FACTORS AFFECTING EFFUSIVITY AND RIPENING BEHAVIOR OF 'EMPIRE' APPLE FRUITS

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ABSTRACT

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Ethylene effusion (J_x, $nl \cdot cm^{-2} \cdot h^{-1}$) was found to be linear with concentration gradient according to Fick's 1st law. The coefficient of effusion of these fruit sections varied between 9×10^{-4} to 5×10^{-3} $cm^2 \cdot h^{-1}$ at P_T = 50 torr. Thus, the fraction of apple epidermal surface available for gas exchange was ca. (1-6) $\times 10^{-7}$. Slight desiccation of the tissue inhibited ethylene transport reversibly. The rate of ethylene effusion from the flesh side (J_x^F) through the epidermis was significantly higher than movement in the opposite direction (J_x^E). This anisotropic effect was enhanced under oxygenated conditions, increased significantly as a function of ethylene concentration and was dependent upon cortical cells subjacent to the epidermis. The addition of ethylene induced more than a 50% increase in the movement of ethane across the tissue sections. These results suggest that ethylene induced an increase in fruit porosity which was O_2-dependent.

'Empire' and 'Idared' apple fruits stored under hypobaric ventilation (P_T = 40 torr, P_{O_2} = 8 torr) or at atmospheric pressure

in He did not ripen at 20 C after 3-5 months in storage. Fruits stored at atmospheric pressure in N_2 with an equivalent O_2 tension (P_{O_2} = 8 torr) did ripen. In 'Empire' apples this slow-ripening phenomenon was found to appear only after 3-5 months in store in a medium which would facilitate intrafruit volatile loss (low pressure or atmospheric pressure in Helium). Neither respiration nor ethylene production were perturbed in these fruits after storage. Only immature fruits at harvest retained this condition after 9 months. Associated with this slow-ripening attribute were attenuated changes in malic enzyme activity and water soluble polyuronides. These attenuated processes were completely reversible upon conditioning previously inhibited fruits in air at 0 C. These data demonstrate an apparent uncoupling of ethylene synthesis and action which supports the hypothesis that a critical level of some native intrafruit volatile is necessary for the retention of ethylene recognition requisite for the stimulation of the ripening process.

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This dissertation is dedicated to those graduate students (including my spouse) who provided a vehicle upon which I could escape the vagaries of pome fruits: Archbold, Cayer, Chamaro, Furutani, Lee, Leon, Long, McCammon, Miranda, Olien, Sams, Wan, Wolpert, Wolk and many others who I have forgotten due to bad chemicals.

Guidance Committee:

The paper-format was adopted for this thesis in accordance with departmental and university regulations. The thesis body was separated into three sections (Section I, II, and Appendix A), which were written in the style of Plant Physiology, Journal of the American Society for Horticultural Science, and Food Biochemistry, respectively.

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INTRODUCTION

Approximately 40% of the fresh U.S. apple crop is stored in controlled atmosphere (CA) conditions. Modern CA storage techniques maintain a low temperature, high relative humidity, and permit control of O_2 and CO_2 at recommended low levels.

S. P. Burg and E. A. Burg have been credited with inventing low pressure (LPS) or hypobaric storage. Both CA and LPS have a rationale in common: if fruit ripening and senescense is mainly a function of intrafruit ethylene, which in turn is a function of fruit ethylene production and porosity, then the storage life of this commodity should be extended if O_2 levels are lowered sufficiently below ambient (ethylene synthesis is highly O_2-dependent). Both systems of storage achieve this result but with differences.

In CA storage there is a very slow atmospheric turnover. Therefore, ethylene accumulates and fruit ripen. In LPS the stored product is continually ventilated with ethylene-free air. Since the diffusivity of gases is inversely proportional to absolute pressue, the intrafruit activity of ethylene in LPS fruit is even lower than could be explained by the high atmospheric turnover (i.e., the maximization of the concentration gradient of ethylene and other volatiles between the inside and outside of these fruits). Reports have been made concerning the slow nature of ripening after removal of apple fruits

from LPS. It is of paramount importance to understand more fully the nature of this slow-ripening phenomenon. Investigations into the factors involved in this 'low pressure effect' might lead to a method of fruit storage which would produce a similar effect but at atmospheric pressure (i.e., in CA).

Since the porosity of fruit tissue determines, in part, how much ethylene and other native fruit volatiles accumulate within the fruit tissues, investigations into some factors which affect fruit porosity were made. In the course of these investigations an unusual phenomenon was observed; the efflux of ethylene was greater than the influx for the same tissue at the same concentration gradient. Because of the potential importance of this finding, investigations were made into the nature of this phenomenon.

LITERATURE REVIEW

Gas Exchange and Transport Properties of Certain Horticultural Commodities

In apple fruits the major barrier to gas exchange is the epidermis (17,27,31,60,62,93). Through the epidermis gas exchange occurs primarily through open lenticels (27), which occupy approximately 1/10000% of the total apple surface area. It has been calculated (27) that each cm^2 peel (epidermis in apple) strip from 'McIntosh' apples contains a total area of open lenticels of about 1×10^{-6} cm^2. Lenticels, according to Clements (31) result from "broken stomata" or from "scars caused by the falling out of trichomes." The number of lenticels found on each apple fruit is a unique characteristic of the cultivated or natural variety of the fruit. For example, 'Black Staymen' was found to possess circa 40 open and 900 closed lenticels while 'Spitzenburger' possessed ca. 200 open and 2000 closed lenticels. Thus, the rate of gas effusion or uptake, which are both a function of actual cellular production or consumption and the size of the open lenticels, of any gas will vary with cultivar (cv.) by a factor of 2-5.

While the epidermis of apples is considered to be the major barrier to gas diffusion (27, 62, 93), in green peppers and tomatoes at least 60% of the carbon dioxide evolution was retarded when a thick lanolin paste was applied to the pedicel (stem) end of the fruit; for

muskmelons and grapes, a decrease of 10% was found (27). However, no effect was noted in oranges, lemons, apples, avocados, limes, pumpkins, bananas, pears, plums, or acorn squash (27). Apple flesh has been found to offer little resistance to gas exchange and no concentration gradient from the outside of the fruit to the inside of the fruit has been discovered (27,44). However, a gradient in the "composition of the internal atmospheres" from the center of the fruit to its periphery has been found in apples (117). If the gas constituencies of apples do change as a function of location within the fruit then the flesh must offer some resistance to effusion or else the "gas constituencies" would reach an equilibrium throughout the fruit intercellular air spaces. In other fruits (avocado and banana), however, the flesh does impose a major barrier to gas exchange (17,27).

It has also been found that the porosity of some climacteric fruits changes with other physiological traits during the ripening process (17,84). Working with avocado fruits both in the peeled and 'intact' condition, a group of investigators (17) noticed that the resistance of the tissues to carbon dioxide (CO_2) effusion (R_{co_2} = $[CO_2]$ in the fruit/rate of CO_2 evolution per fruit fresh wt) changed from the time of the initial climacteric rise (R_{co_2} = 0.7 in intact fruits, R_{co_2} = 0.6 in peeled fruits) to the final, postclimacteric, physiological state of the fruits (R_{co_2} = 2.5 in intact fruits, 2.4 for peeled fruits). A similar result was noted in bananas whereby postclimacteric fruits displayed a gas exchange "impediment" (84).

Porosity also changes with moisture loss. Clements found that once-open lenticels in apple peel could be closed by processes which cause water loss from the outer tissues (31). It has also been observed in avocado and banana that gas exchange was decreased by desiccation of the peel and subjacent tissues (17,128). Fockens and Meffert (44) found that water vapor loss through the apple epidermis was best described as a combination wet surface-gas porous-gas impervious layer model system whereby:

$$m = C_1 B/RT + C_2/RT\ (1/B + D_m t/D)^{-1}$$

m = rate of water loss

C_1 = wet fractional surface

C_2 = porous layer fractional surface

B = coefficient of mass transfer (dependent upon the Reynolds number of the flow along the apple surface)

R = gas constant

T = mean absolute temperature of the boundary layer

D_m = mass of vapor diffusing through a layer of air/mass of vapor diffusing through the porous layer

D = coefficient of diffusion of water vapor in air

t = thickness of the layer.

It was also reported (44) that as water vapor was lost from apple tissues the resistance to H_2O vapor increased. Morphologically, this phenomenon was related to a change in the shape of the epidermal and subtending cells which caused intercellular spaces to become smaller.

Most of the data gleaned from early experiments concerning gas exchange of fruits are interpreted with respect to Fick's 1st law, whereby:

$$J = -D\left(\frac{dc}{dx}\right)$$

J = mass of gas entering the diffusional plane per unit time-unit area

D = coefficient of diffusion ($cm^2 \cdot h^{-1}$) where

$$D = D_o \left(\frac{T}{T_o}\right)^M \left(\frac{P}{P_o}\right)^{-1} \quad (27)$$

$M = 1.75 - 2.00$

$\frac{dc}{dx}$ = mass concentration gradient of the effusing gas per unit distance within or from the diffusional plane.

The quantity of gas effusing across the plane at x in time Δt is equal to $J\Delta t$ whereas the quantity leaving the plane at $x + \Delta x$ in the same time span is $(J + (\frac{dJ}{dx})\ \Delta x)\ \Delta t$. The net gain in the quantity of diffusing gas between these planes may be expressed in terms of the change of concentration in the volume $A\Delta x$ (A = area of plane) and in terms of the difference between these two amounts of transported material:

$$\Delta c \Delta x\ A = (J\Delta t - (J + (\frac{dJ}{dx})\Delta x)\ \Delta t)A$$

$$\Delta c \Delta x = -(\frac{dJ}{dx})\ \Delta x \Delta t$$

$$\Delta c/\Delta t = -(\frac{dJ}{dx})$$

as $\Delta t \to 0$

$$\frac{dc}{dt} = -\frac{dJ}{dx}$$

since $J = -D(\frac{dc}{dx})$ (Fick's 1st law)

$$\frac{dc}{dt} = D\ (\frac{d^2c}{dx^2}) \qquad \text{(Fick's 2nd law)}$$

That is to say, the rate of change of concentration with respect to time is directly proportional to the rate of the concentration gradient change with respect to distance (x). Fick's 1st law may be rewritten as

$$\frac{ds}{dt} = \frac{-xAD(C_{in} - C_{out})}{T}$$

x = fraction of the total area (A) open to gas exchange

T = thickness of the diffusional barrier

C_{in} = concentration of the gas species within the fruit

C_{out} = concentration of the gas species outside the fruit

Kidd and West (76) utilized a simplified form of measurement whereby the porosity factor = $(C_{in} - C_{out})/(\frac{ds}{dt}) = \frac{-T}{xAD}$ for ethylene and carbon dioxide. This equation simplifies to $C_{in}/ (\frac{ds}{dt})$ = r = the resistance coefficient, which was utilized by Trout et al. (125) in their studies concerning internal gas composition of fruits. A similar method was used by Ben-Yehoshua et al. (17) and Burg and Burg (27).

Various mathematical models have been formulated (44) to distinguish types of commodities from which water might evaporate during storage. Mushrooms resemble a simple wet surface model while apples more closely approach a wet surface-porous surface-imporous surface combined model. While it appears that water vapor leaves the fruit via lenticels and "wet surface," in other types of effusion (ethylene and CO_2), the mechanism simplifies to an imporous layer-porous layer model (27).

It has been shown that in apples and other commodities, the skin or epidermis acts as the major source of resistance to diffusion;

porosity often decreases during the ripening process and loss of water decreases porosity. In apples, the flesh adds little to the resistance of diffusion; in avocados and bananas, the flesh is the major barrier to diffusion.

Ethylene Synthesis

The biological history of ethylene (1) can be traced back to Girardin (1864. Jahreshner. Agrikult.-Chem. Versuchssta., Berlin. 72, 235) who discovered that illuminating gas leaking from street lamp gas mains defoliated trees. However, Neljubov (1901. Beih. Bot. Zentralbl. 10, 128) was the first to identify the active component of illuminating gas (1), which was ethylene and, to a lesser degree, acetylene. Neljubov also found that by passing illuminating gas-contaminated laboratory air over a CuO ignition tube the lab air became unable to alter the growth of pea seedlings (1913. Imp. Acad. Sci., St. Petersburg, 31, No. 4, 1).

In 1964, Lieberman and Mapson (88) reported that ethylene could be produced *in vitro* from linolenic acid and other cellular components including the amino acid methionine in a cell-free mixture. These authors also implicated the involvement of H_2O_2 in the degradation of linolenic acid to ethylene. Since this first paper involving precursors to ethylene biosynthesis, methionine has been demonstrated to be the most likely substrate in the fruits of apple (15), avocado (15), tomato (9) and citrus (71); other data has been reported (30) on the involvement of methionine in ethylene production of vegetative tissues.

Lieberman, Kunishi, and Mapson (86) discovered that carbons 3 and 4 of the methionine structure were cleaved and reduced to form the ethylene moiety. Aminoethoxyvinyl glycine (AVG), a derivative of the antibiotic rhizobitoxine (an inhibitor of ethylene synthesis and analog of methionine), strongly inhibited ethylene production by a variety of plant tissues (87). A protein isolated from mung bean hypocotyls (123) has also been found to inhibit 80% or more of the ethylene synthesized by treated hypocotyls: concomitant to suppressed ethylene production the authors observed reduced RNA synthesis (55-60%), protein synthesis (65-80%), and phosphate uptake (60-75%).

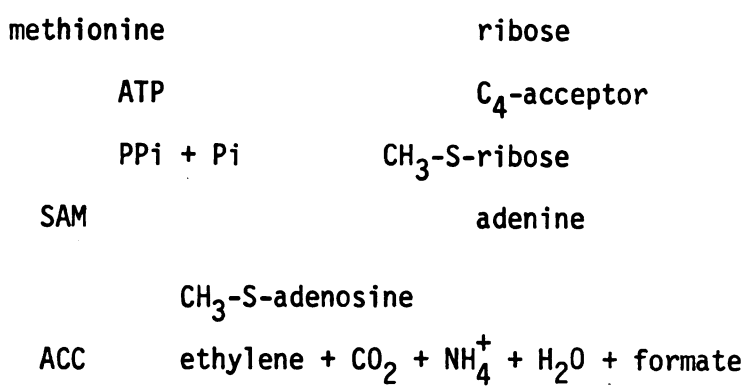
Frenkel, Klein, and Dilley (48) discovered ethylene synthesis required active protein synthesis at an early-climacteric stage and synthesis declined in pear fruit concomitantly with protein synthesis inhibition. These authors also demonstrated that the protein synthesis inhibitor, cycloheximide, inhibited pear fruits from ripening, thus demonstrating that a "coordinated sequence" of biochemical events was triggered by an increase in cellular ethylene activity. At about this same time (52), ethylene production in aged apple peel disks from preclimacteric apples was induced and also found to be dependent upon protein synthesis. However, Galliard et al. (52) found in this system ethylene evolution was stimulated by the peroxidation products of linoleic acid but not methionine. Apple peel disks aged aerobically at 25 C were found to develop a lipid synthesizing system which was protein synthesis dependent (51). Rhodes et al. (108) noticed that during the time when ^{14}C-amino acids were being incorporated at a high rate, the apple peel disks developed a system for producing ethylene.

It has been demonstrated that not only does ethylene production depend on oxygen but so also does ethylene action (68). Baur et al. hypothesized that oxygen was required at some point between methionine and ethylene (15). Carbon dioxide has been found to reduce ethylene production in controlled atmosphere (CA) storage at 5% O_2 (124) since CO_2 is a competitive inhibitor of ethylene action and one of the actions is the stimulation of autocatalysis (2).

The fate of sulfur from methionine must be important biochemically, since Yang and Baur (131) determined that levels of free and protein-bound methionine were excessively low to provide all of the methionine necessary for ethylene production by apple fruits. Homoserine and serine were felt to be requisite for ethylene synthesis (14) and a cyclic system for ethylene production with conservation of sulfur was hypothesized. S-adenosyl methionine (SAM) was found to be an intermediate in the conversion of methionine to ethylene (4, 99). Three years later (5), the sulfur of methionine was observed to recycle back into the cellular methionine pool and SAM was definitely identified as an intermediate in the conversion of methionine to ethylene.

In 1979 the intermediate between SAM and ethylene was discovered to be 1-aminocyclopropane-1-carboxylic acid (ACC) and H_2O_2 was implicated in the further catalysis of this intermediate form to ethylene, CO_2, NH_4^+, H_2O and formic acid (5). Also reported in 1979 was a new assay for ACC (25), which confirmed that SAM was an intermediate of the ethylene biosynthetic pathway. Thus, the evidence

for the main precursor to ethylene (methionine) and the major intermediates (SAM and ACC) in the enzymatic formation of ethylene has come to fruition. The complete pathway (130) has recently been promulgated as follows:



Mattoo and Lieberman have suggested that the ethylene-synthesizing enzyme system is highly structured in the apple cell and is localized in a cell wall-cell membrane complex (96). Ethylene synthesis in mung bean hypocotyl sections was stimulated by certain lipids (73) and reversibly inhibited by some proteins (112) and detergents (102). It has also been demonstrated (72) that ethylene synthesis was disrupted upon "osmotic shock" of these tissues. These same treatments suppressed "membrane activity" as evidenced by suppression of 1-^{14}C-α-aminoisobutyric acid uptake. Imasaki and Watanabe (72) believe that the cell membrane is involved in regulating ethylene production (72,73,102,112).

Ethylene Action in Ripening

Ethylene (ethene) is a C_2 hydrocarbon gas with a double bond and a molecular weight of 28.05. Many plant scientists feel ethylene

is the "ripening hormone" (26). Ethylene appears to act as a triggering mechanism in the ripening process. Figs, not generally associated with climacteric fruits, show a stimulation in both growth and "ripening" (94) due to ethylene. Hulme, Rhodes, Galliard, and Wooltorton (67) explain the ripening process in apples in terms of changes in the "organizational resistance" or permeability of the tissue. These authors feel the respiratory upsurge (respiratory climacteric) associated with the rise in ethylene synthesis in climacteric fruits might be due to mixing of metabolic substrates ordinarily separated by membranes. Ethylene has been found to induce membrane permeability in morning glory flowers (54) and some tissue permeability increases were noted by Harvey in 1915 (57). Mayak, Vaadia, and Dilley (98) found that after treatment of carnation flowers with 2µl/l ethylene there was a decrease in H_2O uptake 4 h after ethylene treatment; this was followed by wilting after an additional 2 h. Carbon dioxide, a competitive inhibitor of ethylene action, inhibited the decline in the H_2O uptake and wilting. Ethylene also enhanced ionic leakage as evidenced by efflux of ^{36}Cl from the tonoplast. These results (54,98) appear to support the hypothesis that ethylene induces ionic leakage. Also, this loss of cell water potential would explain the decreased H_2O uptake after ethylene treatment was observed by Mayak et al. (98). Generally, ethylene is considered to be an accelerator and "integrating agent" in plant senescence (75).

There is a large body of evidence supportive of ethylene's importance in ripening. Ripening and senescence of many fruits and

other plant organs is retarded when endogenous ethylene is removed from the plant parts by hypobaric ventilation (10,38). Inhibition of ethylene synthesis (127) with AVG as a postharvest dip delays ripening in 'Anjou' pears. In apple fruits (11), preharvest sprays of AVG delayed fruit ripening, reduced preharvest drop, and increased fruit removal force. In 'Bartlett' pear (101), one mM AVG inhibited ethylene production and delayed the respiratory climacteric and associated ripening changes in skin color and flesh firmness. These authors found AVG to be less effective on more mature fruit; when exogenous ethylene was applied to AVG-treated tissue the fruits responded by ripening. These data (10,11,38,101,127) support the hypothesis that ethylene is the "ripening hormone."

Silver ions have also been demonstrated to inhibit ethylene synthesis which concomitantly attenuated the ripening process (111). 'McIntoch' apple fruits stored with a "low" ethylene concentration (6 ppm) in the storage chamber were 2 pounds (0.9 kg) more firm at the end of the storage period and with a lessened incidence of brown core than fruits stored in "high" (1570 ppm) ethylene (45). Some authors (28,118) feel ethylene stimulates the respiratory upsurge typical in climacteric fruit which are beginning to ripen. In tomato fruits, dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, blocked ripening (95); this effect was probably due to a decreased $\frac{P}{O}$ ratio in these fruits. Forsyth and Lockhart (47) noted significantly lower CO_2, acetaldehyde, ethanol, ethyl acetate, and other volatile production in low ethylene-treated fruits.

A preponderance of evidence has indicated that the ripening process is dependent upon protein synthesis (37,48,63). When protein synthesis was inhibited in pears with cycloheximide, ripening was prevented while there was no repression of the respiratory climacteric (48). Pearson and Robertson (104) noticed that DNP evoked a large respiratory upsurge only in preclimacteric fruits. These authors proposed that at a critical stage in development, the increased demands of protein synthesis result in a greater concentration of free phosphate acceptors which, in turn, would increase respiration (104). Richmond and Biale (109) studied incorporation of ^{14}C-valine and ^{14}C-leucine in avocado tissue and also observed a sharp rise in amino acid incorporation during an early stage of the climacteric. Puromycin inhibited valine and leucine incorporation but had no effect on O_2 uptake. The authors felt this protein synthesis reflects an induction of a set of enzymes which "catalyse the climacteric process" and final cell breakdown or senescence (109). In pear mitochnodria a decline in ^{14}C-leucine incorporation has been observed during the climacteric rise (110). The increase in malic enzyme activity during the time coincident with ripening was shown (40,48) to be partially due to <u>de novo</u> synthesis, as measued by ^{14}C-phenylalanine incorporation into the electrophoretically purified enzyme. Klein and Dilley (78) found that incorporation of a radioactive amino acid into malic enzyme (40,48) represented total incorporation into 2 isozymes with identical rates of electrophoretic mobility.

Hobson (59) hypothesized that during tomato fruit development various isozymes were formed specifically to carry out the changes associated with ripening. It was observed that isozymes were most diverse qualitatively during the mature green stage and decreased thereafter. Hobson also demonstrated that while specific enzymes increase in activity, overall, total protein content decreases as fruits mature and ripen. To reiterate, enzymes produced during the rise in protein synthesis appear to catalyse various aspects of the ripening process and the climacteric increase in respiration does not appear to be connected since inhibitors do not disturb the respiratory increase, but do stop ripening.

Oxygen is a necessary component for ethylene to act upon plant tissues (68). Burg and Burg (29) have hypothesized that molecular oxygen is required to oxidize the ethylene attachment site and that oxygen is thus required for full "receptiveness" or sensitivity to ethylene. It has also been demonstrated that carbon dioxide acts as a competitive inhibitor with respect to ethylene action (29). These authors have suggested that CO_2 delays fruit ripening by displacing ethylene from its receptor site. All fruits do not show the response to ethylene during maturation and ripening and thus do not fit the classical climacteric fruit pattern displayed by apple and avocado (85). Rhodes et al. (108) give a summary of changes associated with the apple peel disk system upon ageing as follows:

1. respiration rate increases three- to four-fold
2. sensitivity to respiratory inhibition changes

3. development of respiratory increase is dependent upon RNA and protein synthesis (not always so in whole fruit)
4. increase in mitochondria number
5. increase in activity of numerous enzymes (especially malic enzyme)
6. increase in total protein content
7. increase in capacity to incorporate ^{14}C-amino acids
8. increase in total RNA
9. increase in the number of ribosomes
10. increase in fatty acid synthesis
11. increase in the uptake of solutes (electrolytes).

However, other authors have doubted ethylene's role as the "ripening hormone" (43).

Little is known about how ethylene induces or affects the ripening process. Burg and Burg (29) found binding of ethylene to tissues but very little incorporation. Utilizing deuterated ethylene, Abeles et al. (3) found no isotopic exchange of hydrogen with some binding site. It was hypothesized (3) that a dissociable ethylene-receptor complex might act like a switch (conformational change) which "turns on" a "cascade of reactions" when ethylene binds to the receptor. Beyer (18) found no isotopic exchange in deuterated ethylene and felt this was supportive of a ready dissociation of ethylene from a receptor site. Others (115,116) recently discovered the existence of a natural ethylene-binding lipophilic protein; however, no evidence was presented to link the binding capacity to any known physiological action of ethylene. Evidence now indicates ethylene can be

metabolized and incorporated into plant tissues (19,20,21,22,23) even under aseptic (20) conditions. Beyer (23) found that Ag^+ inhibits incorporation of ^{14}C-ethylene into tissue metabolites but not to ^{14}C-carbon dioxide, suggesting that ethylene metabolism is an integral part of its reaction mechanism (21,23).

Malic Enzyme in Ripening Fruits

L-malate is the major C_4 dicarboxylic acid in apple and other pome fruits (69). In 1957 Hulme and Neal (66) injected malate into the branches of apple trees and observed that this treatment induced an "early climacteric." Allentoff et al. (7) made the observation that roughly $\frac{2}{3}$ of the ^{14}C-carbon dioxide incorporated into apple fruits in the last month prior to harvest was found fixed in malic acid. Neal and Hulme (100) discovered that apple peel disks develop a capacity to decarboxylate added malate (the "malate effect") during ripening. The addition of malate caused a large increase in the carbon dioxide output from postclimacteric and senescent fruit but not from preclimacteric fruit. Oxygen uptake of peel tissue was also stimulated by addition of malate, succinate, and α-ketoglutarate (100). Rhodes et al. (106) found that the malate effect required protein synthesis. In these experiments, peel disks from preclimacteric fruit did not exhibit any increase in respiration as did climacteric and postclimacteric peel disks; these disks did, however, show an increased ability to decarboxylate malate oxidatively. It has also been demonstrated (108) that exogenous ethylene reduced the time taken to reach the full malate effect. The authors suggested that ethylene, RNA, and

protein synthesis were involved in the development of the ability of aged apple peel disks to oxidatively decarboxylate added malate. Hulme and Wooltorton (70) suggested that malic enzyme might be involved in the malate effect in the aged peel disk system.

Malic enzyme is one of the dominant proteins in pome fruits (48). Dilley (34) has suggested that not only might malic enzyme be involved in the malate effect but the oxidative decarboxylation of malate via malic enzyme might be the cause of the observed increase in the respiratory quotient (R.Q. = rate CO_2 evoluation/rate O_2 uptake) in certain fruits during the CO_2 climacteric. Hulme and Wooltorton (70) hypothesized that the climacteric in whole fruit might be caused by an increase in malic enzyme activity. Walker (126) suggested that if malate was a storage product accumulating in the tonoplast malic enzyme might provide a mechanism for its mobilization for mitochondrial oxidation under conditions where C_4 acids are plentiful and pyruvate is scarce. Klein (77) postulated that malic enzyme might supply NADPH for biosynthetic purposes and it might also increase the supply of respirable substrate (pyruvate) for enhanced mitochondrial activity during the climacteric rise. Malic enzyme and other enzyme activities increase during the time of the climacteric (34,56,70,107). In early-climacteric fruits (40) incorporation of ^{14}C-phenylalanine increased 4-fold in malic enzyme. Hartmann found no increase in malic enzyme activity (55) in cherries, a non-climacteric fruit. Specific activity (34) of malic enzyme was observed to increase in postclimacteric fruit. Rhodes et al. (108)

found that cycloheximide inhibits the development of the malate effect. Malic enzyme appears to be involved in contributing somewhat to the observed CO_2 evolution increase associated with the "respiratory" climacteric in ripening fruits, malic enzyme activity does increase during this time period in whole fruits, and activity increase of the enzyme is, at least in part, dependent upon specific protein synthesis.

Hulme et al. (65) found that malic enzyme was mainly located in the soluble fraction of the cell and only a small part of the entire activity was found in mitochondria. The reaction mechanism (61) of pigeon liver malic enzyme appears to be of the order bi-ter type as determined by product inhibition patterns with $NADP^+$ and malate as variable substrates. $NADP^+$ binds first with malate entering the active site only after the oxidized pyridine nucleotide has been bound. Carbon dioxide or bicarbonate is the first product liberated followed by pyruvate and NADPH. Dilley (35) was the first to purify and perform kinetic studies on apple fruit malic enzyme.

Changes of Pectinaceous Substances and Associated Enzymes with the Ripening Process

It has been assumed that the loss of flesh firmness in climacteric fruits was due to cell wall-degrading enzymes (36,83). Bartley (12) found that water soluble polygalacturonate residues increased in concentration as the apple fruits softened with ripening. These changes were preceded by loss of galactose residues from the cell wall. Changes in the solubility and properties of pectinic

polysaccharides in apples have also been shown to change during the ripening process (64,74). Knee (82,83) has demonstrated that flesh firmness is inversely proportional to soluble polyuronide content. Gross and Wallner (53) found that isolated cell walls from tomato fruits also contained water soluble polyuronides which tended to increase as the flesh became more soft. In tomato (113), the rise in polygalacturonase activity (PG) did not occur until the onset of ripening and this increase came after the increase in water soluble polyuronides (WSP) had begun. These authors took this to be evidence that enzymes other than PG might be involved in the ripening process.

Albersheim et al. (6) demonstrated that pectic substances are the main component of the middle lamella of secondary plant cell walls and can also be detected in the primary cell wall. In apple (16) and pear, structural alterations do occur cytologically in the cell wall via the dissolution of the middle lamella. Gross and Wallner (53) found that the only components of cell walls declining during ripening were galactose, arabinose, and galacturonic acid. They also determined that the ripening-related decline in galactose and arabinose was a separate biochemical event from polyuronide solubilization which increases during ripening in tomato fruits. Knee (82) found similar results occurring in apples; galactose and arabinose decreased in the cell wall fraction as a function of apple softening and the proportion of polyuronide which was water soluble increased.

Working with tomato fruits (58), Hobson found that cellulase plays only a minor role in the softening process. The author stated

that it was "becoming evident that the physical characteristics of fruit tissues are a reflection of the activities of pectic enzymes to which they are subjected." In tomato fruits, which undergo incomplete ripening ("blotchy ripening") in certain areas, Hobson (58) found much lower polygalacturonase activity in the non-softening (non-red) portion of the fruit. In avocado (8) both cellulase and polygalacturonase activity were low at harvest and increased to a maximum level at about the time of the climacteric peak in respiratory activity; pectin methyl esterase declined from harvest to the climacteric. Awad and Young (8) feel that cellulase is the most important hydrolytic enzyme with respect to fruit textural changes in avocado. In pear (16) no cellulase activity was found. Pear tissue (16) treated with a mixture of cellulase and polygalacturonase led to ultrastructural changes observed in naturally-ripened fruits; in apple, polygalacturonase alone brought similar results. In tomato fruit, polygalacturonase endo-activity appears to be dominant over exo-activity (16). Exo-polygalacturonase is a "terminal cleaving" enzyme while endo-polygalacturonase is a "random cleaving" enzyme (13). In 1974, Bartley (13) found native apple polygalacturonase had only exo-activity. After purification, the author found this enzyme would degrade apple cortical cell wall preparations and low molecular weight uronic acid residues and polyuronides were released. Prior to this time no enzyme had been successfully isolated from apple cortical tissue which was definitely associated with the ripening process (12, 105). Bartley (12) has also isolated β-galactosidase from apple tissue

in both soluble and cell wall preparations which degrades pectin galactan optimally at a pH of 4.0.

The ripening (softening) process thus appears to involve endo- and/or exo-polygalacturonase mainly and cellulase in certain fruits. As ripening continues the amount of enzyme and water soluble polyuronides increases. The increase in ripening-related enzyme activity is probably due to de novo protein synthesis since cycloheximide inhibited the ripening process (48).

Physiological Impact of Low Pressure (LPS) and Controlled Atmosphere (CA) Storage on Fruits

For the purpose of extending the storage life of fruits beyond that observed in refrigerated storage, controlled atmosphere (CA) storage was invented in England ca. 1920. In 1977 about 40% of the fresh U.S. apple crop was stored in CA (97). Modern CA storage techniques maintain a low temperature, high relative humidity, and permit control of oxygen and carbon dioxide at recommended low levels (90). For a major extension of apple storage life, Dewey et al. (33) recommended oxygen and carbon dioxide concentrations to be from 2-3% and 1-5%, respectively. In 1957, Stoddard and Hummel (120) reported some work on storage under low pressure. However, Burg and Burg (28) have been credited with inventing low pressure storage (hypobaric or LPS). Both CA and LPS have a rationale in common; if senescense and ripening of fruit tissue is mainly a function of ethylene production then storage life should be extended if oxygen levels were to be lowered sufficiently below 20-21% (ethylene synthesis is highly oxygen dependent). Both CA and hypobaric systems achieve this; however, in LPS

the fruit are continually ventilated with fresh, ethylene-free humidified air. Since the diffusivity of gases is inversely proportional to total pressure (27), the intercellular activity of these substances decreases as the absolute pressure is decreased. In CA, in order to counteract the effects of accumulating ethylene (CA chambers generally have a low atmospheric turnover), carbon dioxide (a competitive inhibitor of ethylene action) is added into the storage atmosphere, or allowed to accumulate. However, ethylene accumulates to high levels where CO_2 is no longer effective and fruits ripen.

Since the intrafruit ethylene concentration is kept extremely low in LPS (38,92) the apple fruits respond by maintaining flesh firmness (the major criterion for "ripening") similar to their at-harvest condition. Fruits so treated also have a greater chlorophyll content, higher acidity, higher total sugar content, fewer storage disorders, and an increased life after removal from storage. The major disadvantage beyond the costs of employing such a storage technique is slowness to develop flavor upon removal from storage (92). Dilley (38,39) maintains that ripening and senescence are retarded at low pressures most likely as a consequence of ethylene removal. Once fruits are removed from storage they ripen slowly and maximum ethylene production is delayed (38,91). The LPS effect has also been observed in other fruit crops (121,129). Wu (129) observed that tomato fruits "never" ripened and eventually deteriorated if stored at 102 torr (20 torr O_2 partial pressure) for greater than 100 days. Streif and Bangerth (121) noted that "the ripening processes in tomato could be

accelerated by sufficient ethylene concentrations in the storage (LPS) atmosphere, even at low oxygen pressures." It thus appears that the majority of the LPS effects on delaying fruit ripening are due to ethylene removal and are not as dependent upon oxygen partial pressure. Others (119) have reported contradictory results to the above. Utilizing $KMnO_4$ as an ethylene absorbent, Stenvers and Bruinsma (119) were unable to demonstrate any delayed ripening at 150 torr (P_T) in tomatoes; the authors concluded that the concentration of endogenously-evolved ethylene in the storage atmosphere had no effect on the onset and rate of ripening.

Controversy exists (118) as to whether the level of ethylene is important in CA storage. Forsyth et al. (46) report that storage life of 'McIntoch' apples increased if ethylene was removed from the storage vessel. Fruit stored for 189 days at 3.3 C in 3% O_2, 5% CO_2, and 6 ppm ethylene were 0.9 kg (2 pounds) more firm than fruit stored in 1570 ppm ethylene (45,46). These authors noted that flesh firmness persisted for more than a week at room temperature and that acidity and soluble solids content were slightly higher in the low ethylene treatment. Others (24) report no consistent effect of naturally-evolved ethylene in stimulating ripening under low oxygen conditions. Liu (89) reports that ethylene removal from CA frequently, but not always, resulted in greater flesh firmness retention in apple culitvars like 'McIntosh,' but noted little effect on 'Delicious,' 'Golden Delicious,' 'Idared,' and 'Cortland.' For maximum effect the fruits had to be picked in a pre-climacteric state and ethylene should

be "scrubbed" out of the CA chamber. Tomkins and Meigh (124) found that CO_2 in the CA atmosphere would reduce levels of ethylene at 5% O_2.

As far back as 1925 (122) it was recognized that acetaldehyde and ethanol accumulate in apples stored under "excess" CO_2 even in the presence of O_2. Davis et al. (32) noted a stimulation of pyruvate and alcohol dehydrogenase in CA-stored fruit; accumulation of acetaldehyde and ethanol were also noticed. Fidler (41) reported that the degree of carbohydrate plus acid lost in respiring apples was about equal to the amount of CO_2 plus ethanol and acetaldehyde formed. Alcohol accumulation has also been reported in 'Cox's Orange Pippin' under anaerobic conditions (42). Once removed from CA, the fruits produced lower than normal non-ethylene volatiles (103). Other biochemical effects have also been noted (49,50,79,80,81,114) in fruits stored under low oxygen and/or high CO_2. Frenkel and Patterson (50) observed fragmentation, reduction in size, and changes in shape of mitochondria, plastids, and tonoplasts of 'Bartlett' pears stored at high CO_2 concentrations. These ultrastructural alterations were similar to those observed in senescent fruits. Knee (79) could find only slight malate decarboxylation in apples stored at 5% CO_2 and 3% CO_2 at 3.5 C. Carbon dioxide has also been observed to decrease succinic dehydrogenase activity in pears (49) and apples (81,114) during cold storage. Knee (80) found that softening and pectinic changes were similar in CA and control 'Conference' pear fruits.

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SECTION I

FACTORS INFLUENCING THE POROSITY OF 'EMPIRE' APPLE PEEL DISKS

Abstract. Ethylene effusion (J_x, $nl \cdot cm^{-2} h^{-1}$) through 'Empire' apple peel disks was found to be linear with the concentration gradient according to Fick's laws which govern diffusion. The coefficient of effusion ($\emptyset$) of these fruit sections was found to vary between 9×10^{-4} to 5×10^{-3} cm^2 h^{-1} at P_T = 50 torr. From this, the fraction of the apple epidermal surface available for gas exchange was calculated to be ca. $(1\text{-}6) \times 10^{-7}$. Slight desiccation of the tissue inhibited ethylene transport reversibly. The rate of ethylene efflux (J_x^F) was significantly higher than influx (J_x^E). This anisotropic effect was enhanced in O_2 and diminished in N_2. The phenomenon was also found to increase significantly with higher (150-1500µl C_2H_4 per l absolute volume) ethylene concentrations. This condition was dependent upon cortical cells subjacent to the epidermis. The addition of ethylene to the environment around the subepidermal side of peel disks induced an increase in ethane effusion by at least 50%. These results suggest that ethylene induced an increase in fruit porosity which might be O_2-dependent.

In apple fruits the major barrier to gas exchange is the epidermis (1,2,3,5,6,8). Through the epidermis, gas transport occurs

primarily through open lenticels (2) which occupy approximately 1/10000% of the total apple surface area. It has been calculated (2) that each cm^2 of epidermal tissue from 'McIntosh' apples contains a total area of open lenticels of ca. 1×10^{-6} cm^2. Lenticels (3) have been thought to result from "broken stomata" or "scars caused by the falling out of trichomes." The number of open lenticels has been shown (3) to deviate as much as a factor of 4 among cultivars.

Apple cortical tissue offers little resistance to gas exchange and no concentration gradient has been found experimentally (2,4). However, other investigators (10) did discover a gradient in the composition of the internal atmosphere from the center of the fruit to its periphery, thus implying either differing rates of synthesis and/or some resistance of the cortex to gas diffusion within the tissue.

The porosity of some climacteric fruits was shown to change during the ripening process (1,7). Having investigated avocado fruits in both the peeled and intact condition, a group of investigators (1) found that the resistance of the tissues to CO_2 effusion $[CO_2]_{in} / (\frac{dCO_2}{dt})_{fruit}$ increased from 0.7 in intact fruits (0.6 in peeled) at the initial climacteric rise to 2.5 (2.4 in peeled) during the postclimacteric phase; similar results were noted in bananas (7,11). Clements (3) found that apple fruit porosity was diminished by processes which exacerbate water loss from the outer tissues. Fockens and Meffert (4) found that water vapor movement

through the apple epidermis was best described as a combination of a wet surface-gas porous-gas impervious layer model system whereby:

$$m = \frac{C_1 B}{RT} + \frac{C_2}{RT}\left(\frac{1}{B} + \frac{D_m t}{D}\right)^{-1}$$

m = rate of moisture loss

C_1 = wet fractional surface

C_2 = porous layer fractional surface

B = coefficient of mass transfer (dependent upon the Reynolds number of the flow along the surface)

R = gas constant

T = mean absolute temperature of the boundary layer

D_m = mass of vapor diffusing through a layer of air/mass of vapor diffusing through the porous layer

D = coefficient of diffusion of water vapor in air

t = thickness of the layer.

It was also reported (4) that as water vapor was lost, the diffusional resistance increased as the apple epidermis intercellular spaces became smaller due to a change in the shape of the epidermal and subtending cells.

Gas exchange in fruits has been demonstrated to be governed by Fick's law (2) whereby:

$$J = -D\left(\frac{dc}{dx}\right)$$

D = diffusivity

c = concentration

x = distance in the plane of gas movement.

Thus, the amount of gas effusing across a plane at x in time Δt is equal to $J\Delta t$ whereas the quantity leaving the plane at $(x + \Delta x)$ in the same time interval is $(J + (\frac{dJ}{dx})\Delta x)\Delta t$. The net gain in the quantity of diffusing material between these planes may be expressed in terms of the change in concentration in the volume $A\Delta x$ (A = area of the plane) and in terms of the difference between these two amounts of transported material:

$$\Delta c \Delta x A = (J\Delta t - (J + (\frac{dJ}{dx})\Delta x)\Delta t)A$$

$$\Delta c \Delta x = -(\frac{dJ}{dx})\Delta x \Delta t$$

$$\frac{\Delta c}{\Delta t} = \frac{-dJ}{dx} \text{ and as } \Delta t \to 0$$

$$\frac{dc}{dt} = \frac{-dJ}{dx}$$

since $J = -D\ (\frac{dc}{dx})$ Fick's 1st law

$$\frac{dc}{dt} = D(\frac{d^2c}{dx^2}) \quad \text{Fick's 2nd law.}$$

Thus, the rate of change of concentration with respect to time is directly proportional to the rate of the concentration gradient change with respect to distance.

In this paper data are reported which

1. allow for the empirical determination of the coefficient of effusion for ethylene ($\emptyset$ = $cm^2 \cdot h^{-1}$)
2. demonstrate that treatments with facilitate water loss from apple tissue reversibly diminish fruit porosity

3. provide evidence for the hypothesis that ethylene stimulates an increase in porosity.

Materials and Methods

Apparatus (Figure 1)

Experiments were performed at 50 torr absolute pressure and 20-25 C unless otherwise indicated. These conditions were utilized in order to expedite the procedure since effusion would be 15-fold more rapid under these conditions than under atmospheric pressure (2). The rate of ethylene transport across the peel disk was measured by removing 1 ml (at atmospheric pressure) of gas from side B (Figure 1) at various time intervals after the system had been 'started' upon injection of ethylene at E. In order to sample side B at a pressure lower than atmospheric, a device (Figure 1-D) was constructed to increase the absolute volume of side B by ca. 100 ml. This 100 ml sample (P_T = ca. 42 torr) was thus compressed, after isolation from side B at D-3, to about 5 ml and approximately 760 torr. After 1 ml gas was removed, a sample of gas containing equal proportions of O_2 and/or N_2 was injected back into the system (B) so that conditions between sides would remain isobaric.

Peel Disk Chamber (Figure 1,C) and Tissue Preparation

A chamber to hold the tissue in place during the experiments was fabricated from a Plexiglas cylinder (diameter and length ca. 3 cm). Leaks were avoided by having an O-ring seal fitted into

Figure 1. Apparatus for measurement of effusion at various total pressures. Depicted is the device (D-1,-2,-3) designed to collect gas samples from side B under low pressure (50-700 torr) conditions. D-1 and -2 are syringe bodies and concomitant pistons mounted on an aluminum body with pullies (H) and reel (I) for assisting in expanding the absolute volume of side B by ca. 100 ml. J is the air-tight septum for collecting gas at atmospheric pressure from syringes D-1 and -2. D-3 is the location of a clamp used to isolate the syringes from the system B. Within side A the ethylene (or ethane) level was maintained between ca. 150-12000 µl (0.1-8.0 ml ethylene or ethane) per 1 absolute volume. Both sides A and B were kept humidified with moistened filter paper. C is the chamber with apple tissue offering the only resistance to gas exchange between sides A and B. E is the location of an air-tight septum for injection of ethylene into side A. F connects to the vacuum pump. G connects to the mercury manometer. K is the location of a clamp for isolation of side A from side B prior to injection of ethylene or ethane into side A.

the chamber. The entire unit was fastened together with 6 brass screws and wrapped thrice in a paraffin film.

Tissue from the mid-section of 'Empire' apple fruits was selected for experimentation. The apple peel disks with subjacent cortical tissues were cut with a 3.8 cm i.d. cork borer and the plugs were removed from the whole fruit. The cortex tissue was cut flush with the outer edge of the peel leaving only that within the peel concavity. A second cut was made from the subepidermal (flesh) side up to, but not through, the inner epidermis with a 3.2 cm i.d. borer; this ring of cortical cells was removed in such a way that the epidermis and subjacent cortical tissue fit tightly into the chamber with the outer peel tissue compressed against the O-ring and the cortex (ca. 1 mm) inset into the chamber.

The area of the peel disk was calculated after having performed the experiments by removing the flesh and photocopying the disks. The images were cut and weighed against a standard area of photocopy paper. The average peel disk had an area of ca. 8.5 cm^2.

Definition of Terms

J_X^F (efflux) is defined as the rate of gas effusion through the subepidermal or flesh side of the peel disk; J_X^E (influx) is defined as the rate of effusion through the epidermal side of the peel disk (in the opposite direction of J_X^F). The degree of anisotropy or percent increase in effusion from one side to the other was calculated as

$$\left(\frac{(J_X^F - J_X^E)}{J_X^E}\right) \quad 100$$

Measure of Porosity

In certain experiments measurements were taken of J_X^F (ethylene efflux, $nl \cdot cm^{-2} \cdot h^{-1}$) and Q_X^F (flow rate, $\mu l \cdot min^{-1}$). Both of these parameters should be independent measures of tissue porosity or openness of the lenticels. To measure Q_X^F or Q_X^E the peel disk chamber was connected to an O_2 source and pressure gauge. Oxygen was introduced at a pressure of 152.4 cm H_2O on one side (ca. 112 torr above atmospheric pressure) and flow was measured on the opposite side of the chamber with a 100 µl pipette using Kodac photoflow solution. About 5 µl of the photoflow solution was introduced into the pipette and subsequently connected to the tissue chamber. Movement of this fluid across the known volume of the pipette was timed and flow rate calculated. Five readings were made for each Q_X value and the coefficient of variation was observed to be 5% or less ($S_{\bar{x}}$ 100/$\bar{x}$).

Calculation of J_X

At various times after ethylene or ethane was injected into side A (Figure 1), a 1 ml gas sample was taken from side B using the device described previously (Figure 1,D). The gas samples were analyzed chromatographically for ethylene or ethane. The gas chromatograph, a Varian series 1700, used N_2 at 70 C as the carrier gas, was equipped with a 2 mm by 1 m column of activated alumina, and a flame ionization detector. It was capable of detecting ethylene at concentrations of 2 $nl \cdot l^{-1}$ in a 1 ml sample with a retention time of ca. 25 seconds. Ethylene or ethane concentration was correlated with time via linear regression analysis. The correlation coefficients (r)

for these calculations were never less than 0.97 since the process was essentially zero order within the time limits measured. Calculation of J_X was performed as follows:

$$J_X = \frac{60\ \text{min}\cdot h^{-1}\cdot m\left(\frac{(P_T^A V^B)}{P_T}\right)}{A} = \text{nl}\cdot cm^{-2}\cdot h^{-1}$$

m = slope of regression line $\text{nl}(\text{ml}\cdot\text{min})^{-1}$

P_T^A = pressure inside system A (and B)

V^B = absolute volume of system B (ml)

P_T = 760 torr

A = area of peel disk (cm^2).

Experiment 1 (Figures 2 and 3)
Range of Effusivity of
'Empire' Apple Peel
Disks

Twenty-five low pressure-stored fruits were selected at random and kept at 0 C in air until needed. From each apple fruit one peel disk was cut and mounted in the peel disk chamber as previously described. To the subepidermal or flesh side of the chamber was attached a rubber septum-serum cap assembly through which humidified O_2 was introduced at a pressure of ca. 152.4 cm H_2O and flow through the peel disk was measured as previously described. The peel disk chamber was then connected (Figure 1) with the subepidermal or flesh side contiguous with side A. The total pressure of the system was reduced to ca. 30 torr and N_2 added until the pressure reached 100

torr. This procedure was repeated thrice to ensure that the constituents in the system were primarily H_2O vapor and N_2. All treatments within this experiment were performed at P_T = 50 torr. The two sides of the system (A and B) were isolated by the closure of the vacuum tube connection at K (Figure 1). On side A, the pressure was reduced ca. 1.25 torr per ml ethylene added. This was done in order to maintain isobaric conditions between the two sides of the apple peel disk. Ethylene was added to system A according to treatment as follows:

Volume ethylene Added (ml)	Calculated concentration Side A ($\mu l \cdot l^{-1}$)	Time intervals* (min)
0.1	152	10
0.5	760	5
1.0	1520	3
3.0	4560	3
8.0	12158	2

Samples were removed from side B at various time intervals* after ethylene was injected in side A (Figure 1). For each volume of gas removed, an equal amount was added back into system B. Samples were analyzed chromatographically and J_X^F calculated as previously described. After these data were collected $\log_e J_X^F$ was regressed against $\log_e Q_X^F$ at each concentration of ethylene in order to have a predictive equation for J_X^F at some constant Q_X^F.

Experiment 2 (Table 2): Effect of Low Humidity on O_2 Flow Through 'Empire' Apple Peel Disks

Three low pressure-stored fruits were selected for experimentation and one peel disk was cut from each and mounted in the plexiglass chamber as previously described. Q_X^F was then measured as previously described. The side into which O_2 was suffused was reversed and Q_X^E measured in the opposite direction. This procedure was repeated 7 times over a 6-7 h time span for each tissue piece. Between measurements, the chamber, with the peel disk mounted inside, was placed in a desiccator (ca. 12 l) and the system flushed with dry O_2.

Experiment 3 (Table 3): Effect of Alternating Dry and Humid Conditions on Ethylene Efflux

Three peel disks were selected for Q_X^F values ranging between 500-700 $\mu l \cdot min^{-1}$ initially after cutting. Immediately after the flow of O_2 was measured through the peel disk, the rate of effusion of ethylene (J_X^F) was measured at P_T = 50 torr (P_{O_2} = 25 torr, balance N_2 and H_2O vapor) after a concentration gradient of ca. 1500 $\mu l \cdot l^{-1}$ ethylene was established between sides A and B (Figure 1). Between the times 0-3 hours and 21-23 hours the tissue was maintained in dry O_2 (P_T = 200 torr, P_{O_2} = 150 torr, balance N_2). Between times 3-21 and 23-43 hours the tissue was kept in moist O_2 (P_T = 200 torr, P_{O_2} = 150 torr, balance N_2 and H_2O vapor). All treatments were performed at lab temperature (20-25 C).

Experiment 4 (Table 4): Determination of the Effect of Cortex Removal and Relative Humidity on the Efflux and Influx of Ethylene

In these experiments 5 or 6 apples were selected and the tissue mounted in the Plexiglass chamber (Figure 1-C) as before. The fruit used had been stored 4 months in air at 0 C. In one treatment the cortical cells were removed from the subepidermal surface of the peel disks. Either J_X^E or J_X^F were measured at P_T = 50 torr (P_{O_2} = 25 torr, balance N_2) as previously described, but in the absence of water vapor. In the other treatment the flesh was retained on the peel disks and effusion (influx and efflux) measured under humid conditions.

Experiment 5 (Table 5): The Effect of Peel Disk Conditioning on Consecutive Efflux Measurements

The chamber with the peel disk enclosed was wrapped in moist filter paper, placed in a 12 l glass desiccator, and pressure reduced to about 30 torr (T = 20-20 C), whereupon N_2 was added until the pressure reached 100 torr. This process was repeated thrice. The pressure was then increased from 30 to 100 torr with N_2; O_2 was added to reach a total pressure of 200 torr. The tissue was maintained under these conditions for 30 minutes. Efflux (J_X^F) of ethylene was then measured at a concentration gradient of ca. 1500 µl/l (P_T = 50 torr, balance N_2 and H_2O vapor) as previously described (Experiment 1).

Experiment 6 (Figure 4): Effect of Ethylene Concentration and O_2 Level on the Anisotropic Effusion of Ethylene

Four hypobaric-stored 'Empire' apples were selected at random and maintained at 0 C in air until needed. From each apple fruit (block) four peel disks were used. To each disk was assigned, at random, one of four treatments (Table 1). The tissue was handled as previously described in Experiment 5. J_X^F and J_X^E were measured as described in Experiment 1.

Experiment 7 (Table 6): The Effect of Ethylene on Ethane Efflux

The experiment was performed at a randomized complete block with 5 blocks (peel disks or films). As a control, effusion was measured through a Dow Handiwrap-covered aluminum disk which had been perforated with a 1.3 mm diameter syringe needle. Prior to this experiment fruits had been in hypobaric store for ca. 9 months at 0 C. All effusion measurements were performed with the subepidermal or flesh side of the peel disks toward the high ethane (side A) concentration. Chamber pressure (A, B, C Figure 1) was reduced to ca. 30 torr; O_2 was then introduced until the total pressure of the system was 70 torr. In the 700 torr treatments all conditions were identical but N_2 was added until the desired level was reached. The pressure on side A was diminished ca. 1-2 torr to compensate for the ethane injection which followed at septum E. The system was allowed to equilibrate 15 and 60 min for the low and high pressure treatments, respectively. At ca. 4 (100 torr) and 15 (700 torr) min intervals a

TABLE 1.--Treatment assignment for an experiment concerning the effect of ethylene concentration gradient (150 or 1500 $\mu l \cdot l^{-1}$) and O_2 level (27 or 0 torr.) on the anisotropic effusion of ethylene. 2 O_2 levels x 2 ethylene levels x 4 Blocks (peel disks) x 2 sides = 32 treatment combinations

Sequence	Treatment			
	Gas	P_T	P_{O_2}	P_{N_2}[a] (torr.)
Pre-efflux	O_2	200	100	77
	N_2	200	0	177
Efflux	O_2	50	27	0
	N_2	50	0	27
Pre-influx	O_2	200	100	77
	N_2	200	0	177
Influx	O_2	50	27	0
	N_2	50	0	27

[a]Balance H_2O vapor; calculated for T = 25°C, $P^{max}_{H_2O}$ = ca. 23 torr; real lab temperature varied from 23 - 25 C during this experiment.

1 ml gas sample was removed from side B using the device D (Figure 1). To compensate for the pressure loss due to sampling (8 ml total would be removed) a ml of gas containing an equivalent amount of N_2 and O_2 was added back into the system for each sample collected. After 4 samples had been collected, 0.5 ml ethylene was added to side A. At intervals of 4 (100 torr) and 15 (700 torr) min samples were retrieved as before. The gas samples were measured for ethane chromatographically. Rates of effusion ((J_X^F) ethane) were calculated from the slopes of the concentration-time regression equations as previously described.

Experiment 8 (Table 7): Changes in Porosity, Intrafruit Ethylene, and Ethylene Production During Ripening

Seven 'Empire' apples were removed from store (7 months in hypobaric + 1 month in air at 0 C) and allowed to equilibrate for 1 day at 22-25 C. Into the calyx end of each fruit was inserted a 2.5 cm (1.3 mm diameter) syringe needle. After insertion, the passage of the needle was cleaned with a wire. Rubber septa and serum caps were placed over the bases of the syringe needles so that internal gases could not escape. A mixture of lanolin-vasoline was placed around the calyx to insure gas tightness. These fruits were then placed in ca. 1.5 l glass jars into which ethylene-free air was introduced at a rate of 10-20 ml min^{-1}. At daily intervals chamber ethylene was sampled; immediately thereafter, the fruits were removed, immersed in H_2O, and 1 ml of intrafruit gas was withdrawn. All

samples were analyzed chromatographically for ethylene. The rate of ethylene evolution was calculated as follows:

$$\text{rate} = \frac{\text{flow } (\text{ml}\cdot\text{h}^{-1}) \times P_T \text{ (torr)} \times V_i/V_t \text{ (conc. of ethylene)}}{R \text{ (l}\cdot\text{torr /(k}\cdot\text{mole)} \times T \text{ (K)} \times \text{weight (kg)}}$$

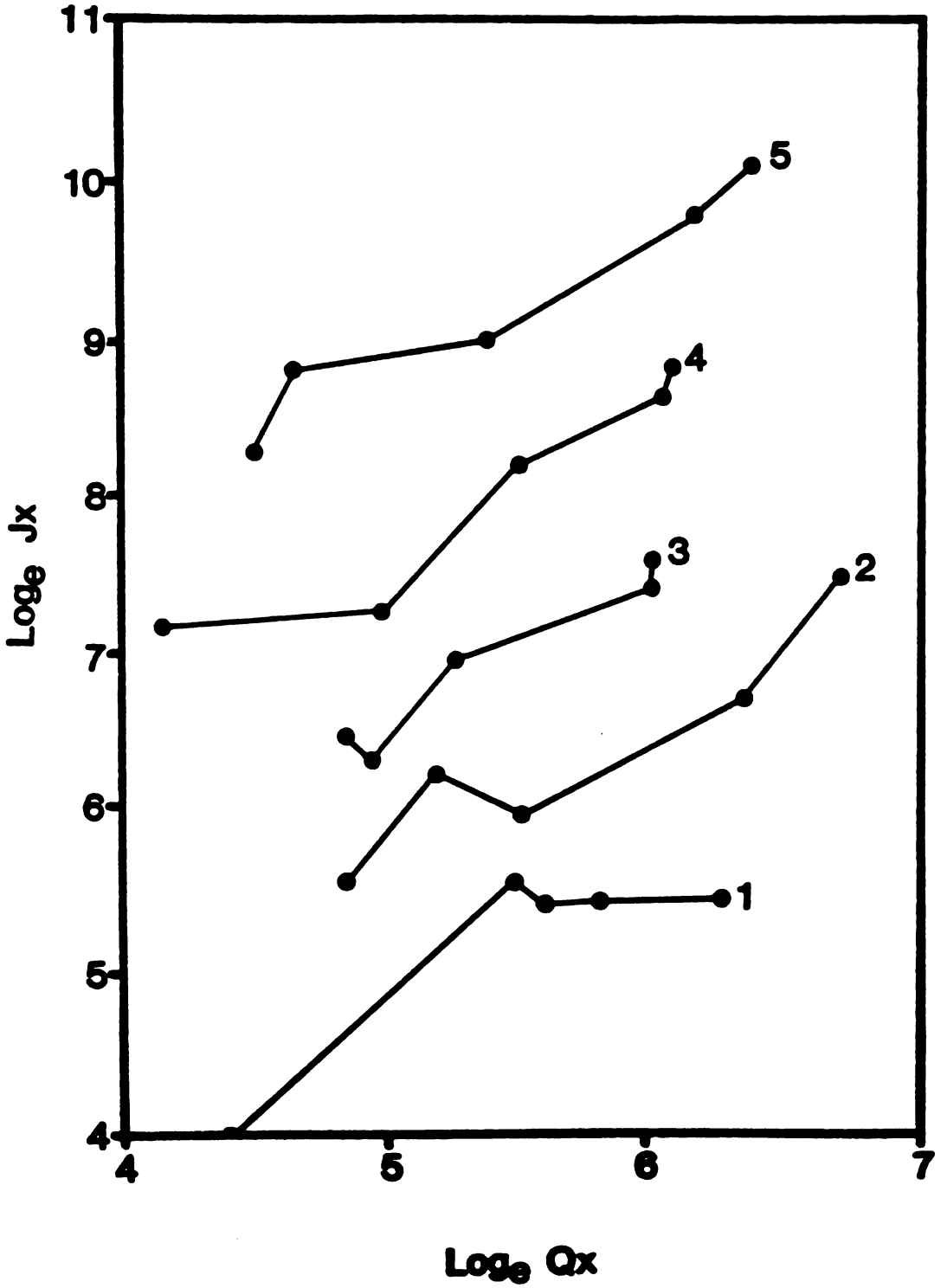
$$= \text{moles}\cdot(\text{kg}\cdot\text{h})^{-1}$$

The purpose of this experiment was to determine if the porosity of whole fruits would change as internal ethylene accumulated. If ethylene does increase the porosity of whole fruit, then, as internal ethylene increases, the ratio of ethylene evolution to internal ethylene might increase.

Results

These data (Figure 2) demonstrate a log-log linear relationship between J_X^F and Q_X^F thus allowing the mathematical prediction of J_X^F at some value of Q_X^F. The median value for Q_X^F across the population of 25 peel disks (25 fruit) was about 200 $\mu l\cdot min^{-1}$. The efflux of ethylene ($(J_X^F)_{200}$) was calculated from the regression equation at each ethylene concentration gradient at Q_X^F = 200 $\mu l\cdot min^{-1}$; data from these calculations are given in Figure 3. The empirical relationships (Figure 2) were log-log linear for no physical reason other than reduction in error variance in J_X^F with respect to Q_X^F. A graph of the calculated J_X^F at Q_X^F = 200 $\mu l\cdot min^{-1}$ as a function of ethylene concentration gradient was linear. This might be expected for gas exchange in apple fruits (2) which has been found to obey Fick's

Figure 2. Changes in $\log_e J_X^F$ as a function of $\log_e Q_X^F$ at 5 concentration gradients (1-5 being ca. 152,760,1520,4559, and 12158 µl ethylene per l absolute volume, respectively). Correlation coefficients for each of the regressions were 1 = 0.87*, 2 = 0.94**, 3 = 0.94**, 4 = 0.94**, 5 = 0.96**. The experiment was a completely randomized design. Effusion was measured from the flesh or subepidermal side of the tissue.


11
10
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law. The slope of this relationship (Figure 3) was 0.77 $cm \cdot h^{-1}$. This value is a function of the coefficient of diffusion of ethylene in N_2 at P_T = 50 torr and the porosity of the epidermis. Data from Table 2 clearly indicate that O_2 flow was favored through the epidermal side (Q_X^F) of the peel disk. These data also demonstrate that flow diminished about 37% for fruit I, 206% for fruit II, and 152% for fruit III. These changes were presumably due to water loss from subepidermal cortical cells which has been shown to diminish porosity (3). To test this hypothesis more thoroughly, efflux measurements were made over various time spans in dry or humid conditions. These data (Table 3) demonstrate that the porosity of the apple peel disks, as measured by J_X^F, declined over time in dry O_2 and recovered when the tissue was maintained in a humid environment. Over the initial 3 h period, porosity diminished ca. 94% and recovered to about the initial value after 18 h in moist O_2. After another period of alternating dry and humid conditioning, J_X^F was about 36% greater than the initial, time 0, value.

In the process of performing certain experiments, it was discovered that the rate of effusion in one direction (J_X^F), was greater than that in the other (J_X^E). Experiments (Table 4) were performed to confirm this anisotropic phenomenon. When subepidermal tissue was left intact, the rate of ethylene efflux (J_X^F) was ca. 65% greater than ethylene influx (J_X^E). When the subtending cortical cells were removed from the peel disks, the process was reversed. Neither observation would be possible if porosity were equivalent at each measurement.

Figure 3. Change in J_X^F as a function of the concentration gradient. These values were calculated at the median value of Q_X^F, 200 $\mu l \cdot l^{-1}$, from the regression of $\log_e J_X^F$ onto $\log_e Q_X^F$ at 5 different ethylene concentrations (Figure 2).

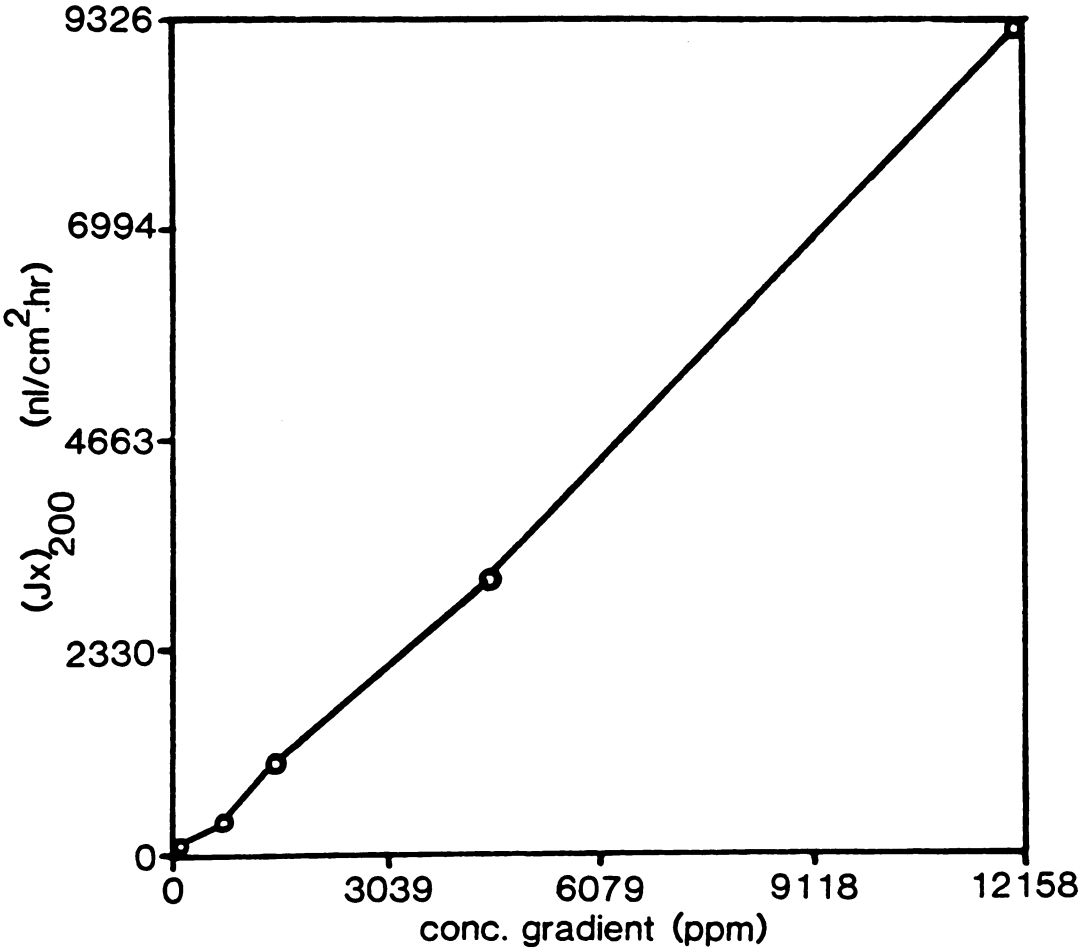
9326
6994
4663
2330
0
$(Jx)_{200}$ (nl/cm^2.hr)
0
3039
6079
9118
12158
conc. gradient (ppm)

TABLE 2.--The effect of time at low humidity on the flow of O_2 through the subepidermal (Q_x^E) side or epidermal (Q_x^F) side of 'Empire' apple peel disks. The O_2 pressure used to induce flow was 152.4 cm H_2O. Between sampling times the peel disks were flushed with dry O_2

	Fruit Number					
	I		II		III	
MIN.	Q_x^E[a]	Q_x^F	Q_x^E	Q_x^F	Q_x^E	Q_x^F
12[b]	424	332	158	107	221	121
55	392	314	151	79	113	41
70	391	319	118	63	103	63
113	382	292	113	57	128	48
322	289	254	96	53	117	38
354	268	247	108	55	136	37
387	272	238	76	39	123	49
417	275	243	87	35	112	48
	19 ± 3[c]		93 ± 10		156 ± 25	

[a] FLOW ($\mu l \cdot min^{-1}$)

[b] Average of 3 different times.

[c] 100 $(Q_x^E - Q_x^F)/Q_x^F$); averaged over time; $\pm s_{\bar{x}}$

TABLE 3.--The effect of alternating dry (0.3 h; 21-23 h) and humid (3-21h; 23-43 h) conditions on ethylene efflux (J_X^F)

time[a]	J_X^F (nl·cm^{-2}·h^{-1})				
	Q_X^F (µl·min^{-1})				
	510	670	760	$\bar{x}$	$s_{\bar{x}}$
0	1781	2178	2415	2125	227
1	1267	1785	1853	1635	226
2	910	1254	1386	1183	174
3	950	1188	1148	1095	90
----------	----------	----------	----------	----------	----------[b]
21	2059	2415	2375	2283	138
22	1267	1901	1782	1650	238
23	989	1267	1505	1254	183
----------	----------	----------	----------	----------	----------
43	2495	2969	3207	2890	257

[a]Hours post cutting.

[b]Dotted lines indicate that the tissue was kept under humid conditions for ca. 19 h.

TABLE 4.--The effect of cortical tissue removal and humidification on the efflux (J_x^F) and influx (J_x^E) of ethylene at a concentration gradient of ca. 1500 $\mu l \cdot l^{-1}$ absolute volume

Peel Disk[a]	w/o cortex			w/ cortex		
	J_x^F-order[b]		J_x^E-order	J_x^F-order		J_x^E-order
I	103-1[c]		281-2	568-2		363-1
II	268-2		1189-1	317-1		136-2
III	243-1		338-2	433-2		191-1
IV	888-2		2153-1	746-1		519-2
V	993-1		1521-2	339-2		251-1
VI	2905-2		4438-1	--		--
$\bar{x}$	900	*[d]	1653	481	**	292
% increase		84			65	

[a]These data reflect two experiments; there are no common blocks across tissue retention treatments.

[b]Refers to the order in which measurements were taken.

[c]J_X^F and J_X^E are expressed in units of $nl \cdot cm^{-2} \cdot h^{-1}$.

[d]*,** Significantly different at the 0.05 and 0.01 level, respectively.

This indicates that these observations were induced by some factor or artifact of the system.

In order to confirm the anisotropic phenomenon previously observed, a method of pre-treating the tissue was required so that consecutive effusion measurements would be consistent. Since porosity increased when tissue was maintained in a humid environment (Table 3), similar treatments were applied in another experiment (Table 5) to determine if consecutive measurements would be similar. These data indicate that conditioning the tissue in a humid chamber 30 min prior to measurement allowed relatively consistent results; porosity increased only about 8% after the initial efflux measurement. This increase was not statistically significant.

In order to determine the effect of O_2 and ethylene on the degree of anisotropy, an experiment (Figure 4) was performed. The anisotropic effect was greater when the ethylene concentration gradient was increased by a factor of 10 (150 to 1500 $\mu l \cdot l^{-1}$). In the presence of O_2, the degree of anisotropy was about 99% greater than the low ethylene level at a concentration gradient of 1500 $\mu l \cdot l^{-1}$; this increase was only about 42% in N_2. Thus, the increase in efflux over influx as a function of ethylene concentration was greater in O_2 than in N_2. These data lead to the hypothesis that ethylene stimulates an increase in fruit porosity. Evidence for this hypothesis is presented in Table 6. The rate of ethane movement (J_x^F) through this apple peel disk system was increased approximately 50 to 100% at P_T = 100 and 700 torr, respectively; these increases were significant statistically. Utilizing identical methods and conditions, no

TABLE 5.--The effect of humidity on consecutive measurements of ethylene efflux at a concentration gradient of ca. 1500 $\mu l \cdot l^{-1}$ absolute volume. Prior to effusion measurements the peel disks were kept in humidified O_2 for 30 minutes (P_T = 200 torr.)

Peel Disk	J_x^F-1[a] ($nl \cdot cm^{-2} \cdot h^{-1}$)	J_x^F-2
I	57	50
II	498	507
III	353	362
IV	353	395
V	1086	1210
$\bar{x}$	469	505[b]

[a]The number represents the order of measurement within block.

[b]Not significant at the 0.05 level.

Figure 4. The effect of ethylene concentration gradients (150 and 1500 $\mu l \cdot l^{-1}$) and O_2 level on the anisotropic effusion of ethylene. Two consecutive measurements were made for each peel disk, first J_X^F followed by J_X^E.

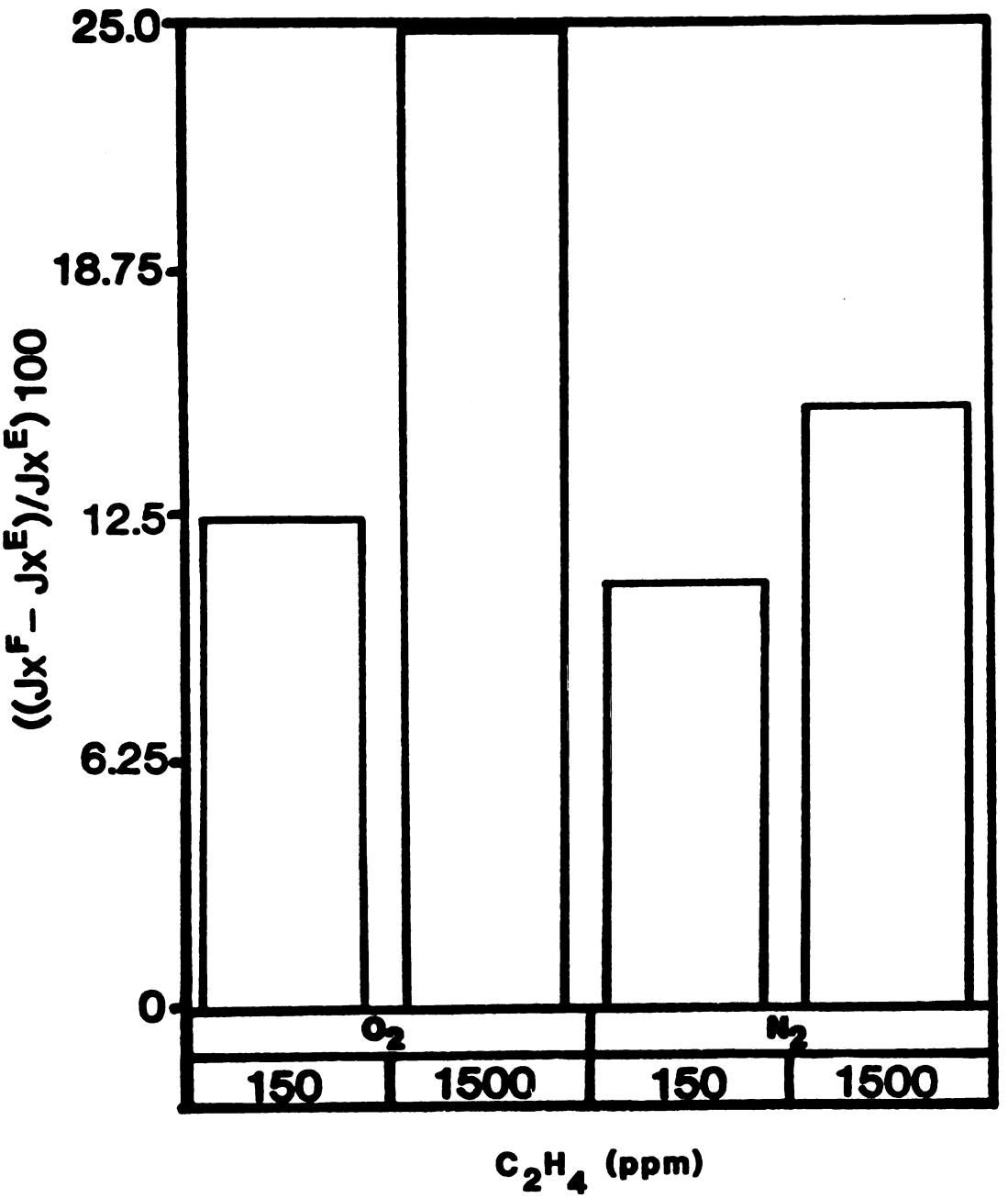
25.0
18.75
12.5
6.25
0
((Jx^F - Jx^E)/Jx^E) 100
O2
N2
150
1500
150
1500
C2H4 (ppm)

TABLE 6.--The effect of ethylene upon ethane efflux (J_x^F) in 'Empire' apple peel disks. As a control, efflux was measured through a film-covered perforated aluminum disk.

Barrier	P_T (torr)	C_2H_4 ($\mu l \cdot l^{-1}$)	J_x^F ($nl \cdot cm^{-2} \cdot h^{-1}$)[a]	Ideal	% Increase
Peel Disk	100	0	212	212	50
Peel Disk	100	760	317**[b]	317	
Film[c]	100	0	72	72	4
Film	100	760	75	75	
Peel Disk	700	0	36	30	119
Peel Disk	700	760	79*	45	
Film	700	0	13	10	8
Film	700	760	14	11	

[a]A concentration gradient of approximately 1500 μl ethane$\cdot l^{-1}$ absolute volume was used.

[b]*,** Significantly different at the 0.05 and 0.01 level, respectively.

[c]Dow 'Handiwrap' covering an aluminum disk perforated once with a 1.3 mm diameter syringe needle.

significant increase in ethane efflux was observed when an inert film was the diffusional barrier. As intrafruit ethylene increased in activity in intact fruits (Table 7), the rate of ethylene evolution increased more than the change in ethylene concentration. The porosity factor increased ca. 25% between 42 and 138 h after storage; this increase was statistically significant at the 0.05 level. However, porosity decreased ca. 39% thereafter.

Discussion

For 'McIntosh' apples, it was determined that the major barrier to gas exchange was the fruit epidermis (2), since upon removal of this barrier 80% of the accumulated ethylene and CO_2 was lost. Comparing flow through pores of known size with 'McIntosh' apple peel slices, Burg and Burg (2) determined that the fraction of the apple surface available (open) for gas exchange was about 1×10^{-6} (F), since

$$J = -D\left(\frac{dc}{dx}\right)$$

$$J_x = -\emptyset\left(\frac{\Delta c}{x}\right)$$

$\emptyset$ = coefficient of effusion = $\frac{D}{F}$

x = thickness of the barrier

$$F = \frac{\emptyset}{D}$$

The diffusivity (D) of ethylene, in N_2 at 20 C and P_T = 760 torr, is 540 $cm^2\ h^{-1}$ (2). Thus, at P_T = 50 torr, this value would be 8208 $cm^2 \cdot h^{-1}$. In Table 8 are given the values of J_X^F at the different concentration gradients of ethylene (152-12158 $\mu l \cdot l^{-1}$) for the observed low, median, mean, and high Q_X^F. From the regression analysis of J_X^F as

TABLE 7.--Changes in the porosity factor ((ethylene evolution/intrafruit ethylene) x 100; P.F.), intrafruit ethylene concentration ($\mu l \cdot l^{-1}$; C_{in}), and ethylene evolution ($\mu l \cdot (kg \cdot h)^{-1}$; et. ev.) in ripening 'Empire' apple fruits. Data represent averages of 6 apples (blocks)

time[a] (h)	P.F.[b]	$C_{in} \pm S_{\bar{x}}$	et. ev. $\pm S_{\bar{x}}$
42	20	43 ± 12	9 ± 3
67	22	100 ± 18	23 ± 4
93	23	154 ± 15	35 ± 3
138	25	143 ± 11	36 ± 3
162	21	170 ± 11	36 ± 3
186	19	180 ± 11	35 ± 3
210	18	209 ± 11	37 ± 3

[a] T = 20-25 C.

[b] $LSD_{0.05} = 3$

TABLE 8.--The predicted values for ethylene efflux at various Q_x^F and the calculation of the coefficient of effusion (Ø) and the fractional surface area (Ø/D) of 'Empire' apple peel disks available for gas exchange. These data were calculated from the regression of ln J_x^F on ln Q_x^F at 5 concentration gradients (c); see Figure 2.

	J_x^F nl·cm^{-2}·h^{-1}			
	Low	Median	Mean	High
$\mu l \cdot l^{-1}$	Q_x^F = 82	Q_x^F = 200	Q_x^F = 300	Q_x^F = 564
152	70	153	217	377
760	197	435	624	1095
1520	463	1062	1548	2784
4559	1383	3058	4387	7694
12158	4410	9326	13111	22280
m^a = $cm \cdot h^{-1}$	0.37	0.77	1.08	1.83
r^b	0.99	0.99	0.99	0.99
Ø = $cm^2 \cdot h^{-1}$	(0.93	1.93	2.70	4.58) x 10^{-3}
Ø/D =	(1.13	2.34	3.29	5.58) x 10^{-7}

[a]Equivalent to the slope from the efflux - c regression equation.

[b]Correlation coefficient.

a function of concentration (c) were calculated the $\emptyset$ ($cm^2 \cdot h^{-1}$) values from the slopes of these equations; $\dot{x}$ was assumed to be 0.0025 cm (2). Knowing the true D for ethylene, the fraction of the apple surface area open ($\emptyset/D$) ranged between 1.13-5.58 x 10^{-7} for relatively low to high porous disks (Table 8).

Data from experiments 2 and 3 (Tables 2 and 3) indicated that as a function of moisture loss the apple epidermis became less porous; both Q_X^F (Table 2) and J_X^F (Table 3) decreased greatly from initial to final values, and J_X^F recovered upon equilibration in a high moisture environment. These data (Table 2) also indicate that flow (Q_X) was favored from the epidermal side ($Q_X^E > Q_X^F$). In 2 of the 3 peel disks tested this flow differential increased as the tissue desiccated. The observed difference in Q_X^E with respect to side could be explained if one were measuring sonic or turbulent flow (Reynolds number greater than 2000). Under these conditions the geometry of the lenticels could explain these results. However, sonic velocities at an orifice only occur when the ratio of the pressures on either side of the orifice are less than 0.53 and the ratio of the cross-sectional areas of the upstream line to the orifice is greater than 25 (9). While the latter criterion was satisfied (ratio = 7 x 10^4 - 3 x 10^6), the former was not (ratio = 0.87). A more likely explanation is that the force exerted against the subepidermal cortex (P = 13332.24 N m^{-2}) was enough to alter the flow via partial blockage of the inner lenticel opening by the subtending cortical cells.

When subepidermal tissue was retained on the apple peel disk (Table 4) efflux (J_X^F) was greater than influx (J_X^E); this was reversed

when the cortical tissue was removed. Since diffusion between two systems which are isobaric must be isotropic, the observed result should be due to an increase in porosity. If ethylene were to increase the porosity of these peel disks, then one might expect to observe an increase in the anisotropic effect (efflux greater than influx) when a greater concentration gradient was used. When the ethylene concentration was increased by a factor of 10 the degree of anisotropy increased 100% in O_2; the same concentration change increased the effect only 42% in N_2 (Figure 4). These data support the hypothesis that ethylene and O_2 are involved in the phenomenon. If ethylene increased the porosity of the apple epidermis, the addition of ethylene to the subepidermal or flesh side of this system (A, Figure 1) would result in an increase in the rate of ethane movement across the peel disk; this observation was made (Table 6). The rate of ethane movement was increased after ethylene injection ca. 50 and 100% at 100 and 700 torr, respectively. The question as to whether this phenomenon occurs in whole fruit was put to a test (Table 7). It was observed that the rate of ethylene evolution was higher than the rate of change in internal ethylene activity between 42 and 138 h post store. However, the porosity dropped sharply thereafter.

The pome lenticel is the major structure for gas exchange (2). These apertures change in size as a function of the state of hydration of the subtending cells (3, 4, these data). It might be possible that ethylene acts as a stimulus for increased water uptake. The limiting component of cell water potential, which physically governs water

sorption, is the lack of cell wall plasticity. If ethylene were to stimulate (enzymatically or perhaps physically at high concentrations) an increase in cell wall plasticity, the subjacent epidermal cells could expand so long as the atmosphere surrounding these cells were nearly saturated with water vapor and the activity of water in the cell remained less than one.

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SECTION II

ATTENUATION OF THE RIPENING PROCESS IN 'EMPIRE' AND 'IDARED' APPLE FRUITS FROM HYPOBARIC STORAGE

Abstract. 'Empire' and 'Idared' apples were harvested at 3 maturities in 1979 and stored (0 C) at ca. 760 or 40 torr total pressure at an equivalent O_2 partial pressure (8 torr) and in air at 760 torr. 'Empire' apples were harvested in 1980 and stored (0 C) at ca. 760 (in $He:O_2$ or $N_2:O_2$) or 40 torr total pressure at equivalent O_2 tensions (8 torr) and in air at 760 torr. Respiration, ethylene production, internal ethylene, flesh firmness, malic enzyme (ME) and alcohol dehydrogenase (ADH) activity, and water soluble polyuronides (WSP) were measured at various durations post store for 8-12 days in air at atmospheric pressure and 20-25 C. Across maturity level, cultivar, and year fruits stored in hypobaric ventilation did not soften significantly post store. Changes in ME and WSP were also diminished indicating that protein synthesis was affected. After 5 months duration in store and 3 days in air at room temperature, the levels of ADH, extracted from the low O_2-treated fruits, were inversely proportional to the relative diffusivity (RD) of the storage medium. Fruits maintained in low pressure or He at an equivalent O_2 tension (8 torr) displayed an attenuated rate of ripening post store as a function of duration and RD in store.

After 5 months duration in store none of the high RD-treated fruits softened significantly; however, none of the low O_2 treatments softened rapidly post store which indicates the 'low pressure effect' can be simulated under normal CA conditions (low RD) by maximizing the concentration gradient of native intrafruit volatiles. These data indicate that some cellular component, which functions in ethylene recognition, was gradually lost or metabolized in store. This apparent uncoupling of ethylene synthesis from ethylene action supports the hypothesis that a critical level of some native intrafruit volatile, possibly ethylene, is necessary for the retention of ethylene recognition requisite for the stimulation of the ripening process.

Flesh softening is a major criterion for 'ripening' of many fruits. The softening process is generally attributed to an increase in activity of cell wall-degrading enzymes (7,18) presumably resulting from ethylene-stimulated protein synthesis (8,13,15). Water soluble polyuronide (WSP) residues increased in concentration as fruits softened (3) and flesh firmness was found to be inversely proportional to soluble uronide content (17). Since flesh firmness change has been shown to be dependent upon protein synthesis (12,13) and soluble polyuronides have been associated with changes in flesh firmness, these aspects were measured in fruit from treatments that allowed or inhibited the ripening process.

One of the dominant proteins in pome fruits which increases in activity during the ripening process (5,16) is malic enzyme (13). This protein catalyzes the oxidative decarboxylation of l-malate to pyruvic acid. It has been hypothesized (25) that this enzyme provides a mechanism by which malate is 'mobilized' for mitochondrial oxidation under conditions when C_4 acids are high in activity and pyruvate is scarse. Apple peel disks (20) developed a capacity to decarboxylate added malate (the "malate effect") during ripening. Exogenous ethylene (23) reduced the time taken to reach a full malate effect. The decarboxylation of malate by pre-climacteric tissue has been demonstrated to require protein synthesis (22,23). Incorporation of ^{14}C-phenylalanine into malic enzyme (ME) was found to increase 4-fold in early-climacteric fruits (12). Thus the measurement of changes in ME activity has been used in this paper as a marker for protein synthesis.

Investigations (12) directed at the dependency of ripening on the activity of newly-synthesized or pre-existing proteins found: fruit ripening was inhibited by cycloheximide only at an early-climacteric stage; ethylene could not overcome this inhibition of ripening or protein synthesis. Once ripening progressed beyond a certain point, it was unaffected by protein synthesis inhibitors. Therefore, normal ripening requires a "co-ordinated synthesis" of enzymes whose activities are rate limiting. The synthesis of these enzymes takes place during the early-climacteric stage prior to any marked physical change in the fruit tissue; and once a capacity to

ripen has been established the inhibition of further protein synthesis is of little consequence on ripening. Ethylene is required in physiologically active concentrations (24,26) in order to initiate this ripening phenomenon which is dependent on specific protein synthesis (12).

Dilley (10) reported that apples did not soften after 7 days at 20 C following 4 months storage in air at a pressure of 76 torr and 0 C. Apple fruits kept at an equivalent O_2 tension but at atmospheric pressure did soften after 7 days. This result was surprising since the production of ethylene was similar to the low O_2 control fruits. This observation led to the conclusion that ethylene synthesis is uncoupled from ethylene action in low pressure-stored apples. The purpose of these experiments were to:

1. test the hypothesis that this hypobaric effect is due to the differential loss of native intrafruit volatiles as a function of storage medium diffusivity;
2. determine if this low pressure effect alters processes other than flesh firmness, which are associated with the elevated rate of specific protein synthesis requisite for the total ripening process;
3. determine the relationship of fruit maturity on the onset and eventual loss of this effect;
4. determine if this effect is reversible under conditions which allow accumulation of fruit volatiles.

Materials and Methods

Fruit Maturity Level (Table 1)

'Empire' apple fruits were harvested at weekly intervals for 3 weeks ('Empire' I, II, III) beginning the last week in September, 1979, at the Graham Experiment Station (GS), Grand Rapids, MI. First maturity 'Idared' fruits ('Idared' I) were harvested during the first week of October, 1979, at the Horticultural Research Center (HRC), East Lansing, MI; two additional levels ('Idared' II, III) were obtained by harvesting at weekly intervals thereafter. For the 1980 season 'Empire' apples (HRC) were harvested beginning the last week in September, 1980. Data (Table 1) are given on the internal levels of ethylene in a random sample of these fruits. After many years of investigation at the post harvest physiology lab at MSU it has been determined that the level of ethylene within the fruit intercellular air spaces is the most critical parameter to assess maturity, ripening and storability (11). The average value for intrafruit ethylene is not as important in making harvest decisions as the range of the upper concentration limit. As fruits mature on the tree, the spread of intrafruit ethylene concentration increases in the sample population. As this change occurs the fruit become more responsive to endogenous ethylene, which acts as a co-ordinator of physiological activity. Therefore, the most mature fruits, those with higher median ethylene values, have the greatest potential to ripen in store. Data from a randomly-selected population of fruit at harvest (Table 1) demonstrate how fruit retention on the tree affects the distribution

TABLE 1.--The effect of maturity on intrafruit ethylene distribution and flesh firmness

Intrafruit ethylene (µl/l)							
'Idared'			'Empire'				
1979			1979			1980	
I (10-5)[a]	II (10-12)	III (10-18)	I (9-28)	II (10-5)	III (10-12)	I (9-25)	II (10-9)
--	0.13	--	0.02	0.05	0.07	0.01	0.15
0.06	0.13	0.14	0.03	0.05	0.15	0.05	0.16
0.07	0.15	0.15	0.03	0.06	0.43	0.06	0.23
0.07	0.20	0.21	0.04	0.06	0.44	0.07	0.27
0.08	0.20	0.22	0.04	0.07	0.47	0.07	0.27
0.09[b]	0.22	0.29	0.05	0.08	0.72	0.08	0.28
0.11	0.25	0.36	0.05	0.10	0.81	0.09	0.28
0.13	0.52	0.40	0.06	0.12	0.85	0.10	0.29
0.18	0.72	0.95	0.07	1.23	1.52	0.12	0.31
0.25	0.79	1.23	0.08	2.78	1.75	0.14	0.50
--	0.85	--	0.10	2.85	4.25	0.18	0.54
7.8[c]	7.4	8.0	8.1	7.7	7.6	7.4	7.2

[a]Maturity level (month-day)

[b]Median value across columns

[c]Flesh firmness (kg)

of intrafruit ethylene. For 'Idared,' the upper levels of internal ethylene at harvest range from 0.11-0.25 µl/l on the 5th of October, 1979 (good for long-term CA storage) and this distribution increased 2- to 5-fold for subsequent harvest dates. Fruits from the last harvest have a greater potential to ripen than the first.

Randomization

Immediately after harvest ca. 200 fruits of 8-10 cm diameter were randomly assigned to storage bins which were subsequently assigned to each storage vessel. At each duration in store, 4 fruits/treatment were selected for experimental observations.

Low Pressure Storage (LPS)

Hypobaric storage treatments were maintained in cylindrical tanks (ca. 1.16 m^3) inside a 0 C storage room (14). The total pressure achieved (40 torr) was brought about by withdrawing air continuously with a Kinney rotary oil seal vacuum pump. The storage vessels were ventilated with ethylene-free air which had been H_2O vapor-saturated at ca. 15-20 C (9,10) at the storage pressure. Ventilating air was regulated at the desired pressure by a Fisher type 98 LD vacuum regulator. The average total pressure obtained over all measurements was 40.4 ± 1.5 torr (± $S\bar{x}$).

Storage Treatments (CA, CA-N_2, CA-He)

For the controlled atmosphere (CA) treatments ca. 1.7 m^3 aluminum drums (14) were utilized and kept in the same refrigerated

chamber as LPS and control treatments. In order to obtain low storage levels of ethylene and other fruit volatiles and maintain an O_2 partial pressure (P_{O_2})of approximately 8 torr (1% O_2 v/v) the chambers were flushed (1-2 standard cubic feet per hour) continuously with N_2 (CA-N_2 or CA) or helium (CA-He). The aluminum tanks were partially-sealed with silicon-rubber caulking compound in order to allow some O_2 leakage at the various background gas flow rates to maintain the desired O_2 level. Ethylene-free air was admitted whenever the O_2 level dropped below 1%. Oxygen and CO_2 levels were monitored daily with an Orsat gas analyser. The CO_2 concentration within the chambers was kept below 0.1% with dry lime. The average O_2 concentration over all measurements was 1.03 ± 0.06% and 1.03 ± 0.03% for Ca-N_2 and CA-He treatments, respectively.

Assuming that the rates of volatile (i.e., ethylene) synthesis are equal between low O_2 treatments, the CA-N_2-treated fruits should contain about 3x more volatiles internally than the CA-He treatment because of the greater gas medium diffusivity. Fruits from the CA-He treatment should have about 6x more volatiles internally than the LPS-treated fruits. The biochemical activity within an apple fruit should differ from one cell to the next with respect to ethylene production, (and hence dissolved ethylene). This same concept should apply to any fruit volatile. Because of this, the partial pressure of ethylene within the fruit intercellular spaces would necessarily determine the minimum amount dissolved across the population of cells. By altering the relative diffusivity (RD) of the

medium surrounding these fruits and maintaining equivalent rates of production, one would change the amount of ethylene, or any fruit volatile, dissolved within that population of apple cells. If the uncoupling of ethylene synthesis from action (the 'low pressure effect') is a function of the loss of fruit volatiles, the maintenance of apple fruits in equivalent levels of O_2, but differing with respect to RD, might result in a ripening response after storage inversely proportional to the RD of the storage medium. These data (Table 2) clearly demonstrate the effect of RD of various gas media on the rate of ethylene movement through an inert film.

Control fruits were kept in air at 0 C in the same refrigerated room as all other storage treatments. In order to avoid excessive desiccation of these fruits, each storage bin was covered in a polyethylene bag punctured with 2 holes to allow some air circulation.

Post Storage Conditions and Treatment

Fruits removed from store were labeled, weighed, put in containers, and ventilated with humidified ethylene-free air at a flow rate of 75-100 ml/kg apple tissue. Ethylene treatment (10 µl/l for 24 hr) was performed between days 0 and 2 after storage in ventilated (1979-80) or static (1980-81) systems. All fruits were maintained between 20-25 C.

Internal Ethylene (int. et.)

A 4 cm syringe needle (18 gauge x 1.5 in) was inserted into the calyx end of each fruit. The needle orifice was unplugged with a

TABLE 2.--The effect of absolute pressure and medium density on the effusion of ethylene through an inert barrier at a constant concentration gradient (1500 µl/l)

	J_x (nl/cm^2 · hr)			
Film[a]	40-N_2	760-N_2	760-He	P_T (torr)[b]
I	278	23	65	
II	309	21	69	
III	310	21	69	
RD[c]: OBSERVED	100	7	22	
RD[c]: IDEAL[d]	100	5	15	

[a]Dow 'Handiwrap' covering an aluminum disk perforated with a 1.3 mm diameter syringe needle. J_x values calculated for total Al disk surface area (ca. 7 cm^2). Multiply each number by 527.38 to convert to actual effusion through film in units of nl/cm^2 · hr.

[b]J_x was measured at 50 & 700 torr. These values were calculated at these P_T by multiplying by 1.25 & 0.92 for 40 & 760 torr, respectively.

[c]Relative diffusivity.

[d]Theoretically, effusion in He should be about 3-fold greater than in N_2.

length of wire, and a syringe body was connected to this inset needle. Upon immersing the fruit in H_2O a 1.2 ml gas sample was withdrawn and subsequently analysed for ethylene chromatographically. The gas chromatograph, a Varian Series 1700, used N_2 at 70 C as the carrier gas, was equipped with a 2 mm by 1 m column of activated alumina, and a flame ionization detector. This instrument was capable of detecting ethylene at concentrations of 2 nl/l in a 1 ml sample with a retention time of ca. 25 sec. Ethylene concentration was determined by comparing peak heights with those produced by a standard gas mixture (ca. 1 µl/l ethylene).

Flesh Firmness (FF)

Flesh firmness was measured with an Effigy penetrometer. The penetration head was 0.79 cm in diameter. The penetrometer was mounted on a drill press equipped with a concave base upon which the apple was stabilized during determinations. Four measurements were made per fruit after removing part of the epidermis.

CO_2/Ethylene Production (R^{CO_2}, R^{et})

During the 1979-80 season, measurements of fruit respiration (ml CO_2/kg·hr) and ethylene production (µl C_2H_4/kg·hr) were made at 20 C and various times after harvest and storage.

After stored fruit had equilibrated at 20 C for 6-8 hr, they were weighed, labeled, and each was assigned to a ca. 2 l glass jar. The fruits were ventilated with humidified, ethylene-free air at 15-25 ml/min using a capillary flow system. Gas samples were withdrawn from

the interior of the chambers and analysed chromatographically. Carbon dioxide was determined by gas chromatography with a Carle 8700 (804-B) Basic gas chromatograph using He at ca. 70 C as the carrier gas. This instrument was equipped with silica gel-molecular sieve columns mounted in parallel and a differential thermal conductivity detector. Ethylene was measured as previously described. Rates of evolution for CO_2 and ethylene were calculated as follows:

$$\text{Rate} = \frac{(\text{Flow rate} \times P_T \times \text{Concentration})}{(R \times T \times \text{fruit fresh weight})} = \text{moles/kg} \cdot \text{hr.}$$

Molar rates of evolution were converted to µl/kg·hr or ml/kg·hr for ethylene and CO_2, respectively.

Malic Enzyme (ME) and Water Soluble Polyuronide (WSP) Extraction

Tissue preparation was as described by Dilley (6), with minor modification. Preparation and extraction was performed in a cold room (0 C). Chilled apples were peeled, cored, cortical tissue cut into ca. 1 cm^3 wedges, and approximately 60 g of the tissue was selected and placed in a ca. 400 cm^2 piece of cheesecloth. The tissue was then dipped in 1 x 10^{-3}M $NaHSO_4$ to inhibit tissue oxidation. The tissue was drained and placed in a beaker with 0.12 g diethyldithiocarbamic acid-sodium salt (DIECA) in 120 ml 0.2M K_2CO_3. The tissue was vacuum infiltrated in this solution for one hour or until the tissue became translucent. The drained tissue was blended 30 sec in 60 ml pH 7.0 H_2O with 0.06 g DIECA. The resultant slurry was vacuum

filtered through a nylon cloth (500 μm mesh) being careful not to squeeze the cell wall material through the filter. The volume of this extract was measured (ca. 70-80 ml) and a $MgCl_2$ solution (400 g/l) was added at a rate of 5 μl/ml extract for the precipitation of soluble pectins. The pH of the solution was adjusted to 9.5 and was from pH 9-10 prior to adjustment. Approximately 25 ml of this solution was added to tubes and centrifuged 15 min at 10000 xg. The resultant solution (pH adjusted to 7.3 with 5N acetic acid) was utilized for determining ME activity. The pellet was dispersed in 10 ml ethanol using a Teflon pestle and centrifuged 5 min at 10000 xg. The supernatant solution was discarded and the pellet was resuspended in ca. 25 ml 100 C distilled H_2O. This extract was cooled to room temperature in an ice-water bath and clarified via centrifugation 5 min at 10000 xg. This clarified solution was utilized in the assay for WSP.

Polyuronide Assay (2,4)

An aliquot (0.1 ml) from each of the apple fruit extracts was added to 3 ml 0.0125M sodium tetraborate in sulfuric acid. These were mixed, heated in a 100 C H_2O bath for 5 min and subsequently cooled to room temperature by immersing in an ice-water bath. Upon cooling, 0.1 ml of 0.2% (w/v) m-phenyl phenol (mpp) in 0.5% (w/v) NaOH was added to the extract-tetraborate mixture. Absorbance was measured at 520 nm (A_{520}) on a Beckman DU monochrometer equipped with a Gilford absorbance meter (model 205) after 1 hr. Duplicate determinations were made for each extract. Reagent blanks contained

extract and 0.1 ml 0.5% (w/v) NaOH instead of the mpp solution. Galacturonate standards (0-100 μg/assay) were measured for approximately every 32 sample measurements. The average slope of the standard regression lines was $0.011\Delta A_{520}/\mu g$ galacturonate (C.V. = 4.5%). The calculation for WSP (μg/g) was performed as follows:

$$\left(\frac{A_{520}^{sample}}{A_{520}^{std}}\right) \times (0.1 \text{ ml/assay})^{-1} \times (\text{Total Volume Extract})$$

$$\times (\text{fruit fresh wt})^{-1} = \mu g \text{ galacturonate equivalents per g fresh weight } (\mu g/g)$$

Malic Enzyme (ME) Assay (6)

The enzyme assay consisted of TRIS-glycylglycinate, 300 μmoles; $MnSO_4$, 3.6 μmoles; $NADP^+$, 0.25 μmoles; l-malate, 11 μmoles; and 0.1 ml extract in a total volume of 3 ml at 20-25 C. Prior to each assay the TRIS-glycylglycinate and $MnSO_4$ mixture was adjusted to pH = 7.3 which is the optimum for this enzyme (6). The assay constituents were mixed in quartz cuvettes and the solution absorbance was read at 340 nm. After this, 0.1 ml extract was added and A_{340} measured at 0, 20, 40, 60, and 80 sec after mixing. Enzyme activity was calculated by the following formula:

$$\left(\frac{(m \times 60 \text{ sec/min})}{(0.01\Delta A_{340} \text{ min}^{-1}/\text{eu})}\right) \times (0.1 \text{ ml extract})^{-1} \times (V_{ext})$$

$$\times (\text{fr. wt.})^{-1} = \text{enzyme units (eu)/g fr. wt. tissue} = \text{eu/g}$$

m = slope of the absorbance-time function = ΔA_{340}/sec

V_{ext} = total volume of extract

The reaction was zero order under these conditions since saturating malate and $NADP^+$ were added (6) and the absorbance-time function was linear (r = 0.97-0.99). The data in Table 3 demonstrate that considerable activity was present in the extract without addition of 11 μmoles l-malate. This result was probably due to native fruit malate in the extract fluid since malate is the major C_4 acid in pome fruits. However, the addition of 11 μmoles/assay l-malate did cause an increase in the initial velocity of the reaction by a factor of about 2. This result indicates that these reactions were being catalyzed by malic enzyme.

Alcohol Dehydrogenase (ADH) Extraction and Assay (21)

In the 1980-81 experiments ADH was measured to determine if this enzyme was induced in fruit from low O_2 storage. From the same population of chilled tissue used in the malic enzyme-soluble polyuronide extraction, 20 g were selected and dipped in 1 x 10^{-3}M $NaHSO_4$. To 60 ml solution (TRIS 24.23 g/l; EDTA 0.29 g/l; sucrose 85.58 g/l; pH = 7.5) 0.60 g polyvinylpyrrolidone (PVP) and 20 g chilled apple tissue were added and blended 30 sec. This mixture was vacuum filtered through a nylon cloth, volume was measured (ca. 60 ml) and ca. 25 ml were put into tubes and centrifuged 15 min. at 10000 xg (0 C). To 2.5 ml buffer (80 μmoles/ml MES; pH = 5.75)

TABLE 3.--The effect of l-malate addition on enzyme activity; extracted from fruits after 9 months store under various conditions

Block			Enzyme Activity (eu/g)[a]	
MAT[b]	P_T	P_{O_2}	w/o l-malate	w/ l-malate
I	40	8	13	24
II	40	8	18	42
III	40	8	25	60
I	760	8	22	44
II	760	8	12	17
III	760	8	40	70
I	760	152	110	210
TOTAL			240	467[c]

[a]Times measured = 0, 20, 40, and 60 sec., pH - 7.3.

[b]Maturity level

[c]Significantly different at the 0.05 level.

was added 0.1 ml acetaldehyde (1700 μmoles per ml) and 0.2 ml extract. The resultant solution was mixed and absorbance measured at 340 nm in a quartz cuvette. The reaction was started by adding 0.1 ml NADPH (2500 μg/ml) and A_{340} was measured at 0, 20, 40, 60, and 80 sec. Enzyme activity was calculated by the following formula:

$$\left(\frac{(m \times 60 \text{ sec/min})}{(0.01 \Delta A_{340} \text{min}^{-1}/\text{eu})}\right) \times (0.2 \text{ ml extract})^{-1} \times (V_{ext})$$

$$\times (\text{fr. wt.})^{-1} = \text{eu/g}$$

Results and Discussion

The Effect of Maturity and Cultivar on Development of Ripening and the Respiratory-ethylene Climacterics

After 3 months in storage (Tables 4 and 5) LPS-treated 'Empire' fruits did not change significantly with respect to flesh firmness (FF) even though ethylene was above the physiologically active level; those stored in an equivalent O_2 tension but at atmospheric pressure softened approximately 4-fold more across cultivar and maturity (Tables 4 and 6). However, the FF change from initial to 8 days post store in 'Idared' fruits (Table 6) from CA was much less. Intrafruit ethylene was initially lower in fruits from LPS than CA (Table 7) after 3 months storage and increased above a physiologically active level after about 2 days. Similar results were noted after 6 months; the average change in firmness of LPS-treated fruits was about 4-fold less than treatments in an equivalent O_2 level at atmospheric pressure. However, the 3rd maturity of 'Empire' (Table 4) softened significantly.

TABLE 4.--Changes in 'Empire' apple flesh firmness (kg) after storage as a function of maturity and storage conditions

Duration of Storage (Months)	P_T	P_{O_2}	Flesh Firmness Change (kg)[a]		
			Maturity		
	(torr)		I (9-28)[b]	II (10-5)	III (10-12)
0	--	--	0.9**[c]	1.0**	0.9**
3	40	8	0.2	0.2	0.2
3	760	8	0.7*	0.9**	0.9**
3	760	152	1.2**	0.6*	0.4
6	40	8	0.0	-0.3	0.7*
6	760	8	0.4	0.2	0.9**
6	760	152	0.4	0.4	0.6*
9	40	8	-0.1	1.3**	2.4**
9	760	8	0.4	2.0**	1.4**

[a]Flesh firmness of fruits directly from store minus flesh firmness after 8 days at 20 C.

[b]Harvest date.

[c]*,**Significant at the 0.05 and 0.01 level, respectively.

TABLE 5.--Changes in 'Empire' apple intrafruit ethylene (µl/l) after storage as a function of maturity and storage conditions

Duration of Storage (months)	P_T (torr)	P_{O_2} (torr)	Day	Intrafruit Ethylene (µl/l)		
				Maturity		
				I (9-28)	II (10-5)	III (10-12)
0	--	--	0	0.1	0.1	0.4
			8	112	102	174
3	40	8	0	0.2	0.9	0.4
			8	42	113	186
3	760	8	0	13	17	16
			8	196	192	197
3	760	152	0	182	68	87
			8	268	190	232
6	40	8	0	0.1	1.3	0.1
			8	25	18	41
6	760	8	0	7	20	1
			8	36	43	200
6	760	152	0	108	39	58
			8	103	178	191

TABLE 6.--Changes in 'Idared' apple flesh firmness (kg) after storage as a function of maturity and storage conditions

Duration of Storage (months	P_T	P_{O_2}	Flesh Firmness Change (kg)[a]		
			Maturity		
	(torr)		I (10-5)[b]	II (10-12)	III (10-18)
0	--	--	0.9*[c]	0.7*	1.3**
3	40	8	0.1	0.3	0.1
3	760	8	0.6*	0.4	0.6*
3	760	152	0.3	0.5	0.5
6	40	8	0.1	0.0	0.2
6	760	8	0.2	0.3	1.1**
6	760	152	0.4	0.0	0.7*

[a]Flesh firmness of fruits directly from store minus flesh firmness after 8 days at 20 C.

[b]Harvest date

[c]*,** Significant at the 0.05 and 0.01 level, respectively.

TABLE 7.--Changes in 'Idared' apple intrafruit ethylene (µl/l) after storage as a function of maturity and storage conditions

Duration of Storage (months)	P_T (torr)	P_{O_2} (torr)	Day	Intrafruit Ethylene (µl/l) Maturity I (10-5)	Maturity II (10-12)	Maturity III (10-18)
0	--	--	0	0.1	0.4	0.3
			8	30	0.4	37
3	40	8	0	2.4	1.0	1.0
			8	50	50	57
3	760	8	0	22	20	21
			8	89	70	95
3	760	152	0	43	23	40
			8	115	71	61
6	40	8	0	0.1	0.1	0.2
			8	61	50	91
6	760	8	0	11	3	11
			8	39	85	78
6	760	152	0	76	15	36
			8	50	81	107

In 'Idared' the last maturity from CA softened significantly (Table 6). After 9 months in store only the least mature fruits from both CA and LPS did not soften. Across maturity and cultivar, LPS-treated fruits softened 2- to 5-fold less while in store (Table 8). Knee (18) observed 2 stages of cell wall breakdown during ripening of apples. In the first stage, firmness declined slowly and cell wall galactan decreased. In the second stage, flesh firmness decreased more rapidly and WSP increased. It was also demonstrated that ethylene did not affect FF change (ΔFF) in the first stage, but removal of ethylene diminished ΔFF and ΔWSP during the second stage. LPS-treated tissue would necessarily have a lower intrafruit ethylene activity than the equivalent treatment at atmospheric pressure; because of this, the observed ΔFF in CA store was probably due to stage 2 ripening (Table 8). These data (Tables 4 and 6) indicate that the lack of a critical concentration of ethylene or other native volatile in LPS-stored fruits (after 3 months) might induce the non-ripening phenomenon since both treatments were at an equivalent O_2 tension (P_{O_2} = 8 torr) and temperature (0 C). It was also found that the 'low pressure effect' was duplicated at atmospheric pressure after a long duration in store. These findings might have resulted from the loss of some ethylene recognition factor which binds or interacts with ethylene to induce the ripening process since ethylene synthesis was uncoupled from ethylene action. The least mature CA-treated fruits eventually obtained this physiological state. This result might indicate that these fruits possessed enough of the factor to ripen after 3 months

TABLE 8.--Changes in 'Empire' and 'Idared' flesh firmness in store as a function of fruit maturity, duration in storage and storage conditions

Duration of Storage (months)	P_T	P_{O_2}	Flesh Firmness (kg)[a]					
			Maturity					
			'Empire'			'Idared'		
	(torr)		I	II	III	I	II	III
3	40	8	-0.3	-0.2	-0.2	0.3	-0.2	0.9**[b]
3	760	8	0.1	0.8*	0.0	0.5	0.3	1.2**
3	760	152	2.0**	2.2**	1.6**	1.5**	1.0**	2.1**
6	40	8	0.2	0.3	-0.8	0.2	-0.2	0.5
6	760	8	1.1**	0.9**	1.0**	0.6*	1.4**	0.9**
6	760	152	3.1**	3.0**	2.0**	1.9**	2.3**	2.4**

[a]Flesh firmness of fruits directly after harvest minus flesh firmness immediately after storage at 0 C.

[b]*,** significant at the 0.05 and 0.01 level, respectively.

in store but due to a gradual dissipation of the hypothesized factor the fruit were unable to ripen as rapidly thereafter.

From the previous results it was observed that the ripening process was perturbed in certain treatments. Because of this, it was critical to determine how these same treatments were affecting fruits with respect to the rate of respiration ($R^{CO}2$; Table 9) and ethylene production (R^{et}; Table 10). Those fruits that display a steady decline in respiration with time have been termed "non-climacteric." Fruits which show an increase in $R^{CO}2$ (2- to 4-fold) coinciding with ripening have been designated as "climacteric" fruits. Fruits with an initial post store respiration rate which is within one standard deviation ($s_{\bar{x}}$) of the initial post harvest value are defined as pre-climacteric. Values above this are termed early-, mid-, or late-climacteric. After 3 months low O_2 storage (LPS & CA) all 'Empire' fruits were determined to be pre-climacteric (Table 9). 'Idared' fruits from identical conditions were generally more advanced metabolically (early- to mid-climacteric). All fruits stored in air at 0 C were mid-climacteric after 3 months and late-climacteric after 6. 'Empire' LPS fruits were pre- to early-climacteric after 6 months duration in store. 'Idared' fruits from these same conditions were mid-climacteric. CA-treated 'Empire' fruits were early- to mid-climacteric and the 'Idared' treatments were mid- to late-climacteric. These data (Table 9) indicate that the respiratory behavior of these tissues was not perturbed. However, the LP-stored fruits were generally less advanced metabolically than comparable treatments at atmospheric pressure.

TABLE 9.--The effect of LPS and CA storage, duration in store, and maturity on 'Empire' and 'Idared' respiratory climacterics after 8 days at 20 C

Duration (Months)	P_T (torr)	P_{O_2} (torr)	Day	$R^{CO_2}_{Day\ 1}(s_{\bar{x}})/R^{CO_2}_{Max}(s_{\bar{x}})/R_{Max} - R_{Day\ 1}$ (ml/Kg. hr)		
				Maturity		
				I	II	III
				'Empire'		
Initial post harvest			1	6.8 (0.7)	6.5 (0.4)	10.8 (0.7)
			t_{max}	13.2 (1.0)	10.7 (0.9)	13.4 (0.5)
				6.4	4.2	2.6
3	40	8	1	6.4 (0.2)	6.8 (0.3)	8.1 (0.5)
			t_{max}	13.1 (2.7)	8.9 (1.8)	14.4 (1.2)
				6.7	2.1	6.3
3	760	8	1	6.6 (0.2)	6.7 (0.6)	11.0 (0.2)
			t_{max}	13.0 (0.5)	10.9 (0.7)	13.8 (0.6)
				6.4	4.2	2.8
3	760	152	1	13.8 (0.7)	12.8 (0.4)	16.8 (0.4)
			t_{max}	15.2 (0.6)	15.3 (1.1)	16.9 (0.3)
				1.4	2.5	0.1
6	40	8	1	7.6 (0.9)	8.6 (0.9)	11.2 (1.2)
			t_{max}	9.1 (1.1)	12.2 (0.4)	16.9 (0.8)
				1.5	3.6	5.7
6	760	8	1	8.9 (0.5)	11.8 (0.7)	14.3 (0.5)
			t_{max}	11.7 (0.3)	13.3 (1.1)	20.3 (0.6)
				2.8	1.5	6.0
6	760	152	1	18.7 (0.3)	19.7 (0.5)	17.8 (1.2)
			t_{max}	18.8 (1.0)	20.2 (0.9)	20.5 (1.3)
				0.1	0.5	2.7

TABLE 9.--Continued

Duration (Months)	P_T	P_{O_2}	Day	$R^{CO_2}_{Day\ 1}$ $(s_{\bar{x}})/R^{CO_2}_{Max}$ $(s_{\bar{x}})/R_{Max}$ $R_{Day\ 1}$ (ml/Kg. hr)		
				Maturity		
	(torr)			I	II	III
				'Idared'		
Initial post harvest			1	5.8 (0.6)	10.1 (1.0)	7.6 (0.5)
			t_{max}	7.7 (1.4)	10.5 (1.1)	12.7 (1.4)
				1.9	0.4	5.1
3	40	8	1	9.0 (0.5)	10.9 (0.4)	13.8 (1.3)
			t_{max}	9.2 (1.0)	14.3 (2.7)	14.0 (0.9)
				0.2	3.4	0.2
3	760	8	1	9.4 (0.4)	12.3 (0.5)	12.6 (0.7)
			t_{max}	12.3 (1.3)	13.7 (0.8)	13.2 (0.4)
				2.9	1.4	0.6
3	760	152	1	11.3 (1.0)	13.2 (0.3)	13.9 (0.9)
			t_{max}	15.9 (1.7)	13.6 (0.5)	14.7 (0.4)
				4.6	0.4	0.8
6	40	8	1	9.7 (0.3)	14.4 (0.5)	13.0 (0.4)
			t_{max}	10.9 (0.4)	17.7 (0.8)	19.4 (0.6)
				1.2	3.3	6.4
6	760	8	1	11.6 (1.0)	18.2 (0.9)	16.8 (0.5)
			t_{max}	11.7 (1.0)	20.7 (1.2)	22.6 (0.7)
				0.1	2.5	5.8
6	760	152	1	14.7 (0.7)	18.8 (0.6)	17.0 (0.4)
			t_{max}	15.9 (1.5)	19.4 (0.8)	19.2 (1.1)
				1.2	0.6	2.2

TABLE 10.--The effect of LPS and CA storage, duration in store, and maturity on 'Empire' and 'Idared' ethylene production after 8 days at 20 C

Duration (Months)	P_T (torr)	P_{O_2} (torr)	Days	$R^{et}_{Day_1}$ (µl/Kg. hr) Maturity I	$(s_{\bar{x}})/R^{et.}_{Max}$ (µl/Kg. hr) Maturity II	$(s_{\bar{x}})/R_{Max} - R_{Day\ 1}$ (µl/Kg. hr) Maturity III
				'Empire' Cv		
Initial post harvest			1	0.4 (0.1)	0.1 (0.0)	0.4 (0.1)
			t_{max}	21.4 (3.9)	22.2 (2.3)	32.3 (3.9)
				21.0	22.1	31.8
3	40	8	1	1.0 (0.6)	0.4 (0.1)	0.3 (0.2)
			t_{max}	32.5 (2.6)	15.0 (8.7)	43.1 (2.3)
				31.5	14.6	42.8
3	760	8	1	0.8 (0.3)	2.1 (1.2)	10.8 (0.8)
			t_{max}	33.4 (0.9)	31.7 (2.2)	41.8 (0.7)
				32.6	29.6	31.0
3	760	152	1	39.8 (3.1)	48.4 (11.2)	40.4 (2.4)
			t_{max}	52.8 (3.7)	49.7 (5.6)	61.6 (4.7)
				13.0	1.3	21.2
6	40	8	1	0.3 (0.2)	0.3 (0.1)	0.1 (0.1)
			t_{max}	15.2 (8.8)	16.2 (2.1)	30.9 (5.2)
				14.9	15.9	30.8
6	760	8	1	2.3 (0.7)	7.1 (0.9)	0.4 (0.5)
			t_{max}	42.3 (3.0)	36.4 (3.2)	50.6 (2.5)
				40.0	29.3	50.2
6	760	152	1	37.2 (3.4)	37.5 (3.6)	33.2 (2.3)
			t_{max}	69.6 (8.2)	60.3 (5.6)	71.4 (2.5)
				32.4	22.8	38.2

TABLE 10.--Continued

Duration (Months)	P_T (torr)	P_{O_2} (torr)	Days	$R^{et}_{Day_1}$ ($s_{\bar{x}}$)/R^{et}_{Max} ($s_{\bar{x}}$)/R_{Max} - $R_{Day\ 1}$ (µl/Kg. hr) Maturity I	Maturity II	Maturity III
				'Idared' Cv		
Initial post harvest			1	0.4 (0.1)	0.3 (0.1)	0.2 (0.1)
			t_{max}	7.1 (2.6)	8.2 (3.0)	19.4 (3.7
				6.7	7.9	19.2
3	40	8	1	2.3 (0.3)	0.9 (0.3)	1.1 (0.4)
			t_{max}	7.0 (0.5)	14.7 (1.3)	24.2 (9.8)
				4.7	13.8	23.1
3	760	8	1	7.5 (1.4)	11.5 (0.6)	16.1 (6.7)
			t_{max}	30.9 (3.5)	31.1 (2.4)	47.2 (13.0)
				23.4	19.6	31.1
3	760	152	1	38.4 (1.5)	42.9 (3.2)	42.9 (4.6)
			t_{max}	50.3 (3.1)	43.7 (5.0)	52.6 (4.5)
				11.9	0.8	9.7
6	40	8	1	0.1 (0.1)	0.6 (0.2)	0.5 (0.3)
			t_{max}	16.3 (7.3)	37.3 (2.1)	38.3 (5.4)
				16.2	36.7	37.8
6	760	8	1	1.8 (0.6)	7.9 (2.1)	11.8 (0.7)
			t_{max}	55.3 (6.4)	53.6 (1.0)	79.3 (4.1)
				53.5	45.7	67.5
6	760	152	1	48.7 (2.3)	38.6 (1.3)	33.9 (2.1)
			t_{max}	66.5 (4.1)	65.2 (2.1)	71.9 (2.8)
				17.8	26.6	38.0

Across maturity, cultivar and durations in storage, apples from LPS were similar to their 'at harvest' condition with respect to ethylene production (Table 10). Fruits directly from CA were producing about 20-fold more ethylene than those one day post harvest. All fruits increased in ethylene production post store. Ethylene production is "autocatalytic"; low levels of intrafruit ethylene naturally induce a change from a low, steady-state to a rapid rate of synthesis (1). Since LP-treated fruits were less advanced physiologically than the equivalent treatment at atmospheric pressure, the level of intrafruit ethylene must be sub-optimal for stimulation of autocatalytic production in store. Because these fruits produce less ethylene they are less advanced with respect to the respiratory climacteric.

The Effect of Time in Storage and Relative Diffusivity of the Storage Media on the Development of the "Low Pressure Effect"

The purpose of this experiment was two-fold:

1. to determine the length of time in store required to observe the non- (slow-) ripening phenomenon
2. to determine if this phenomenon could be induced at atmospheric pressure using helium (He)

Fruits were stored in gas media varying in relative diffusivity (RD, Table 2; from 100:15:5 for LPS:CAHe:CAN_2). Ethylene should accumulate in the fruit intercellular air spaces in a way inversely proportional to the RD, assuming equal rates of synthesis. Thus, a gradient of native intrafruit volatiles would be established between

treatments to determine the involvement of these in the recognition of ethylene for the stimulation of the ripening (softening) process.

After 2 weeks in storage (Table 11) fruits from all treatments were observed to soften significantly after 12 days at 20-25 C. However, after 2 months the rate of softening was lower in LPS- and CA-He-treated fruits than in CA-N_2 or control (C) fruits. The rate of FF change diminished from 2 weeks to 2 months in store by about 67% in the LPS-treated fruits. For the same duration the rate of softening was attenuated 200% in fruits from He at atmospheric pressure. However, after 5 months duration in store neither 100 nor 15 RD treatments changed in FF significantly (0.3 kg in LPS: 0.4 kg in CA-He) after 12 days at room temperature. These data also demonstrate that the level of ethylene within the fruits was far above the physiologically active level. Fruits stored at an equivalent O_2 tension (P_{O_2} = 8 torr) and atmospheric pressure in N_2 did soften significantly. These data confirm that the low pressure effect is mainly due to the loss of some fruit volatile, possibly ethylene. Therefore, these results support the hypothesis that a critical level of some volatile is necessary for the retention of ethylene recognition requisite for the stimulation of the ripening process. Since this phenomenon was only achieved sometime after 2-3 months in store, it appeared that some ethylene recognition factor was gradually lost or metabolized as evidenced by the gradual change in the rate of softening over time.

TABLE 11.--Total change in flesh firmness (kg) from the at harvest condition as a function of relative diffusivity of the storage medium and duration in store

RD[b]	P_T (torr)	P_{O_2} (torr)	Flesh Firmness Change[a] (kg) 2 weeks	2 months	5 months
100	40 N_2	8	2.5**[c]	1.4**	0.4
15	760 He	8	2.3**	1.1**	0.7*
5	760 N_2	8	2.4**	2.2**	1.0**
--	760 N_2	152	2.6**	2.7**	2.6**

[a](Flesh firmness post harvest - FF post store) + (FF day 0 post store - FF day 12 post store).

[b]Relative diffusivity (Table 2).

[c]*,** significant at the 0.05 and 0.01 level, respectively.

The Effect of Time in Storage and RD on ME, WSP, and ADH

The purpose of these experiments were to determine:

1. how ME activity is affected by treatments which allow or inhibit the ripening process
2. if the physical changes in apple fruit texture associated with the slow-ripening syndrome are observed at the cellular level by measurement of WSP
3. if the RD of the storage medium affects ADH activity via loss of some fruit volatile which might cause differential enzyme synthesis or induction of activity

Malic enzyme has been shown to increase in activity markedly during ripening (7). It has also been demonstrated that incorporation of radioactive amino acids into the enzyme increased 4-fold in early-climacteric fruits (12). Therefore, at least part of the observed activity increase is due to de novo synthesis. This experiment (Table 12) will determine if the lack of ethylene recognition observed previously (Tables 4, 5, and 9) with respect to FF changes is carried out in processes other than softening. After 2 weeks or 2 months in store + 12 days at 20-25 C ME activity increased sharply in all low O_2 treatments; LPS fruit enzyme activity increased 130 and 106 eu/g after 0.5 and 2 months in store + 12 days at 20-25 C, respectively; CA-He fruit enzyme activity increased 115 and 108 eu/g; CA-N_2 fruit enzyme activity increased 92 and 148 eu/g at these

TABLE 12.--'Empire' apple malic enzyme activity after 12 days from store at 20-25 C as a function of relative diffusivity of the storage medium and duration in store

RD[b]	P_T	P_{O_2}	Malic Enzyme Activity (eu/g) ± $S_{\bar{x}}$[a]		
	(torr)		2 weeks	2 months	5 months
100	$40N_2$	8	174 ± 9	155 ± 13	59 ± 10
15	760He	8	162 ± 43	174 ± 11	94 ± 13
5	$760N_2$	8	150 ± 4	190 ± 5	79 ± 5
--	$760N_2$	152	145 ± 1	110 ± 3	112 ± 4

[a]ME activity after 12 days at 20-25 C; the initial post storage level was 43 ± 5 eu/g for low O_2 treatments; the initial post storage level was 97 ± 21 eu/g for the air control.

[b]Relative diffusivity.

durations (Table 12). However, there was a sharp drop in ME activity change after 5 months for all the low O_2 (8 torr) treatments. After 12 days, fruit from He and N_2 at equivalent O_2 tensions had about 60 and 30% greater activity, respectively, than the equivalent treatment at low pressure. These data indicate that the lack of ethylene recognition observed for the non-softening phenomenon might apply also to processes other than flesh firmness change. Ethylene and its recognition, therefore, are important in the regulation, probably via protein synthesis, of other biochemcial and physiological changes associated with ripening apples.

Flesh firmness change has also been shown to be dependent upon protein synthesis (12,13) and water soluble polyuronides were inversely proportional to flesh firmness (17; this thesis, Appendix A). Because of these observations, WSP was measured in these treatments which either allowed or inhibited the ripening process. These data (Table 13) should reflect ΔFF attenuation at the chemical level since WSP measurement is probably an indirect measure of cell wall-degrading enzyme activity. After 2 weeks in store, changes in WSP were found to be relatively high for all low O_2 and control treatments. After removal from storage, the increase in WSP over time was about 2-fold for LPS and CA-He-treated fruits; the same measurements increased ca. 3- and 4-fold for CA-N_2 and control fruits, respectively. At this duration, the ΔWSP was observed to be about 60% less in the LPS- and He-treatment than in the N_2 at an equivalent O_2 tension. However, after 2 months in store, the relative change in uronide solubilizing activity was

TABLE 13.--Changes in 'Empire' apple water soluble polyuronide (WSP) activity as a function of relative diffusivity of the storage medium and duration in store

RD[b]	P_T	P_{O_2}	Change in water soluble polyuronides[a] $\pm S_{\bar{x}}$ (µg/g)		
	(torr)		2 weeks	2 months	5 months
100	$40N_2$	8	82 ± 13	79 ± 6	25 ± 2
15	760He	8	82 ± 2	102 ± 3	28 ± 4
5	$760N_2$	8	129 ± 19	104 ± 4	30 ± 3
--	$760N_2$	152	230 ± 13	112 ± 7	49 ± 3

[a]Maximum value over 12 days at 20-25 C minus the initial post store value.

[b]Relative diffusivity.

only 24% higher in N_2 than LPS-treated fruits at equivalent O_2 tensions. After 5 months, all low O_2 treatments had very suppressed soluble uronide increases relative to the previous durations. LPS-treated fruits increased 25 µg/g after 12 days at 20-25 C (ca. 20% less than CA-N_2). With respect to ΔFF (Table 11), equivalent deviations for ΔWSP should have theoretically been about 25, 32, and 39 µg/g for LPS, CA-He and CA-N_2 treatments, respectively. The observed differences in uronide solubilizing activity were about 22% lower than theoretical. These data do confirm observations from other data (Table 11). While intrafruit ethylene was far above the physiologically active level, none of the low O_2-stored fruits responded to the ethylene as rapidly as they had at other durations. Also, the relative rate of change for flesh firmness and WSP were inversely proportional to the diffusive resistance of the storage medium at each low O_2 treatment.

Alcohol dehydrogenase (ADH) is an enzyme not associated with any activity increase during the ripening process. This enzyme was measured to determine if:

1. activity would be increased significantly as a function of low O_2 storage
2. activity would be differentially affected by the RD of the storage media

If volatile compounds, especially ethanol and acetaldehyde, accumulate in the fruit tissues as a function of the storage media RD then ADH activity might reflect these differences, e.g., activation of the

enzyme or promotion of de novo synthesis. These data (Table 14) demonstrate that, after 5 months in store initial post store ADH activity was lowest in control fruits. This value was less than that observed for the LPS treatment by ca. 62%. Fruit stored in He had an ADH activity about 35% greater than that from LPS fruit. Fruits stored in N_2 had an ADH activity about 14% greater than He-treated fruit. Thus, ADH activity, after 5 months duration and 3 days at 20-25 C, was inversely proportional to the RD of the storage medium at equivalent O_2 tensions. After the initial time, activity diminished to similar values after 12 days. These data indicate that ADH was activated or de novo synthesis induced by some intrafruit volatile(s), probably acetaldehyde and/or ethanol.

Reversibility of the 'Low Pressure Effect'

The effect of LPS on changes in FF, WSP, and ME activity has been shown to be related to the loss of some native fruit volatile(s). The "conditioning" of apple tissue, which had previously been inhibited from ripening, at atmospheric pressure (in air) at 0 C might allow these fruits to redevelop ethylene recognition since volatile components would accumulate under these conditions. This appears to be true since both the O_2 concentration and the RD of the storage medium would be increased 20-fold (increased synthesis and decreased effusion). Experiments were performed in the 1980-81 (Figures 1 and 2) storage season to test this hypothesis. Fruits stored continuously 8 months in LPS did not soften significantly (Figure 1). Associated

TABLE 14.--'Empire' apple alcohol dehydrogenase activity as a function of relative diffusivity of the storage medium and duration in store

RD[b]	P_T	P_{O_2}	Alcohol dehydrogenase ± $S_{\bar{x}}$ (eu/g)[a]		
	(torr)		2 weeks	2 months	5 months
100	40N_2	8	11 ± 4	15 ± 3	55 ± 7
15	760He	8	6 ± 1	19 ± 2	74 ± 5
5	760N_2	8	6 ± 2	17 ± 1	84 ± 7
--	760N_2	152	6 ± 1	13 ± 1	34 ± 2

[a]Initial post store values.

[b]Relative diffusivity.

Figure 1. The effect of conditioning (LPS 7+1) 1 month in air at 0 C and continual LP storage on changes in malic enzyme activity, flesh firmness (flesh firm.), and water soluble polyuronides of 'Empire' apples.

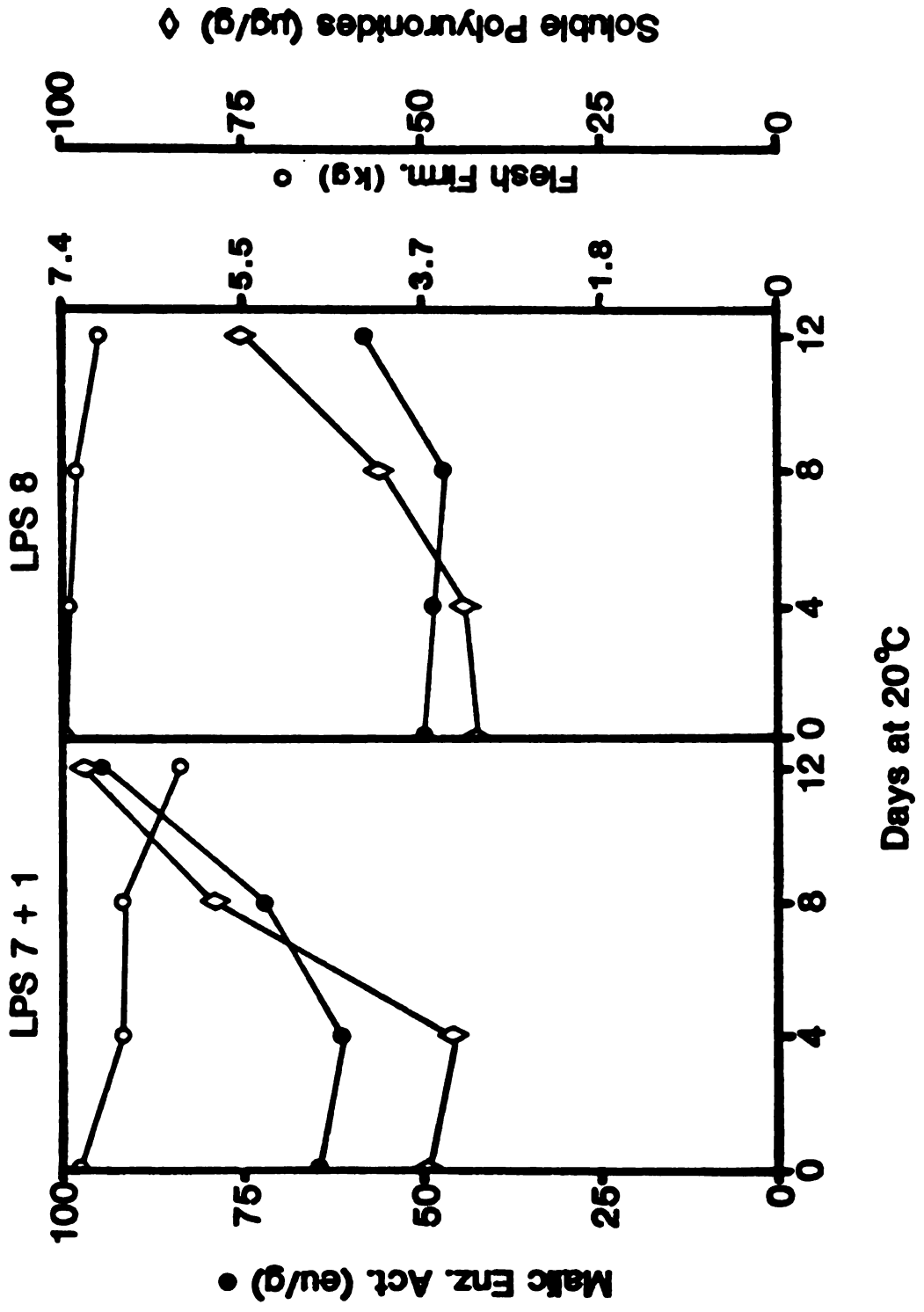
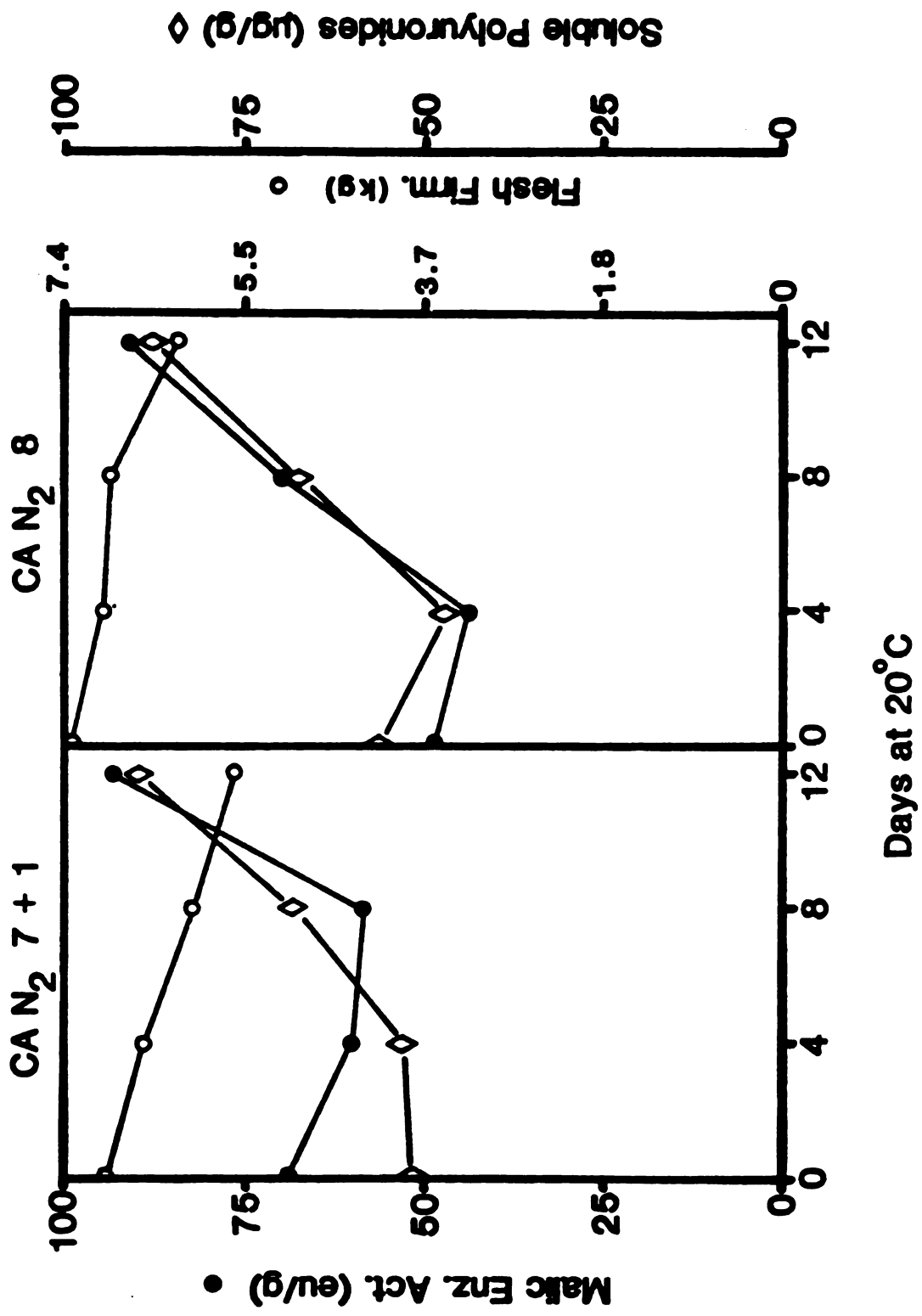

Soluble Polyuronides (ug/g) ◊
Flesh Firm. (kg) o
Malic Enz. Act. (eu/g) •
LPS 7 + 1
LPS 8
Days at 20°C
0
25
50
75
100
1.8
3.7
5.5
7.4
0
4
8
12
0
4
8
12

Figure 2. The effect of conditioning (CA-N_2 7 + 1) 1 month in air at 0 C and continual CA storage on changes in malic enzyme activity, flesh firmness (flesh firm.), and water soluble polyuronides of 'Empire' apples.


Soluble Polyuronides (µg/g) ◊
Flesh Firm. (kg) o
Malic Enz. Act. (eu/g) •
CA N2 7 + 1
CA N2 8
Days at 20°C
100
75
50
25
0
7.4
5.5
3.7
1.8
0
12
8
4
0

with this were attenuated changes in ME (ΔME = ca. 10 eu/g) activity and WSP (ΔWSP = ca. 25 μg/g). After 7 months continuous LPS treatment followed by 1 month conditioning in air at 0 C, these same fruits softened significantly. Also, enzyme activity and WSP increased ca. 60 and 100%, respectively, after 12 days at 20 C. All fruits, after 8 months continuous low O_2 storage or 7 months CA + 1 month conditioning, softened significantly and were observed to increase in WSP and ME activity at about the same rate (Figure 2). These data indicate that the low pressure effect was reversible upon allowing the tissues to equilibrate in air at 0 C and atmospheric pressure. Fruits stored in a low RD relative to air never attained the effect to the same degree as did the LPS fruits.

In summary:

1. the slow-ripening syndrome was found to be a function of the relative diffusivity of the storage medium; fruits stored in a medium of low density, relative to N_2 or air, were observed to gradually, over a 5 month period, lose their ability to react to endogenous ethylene after storage via flesh softening
2. associated with this non-softening attribute were attenuated changes in malic enzyme activity and water soluble polyuronides
3. these attenuated processes were completely reversible after conditioning previously inhibited fruits in air at 0 C and atmospheric pressure

4. the loss of the low pressure effect in long-term store was a function of duration and fruit maturity

These data support the hypothesis that the retention of ethylene recognition is dependent upon ethylene or some other native fruit volatile(s).

The practical implications of these findings are obvious. Commerical CA storage facilities contain some 20000 plus bu of apples per chamber and have a very low atmospheric turnover. Also, O_2 levels are high (2-3%, v/v) relative to what was utilized in these experiments (1%) to induce the low pressure effect. Because of these factors, high levels of ethylene accumulate (11). If ethylene was removed catalytically (11) from the storage atmosphere and O_2 diminished in these storage conditions, then it might be possible to induce a similar slow-ripening physiology commercially, since the concentration gradient would be maximized with respect to all fruit volatiles ($C_{in} - C_{out} = max$). Under these conditions low O_2 storage might only be required for the first 3-5 months followed by air storage for the remainder of the marketing period. Thus, the expense of opening the CA chambers for fruit removal could be eliminated for mid- to long-term CA fruits without appreciable loss of fruit quality.

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APPENDIX A

ASSAY OF WATER SOLUBLE POLYURONIDES BY THE M-PHENYL PHENOL REAGENT METHOD

Abstract: A simple method of extracting water soluble uronides from apple tissue is presented. Data are also presented which indicate that the assay for uronic acids (Blumenkrantz and Asboe-Hansen 1973, Ahmed and Labavitch 1977) results from the binding of m-phenyl phenol (mpp) to the uronic acid moiety (galacturonate and glucuronate in these studies). The pKd (-log of the dissociation constant) for the binding process was 3.96 for galacturonate and 4.08 for glucuronate. The free energy of association of galacturonate to mpp at 20 C was determined to be -5.31 kcal/mole while the value for glucuronate was -5.47 kcal/mole.

Introduction

It has been long presumed that the loss of flesh firmness in climacteric fruits was due to cell wall-degrading enzymes (Dilley 1970, Knee 1974). Water soluble polygalacturonate (WSP) residues have been demonstrated (Bartley 1974, Knee 1973) to increase markedly in activity as apple fruits soften as a function of the ripening process. It has also been reported that flesh firmness is inversely proportional to soluble polyuronide content (Knee 1973, 1974). However, only exo-polygalacturonase activity (Bartley 1978) has been demonstrable in apple fruits. The standard extraction and assay for polygalacturonase is time-consuming and laborious (Pilnik and Voragen

1970), therefore the method of measuring water soluble (Bartley 1974, Knee 1973) and insoluble (Ahmed and Labavitch 1977) polyuronides has become an important indirect measure of enzyme activity and "ripeness" of the fruit.

The purpose of this paper is to discuss the use of the alcohol-insoluble fraction of the pellet from extraction for malic enzyme (Dilley 1966) in determining the relative change of some cell wall-degrading enzyme (presumably polygalacturonase). Evidence is also presented for the binding of m-phenyl phenol to uronide monomers in the assay (Blumenkrantz and Asboe-Hansen 1973) for natural pectinic constituents.

Materials and Methods

Reagents

m-phenyl phenol (meta-hydroxydipenyl; mpp) solution: 0.15% (w/v) mpp (Eastman Organic Chemical, Blumenkrantz and Asboe-Hansen 1973) in 0.5% (w/v) NaOH or 0.2% (w/v) mpp in 0.5% NaOH. For the binding studies with constant galacturonate (2.07×10^{-5} M) or glucuronate (1.63×10^{-5} M) the concentration of mpp varied as follows: 7.56×10^{-4}, 3.78×10^{-4}, 3.03×10^{-4}, 2.27×10^{-4}, 1.51×10^{-4}, 7.56×10^{-5}, 3.78×10^{-5}, 1.89×10^{-5} M.

Sulfuric acid/sodium tetraborate solution: a 0.0125 M solution of sodium tetraborate in concentrated sulfuric acid (Blumenkrantz and Asboe-Hansen 1973) was utilized.

Tissue Preparation

Tissue preparation followed, with some modification, that described (Dilley 1966) for the extraction of apple fruit malic enzyme. All extractions were performed at 0 C. Chilled apples (cv. 'Empire') were peeled, cored, and cortical tissue cut into approximately 1 cm^3 wedges whereupon 60 g of the tissue was selected at random, weighed, placed in a ca. 400 cm^2 piece of cheesecloth, and dipped in chilled 1 x 10^{-3} M $NaHSO_4$ to inhibit tissue oxidation. The cold tissue was then transferred to a beaker with 0.12 grams diethyldithiocarbamic acid (DIECA) in 120 ml of 0.2 M K_2CO_3. The tissue was then vacuum-infiltrated in the K_2CO_3/DIECA solution (0 C) for approximately one hour (or until the tissue took on a translucent appearance).

Extraction

The cold vacuum-infiltrated tissue was then drained of excess K_2CO_3/DIECA solution and blended 30 seconds in 60 ml pH 7 water with 0.06 grams DIECA. The slurry was vacuum-filtered through a nylon cloth (500 μmeter mesh), being careful not to squeeze the cell wall material through the cloth. The volume of the extract was measured (should be between 70-80 ml) and a $MgCl_2$ solution (400 g/liter) was added at a rate of 5 μl/ml extract for the precipitation of dissolved pectins. The pH of the solution was adjusted to 9.5 (should be between 9 and 10 prior to adjustment). Approximately 25 ml of this solution was added to centrifuge tubes and centrifuged 15 min. at 10,000 g. The resultant solution can be utilized for determining malic enzyme activity upon adjusting the pH to 7.3. The pellet was

dispersed in 10 ml absoute ethanol using a Teflon pestle attached to a drill press. The solution was then centrifuged 5 min. at 10,000 g. The supernatant solution was discarded and the pellet resuspended in approximately 25 ml of 100 C distilled water. This solution was clarified by centrifugation 5 min at 10,000 g after having been cooled to room temperature in an ice water bath. This clarified water extract was utilized in the assay for water soluble polyuronides.

Assay (Blumenkrantz and Asboe-Hansen 1973, Ahmed and Labavitch 1977)

The apple fruit extract (or glucuronate and galacturonate standards) was added to the sulfuric acid/tetraborate solution (0.6 ml to 10 ml, respectively), mixed, and heated in a boiling water bath (only for apple extracts) for 5 min. The solutions were allowed to cool to room temperature by immersing in an ice water bath. Upon cooling, 0.6 ml of the 0.2% mpp in 0.5% NaOH (or 0.15% mpp as indicated in Figures 1-5) was added. Absorbance was measured at 520 nm after one hour.

Blanks

Blanks were made for each apple extract assay by adding 0.6 ml extract to 10 ml sodium tetraborate/H_2SO_4 with 0.6 ml NaOH solution (uronides absorb very little without mpp at 520 nm, see Figure 4). Blanks for the binding and absorption spectra studies consisted of 0.6 ml H_2O in 10 ml acid solution with 0.6 ml NaOH solution.

Binding and Adsorption Spectra Studies

Three test tubes were prepared for each treatment combination. Each tube contained 10 ml sulfuric acid solution with 0.6 ml of glucuronate or galacturonate solution and 0.6 ml mpp solution. The final concentrations of all constituents are given in the reagents sections or in Figure headings. The solutions were mixed thoroughly with a glass rod and absorbance was measured at 520 nm after one hour. All absorbance measurements at wavelengths (λ) less than 320 nm were made by diluting the sample (1 ml) with 9 ml of the sulfuric acid solution.

Calculations Utilized for the Binding Study

The calculation for alpha (α, fraction of uronide-mpp dissociated) was as follows:

$$\text{alpha } (\alpha) = 1 - (A_{520} \text{ of each sample}/A_{520} \text{ for the sample with saturating mpp})$$

Thus, A_{520} sample/A_{520} max (assuming all the mpp was bound) is equal to some fraction between 0 and 1. The division of the value (α) by ($1-\alpha$) was assumed to be equivalent to the degree of dissociation for these substances under the particular experimental conditions (20 C). If there was simple (statistical) binding taking place, with little or no cooperativity, the dissociation constant (Kd) would be:

$$[\text{uronide}]_{\text{free}} \times [\text{mpp}]_{\text{free}}/[\text{uronide mpp}]_{\text{bound}}$$

Therefore,

$$-\log[mpp] = -\log Kd + \log \alpha/1-\alpha$$

$$p[mpp] = pKd + \log \alpha/1-\alpha$$

and a plot of p[mpp] versus log $\alpha/1-\alpha$ should yield a straight line. A plot of α versus p[mpp] should be sigmoidal and symmetrical. The slope of the line would be about unity. $\bar{V}$ (v bar, Figure 11) was calculated by determining the amount of galacturonate bound (via the standard curve) and dividing this number by the number of moles mpp in the assay mixture. This was performed by assuming that the absorbance increase at 520 nm was mainly a function of increasing galacturonate concentration and only bound galacturonate could change the absorbance reading since the mpp concentration was kept constant. $\bar{V} = nKa\ [Galacturonate]/1 + Ka\ [Galacturonate]$ (Van Holde 1971); n = the number of binding sites.

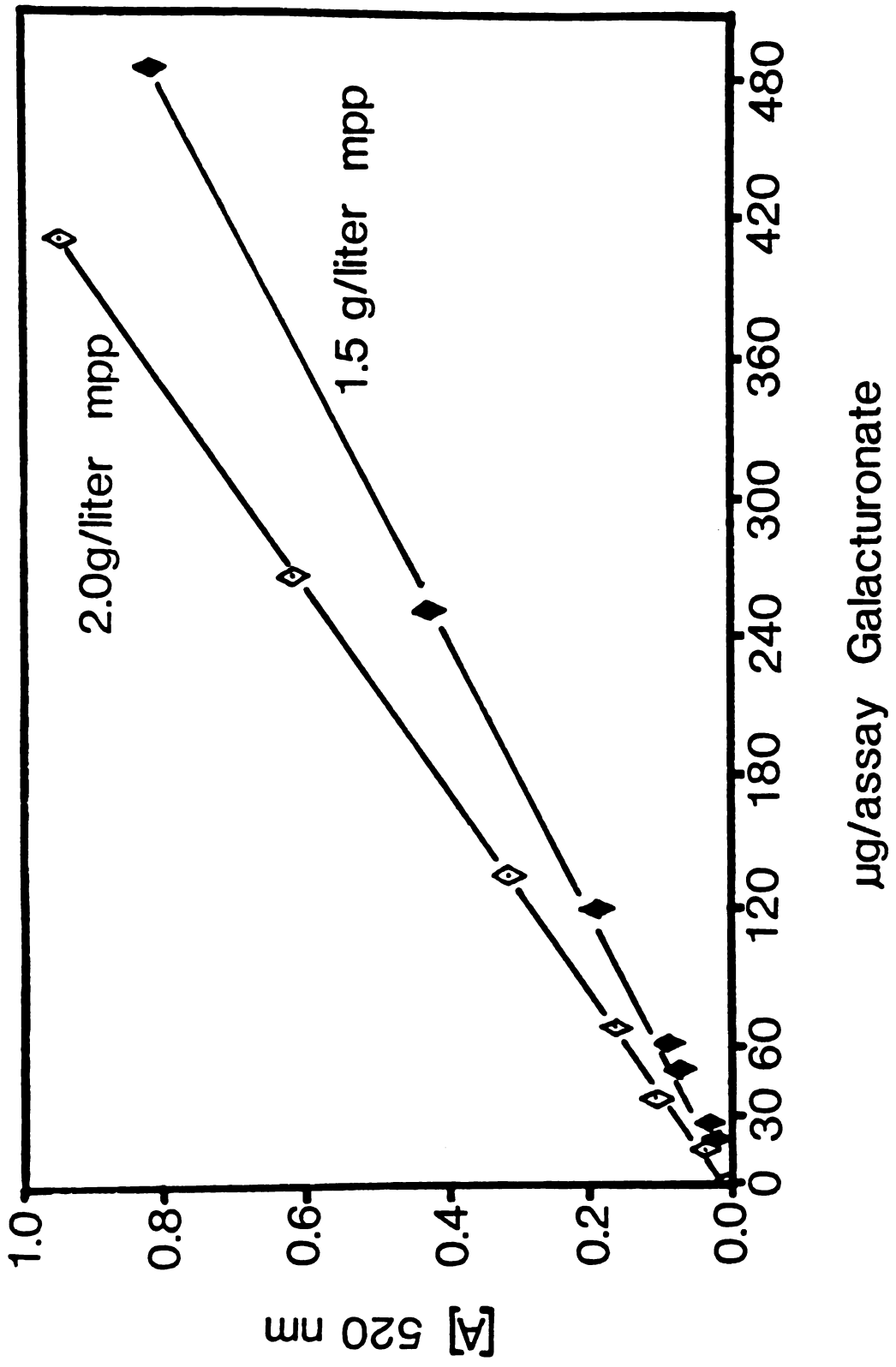
$$1/\bar{V} = 1/n + 1/nKa\ (1/[Gal]).$$

Thus, n can be calculated from the reciprocal of the y-intercept and Ka from the reciprocal of the slope divided by n.

Results and Discussion

Contrary to some reports (Blumenkrantz and Asboe-Hansen 1973) these results (Figure 1) demonstrate that 0.15% m-phenyl phenol (mpp) was not necessarily the optimal concentration. Absorbance at 520 nm was also reported to be linear only up to about 120 µg/11.2 ml

Figure 1. The effect of variable m-phenyl phenol (mpp) concentration on the standard plot for galacturonate. Total assay volume = 11.2 ml; 10 ml sulfuric acid/tetroborate + 0.6 ml galacturonate solution + 0.6 ml of 2.0 or 1.5 g/l mpp; blank of 10 ml acid solution + 0.6 ml H_2O + 0.6 ml mpp mixture.


2.0g/liter mpp
1.5 g/liter mpp
[A] 520 nm
1.0
0.8
0.6
0.4
0.2
0.0
0 30 60 120 180 240 300 360 420 480
µg/assay Galacturonate

(15 μg/1.42 ml) while these data (Figures 1 and 3 insert) clearly show linearity pass 200 μg.

Absorption spectra of the uronide-mpp interaction demonstrated one peak in absorbance at 520 nm (Figures 2,3) and another at 260 nm (Figure 5). The galacturonate absorption spectrum had only one peak at ca. 300 nm while mpp and the uronide-mpp mixture (Figure 4) showed a maximum at 260 nm. No 300 nm absorption maximum was observed when saturating levels of mpp were added to the uronide solution. In the mpp-uronide mixture the 260 nm maximum in absorbance was retained. The previous observation might be due to unbound mpp since the apple extract, which contained much less than 217 μg uronide/11.2 ml total assay volume, had approximately a 17% higher absorbance reading (Figure 5) at this wavelength (260 nm).

The method of utilizing the malic enzyme extraction pellet for study of water soluble polyuronides (WSP) appeared to be valid. The regression (Figure 6) of flesh firmness versus uronide content in the same fruits was highly significant. In this correlation 61% of the observed variation was due to regression ($r^2 = 0.61$). Other data (Knee 1974) concerning equivalent measurements had similar variation and linearity. WSP extraction from ripe tomato fruits (Sawamura et al. 1978) yielded no more than 300 μg/g fresh weight. Knee (1974) reported WSP values ('Cox's Orange Pippin' apples) varying between 100 to 500 μg/g for 13-6 lbs flesh firmness, respectively. These data yield 70-400 μg/g for apples with similar flesh firmness measurements.

Figure 2. Absorption spectrum of galacturonate (18.6, 25.8, and 60 µg/assay; volume = 11.2 ml) from 320 to 600 nm with a constant mpp concentration (0.2 ml/assay of 1.5 g/l mpp in NaOH).

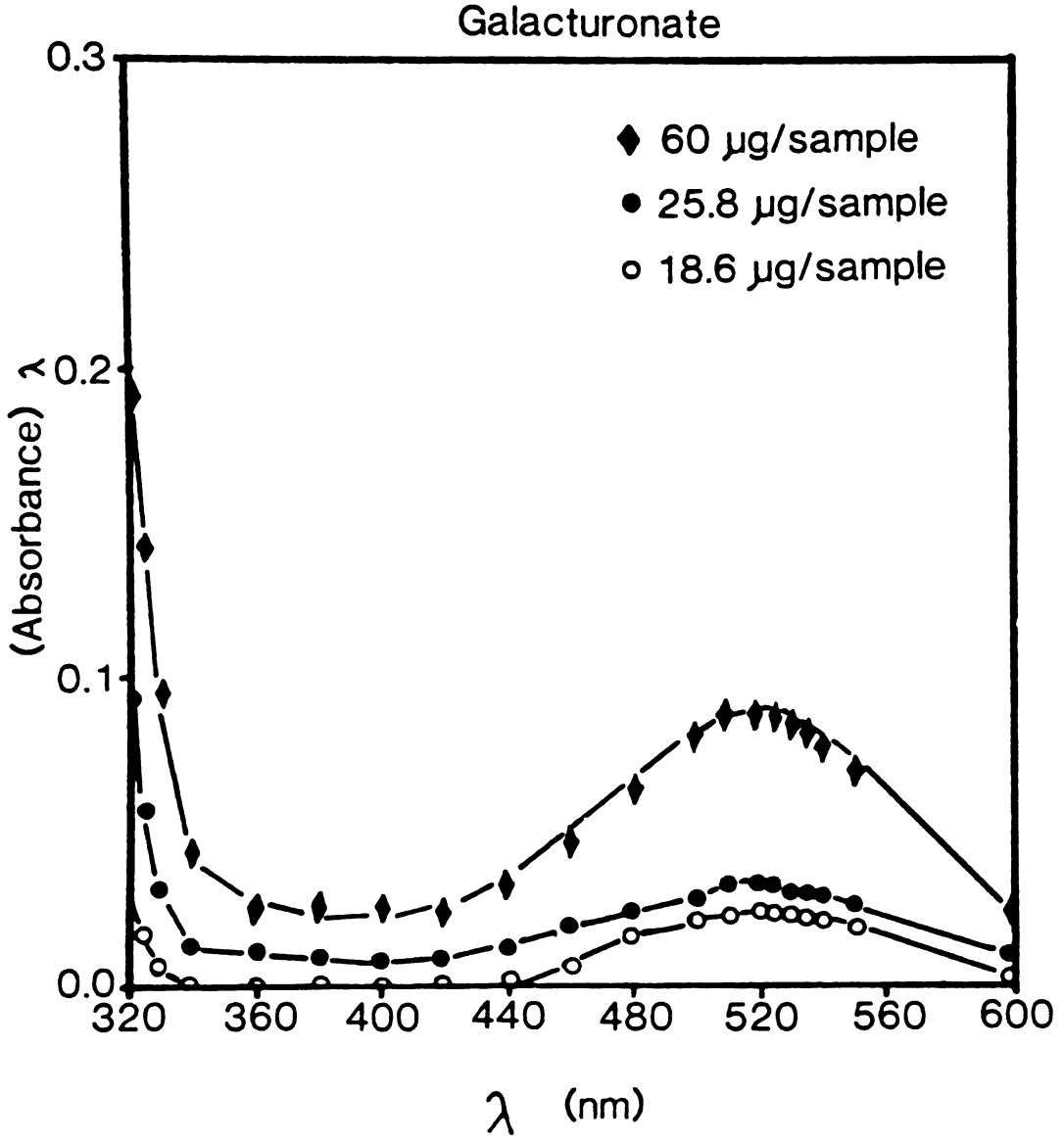

Galacturonate
0.3
0.2
0.1
0.0
(Absorbance) λ
♦ 60 µg/sample
● 25.8 µg/sample
o 18.6 µg/sample
320
360
400
440
480
520
560
600
λ (nm)

Figure 3. Absorption spectrum of glucuronate (21.6, 105.6, and 249.6 μg/assay; volume = 11.2 ml) from 300 to 600 nm with a constant mpp concentration (0.2 ml/assay of 1.5 g/l mpp).

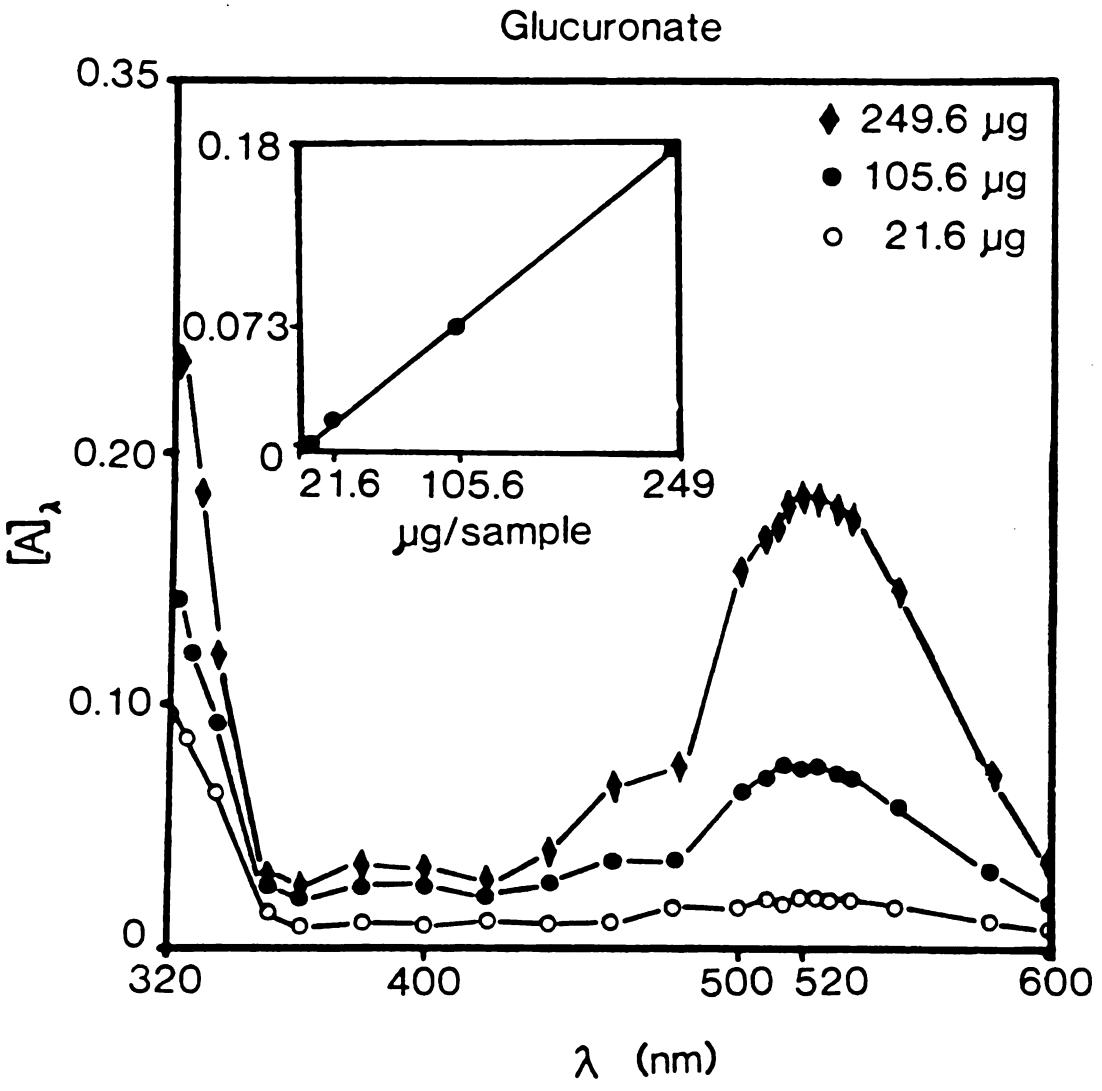

Glucuronate
0.35
0.20
0.10
0
[A]λ
249.6 µg
105.6 µg
21.6 µg
0.18
0.073
0
21.6
105.6
249
µg/sample
320
400
500
520
600
λ (nm)

Figure 4. Absorption spectra of mpp (90 and 372 µg/assay; volume = 11.2 ml) and galacturonate (217 µg/assay) alone. Absorbance at 520 nm for 90 µg/assay mpp was 0.001; 373 µg/assay mpp was 0.002; and, for galacturonate was 0.018. Blank = 10 ml sulfuric acid/tetraborate + 0.2 ml H_2O + 0.2 ml 0.5% NaOH.

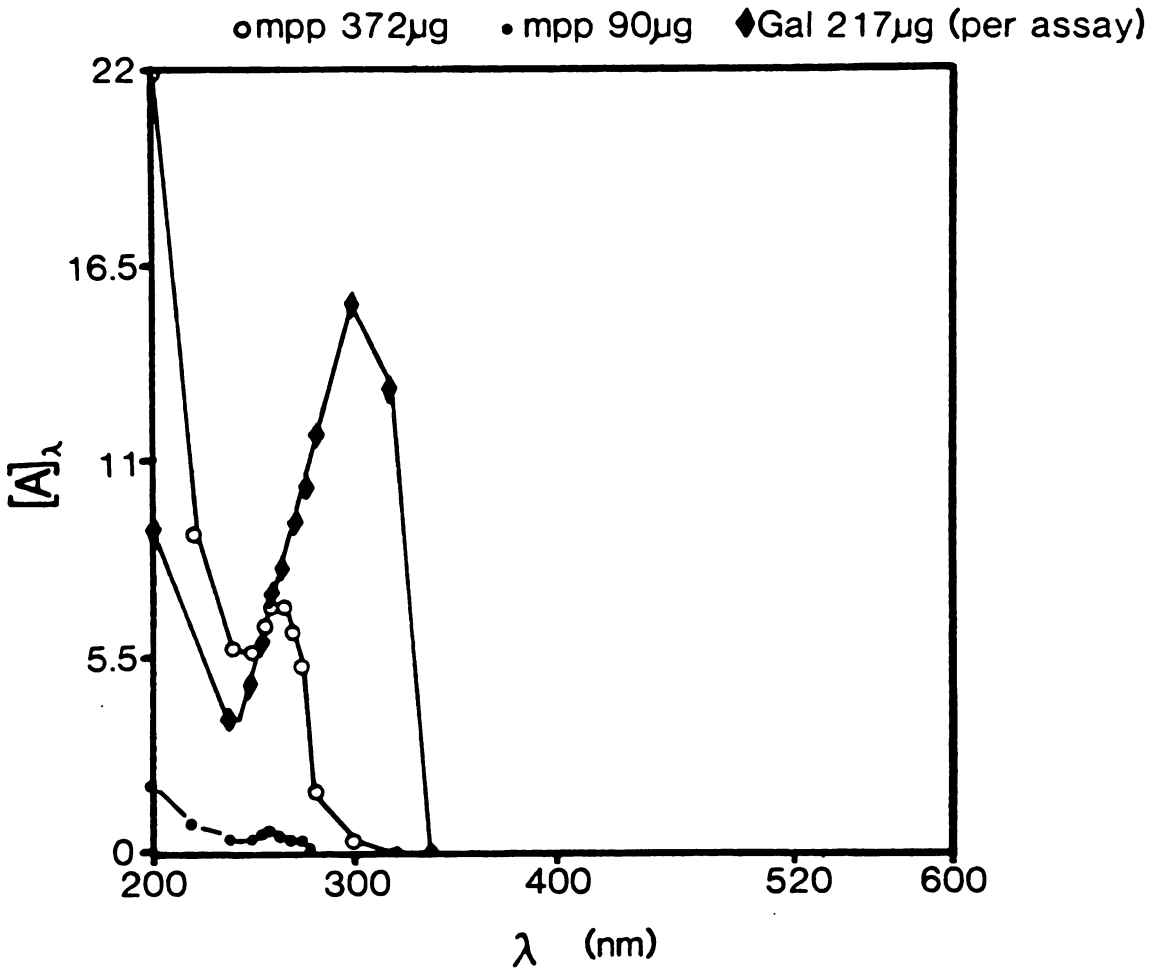

o mpp 372µg
• mpp 90µg
♦Gal 217µg (per assay)
22
16.5
11
5.5
0
[A]λ
200
300
400
520
600
λ (nm)

Figure 5. Absorption spectra of 217.2 µg/assay galacturonate + 1200 µg/assay mpp and apple tissue (cv. 'Empire') soluble polyuronide extract + 1200 µg/assay mpp from 200 to 600 nm. Blank = 10 ml sulfuric acid/tetraborate + 0.2 ml H_2O + 0.2 ml 0.5% NaOH.

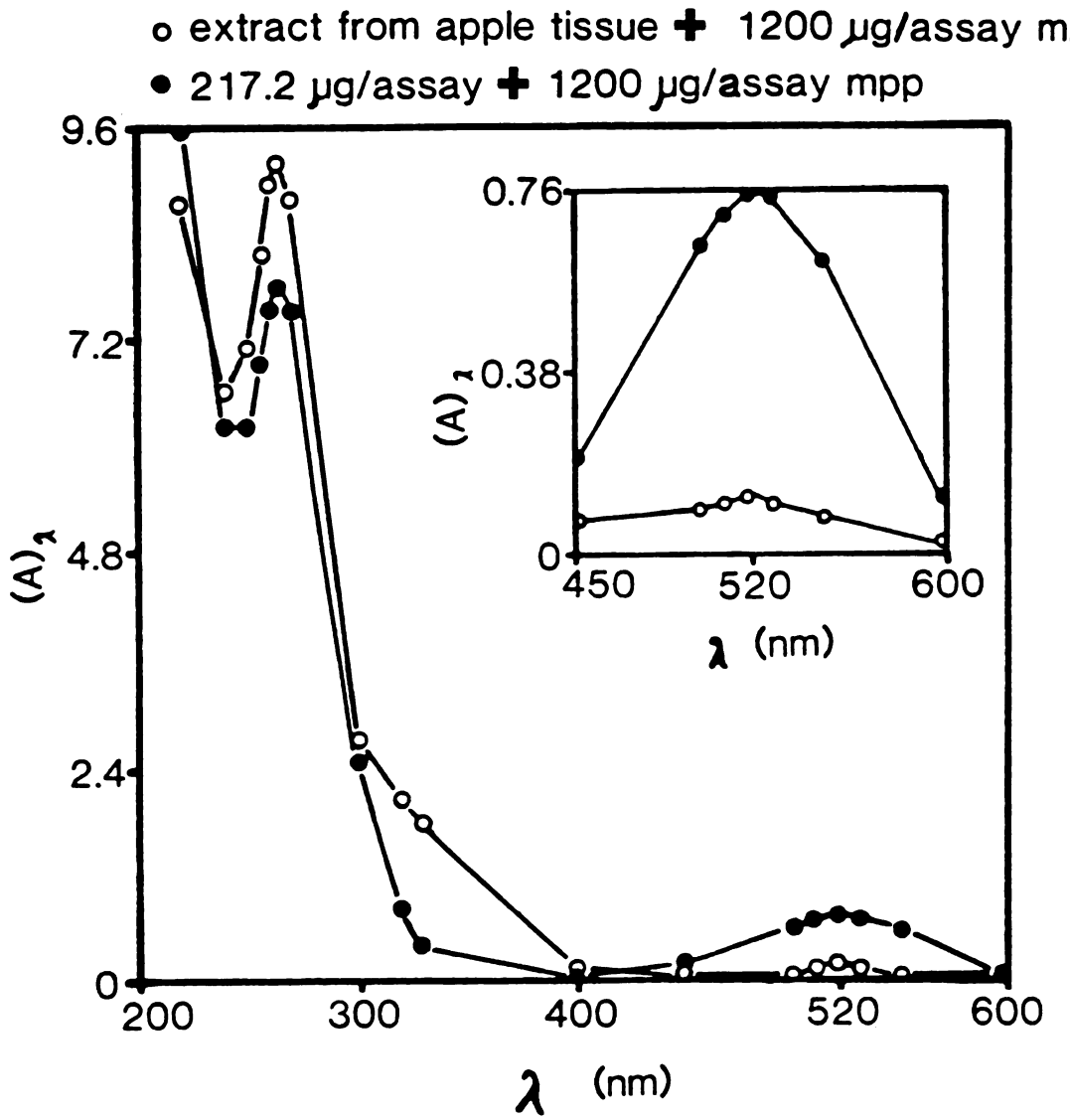
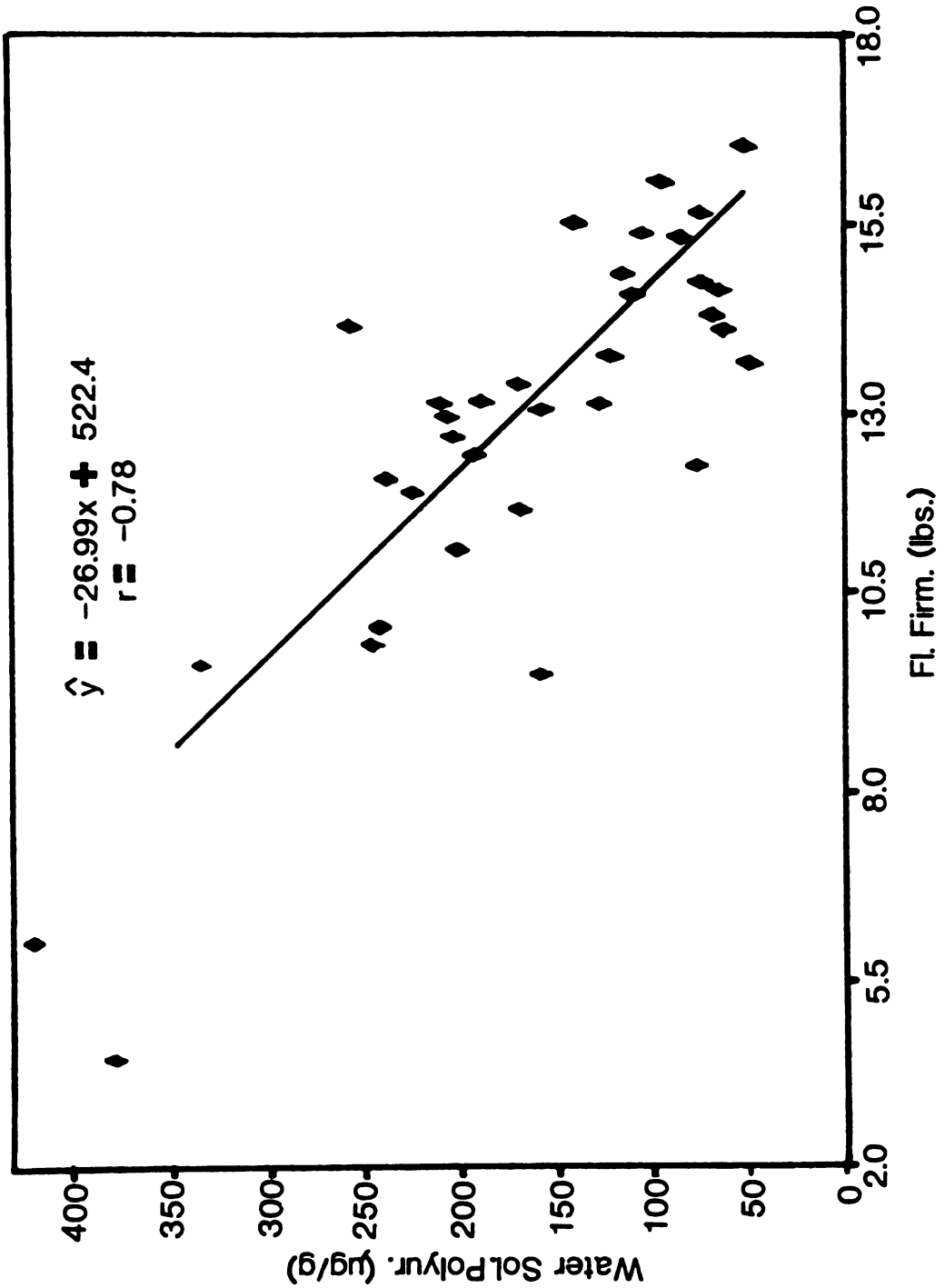

o extract from apple tissue + 1200 µg/assay mpp
● 217.2 µg/assay + 1200 µg/assay mpp
9.6
7.2
4.8
2.4
0
(A)λ
200
300
400
520
600
λ (nm)
0.76
0.38
0
450
520
600
λ (nm)

Figure 6. Changes in 'Empire' apple water soluble polyuronides as a function of flesh firmness.


Water Sol.Polyur. (µg/g)
400
350
300
250
200
150
100
50
0
2.0
5.5
8.0
10.5
13.0
15.5
18.0
Fl. Firm. (lbs.)
ŷ = −26.99x + 522.4
r = −0.78

Data reported in Figures 7 and 8 represent averages of three replications. The coefficient of variation ($S_{\bar{x}}/\bar{x}_{experiment}$) 100 = cv) for the absorbance measurements at 520 nm was 5.3%. Data presented for α at a constant galacturonate concentration (Figure 7) and variable mpp demonstrates that this chemical system is similar to the association of H^+ to weak organic acids. Alpha (α) appeared to approach unity (100% unassociated galacturonate) as mpp concentration was decreased. These data were also plotted in the form of a Henderson-Hasselbach relationship (Figure 8; p[mpp] = pKd + log (α/1-α)) in order to calculate the dissociation constant, Kd, when α = 0.5(log(α/1-α) = 0). The Kd for these data was 1.1 x 10^{-4} (pKd = 3.96); the free energy of association (ΔG^{293}_{ass}) at 293 K was equivalent to -5.31 kcal/mole. A similar experiment was performed (Figures 9 and 10) with glucuronate as the constant component. These results represent the average of 3 replications (cv = 7%) per treatment combination. From the regression equation, pKd at log(α/1-α) = 0 was calculated as before (pKd = 4.08). Since ΔG^{293}_{ass} = -5.47 kcal/mole, it is evident that the binding process is favorable thermodynamically. The Hill coefficient (c) was calculated to be 1.38 and 1.57 for galacturonate and glucuronate respectively. From these same data c was calculated as follows:

$$\bar{V} = (N\ Ka[L]^c)/1 + Ka[L]^c$$

where c = 1 for no cooperativity

c > 1 cooperativity

N = number of binding sites

Figure 7. Changes in the fractional dissociation (alpha) of the mpp-uronide complex as a function of -log(mpp) at a constant galacturonate concentration of 2.32×10^{-5} M.

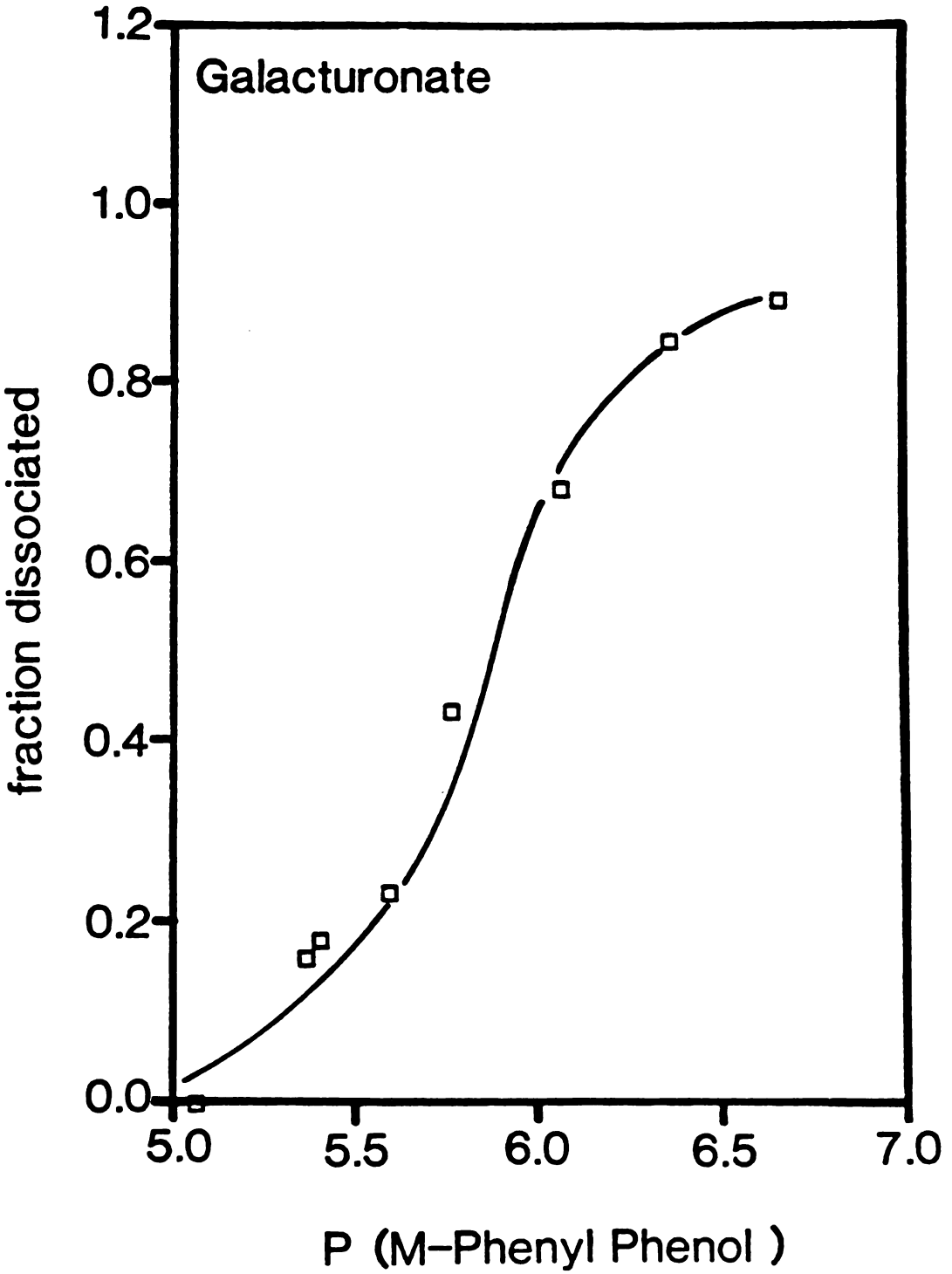

Galacturonate
fraction dissociated
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5.0
5.5
6.0
6.5
7.0
P (M-Phenyl Phenol)

Figure 8. Changes in log(alpha/1-alpha) as a function of -log(mpp) at a constant galacturonate concentration of 2.32×10^{-5}M. The K_d was calculated for molar concentrations and had a value of 1.1×10^{-4}; ΔG_{ass} = -5.31 kcal/mole.

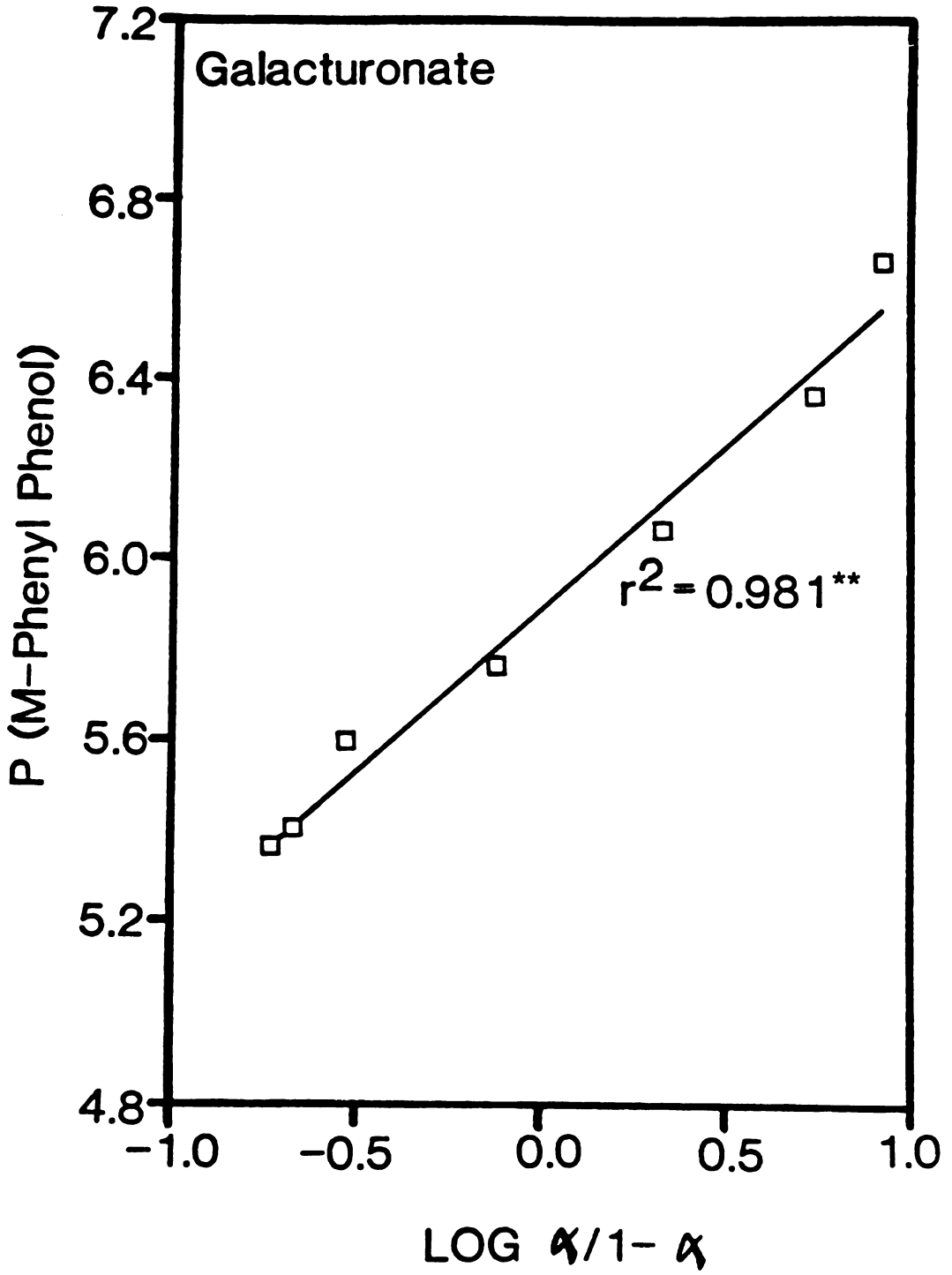

Galacturonate
7.2
6.8
6.4
6.0
5.6
5.2
4.8
P (M-Phenyl Phenol)
$r^2 = 0.981^{**}$
−1.0
−0.5
0.0
0.5
1.0
LOG α/1− α

Figure 9. Changes in the fractional dissociation (alpha) of the mpp-uronide complex as a function of -log(mpp) at a constant glucuronate concentration of 1.63×10^{-5}M.

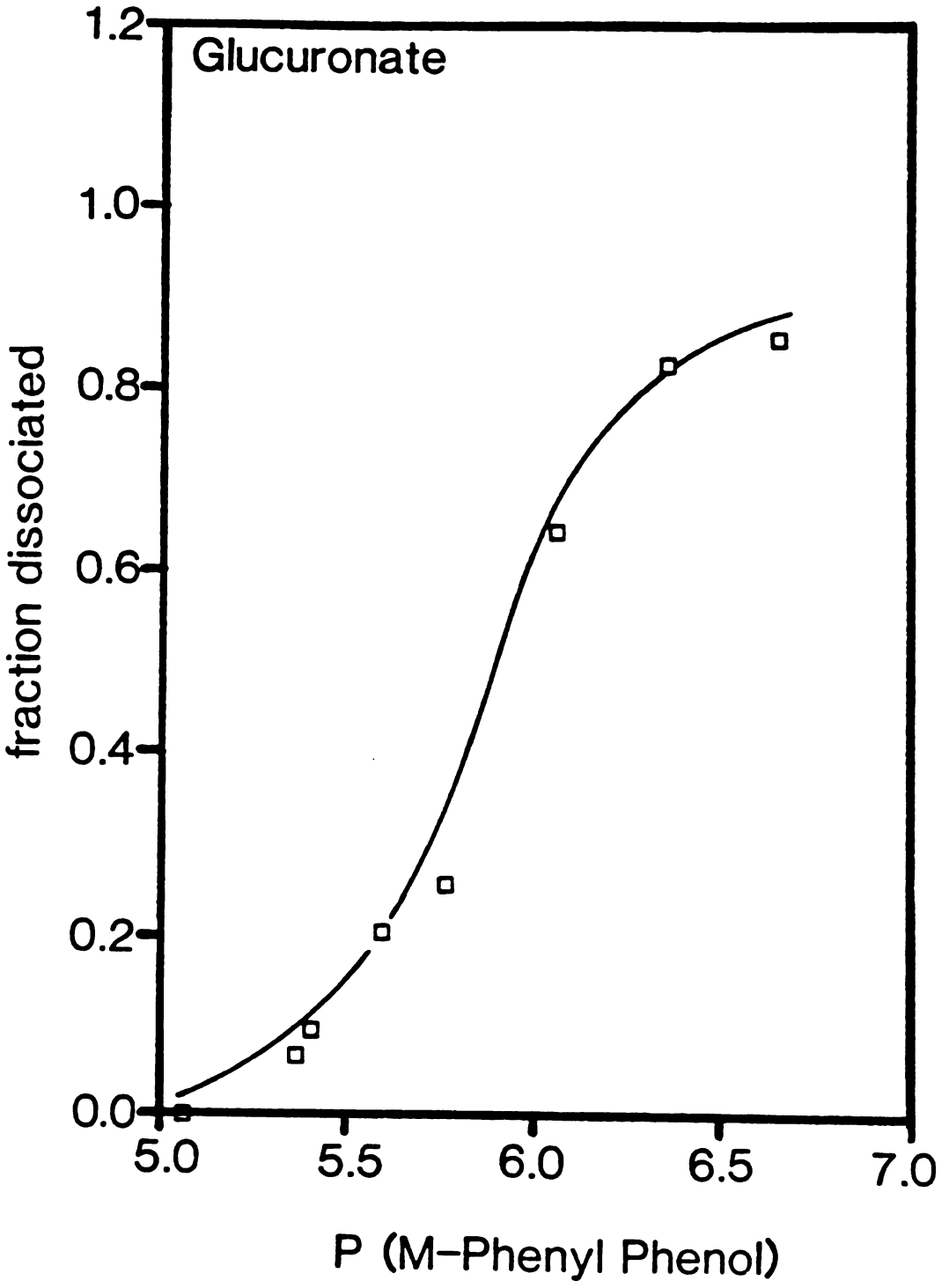
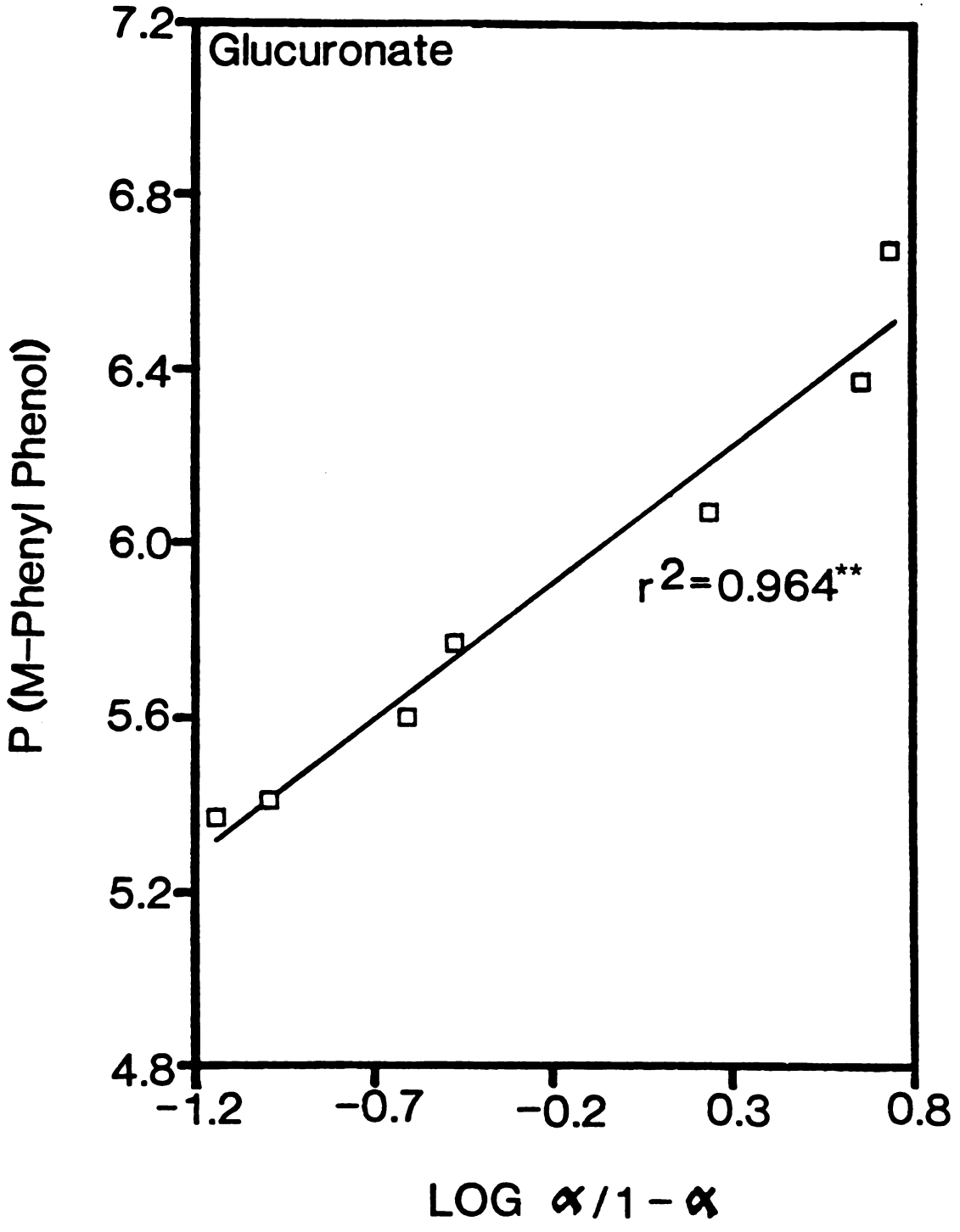

Glucuronate
fraction dissociated
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5.0
5.5
6.0
6.5
7.0
P (M-Phenyl Phenol)

Figure 10. Changes in log(alpha/1-alpha) as a function of -log(mpp) at a constant glucuronate concentration of 1.63×10^{-5}M. The K_d was calculated for molar concentrations and had a value of 8.32×10^{-4}; ΔG_{ass} = -5.1 kcal/mole.


Glucuronate
7.2
6.8
6.4
6.0
5.6
5.2
4.8
P (M-Phenyl Phenol)
$r^2=0.964^{**}$
-1.2
-0.7
-0.2
0.3
0.8
LOG $\alpha/1-\alpha$

$$\bar{V}/(N - \bar{V}) = K[L]^c \qquad \text{(Van Holde 1971)}$$

since $\alpha = N - \bar{V}$ and $(1-\alpha)/\alpha = \bar{V}/(N - \bar{V})$

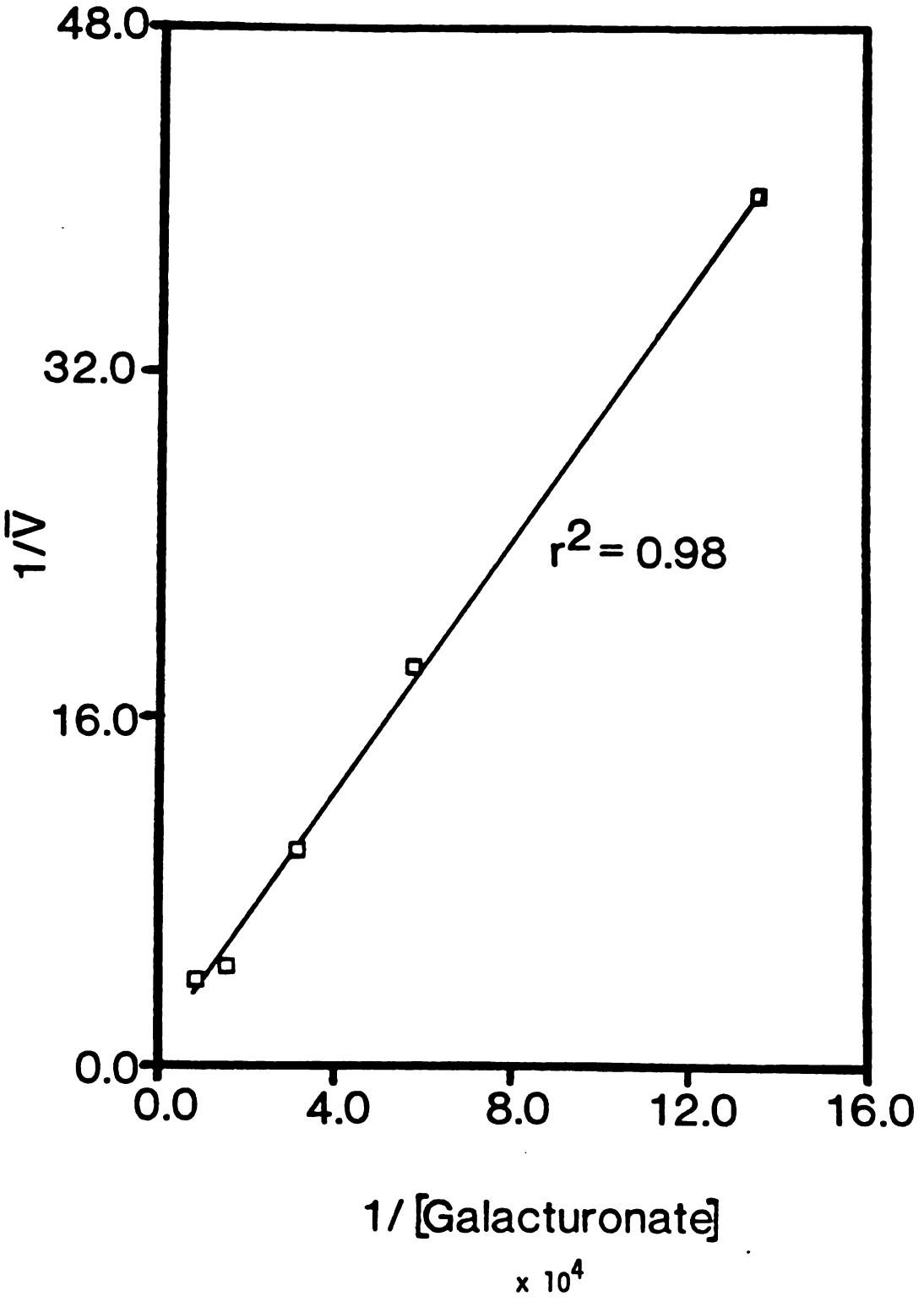
then

$$\log (1-\alpha)/\alpha = c\log[L] + \log K.$$

Thus, the slope of the $\log(1-\alpha/\alpha)$ vs. log L plot is equivalent to c.

From changes in $(\bar{V})^{-1}$ as a function of $[\text{Galacturonate}]^{-1}$ (Figure 11) at a constant mpp concentration it was found that the number of binding sites on mpp was equivalent to about 1.23. From these data the calculation for pKd (3.49) was in close agreement with the value derived previously for galacturonate and mpp (pKd = 3.96). These data support the hypothesis that the observed change in absorbance at 520 nm is due to the binding of the uronide with the m-phenyl phenol moiety. However, data for the number of binding sites (N = 1.23) and the Hill coefficient (c = 1.38 and 1.57) are not close enough to unity to claim either one binding site or non-cooperativity.

Figure 11. Benesi-Hildebrand plot of changes in reciprocal $\bar{V}$ (moles bound uronide/moles mpp) as a function of reciprocal galacturonate concentration at a constant mpp concentration of 4.86×10^{-5}M. The galacturonate concentrations used were: 6.58×10^{-6}, 1.55×10^{-5}, 2.83×10^{-5}, 5.62×10^{-5}, and 1.01×10^{-4}M.


48.0
32.0
16.0
0.0
1/V̄
r² = 0.98
0.0
4.0
8.0
12.0
16.0
1/ [Galacturonate]
x 10⁴

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