STUDIES ON THE MODE OF ACTION OF ETHYLENE IN PLANT TISSUES AND ITS ROLE IN AUTOCATALYTIC ETHYLENE PRODUCTION

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
EVANGELOS MICHAEL SFAKIOTAKIS
1972

This is to certify that the

thesis entitled

STUDIES ON THE MODE OF ACTION OF ETHYLENE IN PLANT TISSUES AND ITS ROLE IN AUTOCATALYTIC ETHYLENE PRODUCTION

presented by

Evangelos Michael Sfakiotakis

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Horticulture

Major professor

Date November 2, 1972

O-7639

ABSTRACT

STUDIES ON THE MODE OF ACTION OF ETHYLENE IN PLANT TISSUES AND ITS ROLE IN AUTOCATALYTIC ETHYLENE PRODUCTION

By

Evangelos Michael Sfakiotakis

Investigations were conducted to determine the mechanism of ethylene action in causing diverse physiological and morphological changes in plant tissues. Studies were also made to determine the processes involved in the regulation of ethylene synthesis during fruit maturation. Several investigative approaches were employed dealing with; pollen germination and tube growth, DNA polymerase activity in etiolated pea, DNA and RNA polymerase activity in potato tuber mitochondria, induction of autocatalytic ethylene production in apple fruits, and control of the internal ethylene concentration in apple fruits during maturation.

The influence of ethylene and CO_2 on pollen germination and tube growth was investigated employing ventilated culture systems. Ethylene had no effect on pollen germinability or tube growth. Germinating pollen did not produce a detectable amount of ethylene (less than 0.1 nl/g/hr). Supplementing the cultures with CO_2 caused a marked increase in germination

and tube growth. The half-maximal response for germination was less than 0.5%. CO_2 levels ranging from 1.08 to 2.22% were found in the internal cavity of lily styles. CO_2 derived from stylar metabolism may, therefore, modulate pollen tube growth thus integrating the events leading to fertilization.

An extract from the apical portion of etiolated seedlings of Pisum sativum L. was used as a test system to examine the action of ethylene on DNA polymerase activity. Extracts from plants previously treated with ethylene showed less activity to synthesize DNA than extracts from untreated plants. Ethylene in vitro showed no activity. Inhibition of cell division by ethylene observed in this and other tissues may be the result of impaired synthesis of DNA polymerase.

RNA and DNA polymerase activity was studied in mitochondria isolated from potato tubers respiring at an accelerated rate in response to treatment with ethylene (10 µl/l). Whole tuber respiration rate increased about 6 hrs after ethylene treatment began and reached a peak value 4.7-fold higher than the initial rate in 24 hrs. The rise in respiration preceded by an increase in DNA polymerase activity initiated within 6 hrs of ethylene application. RNA polymerase activity began to increase after 6 hrs and reached a peak value 2.5-fold higher than the initial rate in 18 hrs. Cytochrome c oxidase activity, although increasing initially

subsequently paralleled the respiration rate pattern of whole tubers. Activity of DNA and RNA polymerase of mitochondria isolated from tubers that did not receive ethylene treatment was not affected by ethylene treatment *in vitro*. Apparently the stimulation of respiration by ethylene is mediated *in vivo* by enhancing mitochondrial DNA, RNA and protein synthesis.

Ethylene biosynthesis becomes autocatalytic as fruit ripening proceeds. Propylene, which fruits do not produce, was employed to determine the stage of maturity apples must attain to autocatalytically produce ethylene and the effect of O_2 tension on autocatalysis. The efficacy of propylene to stimulate ethylene production increased progressively with fruit maturation, but rate of production following treatment with 500 ppm propylene was constant. A shorter lag time to the onset of autocatalytic production was observed for the more mature fruit which reflects a natural increase in sensitivity. Propylene administered at 6.5% O_2 or less did not induce ethylene production, but an anaerobic atmosphere was necessary to completely inhibit ethylene synthesis in fruits once autocatalysis began.

Internal ethylene concentration was followed in apples while attached to or detached from the tree throughout the maturation period. Similar internal gas levels were found in fruits on the tree when compared to levels observed in fruits

immediately after harvest. Harvesting fruits stimulated their ethylene production capacity more quickly as the fruits gained maturity. Isolating the fruit from leaves by girdling and defoliation stimulated an earlier increase in internal ethylene concentration. Leaves apparently provide the fruit with a substance that retards or prevents the onset of auto-catalytic ethylene production.

STUDIES ON THE MODE OF ACTION OF ETHYLENE IN PLANT TISSUES AND ITS ROLE IN AUTOCATALYTIC ETHYLENE PRODUCTION

By

Evangelos Michael Sfakiotakis

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Horticulture

1972

DEDICATION

to my children,
Despina and Michael

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. D. R. Dilley, for his counsel and assistance throughout the course of this work and during the development of the manuscripts contained herein.

The author is also indebted to Drs., F. G. Dennis, M. J. Bukovac, R. S. Bandurski and P. Markakis who served as the guidance committee and for their review of this dissertation. The advice and assistance offered by Drs. J. A. Boezi, N. E. Tolbert and D. H. Dewey are greatly appreciated. Appreciation is also extended to Drs. A. Apelbaum and D. H. Simons with whom the author in collaboration conducted part of this work. Thanks are extended to Dr. J. D. Downes for donating the potato tubers used in the mitochondrial study.

The author gratefully acknowledges the support of the National Science Foundation for providing a portion of the financial assistance which made this study possible.

Special thanks to my wife, Diamanta, for her patience and understanding throughout the course of this graduate study.

Guidance Committee:

The thesis is organized in the journal style with the various sections following the format of the respective journals to which they have been or will be submitted for publication.

"Pollen Germination and Tube Growth: Dependent on Carbon Dioxide and Independent of Ethylene" conducted in cooperation with Dr. D. H. Simons has been published in Plant Physiology. "Inhibition by Ethylene of Soluble DNA Polymerase Activity in Pisum Sativum Seedlings" conducted in collaboration with Dr. A. Apelbaum will be submitted to Science; "DNA and RNA Polymerase Activity of Potato Tuber Mitochondria Enhanced by Ethylene" conducted also in collaboration with Dr. A. Apelbaum will be submitted to Biochemical and Biophysical Research Communications; "Induction of Autocatalytic Ethylene Production in Apple Fruits by Propylene in Relation to Maturity and Oxygen Dependency" and "Internal Ethylene Concentrations in Apple Fruits Attached or Detached from the Tree" will be submitted to Journal of the American Society for Horticultural Science.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES--Continued

FIGURE Page

LIST OF FIGURES--Continued

FIGURE Page

Section V

Appendix

LIST OF FIGURES--Continued

FIGURE Page

PREFACE

Introduction:

Ethylene has been implicated in many stages of growth and development from germination of seeds to senescence of plants. As a normal plant metabolite, the gaseous hormone controls hook formation in etiolation (36,37,54), ripening of fruits (16), sex expression in cucurbits (24) and the onset of senescence of leaves (22,30), flowers (22) and other tissues (65).

Application of ethylene produces a variety of responses characterized by suppression or stimulation of normal morphogenetic events. The list of such responses includes: suppression of the shoot apex growth (17) with inhibition of leaf expansion in several dicots (22,37) or stimulation of growth in a few monocots (52,60,89), flower initiation (18), inhibition of flowering (3), inhibition of bud growth (22), breaking of dormancy in resting tubers (92), corms and bulbs (38,92), stimulation of germination of some seeds (5,34,38,57, 88,90,92) inhibition of root growth (25,26), induction of adventitious roots (59,96), epinasty of leaves (28,29,95) altering of sex expression (24,71), induction of abscission (4,81,95), enhancement of fruit growth (69) and promotion of ripening (10,14,16,41,69,82).

Although knowledge of the physiological role of ethylene has become extensive in recent years little is known about the mechanism of action.

Mechanism of Ethylene Action:

Despite the diversity of phenomena regulated by ethylene nearly all responses have the same dose-response curve (8,67, 82). Another common feature of all ethylene responses is the inhibitory effect of CO_2 (2,4,20,25,56,58,86,94). Lineweaver-Burk plots (20,37) revealed that the CO_2 inhibition of ethylene action is competitive, similar to that observed for competitive inhibitors of enzyme reactions. This observation gave rise to the hypothesis by Burg and Burg (16,20) of a single receptor molecule in a metal containing enzyme requiring oxygen for activation. They suggested that interaction of ethylene with the receptor site caused a primary chemical change. This in turn produced a multiplicity of secondary changes resulting in a variety of physiological responses depending upon the kind and the age of tissue involved (23, 67). While little evidence has been presented to contradict the above proposal, there are a few physiological effects with different apparent kinetics (37,82). Pratt and Goeschl (82) suggest that multiple sites of action may exist.

The mechanism by which ethylene initiates a variety of responses has not been determined. Four possibilities have been proposed; 1) ethylene may act as an allosteric effector

on enzyme activity, 2) it may enhance the permeability of membranes, 3) it may regulate auxin action, and 4) it may regulate the metabolism of nucleic acid and protein synthesis.

In vitro studies have shown no effect of ethylene on the activity of enzymes controlling rate-limiting reactions. The activity of carbonic anhydrase, which contains zinc and has the ability to combine with CO_2, did not change in the presence of ethylene (8, personal communication with H. Ku). Similar results were obtained in vitro studies with β-glucosidase (32), α-amylase (31), invertase (31,85), peroxidase (75) and adenosine triphosphatase (80).

On the contrary, activity of many enzymes increased when the tissue was pretreated in vivo with ethylene. These include peroxidase (42,50,51,68,75,76,84), malic enzyme (48,49, 53,74), phenylalanine ammonia lyase (33,50.78), acid phosphatase (42), chlorophyllase (63), cytochrome c reductase (53), polygalacturonase (70), polyphenol oxidase (42,50,87), protease (73), and cellulase (6,7,27,77). These changes in enzyme activity are probably secondary to the primary action of ethylene.

The hypothesis that ethylene regulates physiological phenomena through an effect on the permeability of membranes has received considerable attention in connection with fruit ripening. The proposal that ethylene may exert its effects by disrupting the membranes and bringing changes of compartmentalization (11) has been criticized because the changes

in the characteristics of membranes may be the result of ripening rather than the cause (8,13,15,22,83).

Lyons and Pratt (64) showed that ethylene caused mitochondrial swelling, suggesting a change in permeability and Olson and Spencer (79,80) found an increase in adenosine triphosphatase when mitochondria were exposed to ethylene. Recent studies have revealed that the effect on mitochondrial swelling was not typical of ethylene action. The concentration of the gas needed for this response was markedly higher than biologically active concentrations, and saturated hydrocarbons like propane and ethane had similar effects (61,72).

The theory that ethylene affects auxin by altering auxin transport, increasing auxin destruction, or inhibiting the synthesis of auxin has received considerable support recently. Burg and Burg observed that ethylene inhibits polar (19) and lateral (17) IAA transport in pea epicotyl. Hall and Morgan (39) showed an increase in IAA oxidase activity in cotton after ethylene treatment. Valdovinos et al. (93), reported that ethylene inhibited the conversion of tryptophan to auxin in Coleus. They concluded that ethylene regulates auxin levels by controlling auxin biosynthesis.

Changes in nucleic acids and proteins are often associated with responses to ethylene. Inhibition of cell division by ethylene was found in fig fruits during stage I of their growth (69). Holm and Abeles (44,45) treated soybean seedlings with ethylene and found that DNA synthesis ceased in

the apex where growth was inhibited, but was promoted in the subapical tissue where swelling took place. In more recent studies with pea Apelbaum and Burg (9) showed that low concentrations of ethylene (50 ppb) prevented cell division in the apical meristem of the shoot and root within a few hours by inhibiting DNA synthesis and preventing the cell from entering prophase. The mechanism whereby ethylene inhibits DNA synthesis has not been elucidated.

Promotion of RNA synthesis was associated with ethylene-induced epinasty in tomato leaves (91), and abscission (1). Ethylene also increased RNA in fig fruits (66). Hulme et al. (49) found an ethylene stimulated synthesis of RNA in apple fruit slices associated with the climacteric phenomenon.

Ethylene increased protein synthesis in ripening (12,35, 43,46,47,62) and abscission (1), and increased the activity of enzymes mentioned earlier.

Application of inhibitors of nucleic acid and protein synthesis diminished the response to ethylene. Frenkel et al. (35) found that fruit ripening was not stimulated by ethylene unless protein synthesis proceeded. Inhibitors of protein and RNA synthesis abolished the suppression of hook opening caused by ethylene (55).

It is not clear whether the above mentioned changes in nucleic acids and protein are due to the direct action of the gas, or whether they occur during the expression of phenomena regulated by ethylene. Thus the observed changes may be the

result rather than the cause of the response induced by ethylene (8). Studies with inhibitors of nucleic acid and protein synthesis cannot resolve the problem because both processes are required in the response studied. Basic studies of the role of ethylene as well as of other hormones in the control of gene activity have suffered for lack of kinetic studies on changes in DNA, RNA, or protein. These would separate cause from effect.

Role of Ethylene in the Autocatalysis of Ethylene Production:

The action of ethylene in ripening of climacteric fruits and other senescing organs is amplified by the fact that the hormone stimulates its own production. Autocatalysis of ethylene production is common to senescing organs detached from the mother plant. However, the phenomenon does not occur in actively growing plant tissues. Fruits attached to the tree exhibit resistance to ripening by exogenous ethylene. This has been attributed to an inhibitor of ethylene action which is transported from the leaves to the fruit (16).

Autocatalysis has been associated with ripening of climacteric fruits (16,58) and senescence of orchid flowers (21,40). The phenomenon may be very common in other tissues as well, but is not well understood. Lack of the appropriate tissue and the difficulties to separate the input from the output gas makes the study more complex.

The mechanism of autocatalysis of ethylene production in detached organs, the nature of the resistance to autocatalysis and ripening in attached organs, and the factors controlling these processes have not been resolved.

Thesis Objectives:

It is quite apparent that ethylene causes many and diverse phenomena in plant growth and development. Plants, explants, and tissue organs respond to the gas they produce normally or are made to produce in response to environmental or chemical stimuli. They also respond to ethylene supplied exogenously. It is possible that ethylene affects a fundamental process and depending upon the kind and age of tissue the response evoked varies accordingly. In general, ethylene activates a potential that exists within the tissue. The potential is governed by the intricate programming of plant growth and development. This suggests that ethylene may affect the process of transcription and the response is observed as the message is eventually translated into enzyme activities which cause the physiological and morphological changes.

The complexity of tissue organs handicaps investigations on the mechanism of ethylene action. Several systems were investigated. A search made to find a simple tissue, reported to respond to ethylene, resulted in studies with pollen germination and tube growth. An hypothesis was formulated

that ethylene would enhance germination and tube growth and that CO_2 would competitively inhibit its action. The hypothesis was not verified but a fundamental discovery on the role of CO_2 in pollen germination and tube growth was made.

Reports of inhibition of cell division by ethylene led to studies on DNA polymerase. An hypothesis was formulated that ethylene inhibits the synthesis or action of DNA polymerase in pea stem. This was, in part, substantiated since ethylene applied *in vivo* to etiolated peas reduced the activity of DNA polymerase to the same extent (nearly 90%) that cell division was inhibited. However, ethylene did not affect DNA polymerase *in vitro*.

These studies led to investigations on DNA and RNA polymerase in mitochondria from quiescent potato tubers since ethylene causes a marked stimulation of respiration in this tissue. An hypothesis was formulated that the respiratory increase in potato in response to ethylene was due to inhibition of DNA synthesis and stimulation of RNA and protein synthesis. Increased activity of RNA polymerase and cytochrome c oxidase were found to precede the respiratory increase but DNA polymerase activity was increased rather than decreased as postulated.

The importance of ethylene in causing fundamental changes in DNA and RNA metabolism which in turn could markedly alter subsequent changes in enzyme activities and resultant physiological responses led to studies of the control of

autocatalytic ethylene production. An hypothesis was formulated that ethylene production in tissues was closely regulated perhaps via feed back control. And loss of this control would lead to autocatalytic production with irreversible consequence such as fruit ripening and senescence. Induction of autocatalytic ethylene production was investigated by employing propylene, a biologically active homolog of ethylene, which allowed for the measurement of endogenous ethylene during maturation of apple fruit. Capacity for autocatalysis was found markedly in advance of the ability of fruits to ripen and that a substance produced by the tree was responsible to delay the onset of ethylene production in fruits until they have gained their potential to ripen.

LITERATURE CITED

1. Abeles, F. B. and R. E. Holm. 1966. Enhancement of RNA synthesis, protein synthesis, and abscission by ethylene. Plant Physiol. 41:1332-1342.

2. Abeles, F. B. and R. E. Holm. 1967. Abscission: The role of protein synthesis. Proc. N. Y. Acad. Sci. 144:367-373.

3. Abeles, F. A. 1967. Inhibition of flowering in Xanthium pensylvanicum Walln. by ethylene. Plant Physiol. 42:608-609.

4. Abeles, F. B. and H. E. Gahagan. 1968. Abscission: The role of ethylene, ethylene analogues, carbon dioxide and oxygen. Plant Physiol. 42:1255-1258.

5. Abeles, F. B. and J. Lonski. 1969. Stimulation of lettuce seed germination by ethylene. Plant Physiol. 44:277-280.

6. Abeles, F. B. 1969. Abscission: Role of cellulase. Plant Physiol. 44:447-452.

7. Abeles, F. B. and G. R. Leather. 1971. Abscission: Control of cellulase activity by ethylene. Planta 97:87-91.

8. Abeles, F. B. 1972. Biosynthesis and mechanism of action of ethylene. Ann. Rev. Plant Physiol. 23:259-292.

9. Apelbaum, A. and S. P. Burg. 1972. Effect of ethylene on cell division and deoxyribonucleic acid synthesis in Pisum sativum. Plant Physiol. 50:117-124.

10. Biale, J. B. 1950. Postharvest physiology and biochemistry of fruits. Ann. Rev. Plant Physiol. 1:183-206.

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

12. Brady, C. J., J. K. Palmer, P. B. H. O'Connell, and R. M. Smilie. 1970. An increase in protein synthesis during ripening of the banana fruit. Phytochemistry 9:1037-1048.

13. 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. Austr. J. Biol. Sci. 23:1143-1152.

14. Burg, S. P. 1962. The physiology of ethylene formation. Ann. Rev. Plant Physiol. 13:265-302.

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

16. Burg, S. P. and E. A. Burg. 1965. Ethylene action and the ripening of fruits. Science 148:1190-1196.

17. Burg, S. P. and E. A. Burg. 1966. The interaction between auxin and ethylene and its role in plant growth. Proc. Nat. Acad. Sci. 55:262-269.

18. Burg, S. P. and E. A. Burg. 1966. Auxin-induced ethylene formation: its relation to flowering in the pineapple. Science 152:1269.

19. Burg, S. P. and E. A. Burg. 1967. Inhibition of polar auxin transport by ethylene. Plant Physiol. 42:1224-1228.

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

21. 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.

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

23. Burg, S. P. and A. Apelbaum. 1971. Physiology and mode of action of ethylene. HortScience 6:359-364.

24. Byers, R. E., L. R. Baker, H. M. Sell, R. C. Herner and D. R. Dilley. 1972. Ethylene: A natural regulator of sex expression of Cucumis melo L. Proc. Nat. Acad. Sci. 69:717-720.

25. Chadwick, A. V. and S. P. Burg. 1967. An explanation of the inhibition of root growth caused by β-indole-3-acetic acid. Plant Physiol. 42:415-420.

26. Chadwick, A. V. and S. P. Burg. 1970. Regulation of root growth by auxin-ethylene interaction. Plant Physiol. 43:192-200.

27. Craker, L. E. and F. B. Abeles. 1969. Abscission: Quantitative measurement with a recording abscissor. Plant Physiol. 44:1139-1143.

28. Crocker, W. and P. W. Zimmerman. 1932. Ethylene induced epinasty and the relationship of gravity to it. Contr. Boyce Thompson Inst. 4:177-218.

29. Crocker, W., A. E. Hitchock and P. W. Zimmerman. 1935. Similarities in the effects of ethylene and the plant auxins. Contr. Boyce Thompson Inst. 7:231-248.

30. Doubt, S. L. 1967. The response of plants to illuminating gas. Botan. Gaz. 63:209-224.

31. Eaglis, D. T. and C. D. Zannis. 1930. The effect of ethylene upon the activity of diastase and invertase. J. Am. Chem. Soc. 52:979-802.

32. Eaglis, D. T. and F. A. Dykins. 1931. The effect of ethylene upon the hydrolysis of salicin by emulsin. J. Am. Chem. Soc. 53:723-726.

33. Engelsma, G. and J. M. H. VanBruggen. 1971. Ethylene production and ethylene induction in excised plant tissues. Plant Physiol. 48:94-96.

34. Esashi, Y. and A. C. Leopold. 1969. Dormancy regulation in subterranean clover seeds by ethylene. Plant Physiol. 44:1440-1472.

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

36. Goeschl, J. D., L. Rappaport and H. K. Pratt. 1966. Ethylene as a factor regulating the growth of pea epicotyl subjected to physical stress. Plant Physiol. 41:877-884.

37. Goeschl, J. D. and H. K. Pratt. 1968. Regulatory roles of ethylene in the etiolated growth habit of Pisum sativum. In Biochemistry and physiology of plant growth substances (Wightman F. and G. Setterfield, eds.), pp. 1229-1242 (Runge Press, Ottawa).

38. Hager, E. S. 1927. A preliminary report on the stimulation of growth of bulbs and seeds with ethylene. Proc. Am. Soc. Hort. Sci. 23:201-203.

39. Hall, W. C. and P. W. Morgan. 1964. Auxin-ethylene interrelationships. In Regulateurs Naturels de la Croissance Végétale (Centre National de la Recherche Scientifique ed.), pp. 727-745 (Gif s/Yuette, Paris).

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

41. Hansen, E. 1966. Post harvest physiology of fruits. Ann. Rev. Plant Physiol. 17:459-480.

42. Herrero, F. and W. C. Hall. 1960. General effects of ethylene on enzyme systems in cotton leaf. Physiol. Plant. 13:736-750.

43. Hulme, A. C., F. Kidd and C. West. 1939. The effect of carbon dioxide on the rise in the protein N of the apple fruit during the climacteric. Gr. Brit. Dept. Sci. Ind. Res. Food Invest. Bd. Rept. 1939:51.

44. Holm, R. E. and F. B. Abeles. 1967. Abscission: The role of RNA synthesis. Plant Physiol. 42:1094-1102.

45. Holm, R. E. and F. B. Abeles. 1968. The role of ethylene in 2,4-D-induced growth inhibition. Planta 78:293-304.

46. Hulme, A. C. 1948. Studies in the nitrogen metabolism of the apple fruit during the normal and ethylene induced climacteric rise in respiration. Biochem. J. 43:343-349.

47. Hulme, A. C. 1954. Studies in the nitrogen metabolism of apple fruits. The climacteric rise in respiration in relation to changes in the equilibrium between protein synthesis and breakdown. J. Exp. Bot. 5: 159-172.

48. Hulme, A. C. 1958. Some aspects of the biochemistry of apple and pear fruits. Advan. Food Res. 8:297-413.

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

50. Imaseki, H., M. Uchiyama and I. Uritani. 1968. Effect of ethylene on the inductive increase in metabolic activities in sliced sweet potato roots. Agr. Biol. Chem. 32:387-389.

51. Imaseki, H. 1970. Induction of peroxidase activity by ethylene in sweet potato. Plant Physiol. 46:170-172.

52. Imaseki, H. and C. J. Pjon. 1970. The effect of ethylene on auxin-induced growth of excised rice coleoptile segments. Plant and Cell Physiol. 11:827-829.

53. Jones, J. D., A. C. Hulme and L. S. Wooltorton. 1965. The respiratory climacteric in apple fruits. Biochemical changes occurring during the development of the climacteric in fruit detached from the tree. New Phytol. 64:158-959.

54. Kang, B. G., C. S. Yocum, S. P. Burg and P. M. Ray. 1967. Ethylene and carbon dioxide; mediation of hypocotyl hook opening response. Science 156:958-959.

55. Kang, B. G. and P. M. Ray. 1969. Effects of inhibitors of RNA and protein synthesis on bean hypocotyl hook opening and their implications regarding phytochrome action. Planta 87:217-226.

56. Kang, B. G. and P. M. Ray. 1969. Ethylene and carbon dioxide as mediators in the response of the bean hypocotyl hook to light and auxins. Planta 87: 206-216.

57. Ketring, D. L. and P. W. Morgan. 1970. I. Regulation of dormancy in Virginia-type peanut seeds. Plant Physiol. 45:268-273.

58. Kidd, F. and C. West. 1945. Respiratory Activity and duration of life of apples gathered at different stages of development and subsequently maintained at constant temperature. Plant Physiol. 20:467-504.

59. Krishnamoorthy, H. N. 1970. Promotion of rooting in mung bean hypocotyl cuttings with Ethrel, an ethylene releasing compound. Plant and Cell Physiol. 11: 979-982.

60. Ku, H. S., H. Suge, L. Rappaport and H. K. Pratt. 1969. Stimulation of rice coleoptile growth by ethylene. Planta 90:333-339.

61. Ku, H. S. and A. C. Leopold. 1970. Mitochondrial responses to ethylene and other hydrocarbons. Plant Physiol. 46:842-844.

62. Lewis, T. L. and D. Martin. 1965. Protein nitrogen content and phosphorylative activity of apple fruits during ripening and senescence. Austr. J. Biol. Sci. 18:1093-1101.

63. Looney, N. E. and M. E. Patterson. 1967. Chlorophyllase activity in apples and bananas during climacteric. Nature 214:1245-1246.

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

65. Mack, W. B. 1927. The action of ethylene in accelerating the blanching of celery. Plant Physiol. 2:103.

66. Maei, N. and R. Romani. 1971. Ethylene stimulated synthesis of ribosomes, ribonucleic acid, and protein in developing fig fruits. Plant Physiol. 48:806-808.

67. Mapson, W. and A. C. Hulme. 1970. The Biosynthesis, physiological effects, and mode of action of ethylene. In Progress in Phytochemistry, Vol. 2 (Reinhold, L. and Y. Liwschitz, eds.), pp. 343-384 (Interscience Publishers, London).

68. Mattoo, A. K. and V. V. Modi. 1969. Ethylene and ripening of mangoes. Plant Physiol. 44:308-310.

69. Maxie, E. C. and J. C. Crane. 1968. Effects of ethylene on growth and maturation of fig Ficus carica L. fruit. Proc. Amer. Soc. Hort. Sci. 92:255-273.

70. McCready, R. M. and E. A. McComb. 1954. Pectic constituents in ripe and unripe fruit. Food Res. 19:530-535.

71. McMurray, A. L. and C. H. Miller. 1968. Cucumber sex expression modified by 2-chloroethane phosphonic acid. Science 162:1397-1398.

72. Mehard, C. W. and J. M. Lyons. 1970. A lack of specificity for ethylene-induced mitochondrial changes. Plant Physiol. 46:36-39.

73. Regeimbal, L. O. and R. B. Harvey. 1927. The effect of ethylene on the enzymes of pineapples. J. Am. Chem. Soc. 49:1117-1118.

74. Rhodes, M. J. C., L. S. C. Wooltorton, T. Galliard and A. C. Hulme. 1968. Metabolic changes in excised fruit tissue. I. Factors affecting the development of a malate decarboxylation system during the ageing of disks of pre-climacteric apples. Phytochemistry 7:1439-1451.

75. Ridge, I. and D. J. Osborne. 1970. Regulation of peroxidase activity by ethylene in Pisum sativum: requirements for protein and RNA synthesis. J. Exp. Bot. 21:720-734.

76. Ridge, I. and D. J. Osborne. 1970. Hydroxyproline and peroxidases in cell walls of Pisum sativum: regulation by ethylene. J. Exp. Bot. 21:843-856.

77. Ridge, I. and D. J. Osborne. 1970. Cell growth and cellulases: regulation by ethylene and indol-3-acetic acid in shoots of Pisum sativum. Nature 229: 205-208.

78. Riov, J., S. P. Monselise and R. S. Kahan. 1969. Ethylene-controlled induction of phenylalanine ammonia-lyase in citrus fruit peel. Plant Physiol. 44:631-635.

79. Olson, A. O. and M. Spenser. 1968. Studies on the mechanism of action of ethylene. I. The effects of ethylene on mitochondria prepared from bean cotyledons. Can. J. Biochem. 46:277-282.

80. Olson, A. O. and M. Spenser. 1968. Studies on the mechanism of action of ethylene. II. Effects of ethylene on mitochondria from rat liver and yeast, and on mitochondrial adenosine triphosphatase. Can. J. Biochem. 46:283-288.

81. Osborne, D. J. 1968. Defoliation and defoliants. Nature 219:564-567.

82. Pratt, H. D. and J. D. Goeschl. 1969. Physiological roles of ethylene in plants. Ann. Rev. Plant Physiol. 20:541-584.

83. Sacher, J. A. and S. O. Salminen. 1969. Comparative studies of effect of auxin and ethylene on permeability and synthesis of RNA and protein. Plant Physiol. 44:1371-1377.

84. Shannon, L. M., I. Uritani and H. Imaseki. 1971. De novo synthesis of peroxidase isozymes in sweet potato slices. Plant Physiol. 47:493-498.

85. Shaw, F. H. 1935. The mechanism of the action of ethylene on cell processes. Austr. J. Exp. Biol. 13:95-102.

86. Smith, W. H. and J. C. Parker. 1966. Prevention of ethylene injury to carnations by low concentrations of carbon dioxide. Nature 211:100-101.

87. Stahmann, M. A., B. G. Clare and W. Woodbury. 1966. Increased disease resistance and enzyme activity induced by ethylene and ethylene production of black rot infected sweet potato tissue. Plant Physiol. 41:1505-1512.

88. Stewart, E. R. and H. T. Freebairn. 1969. Ethylene, seed germination and epinasty. Plant Physiol. 44:955-958.

89. Suge, H. 1971. Stimulation of oat and rice mesocotyl growth by ethylene. Plant and Cell Physiol. 12: 831-837.

90. Toole, V. K., W. K. Bailey and E. A. Toole. 1964. Factors influencing dormancy of peanut seeds. Plant Physiol. 39:822-832.

91. Turkova, N. S., L. M. Vasil'eva and L. F. Cheremukhina. 1965. On the physiology of bending of leaves and stems. Sov. Plant Physiol. 12:721-726.

92. Vacha, G. A. and R. B. Harvey. 1927. The use of ethylene, propylene, and similar compouds in breaking the rest period of tubers, bulbs, cuttings, and seeds. Plant Physiol. 2:187-194.

93. Valdovinos, J. G., L. C. Ernest and E. W. Henry. 1967. Effect of ethylene and gibberellic acid on auxin synthesis in plant tissues. Plant Physiol. 42: 1803-1806.

94. Yamaguchi, S. 1954. Some interrelations of oxygen, carbon dioxide, sucrose and ethylene in abscission. Ph.D. Dissert., University of California, Los Angeles, California.

95. Zimmerman, P. W., A. E. Hitchcock and W. Crocker. 1931. The effect of ethylene and illuminating gas on roses. Contrib. Boyce Thompson Inst. 3:459-481.

96. Zimmerman, P. W. and F. Wilcoxon. 1935. Several growth substances which cause initiation of roots and other responses in plants. Contrib. Boyce Thompson Inst. 7:209-222.

SECTION I

POLLEN GERMINATION AND TUBE GROWTH: DEPENDENT ON CARBON DIOXIDE AND INDEPENDENT OF ETHYLENE

Abstract. The influence of ethylene and CO_2 on pollen germination and tube growth was investigated employing ventilated culture systems. Ethylene had no effect on pollen germinability or tube growth. Germinating pollen did not produce a detectable amount of ethylene (less than 0.1 nl/g/hr). Supplementing the cultures with CO_2 caused a marked increase in germination and tube growth. The half-maximal response for germination was less than 0.5%. CO_2 levels ranging from 1.08 to 2.22% were found in the internal cavity of lily styles. CO_2 derived from stylar metabolism may, therefore, modulate pollen tube growth thus integrating the events leading to fertilization.

INTRODUCTION

Recent reports that ethylene increases pollen germination and tube growth (1,8) prompted us to examine pollen as a simple biological system for studies on ethylene action. Pollen is anatomically simple compared to other highly differentiated tissues and plant organs generally employed. Furthermore, appropriate germination media and culture systems have been exhaustively investigated. Conventionally, hanging drop or other closed environment culture systems are employed. Germinating pollen has a high respiration rate (4) which causes CO_2 to accumulate in closed cultures, and fixation (9) would then be an uncontrolled variable. More importantly, CO_2 is a competitive inhibitor of ethylene (2) whose action was the point of investigation. Nakanishii *et al*. (6) have shown that CO_2 increases the number of pollen tubes penetrating the stigma papilla cells in self-incompatible *Brassica* while ethylene has no effect. Because of these interrelated effects of the production and action of CO_2 and ethylene, the conventional closed or poorly ventilated pollen germination systems were inappropriate for this study and continuously ventilated systems were developed.

MATERIALS AND METHODS

Pollen used in these experiments was collected from freshly opened flowers of greenhouse lily, Lilium longiflorum Thunb, cv Ace, except where otherwise specified (Table III, Fig. 4). Each morning freshly dehiscing anthers were harvested and placed on weighing paper in front of a fan until fully dehisced. The pollen was then scraped from the anthers and mixed prior to storage or use. The medium used was that of Dickinson (4) modified by use of MES buffer as described below. All glassware was washed in dichromate cleaning solution and thoroughly rinsed in distilled water prior to use. Hanging drop cultures were prepared by placing a 10 µl drop of medium on a microscope cover slip; with the use of a dissecting microscope and two sharpened toothpicks approximately one hundred pollen grains were added. All inoculations and experiments were carried out at 27 C in a room where the relative humidity was maintained above 80%. Pollen was always equilibrated in the assay room before inoculation. A thin film of vaseline was used to seal cover slips to a brass cylinder fitted with gas inlet and exhaust ports as illustrated in Figure 1. All gases were humidified by bubbling through water, and the filter paper in each brass cylinder was moistened with 0.2 ml of distilled water to ensure

Figure 1. Hanging drop culture chamber.

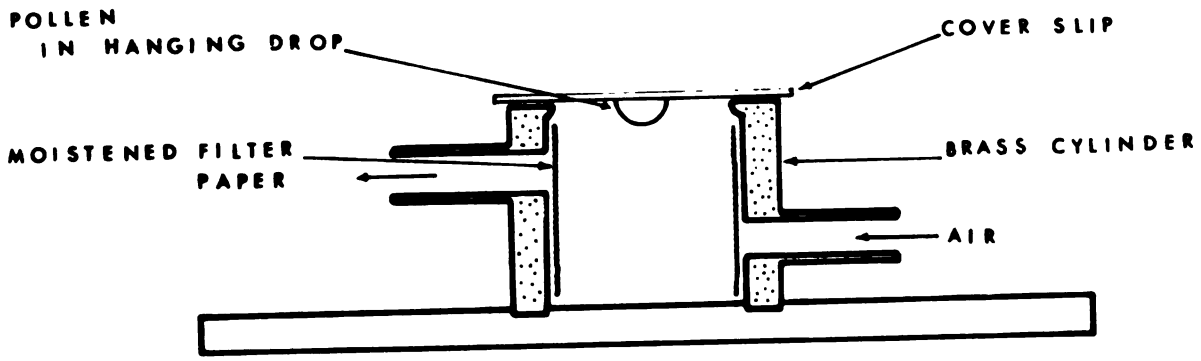
POLLEN
IN HANGING DROP
COVER SLIP
MOISTENED FILTER
PAPER
BRASS CYLINDER
AIR

Figure 1

saturation of the atmosphere surrounding the hanging drop. With gas flows as high as 300 ml/min through these chambers for up to 24 hr no decrease in diameter of the hanging drop could be detected. A metered flow of various measured, constant ethylene and CO_2 mixtures (in cylinders) was admitted to the cultures and the concentration of each gas was monitored at the exhaust from each chamber by gas chromatography. Ethylene was measured by a Varian Aerograph model 1700 equipped with an activated alumiua column and a flame ionization detector. CO_2 and O_2 were measured by gas chromatograph employing silica gel and molecular sieve in parallel and a katharometer. After the appropriate germination period, growth was stopped by rapid freezing at -10 C. Percent germination was measured by examining and counting the grains in the drop under a dissecting microscope. Tube length measurements were made from projections of photographs of the drop.

RESULTS AND DISCUSSION

Buffering of the Medium. Because varying CO_2 concentrations were used, it was necessary to buffer the medium to eliminate any possible effect of CO_2 on pH. Several buffering systems were tested for their effects on percent germination and maintenance of pH in submerged shake cultures with continuous flow of air or 5% CO_2 in air through the cultures. Tulip, Tulipa gesneriara, cv Madam Spoor and Gander, pollen was used for these experiments. High germination was observed in unbuffered cultures at pH 5.0 so this pH was selected. Cultures with 0.01 M MES (pK 6.15) maintained pH 5.0 and had the same germination as unbuffered controls. Buffering at higher pH decreased the germination slightly. High germinability of lily pollen was observed in unbuffered media at pH 5.4 so MES at this pH was used for the CO_2 studies.

Carbon Dioxide effect. The germination of lily pollen in hanging drop cultures increased rapidly when the CO_2 concentration was increased from 0.03% (in air) to 1.3%, with very little further effect at concentrations up to 5% (Fig. 2A). The half-maximal CO_2 concentration was 0.25% to 0.5% as measured by double reciprocal plots (Fig. 2A and 2B). Similar results were obtained with submerged shake cultures where CO_2 concentrations up to 8.4% were used (Fig. 2B).

Figure 2. Effect of CO_2 concentration on percent germination of lily pollen (bar indicates Tukey's ω-test at $p = 0.05$).

A. Ventilated hanging drop culture for 2 hr.

B. Submerged shake culture for 2.5 hr.

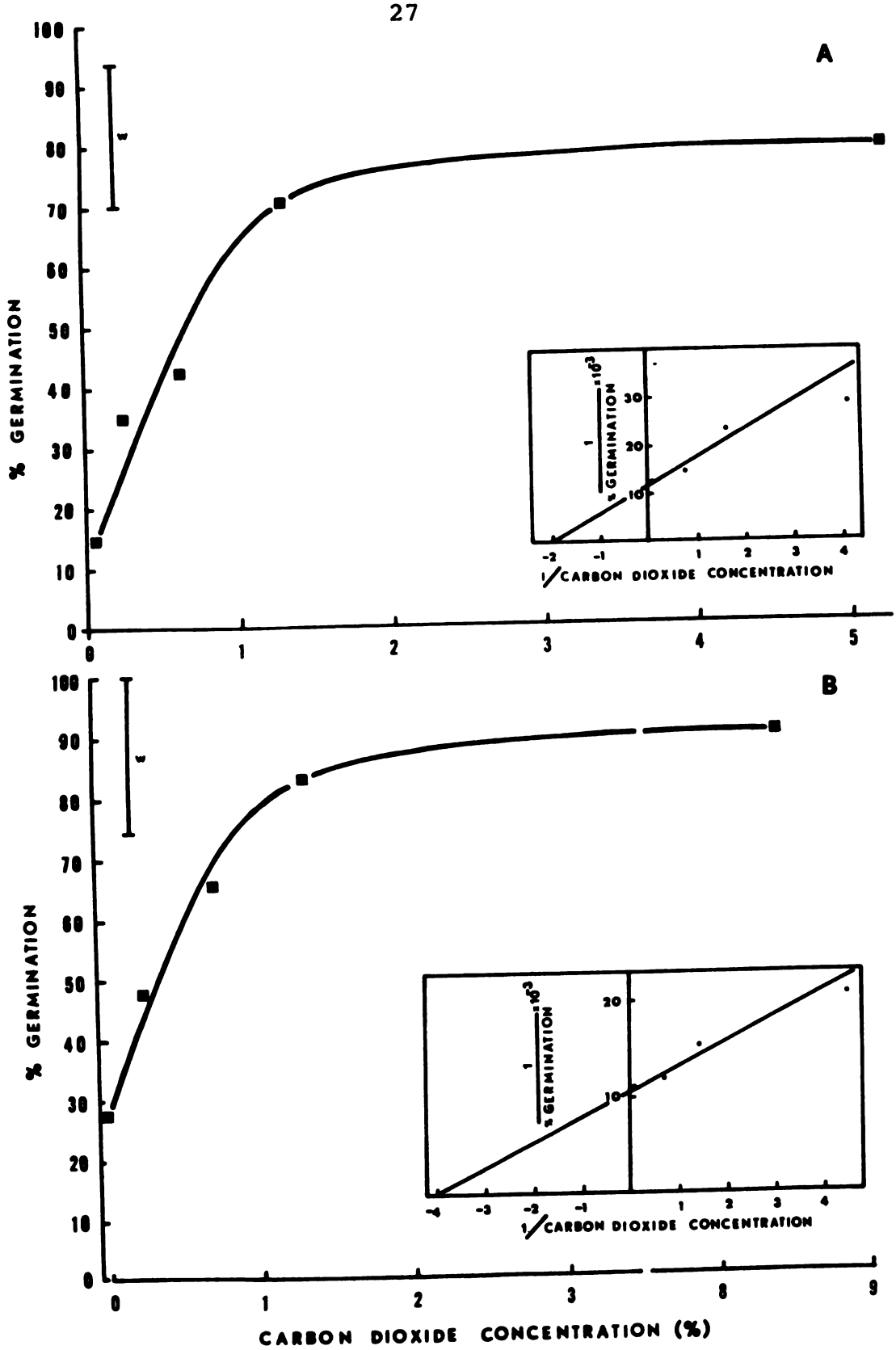
A
B
% GERMINATION
CARBON DIOXIDE CONCENTRATION (%)
1/CARBON DIOXIDE CONCENTRATION
1/% GERMINATION x10³

Figure 2

The internal CO_2 concentration in the stylar cavity of the lily flower was found to be 1.59% (Table I) which is very close to the optimum concentration found for best germination in vitro.

Ethylene Production. The ethylene content in the air stream leaving the hanging drop cultures was the same as the inlet concentration even when the number of pollen grains was increased and the flow rate decreased to a level that respiratory CO_2 accumulated. The minimum ethylene concentration attributable to pollen was observed in closed shake cultures after 3 1/2 hrs, during which 79% germination occurred. These cultures contained 5 mg of pollen in one ml of medium per 25 ml flask. The ethylene concentration in 10 flasks with and without pollen was 19 ppb $\pm$ 3 and 19 ppb $\pm$ 2, respectively.

Response to Applied Ethylene. When air or air containing 10 ppm ethylene was continuously supplied to lily pollen in hanging drop cultures very poor and erratic germination was obtained with no difference between the treatments (Table II). Similarly ethylene had no effect on germination in 5% CO_2 where high germination was obtained. These results contradict those of Search and Stanley (8) and Buchanan and Biggs (1). In an attempt to resolve this paradox we used peach (Prunus persica cv Elberta) and pear (Pyrus communis cv Packham), pollen obtained from a commercial source and stored dry at -10 C. The medium and culture system were those previously

Table I. Concentrations of CO_2, O_2 and ethylene in the stylar cavity of lily flowers at the time of anthesis. Samples (0.5 to 1 ml) were withdrawn from the cavity by a syringe fitted with a hypodermic needle. The flowers were submerged in water while the samples were taken to prevent entrance of outside air.

Sample	Carbon dioxide %	Oxygen %	Ethylene ppm
1	1.46	17.59	0.045
2	2.22	15.36	--
3	1.08	18.60	--
Mean	1.59	17.18	

Table II. Percent germination of lily pollen treated with 10 ppm ethylene and 5% CO_2 in the ventilated hanging drop culture for 2 hr. The data are means of 17 observations.

Treatment	Percent germination*	Range	Standard Deviation
Air	8.2a	0.6 - 28.2	± 8.2
Air + 10 ppm ethylene	10.6a	0.6 - 47.8	±11.5
Air + 5% CO_2	72.2b	44.2 - 91.8	±13.7
Air + ethylene + CO_2	70.9b	17.7 - 94.6	±19.2

*Means followed by different letters differ significantly at P = 0.01, by Tukey's ω-test.

reported (1) except that our air controls were sealed in polyethylene bags. The bags containing the cultures had air inlets and exhausts and were aerated for 5 mins before closing and injecting ethylene into some of them to yield a concentration of 1 ppm. Ethylene did not alter the percent germination (Table III). The increased germination observed by Buchanan and Biggs (1) may have occurred in response to CO_2 produced by the pollen in the sealed plastic bags used for the ethylene treatments. Ethylene was reported (7) to increase pollen germination only in the absence of boron in the medium. Using peach pollen in hanging drops continuously ventilated with 5% CO_2 containing 10 ppm ethylene, we found no effect of 10 ppm boron on percent germination.

Evidence that ethylene did not affect the rate of growth of lily pollen tubes was obtained from tube length distributions in hanging drop cultures (Fig. 3). Adding ethylene did not significantly alter the distribution pattern in air or in 5% CO_2. However, CO_2 with or without ethylene increased pollen tube length. The influence of ethylene on rate of pollen tube growth was checked by measuring single pollen tubes in hanging drop culture on a microscope stage. The incubation chamber was continuously flushed with the appropriate gas mixture which could be changed at will. Adding or removing ethylene did not alter the growth rate of pear pollen tubes (Fig. 4). The slight shifts in the growth curve are due to curvature which introduces an error into the length measurement.

Table III. The influence of 1 ppm ethylene on percent germination of peach and pear pollen cultured on agar and sealed in plastic bags for 5 hr.

Experiment	Peach		Pear	
	Air	C_2H_4	Air	C_2H_4
1.	15.1	19.8	62.5	44.7
2.	21.4	27.4	48.7	65.1
3.	26.4	26.7	52.9	65.2
4.	27.1	27.2	30.2	60.6
5.	25.7	16.1	64.4	61.8
6.	24.8	21.1	52.0	56.7
7.	19.4	20.2	60.4	47.9
8.	12.4	17.1	60.6	59.0
Mean	21.5	22.0	54.0	57.6
S.D.	± 5.5	± 6.5	±11.1	± 7.6

Figure 3. Effect of 10 ppm ethylene and 5% CO_2 on the distribution of lily pollen tube length after 2 hr.

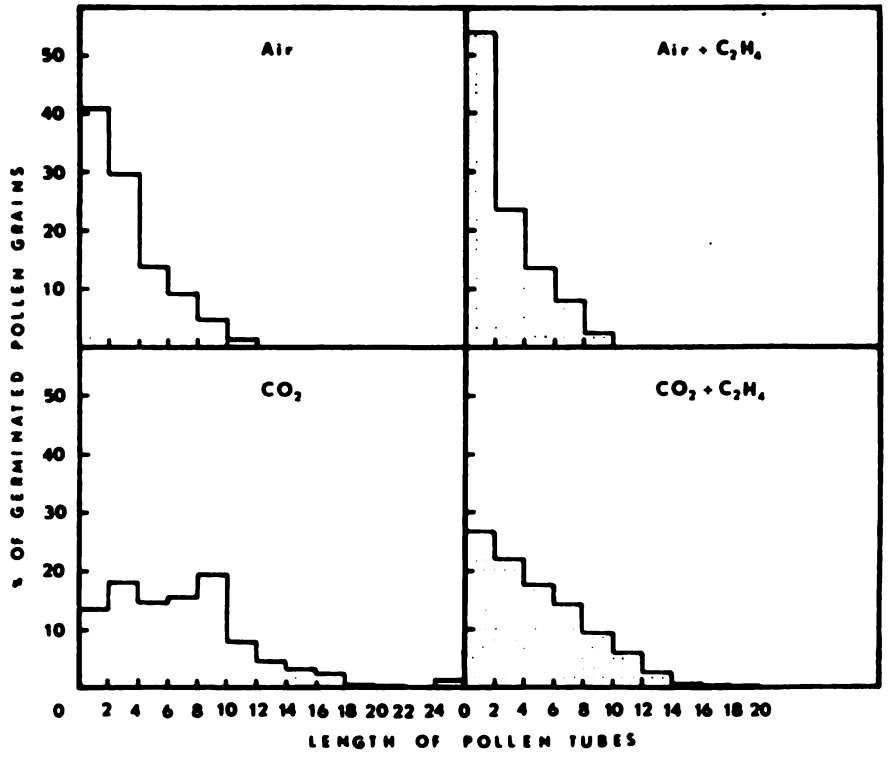
Air
Air + C_2H_4
CO_2
CO_2 + C_2H_4
% OF GERMINATED POLLEN GRAINS
LENGTH OF POLLEN TUBES
0 2 4 6 8 10 12 14 16 18 20 22 24
10 20 30 40 50

Figure 3

Figure 4. Growth of a single pear pollen tube in a hanging drop culture continuously ventilated with 5% CO_2.
↑ indicates a change to a mixture of 5% CO_2 containing 700 ppm ethylene. ↓ indicates return to 5% CO_2 without ethylene.

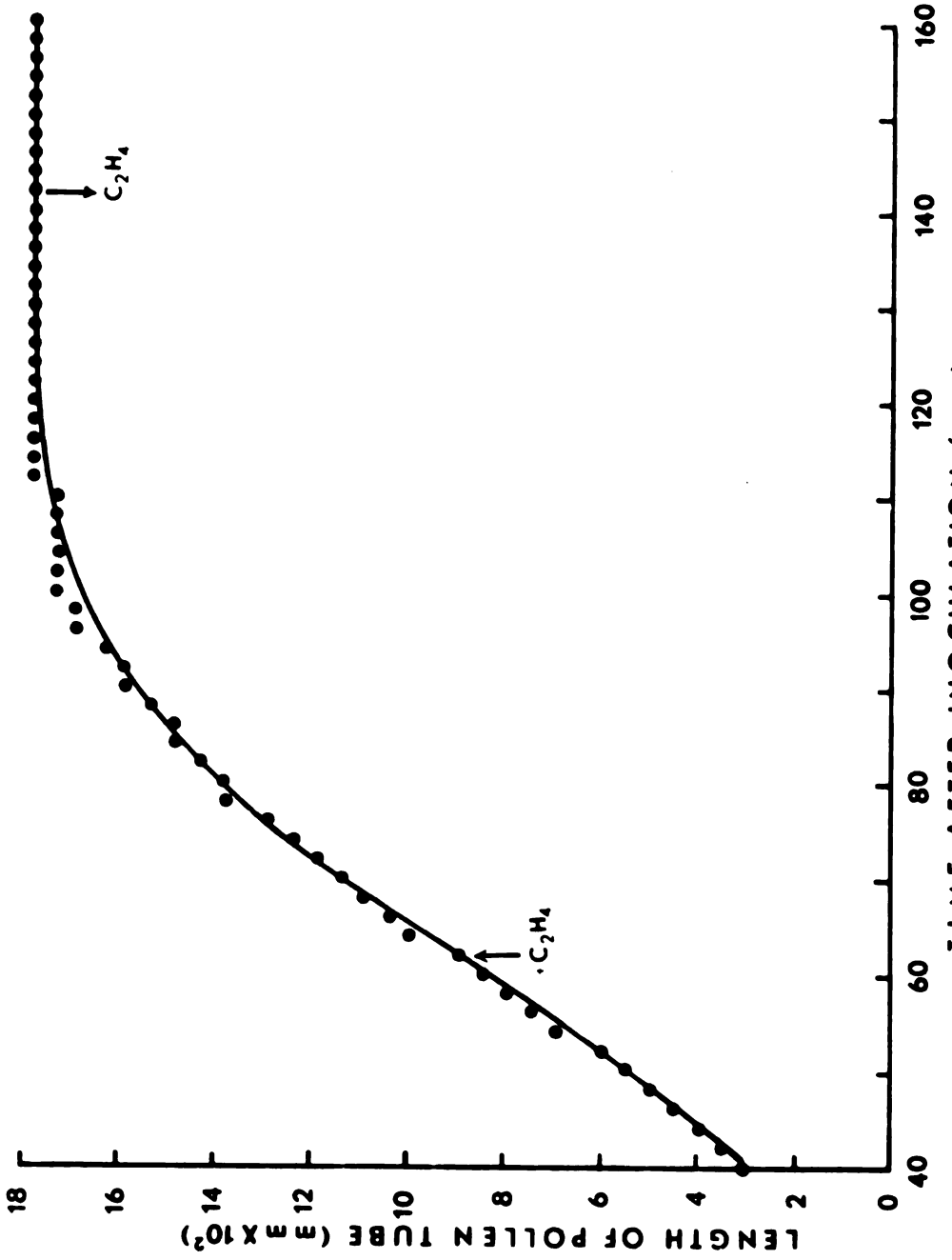
LENGTH OF POLLEN TUBE (mm x 10²)
TIME AFTER INOCULATION (min.)
C_2H_4
C_2H_4
0
2
4
6
8
10
12
14
16
18
40
60
80
100
120
140
160

Figure 4

The extreme variability of all pollen material used was a constant frustration throughout these studies. The range in percent germination within replicates is seen in Table II. Similarly there was extreme variation in pollen tube length both between and within individual replicates whether employing hanging drop, submerged, or shake culture. Tube lengths ranged from just emerging to 20 or more times the grain diameters. Where the growth of two neighboring pollen tubes was measured under the microscope very different rates were obtained. The same variability was observed in hanging drop cultures of lily pollen when undiluted stigmatic fluid was used as the medium. It is doubtful that the artificial growth medium used is the source of the variation. Perhaps the method of collecting the pollen results in a sample of varying maturity. Further experimentation is needed to clarify this point.

The apparent inability of pollen to synthesize ethylene and its insensitivity to exogenous ethylene are unique. Pollen is a rich source of auxin which induces ethylene synthesis in stylar tissue of orchids, and eventually the other floral parts, causing them to senesce (3). Auxin stimulation of ethylene synthesis in vegetative tissue and fruits is a general phenomenon resulting in many diverse morphological and physiological changes. The marked stimulatory effect of CO_2 on pollen germination and tube growth has not been generally recognized. External application of CO_2 apparently enhanced

pollen tube growth in Brassica (6). Participation of endogenous CO_2 in this process is likely because stimulatory levels were found within the stylar cavity of lily (Table I). CO_2 from stylar metabolism may in fact modulate pollen tube growth because the internal CO_2 concentration would reflect respiration rate changes. In addition, a declining concentration of O_2 observed (5) within the style implies a corresponding increase in CO_2 concentration. The biochemical role of CO_2 in germination and tube growth has not been ascertained. CO_2 fixation which is known to occur in pollen (9) may regulate the supply of oxaloacetic acid and hence affect the rate of metabolism.

The marked influence of CO_2 on pollen germination and tube growth may explain the well known "population effect" wherein germination increases as the pollen population increases due to CO_2 accumulation from respiration.

LITERATURE CITED

1. Buchanan, D. W. and R. H. Biggs. 1969. Peach fruit abscission and pollen germination as influenced by ethylene and 2-chloroethane phosphonic acid. J. Amer. Soc. Hort. Sci. 94:327-329.

2. Burg, S. P. and Ellen A. Burg. 1965. Ethylene action and the ripening of fruits. Science 148:1190-1196.

3. Burg, S. P. and M. T. Dijkman. 1967. Ethylene and Auxin participation in pollen induced fading of Vanda blossoms. Plant Physiol. 42:415-420.

4. Dickinson, D. B. 1965. Germination of lily pollen: Respiration and tube growth. Science 150:1818-1819.

5. Linkskens, H. F. and J. Schrauwen. 1967. Measurement of oxygen tension changes in the style during pollen tube growth. Planta 71:98-105.

6. Nakanishii, T., Y. Esashi and K. Hinata. 1969. Control of self-incompatability by CO_2 gas in Brassica. Plant Cell Physiol. 10:925-927.

7. Sauls, J. W. and R. H. Biggs. 1970. Ethrel, boron, sucrose and cellulase effects on peach pollen germination. HortScience 5:341.

8. Search, R. W. and R. G. Stanley. 1968. The effect of ethylene on pollen tube elongation. Plant Physiol. 43:S-52.

9. Stanley, R. G., L. C. T. Young and J. S. D. Graham. 1958. Carbon dioxide fixation in germinating pine pollen (Pinus ponderosa). Nature 182:1462-1463.

SECTION II

INHIBITION BY ETHYLENE OF SOLUBLE DNA POLYMERASE ACTIVITY IN PISUM SATIVUM SEEDLINGS

Abstract. An extract from the apical portion of etiolated seedlings of Pisum sativum L. was used as a test system to examine the action of ethylene on DNA polymerase activity. The extract was capable of catalyzing the polymerization of labeled deoxyribonucleoside triphosphates into a trichloroacetic acid-insoluble product. The system required Mg^{++}, nicked DNA, and all four deoxyribonucleoside triphosphates for maximum activity. Inorganic pyrophosphate and DNAase inhibited the polymerase activity. Extracts from plants previously treated with ethylene showed less activity to synthesize DNA than extracts from untreated plants. Ethylene in vitro showed no activity. Inhibition of cell division by ethylene observed in this and other tissues may be the result of impaired synthesis of DNA polymerase.

Recent studies on the effects of ethylene on growth of etiolated pea seedlings have shown (1) that the gas suppresses growth of the root and shoot apices, lateral branching, and leaf expansion predominantly by inhibiting cell division. Furthermore, ethylene inhibits DNA synthesis in the plumular

hook and subapical region of etiolated pea seedlings (1). Growth inhibition was therefore attributed to inhibition of DNA synthesis by ethylene. DNA polymerase activity is required for DNA synthesis and increases when cells approach the S phase and begin replication (5,6). Nucleic acid polymerases have been isolated from plant tissues (20,22,23) and some plant growth hormones affect their activity (2,11, 14,15). The purpose of this study was to determine whether ethylene controls DNA synthesis by altering DNA polymerase activity.

Seeds of Pisum sativum (cv Alaska) were soaked for 5 hours in tap water, planted in moist vermiculite and grown in complete darkness at 23°C. Seven days after planting, seedlings were treated with 50 µℓ/ℓ ethylene in air. Approximately 500 plants were utilized in each experiment. Plants were placed in an air-tight chamber into which a gas mixture with the desired ethylene concentration was introduced at a flow rate of 470 ml/min. All manipulations were carried out under dim green light, otherwise the seedlings were kept in total darkness. At the end of 24-hour treatment, seedlings were transferred to 2°C, and the apical portions, including the plumule and plumular hook, were excised. The harvested tissue was weighed and kept at 2°C for preparation of the tissue extract.

Five grams of chilled sections were sterilized with 5% sodium hypochlorite (diluted 1:100) and ground for 20 min in

a chilled mechanized mortar and pestle with 2 weights of grinding medium composed of 0.05M Trizma and 0.02M mercaptoethanol at pH 8.0. The suspension was filtered through 4 layers of cheesecloth plus 2 layers of miracloth and centrifuged for 5 min at 400g to remove residual cell wall debris. The supernatant solution was used for DNA polymerase assay.

Nicked DNA was prepared from calf thymus DNA (2.5 mg per ml in 0.01M tris buffer) incubated with pancreatic DNAase I (40 ng DNAase per mg DNA) and 5mM $MgCl_2$ at 37°C for 25 min. The reaction was stopped by heating for 10 min at 60°C. Double stranded DNA treated in this manner is rendered 25% acid soluble (21).

The incorporation of TMP (thymidine monophosphate) into acid insoluble material was used to measure DNA polymerase activity. Unless otherwise stated, 25 µl aliquots of tissue extract containing an appropriate amount of enzyme based on the protein content, were added to 225 µl reaction mixture containing in µmoles: Hepes buffer, pH 7.5, 20; KCl, 12.5; MgCl, 1; Cleland's reagent, 1; dATP, 0.05; dCTP, 0.05; dGTP, 0.05; TTP, 0.02; 6.2 µc ^{3}H-TTP (specific activity 15.5 c/m mole) and 100 µg nicked calf thymus DNA. The incubation was carried out at 37°C. The reaction was stopped by adding 2 ml of 10% cold TCA containing 1% PPi. Fifty microliters of BSA (bovine serum albumin) were added to the mixture and centrifuged for 10 min at 10,000g. The pellet was dissolved in 0.5 ml of 0.2N NaOH and 5 ml of 10% cold TCA + 1% PPi were added.

The acid insoluble pellet was collected on a Whatman FG/C glass filter paper and washed five times with 5% cold TCA and 1% PPi. The filters containing the acid insoluble precipitate were placed in scintillation vials containing 10 ml Bray's solution (3) and ^{3}H was determined with a Beckman scintillation spectrometer with a counting efficiency of 35% for ^{3}H. Incorporation of ^{3}H-TMP into acid insoluble material was obtained by subtracting zero time counts from those of experimental samples. Protein content in the tissue extracts was determined by the method of Lowry et al. (12) on cold TCA precipitated samples.

Soluble DNA polymerase activity as measured by the incorporation of TMP into acid insoluble product was dependent upon the various components of the reaction mixture (Table 1). Highest enzyme activity was obtained in the presence of Mg^{++}, nicked DNA and four deoxyribonucleoside triposphates (dNTP). Omission of Mg^{++} reduced the incorporation rate to 17% of that obtained with the complete mixture. Omission of dATP, dCTP, or dGTP reduced the incorporation to 20-23%, while omitting all three of them reduced the activity to only 10% of that of the complete reaction mixture. The incorporation of TMP into acid insoluble product was dependent upon the addition of DNA to the reaction mixture since only 3% incorporation was observed without added DNA.

Various templates were tested for their priming activity (Table 2). The most active for pea DNA polymerase was

Table 1. Dependency of DNA polymerase activity on components of the reaction mixture.

Reaction Mixture	% Incorporation
Complete	100
$-Mg^{++}$	17
-dATP	20
-dCTP	23
-dGTP	23
-dATP, dCTP, dGTP	11
-Nicked DNA	3

Soluble DNA polymerase was extracted from the apical portion of etiolated pea seedlings. 100% incorporation represents incorporation of 140 pmoles TMP per mg protein.

Table 2. Utilization of different templates by pea DNA polymerase.

DNA Template[1]	% Incorporation[3]
Nicked	100
Nicked denaturated	47
Native	36
Native denaturated	16
poly dT:rA[2]	0

[1]Calf thymus DNA.

[2]Polydeoxythymidylate and polyriboadenylate.

[3]100% incorporation represent incorporation of 140 pmoles TMP per mg protein.

"nicked" DNA, i.e., DNA in which 3' hydroxyl termini have been introduced by the action of pancreatic enzymes. The priming activity of nicked calf thymus DNA was found to be 2.5 fold higher than that of native calf thymus DNA. Heat denaturation of native or nicked DNA reduced the priming activity by about 50%, indicating that the enzyme requires some double stranded structure for optimal priming activity. Utilizing the synthetic template poly dT:rA, which is used for detection of RNA-directed DNA polymerase enzyme (21), resulted in no incorporation of TMP into acid insoluble product. This may indicate that RNA cannot meet the template requirement of pea DNA polymerase. Adding 1 µmole pyrophosphate, one of the reaction products, to the reaction mixture inhibited the incorporation of TMP by 86% (Table 3). Pancreatic DNAase degraded the newly formed DNA and reduced the amount of TMP recovered by 94%.

Gasing the incubation mixture with ethylene (1 ml/l in air) for 30 min prior to the incubation reduced the incorporation rate by only 3-5% (Table 3). In contrast, ethylene treatment of intact pea plants lowered DNA polymerase activity in the apical region to approximately 14% of the activity found in control plants (Fig. 1) at low levels of protein in the assay and to 40% when compared at their respective peak values. The rate of incorporation increased at a decreasing rate with increasing amounts of soluble protein added to the assay mixture, and reached a maximum of 3400 cpm at 100 µg

Table 3. Effect of *in vitro* ethylene treatment, DNAase and pyrophosphate on TMP incorporation into acid-insoluble product.

Treatment	% Incorporation[1]
Control	100
in vitro incubation with ethylene (1 ml/l)	97
in vitro 30 min pre-incubation with ethylene (1 ml/l)	95
pyrophosphate 1 µmole	14
DNAase 20 mg/assay	6

[1]100% incorporation = 4630 cpm/assay

Figure 1. The relationship between incorporation of ^{3}H-TMP and protein content in the assay. DNA polymerase was isolated from 7-day control -o- or 24 hr ethylene treated seedlings -●-.

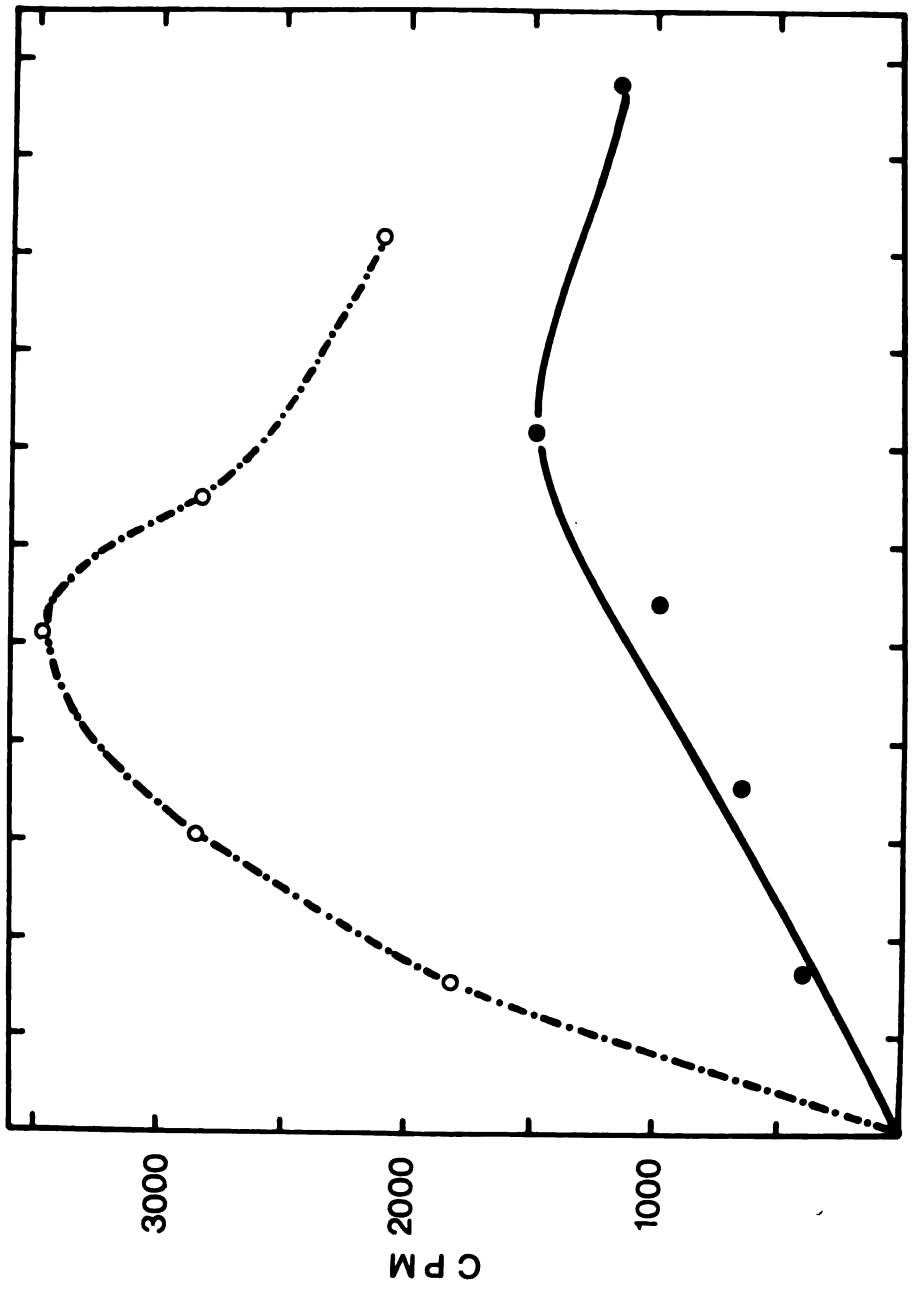

Figure 1

protein per assay in control tissue. Similar incremental additions of enzyme from ethylene treated plants yielded a maximum rate of only 1500 cpm at 140 µg protein in the assay mixture. Control tissue yielded a nonlinear rate of incorporation whereas a linear rate was observed for ethylene treated tissue until the maximum rate was reached. In non-treated tissue, incorporation of TMP was reduced at high protein levels, suggesting degradation of the newly formed DNA by endogenous DNAase. The degradation of DNA appeared to be more rapid in the control than in the ethylene treated tissue, since at 180 µg protein per assay 50% of the newly formed DNA may have been hydrolyzed. Similarly, only 17% of the DNA was degraded by the extract from ethylene treated tissue at a level of 218 µg protein per assay and perhaps less than that up to the maximum since the incorporation rate was linear. The amount of TMP recovered was apparently the net result of DNA polymerase and DNAase activity. Careful examination of Fig. 1 reveals that reduced DNA polymerase activity in ethylene-treated tissue may have been the result of reduced enzyme synthesis. Twenty µg extract protein from ethylene treated tissue was only 1/7 as active as that from the control tissue. At the 140 µg extract protein level (7 times as much) the activity of the ethylene treated tissue was equal to that of 20 µg of control tissue. This proportionality suggests that the enzyme quantity was limiting the incorporation rate in the ethylene treated tissue. Aliquots containing 100 µg

protein per assay were used for subsequent experiments and the results are expressed per mg protein.

The kinetics of the reaction is illustrated in Fig. 2. Incorporation increased with incubation time up to 15 min, reaching maxima of 140 and 90 pmole TMP incorporated for control and ethylene treated tissue, respectively. A 10 min. incubation period was employed for further experiments.

Incorporation of TMP increased sharply with increase in template concentration (Fig. 3) up to 2.5 (ethylene-treated) or 5 µg (control) DNA. Higher concentrations increased incorporation but at a much lower rate, reaching 109 and 70 pmoles TMP incorporated by extracts from control and ethylene treated tissue, respectively.

The data demonstrate activity of soluble DNA polymerase in crude extracts of etiolated pea seedlings. By utilizing the soluble enzyme in the crude extract rather than the chromatin associated DNA polymerase complex, the effect of ethylene on the enzyme activity, per se, was elucidated. This eliminates possible effects of the endogenous template on the reaction, since its activity or availability might be affected by hormones as well (8,9,10). Table 1 shows that very little endogenous template was available in the crude extract where only 3% incorporation of TMP was obtained without exogenously added template.

The almost complete destruction of the acid insoluble product by pancreatic DNAase indicates that the product is a

Figure 2. Time course of TMP incorporation into acid-insoluble product in control -o- or 24 hr ethylene treated seedlings -●-.

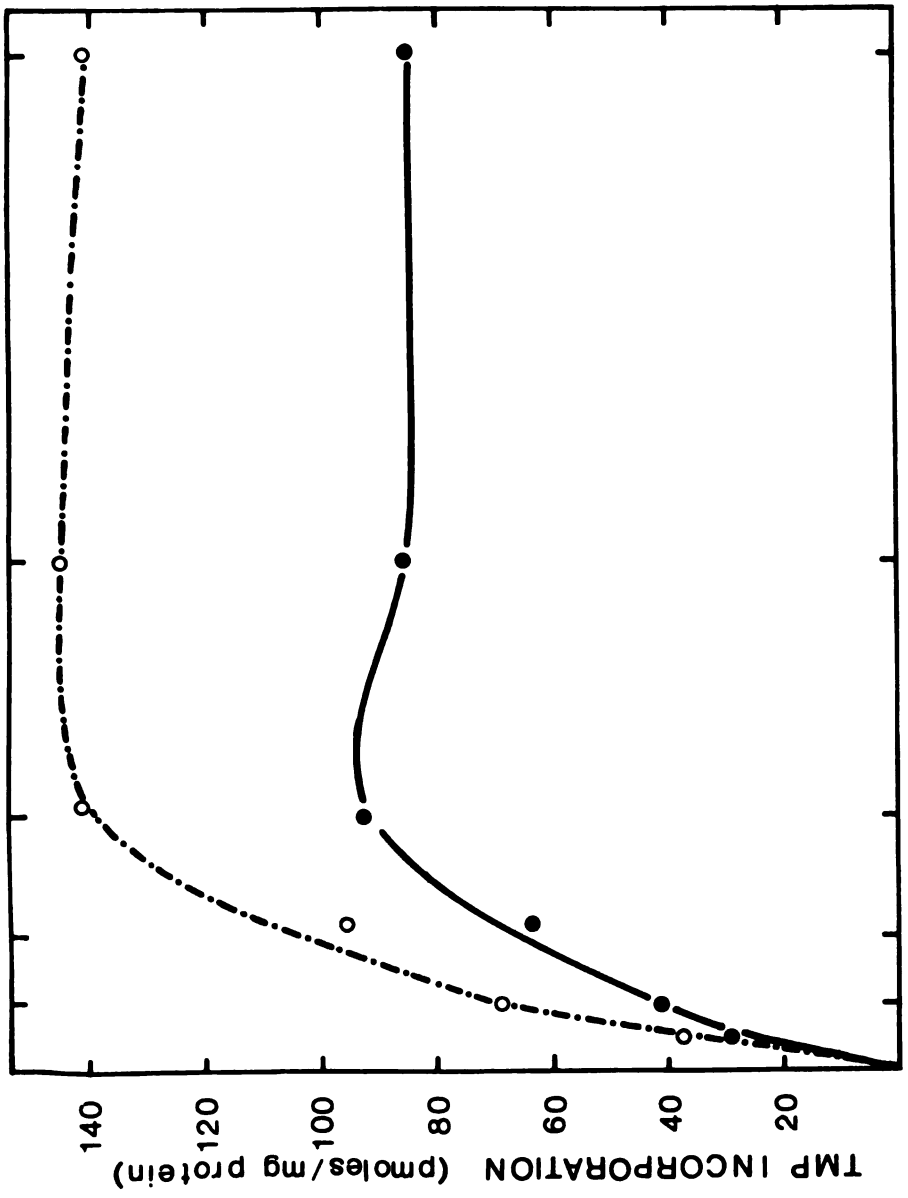
TMP INCORPORATION (pmoles/mg protein)
20
40
60
80
100
120
140
4
8
15
30
60
TIME (minutes)

Figure 2

Figure 3. Effect of template concentration on TMP incorporation Control -o- 24 hr ethylene treated seedlings -●-. Nicked calf thymus DNA was prepared as described in the text.

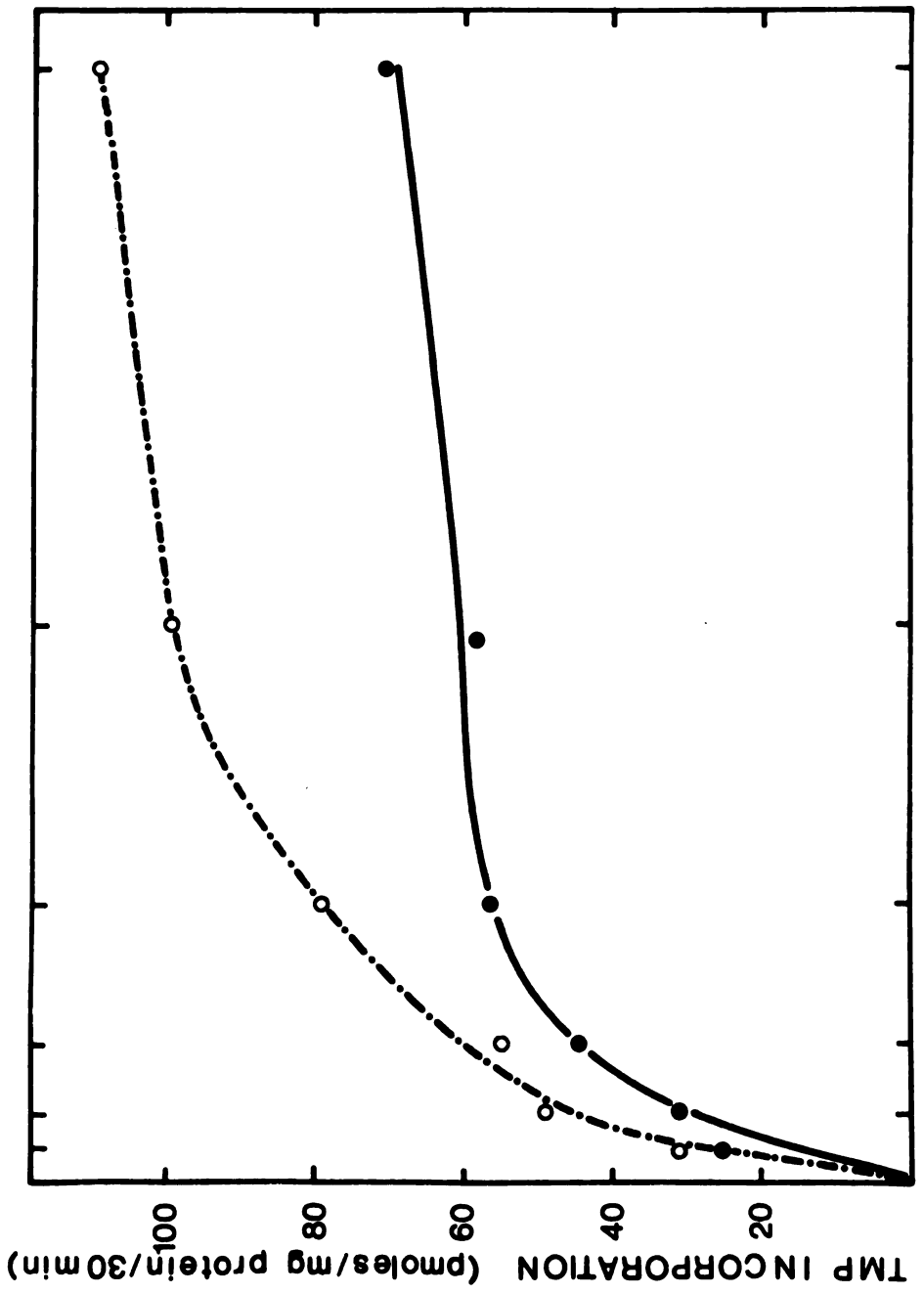
TMP INCORPORATION (pmoles/mg protein/30 min)
20
40
60
80
100
0.62
1.25
2.50
5.0
10.0
Calf thymus nicked DNA (µg)

Figure 3

"DNA-like" polymer. The inhibition of the reaction by pyrophosphate suggests that the polymerization of the nucleotides into a DNA-like polymer liberates pyrophosphate as is commonly recognized. The lack of total dependency of the enzyme activity on the addition of Mg^{++} and the four nucleosides indicates the presence of these substances in the crude extract and is to be expected. However, their concentration was relatively low, since addition of these ingredients to the reaction mixture increased the incorporation rate about 4-fold.

The enzyme from human cells (19) and other tissues (7,16) requires 3' hydroxyl termini on the template for maximal DNA polymerase in vitro activity. Apparently this is also true for pea DNA polymerase, since treatment of native calf thymus DNA with pancreatic deoxyribonuclease I to introduce 3' hydroxyl ends (13) markedly increased its priming activity.

Ethylene or supraoptimal auxin concentration suppress the growth of pea plants by causing almost complete inhibition of cell division in the apical meristems, lateral buds, and the plumular leaf (1). Furthermore, DNA synthesis in the apical tissue is inhibited by ethylene in the same degree as cell division and at similar hormone concentrations (1). The fact that ethylene stops the cell division cycle before prophase led to the conclusion that inhibition of DNA synthesis limits cell division. DNA polymerase activity is required for DNA synthesis (5,6). The present study indicates that the gas inhibits DNA polymerase activity in the cell division zone

of pea seedlings, and suggests that the primary effect of ethylene might be suppression of the activity of this enzyme. Cell division is subsequently inhibited which ultimately results in pronounced growth retardation. Inhibition of DNA polymerase by ethylene is unique. There are many instances where ethylene has been shown to increase other enzyme activities (4,17,18). Ethylene does not affect the enzyme itself since incubation of the crude extract with 1 ml/l ethylene in air had no effect. Response to the gas requires a complete cell or organ system. Inhibition of enzyme synthesis may explain the reduced activity. When compared over the linear range of TMP incorporation versus assay protein, DNA polymerase activity of ethylene treated tissue is only 14% of the control rate. This corresponds closely to the 90% inhibition of cell division observed in this tissue (1).

LITERATURE CITED

1. A. Apelbaum and S. P. Burg, Plant Physiol. 50, 117 (1972).

2. J. H. M. Bex, Planta 103, 11 (1972).

3. G. A. Bray, Anal. Biochem. 1, 279 (1960).

4. G. Engelsma and J. M. H. Van Bruggen, Plant Physiol. 48, 94 (1971).

5. D. L. Friedman, Biochem. Biophys. Res. Commun. 39, 100 (1970).

6. D. W. Foster and H. Stern, Science 128, 653 (1958).

7. R. Greene and D. Korn, J. Biol. Chem. 245, 254 (1970).

8. R. E. Holm, T. J. O'Brien, J. L. Key and J. H. Cherry, Plant Physiol. 45, 41 (1970).

9. K. D. Johnson and W. K. Purves, Plant Physiol. 46, 581 (1964).

10. J. L. Key and J. C. Shanon, Plant Physiol. 39, 360 (1964).

11. H. R. Leffler, T. J. O'Brien, D. V. Glover and J. H. Cherry, Plant Physiol. 48, 43 (1971).

12. O. H. Lowry, N. J. Rosebrough, Farr and R. J. Randall, J. Biol. Chem. 193, 265 (1951).

13. M. Matsuda and H. Ogoski, J. Biochem. 59, 230 (1966).

14. J. M. McComb, A. J. McComb and C. T. Duda, Plant Physiol. 46, 221 (1970).

15. T. J. O'Brien, B. C. Jarvis, J. H. Cherry and J. B. Hanson, In Biochemistry and Physiology of Plant Growth Substances (Wightman and G. Setterfield, eds.), p. 747 (Runge Press, Ottawa, 1968).

16. C. C. Richardson, C. L. Schildkraut, H. J. Aposhian and A. Kornberg, J. Biol. Chem. 239, 222 (1964).

17. I. Ridge and D. J. Osborne, J. Exp. Bot. 21, 720 (1970).

18. J. Riov, S. P. Monselise and R. S. Kahan, Plant Physiol. 44, 631 (1969).

19. A. Schlabach, B. Firdlender, A. Bolden and A. Weissbach, Biochem. Biophys. Res. Comm. 44, 879 (1971).

20. S. Schwimmer, Phytochemistry 5, 791 (1966).

21. S. Spigelman, A. Burny, M. R. Das, J. Keydar, J. Schlorn, M. Travinick and K. Watson, Nature 228, 430 (1970).

22. E. R. Stout and R. J. Mans, Biochem. Biophys. Acta. 134, 327 (1967).

23. E. R. Stout and M. Q. Arens, Biochem. Biophys. Acta. 213, 90 (1970).

SECTION III

DNA AND RNA POLYMERASE ACTIVITY OF POTATO TUBER MITOCHONDRIA ENHANCED BY ETHYLENE

Summary

RNA and DNA polymerase activity was studied in mitochondria isolated from potato tubers respiring at an accelerated rate in response to treatment with ethylene (10 µl/l). Whole tuber respiration rate increased about 6 hrs after ethylene treatment began and reached a peak value 4.7-fold higher than the initial rate in 24 hrs. The rise in respiration was preceded by an increase in DNA polymerase activity initiated within 6 hrs of ethylene application. RNA polymerase activity began to increase after 6 hrs and reached a peak value 2.5-fold higher than the initial rate in 18 hrs. Cytochrome c oxidase activity, although increasing initially subsequently paralleled the respiration rate pattern of whole tubers. Activity of DNA and RNA polymerase of mitochondria isolated from tubers that did not receive ethylene treatment was not affected by ethylene treatment *in vitro*. Apparently the stimulation of respiration by ethylene is mediated *in vivo* by enhancing mitochondrial DNA, RNA and protein synthesis.

Introduction

Respiration rate of plant materials is stimulated by treatment with ethylene (1,2,3,4), particularly in quiescent organs such as mature potato (1,2) and mature but unripe fruits. The increase in respiration has been associated with increased activity of mitochondrial enzymes (5,6,7,8), as a result of either enzyme activation or enzyme synthesis. An increase in respiration rate may lead to an increase in RNA required for protein synthesis, or the converse may be true. We attempted to separate cause from effect by studying DNA and RNA synthesis in mitochondria isolated from quiescent potato tubers following treatment with ethylene.

Materials and Methods

Mature potato tubers (Solanum tuberosum L. cv Kennebec) were obtained from Texas in June and stored at 20°C until used. CO_2 production was monitored (9) with four potatoes in 2 liter containers ventilated with air at 23°C at a flow rate of 100 ml/min. Ethylene treatments were applied by ventilating the containers with a gas mixture of 10 µl/l ethylene in air premixed in pressure cylinders.

Mitochondria were isolated from potato tubers treated with or without ethylene for varying lengths of time in a closed system with a 21% oxygen and 0% CO_2 (Appendix) at 23°C. Using the procedure of Huang and Beevers (10) at 0 to 4°C with

slight modifications. Tubers were washed, chilled at 0 to 4°C for 30 min, peeled and sterilized with 5% sodium hypochlorite (diluted 1:100). The pared tuber was ground with a food grater immersed in grinding media. Differential centrifugation and sucrose density gradient were performed according to the scheme in Figure I.

Cytochrome c oxidase activity used as a marker for the mitochondrial fraction, was measured by the method described by Simon (11). The reaction mixture was composed of 10 µl of mitochondria, 10 µl of 4.0% digitonin, 400 µl of 0.01 M phosphate buffer (pH 7.0) and 100 µl of 1.5 mM cytochrome c reduced with dithionite. The mitochondria were mixed with digitonin and allowed to stand for 1 min, before initiating the reaction by adding the cytochrome c in the buffer. Change in optical density at 550 mµ was measured with a Gilford recording spectrophotometer.

The incorporation of labeled TMP (thymidine monophosphate) and of labeled UMP (uridine monophosphate) into trichloroacetic acid-insoluble material was used to measure DNA and RNA polymerase activity respectively. The reaction mixture for DNA polymerase contained in µmoles: Tris-HCl buffer, pH 8.0, 10; KCL, 5; $MgCl_2$, 2; Cleland's reagent, 1; dATP, dCTP, dGTP, 0.01 each; TTP, 0.0025. Ten µCi ^{3}H-TTP (specific activity 50.8 Ci/mM) and an appropriate amount of the mitochondrial preparation, based on the protein content by the Lowry procedure (12), were added in a total reaction volume of 200 µl.

ISOLATION OF MITOCHONDRIA FROM POTATO TUBERS

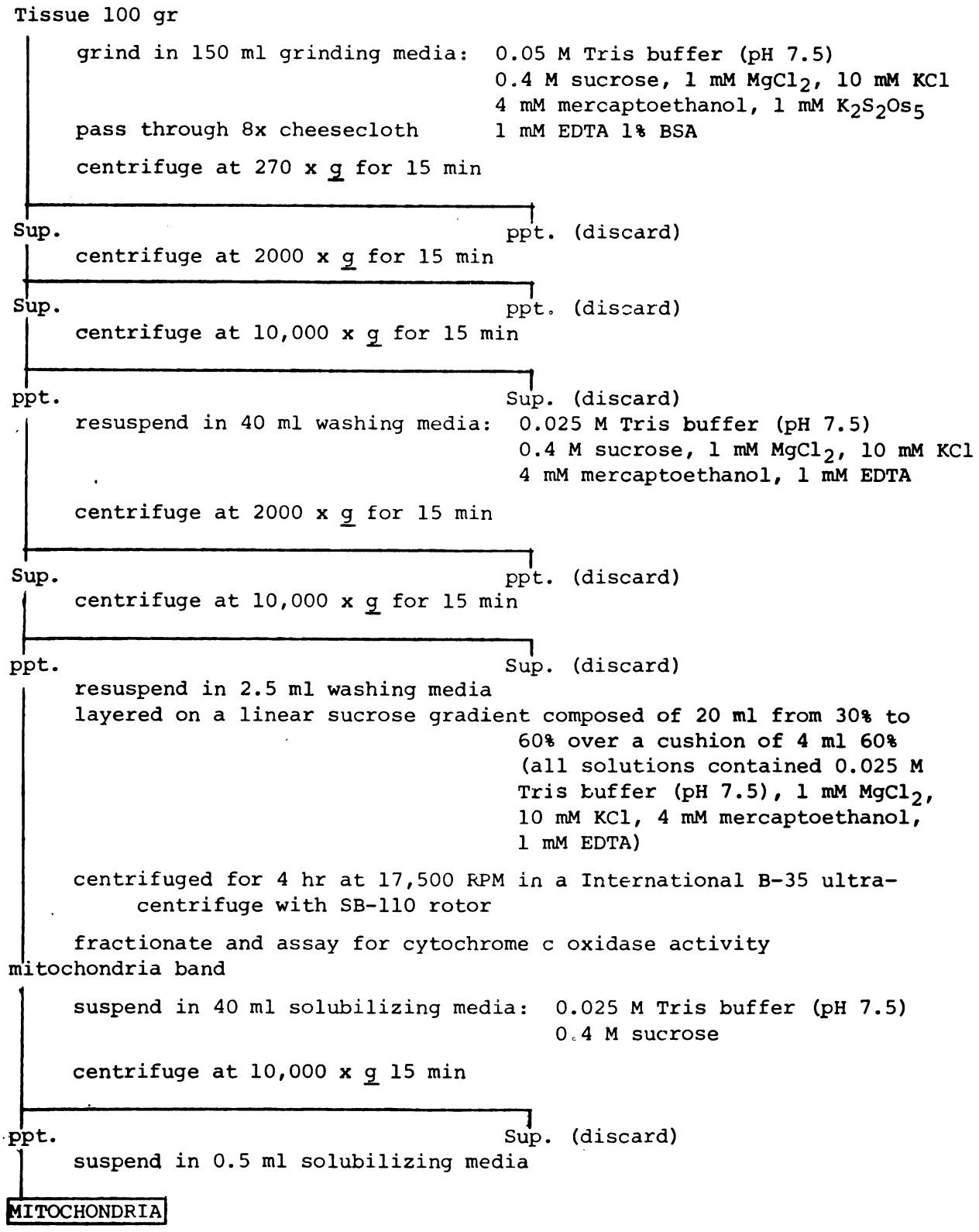

Fig. 1. Scheme for isolation of mitochondria from potato tubers.

For the RNA polymerase the reaction mixture was identical with that described above except that deoxyribonucleoside triphosphates were replaced by ribonucleoside triphosphates, and 0.1 μmole $MnCl_2$ was included in the medium. The incubation was carried out at 37°C for 1 hr, unless otherwise stated. After incubation the reaction was stopped by adding 2 ml of cold 10% TCA containing 1% PPi and centrifuged for 10 min at 10,000 x g. The pellet was dissolved in 0.5 ml of 0.2 N NaOH and 5 ml of cold 10% TCA with 1% PPi were added. The precipitate was transferred onto glass fiber filter disks (Whatman GF/C), and washed five times with 5 ml portions of cold 5% TCA and 1% PPi. The disks were placed in scintillation vials containing 10 ml of Bray's solution (13) and counted in a Beckman scintillation spectrometer with a counting efficiency of approximately 35% for tritium. Unincubated samples served as controls and the counts for these were subtracted from those of experimental samples.

Results

Incorporation of labeled TMP or UMP into acid-insoluble products required the presence of deoxyribonucleoside or ribonucleoside triphosphates respectively (Table I and II). The dependency was more pronounced for the DNA polymerase reaction since omission of any one of the deoxyribonucleoside triphosphates markedly reduced the TMP incorporation rate.

Table I. Dependency of mitochondrial DNA polymerase activity on components of the reaction mixture.

Reaction Mixture	% Incorporated[1]
Complete	100
$-Mg^{++}$	26
-dATP	1
-dCTP	2
-dGTP	7
-dATP, -dCTP, -dCTP	32
+Actinomycin-D (100 µg/ml	0
+Cycloheximide (10^{-5} M)	89
+Ethylene *in vitro* 30 min preincubation with 1 ml/l	96

[1]Mitochondria were isolated from control potato tubers. 100% incorporation represents incorporation of 3.5 pmoles TMP per mg mitochondrial protein.

Table II. Dependency of mitochondrial RNA polymerase activity on components of the reaction mixture.

Reaction Mixture	% Incorporation[1]
Complete	100
$-Mg^{++}$, $-Mn^{++}$	14
-ATP	31
-CTP	47
-GTP	35
-ATP, -CTP, -GTP	35
+Actinomycin-D (100 µg/ml)	37
+Cycloheximide (10^{-5} M)	86
+Ethylene *in vitro* 30 min preincubation with 1 ml/l	107

[1]Mitochondria were isolated from control potato tubers. 100% incorporation represents incorporation of 5.2 pmoles UMP per mg mitochondrial protein.

The RNA polymerase reaction showed less dependency upon the substrates presumably because of the endogenous ribonucleoside triphosphates in nitochondria. Both reactions were dependent on the presence of a divalent cation. Omission of Mg^{++} reduced the rate of incorporation of TMP to 26% and omission of Mg^{++} and Mn^{++} reduced the rate of incorporation of UMP to 14% of that of the complete system. Actinomycin-D (100 µg/ml) completely inhibited TMP incorporation and to a lesser degree the incorporation of UMP. Cycloheximide (10^{-5} M) only slightly reduced the rate of incorporation of either TMP or UMP. Preincubating the mitochondria with 1 ml/l ethylene in air did not influence DNA or RNA polymerase activity.

Incorporation increased with incubation time and the reaction was almost complete by 60 min for both DNA and RNA polymerase assays (Figs. 2 and 3). Subsequent assays were run for 60 min. Incorporation increased rapidly with increasing amounts of mitochondria up to 100 µg mitochondrial protein per DNA polymerase assay and up to 50 µg protein per RNA polymerase assay. Further increase in mitochondrial protein increased the incorporation at a very slow rate (Figs. 4 and 5).

The data presented above were obtained with control potato tubers. Mitochondrial RNA polymerase activity was considerably increased by treatment of tubers with ethylene.

Fig. 2. Time course of TMP incorporation into acid-insoluble product by mitochondria isolated from potato tubers.

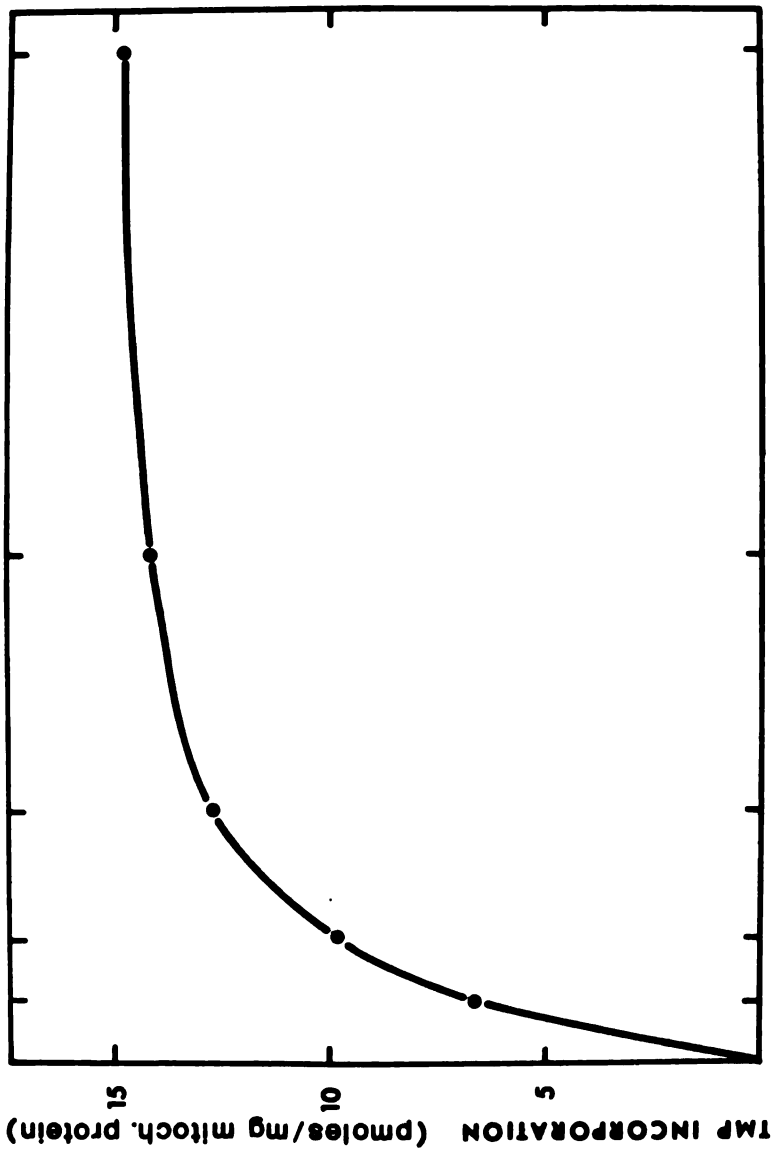
TMP INCORPORATION (pmoles/mg mitoch. protein)
5
10
15
15
30
60
120
180
TIME (minutes)

Figure 2

Fig. 3. Time course of UMP incorporation into acid-insoluble product by mitochondria isolated from potato tubers.

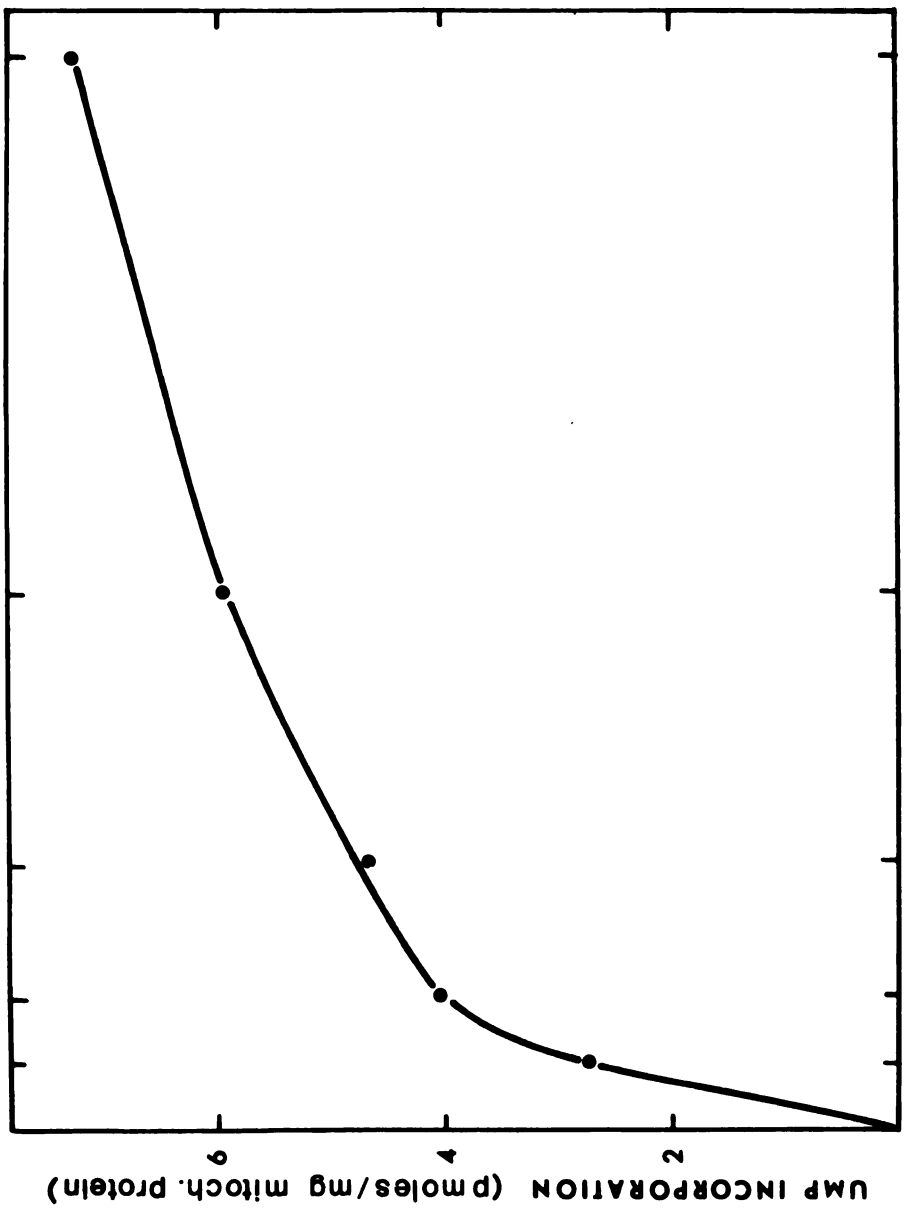
UMP INCORPORATION (p moles/mg mitoch. protein)
2
4
6
0
7.5
15
30
60
120
TIME (minutes)

Figure 3

Fig. 4. Concentration study for the incorporation of TMP into acid-insoluble product by mitochondria isolated from potato tubers.

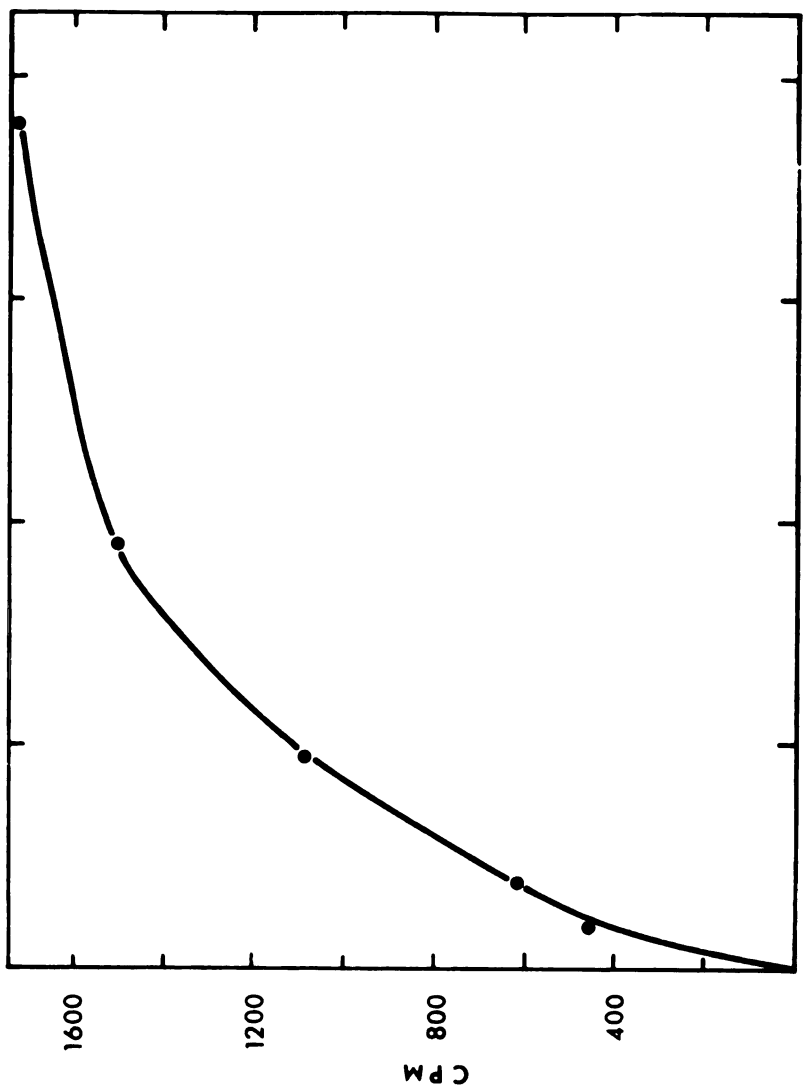
CPM
1600
1200
800
400
50
100
150
200
Mitochondrial protein (µg)

Figure 4

Fig. 5. Concentration study for the incorporation of UMP into acid-insoluble product by mitochondria isolated from potato tubers.

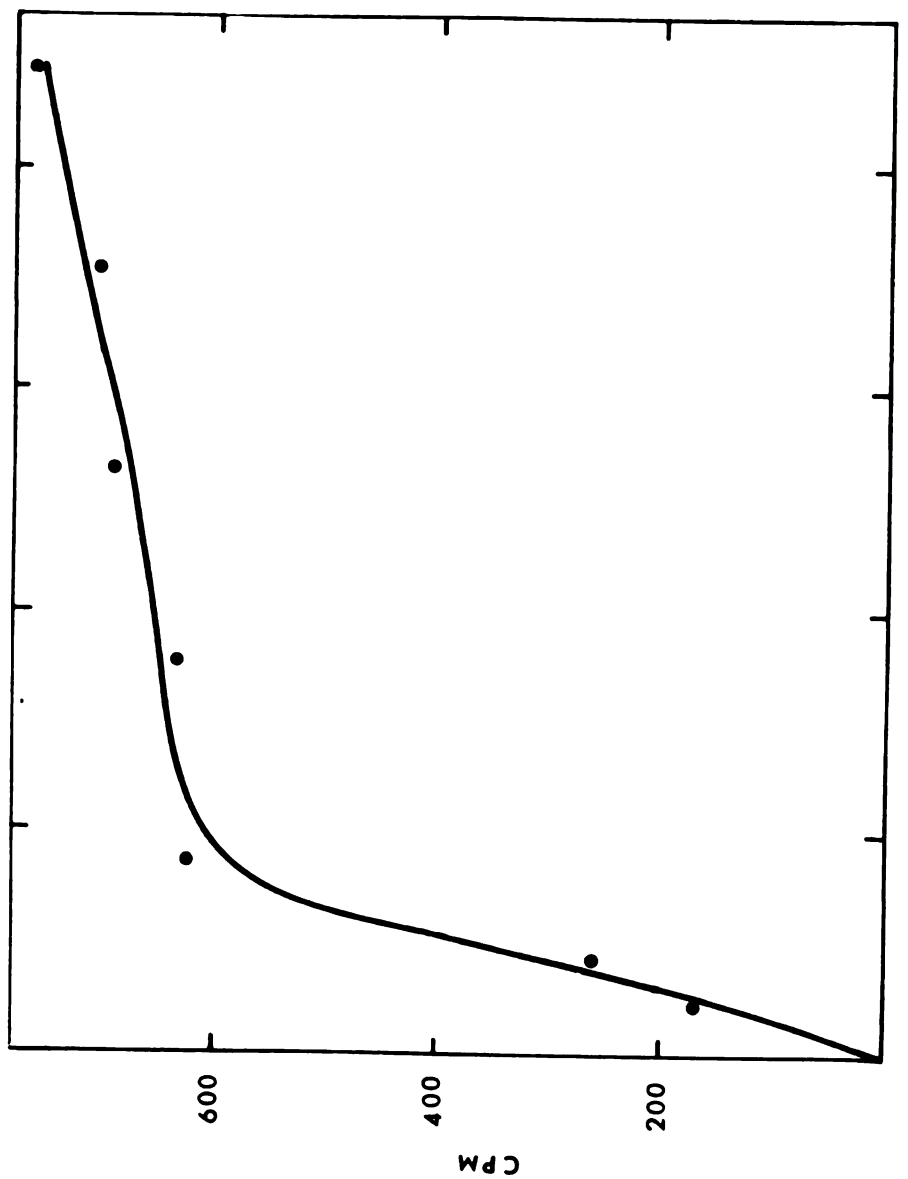

Figure 5

A time course study showed maximal enhancement of the enzyme activity after an 18-24 hr ethylene treatment and a subsequent decline. A three to four-fold increase in activity was obtained at the peak at all three concentrations of mitochondria added per assay (Fig. 6). The lower incorporation rate observed at the highest level of mitochondria used is attributed to the action of ribonuclease.

The effects of ethylene treatment on respiration and activity of cytochrome c oxidase, DNA polymerase, and RNA polymerase in a parallel experiment are shown in Fig. 7. The increase in RNA polymerase activity of mitochondria preceded the increase in respiration rate of intact tubers. The peak in respiration rate occurred almost six hrs later than the peak in RNA polymerase activity (2 1/2-fold increase in the activity). DNA polymerase activity increased six hrs after the initiation of the ethylene treatment, declined at 18 hrs, coinciding with the period of maximum RNA polymerase activity, and subsequently rose.

Ethylene treatment also enhanced the activity of cytochrome c oxidase. With the exception of the 6th hr measurement the increase in cytochrome c oxidase activity slightly preceded but otherwise paralleled the increase in respiration induced by ethylene.

Fig. 6. The effect of ethylene treatment *in vivo* for various durations on the incorporation of UMP into acid-insoluble product by mitochondria isolated from potato tubers after treatment with ethylene for time indicated. ■———■ 100 µl, ●–.–.–● 50 µl, ▼····▼ 25 µ; mitochondria added/assay.

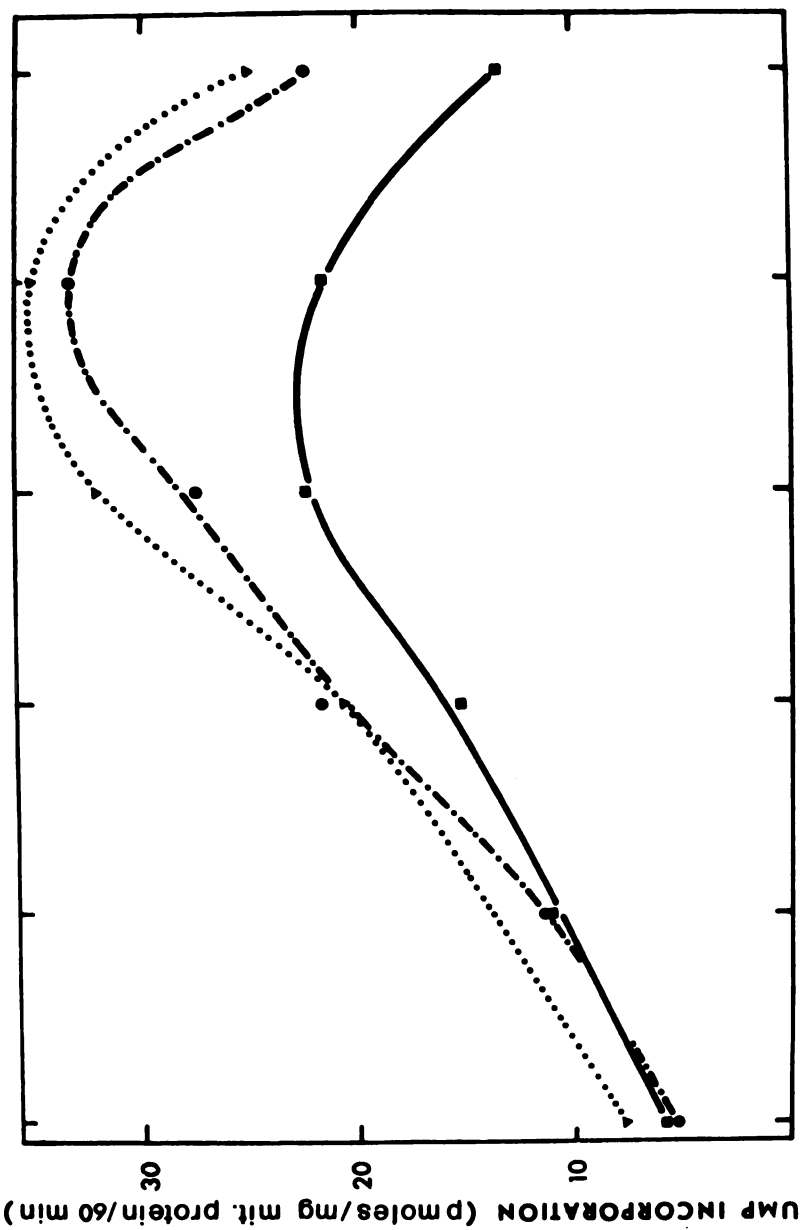
UMP INCORPORATION (p moles/mg mit. protein/60 min)
10
20
30
0
6
12
18
24
30
Hours

Figure 6

Fig. 7. The effect of ethylene treatment on respiration, cytochrome c oxidase, and incorporation of TMP-UMP into acid-insoluble product in mitochondria isolated from potato tubers. ●——● respiration of ethylene treated potatoes, o···o···o respiration of control, □——□ TMP incorporation, ■-·-■ UMP incorporation, *----*cytochrome c oxidase.

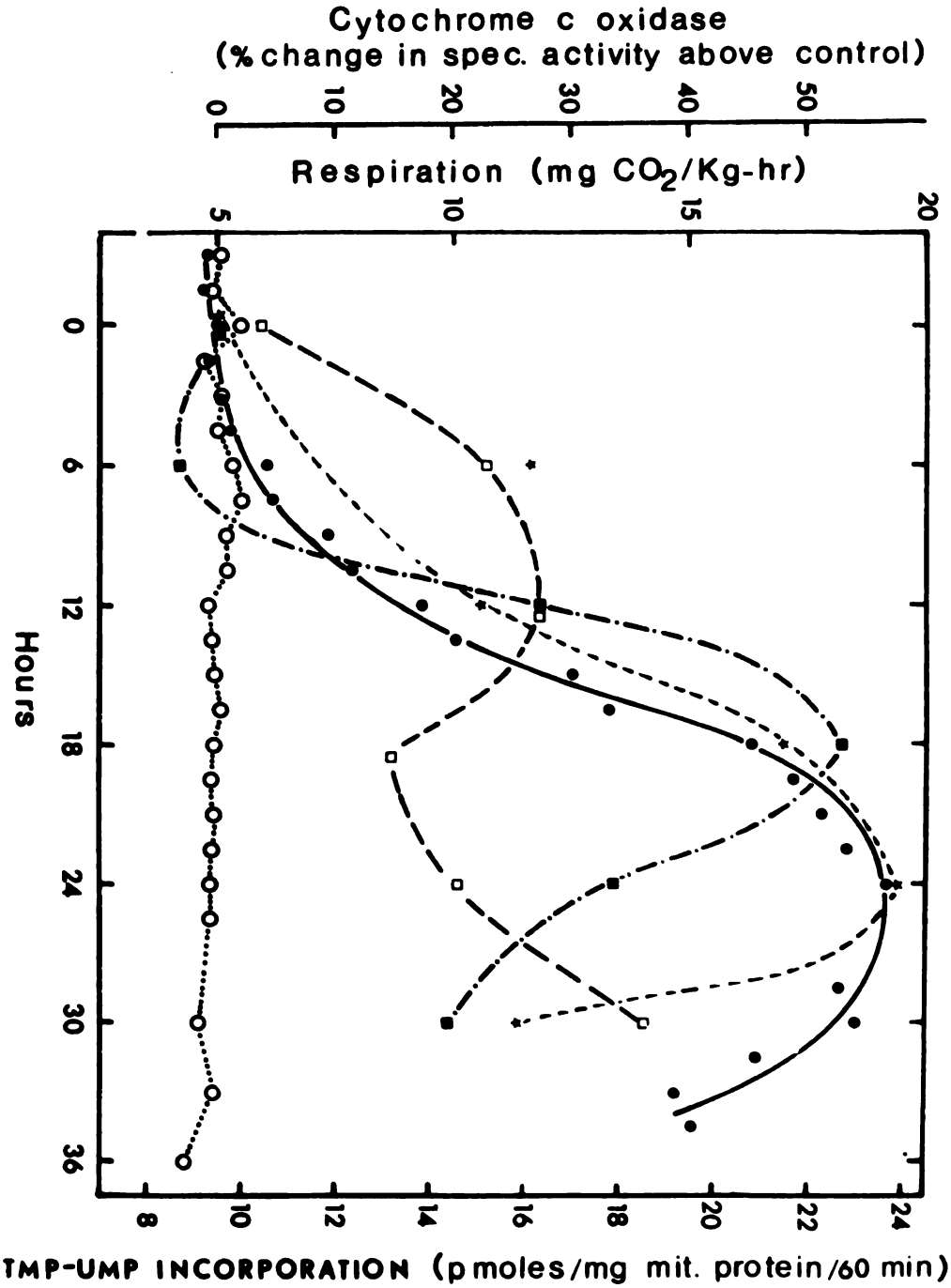
Cytochrome c oxidase
(% change in spec. activity above control)
0
10
20
30
40
50
Respiration (mg CO_2/Kg-hr)
5
10
15
20
Hours
0
6
12
18
24
30
36
8
10
12
14
16
18
20
22
24
TMP-UMP INCORPORATION (p moles/mg mit. protein/60 min)

Figure 7

Discussion

Isolated mitochondria possess many characteristics which enable *in vitro* examination of processes occurring *in vivo*. The technique has revealed increased enzyme activities in mitochondria isolated from both animal (14,15) and plant tissues (16) treated with hormones.

Our data indicate that ethylene, a plant hormone, can induce changes in the activity of mitochondrial DNA and RNA polymerase. RNA polymerase activity in potato tubers rose during the first 18-24 hrs of treatment with ethylene, then declined during an additional 6 hrs. Respiratory activity was greatest when RNA polymerase activity began to decline. Ethylene induced a smaller increase in DNA polymerase activity, noticeable 6 hrs after the initiation of ethylene treatment. However, the relatively long intervals between measurements did not allow determination of the time required for induction.

RNA polymerase may allow the synthesis of mitochondrial proteins necessary for respiratory activity. The increase in specific activity of cytochrome c oxidase slightly before the rise in respiration supports this hypothesis.

The changes in activity of RNA polymerase and cytochrome c oxidase paralleled, but preceded the change in respiration rate of ethylene-treated whole tubers. The decline in enzymatic and metabolic activity suggests that this is of functional significance in mitochondrial metabolism. The specific

mode of action by which ethylene affects nucleic acid metabolism is not known. The failure to induce in vitro, any changes in the enzyme activity of the isolated intact mitochondria suggests the involvement of the nucleus or intact cell in the interaction of ethylene with the site of action.

Potato tubers normally do not encounter ethylene at levels sufficient to cause this striking increase in respiratory activity. Their respiration rate is sensitive to numerous environmental stresses and to circadian rhythm. The sequential rise and fall in DNA and RNA polymerase activity, cytochrome c oxidase activity, and respiration rate in response to applied ethylene suggests that a mechanism exists to restore order and balance to metabolism. Perhaps other stress factors which induce less marked respiratory changes produce similar but more subtle metabolic changes.

LITERATURE CITED

1. Reid, M. S. and Pratt, H. K., Nature, 226, 976 (1970).

2. Reid, M. S. and Pratt, H. K., Plant Physiol., 49, 252 (1972).

3. Wang, C. Y., Mellenthin, W. M. and Hansen, E., J. Amer. Soc. Hort. Sci., 97, 9 (1972).

4. Nichols, R., J. Hort. Sci., 43, 335 (1968).

5. Miller, L. A. and Romani, R. J., Plant Physiol., 41, 411 (1965).

6. Switzer, C. M., Plant Physiol., 32, 42 (1957).

7. Key, J. K., Hanson, J. B. and Bils, R. F., Plant Physiol., 35, 177 (1960).

8. Jones, J. D., Hulme, A. C. and Wooltorton, L. S. C. New Phytol. 64, 158 (1965).

9. Dilley, D. R., Dewey, D. H. and Dedolph, R. R., J. Amer. Soc. Hort. Sci., 94, 138 (1969).

10. Huang, A. H. C. and Beevers, H., Plant Physiol., 48, 637 (1971).

11. Simon, E. W., Biochem. J., 69, 67 (1957).

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 267 (1951).

13. Bray, G. A., Anal. Biochem., 1, 279 (1960).

14. Tata, J. R., Ernster, L., Lindberg, O., Arrhenius, E., Peterson, S. and Herdman, R., Biochem. J., 86, 408 (1963).

15. Boodyn, D. B., Freeman, K. B. and Tata, J. R., Biochem. J., 94, 628 (1965).

16. Baxter, R. and Hansen, J. B., Planta, 82, 246 (1968).

SECTION IV

INDUCTION OF AUTOCATALYTIC ETHYLENE PRODUCTION IN APPLE FRUITS BY PROPYLENE IN RELATION TO MATURITY AND OXYGEN DEPENDENCY

Abstract. Ethylene, the fruit ripening hormone, and other olefinic compounds cause apples and other climacteric fruits to ripen. Ethylene biosynthesis becomes autocatalytic as ripening proceeds. Propylene, which fruits do not produce, was employed to determine the stage of maturity apples must attain to autocatalytically produce ethylene and the effect of O_2 tension on autocatalysis. Red Delicious apples harvested at developmental stages representing 52, 58, 65 and 75% of maturity were gassed with propylene at conc. of 0, 10, 50, 100, 500 and 1000 ppm for one week at 20°C. The ability of propylene to stimulate ethylene production increased progressively with fruit maturation, but rate of production following treatment with 500 ppm propylene was constant. A shorter lag time to the onset of autocatalytic production was observed for the more mature fruit which reflects a natural increase in sensitivity. Propylene administered at 6.5% O_2 or less did not induce ethylene production, but an anaerobic atmosphere was necessary to completely inhibit ethylene synthesis in fruits once autocatalysis began.

Introduction

Autocatalysis of ethylene production (6,7,17) is a striking feature of ripening in climacteric fruits, and is triggered by exposure to ethylene at concentrations above a threshold level (14,28). In most studies of the role of ethylene in ripening of climacteric fruits two processes, ethylene action and production, are interrelated because of the autocatalysis phenomenon. Experimentally it is difficult to study them separately.

One way to assess the role of ethylene in fruit ripening consists of treating the fruit with ethylene at various concentrations and measuring the ripening response (18,21,26,28). Numerous investigations have been undertaken to determine the threshold concentration which triggers ripening (1,2,3,14,25, 27,28). However, it is not clear whether this threshold concentration itself causes ripening or whether it stimulates ethylene production to physiologically active levels which in turn cause the fruit to ripen. Hackett et al. (14) have suggested that the action of ethylene as a ripening hormone in fruits may be separated into two distinct processes; the first is the initiation of ethylene synthesis, the second the physiological response.

Sensitivity to ethylene is not constant throughout the life of the fruit (7), for threshold level needed to cause ripening of climacteric fruits is dependent on the age of the tissue. Fruits attached to the tree are less sensitive to

ethylene than harvested fruits (23), suggesting that ethylene is rendered ineffective by a ripening inhibitor supplied by the parent plant (5).

The threshold sensitivity of fruit to exogenous ethylene may depend upon the supply of oxygen (16,24). This was attributed to the decrease of ethylene production under the low oxygen tension (4,9,11,12,15,16).

In this paper the sensitivity of the apple fruit to autostimulation was studied in relation to the age of the tissue and to O_2 levels required. To avoid complications with the ripening process, premature apples were used starting from a very early stage (52% maturity). In order to measure only endogenous ethylene, propylene was used to trigger ethylene production. Thus, the potential for autocatalysis was determined by the fruit's response to propylene at different stages. Propylene, like ethylene, promotes fruit ripening, inhibits elongation of pea subapical sections and causes epinasty (10). Burg and Burg (8) determined the molecular requirements for ethylene action and found propylene to be the next most active compound to show ethylene-like action. The equivalent concentration of propylene to cause half-maximum ethylene response was found to be 130 times that of ethylene. The role of oxygen in autocatalysis was studied by applying propylene at different oxygen tensions.

Materials and Methods

Plant material: Red Delicious apples were obtained from trees at the Horticultural Research Center at Michigan State University. Fruits were harvested at 52, 58, 65 and 75% maturity, based on a 145-day post-bloom growth period. For experiments on the effect of O_2 in the stimulation of ethylene production by propylene, apples were harvested at 83 and 90% maturity. The fruits used for each experiment were harvested from the same tree and were about equal in size. The experiments described were commenced approximately two hr after harvesting the fruits.

Propylene treatments: Propylene at concn. of 0, 10, 50, 100, 500, and 1000 ppm was applied by injecting known amounts into chambers in the apparatus described in the Appendix. Concentrations were checked by gas chromatography. When propylene was administered at 100 ppm at different O_2 levels the gas was injected into fruit chambers previously flushed with gas mixtures prepared by cylinders.

Measurements of ethylene and CO_2 production: Ethylene production was measured by gas chromatography of samples withdrawn at intervals from the chambers, using a Varian Aerograph 1700 gas chromatograph equipped with a flame ionization detector and a 1/8" x 4' column of activated alumina. The column temperature was 50°C with a N_2 carrier gas flow of 40 ml/min.

The instrument was capable of measuring 10 ppb of ethylene in a 1 ml sample with a peak height : background noise level of 2. Measurements were made every one or two days to determine the rate of ethylene production. Fruit respiration was measured by CO_2 absorption in 2N KOH as described in the Appendix. The two-indicator method was used for the titrimetric determination of carbonate and bicarbonate.

The fruit chambers were aerated every two or three days to avoid excessive accumulation of ethylene.

Results and Discussion

Sensitivity of the tissue to propylene: Propylene triggered the fruits to produce ethylene. The efficacy of each concentration of propylene to stimulate the fruit to produce ethylene increased progressively during maturation (Fig. 1). The minimum concentration of applied propylene and the time required to stimulate ethylene production decreased progressively during maturation (Fig. 1, I, II, III and IV). Propylene at 500 or 1000 ppm applied at 52% maturity stimulated the fruit to produce ethylene by the 7th day at a rate of 50 µl/kg-24 hr (Fig. 1, I) indicating that a rudimentary mechanism for ethylene production existed at this early stage of development. At 75% maturity, treatments with 50, 100 and 500 ppm stimulated the fruit to produce ethylene at the 7th day at the rate of 400 µl/kg-24 hrs, which is very close to the highest rate of

Figure 1. Effect of propylene treatment on ethylene production in Red Delicious apples at 52, 58, 65 and 72% maturity.

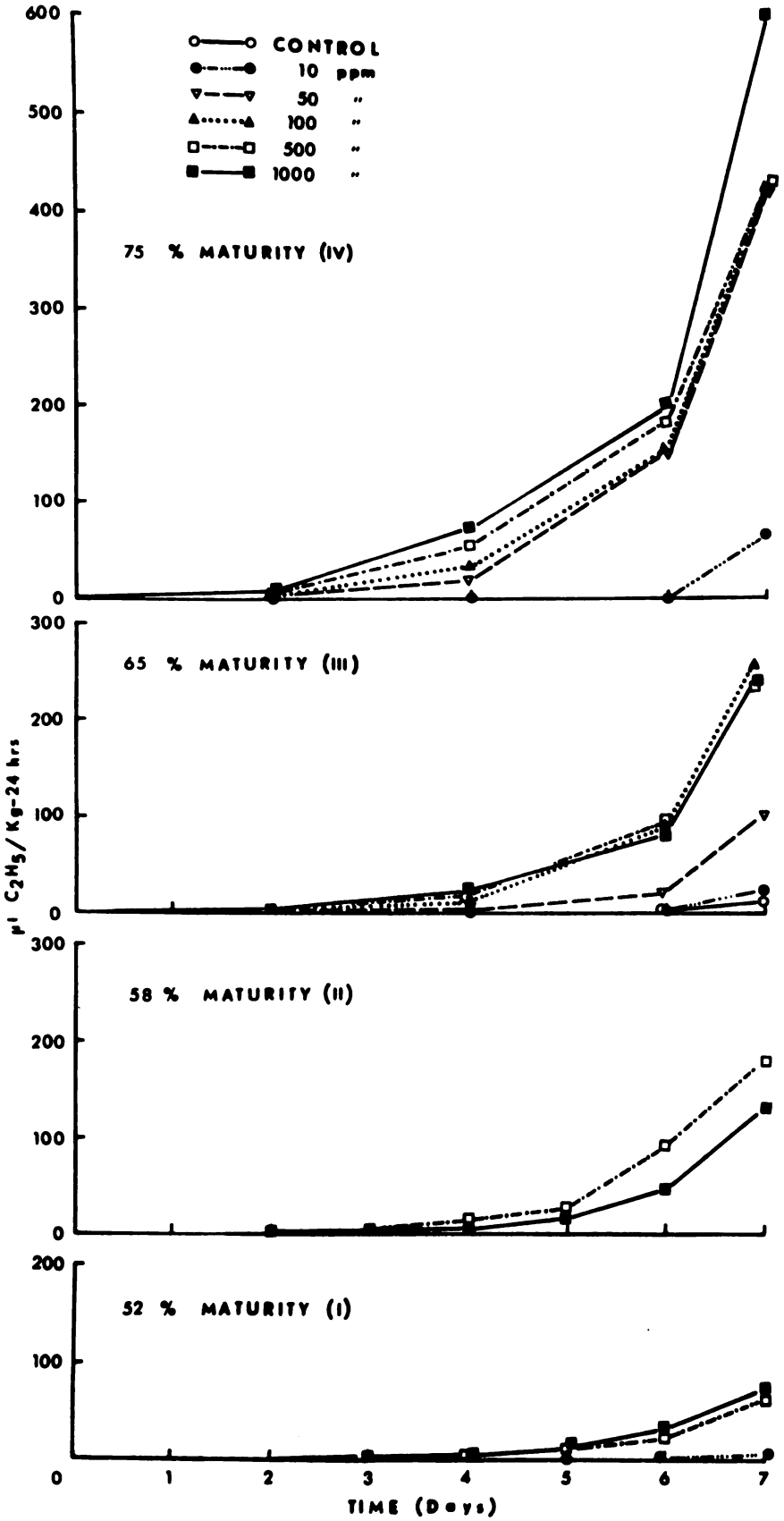
CONTROL
10 ppm
50 "
100 "
500 "
1000 "
75 % MATURITY (IV)
65 % MATURITY (III)
58 % MATURITY (II)
52 % MATURITY (I)
µl C2H5/ Kg-24 hrs
TIME (Days)

Figure 1

ethylene production reported for apples (14,24). Intermediate rates of ethylene production were found for fruit at 58 and 65% of full maturity.

Following treatment with 500 ppm propylene, the rate of ethylene production rose exponentially with time, as illustrated on log scale in Figure 2. This behavior is compatible with the concept of autocatalysis in ethylene production. Rate of production did not vary with stages of maturity as indicated by similar slope. However, more mature fruits required less time to initiate production which may reflect an increase in the sensitivity of the fruit for autocatalysis with maturation.

All fruits of different ages treated with propylene showed moderate increases in respiration rates as measured on the 3rd or 4th day (Fig. 3). The increase in respiration was more marked in the early stages of maturation and at propylene concentrations above 50 ppm.

Propylene even at 1000 ppm had no effect on ripening. Although the gas induced the fruit to produce large amounts of ethylene the 7th day, flesh softening and content of soluble solids were little affected when measured 2-3 weeks after the propylene treatment. This suggests that although the mechanism for autocatalysis of ethylene production is functional in the early stages of maturation the fruit has not obtained the required potential for ripening.

Figure 2. Effect of 500 ppm propylene treatment on rate of ethylene production in Red Delicious apples treated at 52% (I), 58% (II), 65% (III), and 75% (IV) maturity.

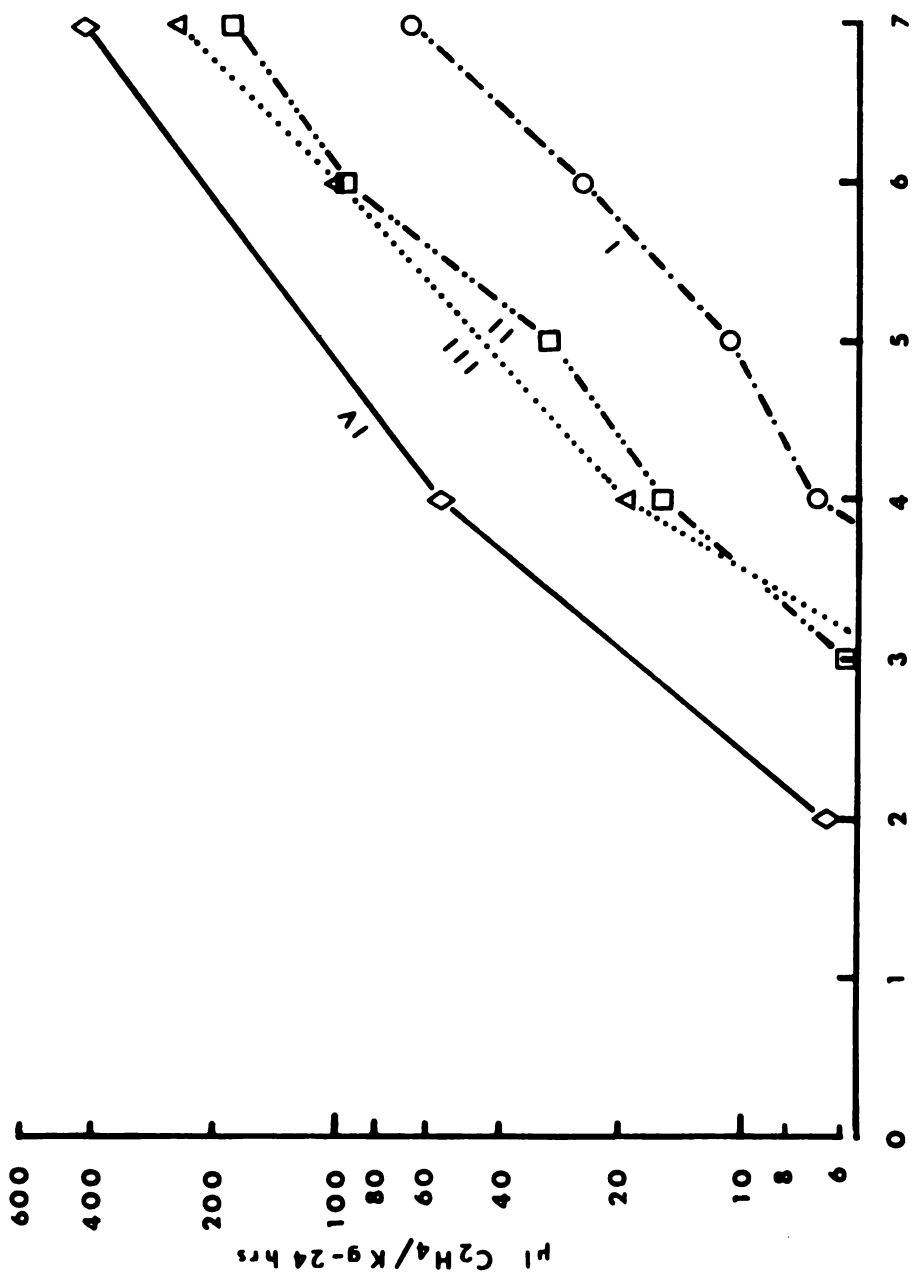
600
400
200
100
80
60
40
20
10
8
6
µl C2H4/kg-24 hrs
0
1
2
3
4
5
6
7
TIME (days)
I
II
III
IV

Figure 2

Figure 3. Effect of propylene concentration on respiration in Red Delicious apples at 30% (A), 35% (B), 52% (C), 58% (D) and 65% (E) maturity, measured the 3rd-4th day after propylene treatment.

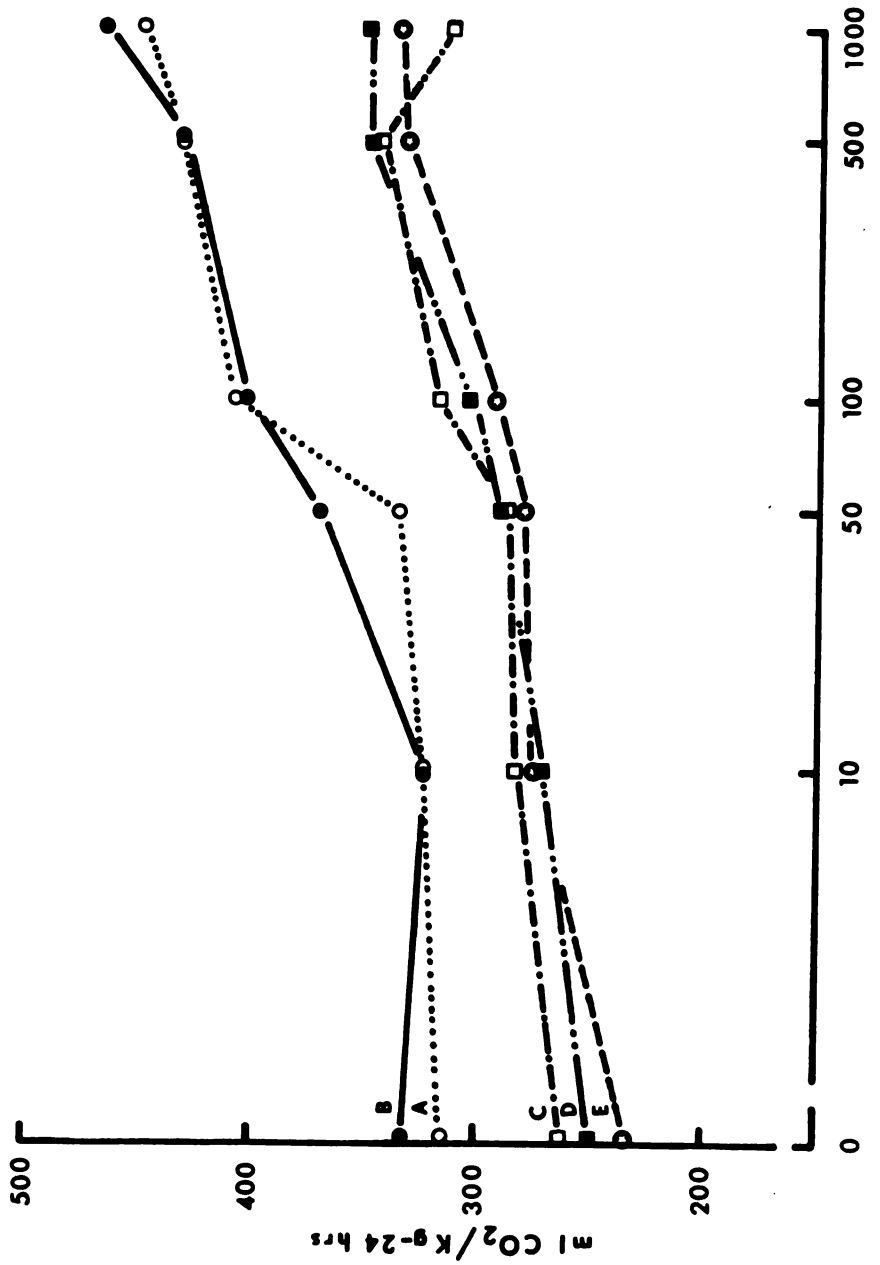
500
400
300
200
ml CO2/kg-24 hrs
A
B
C
D
E
0
10
50
100
500
1000
C3H6 (PPM)

Figure 3

Stimulation of ethylene production by propylene in atmospheres deficient in oxygen: Autocatalysis of ethylene production was retarded by low O_2 levels (Fig. 4). Fruits at 83% of full maturity and treated with 100 ppm propylene required 6.5% or more O_2 in the atmosphere to trigger the mechanism for autocatalysis of ethylene production. Concentrations of O_2 less than 6.5% gave very low rates of ethylene production which was not different from that obtained from fruit not treated with propylene. Above the threshold value of 6.5% a linear dependency upon O_2 concentration was observed, with a saturation level apparently above that of air.

In order to further elucidate the role of O_2, fruits were shifted to low or high O_2 before or after the inductive treatment with propylene. The fruits held in 4.6% O_2 and 100 ppm propylene showed no sign of autocatalysis up to the fifth day (Fig. 5). When they were subsequently shifted to air plus propylene, ethylene production followed the same pattern as in fruit treated initially with propylene in air. Once the fruit gained capacity to produce ethylene, an anaerobic atmosphere was necessary to completely inhibit its synthesis. Transferring the fruit to 4.6% O_2 did not stop production, and even at 1.2% O_2 the fruit produced a considerable amount. At 10.5% O_2 ethylene production was initially about 50% of that observed in air. However, there was little difference after 6 days.

Figure 4. Effect of oxygen concentration in the stimulation of ethylene production by 100 ppm propylene in Red Delicious apples treated at 83% maturity.

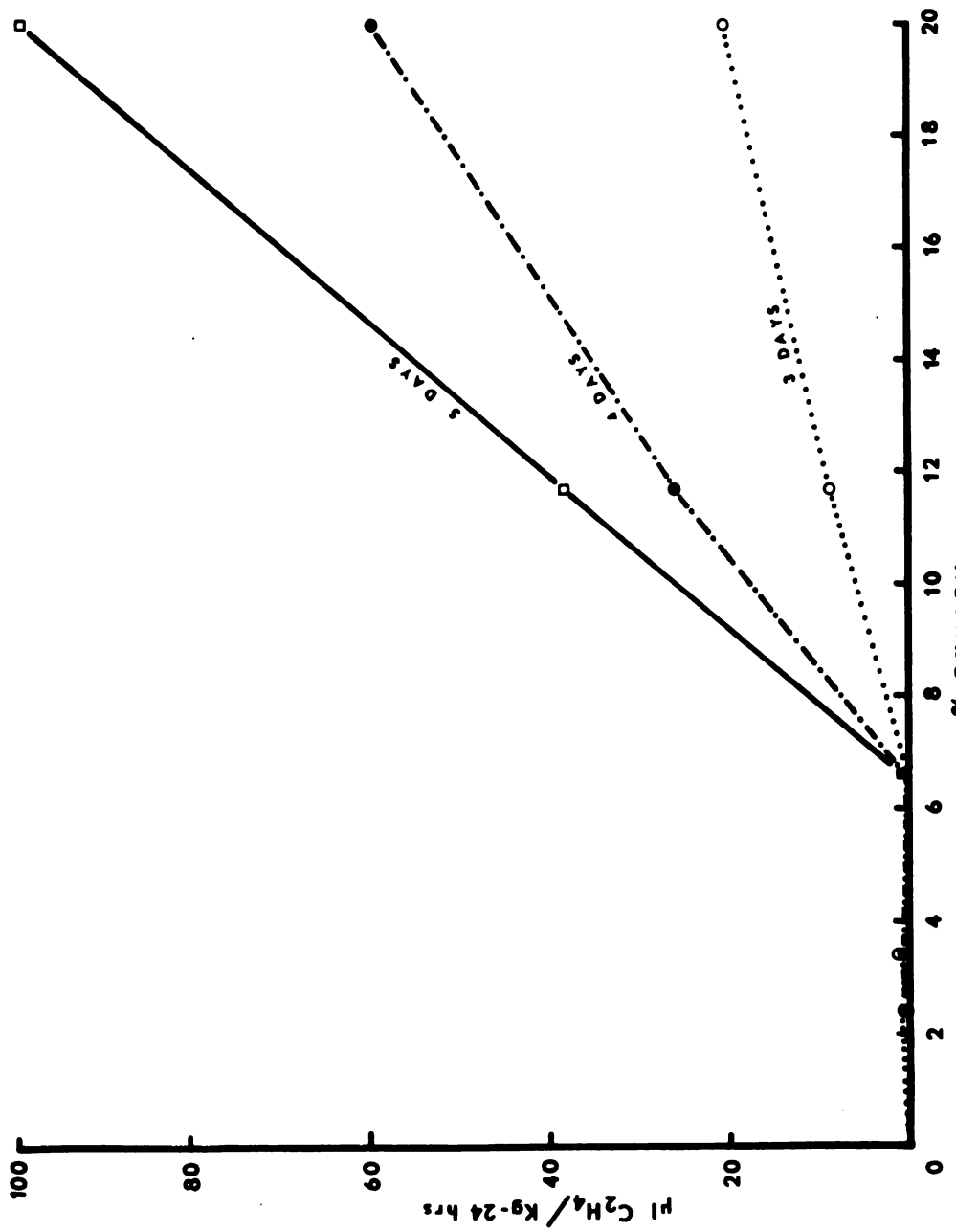
100
80
60
40
20
0
μl C2H4/ kg-24 hrs
0
2
4
6
8
10
12
14
16
18
20
% OXYGEN
5 DAYS
4 DAYS
3 DAYS

Figure 4

Figure 5. Effect of oxygen concentration at the time of exposure in the stimulation of ethylene production by 100 ppm propylene in Red Delicious apples treated at 90% maturity.

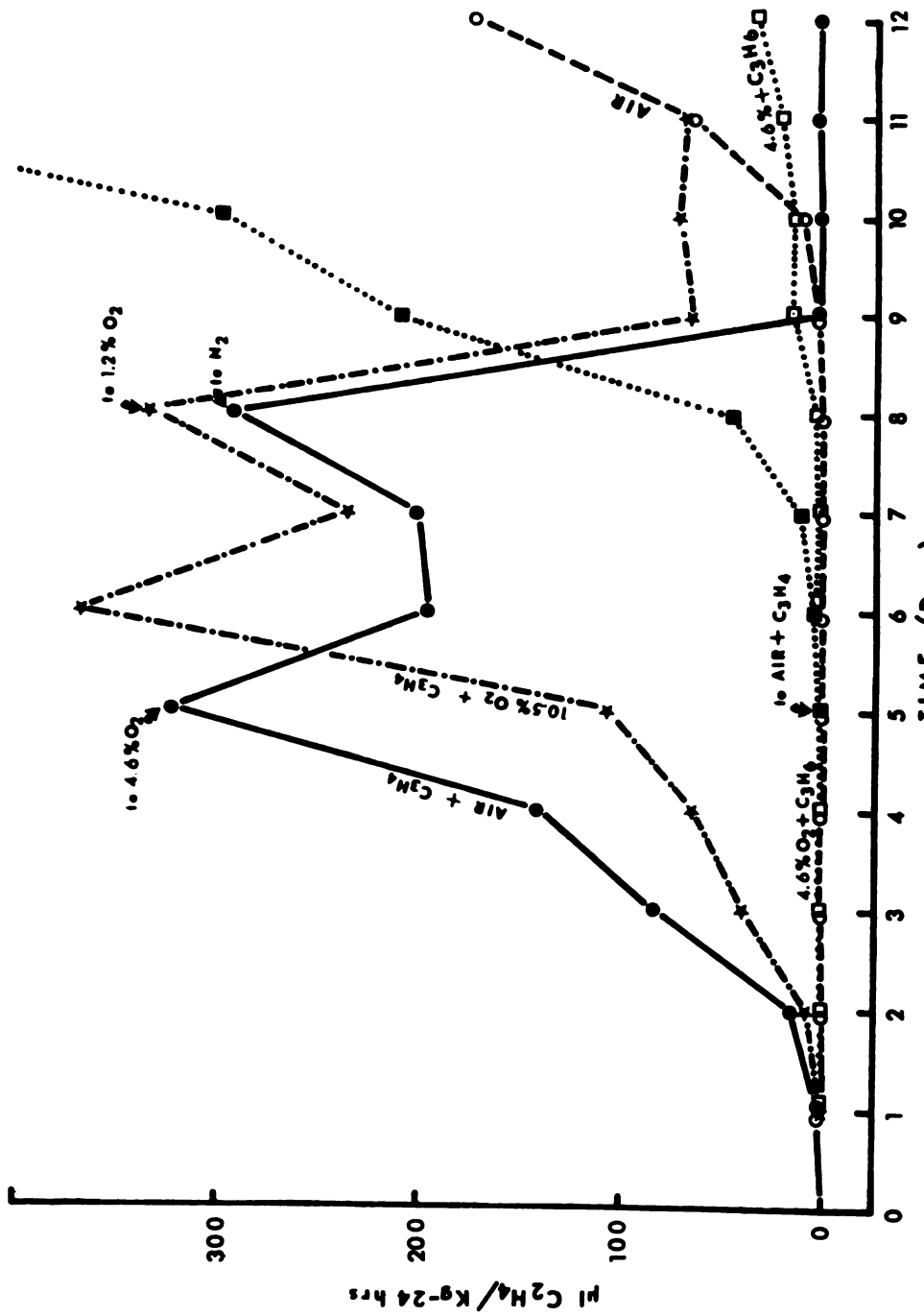
µl C2H4/ kg-24 hrs
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME (Days)
AIR
4.6%+C3H8
1o AIR + C3H4
4.6%O2+C3H8
10.5%O2 + C3H4
AIR + C3H4
1o 4.6%O2
1o 1.2% O2
1o N2

Figure 5

Thus, the stimulation of ethylene production by propylene is in some way linked to the utilization of atmospheric O_2. Fruits kept at the threshold O_2 concentration produced less CO_2 than fruits kept in air (Fig. 6). Our results are in accord with those obtained by other investigators with apples (13,24) and bananas and tomatoes (20) where low O_2 level prevented the onset of the climacteric by inhibiting ethylene production. They clearly indicate the importance of low O_2 levels in retarding the autocatalysis of ethylene production in preclimacteric fruits, vs. their failure to retard ethylene production in fruits which are already synthesizing ethylene at high rates. The results emphasize the importance of placing fruit in controlled atmosphere storage just before they have attained capacity for autocatalysis, and the necessity for a reliable method of predicting the harvest date for fruit intended for long term storage.

While this investigation was being conducted, McMurchie et al. (22), found that propylene induced ethylene production in bananas but not in citrus fruits. The authors suggested the existence of two ethylene producing systems in climacteric fruits, the one responsible for autocatalysis being missing from non-climacteric fruits. This suggestion receives support from the present study for the two ethylene producing systems in climacteric fruits, the non-autocatalytic system is under the control of limited supply of oxygen, while the autocatalytic system is less dependent upon oxygen. This may indicate

Figure 6. Effect of oxygen concentration in the respiration of Red Delicious apples treated with 100 ppm propylene at 99% maturity.

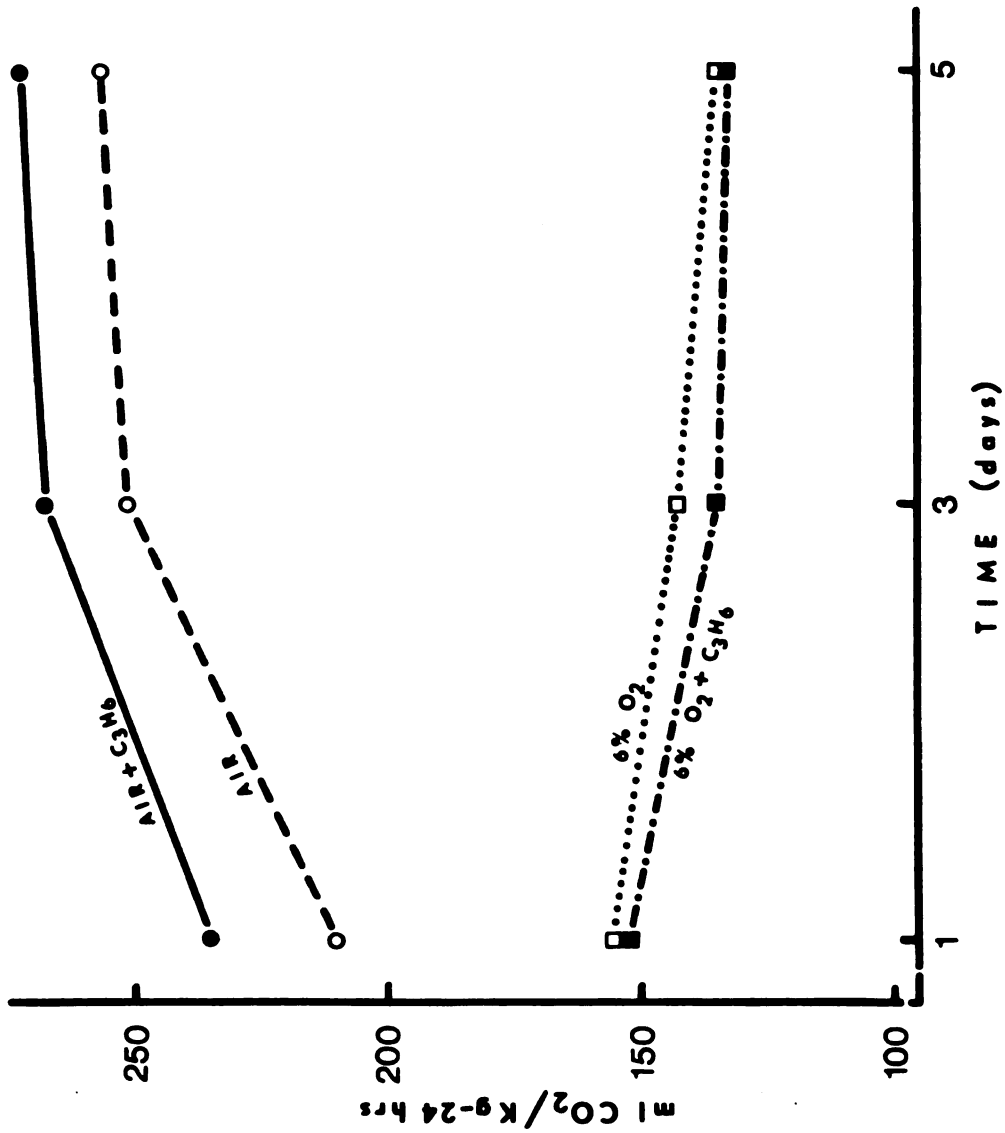
250
200
150
100
1
3
5
TIME (days)
ml CO2/kg-24 hrs
AIR+C3H6
AIR
6% O2
6% O2+C3H6

Figure 6

differences in the affinity of enzymes with oxygen involved in the two ethylene producing systems.

LITERATURE CITED

1. Biale, J. B., R. E. Young, and A. J. Olmstead. 1954. Fruit respiration and ethylene production. Plant Physiol. 29:168-174.

2. Biale, J. B. 1960. The postharvest biochemistry of tropical and subtropical fruits. Advances in Food Res. 10:293-354.

3. 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. Austr. J. Biol. Sci. 23:1143-1152.

4. Burg, S. P., and K. V. Thimann. 1959. The physiology of ethylene formation in apples. Proc. Nat. Acad. Sci. 45:335-344.

5. Burg, S. P. 1963. Studies on the formation and function of ethylene gas in plant tissues. Regulateurs Naturels de la France (Centre National de la Recherche Scientifique, Paris 1964), p. 719.

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

7. Burg, S. P., and E. A. Burg. 1965. Ethylene action and the ripening of fruits. Science 148:1190-1196.

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

9. Craft, C. C. 1960. Ethylene production by tomato tissue. Plant Physiol. 35 (Supp.) vii.

10. Crocker, W., A. E. Hitchcock, and P. W. Zimmerman. 1935. Similarities in the effects of ethylene and the plant auxins. Contrib. Boyce Thompson Inst. 7:231-248.

11. Gane, R. 1934. Production of ethylene by some ripening fruits. Nature 134:1008.

12. Gane, R. 1935. Identification of ethylene among volatile products of ripe apples. Ann. Rept. Food Invest. Board, Lond. 123:3.

13. Hulme, A. C., M. J. C. Rhodes, and L. S. C. Wooltorton. 1971. The effect of ethylene on the respiration, ethylene production, RNA and protein synthesis for apples stored in low oxygen and in air. Phytochemistry 10:1315-1323.

14. Hackett, P. J., A. C. Hulme, M. J. C. Rhodes, and L. S. C. Wooltorton. 1971. The threshold value for physiological action of ethylene on apple fruits. J. Fd. Technol. 6:39-45.

15. Hansen, E. 1942. Quantitative study of ethylene production in relation to respiration of pears. Botan. Gaz. 103:543-558.

16. Kidd, F., and C. West. 1933. The influence of the composition of the atmosphere upon the incidence of the climacteric in apples. Ann. Rept. Food Invest. Board, London, pp. 51-57.

17. Kidd, F., and C. West. 1945. Respiratory activity and duration of life of apples gathered at different stages of development and subsequently maintained at a constant temperature. Plant Physiol. 20:467-504.

18. Lyons, J. M., and H. K. Pratt. 1964. Effect of stage of maturity and ethylene treatment on respiration and ripening of tomato fruits. Proc. Amer. Soc. Hort. Sci. 84:491-500.

19. Mapson, L. W., and J. E. Robinson. 1966. Relation between oxygen tension, biosynthesis of ethylene, respiration and ripening changes in banana fruit. J. Fd. Technol. 1:215-225.

20. Mapson, L. W. 1970. Biosynthesis of ethylene and its control. Proc. Conf. Trop. Subtrop. Fruits, Trop. Prod. Inst., pp. 85-92.

21. McGlasson, W. B., and H. K. Pratt. 1964. Effects of ethylene on cantaloupe fruits harvested at various ages. Plant Physiol. 39:120-127.

22. McMurchie, E. J., W. B. McGlasson and I. L. Eaks. 1972. Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature 237:235-236.

23. Meigh, D. F., J. D. Jones, and A. C. Hulme. 1967. The respiration climacteric in the apple. Production of ethylene and fatty acids in fruit attached to and detached from the tree. Phytochemistry 6:1507-1515.

24. Meigh, D. F., and J. Reynolds. 1969. Effect of low concentrations of oxygen on the production of ethylene and on other ripening changes in apple fruit. J. Sci. Fd. Agric. 20:225-228.

25. Palmer, J. K. 1970. The banana. In The Biochemistry of Fruits and Their Products. (A. C. Hulme Ed.), p. 75. (Academic Press, London and New York).

26. Pratt, H. K., and M. Workman. 1962. Studies on the physiology of tomato fruits. III. The effect of ethylene on respiration and ripening behavior of fruits stored at 20°C after harvest. Proc. Amer. Soc. Hort. Sci. 81:467-478.

27. Pratt, H. K., and J. D. Goeschl. 1968. The role of ethylene in fruit ripening. In Biochemistry and Physiology of Plant Growth Substances (Wightman, F. and G. Setterfield, Eds.), pp. 1295-1302. (Runge Press, Ottawa).

28. Wang, C. Y., W. M. Mellenthin, and E. Hansen. 1972. Maturation of 'Anjou' pears in relation to chemical composition and reaction to ethylene. J. Amer. Soc. Hort. Sci. 97:9-12.

SECTION V

INTERNAL ETHYLENE CONCENTRATIONS IN APPLE FRUITS ATTACHED OR DETACHED FROM THE TREE

Abstract. A method is described for the measurement of internal ethylene concentration in fruits on the tree. Internal ethylene concentration was followed in Red Delicious apples attached to or detached from the tree throughout the maturation period. Levels observed in fruits immediately after harvest were similar to those in fruits on the tree. Harvesting fruits stimulated their ethylene production capacity more quickly as the fruits mature on the tree. Internal ethylene concentrations remained low in fruits left on the tree until ripening changes became evident. Isolating the fruit from leaves by girdling and defoliation hastened the increase in internal ethylene concentration. Leaves left above or below the fruit-bearing spur retarded the rise in ethylene, supporting the concept of a ripening inhibitor originating in the leaf.

Introduction

Many fruits enter the climacteric soon after harvest, whereas they might not ripen for a few months if left on the tree (8,11,12,13). This is especially true for most avocado fruits which do not undergo a climacteric or ripen while attached to the tree.

It is commonly agreed that a substance entering the fruit from the tree inhibits the ripening process (5,6,7,10,17). Burg (7) hypothesized that ethylene is rendered ineffective by a ripening inhibitor supplied by the parent plant, and that harvesting the fruit removes it from the source of this inhibitor. Evidence derived from steam girdling experiments with avocados, mangos and other fruits suggested that the inhibitor is transported in the phloem from leaf to fruit (5,6). Mapson and Hulme (17) suggested that the inhibitor either inhibits ethylene production or raises the threshold value at which ethylene becomes physiologically active in promoting ripening.

The present work was undertaken to determine the effects of removal from the tree and branch girdling and or defoliation on the internal ethylene concentrations in apple fruits. A method was developed to monitor the internal ethylene concentration of fruit attached to the tree.

Material and Methods

Various methods have been used for determining the internal ethylene concentration in tissues (2,4,14,15,18), the most common being the withdrawal of internal gas samples with a hypodermic syringe while the fruit is momentarily submerged in water (4,14,15). In other methods, vacuum is applied to the tissue and ethylene is measured in a sample of the extracted air (2,18). Such techniques (3,9,16) have been criticized on

the grounds that the application of vacuum might upset the equilibrium existing between dissolved gases and the gases in the intercellular spaces (1,3). Furthermore the same plant material cannot be used repeatedly with any of the above methods.

In the present study a modification of the technique described by Williams and Patterson (19) was applied to measure the internal ethylene concentration (IEC) of the same fruit either attached to or detached from the tree. The system consisted of a 22 gauge 1 1/2" hypodermis needle fitted with two serum stoppers as shown in Figure 1. The needle was sterilized and inserted into the fruit from the calyx end to avoid excessive wounding and was sealed with silastic rubber (ARTV. Dow Corning) prepared with a nonphytotoxic catalyst (Herter T I, Wacker Chemie GmbH, Munish, Germany). The tip of the needle was bent to prevent clogging.

Red Delicious apples were used from trees at the Horticulture Research Center at Michigan State University. The needles were inserted into the fruit 80-100 days after bloom and remained for the duration of the season. Gas samples were taken every three to four days with hypodermic syringes and ethylene content was determined by gas chromatography with a Varian Aerograph model 1700 equipped with an activated alumina column and a flame ionization detector. The effect of harvest was examined for some of the fruits equipped with needles by leaving the fruits under the tree or bringing them to the laboratory at 23°C.

Figure 1. Method of sampling the internal ethylene concentration in Red Delicious apple attached to the tree.

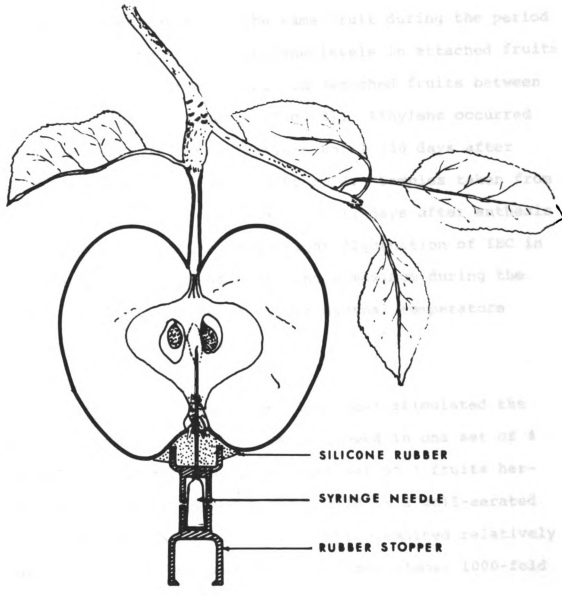

Figure 1

Results and Discussion

Internal ethylene concentration in intact fruit attached to the tree. The technique proved valuable to follow the internal ethylene concentration in the same fruit during the period of maturation on the tree. Ethylene levels in attached fruits agree closely with initial levels in detached fruits between 85 and 150 days after anthesis (Fig. 2). Ethylene occurred at low but measurable concentrations until 130 days after anthesis, then increased about 1000-fold. Samples taken from 4 individual fruits at 4 hr intervals 111 days after anthesis (data not presented) revealed a slight fluctuation of IEC in the fruit with a minimum at night and a maximum during the day. This may have reflected either diurnal temperature changes or a natural rhythm.

Effect of harvest. To determine if harvest stimulated the fruit to produce ethylene, IEC was followed in one set of 4 fruits left on the tree and in another set of 4 fruits harvested 113 days after anthesis and placed in a well-aerated room at 23°C. Following harvest, the IEC remained relatively constant for 1 to 7 days and then increased almost 1000-fold in a 7 to 10 day period (Fig. 3). The difference in lag time may reflect natural differences in maturity or ripening resistance. In contrast, IEC in fruits left on the tree varied from a low of 27 ppb to a high of 776 ppb until 135 days after

Figure 2. Internal ethylene concentrations in Red Delicious apples followed A) by the method with needles attached to the fruit (·-·-·-·-·), and B) by the method of withdrawing a gas sample from harvested fruit submerged in water (————). Values represent means for 4 (attached) or 6 (detached) fruits per sampling date.

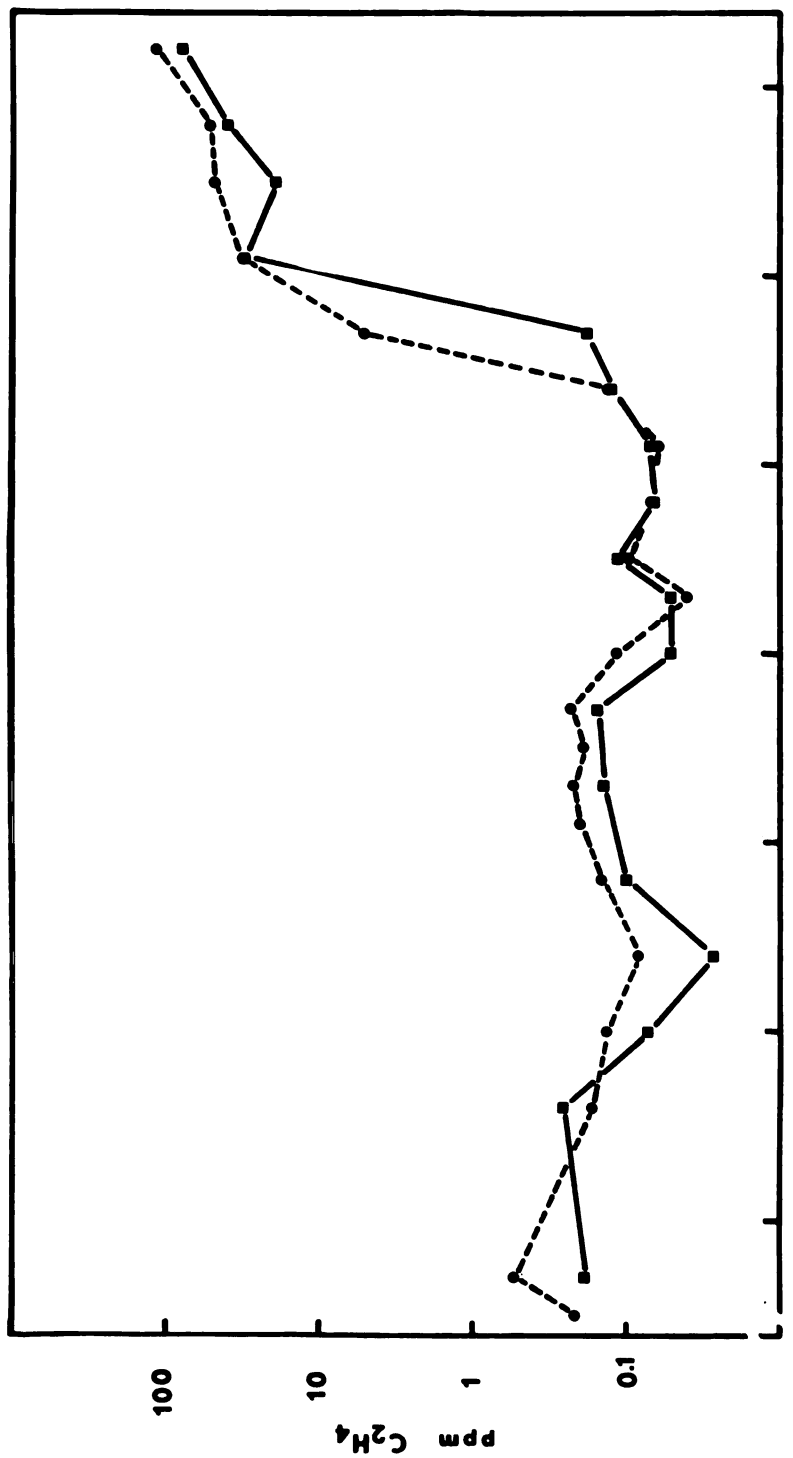
ppm C_2H_4
100
10
1
0.1
90
100
110
120
130
140
150
DAYS AFTER ANTHESIS

Figure 2

Figure 3. Internal ethylene concentrations in four Red Delicious apples left on the tree (A), or harvested 113 days after bloom (B).

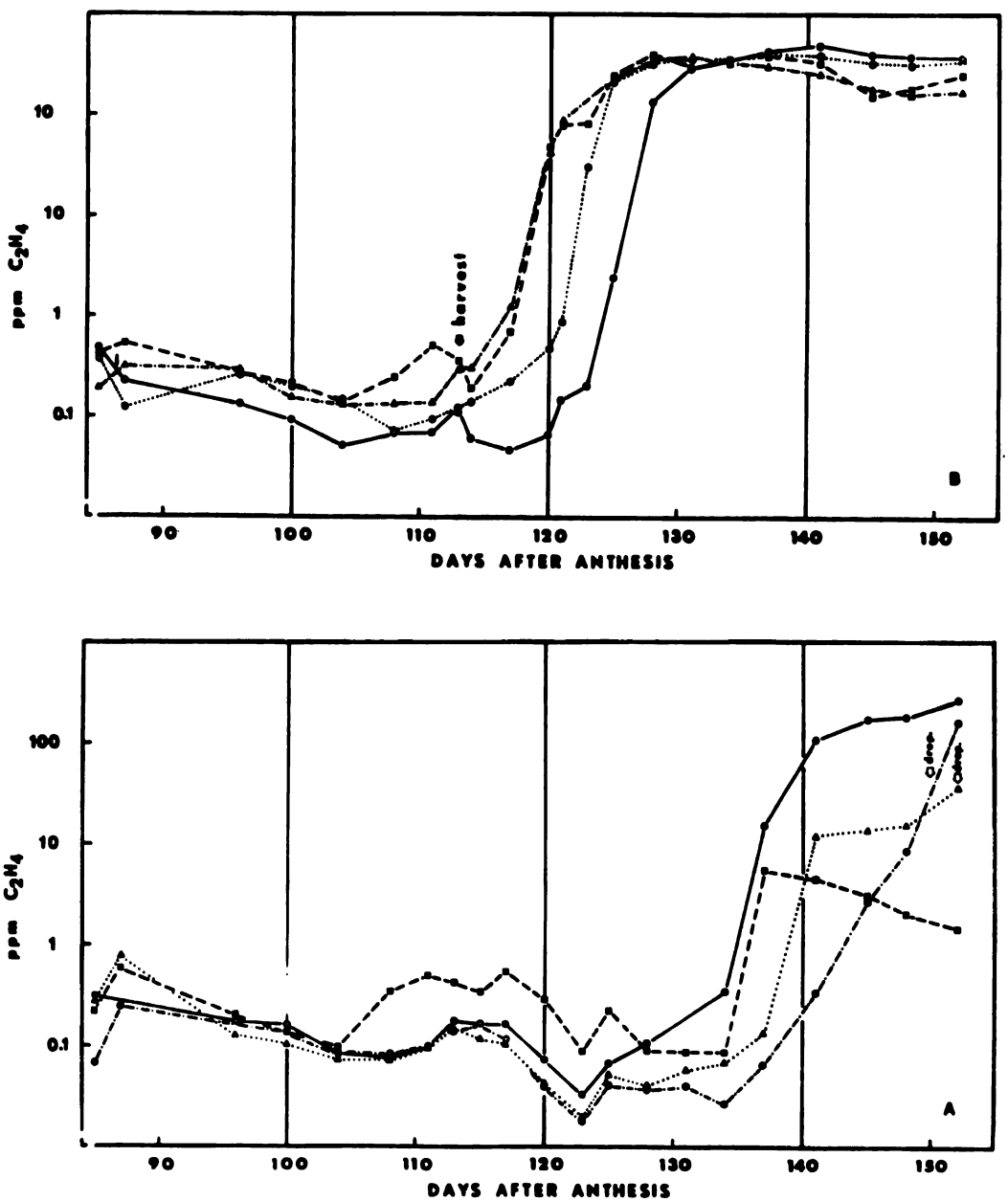
ppm C_2H_4
10
10
1
0.1
harvest
B
90
100
110
120
130
140
150
DAYS AFTER ANTHESIS
ppm C_2H_4
100
10
1
0.1
A
90
100
110
120
130
140
150
DAYS AFTER ANTHESIS

Figure 3

anthesis. Some fruits showed surprisingly high IEC (above 200 ppm) yet did not abscise. The results suggest that an inhibitor supplied by the tree delays autocatalytic ethylene production.

Effect of girdling and defoliation. To determine if the postulated inhibitor originated from the leaves and was translocated through the xylem, 4 treatments were applied 100 days after bloom (Fig. 4). Stems were girdled by removing a section of bark 1.5 cm wide, and gas samples were taken from 4 fruits in each treatment. Complete isolation of the fruit from leaves via phloem transport (D in Fig. 4) hastened ethylene production by 3 weeks, while girdling alone (C) or defoliation alone (B) delayed ethylene production. Both treatments B and C showed high IEC and this discrepancy is not understood.

The data support the concept that a substance originating in the leaves and transported into the fruit through the phloem, prevents autocatalytic ethylene production. The chemical nature of this inhibitor is unknown. The variation between individual fruits, harvested or left on the tree in lag time before accelerated ethylene production (Fig. 3), together with the results of girdling (Fig. 4), suggest that fruits receive differential amounts of the inhibiting substance. The lag in the onset of ethylene production in fruits from defoliated but not girdled limbs (B) compared to fruits

Figure 4. Effect of defoliation and girdling on the internal ethylene concentration in Red Delicious apples.

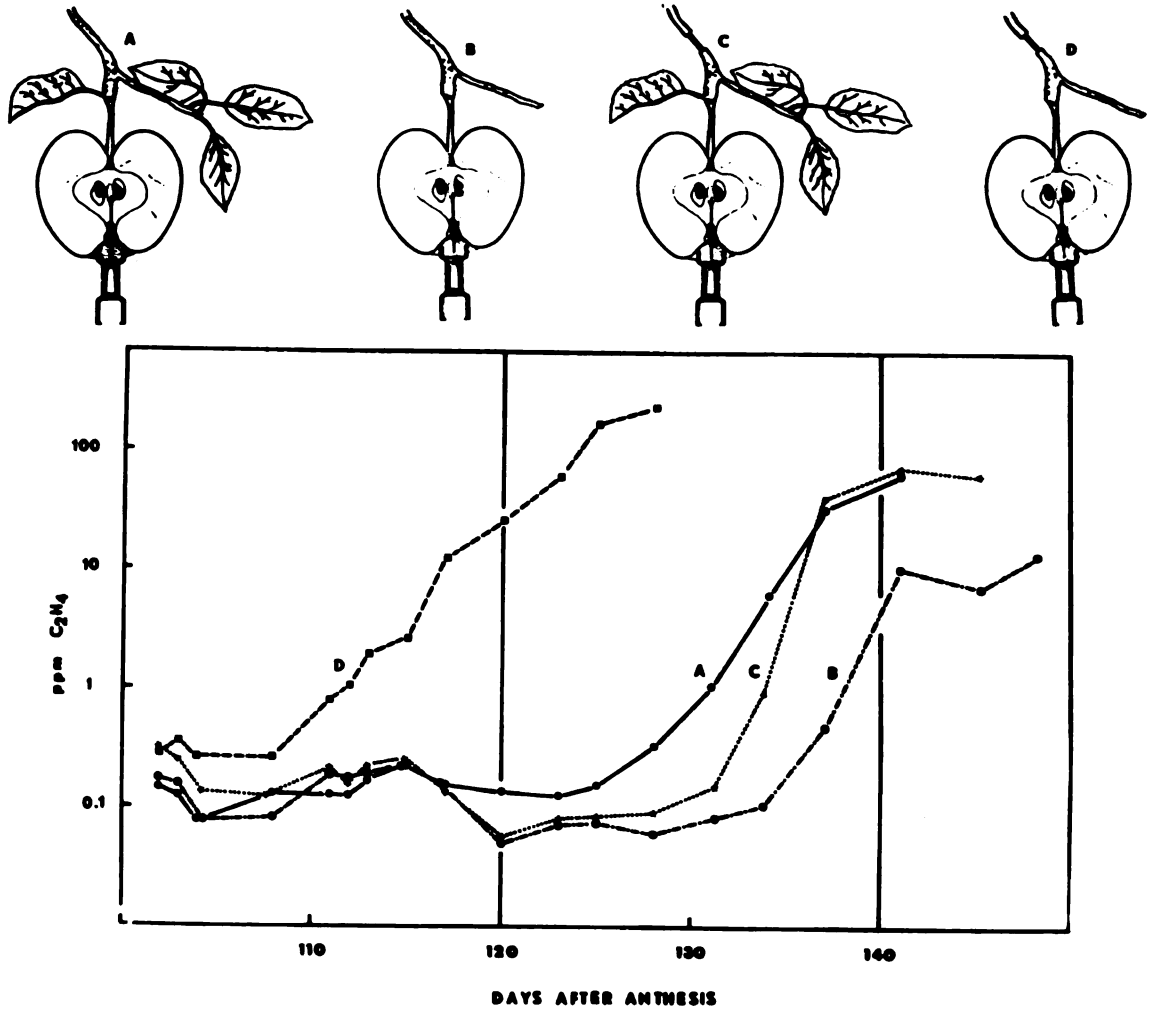

Figure 4

from untreated limbs (A) suggests that availability of substrate (CHO) as well as an inhibitor may be involved in the regulation of ethylene synthesis.

LITERATURE CITED

1. Biale, J. B. 1960. Respiration of fruits. Encycl. Plant Physiol. 12(2):536-592.

2. Blanpied, G. D. 1971. Apparatus for ethylene extraction from plant tissue. HortScience 6:132-134.

3. Brooks, C. 1938. An apparatus for the extraction of internal atmospheres from fruits and vegetables. Proc. Amer. Soc. Hort. Sci. 35:202-203.

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

5. Burg, S. P., and E. A. Burg. 1964. Evidence for a natural occurring inhibitor of fruit ripening. Plant Physiol. 39 (supp.) x.

6. Burg, S. P. 1964. Studies on the formation and function of ethylene gas in plant tissues. In Regulateurs Naturels de la Croissance Végétale (Centre National de la Recherche Scientifique, ed.), pp. 719-728 (Gif s/Yuette, Paris).

7. Burg, S. P. and E. A. Burg. 1965. Ethylene action and the ripening of fruits. Science 48:1190-1196.

8. Burroughs, A. M. 1923. Changes in the respiration rate of ripening apples. Proc. Amer. Soc. Hort. Sci. 19: 225-235.

9. Denny, F. E. 1946. Gas content of plant tissue and respiration measurements. Contrib. Boyce Thompson Inst. 14:257-276.

10. Gazit, S. and A. Blumenfeld. 1970. Response of mature avocado fruits to ethylene treatments before and after harvest. J. Amer. Soc. Hort. Sci. 95:229-231.

11. Gerhardt, F. 1947. A comparison of the ripening of attached and detached apple and pear fruits. Wash. State Hort. Assoc. Proc. 43:57-59.

12. Gore, H. C. 1911. Studies on fruit respiration. U. S. Department Agr. Bur. Chem. Bul. 142, 40 pp.

13. Lewis, C. I., A. E. Murneek, and C. C. Cate. 1919. Pear harvesting and storage investigations in the Rogue River Valley. Oreg. Agr. Expt. Sta. Bul. 162, 39 pp.

14. Lyons, J. M., W. B. McGlasson, and H. K. Pratt. 1962. Ethylene production, respiration, and internal gas concentrations in cantaloupe fruits at various stages of maturity. Plant Physiol. 37:31-36.

15. Lyons, J. M., and H. K. Pratt. 1964. Effect of stage of maturity and ethylene treatment on respiration and ripening of tomato fruits. Proc. Am. Soc. Hort. Sci. 84:491-500.

16. Magness, J. R. 1920. Composition of gases in intercellular spaces of apple and potatoes. Botan. Gaz. 70:308-317.

17. Mapson, W. and A. C. Hulme. 1970. The Biosynthesis, Physiological Effects, and Mode of Action of Ethylene. In Progress in Phytochemistry, Vol. 2 (Reinhold, L. and Y. Liwschitz, eds.), pp. 343-384 (Interscience Publishers, London).

18. Staby, G. L. and A. A. De Hertogh. 1970. The detection of ethylene in the internal atmosphere of bulbs. HortScience 5:399-400.

19. Williams, M. W. and M. E. Patterson. 1963. Internal atmospheres in Bartlett pears stored in controlled atmospheres. Proc. Amer. Soc. Hort. Sci. 81:129-136.

APPENDIX

APPARATUS TO TREAT PLANT MATERIAL WITH GASES AND MEASURE CO_2 AND ETHYLENE PRODUCTION

Abstract. A simple static system is described for treating plant material with olefinic gases and measuring respiration and or ethylene production at a constant O_2 level. The procedure measures CO_2 production by absorption and titration of an alkali scrubber and ethylene by sampling the gas phase of the chamber in which the plant material is enclosed.

Several systems have been devised to treat plant material with gases and measure respiration and ethylene production. The tissue is placed either in sealed containers (2,4,6,7,16, 20) or in ventilated chambers (9,10,15). In the sealed containers CO_2 and ethylene production is measured by withdrawing samples from the gas phase. Monitoring the composition of O_2 and CO_2 in this system is difficult and the method has been used mainly for short term experiments. In ventilated chambers the treatments are applied either by using premixed gases in cylinders (3,18) or by mixing gases in proper proportions in a continuous flowing system (21). CO_2 and ethylene production is measured by sampling the gas stream leaving the chambers. The method offers certain advantages over the sealed

containers. Monitoring the gases is accurate and is considered to be the most appropriate method for measuring rates of emanation of CO_2 or ethylene. However, when CO_2 or ethylene is produced in small amounts the method has to be modified by passing the effluent through an alkali scrubber (1,5) to trap CO_2 or through a mercuric perchlorate absorber (17,24) to remove and measure ethylene. Although ventilated chambers have been the most widely used method for many years, an elaborate apparatus to control flow rates, and an appropriate method to scrub and release ethylene are required. A simple apparatus is described for treating plant material with gases and/or measuring CO_2 and ethylene production.

The system was used by Magness and Diehl (11) and modified by Haller and Rose (8) and by Platenious (14). It is essentially a combination of the apparatus used for determination of CO_2 and O_2 and the Mariotte bottle system with minor modifications. The plant tissue is placed in a chamber (Fig. 1 A) connected to an oxygen bottle (B), which in turn is attached to the Mariotte bottle (C). The O_2 inlets are fitted with small vials containing a saturated solution of ammonium sulfate to prevent backward diffusion of gases into the O_2 supply bottle. As an extra safety for ethylene treatments or measurements and to avoid possible contamination from the oxygen supply, an ethylene absorbent is placed in the O_2 inlet. A manometer is connected to the O_2 container to indicate the

Figure 1. Apparatus for measuring CO_2 and ethylene production of plant material under constant O_2 concentration.

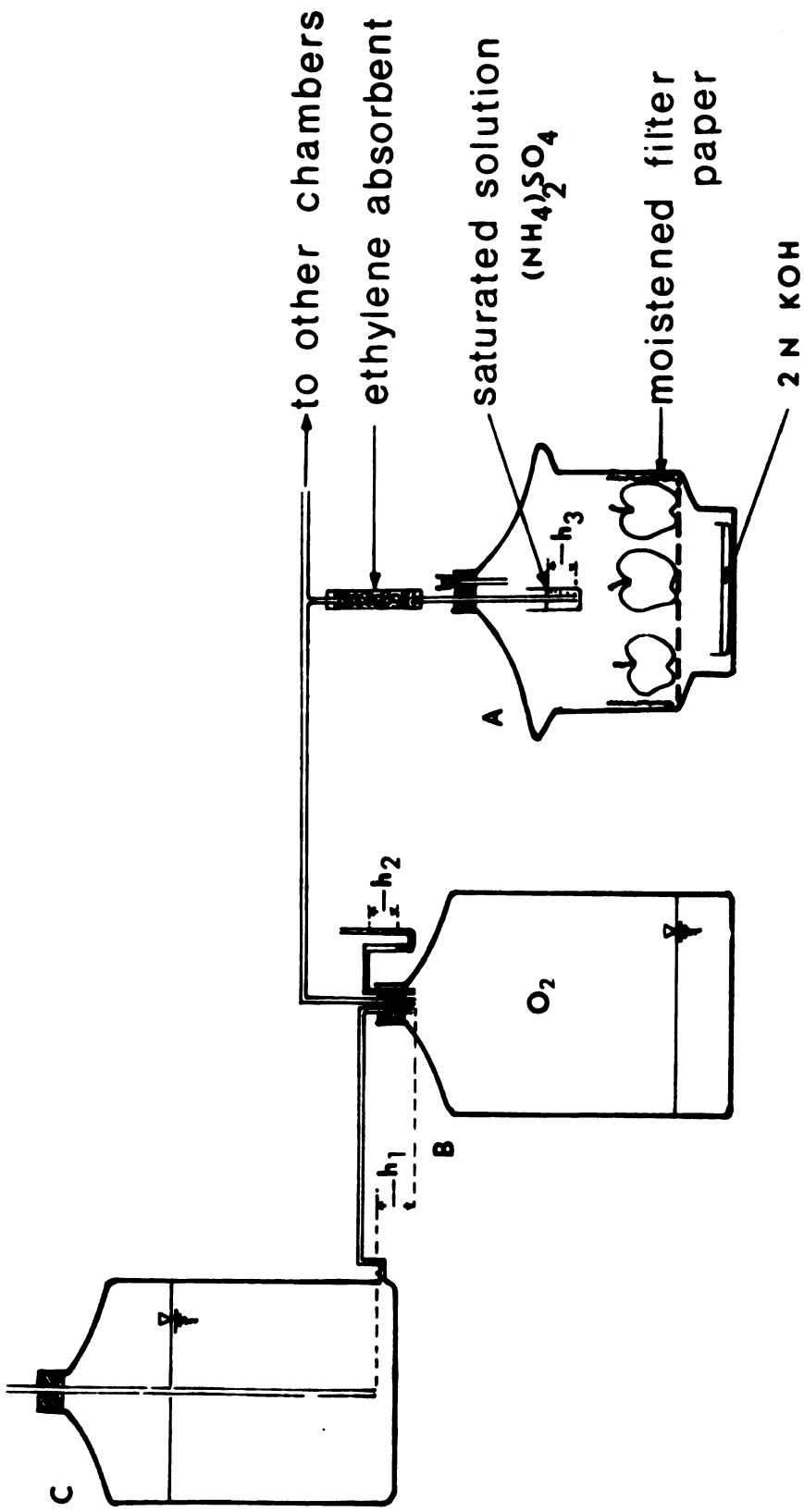
to other chambers
ethylene absorbent
saturated solution
$(NH_4)_2SO_4$
moistened filter paper
2 N KOH
h_3
h_2
h_1
O_2
A
B
C

Figure 1

pressure (h_1) necessary to overcome the pressure (h_3) in the respiratory chambers. This can be controlled by raising the water reservoir (C) and increasing the height (h_2) of the water level at the ambient atmospheric pressure. One Mariotte bottle system can be used for a set of as many as 20 respiratory chambers.

As the plant material respires, CO_2 is given off and O_2 is absorbed. The CO_2 is absorbed by the 2N KOH solution in the petri dish and the O_2 used is replaced by O_2 from the oxygen supply bottle (B). The O_2 withdrawn from the bottle is replaced by water from the water reservoir (A). Thus the system remains very close to atmospheric pressure and the concentration of O_2 in the chamber remains practically unchanged. The system can also be used for O_2 concentration studies since the initial oxygen concentration is maintained during the course of the experiment. Samples can be withdrawn from the chamber and analyzed in a gas chromatograph for ethylene. The system may be opened and aerated every one or two days to avoid excessive accumulation of ethylene in the chamber.

The same system was used for respiration measurements. The CO_2 trapped in the 2N KOH solution was determined by titration with standard 0.5 N or 1 N HCl to the phenolphthalein end point and then to the methyl orange end point (22). In a second method, carbon dioxide was determined by adding excess 1 N $BaCl_2$ and titrating the excess alkali against 0.5 N HCl to the phenolphthalein end point (23). Evaluation of the two

methods showed that the two indicator method was more reliable (Fig. 2).

The amount of sample in the respiratory chamber should be sufficient to produce measurable amounts of ethylene without accumulation of physiologically active levels over a period of one or two days. Enough KOH should be present so that only a third of the alkali is neutralized by the CO_2 evolved during a run. If more than half the KOH is neutralized, the phenolphthalein end point in the titration becomes indistinct. Changes in temperature should be avoided as the resulting pressure changes will affect the O_2 supply.

This procedure was used to determine ethylene production of preclimacteric apples (19 and Fig. 3), and ethylene and CO_2 production of carnation flowers (Fig. 4). Values obtained with carnations are close to those reported by Nichols (13). The same apparatus was used to treat dormant cherry twigs with ethylene to break bud dormancy and measure respiration rate (Fig. 5).

Figure 2. Standard curves obtained from the $BaCl_2$ and the two indicator titration methods for CO_2 determination. Known amounts of CO_2 were injected into closed containers with KOH. The data were corrected for STP conditions.

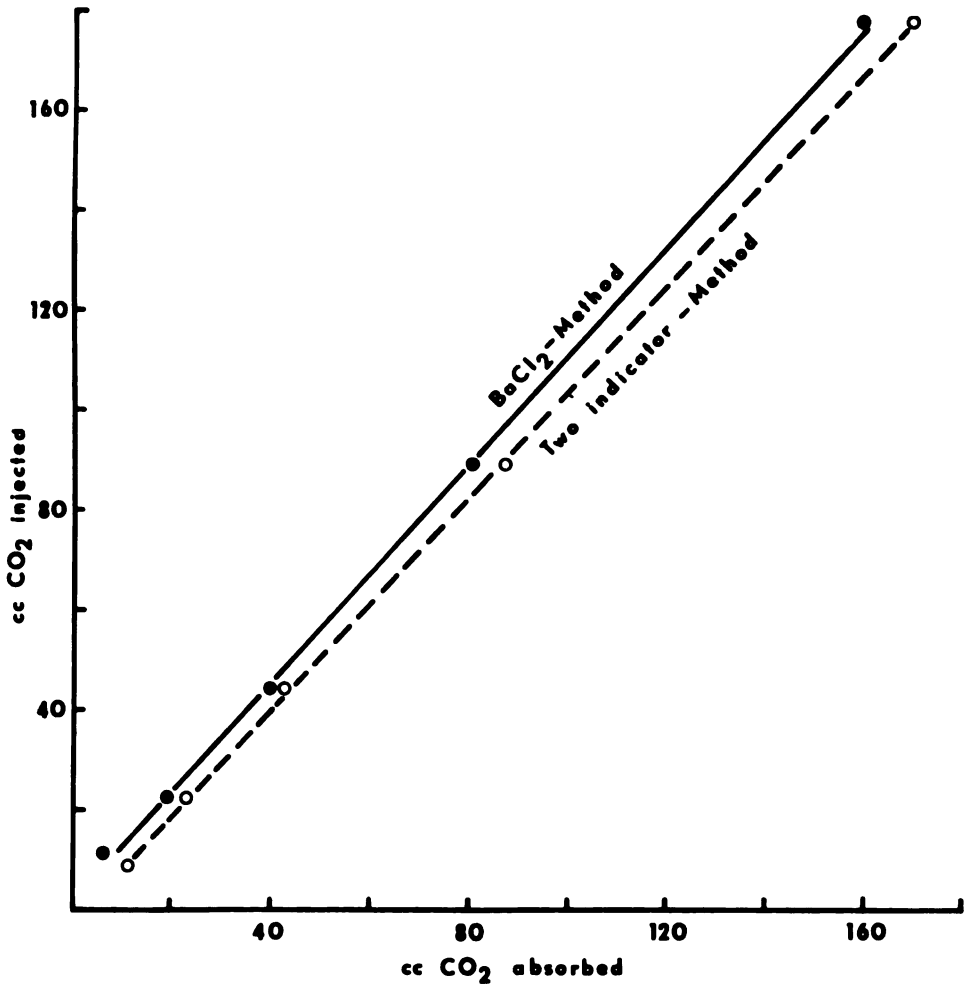
160
120
80
40
cc CO_2 injected
$BaCl_2$-Method
Two indicator - Method
40
80
120
160
cc CO_2 absorbed

Figure 2

Figure 3. Stimulation of ethylene production by propylene in Red Delicious apples treated 126 days after anthesis. The data indicate rate of ethylene production the 6th day after propylene treatment was started.

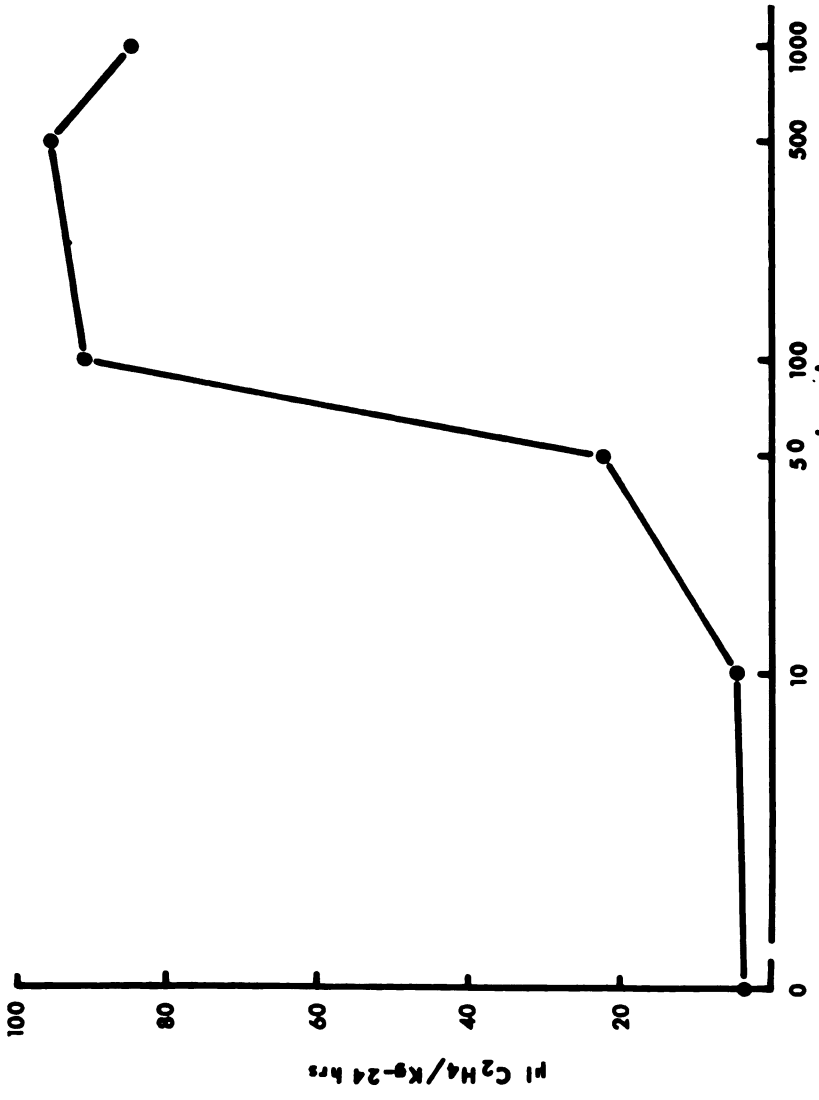

Figure 3

Figure 4. CO_2 and ethylene production by carnation flowers. Three flowers were cut at the tight bud stage and placed in preservative solution (200 ppm 8-hydroxyquinoline and 1.5% sucrose) in each chamber. The data represent the averages of measurements from four replications.

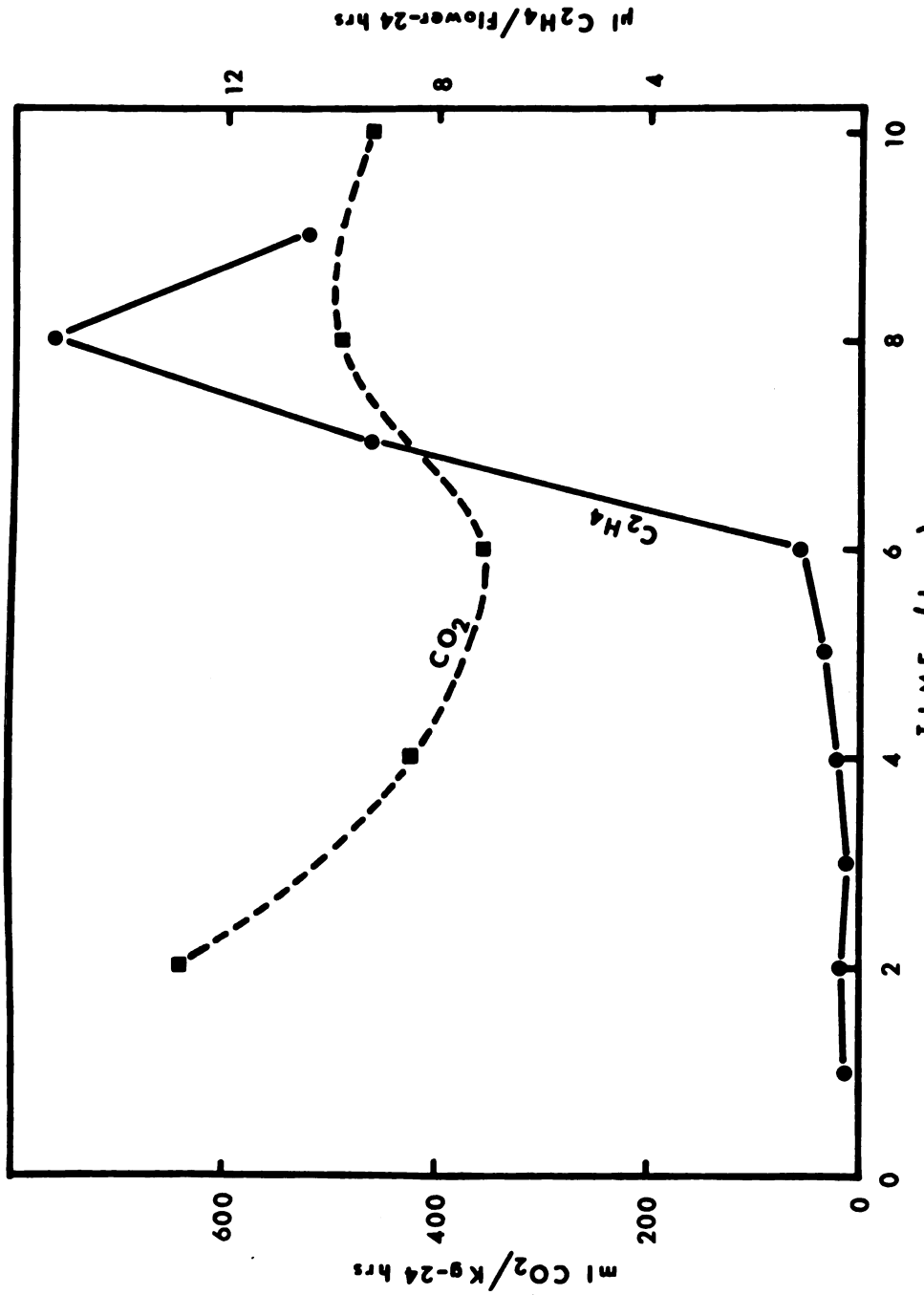
ml CO2/kg-24 hrs
0
200
400
600
pl C2H4/flower-24 hrs
4
8
12
0
2
4
6
8
10
TIME (days)
C2H4
CO2

Figure 4

Figure 5. Effect of ethylene on respiration of dormant cherry twigs collected from orchard on December 21, 1971.

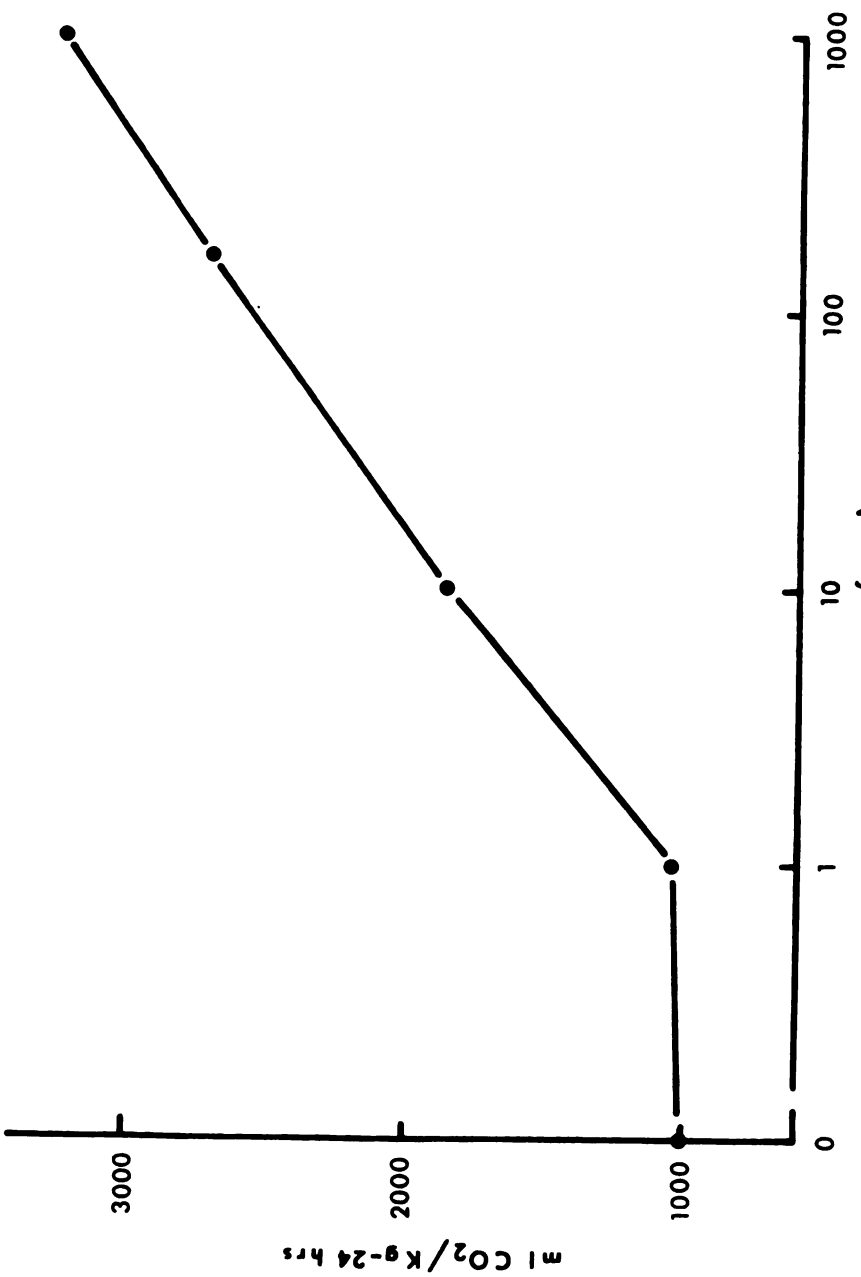

Figure 5

LITERATURE CITED

1. Biale, J. B. 1946. Effect of oxygen concentration on respiration of the fuerte avocado fruits. Amer. J. Bot. 33:363-373.

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

3. Byers, R. E., L. R. Baker, H. M. Sell, R. C. Herner, and D. R. Dilley. 1972. Ethylene: A natural regulator of sex expression of Cucumis melo L. Proc. Nat. Acad. Sci. 69:717-720.

4. Chadwick, A. V. and S. P. Burg. 1970. Regulation of root growth by auxin-ethylene interaction. Plant Physiol. 45:192-200.

5. Claypool, L. L., and R. M. Keefer. 1942. A colorimetric method for CO_2 determination in respiration studies. Proc. Amer. Soc. Hort. Sci. 40:177-186.

6. Eaks, I. L. 1970. Respiratory responses, ethylene production, and response to ethylene of citrus fruit during ontogeny. Plant Physiol. 45:334-338.

7. Fuchs, Y. and M. Lieberman. 1968. Effects of kinetin, IAA, and gibberellin on ethylene production, and their interactions in growth of seedlings. Plant Physiol. 43:2029-2036.

8. Haller, M. H. and D. H. Rose. 1932. Apparatus for determination of CO_2 and O_2 of respiration. Science 75:439-440.

9. Looney, N. E. 1969. Control of apple ripening by succinic acid 2,2-dimethylhydrazide, 2-chloroethyl trimethyl ammonium chloride and ethylene. Plant Physiol. 44:1127-1131.

10. Lyons, J. M. and H. K. Pratt. 1964. Effect of stage of maturity and ethylene treatment on respiration and ripening of tomato fruits. Proc. Amer. Soc. Hort. Sci. 84:491-500.

11. Magness, J. R., and H. C. Diehl. 1924. Physiological studies on apples in storage. J. Agricult. Res. 27:1-38.

12. Maxie, E. C. and J. C. Crane. 1967. 2,4,5-Trichlorophenoxyacetic acid effect on ethylene production by fruits and leaves of fig tree. Science 155:1548-1550.

13. Nichols, R. 1968. The response of carnations (Dianthus caryophyllus) to ethylene. J. Hort. Sci. 43:335-349.

14. Platenious, H. 1942. Effect of temperature on the respiration rate and the respiratory quotient of some vegetables. Plant Physiol. 17:179-197.

15. Pratt, H. K., M. Workman, F. W. Martin, and J. M. Lyons. 1960. Simple method for continuous treatment of plant material with metered traces of ethylene or other gases. Plant Physiol. 35:609-611.

16. Rasmussen, G. K., J. R. Furr and W. C. Cooper. 1969. Ethylene production by citrus leaves from trees grown in artificially salinized plots. J. Amer. Soc. Hort. Sci. 94:640-641.

17. Sakai, S. and H. Maseki. 1970. Quantitative determination of ^{14}C-ethylene produced by plant tissues. Agr. Biol. Chem. 34:1584-1587.

18. Sfakiotakis, E. M., D. H. Simons, and D. R. Dilley. 1972. Pollen germination and tube growth: Dependent on carbon dioxide and independent of ethylene. Plant Physiol. 49:963-967.

19. Sfakiotakis, E. M. and D. R. Dilley. 1972. Stimulation of ethylene production in apple fruits by propylene. HortScience 7:325.

20. Suge, H. 1971. Stimulation of oat and rice mesocotyl growth by ethylene. Plant and Cell Physiol. 12:831-837.

21. Wang, C. Y., W. M. Mellenthiu, and E. Hansen. 1972. Maturation of 'Anjou' pears in relation to chemical composition and reaction to ethylene. J. Amer. Soc. Hort. Sci. 97:9-12.

22. Warder, R. B. 1881. Alkalimetry with phenolphthalein as indicator. Am. Chem. J. 3:55-232.

23. Winkler, C. 1902. "Praktische Übungen in der Massanalyse," 3rd ed., Akademishe Verlagsgesellschaft, Leipzig.

24. Young, R. E., H. K. Pratt, and J. B. Biale. 1952. Manometric determination of low concentrations of ethylene. Anal. Chem. 24:551-555.