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Effects of Aminoethoxyvinylglycine and Countereffects of Ethylene on Ripening of Bartlett Pear Fruits

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ABSTRACT

Pear fruits (*Pyrus communis* L. var. Bartlett) were treated with solutions containing aminoethoxyvinylglycine (AVG) using a modified vacuum infiltration method that introduced 4.3 milliliters solution per 100 grams tissue. At concentrations of 1 millimolar, AVG strongly inhibited ethylene production and delayed for 5 days the respiratory climacteric and accompanying ripening changes in skin color and flesh firmness. AVG was less effective in inhibiting the ripening of more mature fruits. Fruit infiltrated with 5 millimolar AVG had not begun to ripen 12 days after initiation of ripening in the controls. When treated with ethylene the inhibited fruit exhibited a climacteric rise in respiration, softened, and became yellow. Treatment of the AVG infiltrated fruits with ethyelne for 24 hours resulted in no recovery in endogenous ethylene production, but in a stimulation of protein synthesis measured as a 200% increase in leucine incorporation by excised tissue and a 74% increase in the percentage of ribosomes present as polysomes.

Pears which have developed a "ripening capacity" will ripen in response to ethylene that is either applied or which has accumulated endogenously to a critical concentration. As they ripen, the fruit produce a large quantity of ethylene. The gas thus undoubtedly plays a role in the ripening of these and many other fruits, but it is not yet known how its production is triggered nor how it initiates the ripening process (1, 11, 16).

In 1975, Lieberman *et al.* (12) showed that AVG[3], a derivative of the antibiotic rhizobitoxine, strongly inhibited ethylene production by a variety of plant tissues. AVG and related compounds are recognized as potentially useful tools for probing both the biosynthesis of ethylene and its action. The specificity of inhibitors can, however, always be questioned. AVG is believed to block ethylene synthesis between *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (1, 5). It may interfer with other pyridoxal phosphate-dependent reactions (10) and may also inhibit tRNA charging (3).

Wang and Mellenthin (20) infiltrated intact Anjou and Bartlett pears with solutions of AVG. They found that ethylene production was at least partially inhibited in both varieties. However, the ripening of Bartletts was not affected, whereas that of the Anjous was delayed by about 2 days. The present investigation relates an effective delay of Bartlett fruit ripening by AVG and the subsequent release of the inhibition by treatment with ethylene.

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[3] Abbreviation: AVG: aminoethoxyvinylglycine.

MATERIALS

Pear fruits (*Pyrus communis* L. var. Bartlett) were obtained during the 1st week of commercial harvesting from the Sacramento Delta region, and from Lake County, Calif. They were stored at 1 ± 1 C for at least 8 days, but for no more than 4 weeks, to synchronize their ripening before use. The fruit were held at 20 C for 12 to 16 h before infiltration. The [U-^{14}C]leucine was purchased from Schwarz/Mann. AVG was obtained from Hoffmann-La Roche, and Gastrografin (37% iodine) from E. R. Squibb and Sons.

METHODS

Infiltration. The method used was essentially that of Frenkel *et al.* (9) with modifications to improve distribution and to ensure sterility. The fruit were surface-sterilized in diluted household bleach (final sodium hypochlorite concentration 1.3%, w/v) for 15 min then rinsed thoroughly. Subsequent operations were under sterile conditions in a laminar flow hood. The area of the fruit to be injected was wiped with 70% (v/v) ethanol. A 15-gauge needle was inserted 1 cm from the calyx and pushed a predetermined distance to reach the core region. The needle was unblocked and the fruit placed in a vacuum jar. Utilizing nylon luer connectors, the needle was attached to silicone tubing which led to a three-way stopcock positioned outside the vacuum jar (Fig. 1A). Connectors were used to facilitate connecting or disconnecting the tubing without disturbing the inserted needle. The additional outlets of the stopcock were to a syringe barrel containing the solution to be introduced, and to tubing opening back into the vacuum jar (Fig. 1B). Four fruits in each of two vacuum jars were treated simultaneously. With the tap exists to the syringe barrels closed, a vacuum of between 10 and 15 cm Hg was drawn inside the jar. The taps were then turned and the solutions allowed to enter the fruit. This normally required between 5 and 20 min. For each fruit the infusion was followed by an air chase for 6 min before the exit to that syringe barrel was closed. Subsequently, the vacuum was released, the needles removed, and the holes sealed with Vaseline. All of the solutions introduced contained 0.375 M mannitol as an osmoticum, and 4.3 ml were introduced per 100 g fruit tissue.

Radiography. Fruit were infiltrated with a 1:1 (v/v) mixture of 0.375 M mannitol and Gastrografin. Intact fruit and transverse slices 1 cm thick were exposed to x-rays at 100 mamp, 50 kvp, for 0.2 s, with a focal film distance of 76 cm. The Kodak XG-1 film used was contained in a Kodak X-omatic fine cassette, and was developed using standard rapid-processing procedures.

Respiration and Ethylene. The fruit, individually or in groups of three to five, were placed at 20 C in glass jars. A flow of humid air free of ethylene was maintained through the jars at a rate of 24 l/kg·h fruit. Production of CO_2 was measured by the Claypool-Keefer method (8). Ethylene in the outflowing air was measured using a Carle analytical gas chromatograph, model 211. Internal

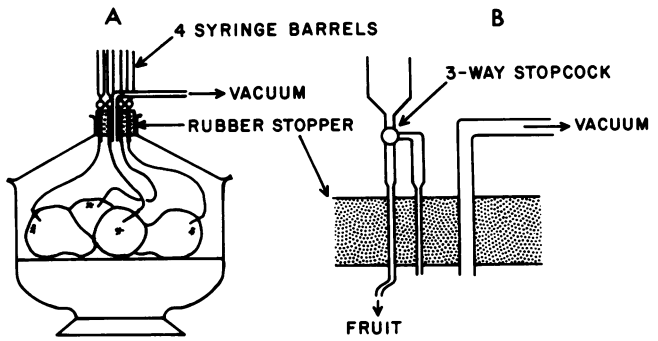

FIG. 1. Apparatus used for the infiltration of pear fruits. Stylized view of vacuum jar and detail of one three-way stopcock which interconnects fruit, solution in syringe barrel, and vacuum inside jar.

ethylene was sampled using a vacuum-immersion technique (13). A needle was inserted into the core of each fruit and unblocked with a fine wire. The fruit were immersed in water which had previously been under a vacuum of 12.5 cm Hg for at least 20 min. The vacuum was reapplied for 3 min, and air drawn from the fruit was analyzed for ethylene content.

Firmness. The force required to puncture the peeled flesh of the fruit was measured using a Univ. of Calif. pressure tester with an 8-mm plunger.

Color. Color was measured with a Gardner XL-23 color difference meter calibrated using a white standard plate ($L = +91.7$, $a = -1.4$, $b = +0.9$). Results presented are a values. Each fruit was marked so that the same two areas per fruit were measured each day.

Radiolabeling. Under sterile conditions, plugs 1.8 cm in diameter were cut radially through the fruit. The flesh portion was cut into discs 2.8 mm thick. These were washed with 0.375 M mannitol, then 12 g were placed in 12 ml of mannitol solution containing 8 μCi [U-^{14}C]leucine, 312 mCi/mmol). The mixtures were incubated at 20 C for 2 h in a shaking water bath. The discs were removed, washed with mannitol solution, and frozen. Duplicate 4-g samples were ground in liquid N_2. The frozen powder was added to 30 ml of 10% (w/v) trichloroacetic acid containing 2 mM leucine. The mixture was homogenized using a Polytron and left at 0 C for 2 h. An aliquot was retained to measure the total radioactivity associated with the discs. The remainder was centrifuged at 45,000g for 15 min and the pellet washed twice with 5% (w/v) trichloroacetic acid, twice with methanol-chloroform-water (12:5:3, v/v), and twice with ethanol. The final residues were dried and combusted. The labeled CO_2 was collected in a scintillation cocktail and counted.

Polysomes. Fruit flesh tissue was sliced into liquid N_2 and stored at −50 C. Ribosomes were extracted and fractionated and the percentage present as polysomes was calculated (15).

RESULTS

Infiltration. A study of this kind depends upon a reasonably good distribution of infiltrated materials since cells not reached by AVG will produce ethylene. Waterlogged regions of pears will not ripen. In preliminary experiments it was determined that infiltration volumes in excess of 5 ml/100 g fruit often resulted in abnormal physiological behavior. The dispersal of infiltrated solutions was considerably improved by the air chase, but the x-radiographs (Fig. 2) show that the final distribution of infiltrated Gastrografin was not entirely homogeneous. Higher concentrations were present in the core with a range of concentrations in the flesh. The extreme calyx and stem ends of the fruit were rarely reached. Eosin red solutions appeared to disperse in a similar way.

The Effects of AVG on Ripening. When fruit were infiltrated with mannitol solution, ethylene production was greatly stimulated (Fig. 3). Respiration was also slightly stimulated. When 10 μM, 50 μM, or 0.25 mM AVG was included in the infiltrated solutions the rate of ethylene production was progressively decreased. With 0.25 mM AVG ethylene production was less than 20% of the mannitol-infiltrated fruit and approximately 50% of the uninfiltrated controls. Concomitant respiratory rates were altered to a much lesser extent, and color and firmness changes (only mannitol and mannitol plus 0.25 mM AVG shown) were not affected.

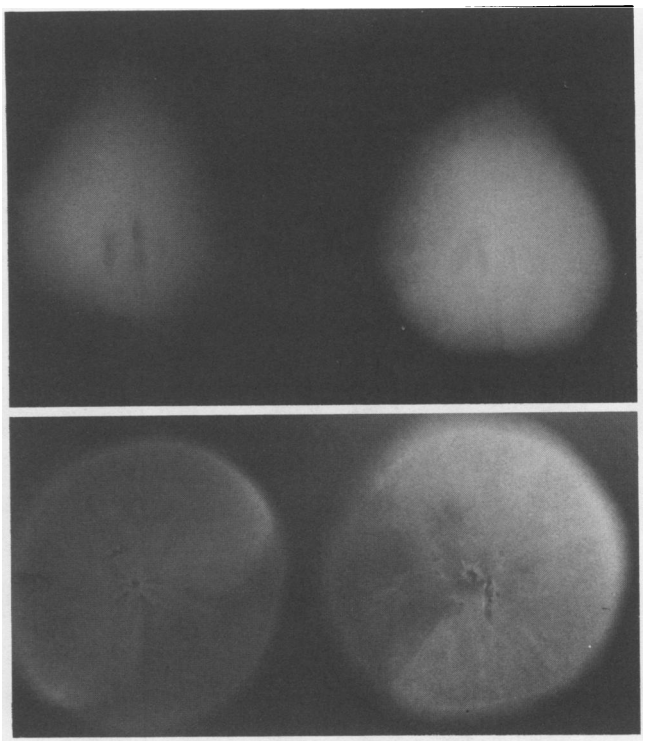

FIG. 2. X-radiographs showing distribution of Gastrografin solution (lighter areas) in infiltrated pear fruit. Upper figure: uninfiltrated control (left) and infiltrated intact fruit (right). Lower figure: transverse slices from infiltrated fruit.

When higher AVG concentrations of 1 and 5 mM were used, the effects on respiration and color change were much more pronounced (Fig. 4). The effect of AVG was significantly influenced by the time of its application. In the early (day 0) application, the fruit were infiltrated with 1 mM AVG 12 h after their removal from cold storage. This caused a 5-day delay relative to mannitol-infiltrated fruits in the peak of ethylene production, the respiratory peak, and the change in skin color (Fig. 4). Ethylene production was inhibited by over 97% and the maximum respiratory rate was 25% less than that of the control. For the late (day 3) application of 1 mM AVG, fruit were infiltrated 3.5 days after removal from cold storage. The fruit, which were physiologically younger than those in Figure 3, were not producing ethylene when infiltrated. Nonetheless, these fruit ripened 2 days earlier than the fruit infiltrated on day 0 (Fig. 4) and produced about twice as much ethylene. Also shown in Figure 4 are the effects of 5 mM AVG on ripening. Seven days after the control fruits had completely ripened, those infiltrated with 5 mM AVG had shown no ethylene production and ripening changes.

Reversal of AVG Inhibition. On day 6, fruit which had been infiltrated with 5 mM AVG were treated with continuous exposure to 8 ± 3 μl/l ethylene. The fruit responded by exhibiting a small climacteric rise in respiration (Fig. 5). They also softened and developed an aroma considered typical of normal ripe Bartletts.

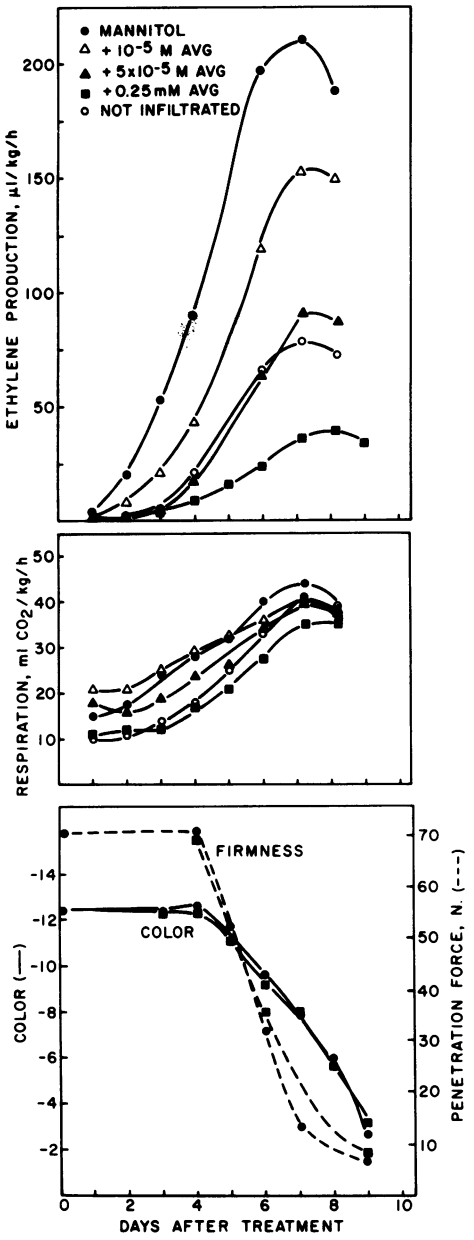

FIG. 3. Effects of infiltration with mannitol and different levels of AVG on ethylene production (upper graph), respiration (middle graph), and color and firmness (lower graph) of pear fruit. At time zero fruit were not infiltrated (○), infiltrated with 0.375 M mannitol alone (●), or with mannitol plus 10^{-5} M (△), 5×10^{-5} M (▲), or 2.5×10^{-4} M (■) AVG. Each treatment comprised four fruits. Mean values are shown. SE for ethylene data (N = 4) average 45% of the mean for values below 15 µl/kg·h, 14% of the mean for values above this. For respiration data (N = 4), SE averaged 9% of the mean throughout. For color values (N = 8), SE averaged 6% of the mean between −13 and −10, and 20% between −6 and 0.

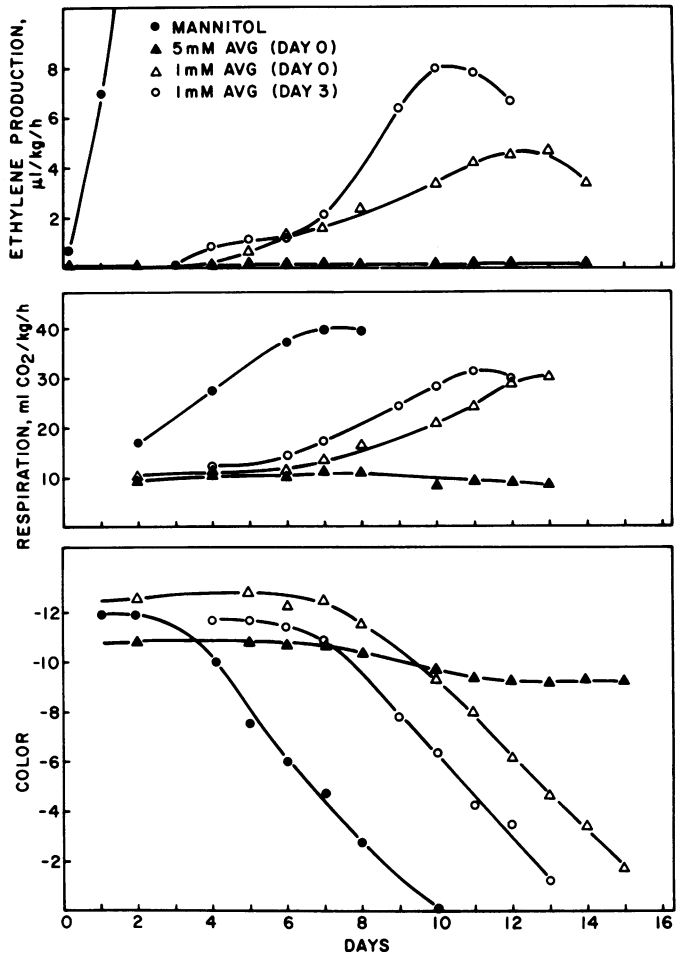

FIG. 4. Effects of 1 and 5 mM AVG on ethylene production (upper graph), respiration rate (middle graph), and color change (lower graph). Pear fruit were infiltrated with mannitol alone (●), or mannitol plus 5 mM AVG (▲), or 1 mM AVG (△) at zero time and with 1 mM AVG on day 3 (○). Measurements were made on four individual fruits, mean values are presented. SE for ethylene were 60% of mean values below 2, and 15% above 3 µl/kg·h. Respiration and color values had SE similar to those in Figure 3.

The rate of color change was similar to that of the controls (Figs. 3 and 4).

In another experiment, fruit were infiltrated with 2.5 mM AVG and 4 days after infiltration they were treated with a continuous supply of either 5 or 10 µl/l ethylene, or with 10 µl/l for 24 h. The inhibition of ripening was overcome in all cases (Fig. 6). The fruit supplied with 5 or 10 µl/l continuously ripened at the same rate, those with a 24 h treatment ripened more slowly. Data on fruit firmness (Table I) show that the pressure changes follow a pattern similar to the color changes.

Ethylene Production. Ethylene production by 2.5 mM AVG-treated fruit and those subsequently exposed to 10 µl/l for 24 h was measured. Three days after the ethylene was administered, *i.e.* day 9 (Fig. 6), no difference in production rate between the ethylene-treated and untreated fruits was discerned. Admittedly, a difference may have been detected using more sensitive methods. By days 11 and 12, the ethylene-treated fruits were producing more ethylene than the controls. On day 12, the internal ethylene of the fruits was measured. All fruit subjected to an ethylene treatment appeared to be producing their own ethylene (Table I). We do not know how soon this had taken place, or whether the ethylene came from cells not exposed to AVG. Utilizing a calculated resistance coefficient of 2.4 and applying Fick's law as done by Burg and Burg (7), we determined that the highest internal ethylene concentration of 31 µl/l (Table I) obtained in the presence of 10 µl/l ethylene represents a production rate of about 9 µl/kg·h, which is very much less than the amounts produced by control fruits (Fig. 3).

Protein Synthesis. Protein synthesis was studied in fruit infiltrated with 2.5 mM AVG and then treated or not treated with 10 µl/l ethylene for 24 h. Discs cut from both sets of fruit took up approximately the same amounts of [^{14}C]leucine (Table II), but those from ethylene-treated fruit incorporated about three times more label into a trichloroacetic acid-insoluble product than did

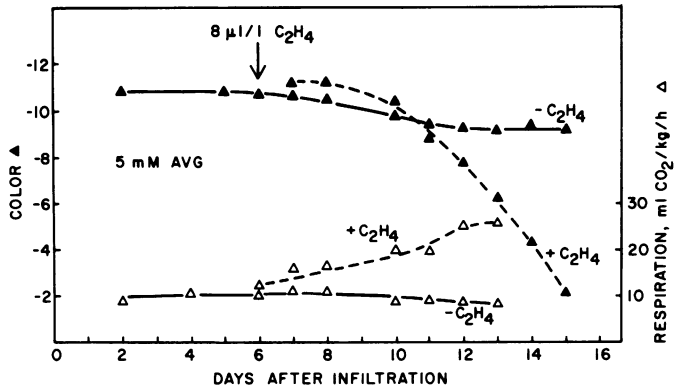

FIG. 5. Effects of ethylene on respiration (△) and color (▲) of fruit infiltrated with mannitol plus 5 mM AVG. (——): No ethylene treatment; (-----): 8 µl/l ethylene supplied continuously beginning on day 6. n = 3. SE as in Figure 3.

Table I. *Firmness and Internal Ethylene Concentrations of Pear Fruit Infiltrated with 2.5 mM AVG and Subsequently (Day 4) Exposed to Ethylene*

Experimental conditions and fruit were the same as in Figure 6.

C_2H_4 Treatment	Firmness		Internal C_2H_4
	Day 8	Day 12	Day 12
	Newtons[a]		*µl/l*
None	67	62	1.2
10 µl/l for 24 h	37	26	4.1
5 µl/l continuous	23	9	14
10 µl/l continuous	25	8	31

[a] One Newton = 0.225 lb force.

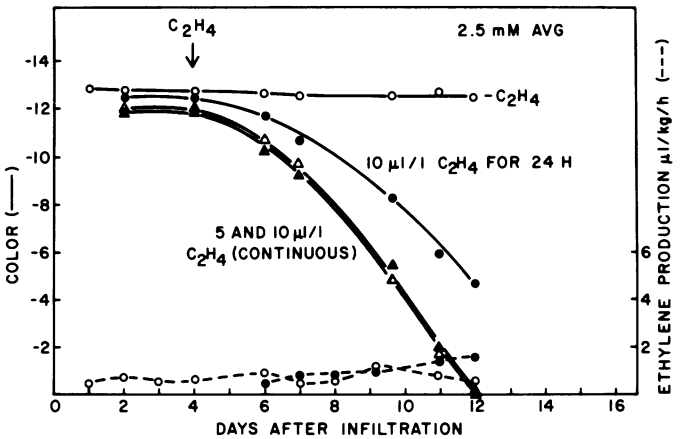

FIG. 6. AVG inhibition of ripening and its reversal by ethylene. Fruit were infiltrated with mannitol plus 2.5 mM AVG and on day 4 were not treated (○) or were treated with 5 µl/l ethylene (▲), or 10 µl/l ethylene (△) continuously, or 10 µl/l ethylene for 24 h (●) only. (——): Color changes; (-----): ethylene production by fruit not exposed to ethylene and those exposed to 10 µl/l C_2H_4 for 24 h.

the control discs. Polysome data (Table II) support the incorporation results. Ethylene treatment caused a 74% increase in the proportion of ribosomes present as polysomes.

DISCUSSION

The results show that when AVG is applied at a sufficiently higher concentration to mature, unripe Bartlett fruits, ripening is prevented. The results indicate that the infiltration technique, despite imperfections in distribution, was effective. We hypothesize that either all of the fruit cells were in contact with AVG, or that the key cells where senescence begins were inhibited, or that precociously senescing cells did not produce enough ethylene to stimulate ripening of the whole fruit. The results should be viewed as an average cellular response to a range of AVG concentrations within one fruit.

Table II. *Amino Acid Incorporation and Per Cent Polysomes in Pear Fruit Cells Infiltrated with 2.5 mM AVG and 4 Days Later Exposed to Ethylene for 24 h*

Treatment	[^{14}C]Leucine Incorporation	^{14}C Uptake	Polysomes / Poly + monosomes × 100
	cpm/4 g fr wt	*%*	*ratio*
None	58,700 ± 1,700	6	39 ± 4
Ethylene (10 µl/l)	167,500 ± 12,000	16	68 ± 4

We have noted that Bartlett pears harvested later in the season or held for several weeks at 0 C could not, as observed by Wang and Mellenthin (20), be prevented from ripening by treatment with AVG. It is likely that in these fruit the internal ethylene concentration, or that of the immediate precursor 1-aminocyclopropane-1-carboxylic acid (2), will have reached a sufficiently high concentration to trigger ripening prior to the injection of AVG. A greater effectiveness of AVG toward less mature fruits has also been reported for tomatoes (4) where the strongest inhibition of ethylene production is in green fruits. Our attempts to assess the effects of AVG on riper pears were not successful as infiltrated solutions are not adequately dispersed as the fruit begins to soften.

Treatment with ethylene overcomes AVG inhibition. This strongly indicates that the AVG effects are mediated solely through inhibition of ethylene synthesis. Partial inhibition by AVG of changes in membrane permeability is also overcome by ethylene (18).

The general pattern of respiratory changes shown in this report (Figs. 3–5) supports the notion that respiration rates are somewhat proportional to ethylene concentrations (14) and that lower ethylene concentrations are needed to initiate ripening than are required for a large climacteric rise (19). Side effects of AVG on respiration, however, should not be ruled out.

A 24-h ethylene treatment of AVG-infiltrated fruit resulted in slower ripening than continuous exposure (Fig. 6), although ripening appeared to begin at the same time. This implies that once the senescence process has begun its rate is influenced by the concentration of ethylene present. However, since ethylene production by fruit ripening in response to a 24-h exposure to ethylene does not appear to be higher than the 1.2 µl/l of nonripening AVG inhibited fruit (Fig. 6 and Table I), it seems that only very low concentrations are required for completion of the ripening process. It is tempting to speculate that the presence of ethylene is not necessary once ripening is underway. The results also indicate that AVG inhibition of endogenous ethylene production could be used to determine ethylene threshold values for specific physiologic responses.

It is widely thought, though not proven, that the synthesis of protein is necessary for the ripening of harvested fruits, and that ethylene treatment will stimulate such synthesis (6, 11, 16). Our results are in agreement. Incorporation studies based on the use of tissue slices could be criticized because of the possibility that wound-induced protein synthesis is taking place (17). It is conceivable that ethylene treatment renders the AVG-infiltrated fruit more responsive to wounding. The polysome experiments, not involving wounded tissue and hence immune to such criticism, support the incorporation data and leave little doubt that protein synthesis in AVG-treated tissues takes place in response to ethyl-

ene. The results lessen, but do not preclude the possibility that AVG partially inhibits protein synthesis (3).

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LITERATURE CITED

1. ABELES, FB 1973 Ethylene in Plant Biology. Academic Press, New York
2. ADAMS, DO, SF YANG 1979 Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Nat Acad Sci USA 76: 170–174
3. ANDERSON J, M LIEBERMAN, A MATTOO, E CHALUTZ 1978 Rhizobitoxine analogs (enol ether amino acids) as inhibitors of ethylene and amino acid incorporation. Plant Physiol 61: S–495
4. BAKER JE, M LIEBERMAN, JD ANDERSON 1978 Inhibition of ethylene production in fruit slices by a rhizobitoxine analog and free radical scavengers. Plant Physiol 61: 886–888
5. BOLLER T, RC HERNER, H KENDE 1979 Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. Planta 145: 293–303
6. BRADY CJ, PBH O'CONNELL 1976 On the significance of increased protein synthesis in ripening banana fruits. Aust J Plant Physiol 3: 301–310
7. BURG SP, EA BURG 1965 Gas exchange in fruits. Physiol Plant 18: 870–884
8. CLAYPOOL L, R KEEFER 1942 A colorimetric method for CO_2 determination in respiration studies. Proc Am Soc Hort Sci 40: 177–186
9. FRENKEL C, I KLEIN, DR DILLEY 1968 Protein synthesis in relation to ripening of pome fruits. Plant Physiol 43: 1146–1153
10. GIOVANELLI JL, LD OWENS, SH MUDD 1971 Mechanism of inhibition of spinach β-cystathionase by rhizobitoxine. Biochim Biophys Acta 227: 671–684
11. LIEBERMAN M 1975 Biosynthesis and regulatory control of ethylene in fruit ripening. A review. Physiol Vég 13: 489–499
12. LIEBERMAN M, AT KUNISHI, LD OWENS 1975 Specific inhibitors of ethylene production as retardents of the ripening process in fruits. *In* Facteurs et regulation de la maturation des fruits. Coll Int CNRS 238, Paris, pp 161–170
13. MAXIE EC, IL EAKS, NF SOMMER, HL RAE, S EL-BATAL 1965 Effect of gamma radiation on rate of ethylene and carbon dioxide evolution by lemon fruit. Plant Physiol 40: 407–409
14. MCGLASSON WB 1970 The ethylene factor. *In* AC Hulme, ed, The Biochemistry of Fruits and Their Products, Vol 2. Academic Press, London, pp 475–519
15. ROMANI RJ, K FRENCH 1977 Temperature-dependent changes in the polysomal population of senescent (ripening) pear fruits. Plant Physiol 60: 930–932
16. SACHER JA 1973 Senescence and postharvest physiology. Annu Rev Plant Physiol 24: 197–224
17. SACHER JA, D ENGSTROM, D BROOMFIELD 1979 Ethylene regulation of wound-induced ribonuclease in turnip root tissues. Planta 144: 413–418
18. SUTTLE JC, H KENDE 1978 Ethylene and senescence in petals of *Tradescantia*. Plant Physiol 62: 267–271
19. WANG CY, WM MELLENTHIN 1972 Internal ethylene levels during ripening and climacteric in Anjou pears. Plant Physiol 50: 311–312
20. WANG, CY, WM MELLENTHIN 1977 Effect of aminoethoxy analog of rhizobitoxine on ripening of pears. Plant Physiol 59: 548–549