ETHYLENE AND FLORAL SENESCENCE IN TRADESCANTIA

by

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1979

ABSTRACT

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Flowers of Tradescantia (Clone 02), which are ephemeral, produce ethylene during senescence with the maximum rates occurring during the initial period of fading. Senescing, isolated petals produce ethylene in a similar manner, exhibit a loss of membrane semipermeability, and exogenous ethylene hastens the onset as well as the subsequent rate of this loss. Aminoethoxyvinylglycine at 0.1 mM completely inhibits ethylene production by isolated petals but only partially inhibits the loss of membrane semipermeability. Isolated petals acquire a sensitivity to ethylene as they mature, becoming fully sensitive on the day of flower opening.

The stimulation of anthocyanin efflux (an indicator of increased membrane semipermeability) by exogenous ethylene occurs after a 1 to 2.5-h lag. Simultaneous application of 0.1 mM cordycepin or cycloheximide with ethylene abolishes the response to ethylene. Analysis of phospholipid levels in these petals during senescence has shown that the increase in membrane semipermeability is accompanied by a massive loss of phospholipids. Factors which enhance or

retard the rate of anthocyanin efflux exert a corresponding effect on the rate of phospholipid loss. The composition of the phospholipid fraction remains constant during senescence. The activity of phospholipase D declines during senescence while that of acyl hydrolase remains unchanged. Lipid peroxidation, as measured by ethane evolution, is absent in these petals during senescence.

Application of 10 mM $CaCl_2$ to ethylene-pretreated petals results in a retardation of the onset and a lowering of the subsequent rate of anthocyanin efflux but stimulates ethylene production in these same petals. Exposure of petals to one hour of anaerobiosis also results in the retardation of anthocyanin efflux while stimulating ethylene production. When petals are cut into basal and apical halves, the initiation of ethylene production in the basal halves is found to precede the onset of anthocyanin efflux by at least 60 min. These results indicate that ethylene production is not dependent on the loss of vacuolar integrity.

When senescing petals are incubated on L-methionine-U-^{14}C, radioactivity is found associated with S-methylmethionine, methionine, CO_2, protein and ethylene. During the course of senescence, there is a large increase in the radioactivity associated with methionine. A substantial portion of this increase in methionine arises from protein degradation. S-Methylmethionine appears to be a storage form of methionine and is not directly involved in ethylene biosynthesis.

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Application of 1-aminocyclopropane-1-carboxylic acid (ACC) to all parts of the flower results in a stimulation of ethylene production. The stimulation of ethylene production by ACC is inhibited by n-propyl gallate but is not inhibited by aminoethoxyvinylglycine (AVG). Analysis of the endogenous content of ACC shows that the level of this compound correlates with the rate of endogenous ethylene production in these petals. Factors which enhance or diminish the rate of endogenous ethylene production exert a corresponding effect on the level of this compound. There is close agreement in the specific radioactivities of ethylene and ACC in petals which have been incubated on L-methionine-U-^{14}C. These results indicate that ACC is the immediate precursor of ethylene in petals of Tradescantia.

ACKNOWLEDGEMENTS

It is both a rewarding and comforting experience to be associated with people who have faith in your ability as well as a desire to see you succeed. In this regard, I wish to thank the three persons who were primarily responsible for the success of this thesis: my parents, Dale and Vivian Suttle, and Nancy Hanson. It is hoped that they too can share in the satisfaction of the completion of this endeavor. Indeed, without their sustained patience and confidence, this work would not have been possible. I would also like to thank Hans Kende and the members of my guidance committee for their contributions as well. Last but not least, my appreciation is extended to all those individuals who were there with a kind word when they were most needed.

"Of all men's miseries the bitterest is this, to know so much and to have control over nothing."

Herodotus

The research reported here was supported by the U.S. Energy Research and Development Administration and the U.S. Department of Energy under Contract EY-76-C-02-1338.

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ABBREVIATIONS

ACC	1-Aminocyclopropane-1-carboxylic acid
AVG	Aminoethoxyvinylglycine
CHI	Cycloheximide
HCTL	Homocysteine-thiolactone
nPG	n-propyl gallate
MET	Methionine
MSO	Methionine sulfoxide
SAM	S-Adenosylmethionine
SEM	Selenomethionine
SMM	S-Methylmethionine
TLC	Thin-layer chromatography

GENERAL INTRODUCTION

The term senescence, as used in these studies, has been defined as "the final phase in ontogeny of the organ in which a series of normally irreversible events is initiated that leads to cellular breakdown and death of the organ" (Sacher, 1973). Early studies concerning plant senescence focussed primarily on the degenerative aspects of senescence. It was noted that massive losses of both RNA and protein accompanied the loss of cellular function during senescence, an observation which indicated to the early investigators (see Varner, 1961) that the whole process of senescence in plants consisted essentially of a loss of cellular function. This conclusion was in agreement with the observed nature of mammalian senescence, a fact which facilitated its general acceptance.

With the advent of the use of radioisotopes as metabolic tracers in biological studies came a reappraisal of many heretofore accepted dogmas of biological science. Through the use of these tracers, it was demonstrated that all biological materials were in a state of continued synthesis and degradation and that the steady-state level of a given molecule represented a balance between these two processes. Thus, the concept of turnover was born. Application of radioisotope techniques to the study of plant senescence

showed that, against a background of degradation, active synthesis of both nucleic acids and proteins continued throughout the senescence process. In addition, application of metabolic poisons, known to interfere with both RNA and protein synthesis, were found to be effective in arresting the senescence process. Thus, not only did synthesis occur during senescence, it was apparently a prerequisite for the process itself.

The acceptance of these observations on plant senescence gave rise to a new way of viewing the overall process of senescence. From this new perspective, plant senescence was seen as an active, highly-controlled aspect of the overall development of the organism or tissue and, as such, senescence became a fruitful area for the exploration of the metabolic control of development.

Leaf Senescence

The initial and most dramatic aspects of leaf senescence occur within the chloroplast and result in a massive reduction of the photosynthetic capacity of the leaf (Wood and Cruickshank, 1944). The underlying biochemical alterations which contribute to the loss of chloroplast integrity and function include: Loss of chlorophyll, which leads to the unmasking of secondary pigments (leaf yellowing), loss of grana and loss of chloroplast membrane lipids (Thimann, 1978b). Interestingly, it has been shown that the loss of chloroplast integrity results from events occurring outside of the organelle itself. Retardation of leaf yellowing by

inhibitors of cytoplasmic protein synthesis (Martin and Thimann, 1972) as well as by enucleation (Yoshida, 1961) all suggest that the chloroplast plays a passive role during leaf senescence. Further support for this hypothesis can be derived from the fact that isolated chloroplasts lose both chlorophyll and protein at a much reduced rate when compared to chloroplasts *in situ* (Choe and Thimann, 1975).

In addition, leaf senescence is associated with reductions in respiration, although a transient increase may occur as senescence proceeds (Woolhouse, 1967). In excised leaf tissue, the increase in respiration can be partially explained by an increase in substrate concentration and by a partial uncoupling of respiration from ATP synthesis (Tetly and Thimann, 1974). However, respiration in leaves which are allowed to senesce on the plant is sensitive to uncoupling agents and leaves retain the capacity to incorporate inorganic phosphate into ATP (see Biale, 1975).

Loss of protein and RNA are also conspicuous features of leaf senescence (see Thimann, 1978a). In excised leaves this hydrolysis is accompanied by an accumulation of amino acids. However, no such accumulation occurs in leaves allowed to senesce on the plant (Thimann et al., 1974). The loss of both protein and RNA is associated with an increase in their respective hydrolases presumably due to *de-novo* synthesis (Balz, 1966). DNA has also been shown to decline during leaf senescence but this appears to be a relatively late event and of minor magnitude (see Thimann, 1978a).

Degradation of the intracellular membrane systems and the resulting loss of compartmentation also occurs during leaf senescence. Loss of vacuolar integrity (as judged by anthocyanin efflux) occurs during senescence of Rhoeo leaf tissue and this loss was shown to precede the onset of respiratory changes (Sacher, 1959). Ultrastructural observations have confirmed that disorganization of the tonoplast is a characteristic attribute of senescence in leaf tissue (Butler and Simon, 1971). Lastly, the ability of calcium salts to delay leaf senescence indicates that loss of membrane integrity is a key event in the overall process of senescence in leaves (Poovaiah and Leopold, 1973).

A major obstacle in arriving at a unified scheme of leaf senescence has been the use of excised leaf tissue. While excision hastens the process of senescence and thereby facilitates its study, the comparative value of such studies is questionable. Ultrastructural observations have shown that the sequence of cytological changes occurring during leaf senescence is not identical in excised and non-excised leaf tissue (Colquhoun et al., 1975).

Fruit Senescence

Fruit senescence (or ripening) is a multifaceted process which has received a great deal of attention because of its commercial importance. Fruits have been classified as either climacteric or nonclimacteric based on the pattern of respiration exhibited during senescence (Biale, 1960). This discussion will focus on the ripening behavior of climacteric fruits.

The most dramatic and well-studied aspect of fruit ripening is the large upsurge in CO_2 evolution or O_2 consumption that occurs during the so-called climacteric period. Initial attempts to explain this surge invoked uncoupling as the biochemical basis for the increase in respiration. However, it has been shown that incorporation of ^{32}P into high-energy compounds was highest during the climacteric (Young and Biale, 1967). Furthermore dinitrophenol, a respiratory uncoupler, has been found to inhibit the subsequent ripening of tomato fruit (Marks et al., 1957). Further studies have shown that cycloheximide, if administered prior to the climacteric period, would greatly inhibit the subsequent ripening processes with little effect on the climacteric process itself (Frenkel et al., 1968). This observation indicated that the capacity for the climacteric rise in respiration was already present within the tissue, and the results of more recent investigations (Theologis and Laties, 1978) confirm this hypothesis.

Fruit ripening is also characterized by changes in both the texture (firmness) and pigmentation of the fruit. Softening is associated with alterations in pectic substances and possibly cellulosic materials as well. Attainment of the characteristic color of the ripe fruit is usually accompanied by loss of chlorophyll with increases in secondary pigments. Ultrastructural observations have shown that the alterations in pigmentation are accompanied by conversion of chloroplasts to chromoplasts (Butler and Simon, 1971).

Also attending fruit ripening are gross changes in tissue permeability. Although the exact timing of these changes is not well established, it is clear that the tissue has lost much of its semi-permeable nature by the onset of the climacteric peak (Hansen, 1966; Sacher, 1967). While the changes in permeability are well documented, the underlying biochemical basis for these changes has received little attention.

Unlike leaf senescence, fruit ripening is accompanied by very small changes in the total levels of both RNA and protein. Some tissues, such as apple, actually show an increase in protein content during ripening (Hulme et al., 1948). Studies with radioactive precursors have shown that both RNA and protein synthesis occur throughout fruit ripening and that a transient increase in the synthesis of both RNA and protein may occur prior to the climacteric period (Richmond and Biale, 1969). In spite of the near-constant levels of protein found throughout ripening, electrophoretic studies have shown that there are marked changes in the nature of the endogenous proteins during ripening (Clements, 1970). Indeed, the activities of many enzymes are altered during the course of ripening with marked increases in many types of hydrolytic activities (see Dilley, 1970). In some cases, there is a good correlation between the onset of increased hydrolytic activity and the decline in substrate content. However, such correlations are not always evident. For example, RNase activity in ripening apples has been shown to increase at the same time when the level of total

RNA is increasing. The physiological importance of the increases in the various enzyme activities is indicated by the effectiveness of various RNA and protein synthesis inhibitors in arresting the ripening process (Frenkel et al., 1968). Application of cycloheximide prior to the climacteric period completely arrests many processes of ripening (Frenkel et al., 1968). Thus, fruit ripening, like leaf senescence, is an active, highly-controlled phase of plant development.

Floral Senescence

Senescence or fading of flowers is a composite phenomenon that includes not only the senescence of the perianth but also the growth of the gynoecium. Early work with orchid flowers (see Burg and Dijkman, 1967) demonstrated that pollination led to a rapid fading of the perianth as well as to an enlargement of the gynoecium. Later it was discovered that an extract of pollen could produce similar symptoms demonstrating that pollination rather than fertilization was the key event. Subsequent investigations (Hall and Forsyth, 1967) showed that auxin was the active principle in pollen which induced these changes. As studies shifted their focus from pollination to the actual senescence of the flowers, greater attention was given to changes occurring within the perianth during fading.

A notable aspect of flower fading is the wilting of the perianth. Examination of the petals showed that wilting was accompanied by liquid logging of the intercellular air spaces

which gave the petals a translucent appearance (Horie, 1961). Studies with carnations (Nichols, 1968; Mayak et al., 1977), rib segments of Ipomoea tricolor flowers (Hanson and Kende, 1975) and Tradescantia (Suttle and Kende, 1978) showed that enhanced efflux of cellular constituents was a characteristic process during petal senescence.

Isolated petals exhibited much the same pattern of respiration during senescence as senescing leaves did (Coorts, 1973). Respiration in petals was at a peak during flower opening and declined subsequently (Biale, 1975). As petals began to show visible signs of deterioration, a transient increase in respiration was noted (Beyer and Sundin, 1978).

A large decline in DNA, RNA and total protein levels was found to occur during petal senescence (Matile and Winkenbach, 1971). In corollas of Digitalis, loss of protein was more extensive and preceded the loss of RNA during senescence (Stead and Moore, 1977). On the other hand, the reverse situation was found to occur in senescing corollas of Ipomoea (Matile and Winkenbach, 1971).

As in senescing leaves and fruits, the activities of several hydrolases were shown to increase during petal senescence in Ipomoea (Matile and Winkenbach, 1971). The activities (on a per corolla basis) of DNase, RNase, glucosidase and phosphatase were found to increase in senescing petals of Ipomoea. The activity of proteases was found to decline during this same period. In addition, both actinomycin D and cycloheximide were found to inhibit the increase in these activities (Matile and Winkenbach, 1971).

Studies on the fate of the newly formed hydrolysis products produced during senescence showed that these substances were actively translocated via the phloem to both the enlarging gynoecium and to the parent plant (Nichols and Ho, 1975). That this recovery is of survival value to the parent plant was indicated by the fact that excision of flowers from Ipomoea plants led to a reduction in the total number of flowers formed by the plant (Wiemken-Gehring et al., 1973).

Loss of Membrane Integrity and Its Significance During Senescence

The frequency of reports demonstrating enhanced leakage of cellular solutes during senescence has left no doubt that this leakage is a normal aspect of senescence of plant tissues. However, the origin of this increased leakage has been interpreted in at least two ways. Most investigators have felt that the increased efflux results from increased membrane permeability (Sacher, 1973). However, Burg et al. (1964) have pointed out that the increase in solute efflux could have arisen from tissue damage that occurs as a result of the hypotonicity of the bathing medium used in these studies (water), or through concentration-dependent diffusion, owing to an increase in the tissue content of the solutes whose efflux is being measured. Subsequent studies with banana tissue have demonstrated that the increased efflux has not been appreciably affected by the inclusion of up to 0.6 M mannitol in the bathing medium (Brady et al.,

1970). Further, it has been shown that cellular constituents whose internal concentration remains constant during senescence also exhibit an increased rate of efflux during senescence (Sacher, 1966). In addition, studies with excised petal tissues have shown that the efflux of preloaded solutes and vacuolar pigments whose content remains constant also increases during senescence (Hanson and Kende, 1975; Suttle and Kende, 1978). Thus, it appears that the increased leakage of solutes observed during senescence results from an increase in membrane permeability.

While the increase in membrane permeability has gained general acceptance, the underlying mechanism which results in the increased permeability is not completely understood. Studies on membrane behavior in model systems have shown that membrane permeability can be affected by at least two different mechanisms. It has been shown that changes in the saturation index of the fatty-acid groups of the membrane lipids can affect membrane permeability properties, presumably through changes in the fluidity of the membrane (Blok et al., 1975). A second, somewhat more fundamental mechanism affecting membrane permeability is the loss of membrane lipids which eventually destabilizes the bilayer configuration of the membrane and thus affects its permeability properties (Simon, 1974).

Examination of the profiles of esterified fatty acids during senescence, with the exception of senescing leaf tissue, has failed to show any significant alteration in the relative amounts of saturated and unsaturated fatty acids.

In senescing leaves, the degree of unsaturation of membrane lipids has been shown to decline, primarily as a result of the loss of linolenic acid (Draper, 1969). Since linolenic acid is found primarily in the chloroplast membranes, its loss probably reflects the loss of chloroplast function rather than the loss of membrane semi-permeability (Mazliak, 1977). On the other hand, large declines in the phospholipid content of both senescing leaf and petal tissues have been shown to occur (Ferguson and Simon, 1973; Beutelmann and Kende, 1977). Comparative studies have demonstrated that the loss of phospholipids roughly accompanies the increase in membrane permeability. Thus, it appears that the loss of membrane function (semi-permeability) during senescence results primarily from the degradation of membrane lipids. Very little information is available concerning the enzymatic basis for the loss of these lipids from senescing tissues.

The next question concerns the physiological significance of the loss of membrane integrity. Metabolic studies have demonstrated that various cellular metabolites and enzymes are not homogeneously dispersed within the cell but rather that they are selectively enriched in one or more subcellular organelles. This spatial separtion is of prime importance to the cell because of the fact that many molecules have multiple fates and because many seemingly opposing reactions (such as protein synthesis and breakdown) occur simultaneously. Thus, the integrated functioning of a cell requires that this spatial separation be maintained.

Therefore, the increase in membrane permeability, which leads to a loss of compartmentation, will certainly result in a modified metabolic pattern which, in turn, could affect the physiological state of the tissue.

Early investigators were quick to grasp this fact and use it to explain many phenomena. In particular, the onset of the respiratory climacteric in fruits has been attributed to the loss of "organizational resistance" or compartmentation (Blackman and Parija, 1928). This notion has been expanded by Sacher (1962, 1973) who has demonstrated that increases in membrane permeability accompany the onset of the respiratory climacteric. Because of this, Sacher has suggested that the increase in permeability is causally related to the respiratory climacteric. However, further studies (Brady et al., 1970) have shown that in some instances, the two phenomena can be dissociated and therefore are not related in a cause-and-effect manner.

Similarly, loss of compartmentation has been used to explain the surge in ethylene production in senescing flower tissue (Hanson and Kende, 1976). In support of this hypothesis it has been shown that the onset of ethylene production in senescing floral tissue coincides with the onset of rolling up of the tissue, a phenomenon, at least initially, driven by permeability-related turgor changes (Hanson and Kende, 1976). Since floating Morning Glory flower tissue produces little ethylene, no direct comparison between loss of compartmentation and ethylene synthesis could be made. Therefore, the validity of this hypothesis remains to be

proven. Along these lines, it has been shown that the onset of ethylene evolution in senescing petals of Dianthus precedes the increase in membrane permeability (Mayak et al., 1977).

The aforementioned increased leakage of anthocyanin pigments during both leaf and petal senescence indicates that loss of vacuolar integrity occurs during senescence. In the past, the vacuole has been considered as a refuse deposit for metabolic end products. However, with the advent of specific cytological stains and the introduction of vacuole isolation techniques, it has become clear that many hydrolytic activities are localized in the vacuole (Matile, 1975; Nishimura and Beevers, 1978; Boller and Kende, 1979). Because of these findings, the vacuole has been proposed as the plant's equivalent to the animal lysosome (Matile, 1975). If this hypothesis holds true for senescing tissues, then the loss of tonoplast integrity gains new importance since it would result in the release of a variety of hydrolases which, in turn, would initiate the numerous degradative changes associated with senescence. This conclusion must remain tentative as the isolation of vacuoles from senescing tissues has never been reported. In addition, it would be necessary to demonstrate that those hydrolases whose activities increase during senescence originate from the vacuole.

The Action of Ethylene During Senescence

The senescence-promoting effects of ethylene were first described nearly a century ago by investigators concerned with the toxic effects of illuminating gas on plants (see Abeles, 1973). However, a role for endogenously produced ethylene as a natural regulator of plant senescence was not established until the introduction of gas chromatographic techniques which allowed for the accurate determination of the small amounts of ethylene normally produced by plants during senescence. Since that time it has become clear that endogenously produced ethylene is a major regulator of senescence in plant tissues.

The exact role of ethylene in senescence has been the subject of an intense debate. Some investigators feel that ethylene is the actual trigger which initiates the entire process of senescence (Burg and Burg, 1968). Support for this hypothesis is derived mainly from the effects of hypobaric treatments on the ripening processes in fruit (Burg and Burg, 1965). Other investigators feel that ethylene synthesis is a result of earlier processes of senescence and that it serves to co-ordinate the final aspects of senescence (Hanson and Kende, 1976). Support for this hypothesis comes from the fact that plant tissues become increasingly more sensitive to applied ethylene as they age, with the youngest tissues often not responding at all (Hanson and Kende, 1976; Suttle and Kende, 1978). In addition, it has been shown (Hanson and Kende, 1976; Suttle and Kende, 1978) that application of inhibitors of ethylene biosynthesis,

such as aminoethoxyvinylglycine (AVG), nearly abolishes endogenous ethylene production without abolishing other physiological events associated with senescence. Regardless of its exact role, there is general agreement that ethylene is a principal, if not the principal, regulator of senescence in most plant tissues.

While not yet understood, the primary action of ethylene has been the focus of some very innovative research. Because of its unique chemical structure, ethylene has two inherent properties which have served as the basis for attempts at explaining its primary action: a) Like other plant hormones, ethylene is soluble in both water and lipids, and b) ethylene is an active ligand which readily forms co-ordination complexes with many metals.

Because of its high degree of lipid solubility, some researchers have felt that the primary action of ethylene is to alter the permeability properties of biological membranes. This effect of ethylene would be similar to the proposed mode of action of a variety of general anesthetics employed in medicine (Miller, 1975). Using isolated plant mitochondria, it has been shown that ethylene, at concentrations exceeding the saturation levels for biological activity in plants (> 10 ppm), caused reversible swelling of this organelle (Lyons and Pratt, 1964). The problems associated with this hypothesis are threefold: a) The very high concentrations of ethylene required to elicit the response, b) the fact that ethylene analogs such as vinyl fluoride, which exhibit biological activity, are not soluble in lipids, and

c) the fact that application of ethylene to many plant tissues has no effect on the permeability properties of these tissues (Burg, 1968).

A second hypothesis concerning the primary action of ethylene contends that its biological activity is mediated through its binding to a metallic receptor site, presumably on an enzyme (Burg and Burg, 1967). Studies on ligand binding in model systems have shown that the attachment of a ligand to a receptor alters the electronic configuration surrounding the receptor. Since many enzyme activities are regulated through conformational changes which result from both hydrophobic and electrostatic interactions, this model of ethylene action is indeed reasonable. Additional support for this hypothesis has been derived from the fact that the biological activity of several ethylene analogs parallels their affinities for certain metals (Burg and Burg, 1967). Recently it has been shown that application of the silver ion results in complete inhibition of many tissue responses to ethylene (Beyer, 1976). This tends to support Burg's contention and suggests that the metallic receptor might be the cuprous ion (Beyer, 1976).

Regardless of the nature of the initial action of ethylene, it has been shown that the activity of ethylene is probably accompanied by its metabolism (Beyer, 1975; Beyer and Sundin, 1978). This metabolism results in both the formation of carbon dioxide and in the incorporation of some part of the ethylene molecule into an unknown compound (Giaquinta and Beyer, 1977). Although the exact details of

this metabolism are not clear, the metabolism of ethylene is tied to its biological activity as immature tissues and tissues treated with silver or CO_2 neither respond to ethylene nor metabolize it to the same extent as do control tissues (Beyer, 1979).

The action of ethylene requires continued protein and in some cases RNA synthesis (see Abeles, 1973). It is a common observation that the action of ethylene is inhibited by cycloheximide and this observation has prompted the suggestion that ethylene action is mediated by protein synthesis. Application of ethylene has been shown to stimulate the incorporation of precursors into both RNA and protein in abscission zones (Abeles, 1968) and in senescing fruit tissues (Hulme et al., 1971). Application of ethylene to various plant tissues has been shown to enhance the activity of many enzymes (see Abeles, 1973). Since in at least two instances the enzymes have been shown to be synthesized de novo (Frenkel et al., 1968; Lewis and Varner, 1970), it is reasonable to assume that ethylene induces their synthesis.

The Biosynthesis of Ethylene

Plant tissues have been shown to convert the following, naturally-occurring compounds into ethylene: acrylic acid (Ghooprasert and Spencer, 1975), linolenic acid (Mapson et al., 1969) and methionine (Lieberman et al., 1965). This list is by no means complete. Most of these precursors were converted to ethylene at low rates, and the degree of metabolic interconversion has usually not been determined. The

peroxidation of linolenic acid which yields both ethane and ethylene probably does occur in natural situations involving stress, such as wounding or pollution damage (Konze and Elstner, 1978). Through the intensive work of several investigators, it is now generally believed that ethylene is derived from carbons 3+4 of methionine in all senescing tissues (Lieberman, 1979). A major complication in the study of ethylene biosynthesis has been that in most tissues the rate of ethylene production is very low. This fact, coupled with the multiple fates of administered methionine, has greatly hindered progress in this area.

The conversion of methionine to ethylene has been viewed in two ways. One view holds that methionine is directly converted to ethylene by an oxidative, free radical-mediated process (Lieberman, 1979). Support for this view comes from the following observations: a) Methionine is readily converted to ethylene in several in-vitro, free radical systems (see Lieberman, 1979), and b) compounds known to scavenge free radicals in-vitro inhibit ethylene production in-vivo (Baker et al., 1978).

The alternative viewpoint has held that methionine is first converted to other compounds which, in turn, are converted to ethylene. Based on the inhibitory action of uncoupling agents and of anaerobiosis, S-adenosylmethionine (SAM) has been proposed as an intermediate in the conversion of methionine to ethylene (Burg, 1973). Indirect evidence for the involvement of SAM in ethylene biosynthesis has been provided by Adams and Yang (1977) and Konze and Kende (1979).

Further progress in this field has awaited the revival and utilization of an old observation concerning the effects of anaerobiosis on ethylene production. In 1942, Hansen found that if apple tissue is placed under nitrogen for several hours (during which time the ethylene production falls to zero) and then is returned to air, ethylene production commences immediately and at a rate several times that of tissues not kept under nitrogen. This observation prompted the search for an intermediate of ethylene biosynthesis which accumulates under nitrogen and subsequently disappears following reintroduction of air. Such a compound, 1-aminocyclopropane-1-carboxylic acid (ACC) has been recently identified (Adams and Yang, 1979).

Subsequent work has shown that ACC is readily converted to ethylene and that this conversion is inhibited by anaerobiosis (Adams and Yang, 1979). Additional research has resulted in the isolation of an enzyme which catalyzes the conversion of SAM to ACC (Boller et al., 1979). Thus, the biosynthetic pathway of ethylene formation in senescing tissues is: Methionine → SAM → ACC → ethylene.

The initiation of ethylene production during senescence could occur through the activation of a pre-existing enzyme system(s) or through de novo synthesis of one or more of the required enzymes. Studies on the effect of mechanical injury or wounding in plants have demonstrated that most, if not all, tissues have the capacity to produce ethylene (Hanson and Kende, 1976; Konze and Elstner, 1978). This observation, coupled with the temporal coincidence of

enhanced cellular leakage and increased ethylene production in senescing flowers, lead Hanson and Kende (1976) to propose that the onset of ethylene production in senescing flowers is dependent on the release of one or more required compounds which occurs as a result of the loss of tonoplast integrity.

The induction of ethylene production in etiolated pea stem sections by auxin and in senescing fruit tissues following ethylene pretreatment has been shown to be inhibited by cycloheximide (Kang et al., 1971; Frenkel et al., 1968). Furthermore, ethylene production in both peas and apples, in which ethylene production has been fully induced, is also sensitive to both RNA and protein synthesis inhibitors (Lieberman and Kunishi, 1975). These observations indicate that the enzymatic machinery for ethylene biosynthesis undergoes turnover and further that treatments which stimulate ethylene production do so through enhanced synthesis of one or more of the required enzymes.

Scope of This Project

The overall aim of this project was to study the involvement of ethylene in the senescence processes of the ephemeral flower of Tradescantia. It was hoped that the use of an ephemeral flower would facilitate the study of the sequence of events that are associated with senescence by reducing the time interval between successive stages of the process. Further, since petals of Tradescantia are heavily pigmented with vacuolar pigments, the use of these petals

should permit an assessment of the role of ethylene in the loss of vacuolar integrity as well as the role of this loss in the overall process of senescence itself.

Specifically, these investigations were undertaken to gain insight into the following questions:

1) the role of endogenously produced ethylene as a regulator of the loss of vacuolar integrity during senescence
2) the mode of action of ethylene in this process
3) the role of vacuolar integrity in ethylene biosynthesis
4) the characteristics of ethylene biosynthesis in these petals.

SECTION I

Ethylene and Senescence in Petals of *Tradescantia*

INTRODUCTION

Ethylene has been shown to be involved in the regulation of senescence in a variety of plant organs including fruits and flowers (Abeles, 1973; Burg and Burg, 1965a; Hanson and Kende, 1975; Morgan et al., 1973; Nichols, 1977). However, its mode of action in this process is not understood. Hanson and Kende (1975) have shown that ethylene enhances loss of membrane semipermeability in mature petal tissue of Ipomoea tricolor. These results are consistent with the hypothesis that the tonoplast is the first membrane to be affected in the course of ethylene action (Hanson and Kende, 1975).

Flowers of Tradescantia are ephemeral and contain delphinidin (Mericle and Mericle, 1971), an anthocyanin pigment which is localized in the vacuole. Preliminary experiments indicated that isolated senescing petals lose this pigment to a bathing medium and that exogenous ethylene hastens the onset and the rate of senescence. Therefore, petals of Tradescantia should be a very suitable material for studying the action of ethylene on the integrity of the tonoplast and the consequences of tonoplast deterioration on the process of senescence itself.

MATERIALS AND METHODS

Plant Material

Cloned plants of a hybrid Tradescantia (02 clone; putative parents T. occindentalis x T. ohiensis; obtained from Dr. L. Mericle, Dept. of Botany and Plant Pathology, M.S.U.) were planted in plastic pots in a 1:1:1 (v/v) mixture of potting soil, sand and perlite. The plants were watered twice daily and maintained under a daily regime of 16 h light, 24°C and 8 h dark, 20°C. The light intensity at plant height was 4 to 6 x 10^4 ergs cm^{-2} sec^{-1} and the relative humidity was maintained between 60 and 70%. Throughout this paper, the following terminology will be used when referring to the plant material: day -2: flowers or flower parts isolated two days prior to flower opening; day -1; flowers or flower parts isolated one day prior to flower opening; and day 0: flowers or flower parts isolated on the day of opening.

Experiments with Intact Flowers

For the determination of ethylene synthesis in whole Tradescantia flowers, single flowers were excised from the plants early in the morning of day 0, and placed with their cut stems into 7 x 15 mm nitro-cellulose tubes containing distilled water. Each tube was inserted into a perforated

foam stopper which was placed into a 50-ml plastic syringe. The plunger of the syringe was adjusted to give an air space of 30 ml, and the syringe was sealed with a serum-vial cap. The progress of flower fading was viewed through the plastic walls of the sealed syringes. In order to study the effect of an exposure of flowers to ethylene, batches of flowers were gassed simultaneously with 10 µl/l ethylene for 90 min in a 50-ml stoppered plastic syringe. Upon completion of this treatment, the flowers were removed and placed into individual 50-ml syringes as described above. The length of time from sealing this syringe until the flowers had completely closed was noted.

Ethylene Production by Isolated Floral Tissue

Flowers were excised from the plant early on day 0 and were dissected into: (i) sepals, (ii) petals, and (iii) the remaining organs (i.e. stamens, gynoecium and receptacle). The respective parts from 3 flowers were placed into a 25-ml Erlenmeyer flask containing 5 ml of 1% agar as support. The flasks were sealed, and ethylene determinations were made throughout the day.

Isolated Petals

Petals were isolated either from buds on day -1 or from flowers on day 0. The petals were floated on 5 ml of glass-distilled water or solutions of the aminoethoxy-analog of rhizobitoxine in 50-ml Erlenmeyer flasks with side-arms; each flask was sealed with a serum-vial cap and was fitted

with a conical cuvette attached with a 2.5-cm rubber tubing to the side arm. This assembly allowed continuous measurement of both ethylene concentration and the absorbance of the bathing solution.

Determination of Pigment Efflux and Ethylene Production

Anthocyanin efflux was monitored in the sealed system by tipping the flask such that a portion of the bathing medium was introduced into the conical cuvette, thereby allowing the measurement of the absorbance of the bathing medium at 575 nm, using a Coleman Junior Colorimeter (Coleman Instruments, Oak Brook, Ill.). Ethylene production was measured by withdrawing a 1.0-ml gas sample from the headspace in the incubation flask and injecting it into a gas-chromatograph as described previously in studies with Ipomoea flower tissue (Kende and Hanson, 1976). Each sample removed was replaced with 1.0 ml of ethylene-free air.

Electrolyte Leakage

Petals were isolated on the appropriate day early in the morning and were floated on glass-distilled water for one hour. They were then placed on 8.0 ml glass-distilled water in 50-ml flasks; the flasks were sealed with serum-vial caps. Ethylene was injected into half of the flasks to give a final concentration of 10 µl/l, and at the appropriate times 5.0 ml of bathing solution was removed and the conductance measured with a Markson Electro Mark Analyzer (Markson Science, Del Mar, Calif.). These 5 ml of solution

were returned to the flask, the flask resealed, and fresh air or ethylene (final concentration 10 μl/l) was reintroduced.

Effects of Ethylene on Endogenous Ethylene Synthesis

Petals of different ages were excised early in the morning and divided into 2 groups. Those to be exposed to ethylene were placed on water soaked cotton inside a 30-ml test tube and the tube was sealed with a serum-vial cap. Following the ethylene treatment, the petals were allowed to stand 5-10 min in laboratory air; after this, both ethylene-pretreated and control petals were placed into 25-ml flasks as described in the experiments on the production of ethylene by isolated floral tissues.

All experiments were repeated no less than 4 times, all giving very similar results.

RESULTS

Figure 1 shows the progress of floral senescence in intact Tradescantia flowers. The buds open early in the morning, becoming fully open by 10:00 h (Stage I). By 16:00 h the petals begin to show signs of wilting beginning at the distal margins and proceeding basipetally (Stages II and III). By 22:00 h the flowers have closed irreversibly (Stage IV), and the petals become translucent because of leakage of cell sap into the intercellular spaces.

When fully open flowers are excised from the plant and placed within a sealed 50-ml syringe, the flowers remain open for 5.25 (± 0.9) h. If similar flowers are pretreated for 90 min with a 10 µl/l atmosphere of ethylene prior to sealing, they remain open for only 3.75 (± 0.5) h. Figure 2 shows the relationship between flower morphology and the rate of ethylene production of flowers which had been kept on the plant until the rate of ethylene evolution was measured. There was an initial increase in the rate of ethylene production occurring slightly before curling of the petals. The rate of ethylene production remained high throughout senescence and fell off as the flowers became fully closed. From Figure 3 it is evident that all floral tissues produced ethylene with the major part originating in the reproductive organs (>70%). There was a substantial

Figure 1. The developmental (morphological) changes during senescence of Tradescantia flowers. Stage I: fully open; Stages II and III: initiation and progression of fading; Stage IV: fully faded flower.

I

II

III

IV

Figure 2. Comparison of the rate of ethylene production and morphology of Tradescantia flowers on day 0. Flowers were excised from the plant throughout the day and were placed inside a stoppered plastic syringe for two hours in order to determine the rate of ethylene production.

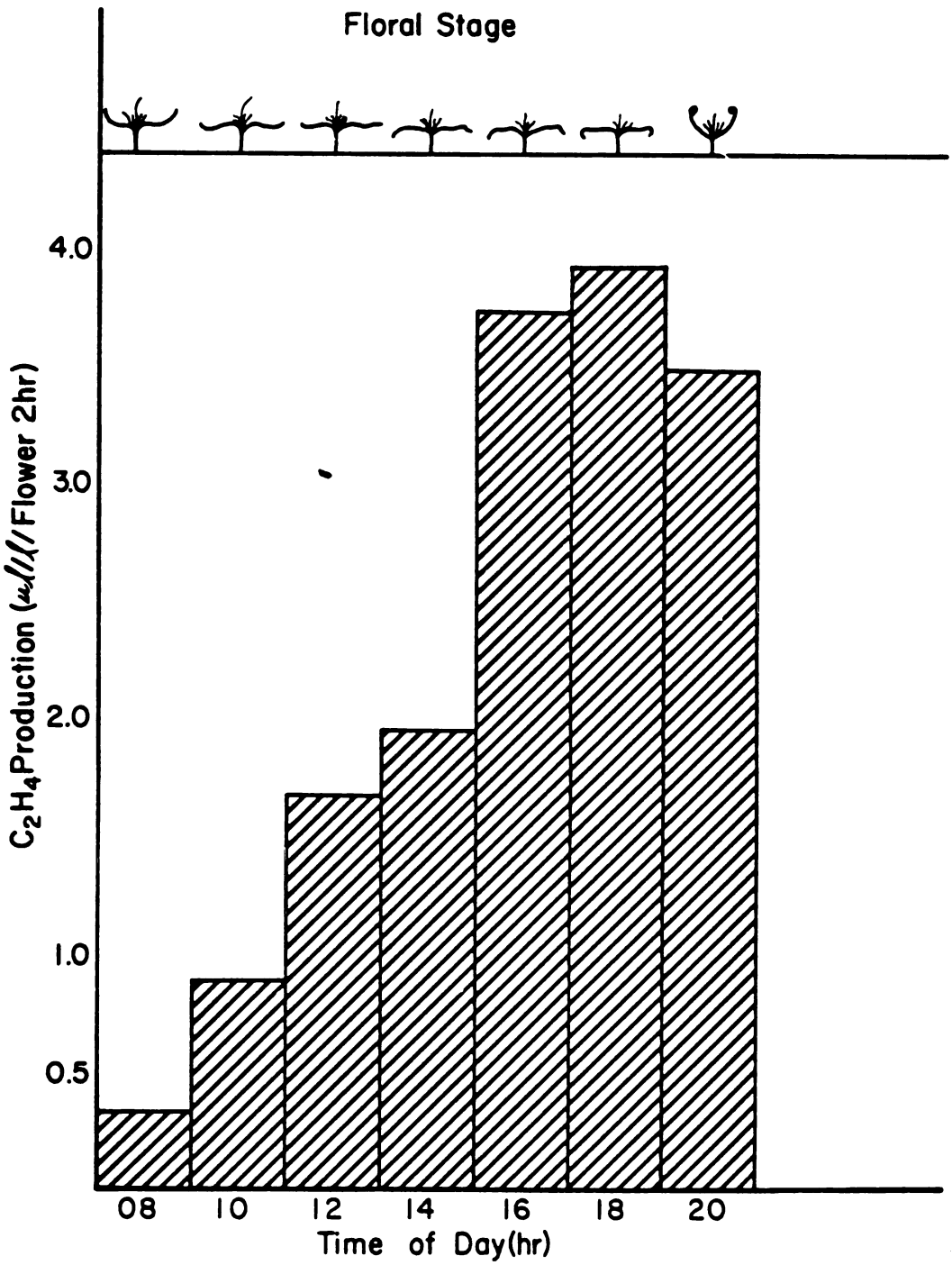
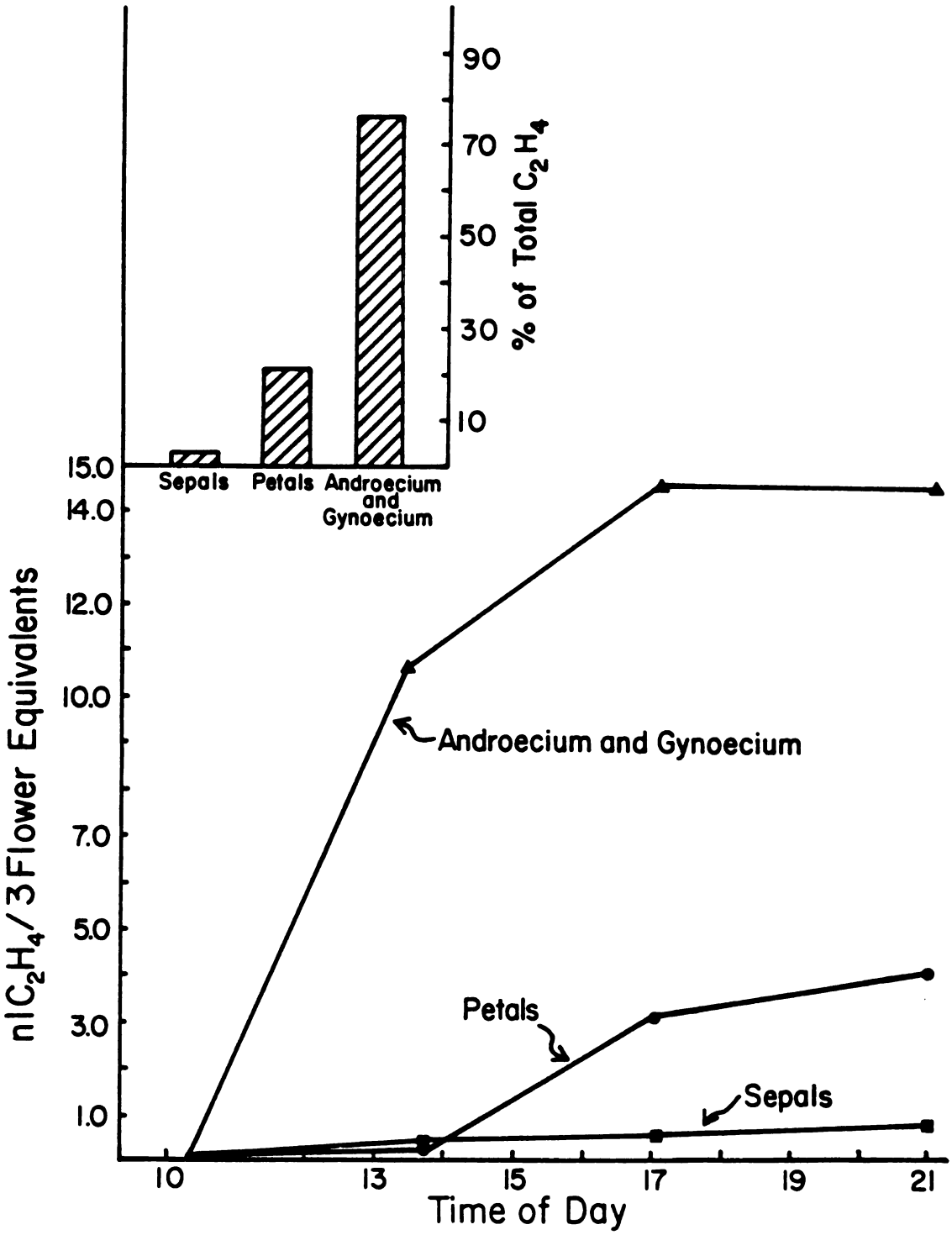

Floral Stage
4.0
3.0
2.0
1.0
0.5
C_2H_4 Production (µl/l/Flower 2hr)
08
10
12
14
16
18
20
Time of Day(hr)

Figure 3. Time course of ethylene production by isolated parts of the Tradescantia flower. Flowers were dissected at 08:15 on day 0 and allowed to stand in a humid chamber until 10:00. The respective organs of three flowers were then placed into a 25-ml stoppered flask. Inset indicates percent production of ethylene by each group of isolated organs.

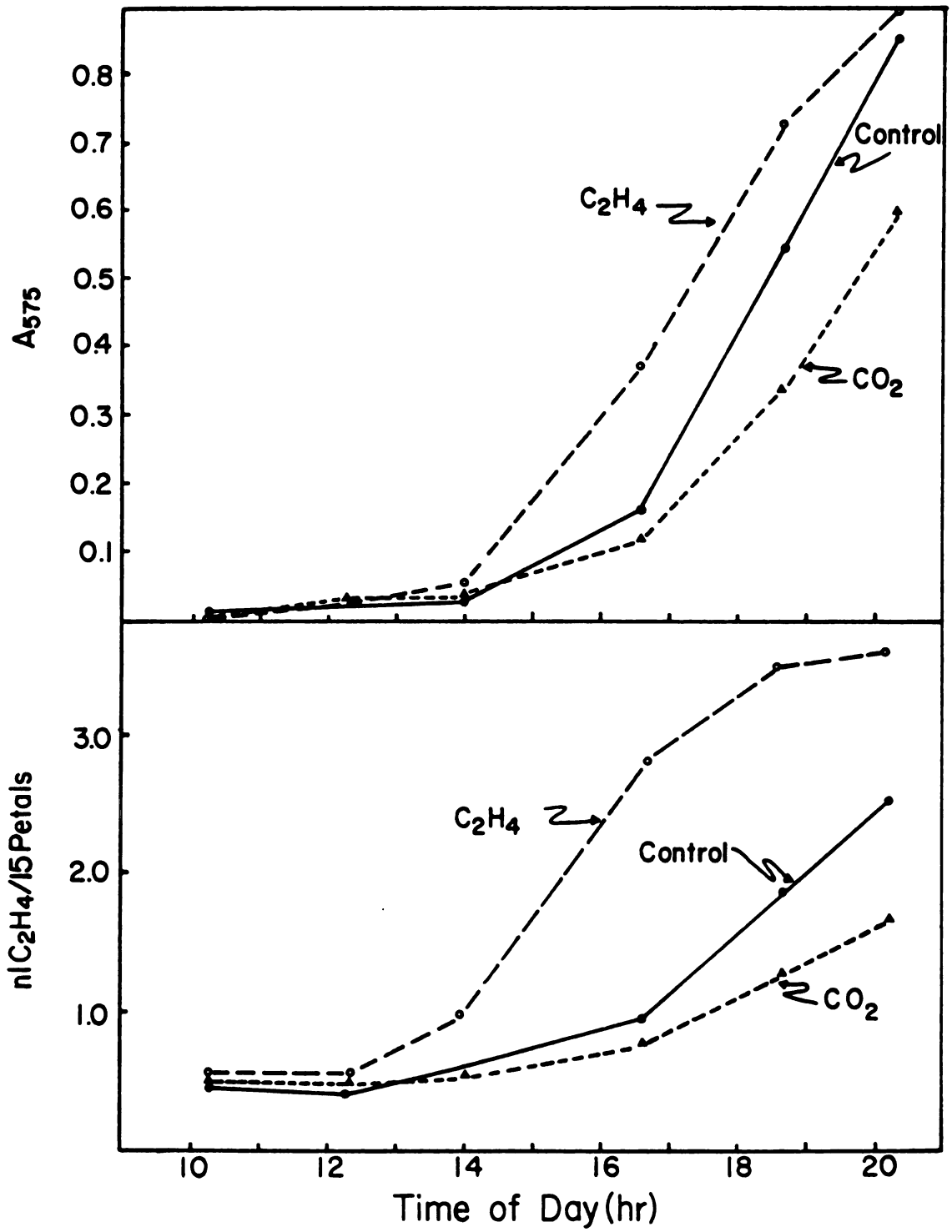

90
70
50
30
10
% of Total C_2H_4
Sepals
Petals
Androecium and Gynoecium
15.0
14.0
12.0
10.0
7.0
5.0
3.0
1.0
nl C_2H_4/3Flower Equivalents
Androecium and Gynoecium
Petals
Sepals
10
13
15
17
19
21
Time of Day

(20%) contribution by the isolated petals as well. Furthermore, both the reproductive organs and the petals produced ethylene throughout the day in a manner analogous to the production by the intact flower. However, the onset of ethylene production in the different organs was not synchronous. Ethylene could be detected in reproductive organs and receptacle tissue by 10:20 h, while the increase in ethylene production by the petals commenced 3 h later. These results show that there are several sources of ethylene production in the intact flower; however, visible signs of fading can be seen only in petals. For this reason, we decided to investigate the relationship between ethylene production and senescence in isolated petals, organs which not only produce ethylene but also respond to it visibly.

While isolated petals produced ethylene during the course of senescence, they did not curl like petals in intact flowers. They did, however, become translucent as the cell sap diffused into the intercellular spaces, and they subsequently lost their pigment to the bathing solution.

Figure 4 shows the relationship between ethylene evolution and pigment efflux in petals isolated on day 0. Both processes started simultaneously between 14:00 and 16:00 h. In petals pretreated for 60 minutes with 10 μl/l ethylene, both pigment efflux and endogenous ethylene production started at least 2 h earlier than in the control petals. Continuous exposure of isolated petals to 4% (v/v) CO_2 resulted in a retardation of both pigment efflux and ethylene production.

Figure 4. Comparison of the time course of ethylene production and pigment efflux in isolated Tradescantia petals on day 0. One group of petals was pretreated with 10 µl/l of ethylene from 09:00 to 10:00 and subsequently transferred to the experimental chamber; one group was placed into a chamber which was maintained at 4% (v/v) CO_2 throughout the experiment. Control petals received no ethylene nor CO_2 treatment.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A575
Control
C2H4
CO2
3.0
2.0
1.0
nlC2H4/15Petals
C2H4
Control
CO2
10
12
14
16
18
20
Time of Day (hr)

If petals were isolated from the bud on day -1 and kept overnight they senesced on day 0 in a manner similar to petals isolated on day 0 (Fig. 5). This fact permits feeding experiments such as those depicted in Figure 5.

When petals were isolated on day -1 and kept overnight on a 0.1 mM solution of aminoethoxyvinylglycine (AVG), they did not produce ethylene. Figure 5 shows that, although there was some ethylene present initially, the toxin-treated petals produced no detectable amounts of ethylene throughout the experiment. The petals still lost pigment, the efflux beginning simultaneously with that in control petals not treated with the toxin. However the total amount of pigment loss throughout the day was considerably less than in control petals. A 60-minute pretreatment with 10 µl/l ethylene restored the pigment efflux from toxin-treated petals to the control level, but ethylene production remained inhibited.

These experiments indicate that factors other than ethylene are also involved in petal senescence. To gain further insight into this, the effect of ethylene on immature petals was determined. Figure 6 illustrates the effect of ethylene on electrolyte leakage from petals which were isolated on day -2, day -1 and day 0. Petals isolated on day -2 showed a constant rate of efflux of electrolytes throughout the day, and continuous exposure of the petals to 10 µl/l ethylene had no effect on this process. Petals isolated on day -1 also exhibited a constant rate of electrolyte efflux through the day, the rate not differing greatly from those isolated on day -2. However continuous exposure

Figure 5. Comparison of the time course of ethylene production and pigment efflux in Tradescantia petals on day 0. Petals were isolated on the evening of day -1 and maintained on either distilled water or a solution of 10^{-4} M AVG. Dashed lines give values for the toxin-treated petals. One group of both AVG-treated and control petals were pretreated for 60 min with a 10 µl/l atmosphere of ethylene prior to being sealed in the experimental chamber.

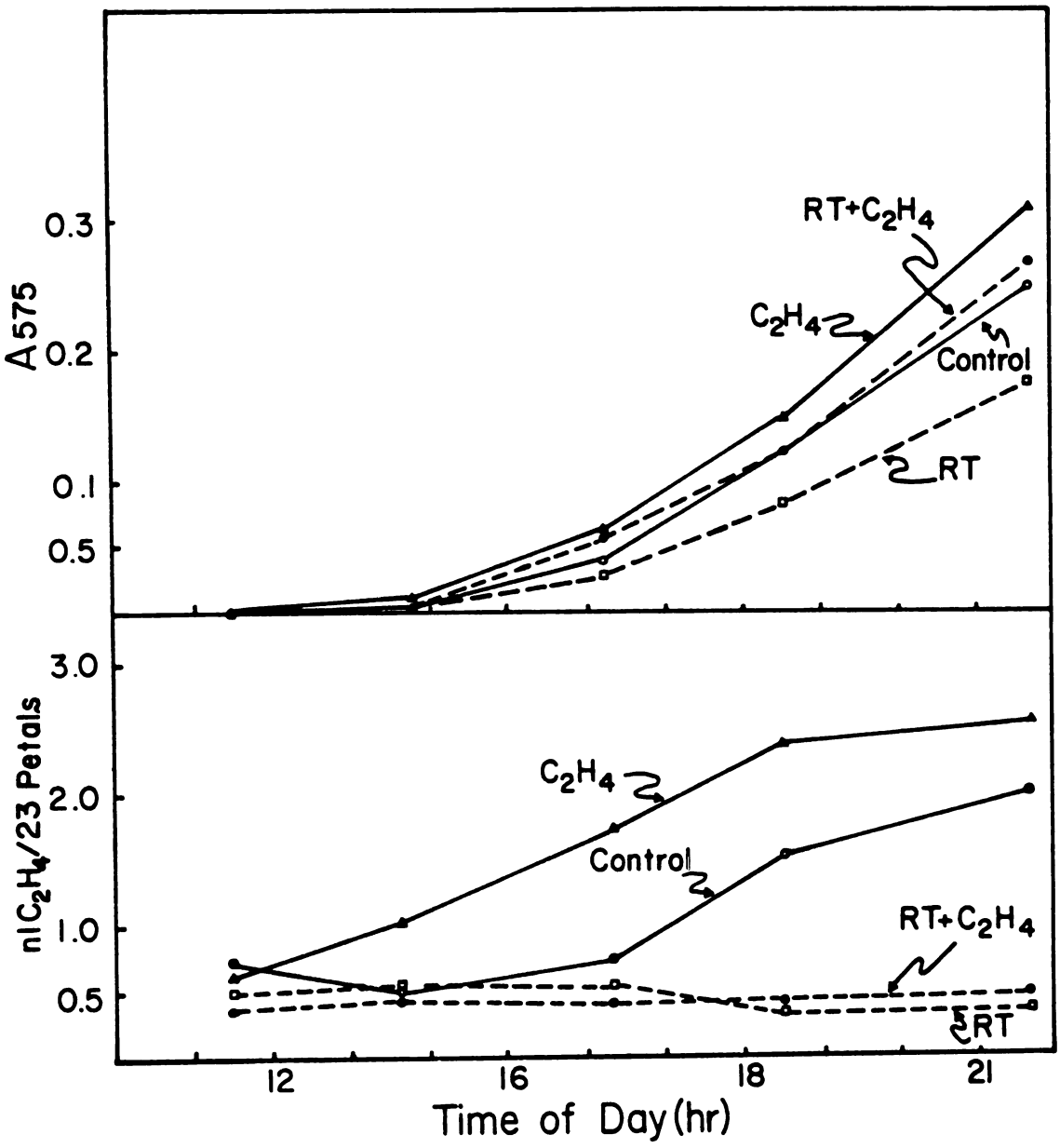
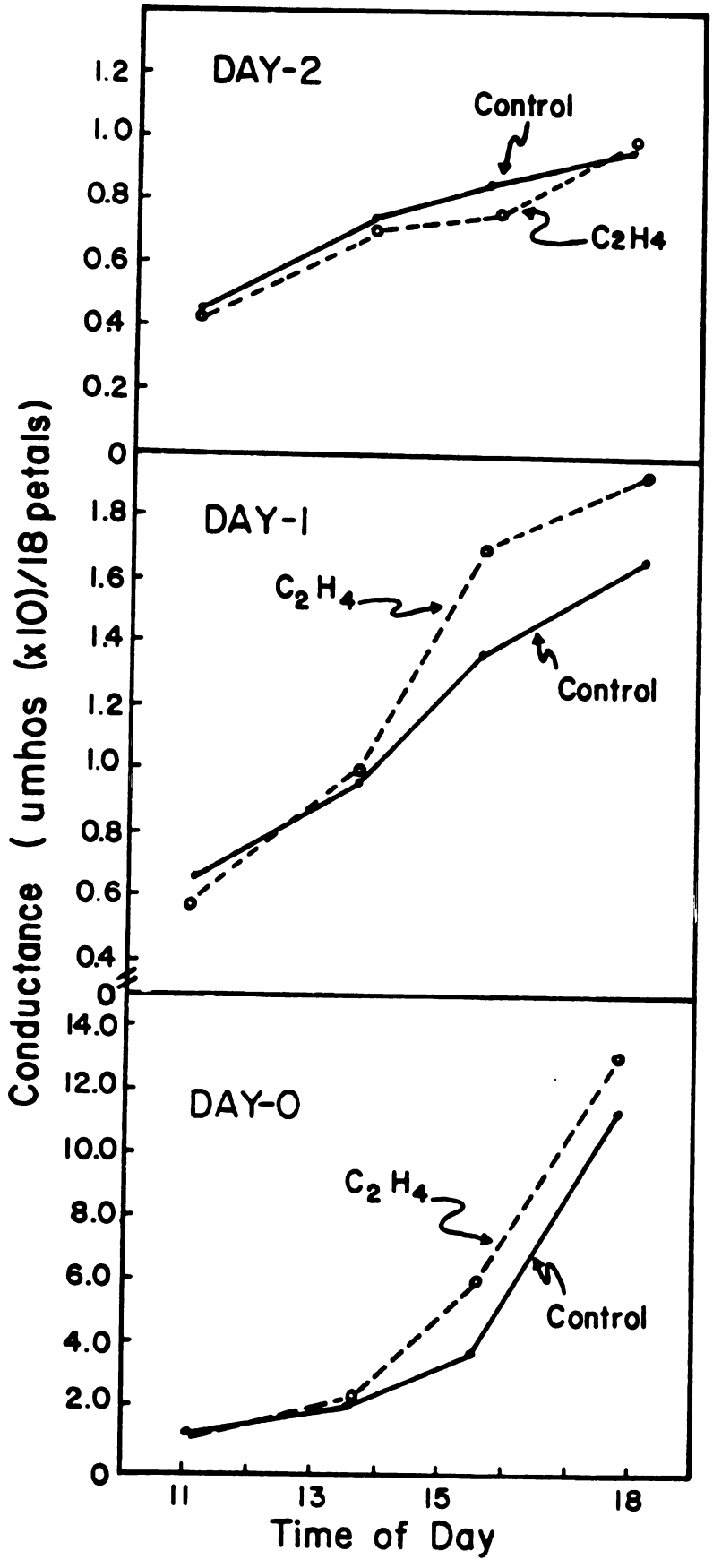

A575
0.3
0.2
0.1
0.5
RT+C2H4
C2H4
Control
RT
nlC2H4/23 Petals
3.0
2.0
1.0
0.5
C2H4
Control
RT+C2H4
RT
12
16
18
21
Time of Day (hr)

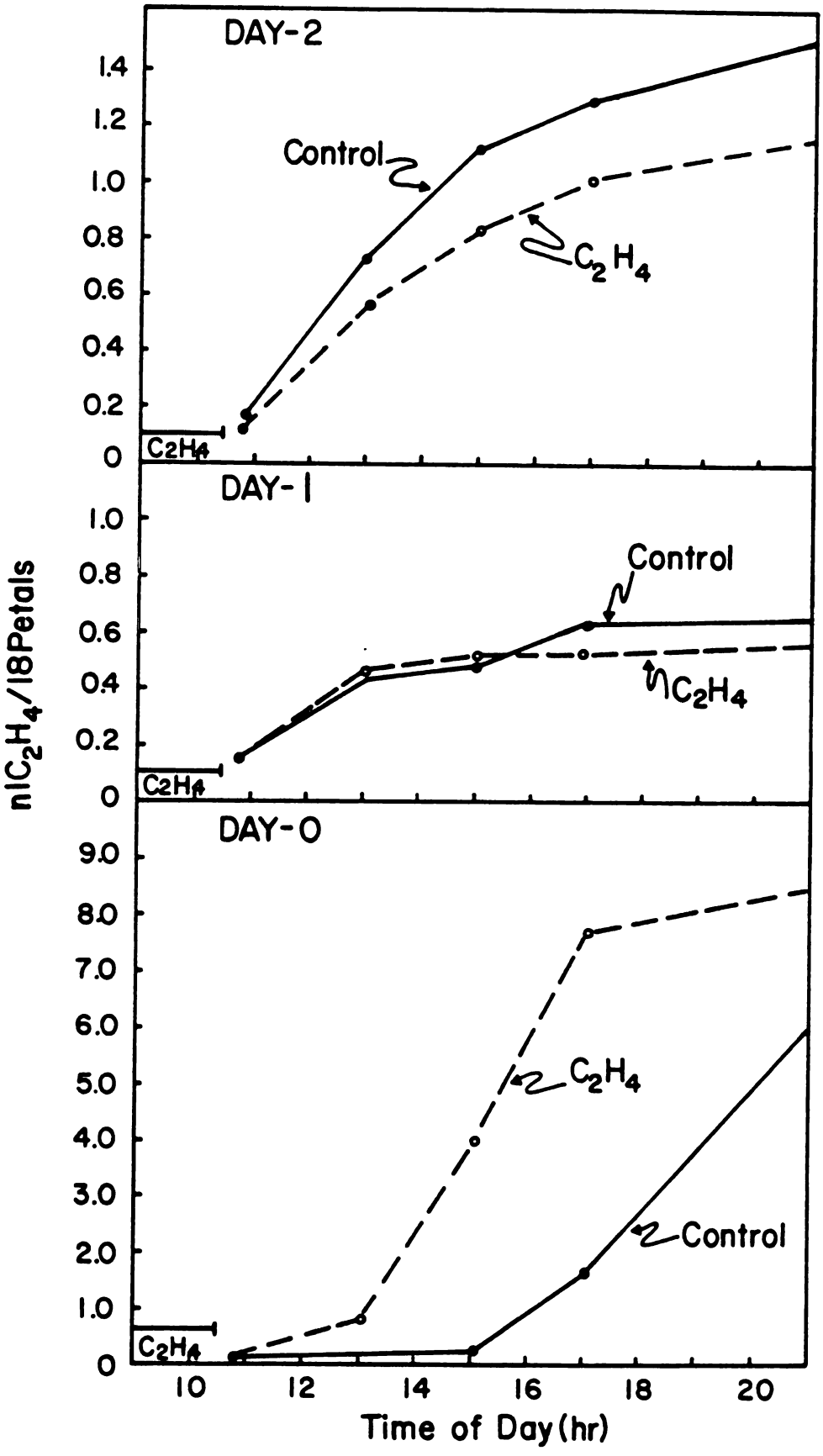
Figure 6. Effect of a 10 µl/l ethylene atmosphere on electrolyte leakage from isolated Tradescantia petals during day -2, -1 and 0. Eighteen petals were floated on 8.0 ml of glass-distilled, deionized water in a sealed 50-ml flask. Dashed lines give values for the ethylene treated petals. Ethylene treatment was begun at 11:00 h.


DAY-2
Control
C2H4
DAY-1
C2 H4
Control
DAY-0
C2 H4
Control
Conductance (umhos (x10)/18 petals)
Time of Day
1.2
1.0
0.8
0.6
0.4
0.2
0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
11
13
15
18

of these petals to 10 µl/l ethylene on day -1 caused an enhanced rate of electrolyte efflux. Although the petals were fully pigmented, there was no anthocyanin efflux from control and ethylene-treated petals on day -1. In petals isolated on day 0, applied ethylene hastened the leakage of electrolytes by 2 h (note difference in ordinate scale). Also in control petals on day 0 there was a spontaneous increase in the rate of electrolyte leakage around 15:30 h, indicating the onset of endogenous senescence.

Figure 7 shows that a 90-minute pretreatment with ethylene had no effect on subsequent ethylene production in petals isolated on day -1 or day -2. In fact, the ethylene pretreatment appeared to depress the rate of subsequent ethylene production in these petals. On day 0, ethylene production in control petals increased spontaneously around 15:00 h. The ethylene pretreatment shifted the onset of ethylene evolution to 13:00 h.

Figure 7. Effect of a 90-min, 10 µl/l ethylene pretreatment on the subsequent rate of ethylene production in isolated Tradescantia petals on day -2, -1, and 0. Eighteen petals were placed into a 25-ml flask containing 5.0 ml of 1% agar. The pretreatment with ethylene was performed in a separate chamber from 09:00 to 10:30 h.


DAY-2
Control
C_2H_4
C_2H_4
DAY-1
Control
C_2H_4
C_2H_4
DAY-0
C_2H_4
Control
C_2H_4
nl C_2H_4/18Petals
Time of Day (hr)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
12
14
16
18
20

DISCUSSION

The results show that ethylene is a regulator of both flower fading and petal senescence in Tradescantia. This is concluded from the fact that exogenous ethylene accelerates the rate of both processes, and that the endogenous production of ethylene sharply increases in both flowers and petals as they deteriorate. The extent to which endogenously produced ethylene is involved in the regulation of petal aging is most clearly seen from the data presented in Figure 5. The rate of anthocyanin efflux, which serves as an indicator of senescence, is retarded in petals which have been treated with AVG and a 60-min pretreatment with 10 µl/l ethylene is sufficient to restore the rate of pigment efflux from AVG-treated petals to the control rate.

The data presented in Figure 3 show that ethylene is produced by all floral tissues with the major contribution originating from the reproductive tissues. These data are consistent with similar observations made with cotton and carnation flowers (Morgan et al., 1973; Nichols, 1977). The role of pollination in initiating ethylene production in flowers is well-documented (Abeles, 1973; Burg and Dijkman, 1967; Hall and Forsyth, 1967), and the substantial ethylene production by the reproductive tissues of Tradescantia flowers may well be another instance of such an interaction.

The role of ethylene in senescence has been interpreted in two, apparently conflicting ways. Some authors consider ethylene as the actual trigger of senescence (see review by Sacher, 1973); others feel that metabolic processes preceding ethylene synthesis lead to the onset of senescence, and that ethylene regulates the rate of the terminal deteriorative changes (Kende and Hanson, 1976; Kosiyachinda and Young, 1975). The results of Figure 5 tend to support the second hypothesis. Although there is no detectable ethylene production in the presence of AVG, the initiation of pigment leakage is not delayed even though the rate of efflux is reduced, indicating that factors other than ethylene may determine the initiation of senescence in isolated petals of Tradescantia.

Further support for the second hypothesis can also be derived from Figures 6 and 7. If ethylene is the trigger initiating senescence in Tradescantia petals, application of ethylene to immature petals which normally produce only small amounts of ethylene on day -2 and day -1 should induce senescence in these tissues as well. Our data show that petals isolated on day -2 are completely insensitive to exogenous ethylene. These petals exhibit no ethylene-enhanced electrolyte leakage nor ethylene-induced ethylene synthesis. On day -1, exogenous ethylene does stimulate electrolyte leakage but has no effect on the rate of subsequent ethylene production. However, on the day 0, exogenous ethylene accelerates the rates of both processes. Thus, as petals mature, they acquire a sensitivity to exogenous ethylene.

The gradual acquisition of ethylene sensitivity has also been observed in flower tissue of Ipomoea tricolor (Kende and Hanson, 1976) and in fruit tissue (Burg and Burg, 1965a).

Results of other experiments concerning the effects of exogenous ethylene applied to petals on day -1 and day 0 point to further differences in the response of mature and immature tissues to the gas. Figures 4 and 6 demonstrate that exogenous ethylene stimulates both anthocyanin and electrolyte leakage from petals on day 0. When measured simultaneously, the rates of efflux of both were found to parallel each other throughout the day (not shown), indicating that ethylene increases membrane permeability in an unspecific fashion. However, exogenous ethylene causes no pigment efflux in petals on day -1, even though they are fully pigmented. Therefore, the effect of ethylene in causing loss of cellular compartmentation is selective in these petals, and components involved in the "autocatalytic" synthesis of ethylene in petals on day 0 may remain sequestered in petals on day -1.

Because petals of Tradescantia are heavily pigmented, the effect of ethylene on membrane permeability can easily be measured. Furthermore, since anthocyanin pigments are localized within the vacuole of all cells, the action of ethylene in enhancing the rate of efflux of this pigment can be interpreted as an effect of the gas on the integrity of the tonoplast. In other studies, utilizing solely electrolytes or radioactive tracers as markers of efflux, the

effect of ethylene on the tonoplast could only be inferred indirectly (Hanson and Kende, 1975). While a definite effect of the gas on the tonoplast has been shown in our present studies, a similar effect of ethylene on the permeability of the plasmalemma cannot be ruled out. It is impossible to say whether the permeability of the plasmalemma would have to increase to permit efflux of anthocyanin from the cell, once the pigment has been released from the vacuole into the cytoplasm.

The question that arises now concerns the physiological significance of the loss of tonoplast integrity. Matile and others, using vacuole preparations from yeast and meristematic tissues, have provided evidence for the vacuolar localization of many hydrolases (Matile, 1977). Based on these data as well as numerous other observations, Matile has proposed the vacuole as the plant's equivalent of the lysosomal compartment of animal cells. Recently, vacuolar localization of hydrolytic enzymes has been shown in mature plant cells (Boller and Kende, 1979). Therefore, the loss of tonoplast integrity would allow the mixing of heretofore sequestered vacuolar hydrolases with their cytoplasmic substrates. Loss of compartmentation between the vacuole and the cytoplasm may, in addition to causing eventual autolysis of the cell, also be the reason for "autocatalytic" ethylene production (Hanson and Kende, 1975).

In conclusion, it was shown that endogenous ethylene is a regulator of senescence in Tradescantia flowers. It appears to play a decisive role in determining the rate of

loss of compartmentation in Tradescantia petals, and this loss of compartmentation could play a central role in bringing about the deteriorative changes in the petals which occur during senescence.

SECTION II

Ethylene Action and Loss of Membrane Integrity During Petal Senescence in *Tradescantia*

INTRODUCTION

Increased membrane permeability is a characteristic attribute of senescing plant tissues (Ferguson and Simon, 1973a; Hanson and Kende, 1975; Sacher, 1973; Suttle and Kende, 1978). Desiccation of leaves, wilting of petals, and enhanced efflux of cellular constituents, such as vacuolar pigments, sugars, and electrolytes are all gross manifestations of more subtle changes in membrane integrity that occur during senescence. Because many aspects of membrane permeability are associated with the composition and organization of the lipid components of membranes (Simon, 1974), considerable attention has been focused on changes in membrane lipids (primarily phospholipids) during senescence. The loss of membrane integrity that occurs during leaf senescence is correlated with a large decline in the phospholipid content (Ferguson and Simon, 1973a). However, because of the long intervals between the determinations, it is difficult to discern whether changes in phospholipid content precede the increase in cellular permeability, or vice-versa.

Ephemeral flowers offer an opportunity to study the temporal sequence of biochemical changes attending senescence. Senescence of isolated segments of Ipomoea flowers is accompanied by an abrupt increase in membrane

permeability (Hanson and Kende, 1975), and a large decline in phospholipid content (Beutelmann and Kende, 1977). However, because phospholipid content has not been directly correlated to the onset of permeability changes within this tissue, it is again difficult to assess the role of phospholipid loss in the observed increases in membrane permeability.

During senescence, anthocyanin is released from isolated petals of Tradescantia, and pre-treatment of the petals with 10 μl/l of ethylene hastens the onset of this process (Suttle and Kende, 1978). Because anthocyanins are localized in the vacuole, these petals offer an opportunity to directly assess the integrity of cellular membranes, in particular that of the tonoplast, as well as the role of ethylene in effecting membrane degradation. This report describes the response of mature petals of Tradescantia to ethylene and the nature of the biochemical changes which result in the loss of membrane integrity during senescence.

MATERIALS AND METHODS

Plant Material

Cloned plants of a hybrid Tradescantia (Clone 02) were grown as described before (see Section I, p. 22). Petals were isolated from fully opened flowers which were excised from the plant early on the morning of flower opening. Prior to use, the petals were stored in a glass petri dish containing a disc of water-saturated filter paper to prevent desiccation.

Simultaneous Determination of Anthocyanin and Electrolyte Leakage

Two groups of eighteen petals each were floated on glass-distilled water for 1 hr. The petals were then placed on 8-ml of glass-distilled, deionized water in 50-ml Erlenmeyer flasks. The flasks were sealed with serum vial caps, and ethylene was added to one flask to yield a final concentration of 10 µl/l in the head space. At the appropriate time, the flasks were opened and both the electrical conductance and absorbance at 575 nm of an aliquot of the bathing medium were determined as described before (Suttle and Kende, 1978). Following the determinations, the aliquot of bathing medium was returned to the flask and the flask resealed. Following resealing, ethylene was again added to

the respective flask to a final headspace concentration of 10 µl/l.

Effect of Cycloheximide and Cordycepin on Ethylene-Induced Efflux

Groups of 15 petals were floated on 5 ml of glass-distilled water or 0.1 mM cycloheximide (Sigma Chemical Co.) in 50-ml Erlenmeyer flasks equipped with side-arm cuvettes as described before (Suttle and Kende, 1978). The flasks were sealed, and ethylene was introduced to a final concentration of 10 µl/l. In one flask only 4.5 ml of distilled water was initially included and after two hours of exposure to ethylene, 0.5 ml of 1 mM cycloheximide was injected through the serum-vial cap. Analogous procedures were used in the study on the effect of cordycepin (Sigma Chemical Co.) and the other inhibitors of transcription.

Determination of Total Phospholipid Levels

Groups of 12 petals were floated on 3 ml of distilled water (or the appropriate inhibitor solution) in 50-ml Erlenmeyer flasks. Ethylene-treated petals were exposed to 10 µl/l of ethylene for 90 min prior to transfer to 50-ml flasks. At the appropriate times the ethylene content of the headspace was determined as well as the absorbance of the bathing unit at 575 nm. The petals were removed and extracted in boiling isopropanol (1 ml) in a 5-ml conical glass-tissue homogenizer. The extract was transferred to a new 18 x 150-mm disposable glass test tube to which 4 ml of

chloroform and 1 ml of methanol were added. The combined organic phase was then purified as described by Beutelmann and Kende (1977). Aliquots of this organic phase were evaporated and the phosphorus determined according to Rouser et al. (1970).

Phospholipid Composition

Batches of 150 petals were extracted as described above in 10 times the volume of each solvent. Following purification, the organic phase was evaporated and then taken up in 1 ml of chloroform. This was applied to a silicic acid column and fractionated according to Beutelmann and Kende (1977), to obtain a phospholipid-enriched fraction. This fraction was evaporated, redissolved in 50 µl of chloroform-methanol (2:1 v/v), and 25 µl was applied to a silica gel TLC plate (pre-coated plastic sheets, Sil Gel, without binders, Brinkman Inst.). The plates were developed in one dimension in chloroform-methanol-acetic acid-water (85:15:10:3.5 v/v). The phospholipids were identified by co-chromatography with authentic standards and by group specific reagents such as molybdenum, ninhydrin, and Dragendorf (Skipski and Barclay, 1969). For quantitative determination, the phospholipids were localized with iodine vapor and scraped from the plate. The phospholipids were eluted, and the phosphorus content was determined as before.

Endogenous Phospholipase Activity

For each phospholipase determination, two groups of 12 physiologically equivalent petals were extracted. One group

was homogenized in 1.0 ml of boiling isopropanol, and the amount of chloroform-methanol soluble phosphorus was measured as described before. The other group was homogenized in 0.3 ml of 0.1 M acetate buffer (pH 5.5) containing 5 mM DTT and 25 mM $CaCl_2$ (extraction buffer). The homogenate was allowed to stand for 1 hr at 28°C at which time 1 ml of boiling isopropanol was added to stop the reaction. The lipid phosphorus was purified and assayed as before. The difference in lipid phosphorus between the two determinations was taken as a measure for endogenous phospholipase activity. Endogenous phospholipases were characterized by homogenizing groups of 12 petals in 0.3 ml of extraction buffer containing 13.5 nCi of ^{14}C-(U)-phosphatidylcholine (specific activity 1.8 Ci/nmol, New England Nuclear). These homogenates were allowed to stand for various lengths of time, and the reaction was stopped by the addition of 1 ml of boiling isopropanol. The organic phase was purified as above and evaporated under a stream of nitrogen. It was re-dissolved in 75 µl of chloroform-methanol (2:1 v/v). An aliquot was chromatographed as before on silica gel plates. Improved resolution was accomplished by two successive developments in the same direction. The first solvent system was acetone-petroleum ether (3:1 v/v), the second chloroform-methanol-acetic acid-water (80:15:10:3.5 v/v). Following TLC, the plates were scanned for radioactivity. The radioactive zones were scraped from the plates and the radioactivity determined by scintillation counting.

Phospholipase-D Activity

Phospholipase-D activity was determined by extracting 1 g (fresh weight) of petals in 10 ml of extraction buffer. The extract was allowed to stand for 1 hr at 4°C and was then centrifuged for 10 min at 13,000 g. The supernatant was used as a source of enzyme. The substrate (phosphatidylcholine) was prepared by adding 0.9 mg of phosphatidylcholine (Sigma Chemical Co.) containing 22.5 nCi phosphatidylcholine-choline-methyl-^{14}C (specific activity 50 mCi/mmol, New England Nuclear) in ether to a 50-ml flask. Following evaporation of the ether, 2 ml of the extraction buffer was added to the flask, and the mixture was sonicated for 10 min. Three ml of the enzyme preparation was added to the flask, and the mixture was incubated at 28°C for 1 h. Following incubation, 1 ml of the reaction mixture was removed and extracted 4 times with petroleum ether. Activity is expressed as water-soluble radioactivity from which the blank control prepared as above but without enzyme has been extracted.

Ethane and Ethylene Determination

Ethane and ethylene contents were determined in 1-ml gas samples by gas chromatography (Elstner and Konze, 1974; Suttle and Kende, 1978).

RESULTS

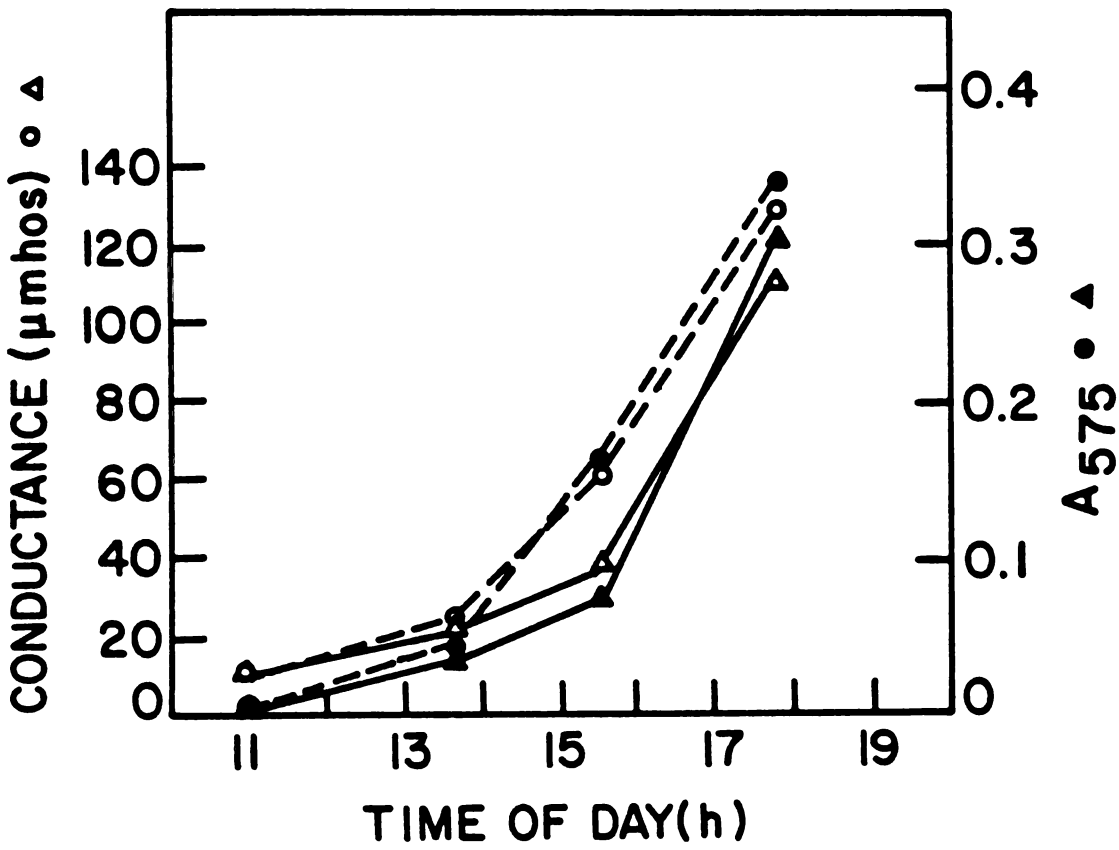
Characteristics of Cellular Efflux During Senescence

During the course of senescence, isolated petals of Tradescantia exhibited large increases in the rates of both anthocyanin and total electrolyte efflux (Suttle and Kende, 1978). The pattern of efflux of both types of cellular constituents mirrored each other during senescence (Figure 8). The onset of the increased rate of efflux in untreated petals began after 15:30 h on the day of flowering. If petals were continuously treated with 10 µl/l of ethylene beginning at 11:00 h, the increased rate of efflux began after 13:30 h or approximately two hours earlier. Again, the rates of efflux of both types of compounds paralleled each other throughout the experiment. Because the content of anthocyanin in these petals remained constant during senescence, the increased rate of efflux of this compound could not be explained on the basis of concentration-dependent diffusion, and must therefore be the result of increased membrane permeability.

The Nature of Ethylene-Enhanced Cellular Efflux

The results presented in Figure 8 also demonstrated that there was a lag between the time of ethylene application (11:00 h) and the onset of increased cellular efflux

Figure 8. Leakage of anthocyanin and electrolytes during natural and ethylene-induced senescence. Efflux of anthocyanin and total electrolytes was determined in two groups of 18 petals floated on deionized, glass-distilled water. Ethylene-treated petals (- -) were continuously exposed to 10 µl/l of ethylene beginning at 11:00 h. Anthocyanin efflux, closed symbols. Electrolyte efflux, open symbols.

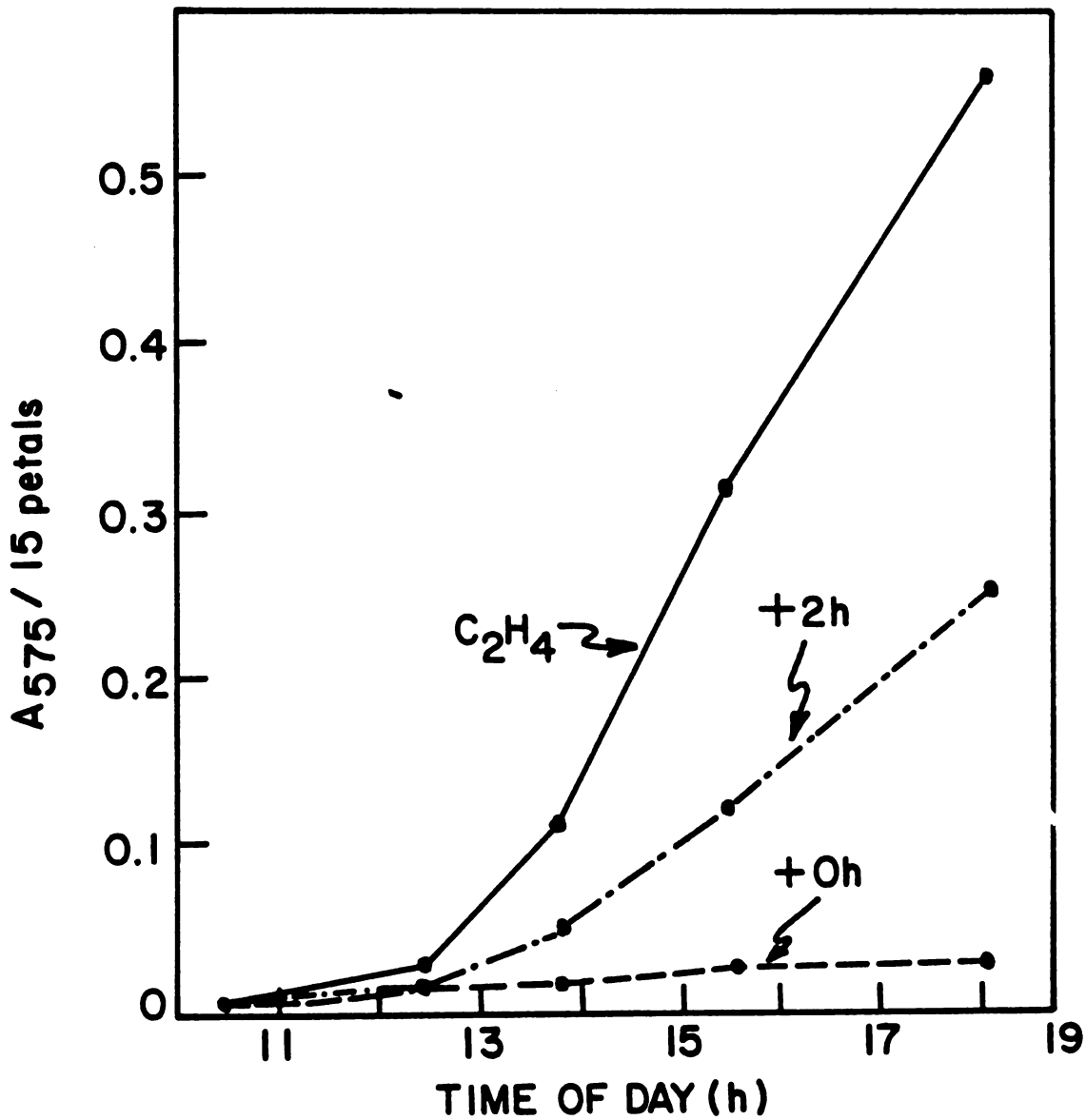

CONDUCTANCE (μmhos) ○ △
140
120
100
80
60
40
20
0
0.4
0.3
0.2
0.1
0
A575 ● ▲
11
13
15
17
19
TIME OF DAY(h)

(13:30 h). Thus, the action of ethylene in enhancing the efflux of anthocyanin appeared to be an inductive phenomenon. The nature of this inductive action of ethylene as well as the nature of the lag period was investigated using the protein synthesis inhibitor cycloheximide. Figure 9 shows that the simultaneous application of 10 µl/l ethylene and 0.1 mM cycloheximide resulted in the complete inhibition of the increase in anthocyanin efflux. However, if petals were exposed to ethylene for two hours prior to the application of cycloheximide (still within the lag period) the response was only partially inhibited. Further experiments (not presented) showed that simultaneous application of 10 µl/l ethylene and 0.1 mM cordycepin, an inhibitor of transcription, also resulted in inhibition of the ethylene-enhanced cellular efflux. On the other hand, simultaneous application of ethylene and the following transcription inhibitors was found to have no effect on the subsequent increase in pigment efflux (results not shown): actinomycin D (15 µg/ml), 5-fluorouracil (0.1 mM), or 6-methylpurine (0.1 mM).

Phospholipid Loss and Anthocyanin Efflux

Because phospholipid loss has been implicated in both ethylene-enhanced and senescence-related increases in cellular permeability (Beutelmann and Kende, 1977; Simon, 1974), we next investigated the relationship between increases in anthocyanin efflux and total phospholipid content in senescing petals of Tradescantia. The onset of membrane

Figure 9. Effect of cycloheximide on anthocyanin efflux in petals continuously exposed to 10 µl/l of ethylene. Ethylene treatment was begun at 10:30 h. Cycloheximide (final concentration 0.1 mM) was added at the time of ethylene application (- - -) or 1 hr afterward (- . -), and the subsequent efflux of anthocyanin monitored.


0.5
0.4
0.3
0.2
0.1
0
A575 / 15 petals
C2H4
+2h
+0h
11
13
15
17
19
TIME OF DAY (h)

deterioration (as judged by increased pigment efflux) began after 12:30 h (Fig. 10). Analysis of phospholipid levels (Fig. 10 C) showed that prior to the onset of increased permeability, the phospholipid content in untreated petals first rose and then fell sharply in parallel with pigment efflux. When petals were pretreated for one hour with 10 µl/l ethylene, the onset of pigment efflux could be detected after 11:30 h, or approximately 60 min earlier than in control petals (Fig. 10 B). In this case, the onset of phospholipid decline commenced with the increase in pigment efflux and the initial rise in phospholipid content was much reduced, as compared to the controls (Fig. 10 C).

Both AVG and a 4% CO_2 atmosphere were shown to retard the rate of anthocyanin efflux in mature petals of Tradescantia (Suttle and Kende, 1978). Petals which were continuously exposed to both of these inhibitors of senescence exhibited a much reduced rate of pigment efflux as compared to control petals, and this was accompanied by a corresponding reduction in the rate of phospholipid loss (Fig. 11).

As mentioned before, cycloheximide is very effective in arresting the increase in pigment efflux normally seen during natural or ethylene-induced senescence in Tradescantia (Fig. 9). Cycloheximide also arrested the decline in phospholipid levels normally observed during petal senescence (Fig. 12).

These results demonstrate a quantitative correlation between phospholipid loss and increased membrane permeability. Further, they indicate that the observed increase in

Figure 10. Effects of a 1-h pretreatment with 10 µl/l ethylene on the subsequent rates of ethylene production (A), anthocyanin efflux (B), and phospholipid levels (C) in mature petals. Ethylene was administered from 09:00 - 10:00 h. Each point represents the assay of a separate group of 12 petals. Values are expressed on a per g fresh weight basis. Ethylene treated petals (- - -); control petals (——).

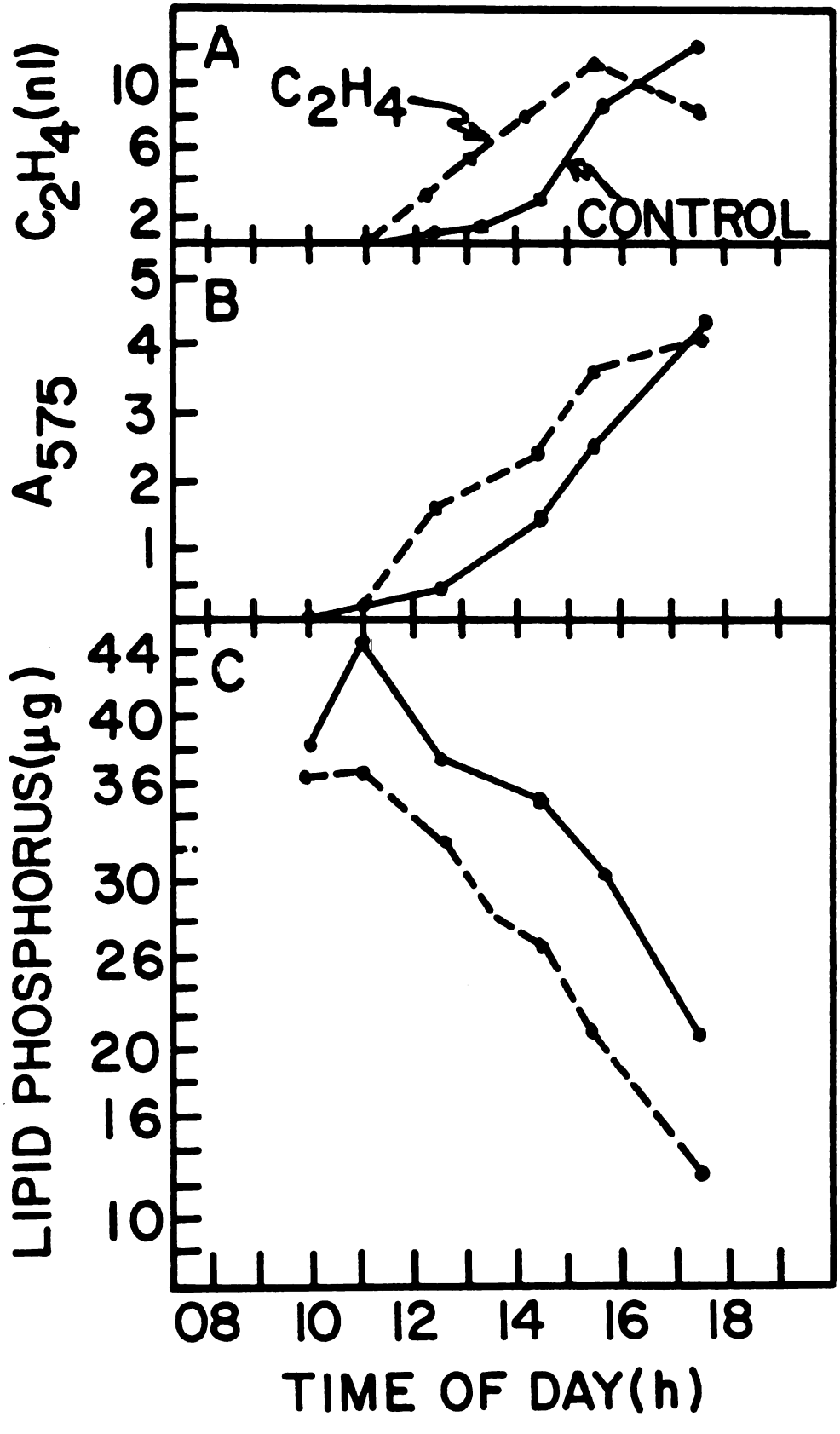

A
C_2H_4
CONTROL
C_2H_4(nl)
10
6
2
B
A_{575}
5
4
3
2
1
C
LIPID PHOSPHORUS(µg)
44
40
36
30
26
20
16
10
08
10
12
14
16
18
TIME OF DAY(h)

Figure 11. Effects of continuous exposure to 0.1 mM AVG and a 4% CO_2 atmosphere on ethylene production (A), anthocyanin efflux (B) and phospholipid levels (C) in mature petals. Each point represents the assay of a separate group of 12 petals. Values are expressed on a per g fresh weight basis. AVG + CO_2 (- - -); control (——).

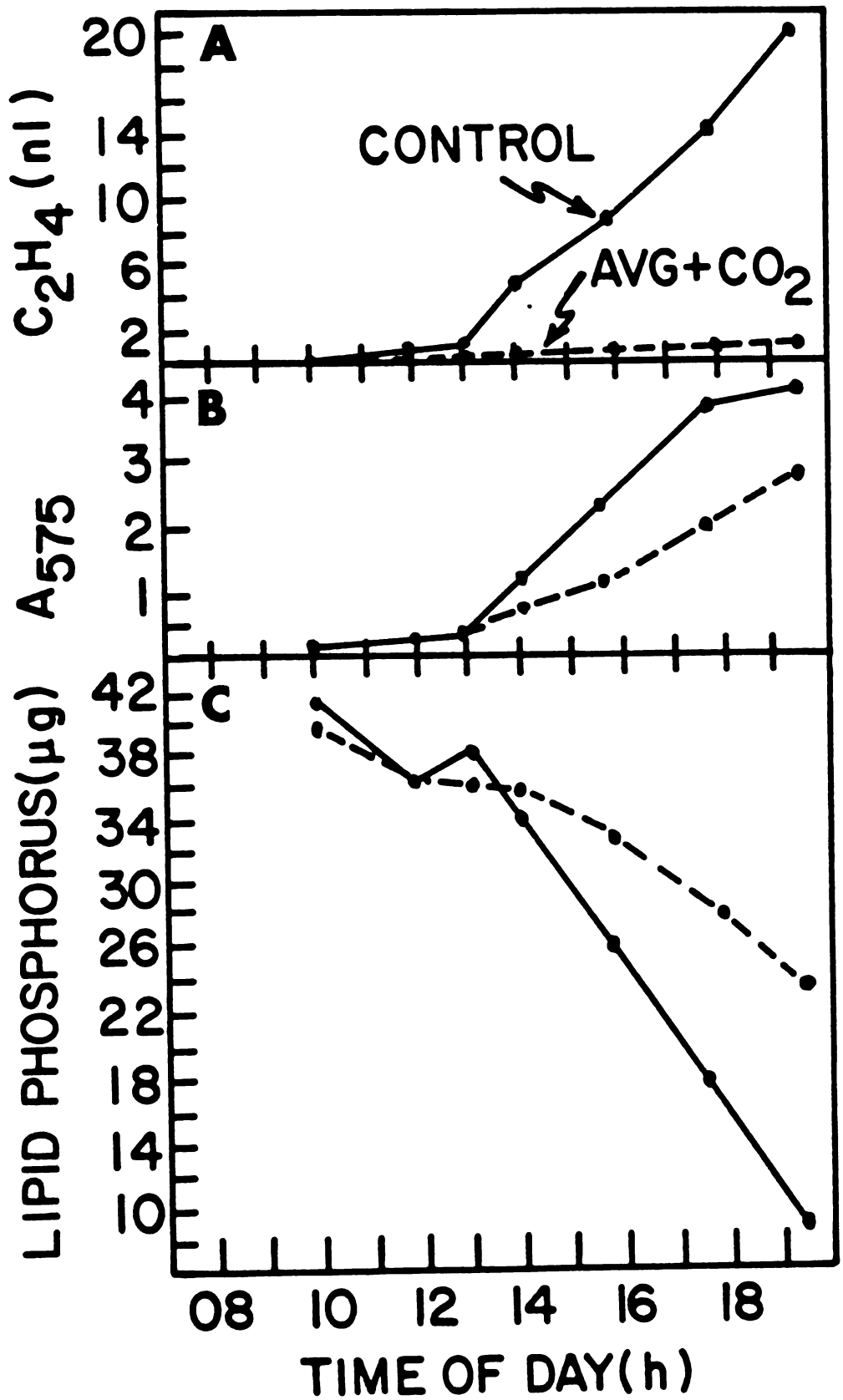
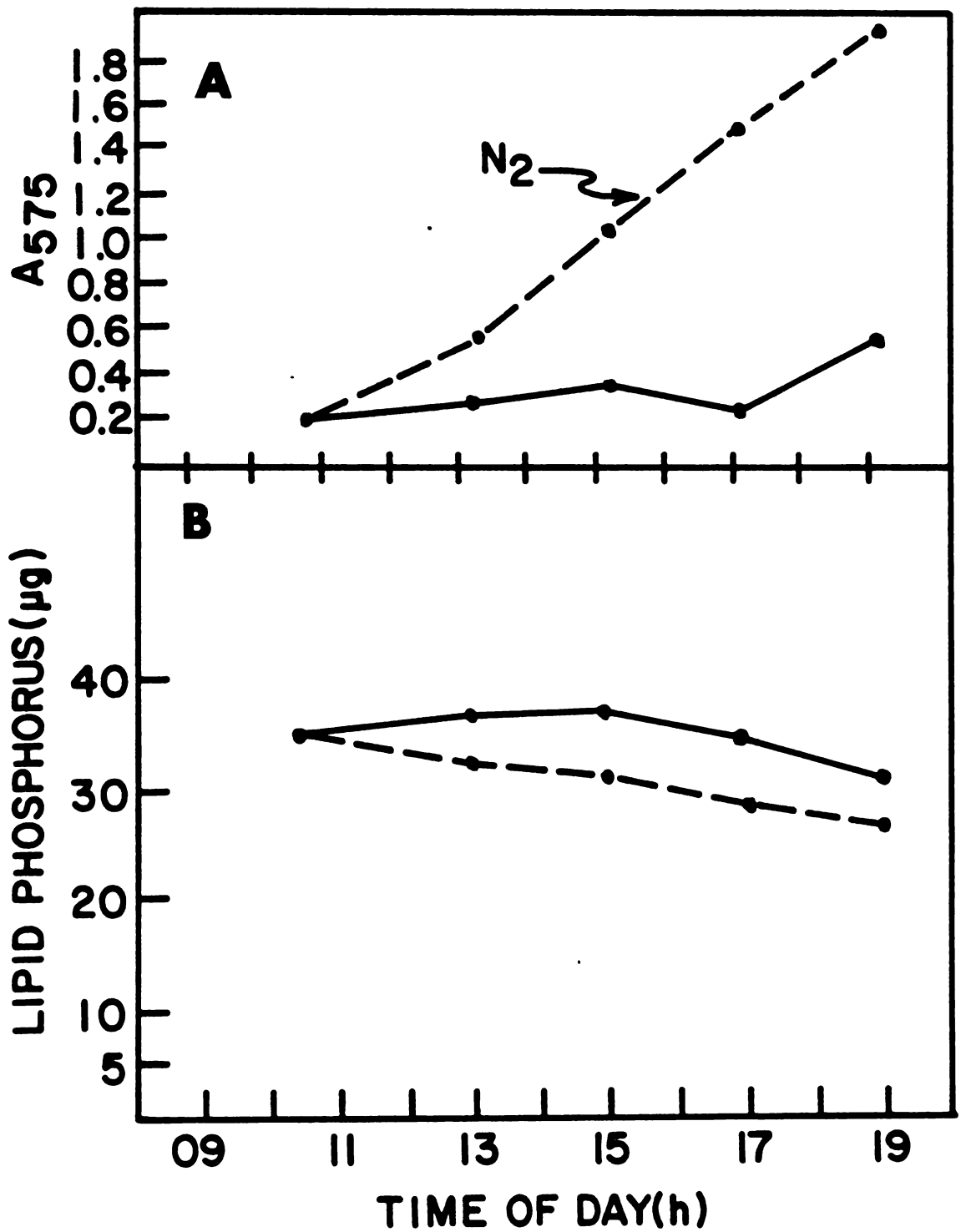

A
CONTROL
AVG+CO2
C2H4(nl)
20
14
10
6
2
B
A575
4
3
2
1
C
LIPID PHOSPHORUS(µg)
42
38
34
30
26
22
18
14
10
08
10
12
14
16
18
TIME OF DAY(h)

Figure 12. Effect of anaerobiosis on anthocyanin leakage (A) and lipid phosphorus content (B) in petals continuously exposed to 0.1 mM cycloheximide. Cycloheximide treatment was begun at 09:00 h and anaerobiosis was initiated at 10:30 h. Each point represents the assay of a separate group of 12 petals. Values are expressed on a per gram fresh wt basis. N_2 + cycloheximide (- - -); cycloheximide (——).


A
B
A_{575}
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
N_2
LIPID PHOSPHORUS(μg)
40
30
20
10
5
09
11
13
15
17
19
TIME OF DAY(h)

permeability was a consequence of the decline in the phospholipid content. However, these results do not exclude the possibility that the increase in membrane permeability leads to a release of phospholipases from a cellular compartment (vacuole?) and that these phospholipases caused the increased rate of phospholipid loss. To test this possibility, the following experiment was performed: Petals were first treated with cycloheximide in order to prevent any increases in phospholipase content and were then placed under a nitrogen atmosphere which induced cellular leakage and loss of compartmentation. While anoxia resulted in a constant, but much increased rate of anthocyanin efflux no substantial loss of phospholipid was observed (Fig. 12). Thus, the decrease in phospholipid content cannot be explained by a release of pre-existing hydrolases from the vacuole.

Phospholipid Composition

Table I shows the composition of the phospholipid fraction both prior to and following the initial increase in membrane permeability. Phosphatidylethanolamine (65%) and phosphatidylcholine (23%) were the major phospholipid species in mature petals. Phosphatidylglycerol (8%) and phosphatidylinositol (5%) were minor species. Analysis of the phospholipid composition following the onset of membrane leakage showed that the percent composition remained unchanged during this phase of senescence.

Table I. Phospholipid composition prior to senescence and at an advanced stage of senescence in isolated petals of Tradescantia.

Groups of 150 petals were homogenized in boiling isopropanol and a phospholipid-enriched fraction was isolated as described in the text. Phospholipid composition was determined after separation by TLC. Values given are percent of total phospholipid.

Time of extraction (h)	PC[1]	PE	PG	PI
09:00	23.0	64.6	7.6	4.8
16:00	22.0	66.0	9.0	3.0

[1]PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol.

Enzymes of Phospholipid Catabolism

Two types of catabolic systems acting on phospholipids have been described in plant tissues: A hydrolytic pathway involving phospholipases and an oxidative pathway resulting in the peroxidation of the fatty acid acyl groups (Galliard, 1970; Galliard, 1973). The latter pathway may or may not be mediated by the enzyme lipoxygenase. The endogenous capacity for phospholipid destruction in petals of Tradescantia was determined at three stages of senescence by taking advantage of the fact that endogenous phospholipids are readily hydrolyzed in crude homogenates of plant tissues unless special precautions are taken (Galliard, 1970). The experimental design was as follows: a) Batches of petals to be assayed were divided into two equivalent groups; b) one group was homogenized in boiling isopropanol to determine the initial phospholipid content; c) the other group was homogenized in buffer and allowed to stand for one hour at 28°C prior to killing in boiling isopropanol; d) the difference in chloroform-methanol soluble phosphorus was taken as a measure of the endogenous phospholipase activity. As Table II shows, the endogenous capacity to degrade phospholipids remained essentially unchanged during petal senescence.

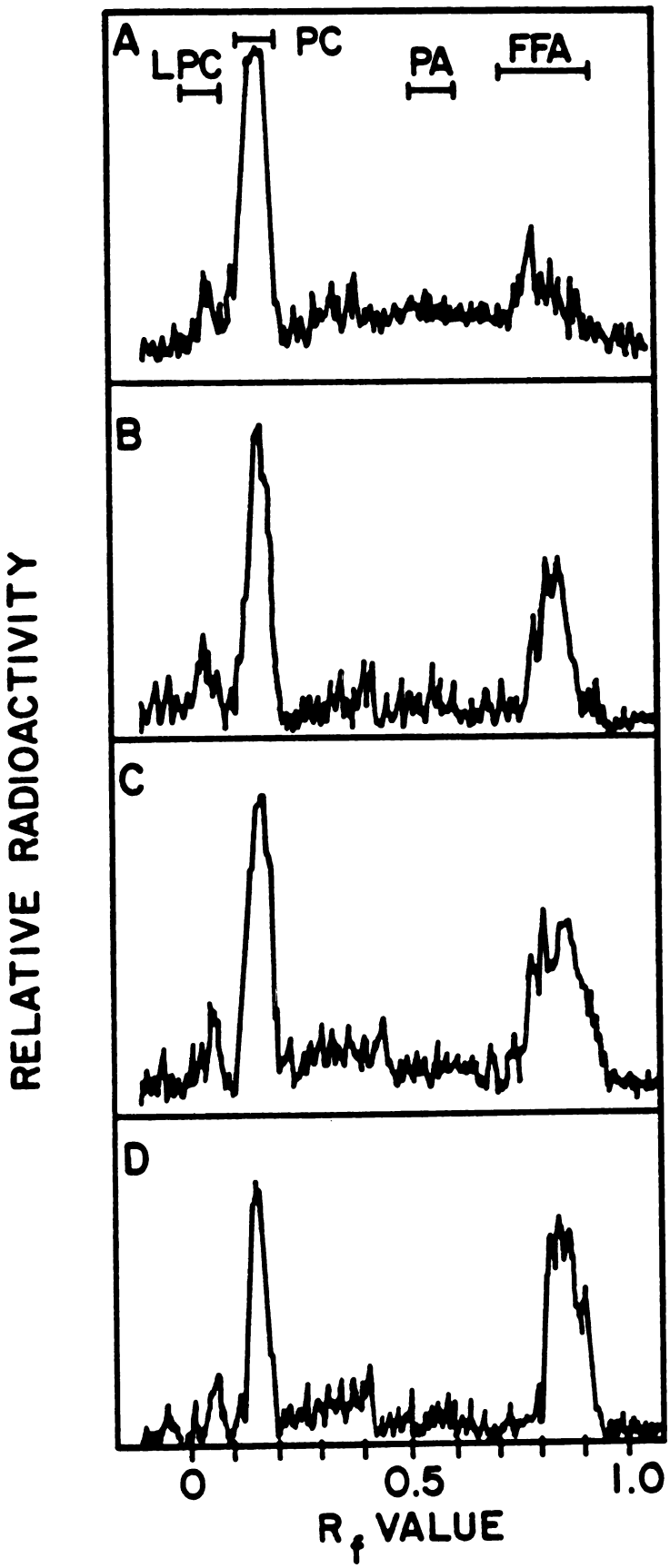
The catabolic sequence of phospholipid breakdown in senescing petals was characterized by homogenizing the petals in a small volume of buffer containing ^{14}C-(U)-phosphatidylcholine (ca. 13.5 nCi). Figure 14 shows the result of a time-course of phospholipid breakdown in crude

Table II. Endogenous phospholipase activity in crude homogenates of petals isolated at various stages of senescence.

Chloroform-methanol soluble phosphorus was determined as before and after a 1 hr incubation (28°C) in 0.3 ml of acetate buffer (pH 5.5) as described in the text. Phospholipase activity is expressed as the difference in chloroform-methanol soluble phosphorus between the initial and final determinations.

Time of Extraction (h)	Lipid Phosphorus ($\mu g\ g^{-1}$ fresh wt) Initial value	Final value	Phospholipase activity
10:30	44.9	29.0	15.9
14:00	38.0	27.2	10.8
16:00	32.8	17.6	15.2

Figure 13. Characterization of endogenous phospholipase activity in crude homogenates of petals isolated at an advanced stage of senescence. Phospholipase activity was assayed by following the fate of ^{14}C-(U)-phosphatidylcholine (13.5 nCi) added to the homogenizing medium after which the homogenates were incubated for 5 min (A), 15 min (B), 30 min (C), and 60 min (D). Bars indicate the position of authentic standards. LPC: lysophosphatidylcholine, PC: phosphatidylcholine, PA: phosphatidic acid, and FFA: free fatty acids.


A
LPC
PC
PA
FFA
B
C
D
RELATIVE RADIOACTIVITY
0
0.5
1.0
R_f VALUE

homogenates isolated from petals at an advanced stage of senescence. With time, there was a decline in the phosphatidylcholine content which was accompanied by a corresponding increase in free fatty acids. Analysis of the rate of hydrolysis in this system showed that it was highest during the first 5 minutes of incubation and then declined. Phosphatidic acid, a possible intermediate of phospholipid breakdown, and lysophosphatidylcholine were not observed which indicated that acyl hydrolase rather than phospholipase D activity predominated during this phase of petal senescence. The activity of phospholipase D was indeed found to decline dramatically during the course of petal senescence (Table III).

Evolution of ethane has been shown to be a reliable indicator of the degree of lipid peroxidation (Riely et al., 1974). During the course of senescence, there was no change in the ethane content in the headspace above the petals. Thus, little if any lipid peroxidation occurred during senescence of petals of Tradescantia.

Table III. Phospholipase D activity during senescence of isolated petals of Tradescantia.

The enzyme was assayed by measuring the release of water soluble radioactivity following a 1-h incubation (28°C) with 1 μmol of phosphatidylcholine containing ^{14}C-choline-methyl-phosphatidylcholine (22.5 nCi). Activity is expressed as water-soluble cpm over an enzyme blank, after 4 successive extractions of the aqueous phase with petroleum ether.

Time of Extraction (h)	Phospholipase D Activity (cpm)	% of Initial Activity
09:00	17,715	100
13:00	2,935	17
15:30	1,590	9

DISCUSSION

Isolated petals of Tradescantia exhibit an increase in both anthocyanin and electrolyte efflux during senescence (Suttle and Kende, 1978). The results presented in Figure 8 show that the rates of efflux of these two compounds proceed in parallel. This fact indicates that the increase in membrane permeability is of a general nature. This indicates that gross alterations in membrane integrity occur during senescence. It is not known if the increase in tonoplast permeability, as demonstrated by the increase in anthocyanin efflux, also leads to an enhanced efflux of other, possibly physiologically more relevant, vacuolar constituents such as enzymes.

As in other senescing tissues (Hanson and Kende, 1975; Sacher, 1973), application of ethylene to isolated petals of Tradescantia has been shown to hasten the onset of the increase in membrane permeability (Suttle and Kende, 1978). The results presented in Figures 8 and 9 demonstrate that ethylene does not directly affect membrane integrity but rather that the gas exerts its effect on permeability through cellular metabolism. This conclusion is consistent with the observed increase in ethylene sensitivity of both flower and fruit tissues as they mature (Hanson and Kende, 1975; Suttle and Kende, 1978). If ethylene were acting

directly on membranes it would be difficult to explain differences in ethylene sensitivity between tissues of different physiological ages.

The lag of 90-150 min between the application of ethylene and the increase in anthocyanin efflux (Fig. 8), together with the inhibitory action of cycloheximide (Fig. 9) indicate that ethylene action in senescing petals of Tradescantia requires protein synthesis. Furthermore, it appears that proteins synthesized within two hours of ethylene application are essential in mediating the increase in membrane permeability because application of cycloheximide after this period only partially inhibits ethylene-enhanced anthocyanin efflux. In addition, the ability of cordycepin, an inhibitor of transcription, to block ethylene-enhanced anthocyanin efflux indicates that RNA synthesis may also be required for ethylene action. The lack of effectiveness of other inhibitors of transcription, such as actinomycin-D, in blocking ethylene-enhanced efflux may be due to insufficient penetration of these inhibitors into the tissue. Thus it appears that the increase in membrane permeability caused by the application of ethylene is a secondary effect of the gas which is mediated by other cellular processes requiring both RNA and protein synthesis.

Protein synthesis has been shown to be a requirement for the completion of the ripening process in fruits (Frenkel et al., 1968; Sacher, 1973). Their studies showed that application of cycloheximide prior to, but not after the respiratory climacteric inhibits the subsequent ripening

processes. As has been pointed out before, abscission and senescence have many features in common (Abeles, 1968). Investigations concerning the mode of action of ethylene in enhancing abscission have also indicated that the action of ethylene requires both RNA and protein synthesis (Abeles, 1968). Of particular interest is the observation that cycloheximide is only effective in arresting ethylene-enhanced abscission if it is administered within four hours of ethylene application. Thus, the effect of ethylene in both senescence and abscission appears to have a similar molecular basis.

The results presented in Figures 10 and 11 indicate that the observed increase in membrane permeability is the result of phospholipid loss. Factors which hasten the onset of pigment efflux (ethylene pretreatment) or inhibit the increase in permeability (CO_2 and AVG or cycloheximide) exert a corresponding effect on the rate of phospholipid loss. It was previously shown (Suttle and Kende, 1978) that exogenous ethylene does not affect the rate of anthocyanin efflux in immature petals of Tradescantia and other experiments (not shown) have demonstrated that the gas also has little effect on the phospholipid content in these petals. Because only total phospholipid levels have been determined, it is not known if the rate of loss of phospholipids in the various subcellular organelles proceeds in parallel or if the membranes of certain organelles are preferentially affected. These results are in general agreement with those of other investigations (Beutelmann and Kende, 1977; Ferguson and

Simon, 1974) which have demonstrated that loss of membrane integrity during senescence is accompanied by losses of phospholipids.

The observed decline in phospholipids during senescence in petals of Tradescantia can be explained by a decrease (or cessation) of phospholipid synthesis, by increased phospholipid catabolism or by both. Inhibitors of respiration have been shown to block the synthesis of phospholipids in plant tissues (Ferguson and Simon, 1973b). As shown in Figure 12, cycloheximide and sustained anaerobiosis together have little effect on phospholipid level in these petals. This observation indicates that the loss of phospholipids during petal senescence is the consequence of an increased rate of catabolism. Further support for this conclusion can be derived from the data presented in Table I. Although there is a 70% reduction in phospholipid content during petal senescence, the phospholipid composition remains essentially unchanged; demonstrating that all phospholipids are lost in parallel.

Surprisingly, the in vitro assays of endogenous phospholipase activities have failed to demonstrate any increase in these activities during senescence (Tables II and III). Analysis of Figure 11 shows that the maximum rate of phospholipid decline during petal senescence is 4.77 μg phospholipid degraded h^{-1} gr^{-1} fresh wt, and the results presented in Table II show that enough phospholipase activity is present in petals prior to the onset of membrane deterioration to account for this maximum rate of phospholipid loss.

Although loss of phospholipids during senescence is well documented, very little information is available concerning the role of endogenous phospholipases in this process. It has been shown that in senescing mung bean cotyledons the catabolic sequence of phosphatidylcholine breakdown is the following: Phosphatidylcholine → phosphatidic acid → lysophosphatidic acid → free fatty acid (Herman and Chrispeels, 1978). The results presented in Figure 13 indicate that a different sequence of catabolism occurs in senescing petals of Tradescantia. In senescing petals, phosphatidylcholine is directly deacylated to yield free fatty acids without accumulation of a lyso intermediate. The addition of 40 µg of unlabeled lysophosphatidylcholine to the in vitro assay system (not shown) did not increase the amount of radioactivity associated with lysophosphatidylcholine indicating that it is not a transient intermediate in this catabolic system. The absence of a lyso intermediate during phospholipid catabolism in these petals is consistent with the fact that no lysophospholipids have been detected during our analysis of the composition of the endogenous phospholipids in petals at an advanced stage of senescence. The fact that no phosphatidic acid was detected during the in vitro characterization indicates that phospholipase D activity is very low in senescing petals of Tradescantia and the results presented in Table III substantiate this conclusion. The observed decline in phospholipase D activity during petal senescence is consistent with the results of earlier investigations (Quarles and Dawson, 1969) which demonstrated that

the activity of this enzyme is highest in young, actively growing tissues and that it declines with age. In contrast to other senescing tissues (Simon, 1974), there appears to be very little lipid peroxidation during petal senescence in Tradescantia.

Senescence in mung beans cotyledon is also associated with a marked decline in phospholipid levels but without any demonstrable increase in phospholipase activities (Herman and Chrispeels, 1978). These results were taken as an indication that phospholipases are sequestered in healthy tissues and that this compartmentation is lost during senescence thereby leading to an enhanced rate of catabolism. The vacuole has been proposed as the plants' equivalent to the animal lysosome (Matile, 1978) and therefore is a prime candidate for the subcellular localization of phospholipase activities. The results presented in Figure 12 show that disruption of the tonoplast (as evidenced by enhanced anthocyanin leakage) is not sufficient to initiate phospholipid catabolism in these petals. However, it is not known if enhanced anthocyanin efflux is also accompanied by enhanced efflux of vacuolar proteins (i.e. hydrolases).

In summary, the action of ethylene in increasing membrane permeability in senescing petals of Tradescantia appears to be mediated by processes requiring both RNA and protein synthesis. Furthermore, these results suggest that the observed increase in membrane permeability appears to be a direct result of an increase in phospholipid degradation presumably caused by an increase in activity of pre-existing phospholipases.

SECTION III

The Role of Vacuolar Integrity in Ethylene Production During Senescence in Isolated Petals of *Tradescantia*

INTRODUCTION

Senescence of the ephemeral flower of Ipomoea tricolor is accompanied by increased membrane permeability and by increased ethylene production (Hanson and Kende, 1975; Kende and Baumgartner, 1974). Application of ethylene to this flower tissue has been shown to accelerate the onset and increase the magnitude of both of these processes (Hanson and Kende, 1975; Kende and Baumgartner, 1974). The temporal coincidence of these two phenomena has led to the hypothesis that changes in membrane permeability, particularly that of the tonoplast, lead to a release of a previously sequestered component of the ethylene-synthesizing system which, in turn, results in the initiation of ethylene production (Hanson and Kende, 1975; Kende and Baumgartner, 1974).

Flowers of Tradescantia contain anthocyanin pigments. Since these pigments are localized in the vacuole, these petals offer a unique opportunity to assess the role of vacuolar leakage on the initiation of ethylene production. Initial studies have shown that the onset of both the increase in vacuolar leakage and the increase in ethylene production coincide (Suttle and Kende, 1978). Application of ethylene hastens the onset of both processes and again the initiation of both phenomena coincide.

The temporal coincidence of these two events tends to support the hypothesis that vacuolar leakage is causally related to the initiation of ethylene production in these petals. However, the temporal coincidence could also arise from the fact that both of these processes, themselves, result from another, more primary event, and as such their coincidence may simply be fortuitous.

During the course of investigations on the effect of ethylene on vacuolar integrity, it was noticed that following certain chemical or physiological manipulations, we were able to separate ethylene production from vacuolar efflux. These results suggest that the two processes are not causally related. In this report the results of several types of experiments in which vacuolar efflux and ethylene formation have been separated are described.

MATERIALS AND METHODS

Plant culture, incubation techniques, and the techniques for the simultaneous determination of anthocyanin efflux and ethylene production have been described before (see Section I, p. 23). In all experiments petals were isolated from fully open flowers between 08:00-08:15 h. For the calcium experiments, one group of petals was immediately floated on an unbuffered solution of 10 mM $CaCl_2$ while the control group was floated on distilled water. After 45 min these petals were transferred to a treatment chamber and were exposed to 10 µl/l of ethylene for 60 min. After ethylene pretreatment, groups of 18 petals were transferred to the incubation flasks. The flasks were flushed, sealed and both the ethylene production and anthocyanin efflux were monitored. The effect of short-term anaerobiosis was determined in essentially the same manner. In this case, groups of petals were treated with either 10 µl/l of ethylene or were subjected to anoxia for 60 min. Anoxia was established by flushing the treatment chamber with nitrogen for 5 min. Control petals were simply floated on water. After pretreatment, groups of 15 petals were transferred to the incubation flasks which were then flushed with ethylene-free air and sealed. Ethylene production and pigment efflux were

monitored. For the comparison of senescence in apical and basal portions of petals, freshly excised petals were cut transversely into two parts of roughly equal area. The two parts were weighed and incubated on distilled water for 60 min. Groups of 16 halves were then transferred to the incubation flasks which were flushed with ethylene-free air and sealed. Ethylene production and pigment efflux were monitored.

RESULTS

Effect of $CaCl_2$

Calcium salts have been shown to delay leaf senescence presumably through stabilization of cellular membranes (Poovaiah and Leopold, 1973). Therefore, the effect of $CaCl_2$ on anthocyanin efflux and ethylene production was examined and the results of one such experiment are shown in Figure 14. Application of 10 mM $CaCl_2$ to ethylene-pretreated petals resulted in a reduction in the rates of anthocyanin efflux during the first 3 h following termination of the ethylene pretreatment. Thereafter, the rates of leakage in both untreated and calcium-treated petals were identical. Figure 14 (lower) shows that, in spite of the initial reduction of anthocyanin efflux, ethylene production was enhanced by application of $CaCl_2$. Other experiments (not shown) demonstrated that magnesium, another divalent cation, had no such effect.

Effect of Short-Term Anaerobiosis

During the course of our investigations concerning the mode of action of ethylene, it was noticed that 1 h of anaerobiosis stimulated ethylene production in petals of Tradescantia. A comparison was made of the effect of 1 h of anaerobiosis on both anthocyanin efflux and ethylene

production in petals of Tradescantia, and the results of one such experiment are shown in Figure 15. Following 1 hour of anaerobiosis, the rates of anthocyanin efflux were reduced compared to control values and this reduction persisted throughout the experiment. On the other hand, brief exposure of petals to anaerobiosis stimulated ethylene production with respect to control petals and this stimulation continued throughout the experiment.

Anthocyanin Leakage and Ethylene Production in Apical and Basal Halves of Petals

As was reported before (Suttle and Kende, 1978), visible signs of petal deterioration appear first in the apical portion of the petals and progress basipetally. Therefore, a comparison of both anthocyanin efflux and ethylene production was made in apical and basal portions of excised petals, and the results of one experiment are shown in Figure 16. Because of differences in fresh weight of the two halves, both anthocyanin efflux and ethylene production are expressed on a per gram fresh weight basis. The onset of increased anthocyanin efflux was detected between 13:00-4:10 h in the apical halves whereas it began between 14:10-15:30 h in the basal halves. On the other hand, the onset of increased ethylene production in the basal halves was detected between 13:00-14:10 h or up to an hour earlier than the onset of anthocyanin efflux in these same halves. Only 13% of the total petal ethylene was produced by the apical halves.

Figure 14. Effect of 10 mM $CaCl_2$ on ethylene-induced anthocyanin efflux and ethylene production in isolated petals of Tradescantia. Following excision from the flowers, petals were floated on either distilled water of 10 mM $CaCl_2$ for 45 min. Both groups of petals were then treated for 60 min with 10 µl/l of ethylene (between 09:00-10:00 h). Following ethylene pretreatment, groups of 18 petals were transferred to the incubation flasks where both anthocyanin efflux and ethylene production were monitored.

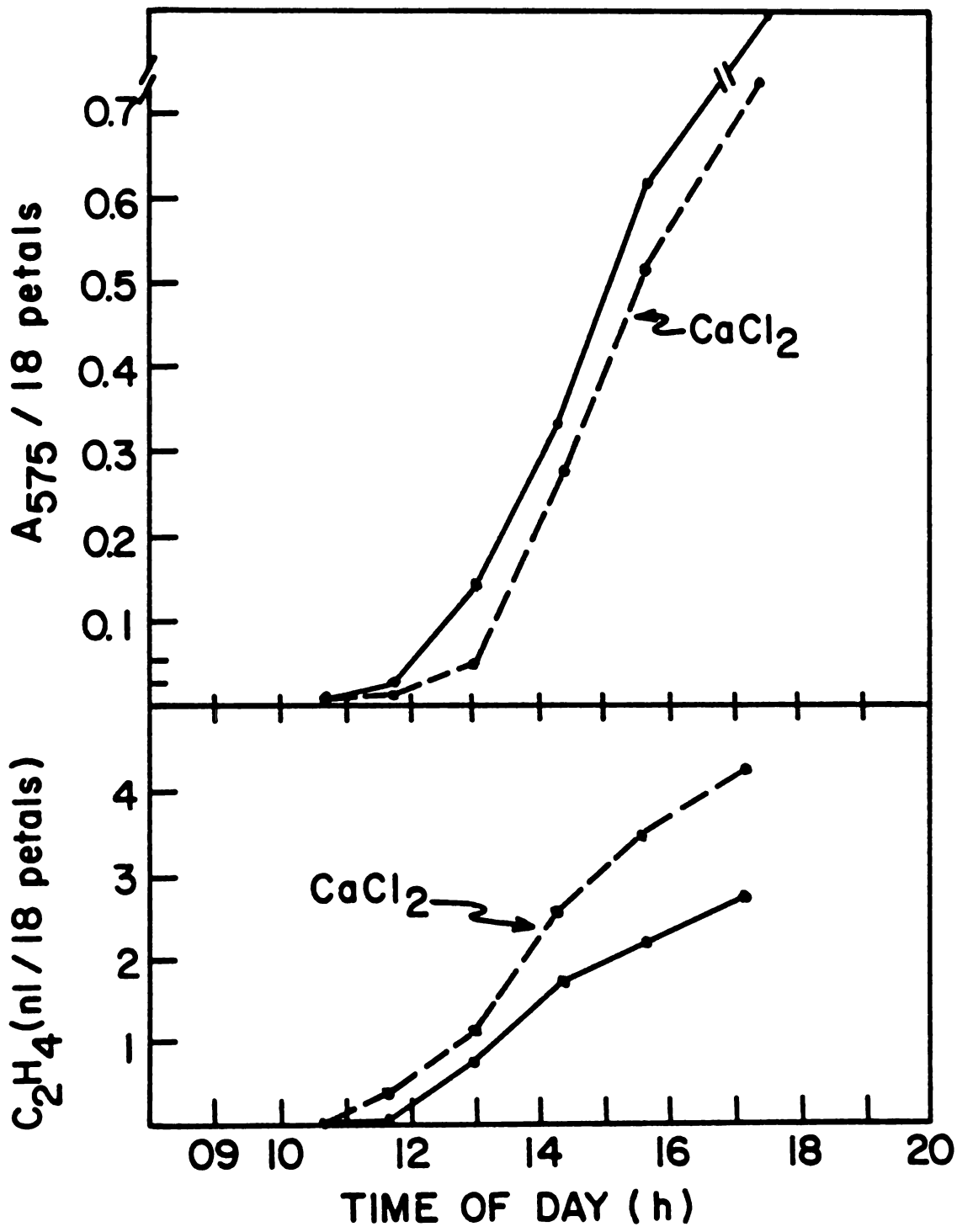

0.7
0.6
0.5
0.4
0.3
0.2
0.1
A575 / 18 petals
CaCl2
4
3
2
1
C2H4(nl / 18 petals)
CaCl2
09
10
12
14
16
18
20
TIME OF DAY (h)

Figure 15. Effect of short-term anaerobiosis on anthocyanin efflux and ethylene production in isolated petals of Tradescantia. Following excision from the flowers, groups of petals were exposed to 10 µl/l of ethylene or were subjected to anoxia for 60 min (between 09:10-10:10 h). Control petals were simply floated on distilled water. Following this pretreatment, groups of 15 petals were transferred to the incubation flasks where both anthocyanin efflux and ethylene production were monitored.

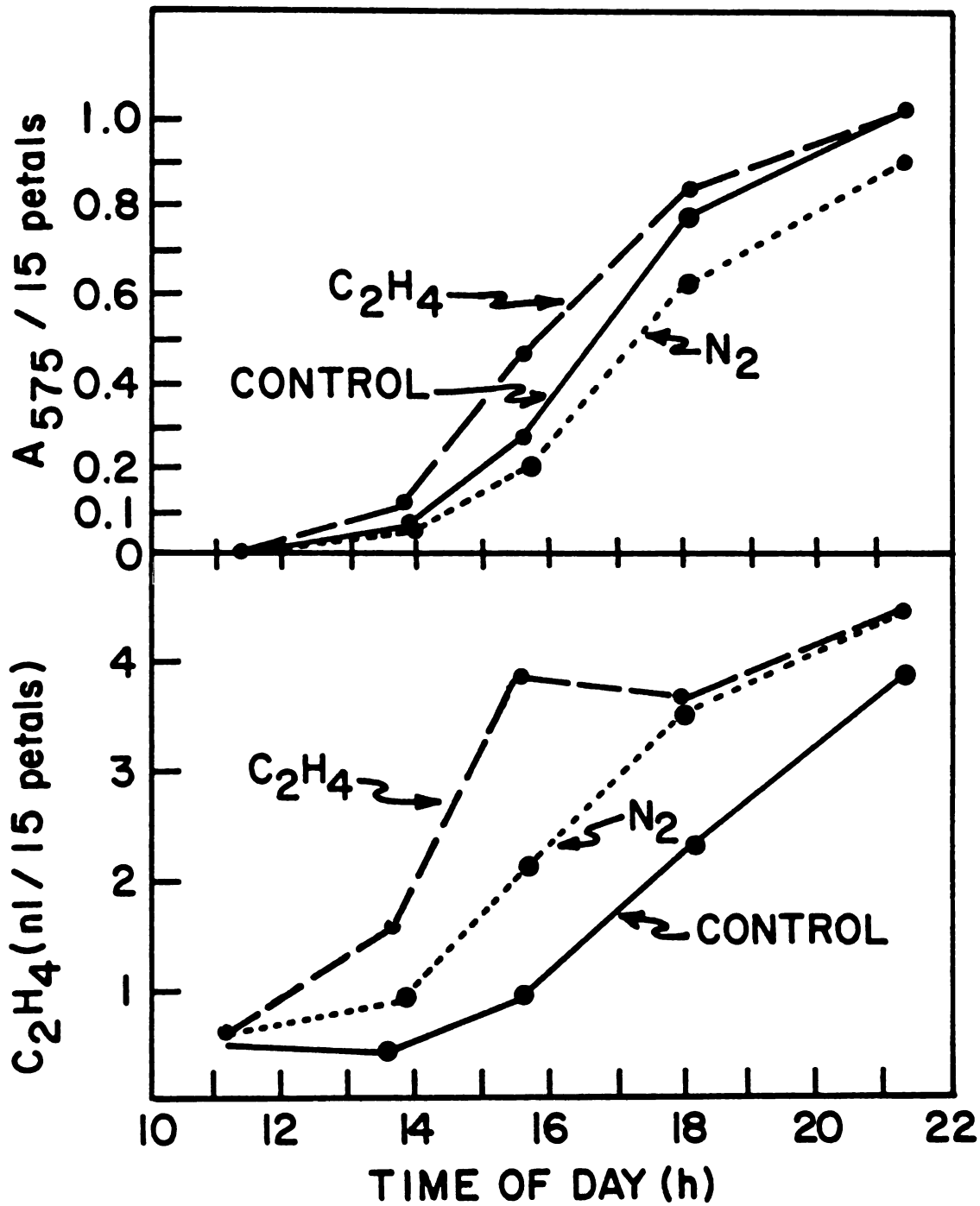
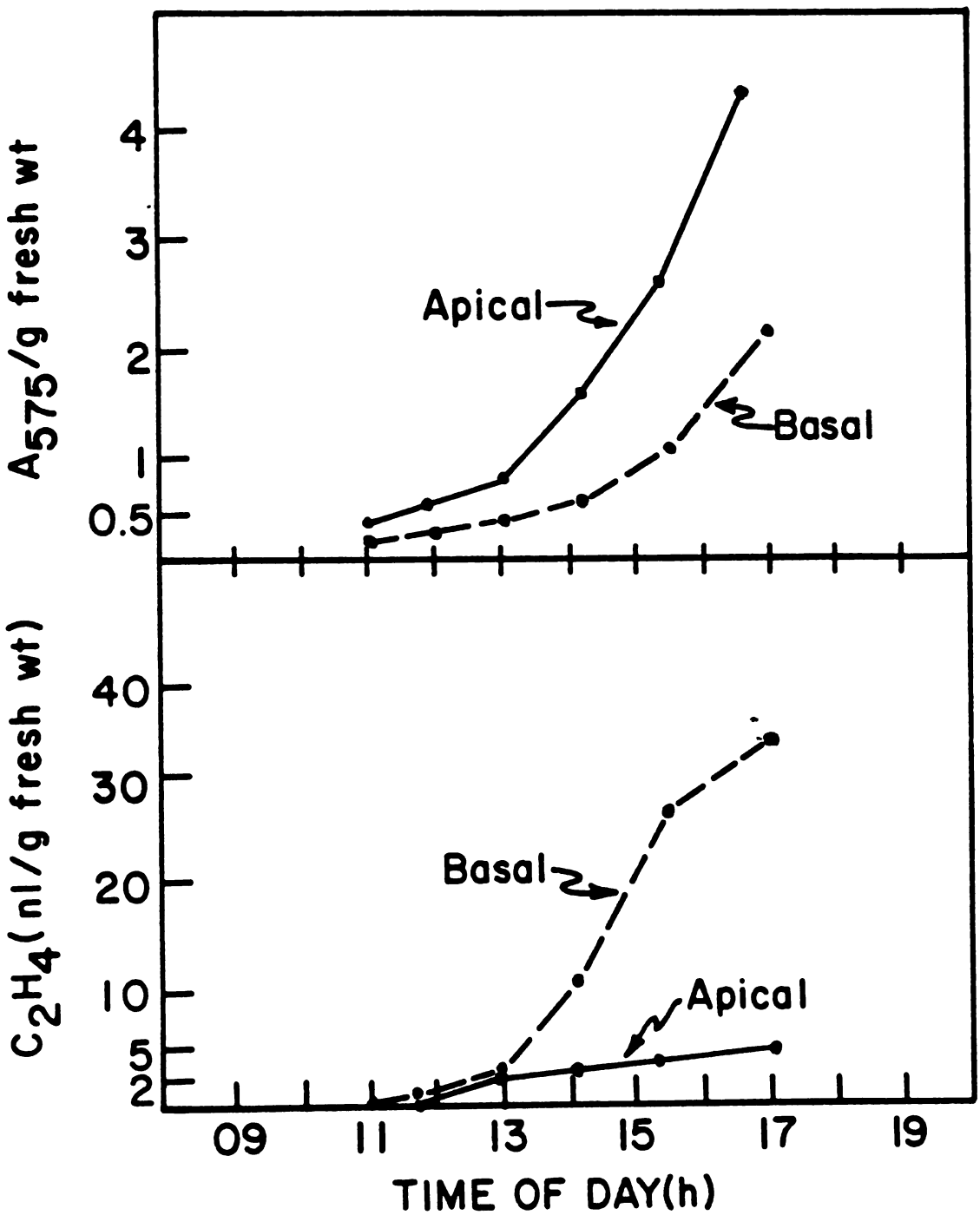

1.0
0.8
0.6
0.4
0.2
0.1
0
A_{575} / 15 petals
C_2H_4
CONTROL
N_2
4
3
2
1
C_2H_4 (nl / 15 petals)
C_2H_4
N_2
CONTROL
10
12
14
16
18
20
22
TIME OF DAY (h)

Figure 16. Comparison of time course of anthocyanin efflux and ethylene production in apical and basal halves of isolated petals of Tradescantia. Following excision from the flowers, petals were cut transversely into apical and basal halves of roughly equal area. Following cutting, petals were preincubated on distilled water for 60 min. After this, groups of 16 halves were transferred to the incubation flasks where both anthocyanin efflux and ethylene production were monitored.


A_{575}/g fresh wt
4
3
2
1
0.5
Apical
Basal
C_2H_4(nl/g fresh wt)
40
30
20
10
5
2
Basal
Apical
09
11
13
15
17
19
TIME OF DAY(h)

DISCUSSION

Increased vacuolar leakage (as judged by anthocyanin efflux) and increased ethylene production could proceed in series as has been suggested by Hanson and Kende (1975) or in parallel. If the former hypothesis is true then it is to be expected that: a) any treatment which retards or diminishes the loss of vacuolar integrity should exert a corresponding effect on the rate of ethylene production and b) the initiation of vacuolar leakage should precede or occur simultaneously with the onset of ethylene production. The results presented in Figures 15 and 16 show that treatments which reduce the rate of vacuolar leakage actually stimulate ethylene production. Furthermore, as can be seen in these figures, the initiation of ethylene production can be observed at least 60 minutes prior to the onset of increased vacuolar leakage. The results presented in Figure 17 demonstrate that these two processes of petal senescence can also be separated spatially. The initial increase in vacuolar leakage occurs in the apical portion of the petals while more than 85% of the total ethylene is produced by the basal half. The data presented in this figure show that the onset of these two processes occurs simultaneously, but in different portions of the petals. Thus, the temporal coincidence of these two phenomena in intact petals does not appear to

be the result of a causal relationship.

The ability of calcium salts to stimulate ethylene production has been noted before (Lau and Yang, 1976). These authors offered no explanation for this observation, but it is conceivable that it too results from a stabilization of membrane integrity. It has been shown that osmotic shock completely inhibits ethylene production and this has been attributed to an effect on membrane integrity (Mattoo and Lieberman, 1977).

Unlike ethylene pretreatment (Suttle and Kende, 1978), the inductive action of brief anaerobiosis stimulates subsequent ethylene production but retards subsequent vacuolar leakage. The ability of short-term anaerobiosis to stimulate ethylene production has not been noted before. When plant tissues are returned to air following long-term (> 3 h) incubation under anoxia there is a transient increase in the rate of ethylene production (Hansen, 1942) and this has been shown to be due to the accumulation of an intermediate of the pathway (Adams and Yang, 1979). However, the stimulatory action of the brief anaerobic pretreatment cannot be attributed to a similar mechanism because: a) the stimulatory effect on ethylene production persists throughout the experiment, and b) the ethylene pathway is not operative at the time of the pretreatment, making it unlikely that an intermediate(s) can accumulate.

The fact that ethylene production is not uniform over the entire petal has been noted before in Dianthus petals (Nichols, 1977). As in Tradescantia, the basal portions of

these petals also produce the greatest amounts of ethylene. The physiological basis for this lack of uniformity is not known, but it may be caused by some factor(s) which originate(s) from elsewhere within the flower (gynoecium?) and diffuse(s) into the petal bases.

SECTION IV

Methionine Metabolism and Ethylene Biosynthesis in Senescing Petals of *Tradescantia*

INTRODUCTION

Methionine has been shown to be the *in vivo* precursor of ethylene in all plant tissues that have been examined thus far (Lieberman, 1979). Investigations on the metabolism of methionine in senescing flower tissue of *Ipomoea tricolor* (Hanson and Kende, 1976a) have shown that, in addition to being converted to ethylene, methionine is also converted to S-methylmethionine (SMM). In this tissue SMM serves as a storage form of methionine and is converted back to methionine during the course of senescence.

As has been discussed in the general introduction, both S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) have been proposed as intermediates in the conversion of methionine to ethylene (Adams and Yang, 1977, 1979). Subsequent work has lead to the isolation of the ACC-forming enzyme from tomato tissue (Boller et al., 1979) and an ACC-dependent, ethylene-forming system from pea stems (Konze and Kende, in press). The biosynthetic pathway of ethylene is thought to proceed as follows:

$$\text{methionine} \longrightarrow \text{S-adenosylmethionine} \overset{\text{AVG}}{\dashrightarrow} \text{ACC} \overset{N_2}{\dashrightarrow} \text{ethylene}$$

In this scheme, AVG, a well-known inhibitor of ethylene biosynthesis (Lieberman, 1979), has been shown to block the formation of ACC (Adams and Yang, 1979; Boller et al.,

1979). The conversion of ACC to ethylene has been shown to require oxygen (Adams and Yang, 1979).

This study was originally intended to investigate the metabolism of methionine in senescing petals of Tradescantia. Of special interest at that time was the occurrence and metabolism of S-methylmethionine. Subsequently, when ACC was proposed as the immediate precursor of ethylene in ripening apples, attention was shifted to the role of this compound in ethylene biosynthesis in petals of Tradescantia.

MATERIALS AND METHODS

Plant Culture and Ethylene Analysis

Clone 02 of Tradescantia was grown as previously described (see Section I, p. 22). Throughout this paper the following terminology will be used: day -1: the day prior to flower opening, day 0: the day of flower opening. Ethylene was analyzed in 1-ml gas samples by gas chromatography as previously described (Kende and Hanson, 1976).

Chemicals

D,L-selenomethionine, cycloheximide, S-adenosyl-L-methionine, n-propyl gallate, and sodium benzoate were purchased from Sigma Chemical Co., L-methionine from Nutritional Biochemicals, S-methylmethionine from United States Biochemicals, dimethylsulfoxide from Aldrich Chemical Co. and ACC and homocysteine-thiolactone from Calbiochem Co. AVG was a gift from Dr. M. Lieberman (USDA-ARS). L-methionine-U-^{14}C was purchased from New England Nuclear. S-methylmethioninemethyl-^{3}H was a gift from Dr. J. Konze.

Treatment with Inhibitors of Ethylene Biosynthesis

Petals were excised from the unopened buds on the evening of day -1 and were floated overnight on 5 ml of the

inhibitor solution or on water in glass Petri dishes. The following morning, the petals were tansferred to 50-ml Erlenmeyer flasks which contained 5 ml of the same solutions as were employed during the overnight incubation. The flasks were flushed with ethylene-free air, sealed, and the ethylene production was monitored.

Treatment with Amino Acids

Petals were excised from unopened buds on the evening of day -1 and were floated on 5 ml of the appropriate amino acid solution, at the concentration noted in Table V, or on water. The following morning, the petals were transferred to 25-ml Erlenmeyer flasks containing 0.3 ml of the same solution as used in the overnight incubation and a disc of filter paper. The flasks were flushed with ethylene-free air, sealed, and ethylene production was monitored.

Treatment with Selenomethionine

Petals were isolated from fully open flowers on the morning of day 0. Petals to be exposed to selenomethionine plus inhibitor solutions were initially pretreated for 90 min by incubation on 5 ml of the inhibitor solution at the concentrations noted in Table III. After this pretreatment, the petals were transferred to 50-ml Erlenmeyer flasks containing 1.5 ml of a solution containing both the inhibitor and selenomethionine. Petals to be exposed to selenomethionine alone were initially floated on water and were subsequently transferred to the incubation flask which contained

1.5 ml of selenomethionine solution. Control petals were exposed to water throughout. Following transfer, the flasks were flushed with ethylene-free air, sealed, and ethylene production monitored.

Amino Acid and Protein Analysis

Petals were excised from fully open flowers on the morning of day 0. Groups of 6 petals were transferred to 25-ml Erlenmeyer flasks containing 5 ml of 1% agar as a support. The flasks were flushed with ethylene-free air, sealed and at the times noted in Table VII, the flasks were opened, the petals removed and extracted with 2 ml of 80% ethanol containing 5 mM β-mercaptoethanol (extraction medium). Following homogenization the extracts were centrifuged at 13,000g for 10 min and the supernatant was decanted. The pellet was re-extracted with an additional 1 ml of extraction medium and recentrifuged. The combined supernatants were evaporated at 40°C under a stream of nitrogen. They were then redissolved in 0.5 ml of 0.1 N HCl. Amino acid levels were determined in aliquots of this 0.5 ml using a modified Technicon autoanalyzer (Lamport, 1969). Methionine and SMM were identified by spiking an aliquot of the plant extract with 25 nmol of authentic L-methionine and D,L-SMM. Protein was determined in the washed, ethanol-insoluble pellet following solubilization in 1 ml of 1 N NaOH.

Metabolism of [^{14}C]Methionine during Petal Senescence

Petals were excised from unopened buds on the evening of day -1 and were floated on a 2-ml solution of 10 μM L-methionine containing 4 μCi of L-methionine-U-^{14}C (256 mCi/mmol). The next morning, the petals were washed with distilled water and were blotted dry. Batches of 12 petals were transferred to 25-ml flasks which contained 5 ml of 1% agar. At the appropriate times, the petals were removed from the flasks and were extracted in ethanol as before. The dried ethanol extracts were redissolved in 75-100 μl of extraction medium. Aliquots of the concentrated extracts were subjected to TLC on precoated plastic-backed TLC plates (0.1 mm MN-cellulose, Brinkman Instruments Inc.). The chromatograms were developed in 1-butanol-acetone-water-diethylamine (30:30:15:6 v/v). Following TLC, the plates were scanned for radioactivity using a radiochromatogram scanner (Packard Instrument Co.) and the radioactive zones were scraped from the plates. For quantitation, these zones were combusted in a sample oxidizer (Packard Instrument Co.), and the radioactivity in the form of trapped CO_2 was determined by scintillation counting. Paper electrophoresis was carried out at 6°C with an applied potential of 700 v using 0.1 M sodium acetate (pH 4.5) as a buffer. For H_2O_2 treatment, equal aliquots of the concentrated plant extract were incubated with 3% H_2O_2 in a 1-ml reaction vial at room temperature. Following 10 min of incubation, the entire reaction mixture was applied to a TLC plate which was developed as before. Acid hydrolysis of the ethanol-insoluble pellet was

carried out by mixing the pellet with a small volume of concentrated HCl. This mixture was transferred to a 1-ml reaction vial which was sealed and flushed with nitrogen. The reaction was allowed to proceed for 24 h at 110 C. Following incubation, the mixture was extracted three times with ethanol, and aliquots of this were subjected to TLC as before.

Determination of Radioactivity in SMM and Ethylene

Petals were excised from fully open flowers on the morning of day 0, and groups of these petals were placed in 50-ml Erlenmeyer flasks containing a disc of water-saturated paper. The flasks were flushed with ethylene-free air, sealed, and ethylene production was monitored. When the petals began producing ethylene, the flasks were opened and 1.25 ml of water containing 3.2 μCi of L-methionine-U-^{14}C (256 mCi/mmol) was introduced into each flask. The flasks were again flushed and sealed with a CO_2 trap inside. The CO_2 trap consisted of a strip of fluted filter paper wetted with a saturated solution of $Ba(OH)_2$ in 1 N NaOH which was suspended in a vial over the petals. After 20, 40, and 120 min of incubation on labelled methionine, an aliquot of the gas phase of each flask was withdrawn and simultaneously replaced with air for the determination of the radioactivity in the ethylene which was produced during that incubation period. The determination of the radioactivity in ethylene was accomplished as described by Hanson and Kende (1976a) with the following modifications: a.) 1 ml of 0.25 M

$Hg(ClO_4)_2$ in 2 M $HClO_4$ was injected into the syringe containing the gas aliquot, and the syringe was shaken at 5°C for at least 4 h, b.) Following adsorption of ethylene, 15 ml of Biofluor scintillation solution (New England Nuclear Co.) was added to the syringe, and the entire mixture was added to a plastic scintillation vial and counted directly. Using this procedure, a counting efficiency of greater than 90% was achieved if the samples were counted within 1 h following addition of the Biofluor solution. After withdrawal of the gas sample, the flasks were opened, and the petals were extracted with ethanol as before. SMM was isolated from these extracts by paper electrophoresis as described above. Following isolation, the radioactivity in the SMM zone and in the ethanol-insoluble fraction (protein) was determined by scintillation counting after combustion of the samples in the sample oxidizer.

Determination of the Specific Radioactivities of Ethylene, Methionine, and SMM

Petals were excised from unopened buds on the evening of day -1 and were floated on 3 ml of water containing 15 μCi of L-methionine-U-^{14}C (256 mCi/mmol). The next morning, the petals were washed and then transferred to a 25-ml Erlenmeyer flask. The flask was flushed with ethylene-free air, sealed, and ethylene production was monitored. When the petals began producing ethylene, the flask was opened and one-third of the petals were removed and extracted with ethanol as before. The flask was flushed with ethylene-free

air and resealed with a CO_2 trap inside. After 5 h, 2 aliquots of the gas phase were withdrawn and the specific radioactivity of ethylene was determined by the method of Hanson and Kende (1976a) with the above-mentioned modifications. The remaining two-thirds of the petals were extracted with ethanol. Methionine was isolated from the extract by TLC, and the specific radioactivity of carbons 3+4 was determined by the method of Hanson and Kende (1976). SMM was isolated by paper electrophoresis and was converted to methionine by acid hydrolysis as described by Hanson and Kende (1976a). The specific radioactivity of carbons 3+4 of this SMM-derived methionine was determined as above.

Dilution Experiments

Petals were excised from unopened buds on the evening of day -1 and were floated overnight on 3 ml of distilled water containing 7 μCi of L-methionine-U-^{14}C. The next morning, the petals were washed and groups of petals were transferred to 25-ml Erlenmeyer flasks. The flasks were flushed with ethylene-free air and sealed with a CO_2 trap inside. When the petals began producing ethylene, the flasks were opened, and 1.25 ml of an unlabelled amino acid solution of water was added to each flask. The flasks were flushed, resealed and placed on a shaker operating at slow speed. After 75 min, an aliquot of the gas phase was withdrawn, and the specific radioactivity of ethylene was determined. The flasks were then again opened, flushed and sealed and were incubated for an additional 95 min. At this

time, a second aliquot of the gas phase was withdrawn and the specific radioactivity of this newly formed ethylene was determined.

Uptake of Methionine and SMM

Petals were isolated from fully open flowers in the morning of day 0 and were placed in 50-ml Erlenmeyer flasks containing a disc of water-saturated paper. The flasks were flushed with ethylene-free air, sealed and ethylene production was monitored. When the petals began producing ethylene, the flasks were opened and a 4.5-ml solution of 0.5 mM L-methionine and D,L-SMM containing 0.093 μCi of L-methionine-U-^{14}C and 0.181 μCi of SMM-methyl-^{3}H was added to each flask. After 45, 90, and 120 min, a group of 12 petals was removed from one flask and was blotted dry. The petals were washed for 20 min with a solution of 5 mM L-methionine, D,L-SMM and $MgCl_2$. Following washing, the petals were combusted in the sample oxidizer which separated the two isotopes as $^{14}CO_2$ and $^{3}H_2O$. The amount of radioactivity of each isotope that was taken up was determined by scintillation counting. The amount of amino acid taken up was calculated by taking into account the specific radioactivity of the two amino acids in the original incubation medium.

Effects of ACC on Ethylene Production

Petals were isolated from fully open flowers in the morning of day 0. Petals to be exposed to ACC in the

presence of either AVG or n-propyl gallate (nPG) were initially floated for 90 min on 5 ml of the following inhibitor solutions: 0.1 mM AVG or 1 mM nPG. After this treatment, the petals were transferred to 50-ml Erlenmeyer flasks containing 1.5 ml of a solution of 0.5 mM ACC plus the inhibitor at one-half of the concentration employed for the pretreatment. Petals to be exposed only to ACC were initially floated on distilled water and were then transferred to 50-ml Erlenmeyer flasks containing 1.5 ml of a 0.5 mM solution of ACC. Control petals were incubated on distilled water throughout. Following introduction of the incubation solution, the flasks were flushed, sealed and ethylene production was monitored. The effect of tissue age and origin on the conversion of ACC to ethylene was determined in freshly excised tissues. Following excision, groups of 9 petals/sepals were transferred to 25-ml Erlenmeyer flasks. 1 ml of a 0.1 mM solution of ACC was added to each flask. The flasks were then sealed, and the ethylene production was monitored.

Endogenous Content of ACC

For the comparison of ACC content with petal age, petals were isolated from either unopened buds or from fully open flowers. Following excision, groups of 30 petals were transferred to 125-ml Erlenmeyer flasks which contained a disc of water-saturated paper. The flasks were flushed and sealed. At 10:30 h day -2, day -1 and a group of day 0 petals were removed from the flasks and were extracted with

5 ml of 80% ethanol. The remaining group of day 0 petals was extracted at 14:00 h. The ethanol extracts were prepared and concentrated as before. Following evaporation, the dried extracts were redissolved in 50 µl of 50% ethanol. Aliquots of this were fractionated by TLC as before. Authentic standards of ACC were run in parallel with these extracts in order to determine the position of the ACC-containing zone. The zones on the developed TLC plates which corresponded to the position of the ACC standards were scraped from the plate and eluted with ethanol. The ethanol was dried under nitrogen and the residue was redissolved in 1 ml of 0.1 M sodium phosphate buffer (pH 11.5). One half of this was used directly in the ACC assay system described by Boller et al. (1979). The remaining half was spiked with 10 nmol of authentic ACC prior to the assay in order to determine the efficiency of the assay. The effect of pretreatment with ethylene or of application of AVG and SEM on ACC levels was studied in essentially the same manner. One group of petals was pretreated with 10 µl/l of ethylene for 60 min prior to transfer to the incubation flask. Petals to be exposed to AVG or SEM were transferred directly after excision to the incubation flasks which contained 2 ml of the AVG or SEM solution at the concentration noted in Table XIII. When ethylene production commenced in the ethylene-pretreated petals, all the petals were removed from the flasks and were extracted as before. The time-course experiment was conducted similarly. In this case, cycloheximide treatment was initiated 60 min prior to the pretreatment

with ethylene. Ethylene pretreatment was performed in a separate flask prior to transfer to the incubation flask. Following pretreatment, groups of 15 petals were transferred to 50-ml flasks which were then flushed and sealed. At various times the ethylene content of a flask was determined, the petals removed and extracted in ethanol for the determination of ACC content as before.

Determination of Radioactivity in Ethylene and ACC

Petals were excised from fully open flowers and were placed in 50-ml Erlenmeyer flasks which contained a disc of water-saturated filter paper. Ethylene production was monitored, and when the petals began producing ethylene, the flasks were opened, and 2.5 ml of distilled water containing 6.3 μCi of L-methionine-U-^{14}C was added to each flask. The flasks were resealed with a CO_2 trap inside. After 40 min, an aliquot of the gas phase was removed from one flask, and the petals within that flask were removed and extracted with ethanol. At this time, a second flask was opened and flushed with ethylene-free air and resealed. After 40 min the procedure was repeated. The third flask was then opened, flushed, and resealed. Following another 40-min period, the procedure was repeated for a third time. The ethanol extracts were prepared and concentrated as before. ACC was isolated by TLC and was converted to ethylene in 10-ml plastic syringes by the method of Boller et al. (1979). The gas phase of this 10-ml syringe was transferred via a hypodermic needle to a 25-ml plastic syringe to which 1 ml of $Hg(ClO_4)_2$

solution was added. The specific radioactivity of ethylene derived from carbons 2+3 of ACC was determined as before.

RESULTS

Characteristics of Ethylene Production

In order to determine the characteristics of ethylene production in mature petals of Tradescantia, the effects of a number of inhibitors of ethylene production in plant tissues were examined, and the results of these experiments are shown in Table IV. Aminoethoxyvinylglycine (AVG) completely suppressed ethylene production in these petals. Also effective in arresting ethylene production were n-propyl gallate (nPG), a free radical scavenger and cycloheximide, a protein synthesis inhibitor. Sodium benzoate, another free-radical scavenger, gave variable results but in general was much less effective in blocking ethylene biosynthesis in Tradescantia. Although not shown, anaerobiosis also completely suppressed endogenous ethylene production.

Effects of Exogenous Amino Acids on Ethylene Production

The ability of AVG to inhibit ethylene production indicated that the ethylene produced was derived from methionine. Therefore, the effect of methionine and other related amino acids on ethylene production was investigated, and the results of those experiments are presented in Table V. Exposure of petals to methionine had no effect on either the

Table IV. Effect of inhibitors on ethylene production in mature petals of Tradescantia.

Petals were isolated from closed flower buds on the evening of day -1 and were floated overnight on the appropriate solution. The next morning, groups of 15 petals were transferred to 50-ml Erlenmeyer flasks containing 5 ml of fresh solution. The flasks were flushed, sealed, and the total amount of ethylene produced by the petals was determined.

Treatment	Concentration	Ethylene Produced (% Control ± SD)
Water	------	100 ± 0
Aminoethoxyvinylglycine (AVG)	0.1 mM	0 ± 0
n-Propyl gallate (nPG)	1.0 mM	2 ± 5
Sodium benzoate	1.0 mM	47 ± 50
Cycloheximide	0.1 mM	1 ± 2

Table V. Effect of exogenously applied amino acids on ethylene production in mature petals of *Tradescantia*.

Petals were excised from closed buds on day -1 and were floated overnight on 1 mM solutions of the amino acids indicated. The next day, groups of 15 petals were transferred to 25-ml Erlenmeyer flasks containing 0.5 ml of fresh solution. The flasks were flushed, sealed, and the total amount of ethylene produced by the petals was determined.

Treatment	Ethylene Produced (% Control ± SD)
Water	100 ± 0
L-methionine	93 ± 8
DL-S-methylmethionine	34 ± 18
Homocysteine-thiolactone	50 ± 12

total amount of ethylene produced or on the initiation of ethylene production in these petals. This observation indicated that the ethylene-producing system was saturated with regard to methionine. SMM and HCTL were found to inhibit ethylene production in Tradescantia. In addition, these two compounds inhibited the enlargement of the petals that was normally observed between the evening of day -1 and the morning of day 0. Therefore, it was concluded that these two compounds were toxic to the petals under these conditions.

The effect of selenomethionine (SEM) on ethylene production was also examined, and the results of one experiment are shown in Table VI. Exposure of petals to SEM resulted in over a 2-fold increase in the amount of ethylene produced. This increased ethylene production was found to be sensitive to both AVG and nPG which indicated that the excess ethylene produced under these conditions was synthesized via the normal pathway of ethylene biosynthesis.

Changes in the Levels of Endogenous Amino Acids and Protein During Senescence

The levels of methionine, SMM, leucine, and protein were determined at various stages of petal senescence, and the results of one such analysis are shown in Table VII. A large increase (from 6.8 to 14.8 nmol/3 petals) in free methionine occurred during senescence of these petals. The level of free leucine was also found to increase as the petals senesced. The level of free SMM, on the other hand,

Table VI. Effect of selenomethionine on ethylene production in mature petals of Tradescantia in the presence and absence of selected inhibitors.

Petals were excised from fully-open flowers on the morning of day 0. Pretreatment with the inhibitor solutions was initiated immediately following excision. After 90 min pretreatment, groups of 10 petals were transferred to 50-ml flasks containing selenomethionine or selenomethionine plus the appropriate inhibitor solution. The flasks were flushed, sealed, and the ethylene produced by the petals was determined. Concentration of the inhibitors employed: AVG: 50 μM; nPG: 0.5 mM.

Pretreatment	Treatment	Ethylene Produced (% Control)
Water	Water	100
Water	SEM	259
Aminoethoxyvinylglycine (AVG)	SEM + AVG	0
n-Propyl gallate (nPG)	SEM + nPG	29

Table VII. Levels of endogenous amino acids and protein in senescing petals of Tradescantia.

Petals were excised from fully open flowers and were incubated in sealed 25-ml flasks until the time of extraction.

Time of Day (h)	Methionine[1]	Leucine[1]	SMM[1]	Protein[2]	Ethylene[1]
09:00	6.80	44.20	7.39	225.0	---
15:00	10.23	67.73	4.27	160.0	---
21:00	14.77	74.17	2.63	175.00	0.04

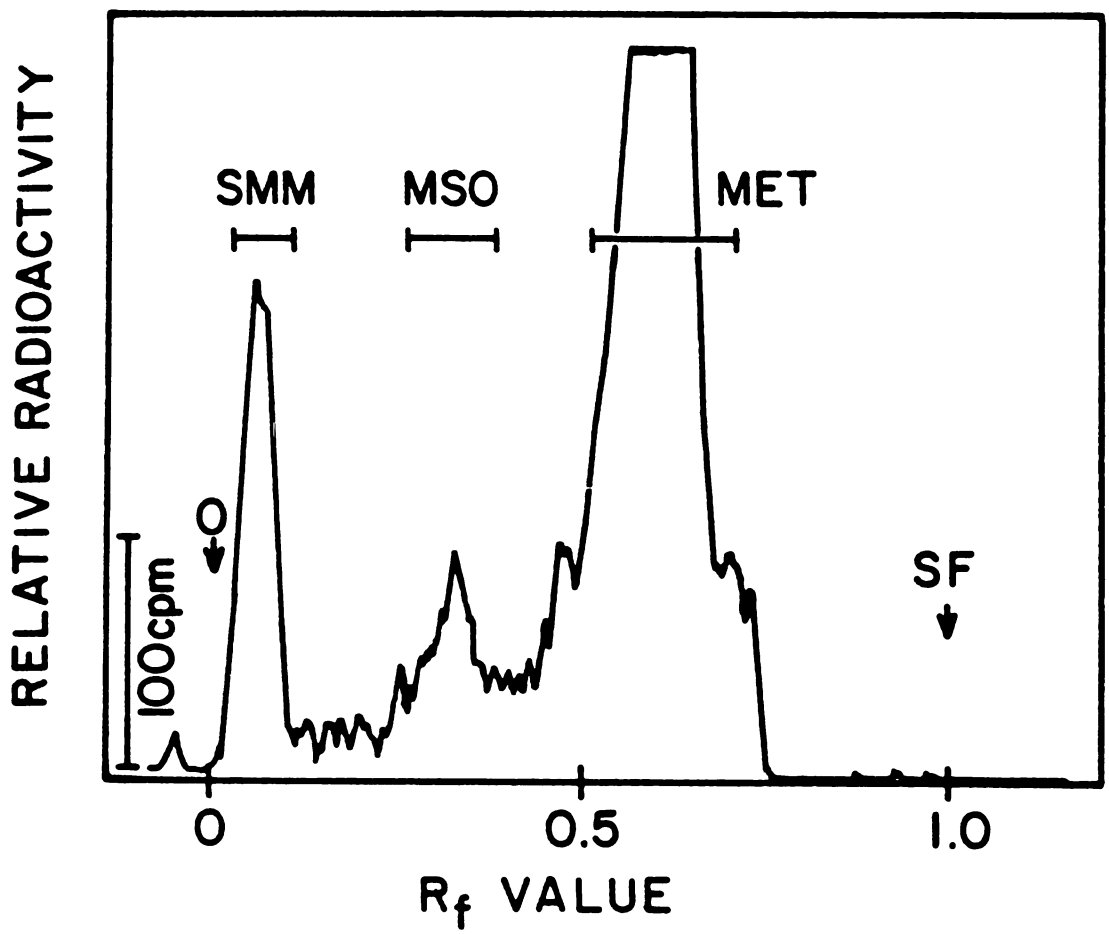
[1]Expressed as nmol/3 petals
[2]Expressed as µg BSA equivalents/3 petals

was found to decline from 7.4 to 2.6 nmol/3 petals as senescence proceeded. The level of protein was also found to decline during senescence with the onset of this decline preceding the onset of ethylene production. As can be determined from the data presented in Table VII, less than 1% of the free methionine present in these petals on the morning of senescence would be needed as a substrate to account for all ethylene produced by these petals during the course of senescence.

Methionine Metabolism During Petal Senescence

In order to investigate the metabolic fate of methionine during petal senescence, petals were incubated overnight between day -1 and day 0 on a solution of labelled methionine. On the next morning of day 0, the petals were washed and placed in Erlenmeyer flasks. At various times during the day the petals were extracted and the radioactivity in various fractions was determined. Radioactivity was found in the following fractions: ethanol-soluble, ethanol-insoluble, carbon dioxide, and in ethylene. Acid hydrolysis of the ethanol-insoluble fraction followed by TLC demonstrated that the radioactivity in this fraction was associated with methionine bound in protein. TLC of the ethanol-soluble fraction followed by scanning of the TLC plates for radioactivity resulted in the separation of three major radioactive zones as shown in Figure 17. The first zone, which had an R_f of 0.05, was found to co-chromatograph with authentic SMM. Elution of this zone from the TLC plate

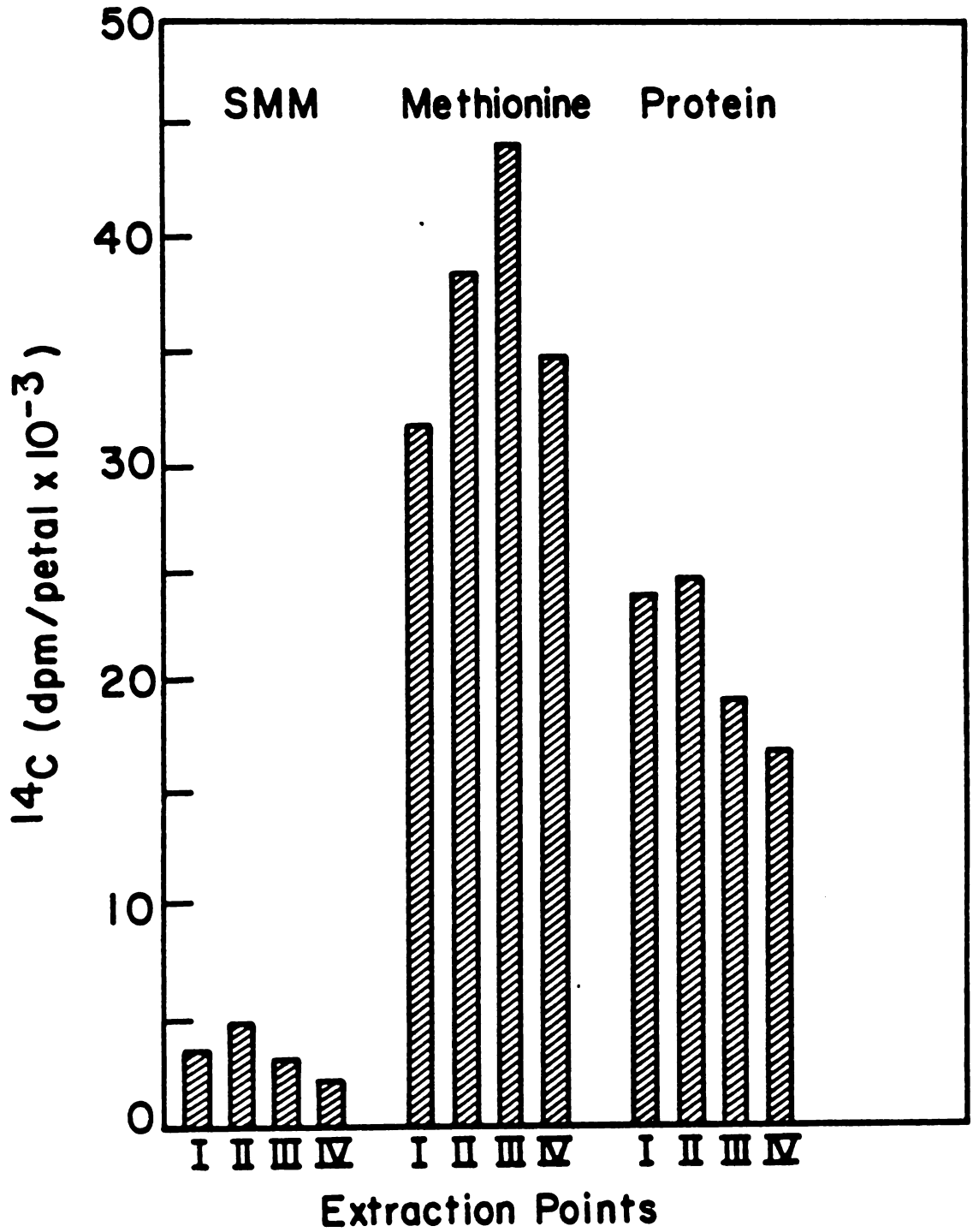
Figure 17. Scan of a radiochromatogram of an extract of Tradescantia petals separated by TLC on cellulose plates in the following solvent system: n-butanol-acetone-diethylamine-water (30:30:6:15 v/v). Petals were excised in the evening of day -1 and were incubated overnight on a 10 mM solution of L-methionine which contained 4 μCi of L-methionine-U-^{14}C. The next morning the petals were washed and extracted with 80% ethanol. O = origin; SF = solvent front.


RELATIVE RADIOACTIVITY
SMM
MSO
MET
O
100cpm
SF
0
0.5
1.0
R_f VALUE

followed by paper electrophoresis at pH 4.5 demonstrated that this compound was a cation with electrophoretical properties identical to that of authentic SMM. Furthermore, treatment of this zone with concentrated HCl under nitrogen yielded a product which co-chromatographed with authentic methionine. It was concluded that zone 1 was SMM. Peak 2 with an R_f of 0.33 co-chromatographed with authentic methionine sulfoxide (MSO). Peak 3, the largest peak, had an R_f of 0.59 and was found to co-chromatograph with authentic methionine. Treatment of the extract with 1.5% of H_2O_2 prior to TLC resulted in the disappearance of Peak 3 with a corresponding increase in Peak 2. This type of behavior was identical to that of authentic methionine which was quantitatively converted to methionine sulfoxide by peroxide treatment. It was therefore concluded that Peak 2 was methionine sulfoxide, an artifact produced by extraction and that Peak 3 was methionine.

The results of a time-course study of methionine metabolism in senescing petals are presented in Figure 18. During the course of senescence, there was a large increase in the level of radioactivity associated with methionine. A small decline in the radioactivity associated with SMM as well as a much larger decrease in the radioactivity associated with protein was also found to occur during petal senescence. These results indicated that the increase in methionine levels observed during senescence was, at least in part, the result of protein degradation rather than the result of the conversion of SMM to methionine as observed in

Figure 18. Distribution of ^{14}C in various fractions isolated from mature petals of Tradescantia at various times during senescence following an overnight incubation on labelled methionine. Extraction times were as follows: I: 09:15 h, II: 12:00 h, III: 16:30 h, IV: 21:15 h.

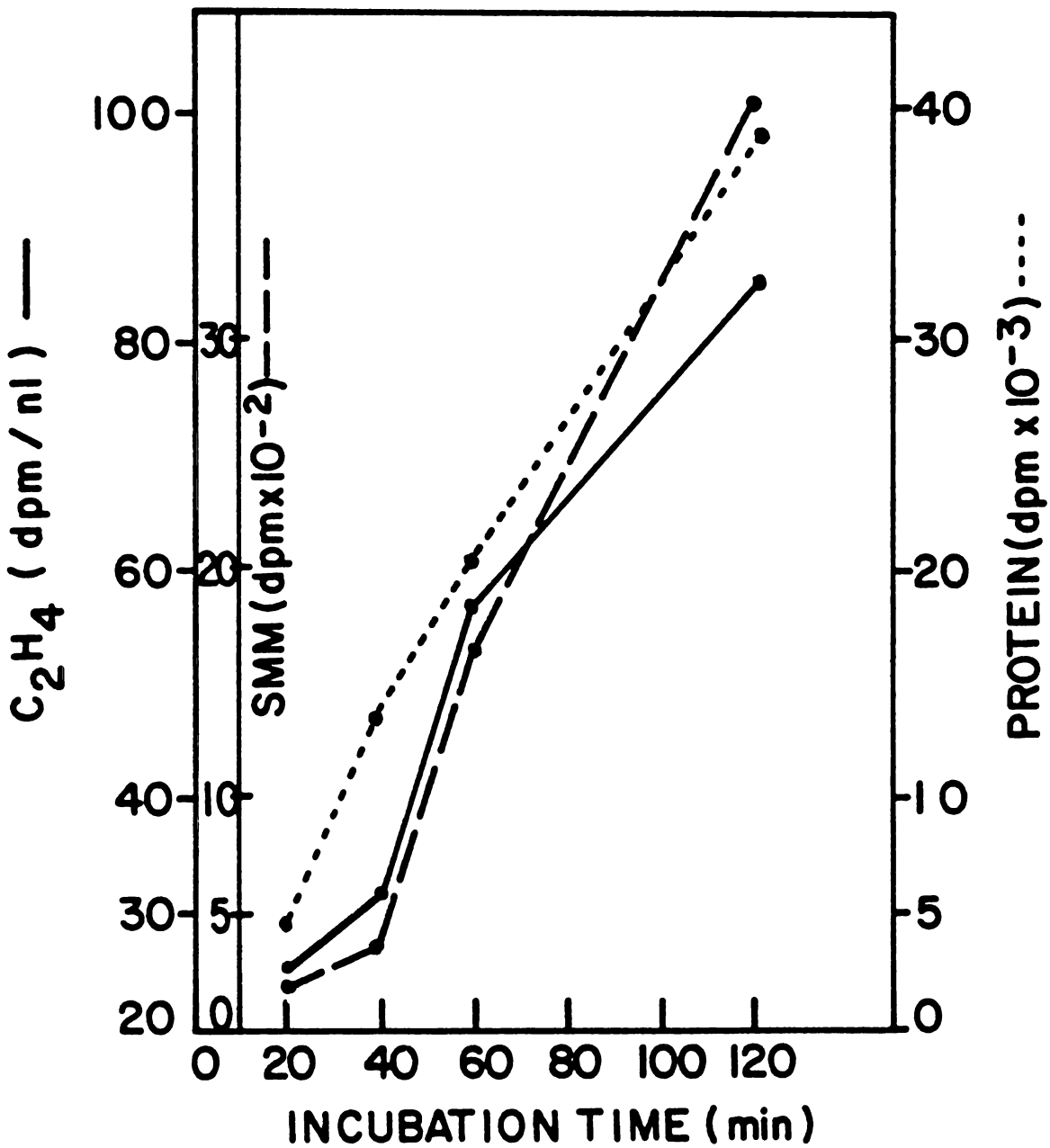

50
40
30
20
10
0
SMM
Methionine
Protein
^{14}C (dpm/petal x 10^{-3})
I II III IV I II III IV I II III IV
Extraction Points

flower tissue of Ipomoea tricolor (Hanson and Kende, 1976a).

Methionine Metabolism in Relation to Ethylene Biosynthesis

Since radioactivity was found in two soluble compounds, SMM and methionine, it was of interest to determine which of these two compounds was the closer precursor of ethylene in petals of Tradescantia. Therefore, a time-course study on the appearance of radioactivity in SMM and ethylene at various times following the application of labelled methionine was conducted. Petals were placed in Erlenmeyer flasks, and the ethylene production was monitored. When ethylene production commenced, labelled methionine was introduced into each flask. After 20, 40, 60, and 120 min of incubation on L-methionine-U-^{14}C, the amount of radioactivity in SMM, ethylene and protein was determined in a group of petals. The results of one experiment are shown in Figure 19. Radioactivity could be detected in all 3 fractions after 20 min incubation. The amount of radioactivity in each compound continued to increase with increasing period of incubation. The subsequent rates of increases of radioactivity in each compound were found to be nearly identical. The fact that no lag was observed in the appearance of label in both SMM and ethylene indicated that both of these compounds were primary products of methionine in these petals. The fact that radioactivity was found in the insoluble (protein) fraction demonstrated that protein synthesis was occurring during this phase of senescence.

Figure 19. Time course of appearance of radioactivity in SMM, ethylene, and protein following application of L-methionine-U-^{14}C (8 μCi) to senescing petals of Tradescantia at 0 time.


100
80
60
40
30
20
40
30
20
10
5
0
0
20
40
60
80
100
120
INCUBATION TIME (min)
C2H4 (dpm/nl) ——
SMM (dpmx10-2) – – –
PROTEIN (dpm x 10-3) ····

Specific Radioactivities of SMM, Methionine and Ethylene

Since applied methionine was readily converted to both SMM and to ethylene without an apparent lag, we felt that a comparison of the specific radioactivity of each of these compounds might allow us to define more accurately which compound was the more immediate precursor of ethylene in petals of Tradescantia. Petals were incubated overnight on a solution of L-methionine-U-^{14}C. On the morning of day 0, the petals were washed and transferred to an Erlenmeyer flask. When ethylene production began, one-third of the petals was extracted with ethanol and the remaining petals were resealed in the flask. After 5 h, aliquots of the gas phase were removed for the determination of the specific radioactivity of the ethylene produced during the trapping period, and the remaining petals were extracted with ethanol. Methionine and SMM were isolated from the extracts, and the specific radioactivities of carbons 3+4 of these two compounds were determined. The results of one such experiment are shown in Table VIII. During the course of the experiment, the specific radioactivity of carbons 3+4 of methionine fell from an initial value of 4.4 to a final value of 3.1 nCi/nmol while that of SMM fell from 11.4 to 9.8 nCi/nmol. The specific radioactivity of the ethylene evolved during this period (7.8 nCi/nmol) was midway between that of methionine and SMM. Thus, these results did not indicate which of the amino acids, methionine or SMM, was a closer precursor of ethylene but they did indicate that either some degree of compartmentation was retained during

Table VIII. Comparison of the specific radioactivity of ethylene produced during petal senescence with those of carbons 3+4 of methionine and SMM.

Petals were incubated overnight on 15 μCi of carrier-free L-methionine-U-^{14}C (256 mCi/mmol). The next morning, the petals were washed, transferred to a 25-ml Erlenmeyer flask, and the ethylene production was monitored. When ethylene production commenced, the flask was opened, one-third (32) of the petals was removed, extracted in ethanol and the flask was resealed. Five h later, two 50-ml aliquots of the gas phase were withdrawn for the determination of the specific radioactivity of ethylene and the remaining petals were extracted in ethanol. Methionine and SMM were isolated from the extracts and the specific radioactivity of carbon atoms 3+4 of these compounds was determined.

Time	Specific Radioactivity (nCi/nmol)		
	Methionine	SMM	Ethylene
Initial	4.4	11.4	---
Final	3.1	9.8	---
Overall	---	---	7.8

this phase of senescence or that a compound other than these two was the actual precursor of ethylene in these petals.

Effects of Unlabelled Amino Acids on the Specific Radioactivity of Ethylene

As a final attempt to differentiate between the two candidates, the ability of methionine, homocysteine and SMM to dilute the specific radioactivity of ethylene was tested. Petals were incubated overnight on labelled methionine and transferred on the next morning to Erlenmeyer flasks, and the ethylene production was monitored. When ethylene production began, solutions of unlabelled amino acids were added to the flasks, and the flasks were resealed. Ethylene was allowed to accumulate between 0-75 min and between 75-170 min after addition of the unlabelled amino acid. At the end of these periods, aliquots of the gas phase were withdrawn, and the specific radioactivity of the ethylene produced was determined. The results of one such experiment are shown in Table IX. Methionine was the best dilutant in either trapping period. Homocysteine was also effective as a dilutant. SMM, on the other hand, exhibited a very marginal capacity to reduce the specific radioactivity of ethylene. These results indicated that methionine was more closely related to ethylene than was SMM.

The low capacity of SMM to dilute could have been caused by a lack of uptake of this amino acid. To ensure that this was not a complicating factor, an uptake experiment was conducted. Petals were floated on a 0.5 mM

Table IX. Reduction of the specific radioactivity of ethylene produced in mature petals of Tradescantia by methionine, SMM and homocysteine-thiolactone.

Petals were excised on the evening of day -1 and were floated overnight on 15 μCi of L-methionine-U-^{14}C (256 mCi/mmol). The next morning the petals were washed, groups of 24 petals were transferred to 25-ml Erlenmeyer flasks which were sealed and the ethylene production monitored. When ethylene production commenced the flasks were opened and 1.25 ml of the following solutions were added, each to a separate flask: a) distilled water, b) L-methionine, c) 2 mM DL-SMM, and d) 1 mM homocysteine-thiolactone. The flasks were flushed and sealed. After 75 min a 50-ml aliquot of the gas phase was withdrawn for the determination of the specific radioactivity of the ethylene evolved. The flasks were then opened and flushed with ethylene-free air and sealed. After 95 min a second aliquot of the gas phase of each flask was removed for the second determination of the specific radioactivity of ethylene.

Collection Period (min)	Dilutant	Radioactivity in ethylene (dpm)	Total Ethylene (nl)	dpm/nl	% Dilution
0-75	Water	458.8	0.81	566.4	0
	Methionine	284.1	1.10	258.3	54
	SMM	420.1	0.86	488.5	14
	Homocysteine	433.1	1.15	376.6	34
75-170	Water	249.2	0.47	530.2	0
	Methionine	178.2	0.77	231.4	56
	SMM	219.4	0.47	466.8	12
	Homocysteine	226.1	0.62	364.7	31

solution of both methionine and SMM which was spiked with both L-methionine-^{14}C and L-SMM-^{3}H. The use of this double-label technique permitted the simultaneous determination of the uptake of both compounds by the same petals, thereby eliminating any problems due to non-uniformity of the tissues employed. The results of such an experiment are presented in Table X. Initially, methionine was taken up to a greater extent than was SMM. However, this difference in uptake declined with time. The determinations of the free pools of both methionine and SMM (Table IV) showed that the free pool of SMM was between 1/5 to 1/2 the size of the free methionine pool. Therefore, in order to achieve the same degree of dilution of the internal pools, only 20-50% as much SMM would have to be taken up as compared to methionine. The results of the uptake experiment demonstrated that this degree of uptake of SMM was achieved. Therefore, failure of SMM to dilute the specific radioactivity of ethylene in these petals cannot be explained on the basis of uptake.

Effect of 1-Aminocyclopropane-1-carboxylic Acid on Ethylene Production

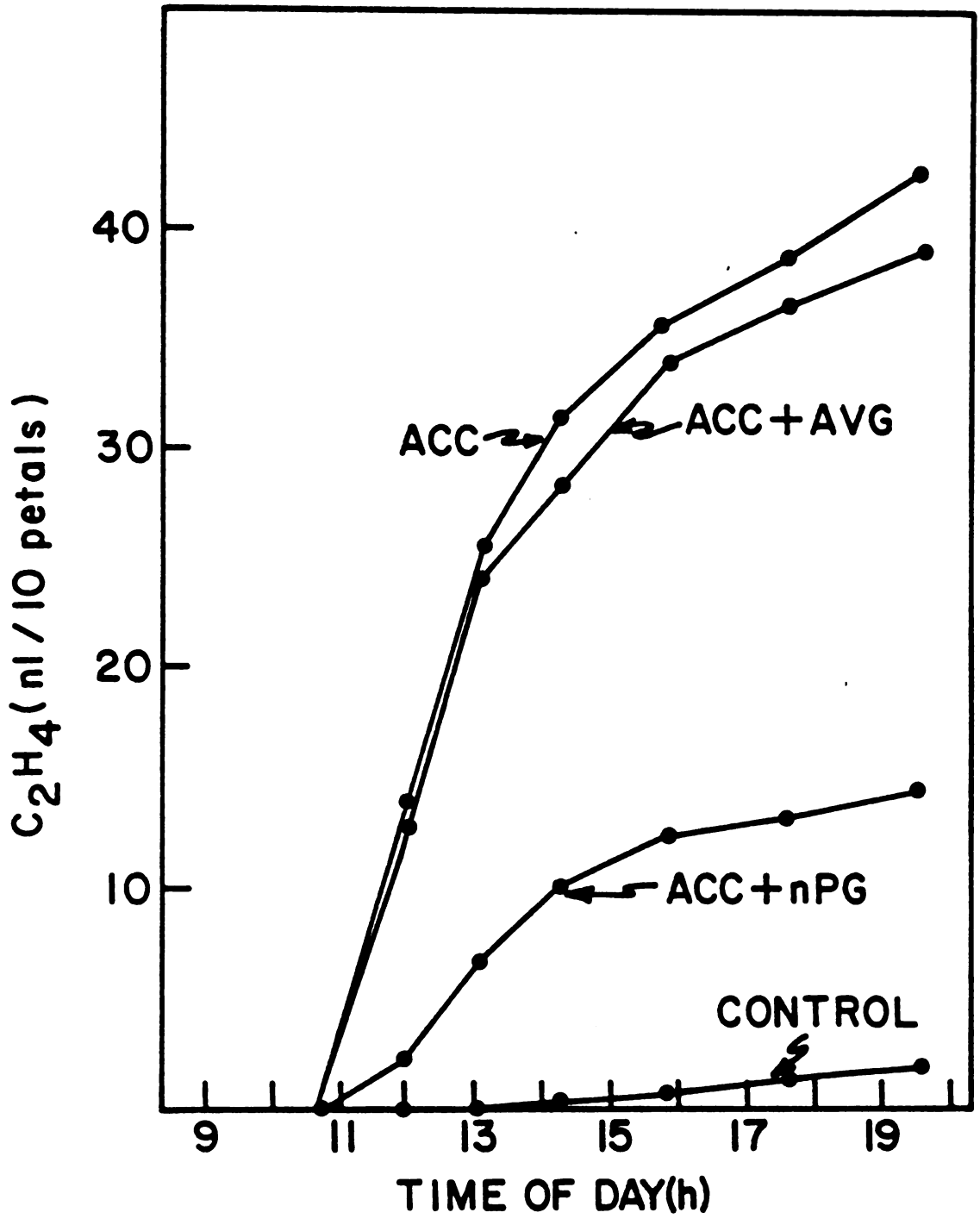
1-Aminocyclopropane-1-carboxylic acid has been identified as the immediate precursor of ethylene in ripening apples (Adams and Yang, 1979). Therefore, it was of interest to determine the effect of this compound on ethylene biosynthesis in petals of Tradescantia. The results of one such experiment are shown in Figure 20. Ethylene production

Table X. Uptake of methionine and SMM during petal senescence in Tradescantia.

Petals were excised from fully open flowers and groups of 12 petals were transferred to 50-ml Erlenmeyer flasks. The flasks were flushed, sealed, and ethylene production was monitored. When ethylene production commenced, 4.5 ml of uptake solution was added to each flask. The uptake solution consisted of 0.5 mM L-methionine and 0.5 mM DL-SMM containing 0.093 nCi of L-methionine-U-^{14}C and 0.181 nCi of L-SMM-methyl-^{3}H. After 45, 90, and 120 min, the petals were removed from a flask, blotted and were then washed in a solution consisting of 5 mM L-methionine, 5 mM-DL-SMM and 5 mM $Mg(Cl)_2$. The petals were reblotted, dried and combusted in a sample oxidizer. The amount of each isotope taken up was determined by scintillation counting. Uptake was calculated on the basis of the amount of each isotope taken up, taking into account the specific radioactivity of the original uptake solution.

Uptake period (min)	Amino acid taken up (μmol)	
	Methionine	SMM
45	0.113	0.022
90	0.167	0.060
120	0.179	0.110

Figure 20. The effect of ACC on ethylene production in mature petals of Tradescantia. Petals were excised from fully-open flowers and were placed on distilled water. Petals to be treated with ACC in the presence of inhibitors were initially floated on solutions containing only the inhibitors. Concentrations of the inhibitors employed were: AVG: 0.1 mM; nPG: 1 mM. Petals that were to be exposed only to ACC and the control petals were floated on distilled water. After 90 min, petals initially incubated on 0.1 mM AVG were transferred to a 50-ml Erlenmeyer flask which contained 50 μM AVG plus 0.5 mM ACC. Petals initially incubated on 1 mM nPG were transferred to a 50-ml Erlenmeyer flask containing 0.5 mM nPG plus 0.5 mM ACC. One-half of the petals initially maintained on water were transferred to a 50-ml flask which contained only 0.5 mM ACC and the remaining one-half was transferred to a flask containing distilled water.


40
30
20
10
C_2H_4(nl/10 petals)
ACC
ACC+AVG
ACC+nPG
CONTROL
9
11
13
15
17
19
TIME OF DAY(h)

could be detected in untreated petals by 14:00 h, and by the end of the experiment these petals had produced 1.93 nl of ethylene. Petals exposed to ACC produced large amounts of ethylene, and the onset of ethylene production could be detected within 15 min of ACC application. Ethylene production by these petals continued at a high rate for the duration of the experiment and reached a final value of 42.7 nl. AVG was found to have little effect on either the initiation or the final amount of ethylene produced by these petals in response to ACC. On the other hand, nPG inhibited the rate of ethylene production in the ACC-treated petals.

As discussed in section I, petals develop the ability to produce ethylene during maturation with only the mature petals being capable of "autocatalytic" ethylene production. It was of interest to determine if the ability of ACC to stimulate ethylene production in flower tissues paralleled the tissues ability to produce ethylene in the absence of ACC. As can be seen in Table XI, application of ACC to all flower tissues, regardless of their endogenous ability to produce ethylene, resulted in the production or large amounts of ethylene. Application of ACC to sepals, a tissue which normally produces very little ethylene, resulted in a 18- to 158-fold stimulation of ethylene production by these tissues. Interestingly, immature petals, which normally produce very little ethylene, produced the greatest amount of ethylene in response to applied ACC. Thus, the ability of ACC to stimulate ethylene production in flower tissues of Tradescantia in no way correlated with the endogenous capacity for ethylene production in the absence of ACC.

Table XI. Stimulation of ethylene production by 1-aminocyclopropane-1-carboxylic acid in sepals and petals of different ages isolated from flowers of Tradescantia.

Flower parts were excised from unopened buds or from fully open flowers early in the morning of the day indicated. Nine petals/sepals were transferred to 25-ml Erlenmeyer flasks containing 1 ml of water of 0.1 mM ACC. The flasks were flushed with ethylene-free air and sealed. Ethylene was collected for 7.5 h, and the total amount of ethylene produced was determined and is expressed as nl C_2H_4/9 petals (or sepals).

Tissue	Age	Treatment	Ethylene produced	% Control
sepals	-2	Water	0.31	100
sepals	-2	ACC	5.71	1,842
petals	-2	Water	0.31	100
petals	-2	ACC	29.95	9,661
sepals	-1	Water	0.23	100
sepals	-1	ACC	6.24	2,713
petals	-1	Water	0.20	100
petals	-1	ACC	36.33	18,165
sepals	0	Water	0.23	100
sepals	0	ACC	11.85	5,152
petals	0	Water	0.95	100
petals	0	ACC	14.26	1,501

Endogenous Levels of ACC in Relation to Endogenous Ethylene Production

Experiments were conducted to determine if the endogenous level of ACC was correlated with the capacity of the tissue to produce ethylene. These results are presented in Table XII. When expressed on a per petal basis, the endogenous level of ACC was low in mature petals prior to the initiation of ethylene production. As the petals senesced and began to produce ethylene, the endogenous content of ACC increased over 5-fold. When expressed on a per petal basis the endogenous levels of ACC in immature petals was found to be similar to that found in mature petals prior to the initiation of ethylene production. The influence of various treatments previously shown to affect the rate of ethylene production in petals of Tradescantia was examined next. Treatments which enhanced ethylene production (SEM, or ethylene pretreatment) lead to elevated levels of ACC (Table XIII). Treatment of mature petals with AVG suppressed both the increase in ethylene production as well as the increase in ACC levels. As a further check of the involvement of ACC in ethylene production in these petals, a time-course study comparing the endogenous content of ACC with the production of ethylene was conducted and the results of one such comparison are shown in Figure 21. The endogenous content of ACC remained low in control petals until the onset of ethylene production when it began to rise. Thereafter, the content of ACC continued to rise as did the rate of ethylene production. Pretreatment of petals with ethylene

Table XII. Endogenous levels of ACC in petals of *Tradescantia* of different physiological ages.

Petals were isolated from closed buds or from fully open flowers early in the morning. At 10:45 h, the day -2, day -1 and one group of day 0 petals were extracted with ethanol. At this time, the second group of day 0 petals was transferred to a 125-ml Erlenmeyer flask which was then sealed. After ethylene production had commenced in these petals (ca. 14:00 h), they were extracted with ethanol. ACC content was determined in the ethanol extracts following separation by TLC.

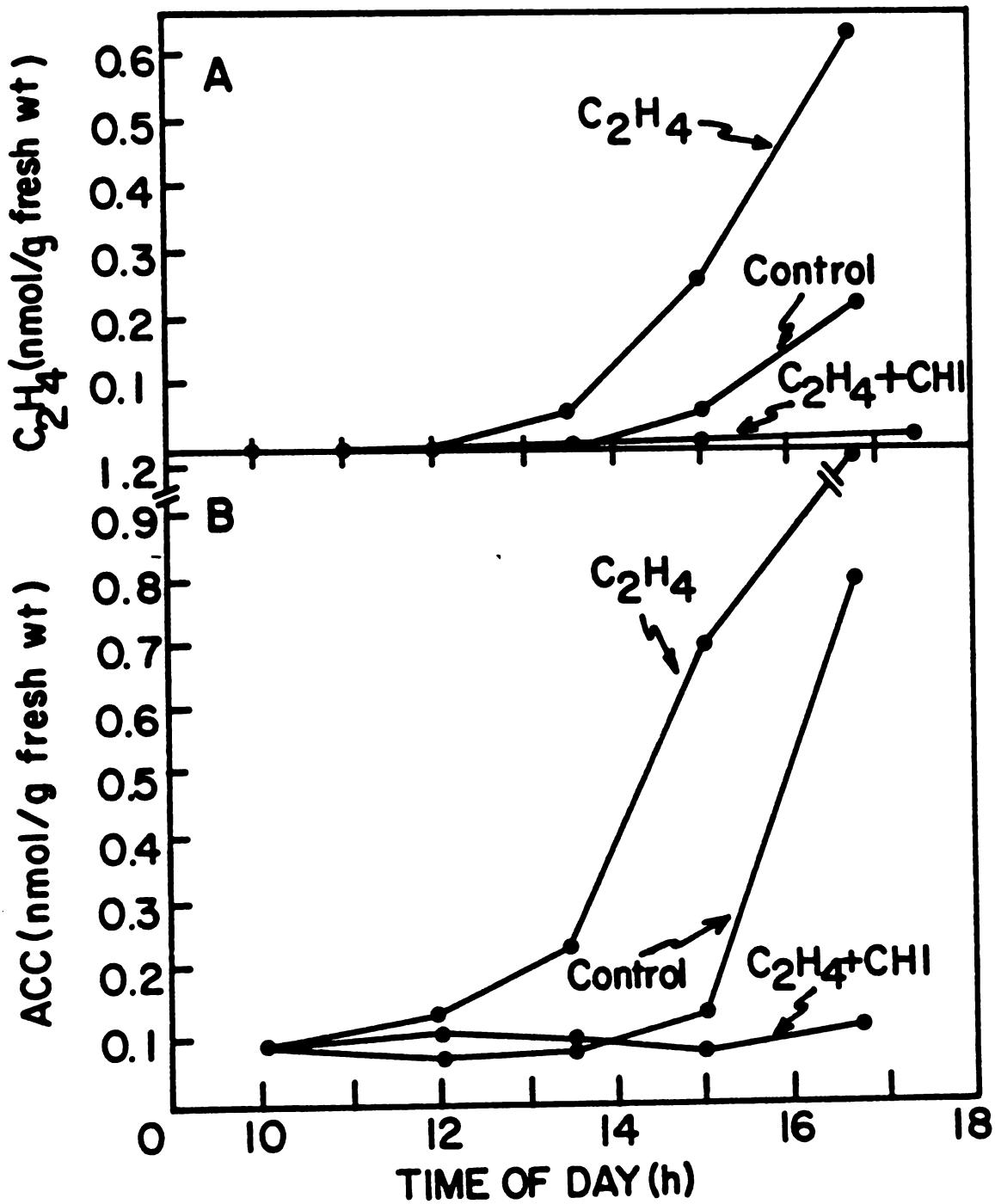
Petal Age	Time of Extraction (h)	ACC Content	
		pmol/30 petals	pmol/g fresh wt
day -2	10:45	37.87	866.52
day -1	10:45	11.87	159.52
day 0	10:45	37.73	89.86
day 0	14:00	214.63	419.89

Table XIII. Endogenous levels of ACC in mature petals of *Tradescantia* following various treatments.

Petals were isolated from fully open flowers early in the morning of day 0. One group of petals was immediately floated on a 0.1 mM solution of AVG and another group was floated on a 0.1 mM solution of selenomethionine. Still another group of petals was exposed to 10 µl/l of ethylene for one hour. Following ethylene pretreatment, the petals were transferred to 125-ml Erlenmeyer flasks which were then sealed. When ethylene production commenced in the ethylene-pretreated petals (ca. 17:00 h), all petals were removed from the flasks, extracted with ethanol, and the endogenous ACC levels were determined.

Petal Age	Treatment	ACC Content (pmol/g fresh wt)
day 0	None	66.60
day 0	Ethylene	716.98
day 0	Aminoethoxyvinylglycine	54.60
day 0	Selenomethionine	319.91

Figure 21. Ethylene production and endogenous levels of ACC in mature petals of Tradescantia. Petals were excised from open flowers and one group of petals was immediately floated on a 0.1 mM solution of cycloheximide. After 60 min, this group of petals as well as another group were treated for 60 min with 10 µl/l of ethylene following this pretreatment, groups of 15 petals were transferred to 50-ml Erlenmeyer flasks. At the appropriate times, the ethylene content in a flask was determined and the petals within that flask were extracted with ethanol to determine the endogenous ACC content. A: ethylene production; B: endogenous content of ACC.


A
B
C_2H_4
Control
C_2H_4+CHI
C_2H_4 (nmol/g fresh wt)
ACC (nmol/g fresh wt)
0.6
0.5
0.4
0.3
0.2
0.1
1.2
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
12
14
16
18
TIME OF DAY (h)

accelerated both the initiation as well as the subsequent rates of increase of ethylene production and also exerted a similar effect on the increase of endogenous ACC content. Petals pretreated with ethylene in the presence of cycloheximide produced no ethylene and showed no increase in the endogenous levels of ACC.

Comparison of the Specific Radioactivities of Ethylene and ACC

The results presented above demonstrated that the endogenous levels of ACC correlated with the tissue's capacity to produce ethylene and indicated that ACC was involved in ethylene production in these petals. In order to establish a precursor-product relationship between ACC and ethylene, a comparison was made between the specific radioactivities of ethylene and ACC at various times following application of labelled methionine. The experimental design was similar to that used in the previous time-course experiments, and the results of one such comparison are shown in Table XIV. The specific radioactivity of ethylene increased with increasing incubation of the petals on labelled methionine. Radioactivity was found in ACC at each determination which demonstrated that it was derived from methionine. Furthermore, the specific radioactivity of both ACC and ethylene closely paralleled each other throughout the experiment. This agreement indicated that these two compounds were metabolically related and that ACC may indeed be the in-vivo precursor of ethylene in mature petals of Tradescantia.

Table XIV. Comparison of the specific radioactivities of ethylene and carbons 2+3 of ACC at various times following addition of L-methionine-U-^{14}C to mature petals of Tradescantia.

Petals were isolated from fully-open flowers, and groups of 15 petals were placed in 50-ml flasks. The flasks were flushed, sealed, and the ethylene production was monitored. When ethylene production commenced, 2.5 ml of water containing 6.3 μCi of L-methionine-U-^{14}C were introduced into each flask. The flasks were flushed with ethylene-free air and sealed. After 40 min, a 50-ml aliquot of the gas phase of one flask was removed for the determination of the specific radioactivity of ethylene and the petals within that flask were removed and extracted with ethanol for the determination of the specific radioactivity of carbons 2+3 of ACC. At this time, the second flask was opened, flushed and resealed. After an additional 40 min, a 50-ml gas sample was removed and petals were extracted as before. The third flask was then opened, flushed and resealed. 40 min later, the procedure was again repeated.

Trapping period (min)	Specific Radioactivity dpm/nl	
	Ethylene	ACC
0-40	25.5	30.6
40-80	64.9	56.4
80-120	94.7	88.9

DISCUSSION

The ability of AVG to inhibit ethylene production in senescing petals of Tradescantia (Table IV) indicates that ethylene production in these petals is similar to that found in the majority of plant tissues. Since AVG has been shown to inhibit ethylene production in tissues which utilize methionine as a precursor of ethylene but not in organisms which utilize other precursors, such as glutamate (Chalutz and Lieberman, 1977), these results indicate that the methionine pathway is operative in these petals. nPG, a compound shown to be effective in inhibiting ethylene production in senescing apple and tomato tissues (Baker et al., 1978), is a very potent inhibitor of ethylene production in these petals. Unlike AVG, nPG is not effective in blocking ethylene biosynthesis in all plant tissues. The inhibitory action of nPG has usually been ascribed to its known ability to scavenge free radicals, particularly O_2^-, and this fact has lead to the proposal that ethylene biosynthesis is mediated by a free radical (Baker et al., 1978). On the other hand, nPG is also a phenolic compound and as such its mode of action may be the result of another, less specific, mechanism. The ability of cycloheximide to inhibit ethylene synthesis indicates that sustained protein synthesis is a prerequisite for ethylene production.

As is true in other ethylene producing tissues (Konze et al., 1978), application of methionine did not affect ethylene production in petals of Tradescantia (Table V). These results indicate that methionine is not limiting ethylene production. In spite of the inability of methionine to stimulate ethylene production, application of SEM to these petals resulted in over a 2-fold stimulation of ethylene production (Table VI). The ability of SEM to enhance ethylene production in tissues which exhibit no response to applied methionine has been noted before (Konze et al., 1978). Their studies have shown that SEM, rather than methionine, is preferentially used as a precursor of ethylene. It is not known if the stimulation of ethylene production by SEM in petals of Tradescantia is the result of a similar preferential utilization, but the results of the inhibitor studies (Table VI) do demonstrate that the excess ethylene produced in response to SEM is probably synthesized via the usual pathway.

The levels of free methionine and leucine increase during petal senescence (Table VII). Concurrently, the level of protein declines, suggesting that the loss of protein, at least in part, gives rise to the increase in free amino acids. The results presented in Table VII show that SMM is a naturally occurring amino acid in these petals. Unlike methionine and leucine, the level of SMM declines during petal senescence. The results presented in Table VII support the previous hypothesis that availability of methionine is not limiting ethylene production in these petals.

In fact, the level of free methionine present in the petals prior to the initiation of ethylene production is more than sufficient to account for all ethylene produced during senescence. This situation is similar to that found in senescing Ipomoea flower tissue (Hanson and Kende, 1976a) but differs from that in ripening apple tissue (Baur and Yang, 1972).

The studies on the fate of labelled methionine (Fig. 18) confirm that the level of free methionine increases during petal senescence. These results also show that SMM is a principal metabolite of applied methionine and confirm that the levels of SMM do decline during senescence. Furthermore, these results support the idea that the increase in methionine results, at least in part, from protein degradation. The loss of radioactivity in SMM cannot account for the increase in methionine as has been shown to occur in senescing Ipomoea flower tissue (Hanson and Kende, 1976a).

The results of the time-course study (Fig. 19) and the comparison of the specific radioactivities of ethylene, methionine, and SMM (Table VIII) did not permit us to determine whether methionine or SMM is the closer precursor of ethylene in these petals. These results confirm the observation that SMM is readily formed from methionine. The fact that the specific radioactivity of SMM is more than double that of methionine indicates that exogenous methionine is preferentially converted to SMM by these petals. Taken on the whole, the results suggest that SMM is a storage form of methionine.

The lack of involvement of SMM in ethylene biosynthesis in these petals is most clearly indicated by the results of the dilution experiments (Table IX). These results show that methionine is the best dilutant in both trapping periods and is therefore more closely related to ethylene than is SMM. The ability of homocysteine to dilute the specific radioactivity of ethylene is consistent with the fact that this compound is the immediate precursor of methionine in most plant tissues. The inability of SMM to reduce the specific radioactivity of ethylene is not due to a lack of uptake (Table X) and must therefore reflect its lack of involvement in ethylene biosynthesis.

In order to demonstrate that ACC is involved in ethylene biosynthesis, three criteria must be met: a) ACC must be converted to ethylene, b) ACC must be an endogenous amino acid, and c) ACC must be formed from applied methionine. The results presented in Figure 20 and in Table XI leave no doubt that ACC is readily converted to ethylene in these petals. The copious amounts of ethylene produced following application of ACC make it unlikely that ACC stimulates ethylene formation in a non-substrate manner. The results presented in Tables XII and XIII demonstrate that ACC is a naturally-occurring amino acid in petals of Tradescantia and that its internal levels correlate with the amount of ethylene being produced. Finally, the results of the comparison of the specific radioactivities of ACC and ethylene at various intervals following application of labelled methionine, (Table XIV) demonstrate that ACC is indeed a metabolite of

methionine, and the close agreement between the specific radioactivities of ethylene and ACC is highly suggestive of the fact that ACC is the immediate precursor of ethylene in these petals.

The ability of applied ACC to stimulate ethylene production in all flower tissues tested (Table XII) demonstrates that the capacity to convert ACC to ethylene does not depend on the physiological age of the tissue. Similar observations have been made by Cameron et al. (1979) who have suggested that the formation of ACC rather than its conversion to ethylene is the rate-limiting step in the biosynthesis of ethylene. The results of the time-course comparison (Figure 21) demonstrate a temporal correlation between the endogenous levels of ACC and the rate of ethylene production. These results are consistent with the above-mentioned hypothesis, namely that ACC formation is the rate-limiting step in ethylene production.

The inability of AVG to inhibit the conversion of ACC to ethylene (Fig. 20) is consistent with the hypothesis that AVG inhibits the formation rather than the utilization of ACC (Adams and Yang, 1979; Boller et al., 1979). The ability of nPG to inhibit the conversion of ACC to ethylene (Fig. 21) suggests a site of action for this inhibitor. However, application of nPG to these petals does not lead to an accumulation of ACC (not shown) as would be expected if only its utilization is being impaired. These results indicate that nPG may interfere at more than one point in the biosynthetic pathway. Indeed, nPG has also been shown to inhibit the

ACC-forming enzyme (Boller et al., 1979). The results with ACC are consistent with the hypothesis that it is the immediate precursor of ethylene in plant tissues, as was first suggested by Adams and Yang (1979).

GENERAL DISCUSSION

A principal question concerning the ability of ethylene to enhance the senescence process lies in ascertaining the site of its primary action. The results presented in Section III demonstrate that the ability of ethylene to stimulate its own synthesis is independent of its action on membrane integrity. A review of the literature shows that many of the physiological processes associated with senescence are functionally independent of one another, and, therefore, apparently proceed in parallel rather than in series. Further, an analysis of the many reports concerning the action of ethylene in senescing tissues shows that the sequence of physiological events in both control and ethylene-treated tissues is essentially identical. This fact, coupled with the observed independence of the various physiological events, suggests that the primary action of ethylene in senescing tissues is directed towards a central mechanism from which the wide array of processes are initiated.

The simplest mechanism which could account for the observed ability of ethylene to affect many independent events would be a primary action at the level of gene expression. Studies in other senescing tissues, such as ripening fruits (Hulme et al., 1971) and abscission zones

(Abeles, 1968), have shown that one of the earliest events following ethylene application is the stimulation of both RNA and protein synthesis. The results presented in Section II demonstrate that the ability of ethylene to enhance vacuolar leakage is dependent on both RNA and protein synthesis and that proteins synthesized during the interval between ethylene application and its initial observable effect (i.e. the lag period) are essential for the manifestation of ethylene action. These facts are consistent with the hypothesis that the primary action of ethylene in senescing tissues occurs at the level of transcription.

However, the inability of ethylene to initiate the senescence syndrome in young or juvenile tissue (Section I, Burg and Burg, 1965; Hanson and Kende, 1976) indicates that ethylene itself does not control the onset of the senescent phase. Rather, it would appear that, as tissues age, they undergo developmental changes which confer ethylene sensitivity to the tissue. The sequential acquisition of ethylene sensitivity in petals of Tradescantia (Section I) is consistent with this hypothesis. Thus, it appears that the initiation of the senescence process in plant tissues is the result of a developmental sequence and that the ability of ethylene to regulate the rate of the subsequent processes is a consequence of the developmental change itself.

Nevertheless, once the tissue becomes competent to respond to ethylene, endogenous ethylene does indeed regulate the rates of the subsequent senescence processes (Section I). Therefore, an understanding of the regulation of its

synthesis is important. The results presented in Section IV indicate that the formation of ACC is the rate-limiting step in the biosynthesis of ethylene. However, it is possible that the formation of SAM also plays a role in controlling the onset of ethylene production in these tissues. In order to differentiate between these possibilities, it would be necessary to extract and assay the activities of both methionine adenosyltransferase and the ACC-forming enzyme at various stages of petal development or at various intervals following ethylene application. Unfortunately, the tissue characteristic which rendered these petals so attractive for the study of compartmentation changes (i.e. high phenolic content) also makes them a poor material for the isolation of active enzymes.

Since flowers exert considerable metabolic drain on the parent plant, it seems logical that their lifespan is directly tied to their physiological function (pollination). The results presented in this thesis demonstrate that in the absence of pollination, the senescence process can be initiated by the petals themselves. This allows the plant to minimize metabolic expenditure in flowers which, for some reason, have failed to be pollinated, and this serves as a physiological failsafe system to ensure that metabolic energy is not wasted on non-functional organs.

REFERENCES

REFERENCES

Abeles, F. B. 1968. Role of RNA and protein synthesis in abscission. Plant Physiol. 43:1577-1586.

Abeles, F. B. 1973. Ethylene in Plant Biology. Academic Press, New York.

Adams, D. O. and S. F. Yang. 1977. Methionine metabolism in apple tissue. Implication of S-adenosylmethionine as an intermediate in the conversion of methionine to ethylene. Plant Physiol. 60:892-896.

Adams, D. O. and S. F. Yang. 1979. Ethylene biosynthesis: Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Natl. Acad. Sci. USA 76:170-174.

Baker, J. E., M. Lieberman, and J. D. Anderson. 1978. Inhibition of ethylene production in fruit slices by a rhizobitoxin analog and free radical scavengers. Plant Physiol. 61:886-888.

Balz, H. P. 1966. Intrazelluläre Lokalisation und Funktion von Hydrolytischen Enzymen bei Tabak. Planta 70:207-23

Baumgartner, B., J. Hurter, and P. Matile. 1978. On the fading of an ephemeral flower. Biochem. Physiol. Pflanzen 168:299-306.

Baur, A. H. and S. F. Yang. 1972. Methionine metabolism in apple tissue in relation to ethylene synthesis. Phytochemistry 11:3207-3214.

Beutelmann, P. and H. Kende. 1977. Membrane lipids in senescing flower tissue of Ipomoea tricolor. Plant Physiol. 59:888-893.

Beyer, E. M. Jr. 1975. ^{14}C-Ethylene incorporation and metabolism in pea seedlings under aseptic conditions. Plant Physiol. 56:273-278.

Beyer, E. M. Jr. 1976. A potent inhibitor of ethylene action in plants. Plant Physiol. 58:268-271.

Beyer, E. M. Jr. 1979. Effect of silver ion, carbon dioxide, and oxygen on ethylene action and metabolism. Plant Physiol. 63:169-173.

Beyer, E. M. Jr. and O. Sundin. 1978. $^{14}C_2H_4$ metabolism in morning glory flowers. Plant Physiol 896-899.

Biale, J. B. 1960. pp. 536-592. In W. Ruhland [ed.] Encyclopedia of Plant Physiology Vol. 12(2). Springer, Berlin.

Biale, J. B. 1975. Fruit ripening and senescence of flowers and leaves. Physiol. Veg. 13:701-708.

Blackman, F. F. and P. Parija. 1928. Analytic studies in plant respiration. I. The respiration of a population of senescent ripening apples. Proc. Roy. Soc. Biol. 103:412-445.

Blok, M. C., E. C. M. Went-Kok, L. L. M. van der Deenen, and J. van Gier. 1975. The effect of chain length and lipid phase transition on the selective permeability properties of liposomes. Biochem. Biophys. Acta 406: 187-196.

Boller, T. and H. Kende. 1979. Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiol. (in press).

Boller, T., R. C. Herner, and H. Kende. 1979. Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. Planta 145:293-303.

Brady, C. J., P. B. H. O'Connell, J. Smydzuk, and N. L. Wade. 1970. Permeability, sugar accumulation and respiration rate in ripening banana fruits. Aus. J. Biol. Sci. 23:1147-1152.

Burg, S. P. 1968. Ethylene, plant senescence and abscission. Plant Physiol. 43:1503-1511.

Burg, S. P. 1973. Ethylene in plant growth. Proc. Nat. Acad. Sci. USA 70:591-597.

Burg, S. P. and E. A. Burg. 1962. Role of ethylene in fruit ripening. Plant Physiol. 37:179-189.

Burg, S. P. and E. A. Burg. 1965. Relationship between ethylene production and ripening in bananas. Bot. Gaz. 126:200-204.

Burg, S. P. and E. A. Burg. 1965b. Gas exchange in fruits. Physiol. Plant. 18:870-884.

Burg, S. P. and E. A. Burg. 1967. Molecular requirements for the biological activity of ethylene. Plant Physiol. 42:144-152.

Burg, S. P. and M. J. Dijkman. 1967. Ethylene and auxin participation in pollen induced fading of Vanda orchid blossoms. Plant Physiol. 42:1648-1650.

Burg, S. P., E. A. Burg, and R. Marks. 1964. Relationship of solute leakage to solution tonicity in fruits of other plant tissues. Plant Physiol. 39:185-195.

Butcher, H. C., G. J. Wagner, and H. W. Siegelman. 1977. Localization of acid hydrolases in protoplasts. Plant Physiol. 59:1098-1103.

Butler, R. D. and E. W. Simon. 1971. Ultrastructural aspects of senescence in plants. Adv. Gerontol. Res. 3:73-129.

Cameron, A. C., C. A. L. Fenton, Y. Yu, D. O. Adams, and S. F. Yang. 1979. Increased production of ethylene by plant tissues treated with 1-aminocyclopropane-1-carboxylic acid. HortScience 14:178-180.

Chalutz, E. and M. Lieberman. 1978. Inhibition of ethylene production in Penicillium digitatum. Plant Physiol. 61:111-114.

Choe, H. T. and K. V. Thimann. 1975. The metabolism of oat leaves during senescence. III. The senescence of isolated chloroplasts. Plant Physiol. 55:828-834.

Clements, R. L. 1970. pp. 159-177. In A. C. Hulme [ed.] Biochemistry of Fruits and Their Products. Vol. 1. Academic Press, New York.

Colquhoun, A. J., J. R. Hillman, C. Crewe, and B. G. Bowes. 1975. An ultrastructural study of the effects of abscisic acid on senescence of leaves of radish (Raphanus sativus L.). Protoplasma 84:205-221.

Coorts, G. D. 1973. Internal metabolic changes in cut flowers. HortScience 8:9-12.

Dilley, D. R. 1970. pp. 179-207. In A. C. Hulme [ed.] The Biochemistry of Fruits and Their Products. Vol. 1. Academic Press, New York.

Draper, S. R. 1969. Lipid changes in senescing cucumber cotyledons. Phytochemistry 8:1641-1647.

Elstner, E. G. and J. R. Konze. 1974. Light-dependent ethylene production by isolated chloroplasts. FEBS Letters 45:18-21.

Ferguson, C. H. R. and E. W. Simon. 1973a. Membrane lipids in senescing green tissues. J. Exp. Bot. 24:307-316.

Ferguson, C. H. R. and E. W. Simon. 1973b. The effects of iodoacetate on phospholipid levels and membrane permeability. J. Exp. Bot. 24:841-846.

Frenkel, C., I. Klein, and D. R. Dilley. 1968. Protein synthesis in relation to ripening in pome fruits. Plant Physiol. 43:1146-1153.

Galliard, T. 1970. The enzymic breakdown of lipids in potato tuber by phospholipid- and galactolipid-acyl hydrolase activities and by lipoxygenase. Phytochemistry 9:1725-1734.

Galliard, T. 1973. pp. 253-284. In G. B. Answell, J. N. Hawthorne, and R. M. C. Dawson [eds.] Form and Function of Phospholipids. Elsevier Scientific Publ. Co., New York.

Ghooprasert, P. and M. Spencer. 1975. Preparation and purification of an enzyme system for ethylene synthesis from acrylate. Physiol. Veg. 13:579-589.

Giaquinta, R., and E. M. Beyer Jr. 1977. $^{14}C_2H_4$: Distribution of ^{14}C-labelled tissue metabolites in pea seedlings. Plant Cell Physiol. 18:141-148.

Hall, I. V. and F. R. Forsyth. 1967. Production of ethylene by flowers following pollination and treatment with water and auxin. Can. J. Bot. 45:1163-1166.

Hansen, E. 1942. Quantitative study of ethylene production in relation to respiration of pears. Bot. Gaz. 103: 5543-5558.

Hanson, E. 1966. Postharvest physiology of fruits. Ann. Rev. Plant Physiol. 17:459-480.

Hanson, A. D. and H. Kende. 1975. Ethylene-enhanced ion and sucrose efflux in Morning Glory flower tissue. Plant Physiol. 55:663-669.

Hanson, A. D. and H. Kende. 1976a. Methionine metabolism and ethylene biosynthesis in senescing flower tissue of Morning Glory. Plant Physiol. 52:528-537.

Hanson, A. D. and H. Kende. 1976b. Biosynthesis of wound ethylene in Morning Glory flower tissue. Plant Physiol. 57:538-541.

Herman, E. and M. Chrispeels. 1978. Catabolism of phospholipids and compartmentation of phospholipases in cotyledons. (Abstr.) Plant Physiol. 61:Suppl. S-15.

Horie, K. 1961. The behavior of the petals in the fading of the flowers of Tradescantia reflexa. Protoplasma 53:663-669.

Hulme, A. C. 1948. Studies in the nitrogem metabolism of the apple fruit during the normal and ethylene induced climacteric rise in respiration. Biochem. J. 434:343-349.

Hulme, A. C., M. J. C. Rhodes, L. S. C. Wooltorton. 1971. The relationship between ethylene and the synthesis of RNA and protein in ripening apples. Phytochemistry. 10:749-756.

Kang, B. G., W. Newcomb, and S. P. Burg. 1971. Mechanism of auxin-induced ethylene production. Plant Physiol. 47:504-509.

Kende, H. and B. Baumgartner. 1974. Regulation of aging in flowers of Ipomoea tricolor by ethylene. Planta 116: 279-289.

Kende, H. and A. D. Hanson. 1976. Relationship between ethylene evolution and senescence in Morning-glory flower tissue. Plant Physiol. 57:523-527.

Konze, J. R. and E. F. Elstner. 1978. Ethane and ethylene formation by mitochondria as indication of aerobic lipid degradation in response to wounding of plant tissues. Biochem. Biophys. Acta 528:213-221.

Konze, J. R. and H. Kende. 1979. Interactions of methionine and selenomethionine with methionine adenosyltransferase and ethylene generating systems. Plant Physiol. 63:507-510.

Konze, J. R., N. Schilling, and H. Kende. 1978. Enhancement of ethylene formation by seleno-amino acids. Plant Physiol. 62:397-401.

Kosiyachinda, S. and R. E. Young. 1975. Ethylene production in relation to the initiation of the respiratory climacteric in fruit. Plant Cell Physiol. 16:595-602.

Lamport, D. T. A. L. 1969. The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymatic degradation of primary cell walls. Biochemistry 8:1155-1163.

Lau, O. and S. F. Yang. 1976. Stimulation of ethylene production in the mung bean hypocotyls by cupric ion, calcium ion and kinetin. Plant Physiol. 57:88-92.

Lewis, L. N. and J. E. Varner. 1970. Synthesis of cellulase during abscission of Phaseolus vulgaris leaf explants. Plant Physiol. 46:194-199.

Lieberman, M. 1979. Biosynthesis and action of ethylene. Ann. Rev. Plant Physiol. 30:533-591.

Lieberman, M. and A. T. Kunishi. 1975. Ethylene-forming systems in etiolated pea and apple tissue. Plant Physiol. 55:1074-1078.

Lieberman, M., A. T. Kunishi, L. W. Mapson, and D. A. Wardale. 1965. Ethylene production from methionine. Biochem. J. 97:449-459.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lyons, J. M. and H. K. Pratt. 1964. An effect of ethylene on swelling of isolated mitochondria. Arch. Biolchem. Biophys. 104:318-324.

Mapson, L. W., J. F. March, and D. A. Wardale. 1969. Biosynthesis of ethylene: 4-methylmercapto-2-oxybutyric acid: and intermediate in the formation from methionine. Biochem. J. 115:653-661.

Marks, J. D., R. Bernlohr, and J. E. Varner. 1957. Esterification of phosphate in ripening fruits. Plant Physiol. 32:259-262.

Matile, P. 1975. The Lytic Compartment of Plant Cells. Springer-Verlag, Vienna.

Matile, P. 1978. The biochemistry and function of vacuoles. Ann. Rev. Plant Physiol. 29:193-213.

Matile, P. and F. Winkenbach. 1971. Function of lysosomes and lysosomal enzymes in the senescing corolla of the Morning Glory (Ipomoea purpurea). J. Exp. Bot. 22: 759-771.

Martin, C. and K. V. Thimann. 1972. The role of protein synthesis in the senescing of leaves. I. The formation of protease. Plant Physiol. 49:64-71.

Mattoo, A. K. and M. Lieberman. 1977. Localization of the ethylene-synthesizing system in apple tissue. Plant Physiol. 60:794-799.

Mayak, S., Y. Vaadia, and D. R. Dilley. 1977. Regulation of senescence in carnation (Dianthus caryophyllus) by ethylene. Mode of action. Plant Physiol. 59:591-593.

Maziak, P. 1977. pp. 48-75. In M. Tevini and H. K. Lichenthaler [eds.] Lipid and Lipid Polymers in Higher Plants. Springer-Verlag, Berlin.

Mericle, L. W. and R. P. Mericle. 1971. Somatic mutations in clone 02 of Tradescantia. J. Heredity 62:322-328.

Miller, K. W. 1975. pp. 341-351. In B. R. Fink [ed.] Molecular Mechanisms of Anesthesia. Raven, New York.

Morgan, P. W., J. I. Durham, and J. A. Lipe. 1973. pp. 1062-1068. In Y. Sumiki [ed.] Plant Growth Substances 1973. Hirokawa, Tokyo.

Murr, D. P. and S. F. Yang. 1974. Inhibition of in vivo conversion of methionine to ethylene by L-canaline and 2,4-dinitrophenol. Plant Physiol. 55:79-82.

Nichols, R. 1968. The response of carnations (Dianthus caryophyllus) to ethylene. J. Am. Hort. Sci. 403:335-349.

Nichols, R. 1977. Sites of ethylene production in the pollinated and unpollinated senescing carnation (Dianthus caryophyllus) inflorescence. Planta 135:155-159.

Nichols, R. and L. C. Ho. 1975. Effect of ethylene and sucrose on translocation of dry matter and ^{14}C-sucrose in the cut flower of the glasshouse carnation (D. caryophyllus) during senescence. Ann. Bot. 39:287-296.

Nishimura, M. and H. Beevers. 1978. Hydrolases in vacuoles from castor bean endosperm. Plant Physiol. 62:44-48.

Poovaiah, B. W. and A. C. Leopold. 1973. Deferral of leaf senescence with calcium. Plant Physiol. 52:236-239.

Quarles, R. H. and R. M. C. Dawson. 1969. The distribution of phospholipase D in developing and mature plants. Biochem. J. 112:787-794.

Richmond, A. and J. B. Biale. 1966. Protein and nucleic acid metabolism in fruits. I. Studies of amino acid incorporation during the climacteric rise in respiration of the avocado. Plant Physiol. 41:1247-1253.

Rieley, C. A., G. Cohen, and M. Lieberman. 1974. Ethane evolution, a new index of lipid peroxidation. Science 183:208-210.

Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5:494-496.

Sacher, J. A. 1959. Studies on auxin-membrane permeability relations in fruit and leaf tissues. Plant Physiol. 34:365-372.

Sacher, J. A. 1962. Relations between changes in membrane permeability and the climacteric in banana and avocado. Nature 195:577-578.

Sacher, J. A. 1966. Permeability characteristics and amino acid incorporation during senescence (ripening) of banana tissue. Plant Physiol. 41:701-708.

Sacher, J. A. 1967. pp. 269-303. In H. W. Woolhouse [ed.] Aspects of the Biology of Aging. Cambridge Univ. Press, London.

Sacher, J. A. 1973. Senescence and postharvest physiology. Ann. Rev. Plant Physiol. 24:197-224.

Simon, E. W. 1974. Phospholipids and plant membrane permeability. New Phytol. 73:377-420.

Skipski, V. P. and M. Barclay. 1976. Thin-layer chromatography of lipids. Methods of Enzymology 14:530-598.

Stead, A. D. and K. G. Moore. 1977. Flower development and senescence in Digitalis purpurea L., cv. foxy. Ann. Bot. 41:283-292.

Suttle, J. C. and H. Kende. 1978. Ethylene and senescence in petals of Tradescantia. Plant Physiol. 62:267-271.

Tetly, R. M. and K. V. Thimann. 1974. The metabolism of oat leaves during senescence. I. Respiration, carbohydrate metabolism and the action of cytokinins. Plant Physiol. 54:294-303.

Theologis, A. and G. G. Laties. 1978. Respiratory contribution of the alternate path during various stages of ripening in avocado and banana fruits. Plant Physiol. 62:249-255.

Thimann, K. V. 1978a. Senescence. Bot. Mag. Tokyo Special Issue 1:19-43.

Thimann, K. V. 1978b. The senescence of leaves. What's New in Plant Physiol. 9:9-12.

Thimann, K. V., R. R. Tetley, and T. V. Thanh. 1974. The metabolism of oat leaves during senescence. II. Senescence in leaves attached to the plant. Plant Physiol. 54:859-862.

Varner, J. E. 1961. Biochemistry of senescence. Ann. Rev. Plant Physiol. 12:245-264.

Wiemken-Gehrig, V., A. Wiemken, and P. Matile. 1973. Mobilisation von Zellwandstoffen in der welkender Blüte von Ipomoea tricolor Cav. Planta 115:297-307.

Wood, J. G. and D. H. Cruickshank. 1944. The metabolism of starving leaves. Aust. J. Exptl. Biol. Med. Sci. 22: 111-123.

Woolhouse, H. W. 1967. pp. 179-213. In H. W. Woolhouse [ed.] Aspects of the Biology of Aging. Cambridge Univ. Press, London.

Yang, S. F. 1968. pp. 1217-1228. In F. Wightman and G. Setterfield [eds.] Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa.

Yang, S. F. 1974. pp. 131-164. In V. C. Runeckles, E. Sondheimer, and D. C. Walton [eds.] Recent Advances in Phytochemistry Vol. 7. Academic Press, New York.

Yoshida, Y. 1961. Nuclear control of chloroplast activity in Elodea leaf cells. Protoplasma 54:476-492.

Young, R. E. and J. B. Biale. 1967. Phosphorylation in avocado fruit slices in relation to the respiratory climacteric. Plant Physiol. 42:1357-1362.