuCl_2$ partially inhibits protein synthesis while nonetheless stimulating ethylene production. In general, ethylene production by the pear cells resembles that of other plant systems. Some differences may reflect the sensitivity of the cells and are discussed. The relatively high levels of ethylene produced and the experimental convenience of the cultured cells should make them especially suitable for further investigations of ethylene production and physiology.

Ethylene production by suspension-cultured plant cells has been generally associated with cell division and found to decrease rapidly after cells enter their stationary phase (2, 15, 19, 23). Dividing, suspension-cultured pear cells also produce low levels of ethylene (28). However, preliminary experiments revealed that, when deprived of auxin and supplied with mannitol, the now quiescent, and perhaps senescent, pear cells produced relatively large amounts of ethylene in response to various stimuli, *e.g.* auxins, $CuCl_2$, and ACC[3]. The auxin-deprived cultured pear cells thus offer prospects of an experimentally very convenient system for the study of ethylene metabolism and physiology. Toward that end, the objective of this paper is to describe the kinetics, inhibitor effects, and other characteristics of the ethylene response along with a brief comparative discussion. In a companion study (unpublished), ethylene production is utilized to illustrate that the quiescent, suspension-cultured pear cells undergo a distinct, senescence-related physiological transition.

A preliminary report of this work has appeared (30).

MATERIALS AND METHODS

Chemicals. IAA was obtained from Eastman Kodak; 2,4-D, Act D, CHI, and NAA from Sigma; ACC from Calbiochem, and [U-^{14}C]leucine (312 mCi/mmol) from Schwarz/Mann. L- and D-MDMP were a gift from Dr. R. Baxter of Shell Biosciences Laboratory and AVG was a gift from Dr. Arthur Stempel of Hoffman-La Roche.

Cells. The strain of cells used in these experiments was established in 1972 by Pech *et al.* (27) from young 'Passe Crassane' pear (*Pyrus communis* L.) fruit.

Cell Culture. Growth media consisted of the mineral nutrients of Murashige and Skoog (25) and the organic nutrients of Nitsch *et al.* (26), as slightly modified by Pech and Romani (28). Cell suspensions were grown at 27°C in 2-L flasks in the dark on a rotary shaker (100–120 rpm) and subcultured every 7 d using about 10% inoculum consisting of a decanted cell suspension. Cells to be used in ethylene studies were first deprived of 2,4-D as described by Pech and Romani (28). In brief, after 7 d in growth medium, the cells were allowed to settle, decanted, and washed twice with an approximately equal volume of fresh growth media less 2,4-D before transferring them into a similar medium. Approximately 2 L of cell suspension in 4-L flasks at final cell densities of 2 to 3 × 10^6 cells/ml were kept for 9 to 10 d in the auxin-deprived growth medium. The cells were then allowed to settle, decanted, washed twice with an equal volume of 'aging' medium, and transferred to aging medium at about 1 to 2 × 10^6 cells/ml. The aging medium of Codron *et al.* (7) consisted of one-fourth concentration of the mineral and organic nutrients present in the standard growth medium, no 2,4-D but with added 0.385 M mannitol and 0.015 M sucrose.

Ethylene Determination. Ethylene was estimated using 125-ml culture flasks containing 50 ml cell suspension in aging medium incubated at room temperature on a rotary shaker at 120 rpm. To measure ethylene, the flasks were flushed for 15 s with a vigorous flow (approximately 10 L/min) of air passed through a 'Balston' borosilicate glass microfiber filter and wool. The resultant ethylene concentration was no more than 10 to 20 nl/l. The flasks were then capped with a septum. After 30 min, or for other periods as indicated, a 5-ml sample of head-space gas was taken for ethylene determination (Carle model 221 analytical GC, alumina column at 80 C, N_2 as the carrier gas, and a flame ionization detector).

Estimation of Per Cent Live Cells. Evans blue (0.5% w/v) was added to 1 ml of cell suspension and after at least 5 min viewed under a light microscope at × 150. As described by Gaff and Okong 'O-Ogola (9) and recently confirmed by Smith *et al.* (36), dead cells were selectively stained by Evans blue. Three fields of at least 100 cells each were counted.

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; Act D, actinomycin D; AVG, aminoethoxyvinylglycine; CHI, cycloheximide; EFE, ethylene-forming enzyme; MDMP, 2-(4-methyl-2,6-dinitroanilino)-*N*-methyl propionamide; NAA, naphthaleneacetic acid; PCV, packed cell volume; SAM, *S*-adenosylmethionine.

Estimation of Cell Count. A 12-ml aliquot of cell suspension was placed in a graduated conical tube and centrifuged for 5 min at 2,000g. Cell count was obtained from a calibration curve relating PCV to number of cells. Two ml PCV equals approximately 10^6 cells and approximately 100 mg dry weight.

ACC Extraction and Assay. The procedure of Lizada and Yang (21) with a few adaptive changes was utilized. Two g of frozen cells were homogenized with a polytron in 4 ml of 10% (w/v) TCA for 1 min and then centrifuged for 20 min at 27,000*g*. The supernatant fraction was loaded on a column of approximately 5 g Dowex 50 W-x8, washed with water, eluted with 2 N NH_4OH, and the eluate concentrated and assayed as described by the above authors. The efficiency of ACC conversion to ethylene ranged between 65 and 85%.

Determination of Per Cent Polysomes. Cell suspensions (50 ml) were vacuum filtered, immediately frozen in liquid N_2, and stored at −50°C. The procedure described by Romani and French (33) used for the isolation of ribosomes for pear fruit tissue was utilized with few modifications. A 1- to 2-g sample of frozen cells, yielding enough ribosomes for one gradient, was homogenized in the presence of liquid N_2, 0.1 to 0.2 g sand, and 13 ml isolation medium. No additional base was needed to maintain the pH of the homogenate at 8.5. Following centrifugation, the gradients were scanned at 254 nm and the monosomal and polyribosome fractions collected separately for the determination of volume and *A* at 260 nm with 30% sucrose solution as a blank.

Incorporation of [^{14}C]Leucine. Protein synthesis was estimated by the method of Ferrari and Widholm (8) with some modifications. Cell suspensions in aging media were first diluted with fresh aging medium to a cell density of 2 to 4 × 10^5 cells/ml and 5-ml aliquots of this suspension were incubated with 0.02 μCi [U-^{14}C]leucine in 25-ml flasks on a rotary shaker (100 rpm). Specific treatments are described under "Results."

At the end of the incubation period, the cells were collected on a 2.4-cm glass fiber disc. Total isotope uptake was estimated by washing the cells three times with 5-ml aliquots of water containing 0.1 mM cold leucine. Incorporated counts were estimated by removal of unincorporated and indiscriminately bound isotope with three washings using a CH_3OH:$CHCl_3$:H_2O (12:5:3, v/v/v) mixture. The discs were placed in a scintillation vial with 5 ml Aquasol.

RESULTS

Ethylene Production in Response to Auxins. Ethylene production in response to different stimuli was first examined for pear cells in three different states: growing (in growth medium) quiescent (in auxin-deprived growth medium), and 'senescent' (in aging medium). As confirmed by subsequent, definitive comparative experiments (30), senescent cells are several-fold more responsive and were therefore chosen for the kinetic studies reported below.

After a few days in aging medium, unperturbed suspension cultures of pear cells evolve small amounts of ethylene, in the order of 0.5 to 4 nl $C_2H_4/10^6$ cells·h. However, ethylene production increases several-fold in response to IAA (Fig. 1) with rates of approximately 40 nl/10^6 cells·h attained 12 h after the addition of 50 to 250 μM IAA. At the higher IAA concentrations (500 and 1,000 μM), there is a concentration-dependent lag.

Ethylene production in response to NAA and 2,4-D (Fig. 1) is, in general, similar to that observed with IAA. However, there were no lag periods at higher than optimal concentrations. Rather, NAA concentrations of 250 μM or above and 10 μM 2,4-D or above induced an early but transient increase in rate of ethylene production which reached a maximum in 2 to 4 h. Progressive browning of the suspension followed by cell death was more pronounced with 2,4-D than with NAA. At intermediate concentrations of NAA or 2,4-D, the initial ethylene burst was followed by a period of higher and sustained ethylene evolution.

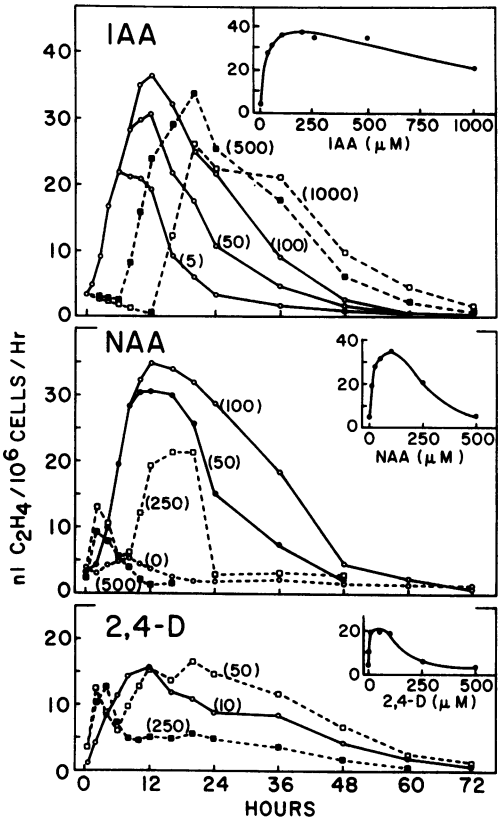

FIG. 1. Ethylene production by suspension-cultured pear fruit cells in response to increasing concentrations (numbers in parentheses) of IAA, or 2,4-D. Response to sub- and optimal (——) or to supra-optimal (---) auxin concentrations. Insets: Maximum rates of ethylene production (measured at hour 12) as a function of stimulator (IAA, NAA, or 2,4-D) concentration.

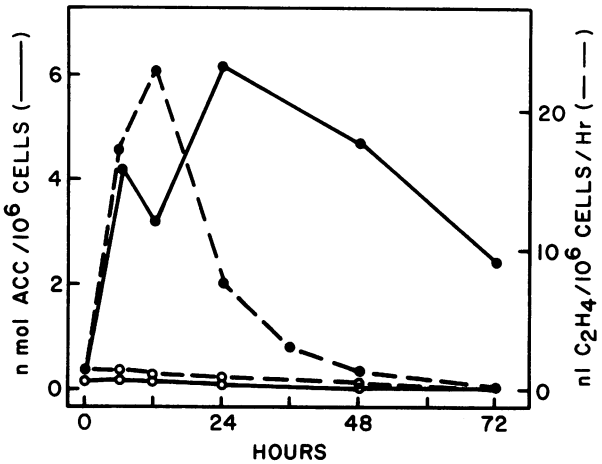

FIG. 2. Ethylene evolution (---) and ACC accumulation (——) by control (○) and IAA (50 μM) treated (●) pear cells.

Maximum ethylene production rates induced by a range of IAA, NAA, or 2,4-D concentrations are summarized in the insets of Figure 1. A half-maximal response is attained with 5 μM IAA or NAA and with 1 μM 2,4-D. Inhibitory effects of higher concentrations of NAA and 2,4-D are clearly seen.

IAA-Induced Ethylene Production as Related to ACC. The rise in rate of ethylene production following addition of IAA is

Table I. *Inhibition of IAA-Induced Ethylene Production by AVG*

Treatment	C_2H_4 Production[a]	Inhibition
	nl/10⁶ cells·h	%
Control	2.3	
Control + AVG, 50 μM	0.4	83
IAA, 100 μM	34.0	0
IAA + AVG, 10 μM	10.3	70
IAA + AVG, 25 μM	4.3	87
IAA + AVG, 50 μM	2.1	94
IAA + AVG, 100 μM	1.1	97

[a] Rates were measured 12 h after the addition of IAA when ethylene production rates were maximal.

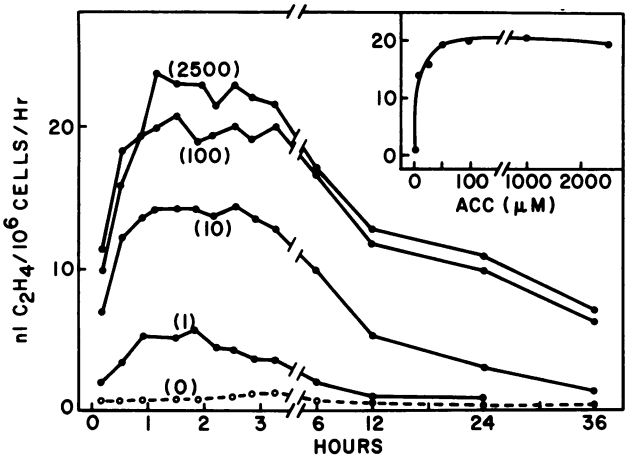

FIG. 3. Pattern of ethylene production by pear cells in response to increasing concentrations (numbers in parentheses) of ACC. Inset: Maximum rates of ethylene production (measured at hour 2) as a function of ACC concentration.

accompanied by an increase in ACC (Fig. 2). The formation of ACC as an intermediate is corroborated by the effects of AVG (Table I), known to inhibit the formation of ACC (41). ACC levels remain relatively high (Fig. 2) during the later period of decreasing ethylene production raising the possibility that EFE, required for the conversion of ACC to ethylene (39), may become limiting.

This possibility was tested with exogenous ACC which does itself induce a 20- to 30-fold increase in ethylene production by non-IAA treated senescent pear fruit cells (Fig. 3). The response is rapid, reaches a maximum in 1 h, is sustained for an additional 1 or 2 h, and then declines. Maximum rates of ethylene evolution follow addition of about 50 μM ACC and concentrations as high as 2,500 μM cause neither additional stimulation nor inhibition (inset, Fig. 3). Based on these observations, 100 μM ACC was used in subsequent studies.

The rapid response to ACC (Fig. 3) assures that the relatively gradual rise in ethylene evolution following the addition of auxins (Fig. 1) is not constrained by a shortage of EFE. Since additions of ACC at any time to IAA-treated cells result in a burst in rate of ethylene production (Fig. 4), it would appear that EFE is also not limiting during the decline of the IAA response. The apparent contradiction, *i.e.* exogenous ACC is converted to ethylene (Fig. 4) by cells that normally are accumulating ACC (Fig. 2), is discussed below. It is clear, however, that the responsiveness to ACC is greatly diminished 60 h after IAA treatment.

Effects of Medium Renewal and Ethylene on Ethylene Production. To test if continued ethylene production is constrained by the accumulation of inhibitory substances, auxin-treated cells were filtered and transferred to fresh medium, with or without 100 μM IAA, at designated intervals after initial treatment with

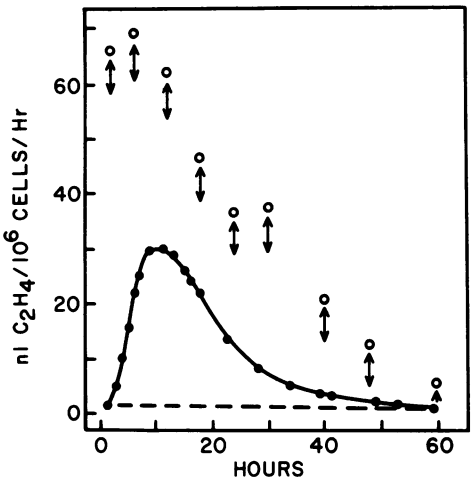

FIG. 4. Ethylene production in response to ACC added to pear fruit cells at increasing time intervals after treatment with IAA. Rates of ethylene production by non-IAA treated controls (---) and following the addition of 100 μM IAA at time 0 (●——●). Burst (↕) in ethylene production measured 2 h after the addition of 100 μM ACC at 0, 6, 12, 18, 24, 30, 40, 48, or 60 h after IAA (O).

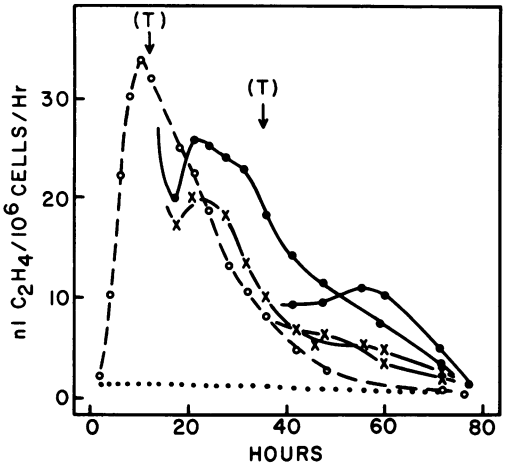

FIG. 5. Ethylene production by IAA-treated pear cells (O--O) as affected by transfer (T) to fresh medium lacking (×——×) or containing (●——●) 100 μM IAA 12 or 36 h after the initial IAA (100 μM) stimulus. Non-IAA treated control (·····).

IAA. Only a small increase in ethylene production followed transfer to fresh medium lacking IAA and a slightly higher production occurred in the presence of IAA (Fig. 5). It appears that the decline in the ethylene response cannot be attributed to either toxic by-products or insufficient levels of IAA. Nor, is the decline in ethylene production merely a function of cell age since control cells first supplied with IAA after 12 or 36 h (29) produced C_2H_4 at near maximal rates. Finally, the decline is not due to ethylene self- or feedback inhibition (31) since cells exposed to 60 or 250 μl/l ethylene for 18 h still produced large amounts of ethylene in response to stimulus (Table II).

Copper-Induced Ethylene Production. $CuCl_2$ was employed to more directly assess the effects of toxic stress (Fig. 6). Following a lag period of 0.5 to 1 h, there was an almost linear increase in rate of ethylene production in response to $CuCl_2$ concentrations ranging from 25 to 250 μM with a resultant 30- to 40-fold enhancement over control levels. Maximum ethylene production in response to $CuCl_2$ was reached in 4 to 5 h, and this temporal relationship holds over the 50 to 175 μM of $CuCl_2$ concentrations yielding maximum or near maximum rates of ethylene produc-

Table II. *Ethylene Production by Pear Fruit Cells in Response to 100 μM $CuCl_2$ with and without Prior 18-Hour Exposure to Ethylene*

Cells (50 ml) on their 5th or 6th d in aging medium were placed in 125-ml Erlenmeyer flasks and gassed with a continuous flow (30 ml/min) of 60 or 250 μl/l ethylene in air.

Treatment	C_2H_4 Production
	μl/10⁶ cells·h
Experiment 1[a]	
Control	0.36 ± 0.05[b]
+ $CuCl_2$	22.0 ± 2.0
C_2H_4, about 250 μl/l	0.54 ± 0.04
+ $CuCl_2$	21.0 ± 1.5
Experiment 2[a]	
Control	0.16 ± 0.03
+ $CuCl_2$	16.4 ± 1.1
C_2H_4, about 60 μl/l	0.24 ± 0.05
+ $CuCl_2$	19.4 ± 3.5

[a] Two different subcultures of Passe Crassane pear cells.

[b] Mean and range of three replicate flasks. Maximum rates of ethylene production measured 5 to 6 h after the addition of $CuCl_2$.

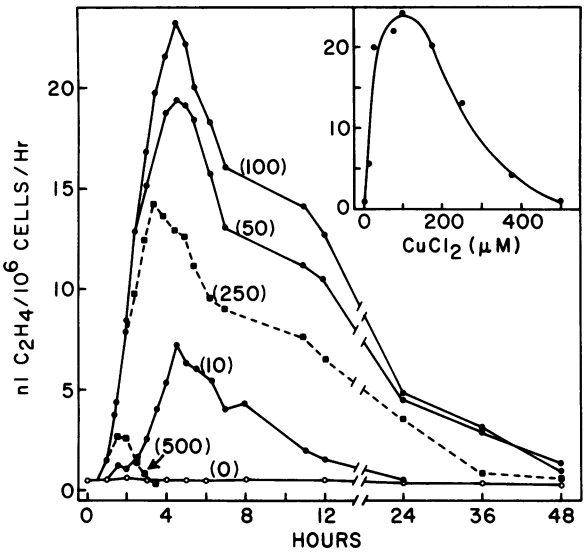

FIG. 6. Ethylene production by cultured pear fruit cells in response to increasing μM concentrations (number in parentheses) of $CuCl_2$. Inset: Maximum rates of ethylene production (measured at hour 4) as a function of $CuCl_2$ concentration.

tion. Toxicity and inhibition of ethylene production occurs at higher concentrations (Fig. 6, inset). Harmful effects of the higher copper concentrations, a browning of the cell suspension and accompanying cell death, can be observed within 6 to 12 h.

The Requirement for RNA and Protein Synthesis. Protein and RNA synthesis have long been implicated as requisites for ethylene production (18). More than 90% inhibition of IAA-stimulated ethylene production was observed when any one of several inhibitors, 10 μM D-MDMP, CHI, Act D (29), or 50 μM AVG (Table I), was added together with IAA. The K_i for D-MDMP, Act D, and AVG was approximately 0.5, 2.5, and 5 μM, respectively. Equally pronounced inhibition was observed if D-MDMP, CHI, or AVG were added together with $CuCl_2$ (-ACC column of Table IV). However, as seen by the response to inhibitors of transcription (Table III), RNA synthesis is not a prerequisite for the response to $CuCl_2$. Low levels (10–30%) of inhibition when Act D or cordycepin were added with, or 1 h after $CuCl_2$, are attributed to indirect effects on protein synthesis (20).

Protein synthesis inhibitors (D-MDMP or CHI) or an RNA synthesis inhibitor (Act D) added about midway in the response

Table III. *Inhibition of $CuCl_2$-Stimulated Ethylene Production by Act D and Cordycepin Added at Different Times after 100 μM $CuCl_2$*

Time of Addition	Inhibition[a]	
	Act D (10 μM)	Cordycepin (10 μM)
h	%	
0	29	23
1	13	10
2	2	8
3	0	0
4	0	1

[a] Per cent inhibition was based on ethylene production rates in the presence and in the absence of the inhibitor measured 4 to 5 h after the addition of the inhibitor.

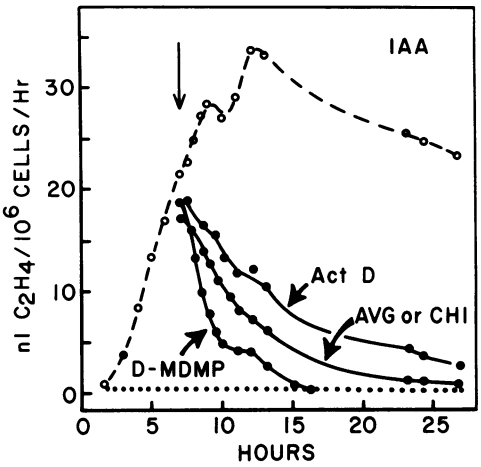

FIG. 7. Inhibited rates of IAA-stimulated ethylene production following the addition (↓) of 10 μM D-MDMP, 10 μM CHI, or 50 μM AVG, or 10 μM Act D 7 h after IAA (●——●). Nontreated (·····) and IAA-treated (O- - -O) controls.

to IAA caused an immediate decline in rates of IAA-stimulated ethylene production (Fig.7). This observation implies that continuous protein and RNA synthesis are required for a maximal response to IAA. Very similar inhibitory effects of protein but not RNA synthesis inhibitors were observed for $CuCl_2$-stimulated ethylene production (29).

When IAA or $CuCl_2$-treated cells were held for several hours in the presence of either AVG or protein synthesis inhibitors and then supplied with ACC, varying increments in ethylene production were observed (Table IV). Cells treated with AVG responded similarly to the nontreated controls affirming that AVG affects neither EFE synthesis nor, as shown by Yu *et al.* (39), its action in converting exogenous ACC to ethylene. Absence of significant inhibition by AVG implies that stimulation of EFE synthesis by IAA and especially by $CuCl_2$ is not in response to increasing levels of ACC. The D-MDMP and CHI-treated cells exhibited a 60 to 75% reduction in their ability to convert ACC to ethylene irrespective of IAA or $CuCl_2$ treatment. This is further confirmation that protein synthesis is required for maintenance of EFE. The absence of an effect by Act D or cordycepin (data not shown) implies that the requisite mRNA is stable over this period of time.

Inhibitor Effects on Polysomes and Protein Synthesis. Typical polysomal profiles and data for the effects of IAA, $CuCl_2$, and various inhibitors are shown in Figure 8 and Table V. D-MDMP caused a dramatic decrease in polysomes whereas, as expected, the inactive isomer L-MDMP had no effect. CHI had a much less pronounced effect on polysomes, consistent with its 'locking'

Table IV. *Conversion of ACC to Ethylene as Affected by Prior Exposure to IAA or $CuCl_2$ with or without the Added Presence of Inhibitors*

Treatment	Rate of C_2H_4 Formation		
	− ACC	+ ACC	Increment
	nl/10⁶ cells·h		
Experiment 1[a]			
Control	0.3	10.7	10.4
+ IAA	23.4	28.6	5.2
+ IAA + D-MDMP, 10 μM	0.8	4.3	3.5
+ IAA + CHI, 10 μM	1.6	5.6	4.0
+ IAA + AVG, 50 μM	1.3	13.4	11.1
Experiment 2[b]			
Control	0.5	21.8	21.3
+ $CuCl_2$	12.0	20.4	8.4
+ $CuCl_2$ + D-MDMP, 10 μM	0.8	6.2	5.4
+ $CuCl_2$ + CHI, 10 μM	0.5	5.3	4.7
+ $CuCl_2$ + AVG, 50 μM	0.3	22.8	22.5

[a] Inhibitors added at 7 h and ACC at 28 h after stimulation by IAA.
[b] Inhibitor added together with $CuCl_2$. ACC 18 h later.

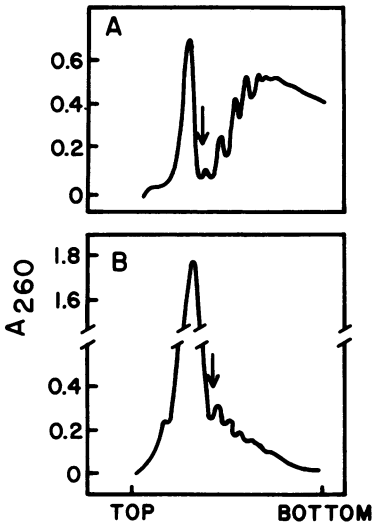

FIG. 8. Sucrose density gradient polyribosomal profiles. Control cells (A) and cells 1 h after exposure to 10 μM D-MDMP (B). Arrow (↓) indicates the separation point for quantitative estimates of monosomes and polysomes.

of ribosomes on the mRNA (10). AVG had no effect on the polysomal population and Act D caused a slight decrease in polysomes. $CuCl_2$ reduced the percentage of polysomes by about half.

The inhibitory effects of copper and D-MDMP on protein synthesis were also assessed via the incorporation of [^{14}C]leucine into protein. Uptake and incorporation of [^{14}C]leucine is linear for cell densities in the range 0.25 to 3×10^5 cells/ml from about 10 min to 1.5 h (29). The incorporation to uptake ratios (Table VI) indicate a significant reduction in protein synthesis whose magnitude fully corroborates the previous results obtained via polysomal analyses. It is noteworthy that D-MDMP effectively inhibited both protein and ethylene synthesis, whereas $CuCl_2$ partially inhibits protein synthesis while stimulating ethylene production.

DISCUSSION

In keeping with the objectives of this study, a brief comparison of ethylene production by the cultured fruit cells with that by other plant systems seems appropriate.

Table V. *Effect of CHI, D-MDMP, AVG, and Act D on the Polysome Levels in IAA or $CuCl_2$-Treated Pear Fruit Cells*

Treatment (Time Lapse)[a]	Polysomes	Decrease in Polysomes
	%	
Control, 7 h	84	0
+ Act D, 6 h	81	5
+ AVG, 7 h	87	0
+ IAA, 7 h	86	0
+ IAA, 7 h, + CHI, 6 h	61	28
+ IAA, 7 h, + D-MDMP, 1 h	17	80
+ IAA, 7 h, + D-MDMP, 6 h	11	87
+ IAA, 7 h, + L-MDMP, 6 h	86	0
+ $CuCl_2$, 3.5 h	44	50
+ $CuCl_2$, 2.5 h, + D-MDMP, 10 min	22	74
+ $CuCl_2$, 2.5 h, + D-MDMP, 30 min	14	84
+ $CuCl_2$, 2.5 h, + CHI, 30 min	65	23

[a] Time lapse between the start of the incubation, addition of the inhibitor, and/or measurement of polysomal level.

Table VI. *Effect of $CuCl_2$ and D-MDMP on the Uptake/Incorporation Ratio of [^{14}C]Leucine by Cultured Pear Cells*

Incubation Time	Incorporation/Uptake		
	Control	$CuCl_2$	$CuCl_2$ + D-MDMP
min			
10	51	30 (41)[a]	14 (73)
20	51	23 (55)	7 (87)
30	50	22 (54)	8.5 (83)

[a] Numbers in parentheses, per cent inhibition.

The Auxin Response. In general, the time and concentration dependence of ethylene production by pear fruit cells in response to IAA, NAA, and 2,4-D at low and saturating concentrations (Fig. 1) is similar to that observed in other plant (6) and cell (23) systems. At higher concentrations, however, one deserves distinct kinetic differences. The initial burst of ethylene elicited by 2,4-D and NAA maximizes in 2 to 4 h and is comparable, in this respect, to that elicited by $CuCl_2$ stress (Fig. 6). In contrast, the response to high IAA concentrations exhibits an initial lag whose duration is a function of IAA concentration. Implied is an ability of the cells to lower the effective concentration of IAA to a stimulatory level. Auxin conjugation and decarboxylation have been observed in other plant (16) and cultured cell (36) systems.

Auxin removal could also account for the decrease in ethylene evolution that is observed about 12 h after induction (Fig. 1A). However, that seems unlikely since a second addition of IAA 24 or more hours after the first addition never resulted in a significant increase in rate of ethylene evolution. Restraints by virtue of metabolite depletion or by-product accumulation appear unlikely since transfer of cells showing a decreasing ethylene production to a fresh medium (Fig. 5) does not result in a significant recovery of ethylene output. Finally, as noted in Table II, ethylene autoinhibition is also unlikely.

It has been shown by Yu and Yang (40) that auxin enhances ethylene production by stimulating the activity of ACC synthase and the formation of ACC. Since a rapid increase in ACC is also observed upon the addition of IAA to the senescent pear cells (Fig. 2) and ethylene evolution is inhibited by AVG (Table I), it appears that ethylene biosynthesis in the cultured pear cells occurs via SAM and ACC as described by Adams and Yang (1). It is noteworthy that IAA- or $CuCl_2$-stimulated EFE formation occurs even if ACC synthesis is inhibited by AVG (Table IV).

The Copper Response. $CuCl_2$-induced ethylene evolution by

cultured pear cells exhibited a lag phase of about 30 min (Fig. 6), roughly similar to that observed following mechanical injury to other systems (4, 35). A maximal response is reached in 4 to 5 h, also analogous to that observed upon $CuCl_2$ treatment of mung bean hypocotyls (17) and following mechanical injury of fruits (24), or flower buds (11). Finally, $CuCl_2$-stimulated ethylene evolution in cultured pear cells is accompanied by an increased ACC content as observed following $CuCl_2$ treatment of mung bean hypocotyls, or orange albedo discs (41), the mechanical injury of fruits (4) and leaves (14), waterlogging in tomato plants (5), and drought stress in wheat leaves (3). Pear cells may be said to exhibit a characteristic plant stress response.

Noting that AVG added 30 or 40 min after wounding of bean leaves did not inhibit further ethylene production, Konze and Kwiatkowski (14) suggested that all or most of the ACC is synthesized during an initial period following wound stress. That does not appear to be the case in the copper-treated pear cells. The burst in ethylene evolution, which reaches a maximum 4 to 5 h after the addition of copper, is dependent upon a concomitant increase in rates of ACC synthesis. This is evidenced by the fact that addition of AVG at any time up to the point of maximum ethylene evolution results in a rapid reduction in rate of ethylene production (29). Moreover, inclusion of ACC with copper results in relatively rapid increase in rates of ethylene evolution confirming that the EFE is not limiting or conversely, that ACC formation is the rate limiting step.

Response to ACC. The addition of ACC to the pear fruit cells results in a rapid rise in rate of ethylene evolution (Fig. 3) and, as in other systems (14, 38), may be taken as an indication that EFE is present at all times. The nearly similar pattern of increase in rates of ethylene evolution for all ACC concentration above 10 μM (Fig. 3) also implies that rate of ACC uptake may be the determining factor.

Transient Nature of the Response. It would be well to know that the observed response to auxins, chemical stress, or precursor is characteristic for each stimulus and not normalized by other controlling factors. The rapidity of the response to ACC assures that the lag phases in response to auxins and $CuCl_2$ are distinctive and not attributable to a deficiency of EFE activity. However, the close parallel in fall-off of ethylene production rates regardless of the initial stimulant raises the possibility that declining EFE activity is the common limiting factor. Relatively high residual levels of endogenous ACC present well after ethylene evolution in response to IAA has declined (Fig. 2), also imply limiting EFE activity.

Alternative, not mutually exclusive possibilities include Lurrsen's (22) suggestion of regulation at the ACC uptake and transport site(s). ACC compartmentalization has not been investigated, its potential regulatory effect on ethylene evolution cannot be disregarded. ACC metabolism by some pathway other than conversion to ethylene could also account for internal control of ACC levels (12). Some conjugation seems to occur in cultured pear cells, since ethylene is obtained upon acid hydrolysis of an ACC-free fraction obtained by ion exchange chromatography (N. Hoffman, personal communication).

The question is not resolved. The ethylene-producing response is, in some way, self-limiting though apparently not via ethylene-mediated feedback mechanisms (Table II).

Inhibitor Effects. Several studies (13, 37, 39) have shown that continued synthesis of EFE is essential for continued ethylene production in response to ACC. An involvement of protein and RNA synthesis in the IAA response is substantiated by the almost complete inhibition of ethylene evolution with the concomitant addition of D-MDMP, CHI, or Act D. An inhibitory effect is also observed if the same inhibitors are added 7 h after IAA (Fig. 7), implying that full manifestation of the IAA response is dependent on a continual synthesis of protein and RNA. However, pear cells exposed to CHI and D-MDMP for 18 to 21 h retained 25 to 50% of their capacity to convert exogenous ACC to ethylene (Table IV). Based on these limited data, the half-life of EFE in cultured pear fruit cells is estimated at 12 to 20 h, much longer than the 2 to 2.5 h estimated for the enzyme in citrus albedo tissue (13).

The effectiveness of the protein synthesis inhibitors is indicated by data on polysomes (Table V) and amino acid incorporation (Table VI). However, the efficacy of D-MDMP as an inhibitor of C_2H_4 synthesis decreases with time of addition. The inhibitor has virtually no effect when added 12 h after IAA or 3 h after $CuCl_2$, *i.e.* at or near the point of maximum rates of ethylene production. Accordingly, the transient nature of the ethylene response may either result from the arisal of internal constraints on protein synthesis, or factor(s) not related to protein synthesis may be determinative.

Polysomal analyses (Table V) and incorporation/uptake data (Table VI) confirm that $CuCl_2$ also inhibits protein synthesis. The inhibition must be selective for it does not appear to deter the synthesis of enzymes participating in ethylene biosynthesis.

Remarks. Senescent, suspension-cultured, Passe Crassane pear fruit cells can clearly be induced to produce readily measurable amounts of ethylene (20–40 nl/10^6 cells·h or about 250 to 500 nl/g cells). Maximum rates do vary from culture to culture due, in part, to what may be a senescence phenomenon (unpublished) which was not closely controlled in these experiments. Over a period of 2 years, the cells would have undergone approximately100 subculturings and 3 to 4 times that number of divisions. With few exceptions, all subcultures could be stimulated to produce ethylene once placed in aging media. Some, inexplicably, did not. A cell line initiated from 'Bartlett' pear fruit could not be stimulated to produce large amounts of ethylene.

Though subject to some instability and variation, the tractability and responsiveness of cultured cells should make them a convenient system for the study of ethylene synthesis. As physiological transitions accompanying varying stages of culture growth, quiescence, and death are better understood, the suspension cell cultures should also prove useful in examining the physiological role(s) of ethylene.

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