

Inhibition of Ethylene Biosynthesis by Aminoethoxyvinylglycine and by Polyamines Shunts Label from 3,4-[¹⁴C]Methionine into Spermidine in Aged Orange Peel Discs¹

Received for publication April 9, 1981 and in revised form September 15, 1981

ZEEV EVEN-CHEN², AUTAR K. MATTOO³, AND RAPHAEL GOREN

Department of Horticulture, The Hebrew University of Jerusalem (Z. E.-C., R. G.); and Department of Plant Genetics, The Weizmann Institute of Science (A. K. M.); Rehovot, Israel

ABSTRACT

The flux of radioactivity from 3,4-[¹⁴C]methionine into *S*-adenosyl-L-methionine (SAM), 1-aminocyclopropane-1-carboxylic acid (ACC), spermine, and spermidine while inhibiting conversion of ACC to ethylene by 100 millimolar phosphate and 2 millimolar Co²⁺ was studied in aged peel discs of orange (*Citrus sinensis* L. Osbeck) fruit. Inhibition up to 80% of ethylene production by phosphate and cobalt was accompanied by a 3.3 times increase of label in ACC while the radioactivity in SAM was only slightly reduced. Aminoethoxyvinylglycine (AVG) increased the label in SAM by 61% and reduced it in ACC by 47%. Different combinations of standard solution, in which putrescine or spermidine were administered alone or with AVG, demonstrated clearly that inhibition of ethylene biosynthesis—at the conversion of SAM to ACC—by AVG, exogenous putrescine or exogenous spermidine, stimulated the incorporation of 3,4-[¹⁴C]methionine into spermidine.

plant metabolism differ diametrically. Although ethylene is a plant-aging hormone leading to retardation of growth and promotion of senescence (1, 6), polyamines delay senescence in excised leaves and protoplasts (3, 13, 20). Polyamines also inhibit ethylene biosynthesis in several plant tissues (4, 25), but the mechanism of this inhibition was not yet determined. The possibility that the utilization of SAM in the biosynthesis of either ethylene or polyamine is regulated in the plant cell may have important physiological implications and therefore needs further investigation.

Ethylene production by albedo tissue of mandarin fruit (11), and by wounded tissue of citrus fruit (12, 29) has been previously reported. The induction of ethylene formation by ABA and the inhibitory effect of AVG on its production in citrus bud culture and leaf explants were also studied (10, 21). In the present investigation, we have used orange peel tissue to study relative incorporation of radioactivity from 3,4-[¹⁴C]methionine into SAM, ACC, spermine, and spermidine while inhibiting biosynthesis of ethylene from ACC by cobalt and phosphate ions. By specifically inhibiting the terminal step of ethylene biosynthesis (Fig. 1), unwanted effects of excess ethylene on tissue metabolism were kept to a minimum. Under these conditions, we show that inhibition of ethylene biosynthesis at the step of conversion of SAM to ACC by AVG and the polyamines, putrescine and spermidine, stimulates 3,4-[¹⁴C]methionine incorporation into spermidine, while lowering the concentration of ACC.

Biosynthesis of ethylene in higher plants has been extensively studied and reviewed (14). Following the discovery in 1966 by Lieberman *et al.* (15) of methionine as a precursor of ethylene in higher plants, Adams and Yang (2) and Lurssen *et al.* (17) have shown independently that ACC, a metabolite of SAM⁴ (5, 27), is efficiently converted to ethylene. These observations have now been extended to other plant tissues and confirmed elsewhere (7, 9, 18, 26). It seems likely that ethylene is synthesized from methionine via the following metabolic sequence: methionine → SAM → ACC → ethylene (Fig. 1). Thus, SAM metabolism in plants assumes a particular significance, inasmuch as it is also a well established precursor of polyamines (19, 23) (Fig. 1).

SAM may be utilized by plants for the biosynthesis of either polyamines or ethylene, or both, depending upon the conditions that regulate its pathway. However, the conditions appropriate for channeling SAM towards either of the two are not yet determined. Interestingly, the functions of polyamines and ethylene in higher

MATERIALS AND METHODS

Plant Material. Mature Shamouti orange fruits (*Citrus sinensis* L. Osbeck) were picked, washed with water, surface-sterilized with 70% ethanol, and peeled. The peelings were then put in a dark humid chamber for 72 h at 25°C to age, and used to prepare discs of 5 mm in diameter and 5 mm thickness. The discs were incubated in test tubes (four per tube, tissue weighing approximately 0.8 g) with 0.75 ml of a medium (pH 5.5) containing 0.25 M sucrose and 0.75 μCi of 3,4-[¹⁴C]methionine (49 mCi/mmol) in H₂O; when indicated, 2 mM Co²⁺, 100 mM Na-phosphate, 2.6 mM AVG, 10 mM putrescine, 10 mM spermidine, or 5 mM Ca²⁺ were added either alone or in various combinations. Each medium was infiltrated into the tissue under vacuum, and the tubes were then placed in a vial with two compartments (one of the compartments contained 2 ml saturated KOH for trapping total CO₂ including [¹⁴C]O₂ and the other contained 1 ml of 0.25 M mercuric perchlorate to absorb ethylene). After 20 h incubation, radioactive CO₂ and ethylene were determined and the tissue was frozen at -20°C until analyzed. The experiments were repeated twice and averages of the data are presented. Standard errors between experiments were generally in the range of 5 to 10% of the means.

¹ Supported by a grant from the Israeli Ministry of Absorption to Z. E. C. and by a grant from the Israeli Ministry of Agriculture to R. G.

² Present address: Department of Biochemistry, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva Israel 84105.

³ Present address: Plant Hormone Laboratory, Room 205, Building 002, Beltsville, Agricultural Research Center (West), United States Department of Agriculture, Beltsville, MD 20705.

⁴ Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; SAM, *S*-adenosyl-L-methionine.

Table I. Effect of Phosphate (100 mM) and Co^{2+} Ions (2 mM) in the Presence of Sucrose (0.25 M in H_2O) on the Incorporation of 3,4- ^{14}C Methionine into Ethylene, CO_2 , SAM, and ACC in Orange Peel Discs

Figures in parentheses indicate values relative to control which was considered as 100%.

Treatment	$^{14}\text{C}_2\text{H}_4$	$^{14}\text{CO}_2$	^{14}C SAM	^{14}C ACC	ACC:SAM	ACC: C_2H_4
	<i>nCi/g fresh wt</i>				<i>ratio</i>	
Sucrose	51.30 (100)	6.71 (100)	37.63 (100)	23.23 (100)	0.62	0.45
Sucrose, phosphate	34.84 (68)	6.03 (90)	31.31 (83)	34.99 (151)	1.12	1.00
Sucrose, phosphate, Co^{2+}	10.44 (20)	5.88 (88)	35.72 (95)	76.73 (330)	2.15	7.35

Chemicals. L-3,4- ^{14}C Methionine was purchased from Center d'etudes Nucleaires de Saclay, Gif-Sur Yvette, France. AVG was a gift from J. P. Scannel (Hoffman LaRoche). Most of the chemicals used were of analytical grade and were purchased from Sigma.

Analysis of ^{14}C SAM. SAM was extracted from peel discs by the method of Pegg and Williams-Ashman (19). After applying samples to a Dowex column, SAM was eluted with 5 N HCl. The eluates were evaporated to dryness under vacuum at 40°C, dissolved in 0.1 M HCl, and then spotted on Whatman No. 3 MM paper. The developing solvent system used was 1-butanol:acetic acid:water (4:1:5, v/v/v) and chromatography was performed at 25°C. Only one radioactive zone comigrating with an authentic SAM standard was detected. This fraction was tested as a substrate for an active soluble extract of pink tomato fruit for conversion to ^{14}C ACC, using the assay medium of Boller *et al* (5). Tomato fruit extract converted, as expected, by using this procedure, 15% of the previously identified ^{14}C SAM into radioactive ACC, confirming the paper chromatographic identification of SAM.

Determination of ^{14}C ACC. Radioactive ACC was determined by the method of Lizada and Yang (16), after extracting the tissue for ACC by the method of Yu and Yang (28).

Analysis of Polyamines. Polyamines were analyzed by the procedure described by Seiler and Wiechmann (22). Orange peel discs were extracted with 4% HClO_4 , dansylated, and separated by TLC, using the solvent system ethylacetate:cyclohexane (2:3, v/v). The zones on the TLC co-migrating with authentic dansylated samples of spermidine and spermine were scraped off the TLC plates and radioactivity was determined in a scintillation spectrometer.

RESULTS AND DISCUSSION

Carbons 3 and 4 of methionine are specifically converted into the carbon skeleton of ethylene in most of the higher plants tested (14). Under the experimental conditions, aged orange peel discs converted 3,4- ^{14}C methionine into ^{14}C ethylene (Table I), and a good proportion of the label was seen in SAM and ACC, the intermediates of ethylene biosynthesis (Fig. 1). The amount of radioactive CO_2 produced was relatively very low. Addition of 100 mM phosphate to the incubation medium reduced the incorporation of methionine into ethylene by 30%, while 50% more ACC accumulated in the tissue. However, an addition of Co^{2+} together with phosphate caused 80% inhibition of ethylene synthesis and a 3.3-fold increase in ^{14}C ACC (Table I). Also, incorporation into radioactive SAM was not changed by these treatments. The findings suggest that inhibitory effects of phosphate and Co^{2+} are more pronounced at the conversion of ACC to ethylene. Indeed, the ratios of both ACC:SAM and ACC:ethylene increased from 0.62 and 0.45 in the control to 1.12 and 1.00 in the presence of phosphate, and to 2.15 and 7.35 in that of phosphate and Co^{2+} (Table I). These results complement previous reports in which phosphate (8, 9) was shown to inhibit ethylene synthesis in tomato fruit plugs, pea seedling segments, and carrot slices, and Co^{2+} (28) was found to inhibit the conversion of ACC to ethylene in auxin-induced ethylene production.

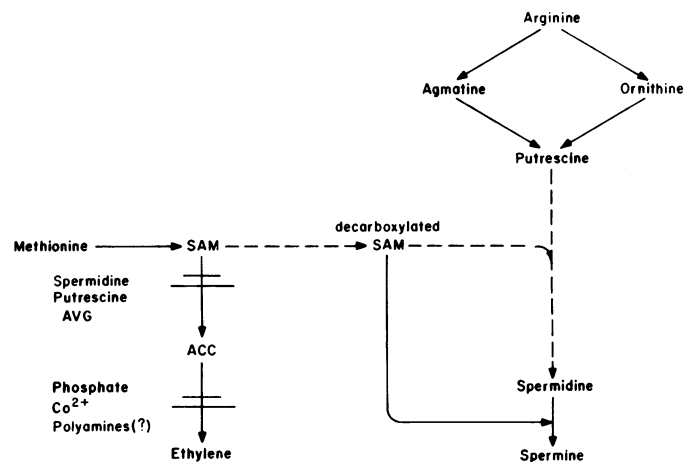


FIG. 1. The pathways of ethylene and polyamine biosynthesis from methionine via SAM (→); alternative pathway of polyamine biosynthesis from methionine, and arginine via putrescine (---→).

When incorporation of labeled methionine into ^{14}C ethylene was inhibited by 80% in the presence of Co^{2+} and phosphate, some label from methionine was recovered in spermidine and spermine; however, the label incorporated into spermine was twice as much as that incorporated into spermidine (Table II). AVG, which is a potent inhibitor of ethylene biosynthesis in higher plants (14) including citrus (10, 21), was shown recently to inhibit the conversion of SAM to ACC (5, 27). In orange peel tissue also (Table II) the presence of AVG resulted in 60% more ^{14}C SAM than the control, apparently at the cost of label in ^{14}C ACC which was reduced by 50% as compared with control. Concomitant with the inhibition of ethylene formation at the stage of SAM conversion to ACC by AVG, a dramatic increase (475%) in label from 3,4- ^{14}C methionine was seen in ^{14}C spermidine, whereas in spermine radioactivity was slightly reduced. In fact, the radioactivity in spermine remained very nearly the same under different treatments, an interesting finding which may mean that its level is under strict metabolic regulation; this, however, was not explored further. When the incubation medium contained both unlabeled putrescine and AVG, the incorporation of 3,4- ^{14}C methionine into radioactive spermidine increased 2-fold as compared with AVG alone, and about 10-fold as compared with control (Table II). Moreover, putrescine prevented the AVG-mediated accumulation of label in SAM and caused further decrease of label in ACC. Incubation medium containing unlabeled spermidine and AVG also prevented the AVG-mediated increase in the transfer of label from methionine into SAM; however, unlabeled spermidine in the presence of AVG could not cause further increase in radioactive spermidine as was seen with putrescine (Table II).

It seems that although AVG inhibited the incorporation of SAM into ACC it did not inhibit the conversion of 3,4- ^{14}C methionine into either spermidine or spermine. Furthermore, we have shown that orange peel tissue possesses the enzymic machinery required to convert 3,4- ^{14}C methionine to spermidine via

Table II. Effect of AVG, Putrescine, and Spermidine on the Incorporation of 3,4-¹⁴C]Methionine into Labeled SAM, ACC, Spermidine, and Spermine in Orange Peel Discs in the Presence of Phosphate and Co²⁺

Incubation Medium	¹⁴ C]SAM	¹⁴ C]ACC	¹⁴ C]Spermidine	¹⁴ C]Spermine
	<i>nCi/g fresh wt</i>			
Sucrose, phosphate, Co ²⁺ (Control)	35.72	76.73	0.29	0.71
	<i>% of control</i>			
Control + AVG	161	53	475	84
Control + AVG + putrescine	89	41	980	107
Control + AVG + spermidine	93	47	420	97
Control + putrescine	115	40	470	102
Control + spermidine	85	35	350	91
Control + spermidine + Ca ²⁺	94	64	260	99

SAM, and that this process is amplified when the conversion of SAM to ACC is decreased, for instance in the presence of AVG, spermidine, or putrescine (see below). However, it seems that below a certain threshold, putrescine may limit the conversion rate of SAM to spermidine, inasmuch as when it was supplied exogenously, 3,4-¹⁴C]methionine incorporation into spermidine was further increased in the presence of AVG. This conclusion is supported by other data (Table II), showing that when no AVG was present, putrescine increased the incorporation of label from 3,4-¹⁴C]methionine into spermidine 5-fold, while inhibiting the conversion of SAM to ACC.

AVG is known also to inhibit endogenous synthesis of methionine by affecting β -cystathionase activity (14). Therefore, under these conditions, when methionine is limiting, any exogenously provided substrate, for example labeled methionine, would be expected to get utilized for either the formation of ethylene or/and polyamines via SAM. Since AVG also prevents the conversion of SAM to ACC, the flux of label from 3,4-¹⁴C]methionine would be expected to shunt away into spermidine/spermine. This is precisely what was observed (Table II). However, inasmuch as only the flux of radioactivity was determined in the present investigation, proof that spermidine/spermine synthesis is promoted is not complete. How far AVG or polyamines affect the specific activity of various intermediates and biosynthetic end products remains to be investigated.

Spermidine alone also inhibited the conversion of SAM to ACC while increasing the formation of labeled spermidine (Table II), indicating that it may be acting as a feed-forward activator (autocatalyst) of its own synthesis from methionine. It might therefore be that one metabolic site of polyamine-mediated inhibition observed previously (4) is at the conversion of SAM to ACC. Because these experiments were not designed to study the stage at which the conversion of ACC to ethylene occurs, we do not rule out the possibility that polyamines inhibit not only the conversion of SAM to ACC but perhaps also the conversion of ACC to ethylene.

Although AVG, putrescine, and spermidine all increased the incorporation of labeled methionine into spermidine, it is possible that the mechanism of action for AVG may be different from that for putrescine and spermidine. AVG decreased the incorporation of SAM into ACC and caused SAM to accumulate. This accumulation of SAM may also enhance the conversion of methionine into spermidine through a mass-action effect. However, spermidine appeared to inhibit slightly the conversion of 3,4-¹⁴C]methionine into SAM while putrescine was slightly stimulatory (Table II). Therefore, the methionine to SAM step does not appear to be a metabolic site of polyamine action. The stimulation of methionine incorporation into spermidine by polyamines seems to be a result of their effect at a later step in the biosynthetic pathway.

An earlier work showed that Ca²⁺ reversed the inhibitory effect of polyamines on ethylene production partly or in full (4), and that Ca²⁺, supplied together with polyamines, diminished their action, indicating probable involvement of an initial ionic attach-

ment mechanism (13). We have found (Table II) that Ca²⁺ lowers spermidine-mediated inhibition of 3,4-¹⁴C]methionine incorporation into ACC and the incorporation into radioactive spermidine by 30%. In other words, Ca²⁺ allowed the channeling of 3,4-¹⁴C]methionine into the ethylene pathway by allowing for more ACC formation and by inhibiting spermidine formation—both actions being contrary to what an exogenous addition of spermidine does. Thus, our data confirm the finding (4, 13) that spermidine action is partly blocked by Ca²⁺, which affects the conversion of SAM to ACC.

The data presented may suggest that there is a close physiological link between ethylene and polyamine biosynthesis. This indicates that hormonal interactions with one of the two biosynthetic pathways may affect the other and be reflected in a physiological response. For example, it was found (10, 21) that ABA-induced ethylene formation is inhibited by AVG. Does this mean that polyamine biosynthesis is increased concomitantly? Or is the reduction of both the activity of arginine decarboxylase and putrescine level by ABA (24) accompanied by increased ethylene formation? Further work with various plant systems at different stages of development will provide the answers to these intriguing questions. In fact, putrescine and AVG, supplied independently, were found recently to increase the incorporation of label from 3,4-¹⁴C]methionine into spermidine and spermine in mature green tomato fruit tissue (Adams, Wang, and Lieberman, personal communication). In the tomato experiments cited above (Adams et al, personal communication) the last step from ACC to ethylene was not inhibited as was done in the present study, thus showing that under conditions in which ethylene production from ACC is not blocked, the observed effects of AVG and polyamine can be as well obtained. That polyamines are capable of directly inhibiting ethylene formation (4) further emphasizes the importance of the proposed interactions of plant growth regulators with biosynthetic pathways of polyamines and ethylene (Fig. 1).

Acknowledgments—A. K. M. thanks the German Academic Exchange Service (DAAD), Bonn, West Germany for a fellowship. We thank D. O. Adams, S. Y. Wang, and M. Lieberman for providing us with their unpublished data cited in the text. The cooperation of A. Altman in identification of polyamines is appreciated.

LITERATURE CITED

1. ABELES FB 1973 Ethylene in Plant Biology. Academic Press, New York, pp 206
2. ADAMS DO, SF YANG 1979 Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as a intermediate in the conversion of methionine to ethylene. Proc Natl Acad Sci USA 76: 170-174
3. ALTMAN A, R KAUR-SAWHNEY, AW GALSTON 1977 Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. Plant Physiol 60: 570-574
4. APELBAUM A, AC BURGOON, JD ANDERSON, M LIEBERMAN, R BEN-ARIE, AK MATTOO 1981 Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. Plant Physiol 68: 453-456
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. BURG SP 1968 Ethylene, plant senescence and abscission. *Plant Physiol* 43: 1503-1511
 7. CAMERON AC, CAL FENTON, Y YU, D ADAMS, SF YANG 1979 Increased production of ethylene by plant tissue with 1-aminocyclopropane-1-carboxylic acid. *HortScience* 14: 178-180
 8. CHALUTZ E, AK MATTOO, Y FUCHS 1980 Biosynthesis of ethylene: the effect of phosphate. *Plant Cell Environ* 3: 349-356
 9. FUCHS Y, AK MATTOO, E CHALUTZ, I ROT 1981 Biosynthesis of ethylene in higher plants: the metabolic site of inhibition by phosphate. *Plant Cell Environ* 4: 291-295
 10. GOREN R, A ALTMAN, I GILADI 1979 Role of ethylene in abscisic acid-induced callus formation in citrus bud cultures. *Plant Physiol* 63: 280-282
 11. HYODO H 1977 Ethylene production by albedo tissue of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. *Plant Physiol* 59: 111-113
 12. HYODO H 1978 Ethylene production by wounded tissue of citrus fruit. *Plant Cell Physiol* 19: 545-551
 13. KAUR-SAWHNEY R, AW GALSTON 1979 Interaction of polyamines and light on biochemical processes in leaf senescence. *Plant Cell Environ* 2: 189-196
 14. LIEBERMAN M 1979 Biosynthesis and action of ethylene. *Annu Rev Plant Physiol* 30: 533-591
 15. LIEBERMAN M, AT KUNISHI, LW MAPSON, DA WARDALE 1966 Stimulation of ethylene production in apple tissue slices by methionine. *Plant Physiol* 41: 376-382
 16. LIZADA C, SF YANG 1979 A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. *Anal Biochem* 100: 140-145
 17. LÜRSSEN K, K NAUMANN, R SCHRODER 1979 1-Aminocyclopropane-1-carboxylic acid—a new intermediate of ethylene biosynthesis in higher plants. *Z Pflanzenphysiol* 92: 285-294
 18. MATTOO AK, Y FUCHS, E CHALUTZ 1981 Regulatory aspects of ethylene biosynthesis in higher plants and microorganisms. *Israel J Botany* 30: 55
 19. PEGG AE, HG WILLIAMS-ASHMAN 1969 On the role of S-adenosyl-L-methionine in the biosynthesis of spermidine by rat prostate. *J Biol Chem* 244: 682-693
 20. POPOVIC RB, DO KYLE, AS COHEN, S ZALIK 1979 Stabilization of thylakoid membrane by spermine during stress induced senescence of barley leaf discs. *Plant Physiol* 64: 721-726
 21. SAGEE O, R GOREN, J RIOV 1980 Abscission of citrus leaf explants. Interrelationships of abscisic acid, ethylene, and hydrolytic enzymes. *Plant Physiol* 66: 750-753
 22. SEILER N, M WIECHMANN 1965 Zum Nachweis von Aminen im 10^{-10} -molar-Bereich Trennung von 1-Dimethylaminonaphthalin-5-sulfonsäure-amiden auf Dunnschichtchromatogrammen. *Experientia* 21: 203-204
 23. SMITH TA 1975 Recent advances in the biochemistry of plant amines. *Phytochemistry* 14: 865-890
 24. SURESH MR, S RAMAKRISHNA, P ADIGA 1978 Regulation of arginine decarboxylase and putrescine levels in *Cucumis sativum* cotyledons. *Phytochemistry* 17: 57-63
 25. SUTTLE JC 1980 Effect of polyamines on ethylene production. *Plant Physiol* 65: S-34
 26. YOSHII H, A WATANABE, H IMASEKI 1980 Biosynthesis of auxin-induced ethylene in mung bean hypocotyls. *Plant Cell Physiol* 21: 279-291
 27. YU Y, DO ADAMS, SF YANG 1979 1-Aminocyclopropanecarboxylic acid synthase, a key enzyme in ethylene biosynthesis. *Arch Biochem Biophys* 198: 280-286
 28. YU Y, SF YANG 1979 Auxin-induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. *Plant Physiol* 64: 1074-1077
 29. YU Y, SF YANG 1980 Biosynthesis of wound ethylene. *Plant Physiol* 66: 281-285