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The Effect of Hypobaric Storage and Temperature on Ethylene Biosynthesis and Changes in Protein Synthesis During Apple (Malus sylvestris, L.) Ripening

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Major professor

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THE EFFECT OF HYPOBARIC STORAGE AND TEMPERATURE ON ETHYLENE BIOSYNTHESIS AND CHANGES IN PROTEIN SYNTHESIS DURING APPLE (*MALUS SYLVESTRIS* L., cv. 'MUTSU') RIPENING

By

Liming Li

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ABSTRACT

THE EFFECT OF HYPOBARIC STORAGE AND TEMPERATURE ON ETHYLENE BIOSYNTHESIS AND CHANGES IN PROTEIN SYNTHESIS DURING APPLE (Malus sylvestris L., cv. 'Mutsu') RIPENING

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Preclimacteric apple fruits were ripened at 20°C in air immediately after harvest or following hypobaric storage (0°C at 0.05 atm) for 4, 6 or 9 months. Factors investigated included the effects of hypobaric storage and low temperature on development of ethylene biosynthesis.

Following 6 month's hypobaric storage ethylene production and EFE activity in preclimacteric apples increased sharply in fruits held in air at 20°C but remained minimal in fruits held at 0°C for 16 days. 1-aminocylcopropane-1-carboxylic acid (ACC) content gradually accumulated in apples held at 0°C. The results suggest that the rate limiting step in ethylene production in preclimacteric apples stored at 0°C is the conversion of ACC to ethylene.

Two-dimensional polyacrylamide gel electrophoresis of 'Mutsu' fruit proteins revealed that nineteen polypeptides underwent changes in concentration during the ripening process indicating that protein synthesis and degradation are involved in apple ripening.

To my son, Xiaofeng

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major professor, Dr. D. R. Dilley, for his counsel and assistance throughout the course of this work. Appreciations are also expressed to Dr. J. F. Hancock, and Dr. M. Thomashow for serving as my committee members.

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LIST OF ABBREVIATIONS

1. ACC: 1-aminocyclopropane-1-carboxylic acid
2. AOA: aminooxyacetic acid
3. AVG: aminoethoxyvinylglycine
4. EFE: ethylene forming enzyme
5. IAA: indole-3-acetic acid
6. IE: internal ethylene
7. MTA: 5'-methylthioadenosine
8. MTR: 5'-methylthioribose
9. SAM: s-adenosyl methionine
10. SDS: sodium dodecyl sulfate

INTRODUCTION

It is well documented that ethylene plays an important role in initiating ripening of climacteric fruits. In apples the onset of ripening is marked by a sharp increase in ethylene production. Techniques which serve to delay the initiation of ethylene production or retard the rate of ethylene biosynthesis would therefore delay or retard fruit ripening. Hypobaric storage, first introduced by Burg and Burg in 1966 in which the partial pressures of ethylene, oxygen and certain other gaseous metabolic waste products are maintained at very low level, is currently the most advanced technology available for extending the storage life of apples. Moreover, the onset of ethylene production of long-term hypobarically stored fruits is significantly delayed compared to non-stored fruit.

Low temperature decreases both of the rate of ethylene biosynthesis and the sensitivity of fruit to ethylene. The behavior of ACC synthase and EFE at low temperature seems different among different species. The rate limiting reaction in the ethylene biosynthesis pathway of apples at low temperature remains unknown.

Significant changes in both physiology and biochemistry follow the initiation of ethylene production by apples. It

is believed that the enzymatic reactions involved in fruit ripening are under the control of ethylene. Protein profile of one dimensional electrophoresis gels undergoes changes during apple ripening. There has been no protein profile obtained by two dimensional electrophoresis from apples during ripening to date.

The first objective of the thesis was to determine the effects of low temperature (0°C) and hypobaric storage on ethylene biosynthesis. The second thesis objective was to establish and analyze differences between two dimensional polypeptide maps of apples obtained at four ripening stages.

LITERATURE REVIEW

A: HYPOBARIC EFFECT ON ETHYLENE BIOSYNTHESIS

Hypobaric storage (subatmospheric storage, low pressure atmosphere storage) invented by Burg and Burg (1966) in 1965 provided the most significant advance in postharvest preservation technology since controlled atmosphere storage was introduced in England in the 1930s. The storage life of a wide range of fresh fruits, vegetables, and cut flowers can be extended beyond that achieved by conventional storage methods. Hypobaric storage was also found to markedly extend the storability of fresh meats, poultry and fish.

According to Dalton's law, the total gaseous pressure of a system is the sum of the partial pressures of its component gases. Thus as the pressure of a sealed chamber is lowered, the partial pressure of each gaseous component within the chamber is proportionally reduced. The reduction of the partial pressure of oxygen leads to the inhibition of both respiration and the biosynthesis and action of ethylene by plant tissues since these are all oxygen dependent process. Moreover, the rate of diffusion of ethylene and other volatiles is inversely related to the absolute pressure of the system. This reduces the equilibrium concentration of ethylene, oxygen, carbon dioxide and other

volatile metabolic gases thereby extending the storage life of many plant tissues. It has been suggested that the hypobaric effect is a multiple effect of reduction in ethylene, oxygen, and total pressure rather than simply a low oxygen effect (Burg and Burg, 1966; Dilley, 1982; Dilley et al., 1982). This hypothesis has been confirmed by the observations that hypobarically stored fruits gain the capacity to produce ethylene more slowly than fruits from low oxygen CA storage at an equivalent oxygen partial pressure. The activity of malic enzyme, a ripening related enzyme, is likewise reduced by hypobaric storage. The role of the verified atmosphere in promoting diffusion of gases from the tissues was confirmed in experiments where helium was substituted for nitrogen in experiments conducted at equivalent oxygen partial pressures at normal atmospheric pressure (Dilley et al., 1982).

The effect of hypobaric storage on ethylene biosynthesis has been investigated by a number of researchers. Dilley (1982) reported that the rate of ethylene production by hypobarically stored apples was initially much lower than that of apples stored in air or in controlled atmospheres upon transferring the fruits to air at 20°C. Hypobarically stored apples eventually regained a normal rate of ethylene production. In some instances hypobarically stored apples were found to ripen more slowly at 20°C in air storage than samples of the same

fruits did immediately following harvest (Dilley, 1982). In these instances it was observed that normal ripening was achieved when fruits were returned to air at 0°C for several weeks prior to ripening them at 20°C. These results indicate that the ability of apples to synthesize and respond to ethylene can be altered by hypobaric storage. Bufler and Bangerth (1983) observed that apples stored at 4°C and 6.6 kpa (0.065 atm) did not ripen for 4 months and that ACC synthase activity and ethylene production were not induced. ACC synthase activity and ethylene production, however, began to increase rapidly when apples were transferred to 20 kpa (0.197 atm) and propylene appeared to promote the increase of ACC synthase activity and ethylene production after the transfer. Rapid increase in ACC synthase activity, ACC content and ethylene production also occurred when apples were stored at 4°C, 6.6 kpa and ventilated with oxygen or ethylene. These results indicate that both oxygen and ethylene play important role in ripening. The authors suggest that a ripening factor (inhibitor or promotor) may be influenced by oxygen and ethylene/propylene resulting in the onset of ripening; induction of ACC synthase activity and consequently autocatalytic ethylene production.

It has been suggested that the optimum absolute pressure varies according to the commodity but ranges generally from 0.013 to 0.1 atmosphere. The optimum

conditions for the hypobaric storage of apples is 0°C, 0.05 atm with exception of 'McIntosh' and perhaps other chilling sensitive varieties which require 3 to 4°C during storage.

B: THE PATHWAY OF ETHYLENE BIOSYNTHESIS BY PLANT TISSUE

Plant physiologists had searched many years for the precursors of ethylene in higher plants before Liebermann et al. reported that methionine seemed to be the best candidate (Liebermann, 1979). Direct evidence in support of the role of methionine as an ethylene precursor in vivo was first reported by the same workers who found that labeled methionine was efficiently converted to ethylene by apple fruit tissue. The ethylene was derived from C-3,4 of methionine (Liebermann et al., 1966). These findings were confirmed by the observations that the ability of plant tissues to convert methionine into ethylene parallels their ability to produce ethylene endogenously and that the specific radioactivity of ethylene recovered approached that of the administered methionine (Liebermann, 1979). These results indicated that methionine is directly in the biosynthetic pathway for ethylene.

Burg (1973) and Murr and Yang (1975) suggested that s-adenosyl methionine (SAM) may be an intermediate in ethylene biosynthesis. It was reasoned that 5'-methylthioadenosine

(MTA) would be a degradation product if ethylene is derived from SAM (Adams and Yang, 1977). These speculations were later borne out by the experimental evidence that MTA and 5'- methylthioribose (MTR, hydrolytic product of MTA) were formed from methionine in parallel with ethylene production when labeled methionine was fed to apple tissue.
The intermediate between SAM and ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), was identified by Adams and Yang (1979) who compared the metabolism of methionine by apples in air and in a nitrogen atmosphere. They found that methionine was efficiently converted to ethylene in air but in nitrogen it was metabolized to MTR and ACC. In apple tissue the pathway of ethylene biosynthesis has been established as follows:

Methionine --> SAM --> ACC --> ethylene

Since SAM is an intermediate for many metabolic processes great attention has been given to the reactions from SAM to ethylene by plant hormone researchers.

ACC synthase, the enzyme which converts SAM to ACC, requires pyridoxal phosphate and the activity can be strongly inhibited by aminoethoxyvinylglycine (AVG), aminooxyacetic acid (AOA) but stimulated by indole-3-acetic acid (IAA), ripening and stresses (Adams and Yang, 1979; Boller et al., 1979; Young and Meredith, 1971; Yu et al.,

1979). ACC synthase in tomatoes (Boller et al., 1979) is water soluble, however, no extractable ACC synthase activity has been found in extracts of apples without the addition of a detergent to the extraction medium (Bufler and Bangerth, 1983).

Ethylene forming enzyme (EFE) catalyzes the conversion of ACC to ethylene (Kende et al., 1985). It has been suggested that EFE is a highly structured enzyme requiring the maintenance of membrane integrity for its activity since various lipophilic compounds such as phosphatidylcholine, Tween 20 and Triton X-100 and osmotic shock treatment, all of which could modify membrane structure, greatly reduce the rate of ethylene synthesis in plant tissues (Apelbaum et al., 1981; Mayne and Kende, 1986). The conversion of ACC to ethylene is also an oxygen dependent (Adams and Yang 1979) and heat sensitive process (Field, 1981$_b$; Yu et al., 1980). To date EFE has not been isolated and purified. EFE activity can be only determined <u>in vivo</u> by measuring the conversion of administered ACC to ethylene.

C: LOW TEMPERATURE EFFECT ON ETHYLENE BIOSYNTHESIS

Field (1981$_b$) summarized the low temperature effect on plants in three categories:

(1): a non-damaging reduction in temperature,

(2): a response to chilling in those species that are

sensitive to temperature below 10-12°C but above 0°C, (3): the effect of a short or long duration of exposure to a freezing temperature of 0°C or below. All three categories of low temperature effect ethylene biosynthesis by plant tissue.

The effect of a non-damaging reduction in temperature on the rate of ethylene production by plant tissue can be generally considered as a thermal effect. Saltveit and Dilley (1978) reported that lowering the incubation temperature below the growing temperature of 24°C markedly reduced ethylene production and delayed the detection of a wound ethylene peak in pea epicotyls. When the temperature was increased from 10 to 25°C there was approximately a ten-fold increase in ethylene production from 40 to 400 pmol $g^{-1}h^{-1}$. When leaf discs cut from primary leaves of Phaseolus vulgaris L. grown at 25°C were incubated at temperatures below 25°C, basal and wound ethylene production continued at reduced rates (Field 1981_a). Similar activation energies for ethylene biosynthesis of dwarf bean leaf discs (Field 1981_a), etiolated pea stem sections (Saltveit and Dilley, 1978) and apple fruit cortical tissue (Hoffman and Yang, 1980) were obtained over a similar temperature range of 11.4°C to 30°C, corresponding to Q_{10} values ranging from 1.7 to 2.8.

The lowest temperature of detectable ethylene production varies over a wide range in different plant

species. Ethylene production by cucumbers (Wang and Adams, 1982a) and honeydew melons (Lipton and Wang, 1987) was not detectable when fruits were held at a chilling temperature (below 10 -12°C) but increased markedly when they are transferred to warm temperature. Leaf discs from Phaseolus vulgaris L. and cut carnation flowers were found to produce ethylene at 2.5°C and 1.6°C, respectively (Field, 1981a; Nichols, 1966). Most plants produce negligible amounts of ethylene at 0°C (Field, 1981b). Pome fruits such as apples and pears gain capacity to produce ethylene at 0°C.

The induction of ethylene biosynthesis by cold stress has been reported by several of researchers(Wang and Adams, 1982a; Wang and Adams, 1982b; Field, 1985). Ethylene production of cucumbers that had been exposed to chilling temperature of 2.5°C for one week increased sharply after the fruits were transferred to warm temperature while that of cucumbers held continuously at 13°C was negligible(Wang and Adams, 1982a). The ripening of 'Bosc' pear requires a certain period of cold exposure to gain the capacity to produce ethylene (Sfakiotakis and Dilley, 1974) which in turn initiates other ripening reactions upon transfer to optimal temperature for ripening.

The effect of freezing temperature on ethylene production is poorly documented (Field, 1985). Cell damage associated with freezing increases ethylene production of adjacent undamaged cell (Elstner and Konze, 1976; Kimmerer

and Kozlowski, 1982). Young and Meredith (1971) reported that exposure of citrus leaves to subfreezing temperatures induced ethylene production at rates between 0.1 to 38.3 μl $kg^{-1}h^{-1}$. The induction of high ethylene levels was associated with freeze-injury and abscision of the leaves. The author suggested that the freeze-induced ethylene is involved with freeze-induced leaf abscision but the induction of ethylene production was not the only requirement for leaf abscision. Here again, the increase in ethylene production occurred only after the tissue was warmed. It has been suggested that cold or freezing exposure activates the gene coded for ACC synthase in plant tissue but the translation of ACC synthase only occurs at warm temperatures (Wang and Adams, 1982$_b$). However, no evidence has been gathered to support this hypothesis.

When ethylene production data are presented in the form of an Arrhenius plot, which typically shows a discontinuity at the critical temperature leading to two different activation energies for a given process, there is a marked discontinuity at 11.4°C for dwarf bean (Field 1981$_a$), and at 12°C for tomato (Mattoo et al., 1977). Non-chilling sensitive plants such as apple (Apelbaum et al., 1981) and pea (Saltveit and Dilley, 1978) also demonstrate similar temperature breaks. Though it has been suggested that discontinuous Arrhenius plots need not indicate a membrane phase change in chilling-sensitive species these results

demonstrate that there is a pronounced change in ethylene synthesis at a critical temperature, supporting the hypothesis that a membrane-bound enzyme may be involved in the biosynthesis of ethylene (Field, 1981b).

D. HIGH TEMPERATURE EFFECT ON ETHYLENE PRODUCTION

The effect of high temperature on ethylene production is well documented. The optimum temperature for ethylene biosynthesis in most species is 30 to 35°C (Field, 1981b). Above the optimum temperature, ethylene production abruptly decreases and stops at approximately 40°C (Field, 1981b; Saltveit and Dilley, 1978; Yu et al., 1980). Saltveit and Dilley (1978) reported that ethylene production is strongly suppressed at temperature above 38°C in excised segments of etiolated pea seedlings (Pisum sativum L.). Field (Field, 1981b) observed that both basal and wound ethylene production by leaf discs cut from primary leaves of Phaseolus vulgaris L. increased up to temperature of 35 to 37.5°C and then declined rapidly. There was no detectable ethylene production at temperature above 42.5°C. Moreover, there was a marked increase in electrolyte leakage corresponding to the reduction in ethylene production between 37.5°C and 42.5°C. These results suggested that high temperature inhibition of ethylene biosynthesis may be associated with membrane integrity. Studying high

temperature effects on ethylene production by mung bean hypocotyl sections, Horinchi and Imaseki (1986) showed that both IAA-induced and ACC-dependent ethylene biosynthesis were almost completely suppressed at 42.5°C. Maxie et al. (1974) pointed out that the suppression of ethylene production at high temperature is responsible for the failure of 'Bartlett' pear to ripen at high temperature.

Inhibition of ethylene production at high temperature has been reported to be a reversible process for apple (Burg and Thimann, 1959), banana (Burg and Burg, 1962) and leaf tissue of Phaseolus vulgaris L. (Field, 1981b). These results indicate that high temperature does not damage the ethylene biosynthetic system permanently. Field (1981b) suggested that high temperature causes perturbation of membranes by altering the conformation of membrane-bound enzymes or other factors and therefore inhibits ethylene production. Those changes are reversible when the temperature is lowered from the inhibitory range.

E. THE INFLUENCE OF TEMPERATURE ON THE PATHWAY OF ETHYLENE BIOSYNTHESIS

It has long been known that temperature has a great influence on ethylene production in plant tissue. With the establishment of the pathway of ethylene biosynthesis by Adams and Yang (1979) plant researchers have had the

opportunity to determine which steps in the pathway are temperature-sensitive and to further understand the mechanism of ethylene regulation of ethylene by plant tissue under temperature stresses.

Yu et al. (1980) observed that the inhibition of ethylene production by hypocotyl segments of mung bean at high temperature is accompanied by accumulation of ACC in the tissue. These results indicate that the conversion of ACC to ethylene is highly susceptible to high temperature while ACC synthase is not. Therefore the author suggests that the conversion of ACC to ethylene is the rate-limiting step at high temperature. Field (1981b) reported that the rapid reduction of ethylene production by leaf tissue of Phaseolus vulgaris L. at high temperature was possibly due to temperature-induced perturbation of membranes since there was a marked increase in electrolyte leakage corresponding to the inhibition of ethylene production. Considering the suggestion that EFE is an enzyme system containing membrane-bound enzymes (Apelbaum et al., 1981), it is reasonable to postulate that EFE might be non-functional at high temperatures. The inhibition of ethylene production at high temperature has been also reported by Saltveit and Dilley (1978), Horiuchi and Imaseki (1986) but they did not establish the rate-limiting step at the inhibitory temperature.

The effect of low temperature on the ethylene

biosynthetic system is more complex. It appears that ACC synthase and EFE behave differently in different species. Wang and Adams (1982b) found that low ethylene production by cucumbers at a chilling temperature (2.5°C) was associated with correspondingly low levels of ACC and ACC synthase activity, suggesting that the synthesis of ACC was the rate-limiting step in ethylene production. Field (1981a) also concluded that the rate-limiting step in ethylene production by leaf tissue of Phaseolus vulgaris L. at 5°C was ACC synthesis since the addition of 1 mM ACC enhanced ethylene production at 5°C. In the temperature range of 4 to 35°C, the addition of 1 mM ACC to apple fruit tissue did not change the qualitative pattern of ethylene production, while ACC dependent ethylene production increased 2.5 times over this temperature range (Apelbaum et al., 1981).

Lipton and Wang (1987) recently discovered that substantial amounts of ACC accumulated in honeydew melons held for two and half weeks at a chilling temperature of 2.5°C and there was associated with a low level of ethylene production. They also observed that the ACC content rapidly decreased corresponding with a sharp increase in ethylene production following the transfer from the chilling temperature to 25°C. The ACC content in melons held continuously at 10°C remained low. These results indicate that chilling exposure stimulates ACC synthase activity in honeydew melons but the conversion of ACC to ethylene only

occurs at warm temperatures leading to the accumulation of ACC in tissue at chilling temperatures and the subsequent decrease in ACC content upon warming the tissue. It can be concluded from these observations that the rate-limiting step in ethylene production of honeydew melon at chilling temperature is the conversion of ACC to ethylene rather than the lack of substrate.

In summary, it appears that the inhibition of ethylene production can occur at different points in the biosynthetic pathway in different tissues in response to temperature stresses. High temperature inhibition results from the inactivation of EFE while no such clear statement can be made about low temperature effect since EFE and ACC synthase act differently in different species under low temperature stresses.

F. CHANGES IN PROTEIN COMPOSITIONS DURING FRUIT RIPENING

Fruits are generally low in protein as well as in total nitrogen compared to seeds, leaves and other plant parts (Hansen,1970). Apples had the lowest protein concentration among the thirty four different fruit species analyzed by Watt and Merrill (1963). The protein constituents of fruits, however, are of great importance since they are not only components of nuclear and cytoplasmic structures but

also represent the full complement of enzymes involved in metabolism during growth, development, maturation and senescence (Hansen, 1970). Not surprisingly, the content and composition of proteins and activities of some enzymes have been observed to undergo changes during fruit ripening (Hansen, 1970; Clements, 1970; Klein, 1969).

The existence of high levels of phenolics and organic acids and the low level of protein content in apples have made studies of proteins more difficult than with other fruits. Although it has been found that some bands in protein patterns of one dimensional electrophoresis gel undergo changes during apple ripening (Clements, 1970; Klein, 1969), no further work in identification has been published and no protein profiles of two dimensional electrophoresis gel apple proteins have been obtained to date.

SECTION 1

THE EFFECTS OF HYPOBARIC STORAGE AND TEMPERATURE ON ETHYLENE BIOSYNTHESIS BY APPLES (cv. 'Mutsu')

INTRODUCTION

The ripening of climacteric fruit is marked by a sharp increase in ethylene production which is autocatalytic in nature and this distinguishes them (Dilley,1982). The role of ethylene in fruit ripening is well documented (Abeles,1973). Adams and Yang (1979) established the pathway of ethylene biosynthesis in ripening apples: SAM --> ACC --> ethylene. The conversion of SAM to ACC is catalyzed by ACC synthase, a pyridoxal enzyme. In most cases the rate of ethylene synthesis in plant tissues is limited by the activity of ACC synthase (Kende et at., 1985). The ripening of apples, however, requires the activation of both ACC synthase and ethylene forming enzyme (EFE) which converts ACC to ethylene (Hoffman and Yang, 1980).

The effect of temperature on the production of ethylene by plant tissue has also been widely researched (Field, 1985). Lowering storage temperature can reduce both the rate of ethylene production and the sensitivity to ethylene action in plant tissue. Saltveit and Dilley (1978) reported that lowering the temperature below 24°C markedly reduced the production of ethylene from excised subapical stem segments of pea seedlings and delayed the onset of the wound

ethylene response after excision. There was approximately a ten fold increase in ethylene production, from 40 to 400 $pmol \cdot g^{-1} \cdot hr^{-1}$, when the temperature was increased from 10°C to 25°C. Chilling sensitive fruits such as cucumber (Wang and Adams, 1982b) and honeydew melon (Lipton and Wang, 1987) have a very low capacity to produce ethylene when held at low but non-chilling temperature. Fields (1985) assumed that ethylene production is negligible in most plant systems at 0°C. All apple cultivars tested, however, produce ethylene at 0°C.

Hypobaric storage extends the postharvest preservation period of fruits by reducing the partial pressure of oxygen and ethylene. The rate of ethylene production by apples taken from hypobaric storage is much lower than that of air-stored apples when ripened at 20°C (Dilley, 1982). In addition, fruits ripen more slowly after long term hypobaric storage at 0.1 atm than they do at harvest (Dilley, 1982). Dilley et al. (1982) pointed out that the hypobaric effect is not simply due to the low oxygen level but may involve the uncoupling of ethylene synthesis and action.

A number of investigators have attempted to determine the temperature-sensitive step in the pathway of ethylene synthesis, especially the rate limiting step at extreme temperatures (Apelbaum et al., 1981; Field, 1981a; Field, 1981b; Field, 1984; Lipton and Wang,1987; Wang and Adams,

1982b; Yu and Adams, 1980). Ethylene production is inhibited at high temperatures due to the inhibition of ethylene forming enzyme (Apelbaum et al.,1981). However the effect of low temperature on the pathway of ethylene biosynthesis is different among different species (Wang and Adams, 1982b; Wang and Sams, 1985; Lipton and Wang, 1987).

The present study was undertaken to investigate the effects of low temperature (0°C) on the pathway of ethylene biosynthesis in apple fruits and to determine the hypobaric effects on ethylene production in apples.

MATERIALS AND METHODS

Preclimacteric 'Mutsu' apples were harvested in October 1986 at the Horticulture Research Center, Michigan State University, East Lansing, Michigan and immediately placed into hypobaric storage chambers maintained at 0.053 atm and 0°C or allowed to ripen naturally at 20°C. Apples were removed for analyses after storage for four, six and nine months.

Ethylene production: Apples were placed individually into 1.7 liter glass jars and ventilated with air 100cc·min^{-1} at 0 or 20°C, as indicated. There were six replicates per treatment. Air flow was interrupted and jars were sealed one hour prior to sampling after which the air flow was restored. The ethylene concentration with gas sample was

determined once daily by gas chromatography employing a Varian Model 3400 equipped with a flame ionization detector.

ACC measurement: Frozen (-20°C) cortical tissue was used for ACC content measurement. Four grams of cortical tissue were extracted with 10 ml cold methanol for 1 min and then centrifuged for 15 min at 15,000 g. ACC in the supernatant was assayed by the method of Lizada and Yang (1979) except the time of incubation for the final reaction was five minutes. ACC content was determined with 4 apples per treatment once every three days.

Assay of EFE: EFE was determined <u>in vivo</u> by measuring the conversion of administered ACC to ethylene (Yu et al., 1985). One gram of fresh weight of cortical tissue discs (0.7 cm in diameter) was reacted at 30°C with one ml 50 mM Mes buffer (pH 6.1) containing 0.1 mM CHI, 2% sucrose, 50 ug chloramphenicol and 2 mM ACC in a 25 ml glass flask sealed with a rubber stopper. After one hour one ml gas sample was withdrawn and ethylene was determined by gas chromatography. Like ACC measurement, EFE activity was determined once every three days using four individual apples as treatment per replicate.

Temperature transfer experiment: Apples were taken from hypobaric conditions after six or nine month storage and immediately treated as follows: 1) Held continuously at 0°C for 15 days, 2) held continuously at 20°C for fifteen days, 3) held initially at 0°C and transferred to 20°C

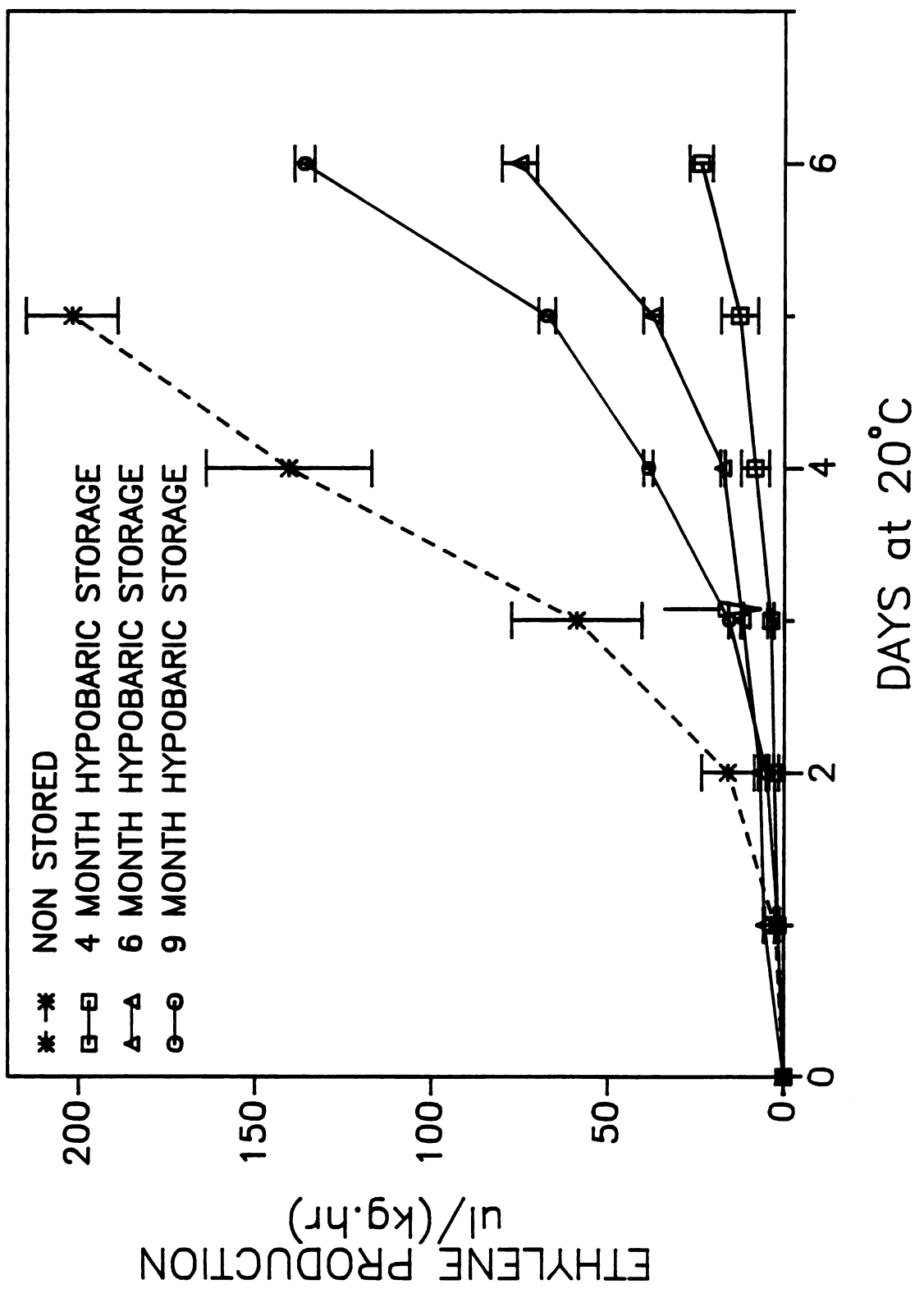
after two, four or seven days. Ethylene production, ACC content and EFE activity were determined as described above.

RESULTS

Hypobaric Effects on Ethylene Production: Ethylene production in nonstored apples increased rapidly at 20°C after a two day lag upon removal from the tree reaching a rate of 200 $\mu l \cdot Kg^{-1} \cdot hr^{-1}$ five days after harvest (Fig.1). Compared to nonstored apples the onset of ethylene production in hypobarically stored apples was delayed when they were placed under conditions favorably for ripening. Apples examined after 4 months of hypobaric storage produced minimal amount of ethylene during the first 6 days at 20°C. There was about a four day difference in the onset of accelerated ethylene production in apples after four months hypobaric storage compared with nonstored apples. The delay eventually diminished after longer term hypobaric storage. The lag periods before accelerated ethylene production for apples stored for six and nine months were only three and four days respectively. Ethylene production of all hypobarically stored apples finally reach a rate of 130 $\mu l \cdot Kg^{-1} \cdot hr^{-1}$ (Fig.2) and ripened normally.

Temperature Effect On Ethylene Production: Apples examined after 6 months of hypobaric storage and allowed to

Figure 1: The effect of hypobaric storage on ethylene production of apples (cv. Mutsu) at 20°C. Apples were either held at 20°C immediately after harvest (*-*) or placed in hypobaric storage for 4 months (□—□), 6 months (△—△), or 9 months (○—○) then held at 20°C.


ETHYLENE PRODUCTION
ul/(kg.hr)
200
150
100
50
0
0
2
4
6
DAYS at 20°C
NON STORED
4 MONTH HYPOBARIC STORAGE
6 MONTH HYPOBARIC STORAGE
9 MONTH HYPOBARIC STORAGE

ripen at 20°C exhibited a lag period after which ethylene production rose sharply eventually reaching 130 $\mu l \cdot kg^{-1} \cdot hr^{-1}$ on the eighth day (Fig. 2A). Fruits held continuously at 0°C produced negligible amounts of ethylene for the entire sixteen day duration of the experiment. Like apples held continuously at 20°C, the ethylene production of apples transferred to 20°C after two, four or seven days at 0°C eventually reached 130 $\mu l \cdot kg^{-1} \cdot hr-^{1}$. Once ethylene production began to increase, the rates (slope of the linear portion of the curve) were approximately equal (Table 1). In order to quantify the temperature effect on the delay in the onset of ethylene production caused by hypobaric storage the linear portion of the ethylene production curve was extent to x axis and the lag period of each treatment was measured and summarized in Table 1. The lag period of ethylene production by apples held at 20°C was 3.5 days. The lag periods for apples held at 0°C for two, four and seven days and then transferred to 20°C were less and were 2.4, 2.2 and 1.3 days, respectively. These results indicate that the delay in ethylene production caused by hypobaric storage diminishes while apples are held at 0°C, 1 atm. Similar results were obtained for apples examined after nine months of hypobaric storage except that the onset of ethylene production occurred one day earlier than apples stored for six months which, again, demonstrates that the lag period decreases with longer term storage. Temperature

Figure 2: The effect of temperature on ethylene production by apples (cv. 'Mutsu'). After 6 months (A) and 9 n=months (B)' storage, apples were held at either continuous 20°C (*-*), continuous 0°C (o—o) or transferred to 20°C from 0°C at 2 days (□—□), 4 days (△—△) or 7 days (◇—◇).

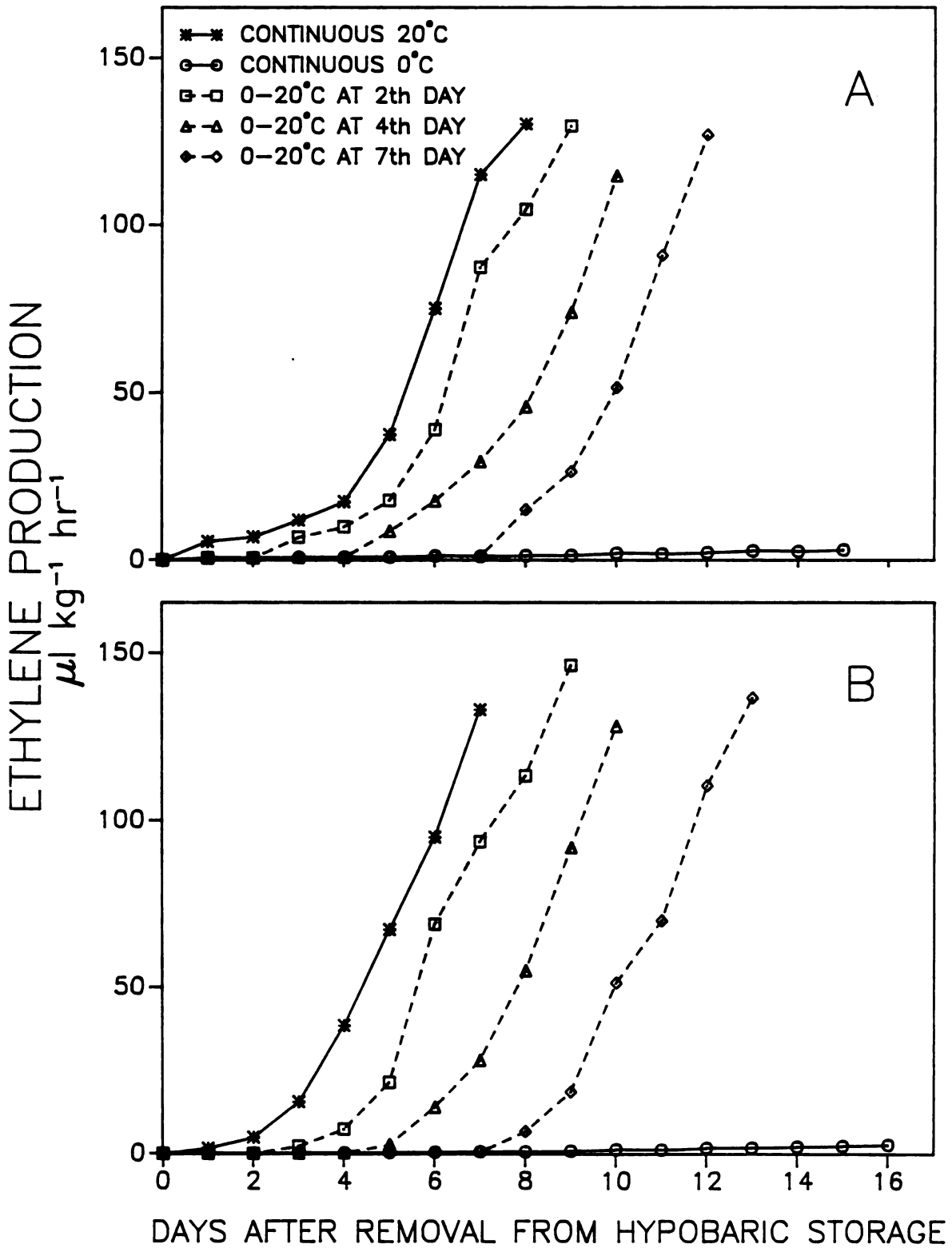

CONTINUOUS 20°C
CONTINUOUS 0°C
0–20°C AT 2th DAY
0–20°C AT 4th DAY
0–20°C AT 7th DAY
A
B
150
100
50
0
0
2
4
6
8
10
12
14
16
ETHYLENE PRODUCTION
μl kg⁻¹ hr⁻¹
DAYS AFTER REMOVAL FROM HYPOBARIC STORAGE

TABLE 1. THE EFFECT OF TEMPERATURE ON ETHYLENE PRODUCTION BY HYPOBARICALLY STORED APPLES (cv. MUTSU.)

STORAGE TIME MONTHS	TRANSFER TREATMENT	r^2	SLOPE*	STD OF SLOPE	LAG DAYS
6	20° continuous	0.99	30.3	2.5	3.5
6	0° to 20° 2nd day	0.95	28.9	2.7	2.4
6	0° to 20° 4th day	0.98	28.4	3.8	2.2
6	0° to 20° 7th day	0.99	34.1	2.1	1.3
9	20° continuous	0.99	29.1	1.5	2.6
9	0° to 20° 2nd day	0.98	29.4	2.7	2.0
9	0° to 20° 4th day	0.99	29.2	2.8	1.8
9	0° TO 20° 7th day	0.99	29.5	1.6	1.4

*The last 5 points of each ethylene production curve in Figure 2 were used for linear regression.

Effects On the Pathway of Ethylene Biosynthesis:

Ethylene forming enzyme (EFE) activity increased rapidly when apples were examined after six months of hypobaric storage and held continuously at 20°C (Fig.4). Apples held continuously at 0°C had very low activity for the 16 day duration of the experiment. When apples were transferred to 20°C after seven days at 0°C, however, EFE activity increased sharply in a pattern similar to that of apples held continuously at 20°C.

ACC gradually accumulated in apples held continuously at 0°C after six months of hypobaric storage (Fig. 5). This indicates that ACC synthase is active at 0°C. The ACC content of apples held at 20°C after hypobaric storage accumulated for six days then gradually declined to the basal level of 5 $nmol.g^{-1}$.

DISCUSSION

When 'Mutsu' apples were allowed to ripen naturally after harvest at 20°C their rate of ethylene production increased almost immediately. There was a lag in the initiation of ethylene production of two to four days for apples of identical physiological maturity if they were first stored in hypobaric conditions and then allowed to ripen at 20°C. It appears that the delay in ripening at 20°C, 1 atm induced by hypobaric storage was maximal shortly

Figure 3. The effect of temperature on the activity of ethylene forming enzyme of apples (cv. 'Mutsu'). Apples from 6 month hypobaric storage were held at either continuous 0°C (O—O), continuous 20°C (□—□) or transferred to 20°C after being held at 0°C for 7 days (Δ-Δ). Arrow indicates transfer from 0°C to 20°C.

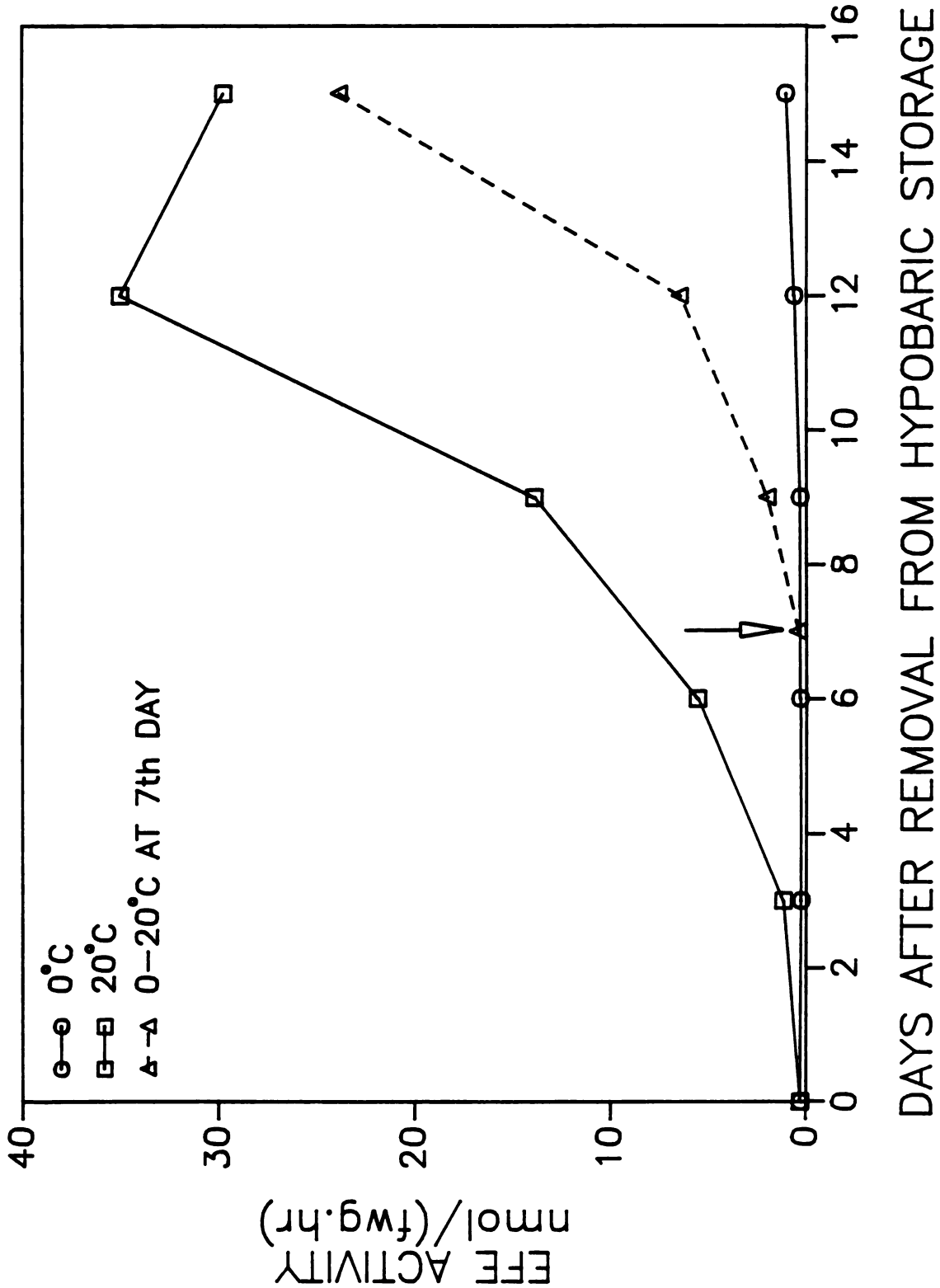

EFE ACTIVITY
nmol/(fwg.hr)
40
30
20
10
0
0
2
4
6
8
10
12
14
16
DAYS AFTER REMOVAL FROM HYPOBARIC STORAGE
0°C
20°C
0–20°C AT 7th DAY

Figure 4. The effect of temperature on the content of ACC of apples (cv.'Mutsu'). Apples from 6 month hypobaric storage were held at 0°C (O—O) or 20°C (□—□).

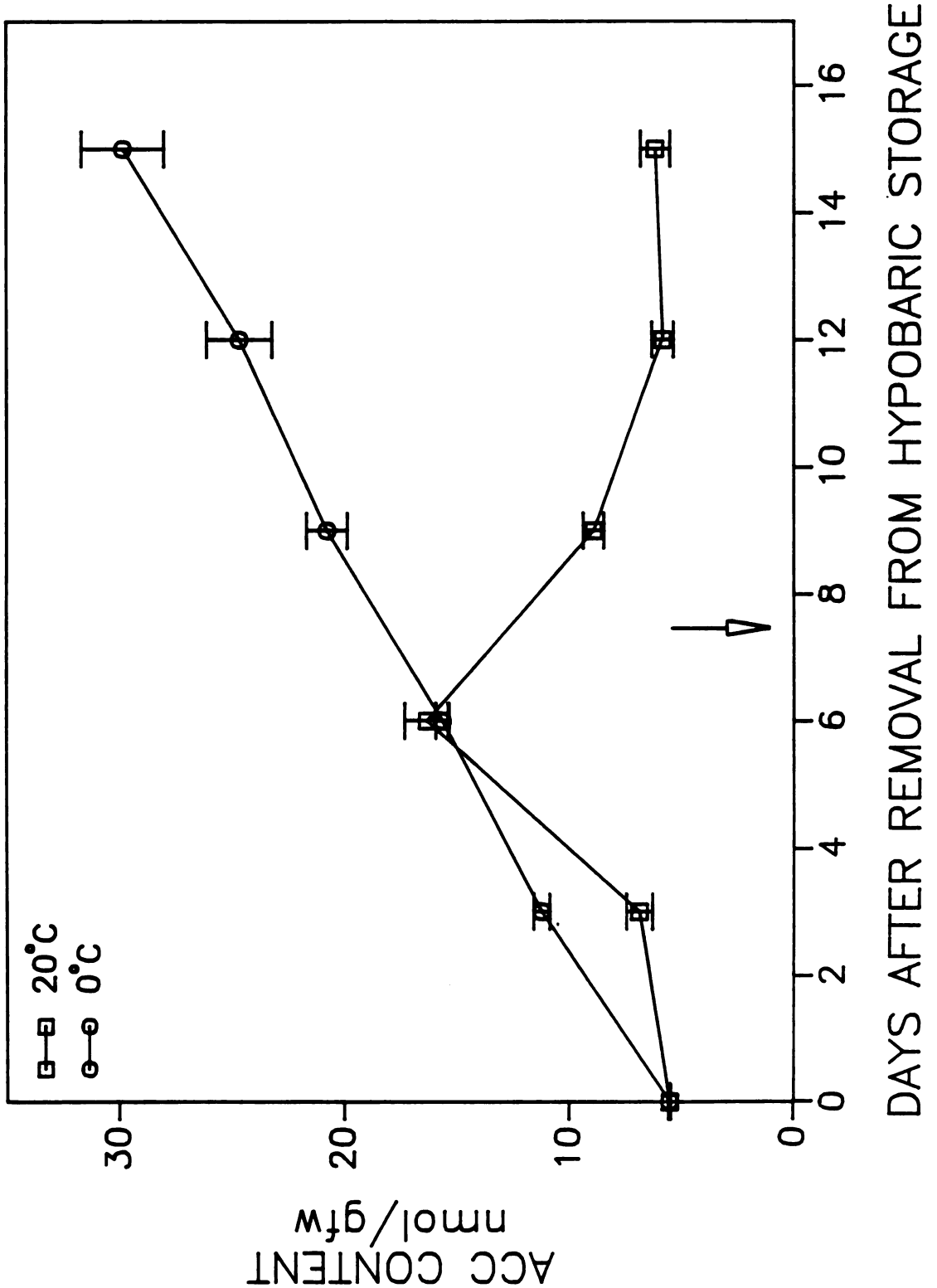
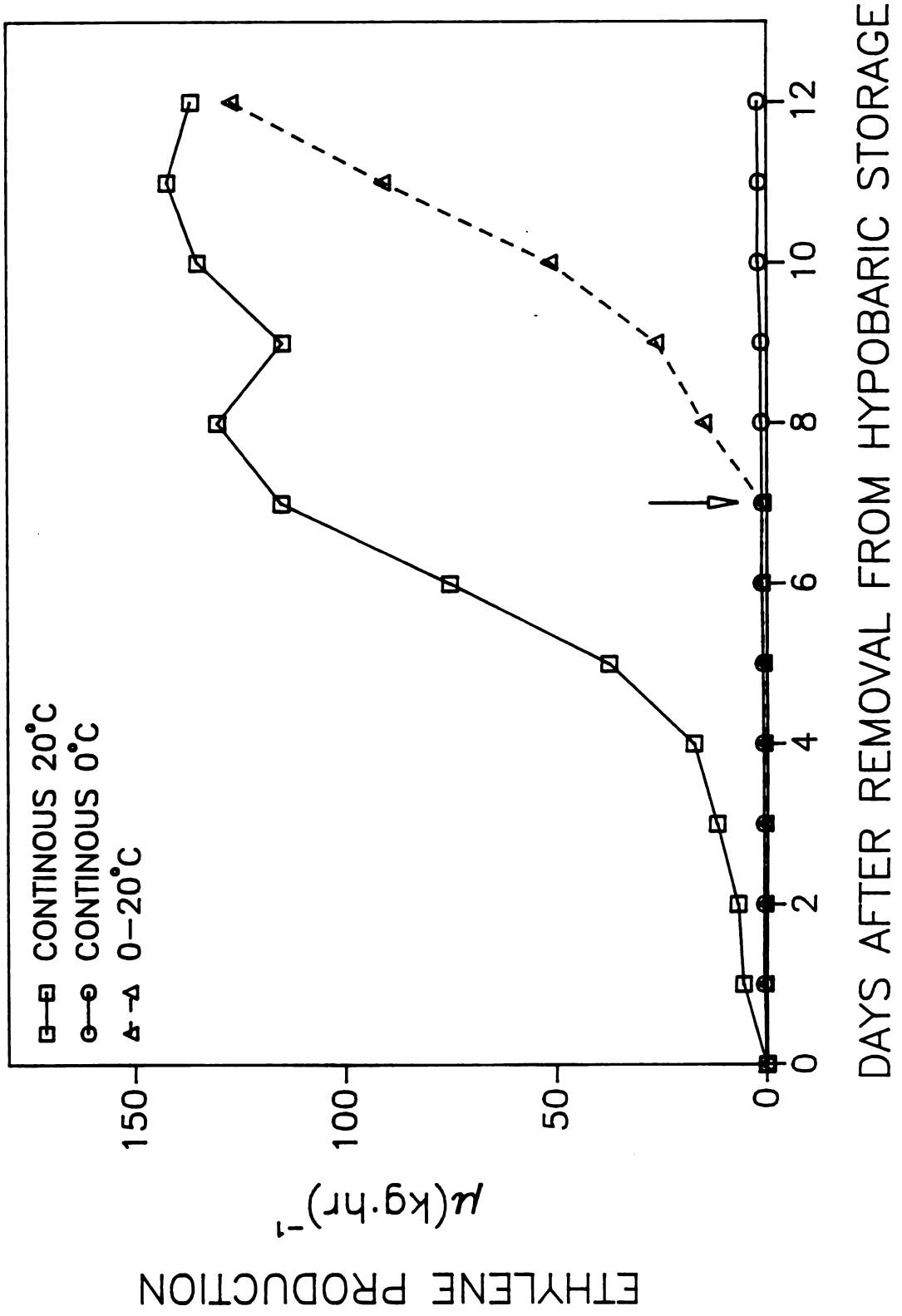

ACC CONTENT
nmol/gfw
30
20
10
0
0
2
4
6
8
10
12
14
16
DAYS AFTER REMOVAL FROM HYPOBARIC STORAGE
20°C
0°C

Figure 5. The effect of temperature on ethylene production of apples (cv. 'Mutsu'). Apples from 6 month hypobaric storage were held either at continuous 0°C (O—O), continuous 20°C (□—□) or transferred to 20°C after being held at 0°C for 7 days (△-△). Arrow indicates transfer from 0°C to 20°C.


ETHYLENE PRODUCTION
μ(kg.hr)⁻¹
150
100
50
0
0
2
4
6
8
10
12
DAYS AFTER REMOVAL FROM HYPOBARIC STORAGE
CONTINOUS 20°C
CONTINOUS 0°C
0–20°C

after the fruits are placed in storage and gradually diminishes over time. This suggests that apple fruits temporarily lose the ability to produce ethylene as a result of hypobaric storage. This observation corresponds to those of Dilley and coworkers (Dilley, 1982; Dilley et al., 1982) who found that apple fruits ripened more slowly after long term hypobaric storage at 0.1 atm than they do at harvest. It is noteworthy that apples regain the ability to produce ethylene more rapidly after a longer term (nine months) than they do after a shorter term (four months) hypobaric storage. It is reasonable to propose that there is a period when stored apples exhibit a minimal sensitivity to ethylene. It would appear that for 'Mutsu' fruits this period is less than four months. Our data here can not used to explain how the capacity for ethylene production increases during a long term hypobaric storage.

Ethylene production increased sharply only at 20°C whether in naturally ripened or hypobarically stored fruits. As long as preclimacteric fruits were held at 0°C ethylene production was minimal. Ye and Dilley (1986) reported that the production of ethylene in apples removed from hypobaric storage remained very low at 1°C over a 15 day duration. The present study confirms this inhibitory effect of low temperature on ethylene production by apples. However, the possibility that apples might begin to increase levels of ethylene after a longer period at 0°C (>16 days) can not

be excluded. Pears (cv. 'Eldorado') exhibit an increase in internal ethylene during a long term storage (Wang et al., 1985). The inhibition of ethylene production and the accumulation of ACC content in apple tissue at 0°C indicate that ACC synthase is active at 0°C while EFE is inhibited to the low temperature. These results differ from those of Wang & Adam (1982b) who found that ACC content, ACC synthase and ethylene production in cucumber tissue were all kept low at subchilling temperature. They suggested that the synthesis of ACC was the rate limiting step in ethylene production at low temperatures. Our results, however, correspond to those of Lipton and Wang's (1987) that 'honeydew' melons accumulated substantial amounts of ACC during a 2.5 week chilling exposure at 2.5°C. It has also been reported that preclimacteric pears (cv. 'Eldorado') (Wang et al., 1985) accumulate ACC in long term cold storage but at a much lower rate than do apples. It is reasonable to conclude that the inhibition of ethylene production is due to the lack of ability of apples to convert ACC to ethylene at 0°C and not due to the low levels of ACC since EFE activity was inhibited at 0°C but ACC accumulated in apple tissue. The behavior of ACC synthase and EFE enzyme in apples at 0°C is very much like at high temperature in that EFE activity is inhibited and ACC synthase remains active leading to an accumulation of ACC. The accumulation of ACC in apple tissue at 0°C can be also

used to explain why the delay in ethylene production caused by hypobaric storage gradually diminishes over time at 0°C. It is possible that the ACC accumulated in apple tissue at 0°C accelerated ethylene production upon the subsequent transfer to 20°C. Our results would have been clearer if the experimental design had included measurement of the ACC synthase activity at both 0°C and 20°C. However, even lacking those observations, the data clearly indicate that the rate limiting step of ethylene production in apples at 0°C is the conversion of ACC to ethylene.

The ACC content of apples held at 20°C after hypobaric storage accumulated for six days then gradually declined to the basal level of 5 $nmol.g^{-1}$. The peak of ACC content is explained by the fact that the increase in ACC synthase activity occurred prior to that of EFE activity when apples were taken from hypobaric condition and held at continuous 20°C. Since EFE was activated more slowly than ACC synthase after apples were removed from hypobaric storage, not all the ACC synthesized in first six days is converted to ethylene. This implies that the conversion of ACC to ethylene in the apples was the rate-limiting step in the first 6 days after removal from hypobaric storage. ACC deficiency, however, limited ethylene production in the later stages since ethylene production reached its peak five days earlier than that of EFE (Fig.3, Fig.4).

SECTION 2

CHANGES IN PROTEIN COMPOSITION DURING APPLE RIPENING, (cv. MUTSU)

INTRODUCTION

Significant physiological and biochemical changes occur during apple ripening. It has been demonstrated (Abeles, 1973) that ethylene plays an important causal role in initiating these changes. How ethylene initiates ripening and what enzymatic reactions related to ripening are directly or indirectly affected by ethylene remain to be elucidated. There are two main theories of ripening control to explain the ripening phenomenon. One argues that ripening involves a change in the 'organizational resistance' of the cell. It has been proposed that this change is caused by an alteration in membrane permeability which leads to leakage of ions and metabolites and the release or activation of hydrolytic enzymes. Another explanation, that ripening involves the expression of specific genes which causes an initial sequence of biosynthetic changes leading to ripening, has recently been gaining support (Meyer and Chartie, 1981). There is evidence showing that the ripening of tomatoes (Grierson et al., 1984) and avocadoes (Grierson et al., 1985) involves gene expression but no similar work has been done on apples. Although it has been observed that changes in protein bands on one dimensional electrophoresis patterns and changes in

some enzyme activities occur during apple ripening (Klein, 1969; Clements, 1970), so far no protein patterns by two dimensional electrophoresis have been made by apples at different ripening stages. Changes in the protein distribution by two dimensional electrophoresis could be helpful to identify certain proteins that may be closely associated with the ripening process. This information may be useful in subsequent studies to identify the gene products involved in ripening which would help to understand ethylene's causal role in ripening.

The objective of this experiment was to examine protein composition changes during apple ripening.

MATERIALS AND METHODS

Plant Material: 'Mutsu' fruits were harvested at different ripening stages at the Horticultural Research Center at Michigan State University, October 1986. Apple ripening stages were divided according to their internal ethylene levels as follows:

Ripening stage 1: (preclimacteric stage): internal ethylene < 0.1 ppm;

Ripening stage 2: internal ethylene of about 1 ppm;

Ripening stage 3: internal ethylene of about 10 ppm;

Ripening stage 4: (postclimacteric stage) internal ethylene of about 100 ppm.

Cortical tissue of 'Mutsu' fruit was sliced and frozen in liquid nitrogen immediately after sampling and dried under vacuum. The vacuum-dried tissue was then ground to a fine powder and stored at -20°C.

Protein Extraction: One hundred twenty five mg of freeze-dried apple powder was extracted with O'Farrell' lysis buffer (9.5M urea, 5% 2-mercaptoethanol, 2% Nonidet P40, 2% Ampholytes pH 3-10) (O'Farrell, 1975). After two centrifugations of the sample (35,000g for 15 and 10 minutes), the extraction can be loaded on gels immediately or stored at - 80°C.

Electrophoresis: The first dimension (nonequilibrium pH gradient electrophoresis) was done according to O'Farrell' et al. (1977). The corresponding volumes of supernatant solution containing about 50 ug protein were loaded onto each tube gel (length 12 cm, internal diameter 3 mm) containing 4% T (w/v) polyacrylamide, 9.15M urea, 2% (w/v) Nonidet P-40, 2% Ampholines (pH 3-10). Samples were loaded on the acidic end of the gel covered with a 15 µl overlay solution (8M urea and mixture of 0.8% pH 5-7 and 0.2% pH 3-10 Ampholines). Electrophoresis was conducted at 100V for 30 min, 200V for 60 min, 300V for 30 min, 400V for 60 min and 500V for 120 min. Gels were removed from the tubes and stored at -80°C without equilibrium in SDS sample buffer after first dimensional electrophoresis.

The second dimension electrophoresis was performed

according to O'Farrell' (1977). The separation gel consisted of 12% T (w/v) and the stacking gel was 4% T (w/v). The thickness of the gel was 0.75 mm. The tube gel was equilibrated for 30 min in SDS sample buffer (10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 0.0625 M Tris-Cl pH 6.8) and washed briefly with double deionized water before loading on the slab gel. The running conditions of the slab gel were 15 mA constant current per gel until the dye front reached the bottom of the gel. The running time was about five hours.

Staining and destaining: The gel was first stained in 40% methanol, 10% acetic acid, 0.04% Coomassie Brilliant Blue for at least two hours (better overnight). Destaining is carried on by two changes of 50% methanol, 12% acetic acid followed by several changes of 10% methanol and 5% acetic acid. When the destaining was completed, the gel was then silver-stained by a modified Positive-image Silver Stain method described by Merrill et al. (1984). After washing the gel twice with 10% methanol containing 5% acetic acid for 15 minutes each, the gel was soaked in 3.4 mM potassium dichromate containing 3.2 mM nitric acid for 5 minutes and washed twice for five minutes each with double deionized water. The gel was then placed in 12 mM silver nitrate for 20 minutes followed by a brief wash with double deionized water for one minute. This was followed by an addition of a solution containing 0.28 M sodium carbonate

containing 0.5 ml of formaldehyde (a commercially available solution of 37% formaldehyde) per liter. After 30 seconds a precipitate of silver salts formed and the solution turned to yellow. The yellow solution was discarded and replaced by fresh one to prevent the precipitate from adsorbing to the surface of the gel surface. The development was stopped by 5% acetic acid. The gel was then washed twice, twenty minutes each, with water before storage. The silver-stained gel can be stored in water for indefinite time.

RESULTS AND DISCUSSION

The two dimensional patterns of buffer-extractable proteins in cortical tissue of 'Mutsu' apples at different ripening stages are shown in Figure 1 through Figure 4. There were about 200 polypeptides detected on the two dimensional polyacrylamide gel maps by use of Coomassie blue and silver double staining technique. Nineteen protein reactive regions (spots) on the gels underwent changes during the ripening process (Table 1) while the remainder of the protein did not change in relative intensity. These proteins were used as references for the examinations. Spots numbered 1 to 8 were absent at the preclimateric stage but appeared at later stage of ripening. It is noteworthy that the intensity of spot number 4 increased very strongly at the postclimacteric stage (about 1% of total protein

content). Spot number 8 absent throughout the whole ripening process but appeared at the postclimacteric stage. Spot number 12 gradually decreased during ripening and disappeared at postclimacteric stage. Another four spots (numbered 13, 14, 15, 16) also decreased their intensities during ripening and the postclimacteric stage. Three spots (numbered 17, 18, 19) increased their intensities at early ripening stage (ripening stage 2) then decreased afterwards.

The changes of the intensities of the nineteen spots during the ripening of 'Mutsu' apples indicate that the synthesis and degradation of proteins are involved in apple ripening. It has been shown that there are significant changes in the qualities and quantities of particular mRNAs during the maturation and ripening of tomato (Grierson et al., 1984)and avocado (Tucker and Laties, 1984) which suggest that fruit ripening results in the regulation of gene expression. Although it is reasonable to hypothesize that apple ripening is regulated in the same way, further proof is needed.

The procedure of first dimensional electrophoresis in the present study consisted of loading protein samples at the acid end of the tube gels. When the protein extracts were loaded at the basic end as for isoelectric focusing, only a few proteins entered into the gel. This is probably due to the fact that apples contain predominantly acidic proteins which remain positive charged in O'Farrell lysis

Figure 1. Two dimensional protein profile of preclimateric IE < 0.1 ppm apples cv. 'Mutsu'.

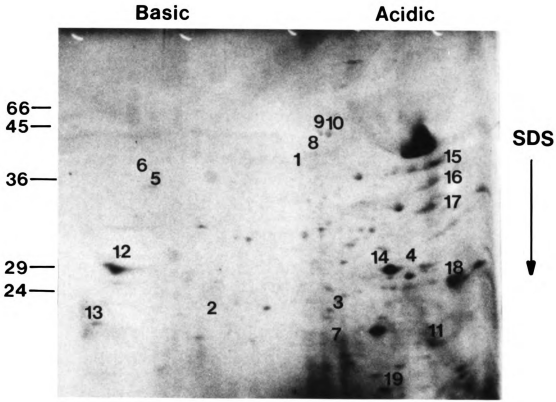

Basic
Acidic
66
45
36
29
24
SDS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure 2. Two-dimensional protein profile of apples cv. 'Mutsu' at ripening stage 2, IE = 1 ppm.

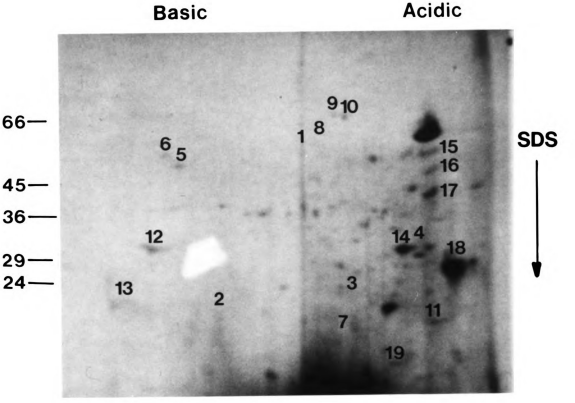

Basic
Acidic
66
45
36
29
24
SDS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure 3. Two-dimensional protein profile of apples cv. 'Mutsu' at ripening stage 3, IE = 10 ppm.

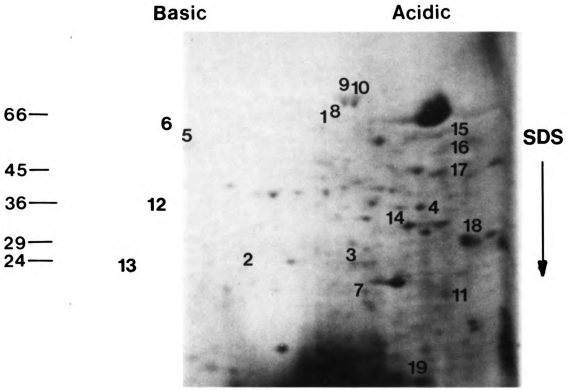

Basic
Acidic
SDS
66
45
36
29
24
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure 4. Two-dimensional protein profile of postclimacteric, IE > 100 ppm apples, cv. Mutsu.

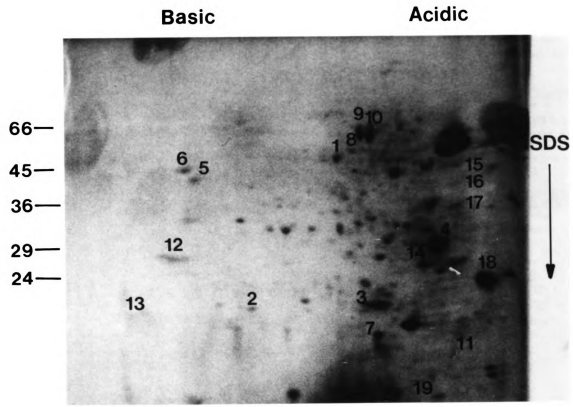

Basic
Acidic
66
45
36
29
24
SDS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Table 1. Behavior of the 19 spots related to apple (cv. Mutsu) ripening. Arrows indicate unchanged (→), increased (↗), or decreased (↘), Symbol (--) represents nonexistent spots.

Spot	MW(kD)	Ripening 1 IE < 0.1ppm	Ripening 2 IE = 1ppm	Ripening 3 IE = 10ppm	Ripening 4 IE > 100ppm
1	61	--	--	↗	↗
2	34	--	--	↗	↗
3	34.5	--	--	↗	↗
4	44	--	--	↗	↗
5	56	--	↗	↗	↗
6	59	--	↗	↗	↗
7	29	--	--	--	↗
8	63	--	--	--	↗
9	67	→	--	↗	↗
10	67	→	↗	↗	↗
11	24	→	↗	→	↘
12	29.5	→	↘	↘	→
13	37.5	→	↘	↘	↘
14	57	→	↘	↘	↘
15	23.5	→	→	↘	↘
16	52	→	→	→	↘
17	36	→	↗	↘	→
18	37.5	→	↗	↘	↘
19	48	→	↗	↘	↘

buffer. These proteins migrate to the cathode. Our results are similar to those of Meyer and Chartier who could only obtain good resolution of protein patterns by loading protein extracts of tobacco mesophyll protoplasts onto the acid end of the first dimensional gel (Meyer and Chartie, 1981).

The major drawback of the analysis of compositional changes of proteins by two-dimensional gel electrophoresis is that no function can ascribed to specific proteins. However, this technique is the most sensitive method to gain a general overview of protein synthesis and to analyze the expression of genes of as yet unknown function (Meyer and Chartie, 1981). Moreover, the technique may not reveal the presence of proteins at subliminal levels. Though previous workers (Klein, 1969; Clements, 1970) using one-dimensional gels reveal some differences in protein patterns in apples at different ripening stages, the present study provides much more information on many polypeptides affected during apple ripening. This could be helpful in identifying some of the polypeptides whose syntheses is markedly altered during the ripening process.

CONCLUSIONS

1. Hypobaric storage causes a delay in ethylene production of 'Mutsu' apples at 20°C. The delay eventually diminishes under hypobaric conditions during long term storage. The delay diminishes rapidly at 0°C, 1 atm.

2. Ethylene production and EFE activity are inhibited in apples held at 0°C while ACC accumulates in those fruits, suggesting that the inhibition of ethylene production at 0°C is caused by inhibition of ethylene forming enzyme activity rather than by lack of substrate.

3. Nineteen polypeptides underwent changes in concentration during ripening, suggesting that both synthesis and degradation of proteins are involved in the apple ripening.

LITERATURE CITED

LITERATURE CITED

1. Abeles FB 1973 Ethylene in Plant Biology. Academic Press, N.Y.

2. Adams DO, SF Yang 1977 Methionine metabolism in apple tissue: implication of S-adenosylmethionine as an intermediate in the conversion of methionine to ethylene. Plant Physiol 60: 892-896

3. Adams DO, SF Yang 1979 Ethylene biosynthesis: Identification of 1-amino-cycloproprane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene Proc Natl Acad Sci USA 76: 170-174

4. Apelbaum A, AC Burgoon, JD Anderson, T Solomos, M Liebermann 1981 Some characteristics of the system converting 1-aminocyclopropane-1-carboxylic acid to ethylene. Plant Physiol 67:80-84

5. Biggs MS, RW Harriman, AK Handa 1986 Changes in gene expression during tomato fruit ripening. Plant Physiol 81: 395-403

6. Boller T, RC Herner, H Kende 1979 Assay for and enzymatic formation of an ethylene precursor, 1-amino -cyclopropane-1-carboxylic acid. Planta 145: 293-303

7. Bradford MM 1976 A rapid and sensitive method for the quantification of protein-dye binding. Anal Biochem 72: 248-254

8. Bufler G, F Bangerth 1983 Effects of propylene and oxygen on the ethylene-producing system of apples. Physiol Plant 58: 486-492

9. Burg SP, KV Thimann 1959 The physiology of ethylene formation in apples. Proc Nat Acad Sci USA 45: 335-344

10. Burg SP and EA Burg 1962 Role of ethylene in fruit ripening. Plant Physiol 37: 179-189

11. Burg SP, EA Burg 1966 Fruit storage at subatmospheric pressure. Science 153: 314-315

12. Burg SP 1973 Ethylene in plant growth. Proc Natl Acad Sci USA 70: 591-597

13. Clements RL 1970 Proteins. in The Biochemistry of Fruits and their Products. Hulme AC eds, Academic Press, London and N.Y. vol.1: pp 159-177

14. Dilley DR 1982 Principles and effects of hypobaric storage of fruits and vegetables. Amer. Soc. Heating Refri. and Air Conditioning Engineering Transactions 88 (1): 1461-1478

15. Dilley DR, P Irwin, MW Mckee 1982 Low oxygen, hypobaric storage and ethylene scrubbing. Controlled Atmospheres for Storage and Transport of Perishable Agricultural Commodities. Eds. D. G. Richardson and M. Maheriuk. Timber Press, Beaverton, Or. 317-329

16. Elstner EF, JR Konze 1976 Effect of point freezing on ethylene and ethane production by sugar beet leaf disks. Nature 263: 351-352

17. Field RJ 1981a The effect of low temperature on ethylene production by leaf tissue of Phaseolus vulgaris L. Annuals Botany 47: 215-223

18. Field RJ 1981b A relationship between membrane permeability and ethylene production at high temperature in leaf tissue of Phaseolus vulgaris L. Annals Botany 48: 33-39

19. Field RJ 1984 The role of 1-aminocyclopropane-1 -carboxylic acid in the control of low temperature induced ethylene production in leaf tissue of Phaseolus vulgaris L. Annals Botany 54: 61-67

20. Field RJ 1985 The effect of temperature on ethylene production by plant tissues. in Ethylene in plant Development Roberts and GA Tucker eds Butterworths pp 47-69

21. Grierson D, A Slater, J Speirs, GA Tucker 1985 The appearance of polygalacturonase messenger-RNA in tomatoes - one of a series of changes in gene-expression during development and ripening. Planta 163: 263-271

22. Grierson D, A Slater, M Maunders, P Crookes 1985 Regulation of the expression of tomato fruit ripening genes: the involvement of ethylene. in Ethylene and Plant Development Roberts JA, GA Tucker eds Butterworths pp 147-162

23. Hoffman NE, SF Yang 1980 Changes of 1-amino -cyclopropane-1-carboxylic acid content in ripening fruits in relation to their ethylene production rates. J Amer Soc Hort Sci 105 (4): 492-495

24. Horinchi Y, H Imaseki 1986 High temperature sensitivity of auxin-induced ethylene production in mung bean hypocotyl sections. Plant Cell Physiol 27(3): 453 -461

25. Jones JF, H Kende 1979 Auxin-induced ethylene biosynthesis in subapical stem sections of etiolated seedings of Pisum sativum L. Planta 146: 649-656

26. Kende H, MA Acaster, M Guy 1985 Studies on the enzymes of ethylene biosynthesis. in Ethylene and Plant Development Roberts and GA Tucker eds Butterworths pp 47-69

27. Kimmerer TW, TT kozlowski 1982 Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. Plant Physiol 69: 840-847

28. Konze JR, H kende 1979 Ethylene formation from 1-aminocyclopropane-1-carboxylic acid in homogenates of etiolated pea seedling. Planta 146: 293-302

29. Liebermann M, A Kunishi, LW Mapson, DA Wardale 1965 Ethylene production from methionine. Biochem J 97: 449-459

30. Liebermann M, A Kunishi, LW Mapson, DA Wardale 1966 Stimulation of ethylene production in apple tissue slice by methionine. Plant Physiol 41: 376-382

31. Liebermann M 1979 Biosynthesis and action of ethylene. Ann Rev Plant Phyiol 30: 538-591

32. Lipton WJ, CY Wang 1987 Chilling exposures and ethylene treatment change the level of ACC in 'honey dew' melons. J Amer Soc Hort Sci 112 (1): 109-112

33. Lizada MC, SF Yang 1979 A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Analytical Biochemistry 100: 140-145

34. Mattoo AK, JE Baker, E Chalutz, M Liebermann 1977 Effect of temperature on the ethylene-synthesizing system in apple, tomato and Penicillium digitatum. Plant and Cell Physiol 18: 715-719

35. Maxie EC, FG Mitchell, NF Sommer, RG Snyder, HL Rae 1974 Effect of elevated temperature on ripening of 'Bartlett' pear, Pyrus communis L. J Amer Soc Hort Sci 99: 344-349

36. Mayne RG, H Kende 1986 Ethylene biosynthesis in isolated vacuoles of Vicia faba L. - requirement for membrane integrity. Planta 167: 159-165

37. Merril CR, D Goldman, ML Van keuren 1984 Gel protein stains: silver stain. Methods Enzymol 104: 441-447

38. Meyer Y, Y Chartie 1981 Hormonal control of mitotic development in tobacco protoplasts. Plant Physiol 68: 1275-1278

39. Murr DP, SF Yang 1975 Inhibition of in vivo conversion of methionine to ethylene by L-canaline and 2,4-dinitrophenol. Plant Physiol 55:79-82

40. Nichols R 1966 Ethylene production during senescence of flowers. J Hort Science 41: 279-290

41. O'Farrell PH 1975 High resolution two-dimensional electrophoresis of proteins. J Bio Chem 250: 4007-4021

42. O'Farrell PZ, HM Goldman, PH O'Farrell 1977 High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12: 1133-1142

43. Saltveit ME, DR Dilley 1978 Rapidly induced wound ethylene from excised segment of etiolated Pisum sativum L., cv. Alaska. Plant Physiol 61: 675-679

44. Tucker ML, GG Laties 1984 Interrelationship of gene expression, polysome prevalence, and respiration during ripening of ethylene and/or cyanide-treated avocado fruit. Plant Physiol 74: 307-315

45. Wang CY, DO Adams 1982a Ethylene production by chilled cucumbers (Cucumis sativum L.). Plant Physiol 66: 841-843

46. Wang CY, DO Adams 1982b Chilling-induced ethylene production in cucumbers (Cucumis sativum L.). Plant Physiol 69:424-427

47. Wang CY, CE Sams, KC Gross 1985 Ethylene, ACC, soluble polyuronide, and cell wall noncellulosic neutral sugar content in 'Eldorado' pears during cold storage and ripening. J Amer Soc Hort Sci 110: 687-691

48. Yang SF 1980 The biochemistry of ethylene: biosynthesis and metabolism. Recent Adv Phytochem 7: 131-164

49. Ye W, DR Dilley 1986 Biochemistry of maturation and ripening in apple fruit. HortScience 21 (3): 837

50. Young RE, F Meredith 1971 Effect of exposure to subfreezing temperatures on ethylene evolution and leaf abscission in citrus. Plant Physiol 48: 724-727

51. Yu L, Hoffman NE, SF Yang 1985 Promotion by ethylene of the capability to convert ACC to ethylene in preclimateric tomato and cantaloupe fruits. Plant Physiol 77: 407-411

52. Yu YB, DO Adams, SF Yang 1979 1-Aminocyclopropane-carboxylate synthase, a key enzyme in ethylene biosynthesis. Arch Biochem Biophys 198: 280-286

53. Yu YB, DO Adams, SF Yang 1980 Inhibition of ethylene production by 2,4-dinitrophenol and high temperature. Plant Physiol 66: 286-290