PHYSIOLOGICAL STUDIES OF BITTER PIT IN APPLE

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(ABSTRACT)

In a series of related experiments some aspects of the histology and physiology of the disorder bitter pit in apple (***Malus domestica*** Borkh.) were studied.

A two year field study was conducted to induce consistent bitter pit development in spur type 'Delicious' (D) and 'Golden Delicious' (GD) apple fruit. Multiple spray treatments of $CaCl_2$ and $MgCl_2$, combined with paper bag fruit covers, were applied and subsequent bitter pit development examined. The main effect of bags in both cultivars was increased pit development, decreased Ca in fruit and increased fruit K. $CaCl_2$ sprays resulted in less pit development, increased Ca in fruit, and less fruit Mg. $MgCl_2$ sprays resulted in increased bitter pit, decreased fruit Ca, and increased fruit Mg. Overall, field treatments provided a wide range of bitter pit incidence after storage, from 100% (bag and $MgCl_2$ spray combined) to 3% ($CaCl_2$ spray alone) in both D and GD.

The cellular morphology of pitted apple fruit from field trees was examined using transmission and scanning electron microscopy. The overall tissue morphology of both cultivars was similar, but in pitted tissues differences were observed in tannin localization, starch hydrolysis, and cell wall morphology. Cation levels in the tissues were examined using X-ray microanalysis. High Mg levels were localized in pit cells, while K levels were similar in both healthy and pitted cells. Ca levels in both tissue types were too low to be detected by this method.

Using 'Golden Delicious' fruit from the field study, the relationship between pyruvate kinase activity, fruit cation concentration and bitter pit was investigated. Pyruvate kinase activity during early fruit growth was higher in fruit which developed 100% bitter pit after storage ($MgCl_2$ spray

+ bag), than in fruit that developed 3% bitter pit ($CaCl_2$ spray). Fruit with a high bitter pit incidence had a lower Ca: Mg + K ratio than fruit with a low level of the disorder. There was a strong positive correlation between enzyme activity early in the season and bitter pit incidence after storage. An assay for pyruvate kinase may be valuable for early prediction of postharvest bitter pit development.

Finally, the qualitative electrophoretic patterns of soluble fruit proteins from each treatment were examined starting early in the season and continuing until termination of fruit storage. Patterns from all treatments were almost identical throughout the season.

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Table of Contents

List of Illustrations

CHAPTER 4

CHAPTER 5

CHAPTER 6

List of Tables

CHAPTER 3

CHAPTER 5

CHAPTER 6

CHAPTER 1

INTRODUCTION

Bitter pit of apple fruit is found in most apple growing regions of the world and is of major economic importance. The affected fruit displays brown, irregular pitted areas where skin cells die as the tissue below collapses. Occasionally tissues deeper in the flesh are affected, although this normally accompanies surface pitting. Generally, disordered tissues are not consumed but are reportedly bitter to the taste.

Bitter pit is recognized as a postharvest physiological disorder, developing primarily in storage. However it is regularly observed in the orchard just before harvest. It can be a very erratic problem and it is not well understood. It may be rife one year and absent the next, and affect most fruit on one tree while a neighboring tree is not affected. It may be scattered all over a tree, on any quadrant, high up or low down, on the tree periphery or the interior.

Most cultivars have records of susceptibility to bitter pit, even though this may be rare in some 'resistant' types. The disorder is not easily predicted with reliability. An apparently pit free crop may be harvested and then develop severe pitting after two or three months of storage. This may damage the reputation of fruit with substantial cost to the growers or their production areas.

Considerable evidence has accumulated that bitter pit is associated with low fruit calcium levels. Currently, one of the most successful methods of reducing bitter pit is by applying calcium sprays in the field. However, control is rarely complete, and a number of sprayings is necessary to obtain commercially useful results. Postharvest calcium treatments, including dips, injections, and vacuum infiltration have had moderate success in reducing bitter pit. Various other mineral elements have also been implicated in bitter pit development including high levels of magnesium, potassium, and nitrogen. Generally, conditions which interfere with calcium accumulation in the fruit, such as water stress, nutrient antagonists, and high leaf to fruit ratios, result in increased bitter pit.

Currently, a basic explanation of the cause of bitter pit remains obscure. The large volume of literature generated over the last 120 years since the disorder was first described, has resulted in a better understanding of the disorder, yet it remains a serious industry problem.

The objective of this study was to examine what was known about the disorder, and conduct a series of experiments which encompass both known aspects of bitter pit physiology, and introduce new concepts and techniques to further

elucidate its nature. The overall approach was to relate individual experiments closely to one another so that fairly broad interpretations could be made.

A field experiment was conducted to examine the effects of fruit bagging, and multiple calcium and magnesium fruit sprays on subsequent pit development. The objective of this study was to develop a reliable system of inducing bitter pit in the field. In this way, fruit predisposed to bitter pit could be examined over the growing season, before visible symptoms of the disorder appear. In an associated study, the histology of bitter pit was re-examined using modern microscopy techniques. While several descriptions of bitter pit on a cellular level exist, no published electron micrographs appear in the literature. Both scanning and transmission electron microscopes were used to photograph samples, coupled with X-ray microanalysis of the tissues. The objective was to provide a more complete picture of sub-cellular symptoms.

Fruit from the field experiment were examined from early fruit development through storage in two separate physiological studies. The first study focused on an important respiratory enzyme, pyruvate kinase. This enzyme had been reported to show sensitivity to cation levels in unrelated crops. Using an assay for pyruvate kinase, the relationship between enzyme activity, fruit cation ratios, and bitter pit development was examined. The objective was to determine whether an assay for pyruvate kinase activity had potential as an early predictor of potential bitter pit problems. The second study was an examination of the protein populations of developing fruit from the early stages of growth, through matura-

tion and storage. The objective was to identify protein differences associated with pit initiation or development.

Each study is treated in a separate chapter and appears as a complete paper. For this reason, some repetition of methods was inevitable. Papers were formatted according to the current requirements of Journal of the American Society for Horticultural Science.

CHAPTER 2

LITERATURE REVIEW

Bitter pit of apple has been known for 120 years, yet it remains a serious postharvest disorder (Perring, 1986). Despite hundreds of investigations aimed at its cause and control, it has become of increasing concern because many modern orchard practices tend to favor its occurrence. Bitter pit may develop in the orchard and appear on fruit just before harvest; however, it more commonly appears on fruit during storage or marketing (Faust and Shear, 1968; Perring, 1986). The disorder disfigures fruit and therefore destroys its fresh market value. Often it is not detected during packing, and a few fruit may develop bitter pit while moving in the marketing chain, putting the pack out of grade.

Historical background

A German research worker, Jager (1869), was the first to publish a description of the disorder and suggested it was caused by rapid transpiration in susceptible cultivars. Several years later Jones (1891) described bitter pit in Vermont, and Cobb (1895) described it in New South Wales. McAlpine (1921) studied the disorder in Australia from 1911 to 1921 and published over 900 pages of text which contributed greatly to early bitter pit knowledge, but never fully defined its cause. By 1924 it was acknowledged to exist in most apple growing regions throughout the world (Hessler and Whetzel, 1924).

Symptoms

The first visible sign of the disorder may only be slight indentations in the skin with no change in color. The skin over these depressions usually takes on a deeper color than the surrounding skin. As the tissue just below the skin collapses, the epidermal cells die, and the disorder appears as small brown desiccated pits approximately 3 to 5 mm in diameter. The pits are often more numerous on the calyx end of the fruit, and are rarely seen on the shoulder or stem end. A transverse section through a pit will reveal brown, dry, spongy tissue just beneath the skin. Occasionally the affected tissue may be deeper in the flesh and not associated with the skin. This form of pitting is often confused with internal cork, another physiological disorder of apples (Perring, 1986; Faust and Shear, 1968; Tukey, 1971).

Histology and biochemistry

Bitter pit has been reported to be associated with the vascular bundles of apple fruit (MacArthur, 1940). In light microscope studies, cell rupture and abnormalities in the pectic substances of the cell walls appeared in pit areas (Buchloch et al., 1961; MacArthur, 1940). Electron micrographs revealed cell separation at the middle lamella (Buchloch et al., 1961). Farmer (1906, 1907) reported an accumulation of starch in pit cells, which was later confirmed by several workers (Hopfinger and Poovaiah, 1979; MacArthur, 1940; Simons, 1962; Smock, 1941).

Several authors have attempted to differentiate between bitter pit on the tree and that which develops in storage (Faust and Shear, 1968), however histological studies attest that there are no indications of any differences between the two (MacArthur, 1940; Simons, 1962; Smock, 1941; Smock and van Doren, 1937). It appears then that storage pit is a later appearance of the disorder in predisposed fruit.

A rise in ethylene production (Faust and Shear, 1968), increased respiration (Martin et al., 1962), increased protein synthesis (Feucht and Waldes, 1964), and an accumulation of inorganic ions (Askew, 1960; Hopfinger and Poovaiah, 1979)) have all been reported in pitted tissues. However, similar observations have been made in apple tissues with fungal, viral, or bacterial infections (Perring, 1986; Faust and Shear, 1968), and are considered to normally accompany tissue damage.

Development of theories on the cause of bitter pit

In spite of Jager's (Jager, 1869) first description of bitter pit, and his suggestion that it was caused by excess transpiration, many other theories have evolved. For the next 30 years bitter pit was regarded as a fungal disease (Lamson, 1899), until Zchokke (1897) was able to show that it was physiological in nature. Later studies by Lafar (1898) suggested that pitting was due to cell rupture and the subsequent oxidation of tannins; Crabill and Thomas (1916) favored the idea that pit was caused by poisoning from toxic sprays; Kidd and West (1923) suggested that it resulted from extremes in fruit temperature; while Carne (1927) theorized that bitter pit was the necrosis of starch-filled-cells resulting from dehydration. Using the interpretation of existing literature, Atanasoff (1935) concluded that bitter pit was a virus disease, and this theory was not fully discredited until the grafting experiments of Campbell and Luckwill (1962) showed no virus transmission from 'infected' to 'healthy' trees. Theories went a full circle when Smock (1941) convincingly associated excessive leaf transpiration with fruit bitter pit development, confirming the suggestion first proposed by Jager (1869).

In the mid-1930's the first pioneering studies relating fruit mineral content and bitter pit incidence were made (Delong, 1936). Low calcium and boron in the fruit were associated with an increase in the disorder (Delong, 1937). Following these findings much of the next two decades were spent studying the effect of boron deficiencies on bitter pit (Burrell, 1940; Burrell et al., 1956; Magness, 1942). It was not until the discovery by Garman and Mathis (1956) that calcium

reduced, while magnesium and potassium increased pitting in apples, that the role of calcium in the development of postharvest disorders received major attention (Baxter, 1960; Beyers, 1962; Drake, 1966; Jackson, 1961; Oberly and Kenworthy, 1961; Pienar et al. 1964; Raphael and Richards, 1962; Stiles, 1964; Yamazaki and Mori, 1961).

Despite increased interest in bitter pit, calcium nutrition and related subjects during the 1960's, Bunemann (1972) concluded, after reviewing more than 700 publications, that 'the true mode of action of the cation imbalance is unknown'. In a subsequent review of further publications on bitter pit in apple, it was concluded that a basic explanation of the causes of bitter pit was lacking (Bunemann et al., 1979).

From the first pioneering work relating calcium nutrition to bitter pit incidence (Delong, 1937; Garman and Mathis, 1956) to date, most studies have involved field and postharvest manipulation and measurement of fruit cation levels and their effect on bitter pit development. In spite of a continued international research effort, Perring (1986) concluded that the cause of pitting was still obscure, and suggested that future research should concentrate on metabolic processes in fruit where the demand for calcium arises.

Present understanding of bitter pit causes

Susceptibility to bitter pit is currently associated with low calcium (Hewett and Thomson, 1988; Perring, 1986; Perring and Pearson, 1986a, 1986b, 1987),

and high magnesium and potassium (Conway and Sams, 1987; Fallahi, et al., 1987; Hopfinger et al., 1984; Sharples, 1980; Terblanche et al., 1980) concentrations in the fruit. Much emphasis is placed on the importance of calcium, since various physiological disorders including bitter pit have been associated with calcium- deficient fruit (Ferguson, 1984). However, the concentration of other major elements including high magnesium, potassium, and nitrogen aggravate the problems caused by a lack of calcium (Fukumoto and Nagai, 1983; Johnson et al., 1987).

Many orchard factors can contribute to low fruit calcium and bitter pit development. Calcium transport to the outer cell layers in fruit is heavily influenced by uptake, distribution, and competition within the tree (van der Boon, 1980a, 1980b). Conditions which result in a large number of leaves in proportion to the number of fruit increase the disorder (Ferguson, 1980). In addition, conditions which increase the stress between the leaves and fruit on the tree, can be anticipated to increase bitter pit development (Terblanche et al., 1980). Unseasonably warm dry weather, cycles of summer rain and drought, or other situations leading to water stress in the orchard, are more likely to result in bitter pit (Irving and Frost, 1987; Schumacher et al., 1986). Generally, the larger the number of leaves and the smaller the number of fruit, the greater the calcium stress placed on fruit (van der Boon, 1980a, 1980b). It has also frequently been observed that larger fruit are more likely to develop bitter pit than smaller fruit (Perring, 1986).

Most cultivars of apple have been reported as being susceptible to bitter pit, some more so than others. Cultivars such as 'York' and 'Baldwin' are considered very susceptible, 'Delicious' and 'Golden Delicious' moderately susceptible, and 'McIntosh' and 'Rome Beauty' appear to be unaffected (Tukey, 1971). Trees just coming into bearing appear to be most susceptible, mainly because they are more vigorous. Generally, high vigor, whether it involves the whole tree or individual limbs, is associated with increased bitter pit (Schumacher et al., 1986; Terblanche et al., 1980). In addition factors which stimulate shoot growth, such as high nitrogen application and heavy pruning increase the susceptibility to bitter pit (Razeto, 1984).

The association of bitter pit with a lack of calcium and the suggestion that calcium sprays (Sharples et al., 1979), dips (Fallahi et al., 1987), core injections (Perring and Pearson, 1987), and vacuum infiltration (Conway and Sams, 1987) may reduce the severity of the disorder have probably led to misunderstandings about its cause. These treatments imply that the cause of bitter pit is a calcium deficiency, which can be likened to a deficiency of any other nutrient, and remedied by gross nutrient applications. The many contradictory results in the literature (Bunemann, 1972; Bunemann et al., 1979) suggest that this has been an oversimplified approach, and new avenues as to the cause of bitter pit need to be explored (Perring, 1986).

Predicting bitter pit

Many studies have used the relationship between bitter pit and the mineral composition of apples at harvest to predict potential for bitter pit development (Autio et al., 1986; Bramlage et al., 1985; Chin and Bould, 1977; Johnson et al., 1987; Knee and Bubb, 1975; Lewis et al., 1977; Martin et al., 1975; Perring, 1976; Sharples, 1980; Sharples et al., 1979).

In general, prediction models based on the concentration of a single nutrient (calcium) have been only moderately successful (Bunemann et al., 1979). Predictions based on the concentration of more than one mineral have met with better success, especially when using ratios of calcium, potassium, magnesium, nitrogen and phosphorus, which are known to have a major influence on bitter pit (Bramlage et al., 1985; Perring, 1986). The best potential prediction systems involve the collection of a wide range of information. Measurements including fruit nutrient levels, fruit size, respiration rate, maturity, and orchard climate data have been used to formulate unique prediction equations for specific orchards (Autio et al., 1986). This approach has shown reasonable success, but would involve high costs and considerable expertise. Prediction of bitter pit will remain difficult unless a better understanding of the cause of the disorder is reached.

Pyruvate kinase and bitter pit

Pyruvate kinase has been characterized in a number of plant tissues (Duggleby and Dennis, 1973a, 1973b; Miller and Evans, 1957; Turner and Turner, 1980) as a major regulatory enzyme in glycolysis. There is a growing in-

terest in an assay for pyruvate kinase as an indicator of physiological disorders in fruit trees with an imbalance of K, Ca and Mg .

Miller and Evans (1957) established that pyruvate kinase from higher plants requires K^+ or NH_4^+ for activity. Duggleby and Dennis (1973a, 1973b) and Tomlinson and Turner (1973) showed that the enzyme had a requirement for K and Mg for activation, while Ca was a strong inhibitor of the enzyme in peas and carrots. Meli and Bygrave (1972) have shown that rat liver mitochondria can determine the rate of pyruvate kinase by altering the ratio of Mg to Ca *in vivo*. Bar-Akiva et al. (1976) showed that low Mg and K accompanied low pyruvate kinase activity in lemon leaves, whereas low Ca caused an increase in enzyme activity. Lavon et al. (1988a, 1988b) conducted a detailed study of the effect of K, Mg, and Ca on pyruvate kinase activity in citrus. Their results clearly demonstrated the inhibitory effect of Ca ions on pyruvate kinase activity and confirmed Bar-Akiva's suggestion that the enzyme possessed promise as an indicator of Ca deficiencies.

While pyruvate kinase activity has not been studied in apple fruit, it has potential to indirectly measure the relative ratios of available cations in tissues, which could prove to be an invaluable tool for bitter pit study, and early prediction.

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CHAPTER 3

A FIELD SYSTEM FOR INDUCING BITTER PIT IN APPLE

Additional index words. ***Malus domestica***, postharvest physiology, disorders, cations

Abstract. A two year study was conducted to induce bitter pit development in spur type 'Delicious' (D) and 'Golden Delicious' (GD) apple (*Malus domestica* Borkh.). In 1987 1% (w/v) $CaCl_2$ was sprayed on fruit every 10 days; fruit were covered with Kraft paper bags; or the two treatments were combined. In 1988 a 1% (w/v) $MgCl_2$ spray treatment was added. All treatments began 5 days after full bloom. The main effects of bags in both GD and D were increased pit development, decreased Ca in fruit, increased fruit K, smaller fruit and more fruit per spur (GD only). $CaCl_2$ sprays resulted in less pit development, increased Ca in fruit, less fruit Mg, smaller fruit and more fruit per spur (GD only). $MgCl_2$ sprays resulted in increased bitter pit, decreased fruit Ca, increased fruit Mg, larger fruit and less fruit per spur (GD only). Overall, field treatments provided a wide range of bitter pit incidence after storage, from 100% (bag and $MgCl_2$ spray combined) to 3% ($CaCl_2$ spray alone) in both D and GD.

Bitter pit in apples remains a serious postharvest disorder despite an international research effort to control its development (Bunemann et al., 1979; Perring, 1986). One of the problems associated with studying the disorder is the large variability in its severity from season to season. Incidence of the disorder can range from severe to non-existent, depending on locality, cultivar, orchard management, and growing season (Perring, 1986). If the postharvest physiology of the disorder is to be studied systematically, a reliable method for consistently inducing the disorder should be developed. Such inductive treatments must be able to provide a wide range of symptoms from severely pitted to healthy fruit from a given set of trees. This would enable developmental studies of fruit predisposed to bitter pit.

It is well known that fruit Ca, Mg and K have a major influence on the severity of bitter pit. Low Ca and high Mg and K tend to promote pit incidence while the converse is true for healthy fruit (Perring, 1968; Perring and Plocharski, 1975; Hopfinger et al., 1984). Generally apple trees and fruit have adequate K, which limits its manipulation. However, Ca and Mg levels in fruit tend to be lower and there is therefore a potential for modifying their ratios.

Fruit transpiration rate influences the transport of cations, particularly Ca, into the fruit. Shaded fruit with low transpiration tend to have less Ca and greater pit incidence than rapidly transpiring fruit (Perring, 1979). In this study, Kraft paper bags were used to cover fruit and reduce transpiration in some treatments. The objective was to combine Ca and Mg sprays with bagging of

fruit to consistently produce bitter pit in apple. A combination of field treatments were developed which resulted in severe pit development after storage.

Materials and Methods

Fruit used in this study were on fifteen-year-old 'Sundale Spur Golden Delicious' and 'Red Chief Delicious' (***Malus domestica*** Borkh.) apple trees at the Horticulture Farm, Virginia Polytechnic Institute and State University, Blacksburg.

Experiment 1 - 1987

The experiment was a 2 X 2 factorial with 16 replications of 5 fruiting spurs each in a randomized complete block design. Treatments were 1% (w/v) $CaCl_2$ spray/no $CaCl_2$ spray and bags/no bags; treatments began 5 days after full bloom. Sprays were applied every 10 days with a hand held sprayer with Tween 20 as surfactant. Bags were Kraft 3 lb brown paper and were carefully stapled over spurs without damage to vegetative or floral structures. Bags were removed and replaced at each spray date, and removed completely 4 weeks before harvest. Fruit were harvested 148 days after full bloom and placed in storage ($-1°C$) for 6 weeks. Following storage an assessment of bitter pit incidence was made. For cation analysis, fresh tissues (a composite sample of the distal 1 cm of 3 fruit per replicate with skin) were freeze dried and ground. Each tissue sample (3g) was combusted at 490°C for 6 hr. The ash was disolved in 10 ml of 6 N HCl, and diluted to 50 ml with distilled water. Cations (Ca, Mg, K) were then determined by atomic absorption spectrophotometry.

Experiment 2 - 1988

The experimental design was the same as in the previous year except two levels of a third factor, 1% (w/v) $MgCl_2$ spray/no $MgCl_2$ spray, were added to form a 2 X 2 X 2 factorial.

Results

The initial field trial in 1987 indicated that bagging increased bitter pit, and decreased calcium content and fruit weight (Tables 1 and 2) in both cultivars. The observed range in bitter pit was 51% affected fruit for bagged 'Golden Delicious', to 2% for calcium sprayed 'Delicious' fruit. Bags had a significant effect on the number of fruit per spur (increased in 'Golden Delicious' and decreased in 'Delicious'), and significantly elevated fruit Mg concentrations.

In 1988, bitter pit incidence in both 'Golden Delicious' and 'Delicious' ranged from 3% for fruit sprayed with Ca to 100% in bagged fruit sprayed with Mg (Tables 3 and 4). The main effects of bags and Mg spray were to significantly increase bitter pit in both cultivars. These treatments also interacted to enhance bitter pit. The effect of Ca sprays in both cultivars was to lower bitter pit frequency. Calcium had a large effect when applied to bagged fruit with significant interaction between Ca and bags.

The effect of Mg sprays on bitter pit appeared to be greater than that of Ca sprays (Tables 3 and 4). In 'Golden Delicious' the main effect of Mg sprays had a higher order of significance than Ca sprays, and in both cultivars Ca and Mg sprays combined had a greater incidence of bitter pit than unsprayed fruit. Bags and Ca spray treatments had the greatest effect on fruit Ca concentrations of

Table 1. Effects of $CaCl_2$ sprays and paper bags on fruit bitter pit incidence, cation concentration, weight, and number per spur of 'Golden Delicious' apple trees.

	Bitter pit (%)	Ca	Mg	K	Weight (g)	Fruit per spur
		($mg.kg^{-1}$ dry wt)				
Treatment means:						
No Ca, no bag	4	376	199	7150	136	1.9
Bag	51	234	235	7210	114	2.7
Ca	3	471	180	7020	138	2.8
Bag + Ca	21	289	197	7100	123	3.3
Main effects and interactions:						
Bag	**	**	*	NS	**	*
Ca	*	**	*	*	*	*
Bag x Ca	*	*	NS	NS	*	*

NS, *, ** Nonsignificant or significant at the 5% or 1% levels respectively.

Table 2. Effects of $CaCl_2$ sprays and paper bags on fruit bitter pit incidence, cation concentration, weight, and number per spur of 'Delicious' apple trees.

	Bitter pit (%)	Ca	Mg	K	Weight (g)	Fruit per spur
		($mg.kg^{-1}$ dry wt)				
Treatment means:						
No Ca, no bag	6	336	221	7770	149	1.1
Bag	29	209	256	7590	118	0.8
Ca	2	389	201	7470	150	1.2
Bag + Ca	9	278	220	8070	122	0.8
Main effects and interactions:						
Bag	**	**	*	NS	**	*
Ca	*	**	NS	NS	*	NS
Bag × Ca	*	*	NS	*	NS	NS

NS, *, ** Nonsignificant or significant at the 5% or 1% levels respectively.

Table 3. Effects of $CaCl_2$, $MgCl_2$ sprays and paper bags on fruit bitter pit incidence, cation concentration, weight, and number per spur of 'Golden Delicious' apple trees.

	Bitter pit (%)	Ca ($mg.kg^{-1}$ dry wt)	Mg	K	Weight (g)	Fruit per spur
Treatment means:						
No bag, Ca, or Mg	5	372	214	6950	134	2.3
Bag	42	248	230	7120	108	2.8
Ca	3	455	189	6780	129	3.1
Mg	36	266	257	7220	140	0.7
Bag + Ca	13	309	221	8090	95	4.9
Bag + Mg	100	204	288	7850	109	1.3
Ca + Mg	12	348	209	6310	137	1.9
Bag + Ca + Mg	39	269	245	7630	101	2.4
Main effects and interactions:						
Bag	**	**	NS	*	**	*
Ca	*	**	*	NS	*	*
Mg	**	*	**	*	*	*
Bag × Ca	*	*	*	NS	*	*
Bag × Mg	**	*	**	NS	NS	NS
Ca × Mg	NS	*	NS	NS	NS	NS
Bag × Ca × Mg	*	NS	*	NS	NS	NS

NS, *, ** Nonsignificant or significant at the 5% or 1% levels respectively.

Table 4. Effects of $CaCl_2$, $MgCl_2$ sprays and paper bags on fruit bitter pit incidence, cation concentration, weight, and number per spur of 'Delicious' apple trees.

	Bitter pit (%)	Ca	Mg	K	Weight (g)	Fruit per spur
		($mg.kg^{-1}$ dry wt)				
Treatment means:						
No bag, Ca, or Mg	7	321	232	7640	141	0.9
Bag	32	199	242	8550	113	1.1
Ca	3	441	187	7880	140	0.8
Mg	32	230	271	7960	149	0.9
Bag + Ca	9	312	210	8430	112	1.1
Bag + Mg	100	185	227	8090	119	0.8
Ca + Mg	9	321	213	6980	148	0.9
Bag + Ca + Mg	29	290	255	8810	116	1.0
Main effects and interactions:						
Bag	**	**	NS	*	**	NS
Ca	*	**	NS	NS	**	NS
Mg	*	*	**	NS	*	NS
Bag x Ca	*	*	NS	NS	*	NS
Bag x Mg	**	NS	*	NS	NS	NS
Ca x Mg	NS	*	*	NS	NS	NS
Bag x Ca x Mg	NS	NS	NS	NS	NS	NS

NS, *, ** Nonsignificant or significant at the 5% or 1% levels respectively.

both cultivars. The main effect of the bags was to decrease fruit Ca, while Ca sprays elevated fruit Ca concentrations, and Mg sprays decreased fruit Ca. In both cultivars first order interactions were significant except the interaction between bags and Mg in 'Delicious' (Table 4).

In 1988 bags had no significant effect on fruit Mg concentrations in either cultivar (Table 3 and 4). Ca sprays resulted in decreased Mg in 'Golden Delicious' fruit, while Mg sprays significantly increased fruit Mg levels in both cultivars. In addition, there was an interaction between bags and Mg sprays resulting in an elevation of fruit Mg. Both bags and Mg sprays significantly increased K levels in 'Golden Delicious' apples (Table 3), In 'Delicious', bags increased K levels, while the main effects of other treatments were not significant (Table 4).

Bagging fruit reduced fruit weight in both cultivars (Tables 3 and 4). The same effect was observed for Ca sprays, and the two treatments interacted resulting in the greatest reduction in fruit weight. The main effect of Mg sprays was significant and resulted in an increase in fruit weight in both cultivars (Table 3 and 4). The number of fruit per spur was influenced by treatments in 'Golden Delicious' (Table 3), while treatments had no effect on fruit number in 'Delicious' (Table 4). Bags and Ca sprays both increased the number of fruit retained per spur on 'Golden Delicious'; Mg sprays reduced the number of fruit per spur (Table 3).

Discussion

The observation in 1987 that fruit with a high incidence of bitter pit were high in magnesium, agreed well with previous records (Hopfinger and Poovaiah, 1979; Hopfinger et al., 1984; Lewis et al., 1977). This led to the modification of field treatments in 1988 to include magnesium chloride in the spray treatments. The primary objective of this study, to induce an array of bitter pit symptoms in treated fruit, was most successfully achieved in the second year of treatment.

In both cultivars studied, covering the fruit with paper bags during fruit development caused an increase in bitter pit in both years. The most likely effect that bags had on fruit physiology, was to reduce the total amount of water transpired over the season. As calcium is largely carried to fruit in the transpirational stream (Shear and Faust, 1970) bagging would have reduced total calcium accumulation in fruit over the season. Results clearly showed reduced calcium at harvest in fruit which were covered.

Magnesium accumulation in the fruit showed an opposite trend, with bagged treatments having higher Mg levels than unbagged fruit. This may be the result of differential phloem/xylem transport leading to greater magnesium accumulation, or a direct cation antagonism effect of low Ca levels allowing increased Mg uptake to balance the ionic charge of the tissue. However, this is speculation

as statistical treatment of results did not show a clear effect of bagging on Mg accumulation.

Calcium sprays increased fruit calcium and reduced bitter pit as has previously been described (Johnson et al., 1987). Calcium sprays appear to be of much more practical importance when fruit are predisposed to bitter pit than when not. This is well illustrated by comparing the base level of orchard bitter pit in this study (no spray/no bag) with highly inductive treatments (Mg spray/bag). In both cases, including Ca sprays reduces bitter pit by about one half. If a large percentage of the crop has potential to develop bitter pit, reducing pitting by 50% with Ca sprays would be economically important.

In agreement with previous observations that postharvest magnesium dips induce bitter pit symptoms (Hopfinger and Poovaiah, 1979) and that bitter pit development is associated with high fruit Mg (Perring, 1986); magnesium sprays increased fruit magnesium, reduced fruit calcium and increased bitter pit.

The effect of field treatments on fruit weight was complex. Bags generally resulted in reduced fruit size, probably by reducing leaf and fruit photosynthesis on the enclosed spurs. Calcium sprays increased while magnesium sprays decreased fruit number retained on spurs in 'Golden Delicious'. This effect was not observed in 'Delicious'. It is possible that in 'Golden Delicious,' Ca inhibited fruit abscision while Mg enhanced abscision, perhaps by direct modification of respiration rate (Turner and Turner, 1980).

The selection of field treatments, and their subsequent interaction, provided an excellent range of bitter pit percentages in fruit of both cultivars. Using this set of treatments, the relative contributions of Ca, Mg and fruit bagging on bitter pit development were determined. Knowing with some assurance that the fruit will develop bitter pit, this type of trial could be used to study developmental changes associated with bitter pit initiation and manifestation.

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CHAPTER 4

CHARACTERIZATION OF APPLE BITTER PIT SYMPTOMS

Additional index words: ***Malus domestica***, physiological disorders, cations, tannins, electron microscopy, x-ray microanalysis

Abstract. **The cellular morphology of pitted apple (*Malus domestica* Borkh.) fruit from 'Golden Delicious' and 'Delicious' trees were examined using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The overall tissue morphology of both cultivars was similar. TEM revealed differences in the localization and conformation of tannins in pitted versus healthy tissue, and small differences in cell wall morphology. In SEM, the cell walls of pitted cells were irregular and appeared to be collapsed compared to cell walls in healthy tissue. SEM also revealed an accumulation of granular starch bodies in pit zones. An examination of cation levels using X-ray microanalysis revealed elevated Mg levels localized in pit cells, while K levels were similar in both healthy and pitted cells. Ca levels in both tissue types were too low to be detected by this method.**

Bitter pit is a physiological disorder of apples that may occur on the tree or develop during or after cold storage and is characterized by brown necrotic depressions in the fruit surface and flesh (Cobb, 1895). Despite more than a century of concerted research, bitter pit remains a serious problem in world apple production (Bunemann, 1972; Bunemann et al., 1979). It is known that the disorder is not the result of pathogen infection, but is inherent in some cultivars and it is physiological in nature (Wilkinson and Fidler, 1973).

Most studies have been aimed at the nutritional status of the tree and crop (Bunemann et al., 1979; Garman and Mathis, 1956; Perring, 1986; Wilkinson and Fidler, 1973) and have implicated Ca as playing a key role in the development of bitter pit (Ferguson et al., 1979; Hopfinger and Poovaiah, 1979, 1984; Meyer et al., 1979; Perring, 1968, 1985, 1986; Perring and Sharples, 1975). Apples which exhibit symptoms of the disorder are generally lower in Ca than sound apples (Ferguson et al., 1979; Holland, 1980; Perring and Sharples, 1975). Increasing fruit Ca concentrations with fertilizers, sprays and dips has achieved only moderate success in reducing bitter pit (Johnson, 1979; Wilkinson and Fidler, 1973). After reviewing several hundred publications on bitter pit, calcium nutrition, and related subjects Bunemann et al. (1979) concluded that a basic explanation of the causes of bitter pit is lacking.

Bitter pit has been described previously on a microscopic level, nevertheless little is understood about the cellular and sub- cellular nature of the disorder (Bunemann, 1972; Bunemann et al., 1979; Chamel and Bossy, 1981; Hopfinger

and Poovaiah, 1979; MacArthur, 1940; Perring, 1986; Smock and Van Doren, 1937; Simons, 1962, 1968). Evidence that Ca is involved in the development of the disorder has resulted mainly from applications and measurements of nutrient levels in whole fruit or fruit sections (Ferguson et al., 1979; Kahn, 1977; Perring and Sharples, 1975), as well as microscopic localization of nutrients (Chamel and Bossy, 1981; Hopfinger and Poovaiah, 1979). The objective of this study was to describe bitter pit symptoms on a microscopic level using a multifaceted approach, namely transmission electron microscopy, scanning electron microscopy (critical point dried and freeze dried tissues) and X-ray microanalysis, in the hope that this would provide a more complete view of the histology of bitter pit.

Materials and Methods

Plant material

Apple fruit from 8-year-old 'Sundale Spur Golden Delicious' and 'Triple Red Delicious' (***Malus domestica*** Borkh.) trees were harvested from the Horticulture Farm, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Fruit was harvested at commercial maturity and placed in cold storage (-1°C) for 12 weeks.

Tissue preparation

In 1987, selected apple fruits with bitter pit symptoms were sectioned with a blade into 1 mm thick slices (tangential section taken from within 2 mm of the fruit surface). For transmission electron microscopy (TEM), tissue samples were immediately placed in a primary fixative of 3% (v/v) glutaraldehyde and 0.1 M Na cacodylate buffer (pH 7.3) for 3 hrs. Samples were then washed in 0.2 M phosphate buffer and post-fixed in 0.1% (w/v) osmium tetroxide. The tissue was dehydrated through an ethanol/acetone series, embedded in Spurr's plastic and sectioned with a glass knife. Sections were placed on copper grids and stained with uranyl acetate followed by lead citrate then examined in a Zeiss 10C transmission electron microscope (Carl Zeiss, Oberkochen, West Germany), set at 60 kV (Echeverria, 1987).

For scanning electron microscopy (SEM), sectioned tissues were rapidly frozen to −80°C followed by freeze drying (freeze drier; Virtis Inc., Gardiner, New York, USA) for 48 hrs. The samples were then fractured across selected planes and mounted on metal stubs. The sample surfaces were sputter-coated (sputter coater; Hummer X, Alexandria, Virginia, USA) to 20 nm with gold/paladium and examined with a Philips 505 SEM (Phillips, Eindhoven, Holland) set at 20 kV (Lee et al., 1988). For measurement of cation levels, samples were mounted on carbon stubs, carbon coated under vacuum (Kinney Vacuum Co., Boston, Mass. USA) and examined with a Phillips 505 SEM using energy dispersive X-ray analysis (EDAX 9100 system; EDAX Int. Inc., Prarie View, Illinois, USA) at a tilt angle of 30° and at 15 kV.

To study differences between freeze drying and conventional critical point drying, selected samples were fixed in 3% (v/v) glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3) and then dehydrated in an ethanol series. The tissue was then critical point dried (critical point drier; Ladd Research Industries, Burlington, Vermont, USA) through liquid CO_2 , mounted on stubs, sputter coated with gold/palladium, and examined under the same SEM conditions described above.

Results

Results obtained for 'Golden Delicious' and 'Delicious' were similar. Transmission electron micrographs of healthy cells showed highly vacuolated cells with chloroplasts, starch grains, a granular cytoplasm, and regular wall structure (Fig. 1). The vacuoles contained a large amount of structured tannin (identified by affinity for osmium stain, propensity and localization) oppressed to the tonoplast in a continuous coating and aligned in stacks perpendicular to the membrane. In the cells on the periphery of the pit (Fig. 2), vacuolation appeared to have progressed to a maximum, with only a very thin layer of cytoplasm visible and no organelles present. These cells (Fig. 2) were still high in tannin, but the tannin orientation appeared to be parallel to the tonoplast and the cell wall. In addition, the walls of these cells had evidence of slight distortion. In the pit zone (Fig. 3), the cells had become completely vacuolated and appeared to be void of any protoplasmic constituents except for residual tannins. Tannins appeared as globular bodies lacking visible internal structure oppressed to the cell wall. The cell walls had some evidence of distortion particularly at the middle lamella (Fig. 3).

No large granular bodies were observed in the TEM study, as were observed in the SEM micrographs. Considerable difficulty was experienced in sectioning samples of pitted tissues with glass blades, and most thin sections disintegrated while being cut. This may have been caused by heavy starch accumulation

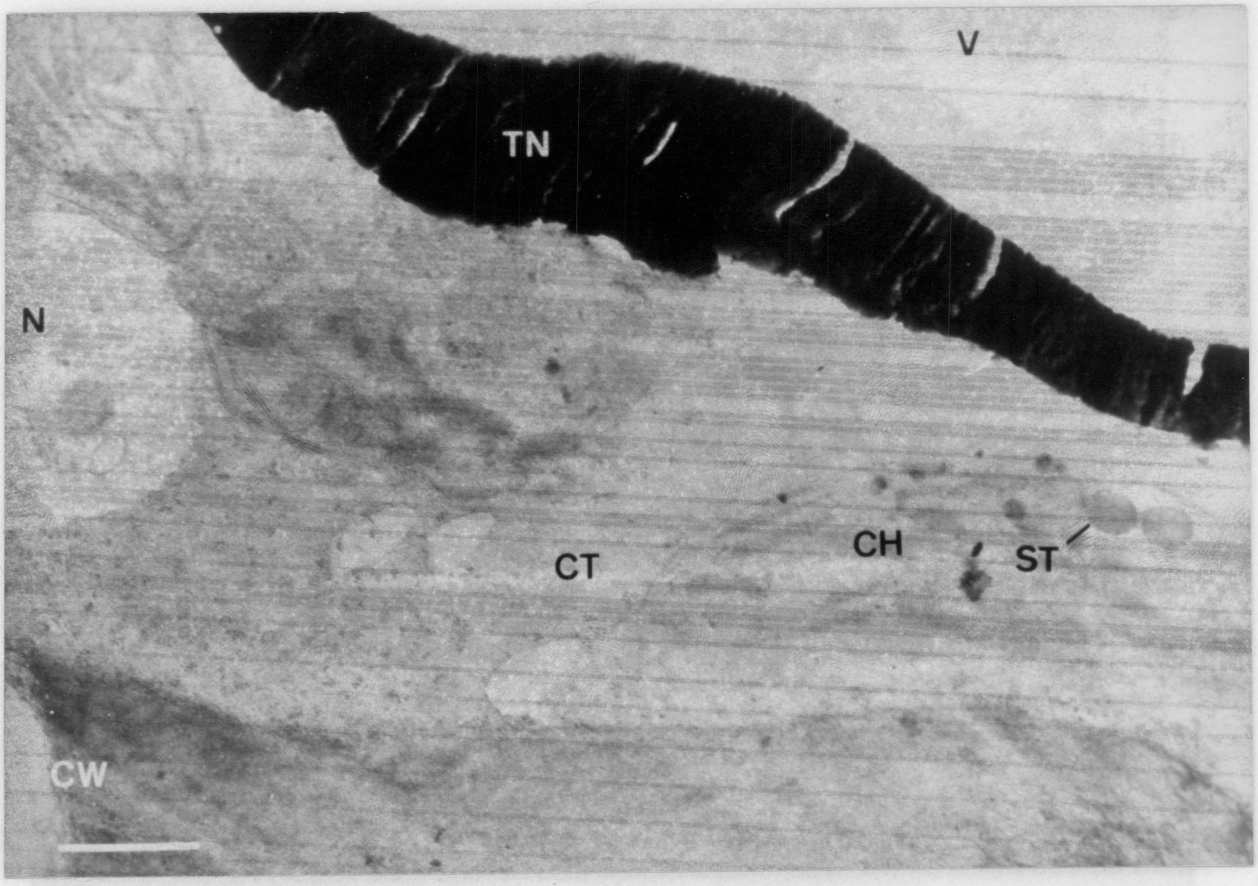

Fig. 1. Detailed section of a healthy 'Delicious' apple cell from a fruit with bitter pit symptoms. Structured tannin (TN), the vacuole (V), cytoplasm (CT), chloroplasts (CH), starch grains (ST), nucleus (N) and the cell wall (CW) are visible; bar = 1 μm.

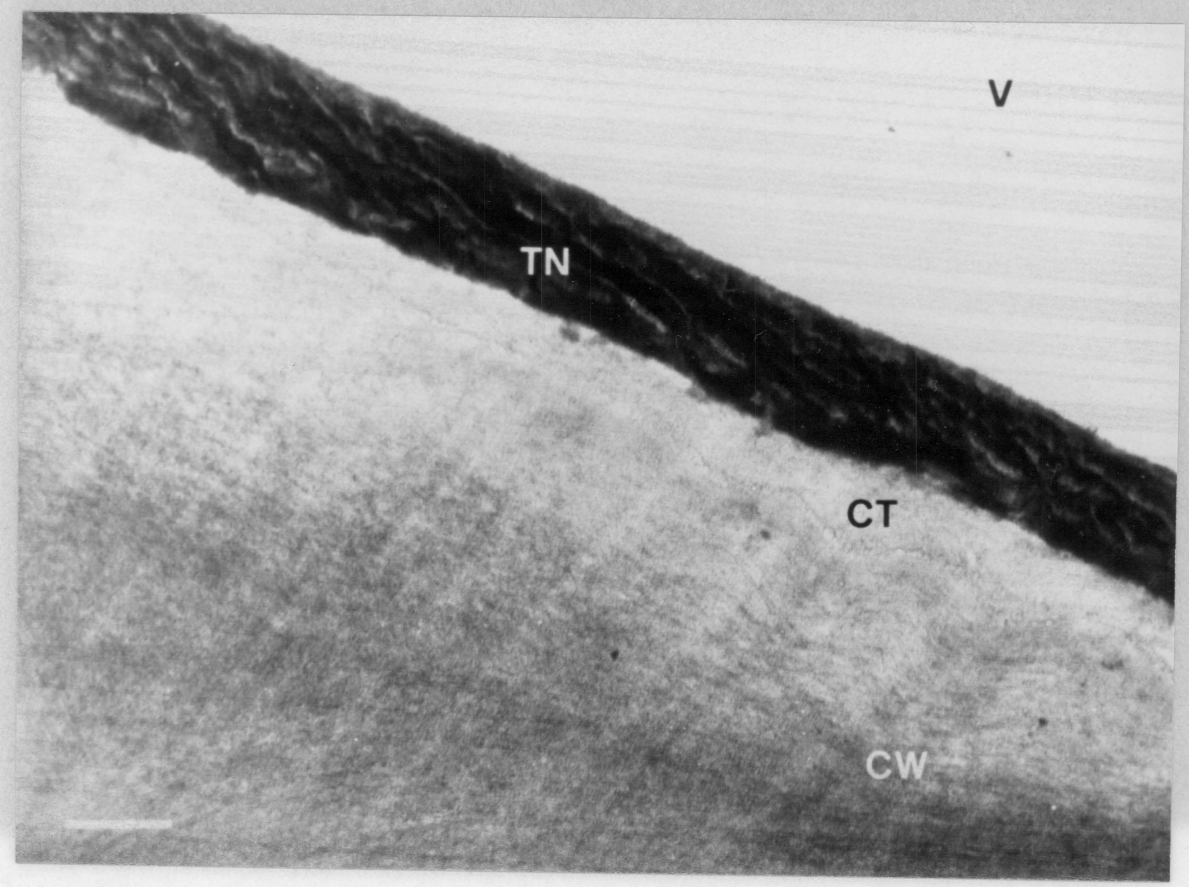

Fig. 2. A detailed view of the tannin (TN) conformation in the vacuole (V) of a cell close to bitter pit tissue in 'Delicious' fruit. Note the tightly oppressed cytoplasm (CT) and wave-like pattern in the cell wall (CW) matrix; bar = 0.5 μm.

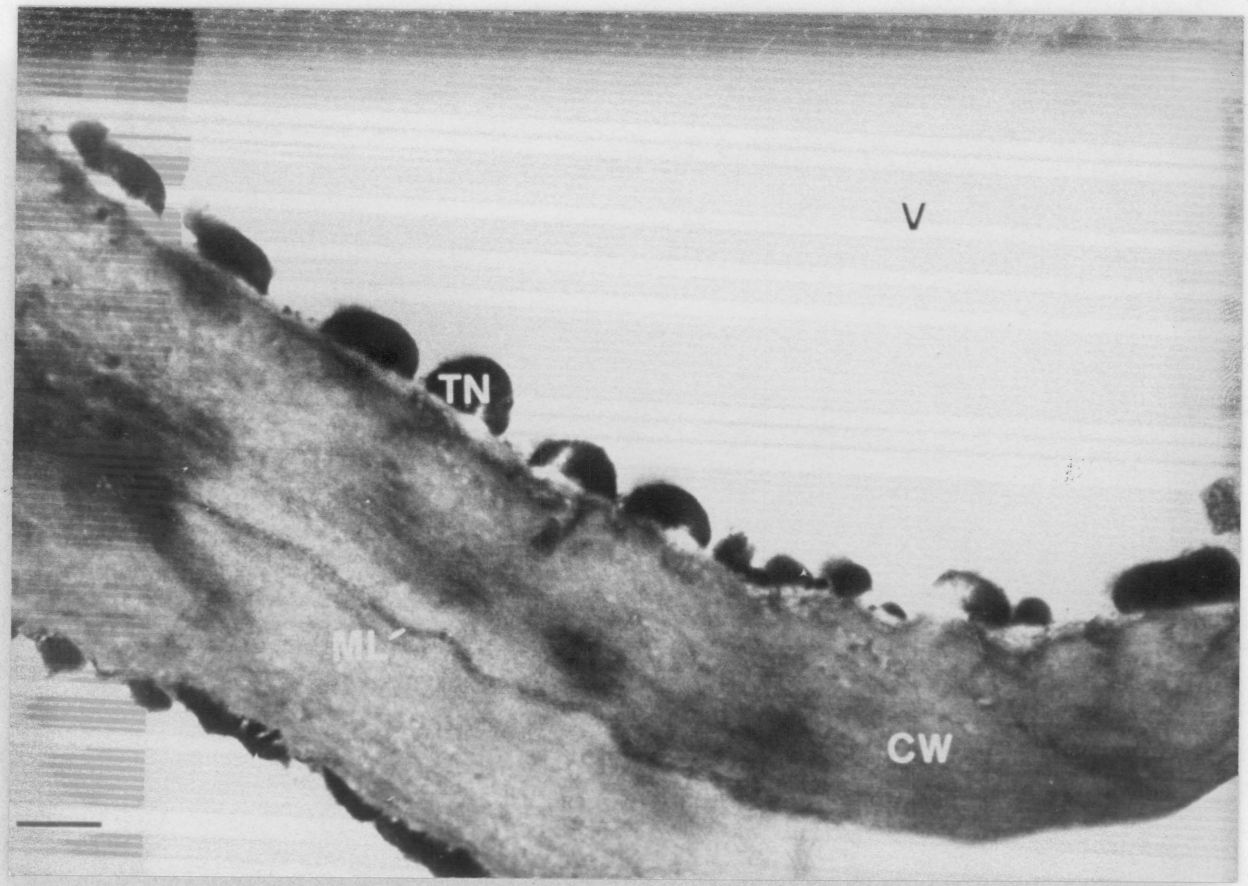

Fig. 3. A tissue section in the pit zone of a 'Delicious' apple fruit. Tannins (TN) have a globular structure, and are adsorbed on the cell wall. Note the distortions of the cell wall (CW) at the middle lamella (ML) and the high degree of vacuolation (V); bar = 0.5 μm.

in these tissues (MacArthur, 1940; Simons, 1962, 1968; Smock, 1934; Smock and Van Doren, 1937). Sections which remained intact were probably made through planes of the cell which missed cutting through starch deposits. This could account for the discrepancy observed when comparing TEM and SEM micrographs.

In scanning electron micrographs, healthy cells appeared to have rigid geometric walls (Fig. 4) and to lack other visible constituents. The cells immediately adjacent to a pit had walls which appeared to have normal structural integrity but had granular bodies adhering to the inner surface (Fig. 5). These were identified as starch by comparison with other micrographs and reports (Hopfinger and Poovaiah, 1979). In the pit area cell walls appeared to have collapsed and there was a large amount of open space in the pit area (Fig. 6), while the intercellular spaces were filled with large starch bodies. Both freeze drying and critical point drying resulted in similar tissue morphology under SEM conditions (comparative data not presented).

A study of the cationic composition of the cells in each tissue zone using energy dispersive X-ray analysis revealed high potassium levels in healthy and in pitted tissues (Fig. 7, 8). In contrast, levels of Mg were too low to be detected in healthy tissues (Fig. 7) but were high in the pit zone (Fig. 8) as has been observed previously (Hopfinger and Poovaiah, 1979). Calcium concentrations in both tissues were too low to detect by X-ray analysis.

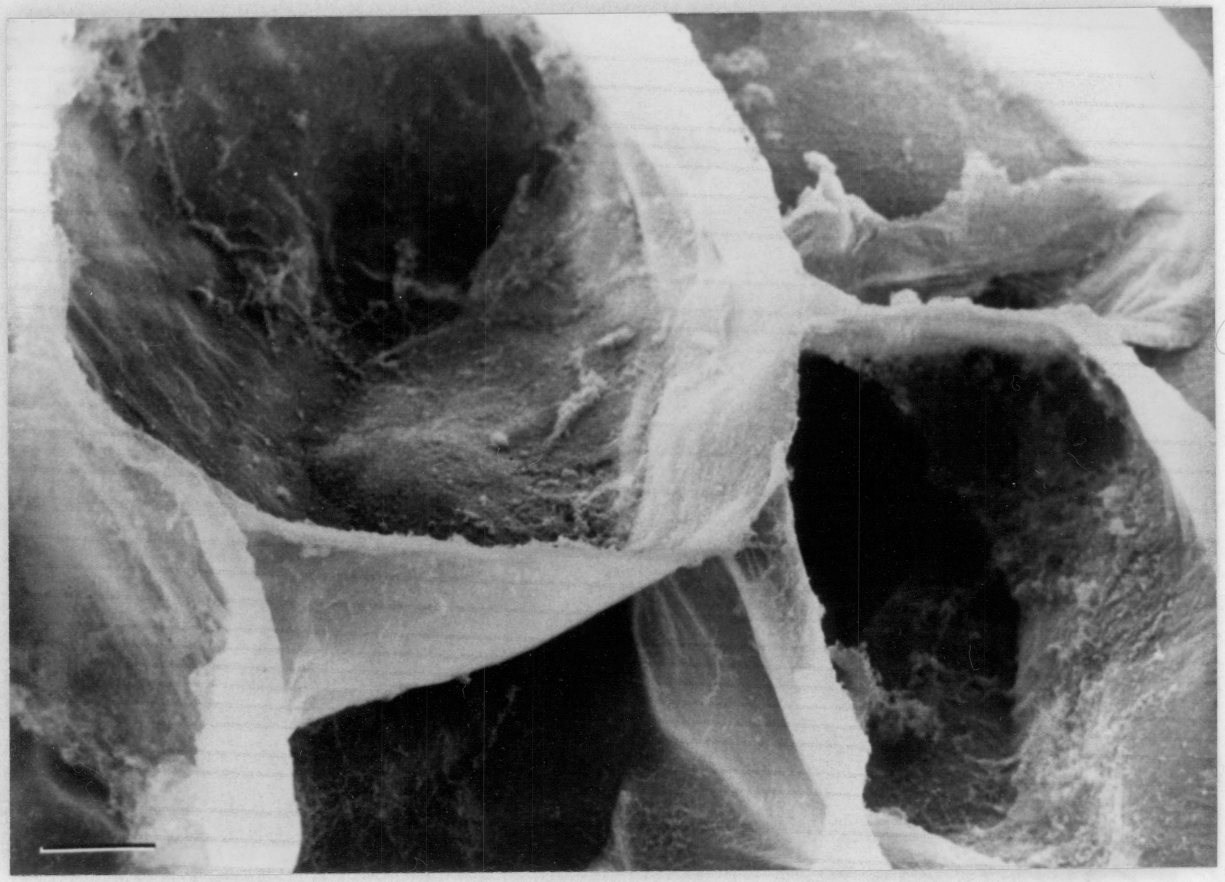

Fig. 4. Healthy 'Golden Delicious' apple cells. Cell walls have a regular helical shape. Remnants of cell debris can be seen, most cell contents were lost during preparation; bar = 10 μm.

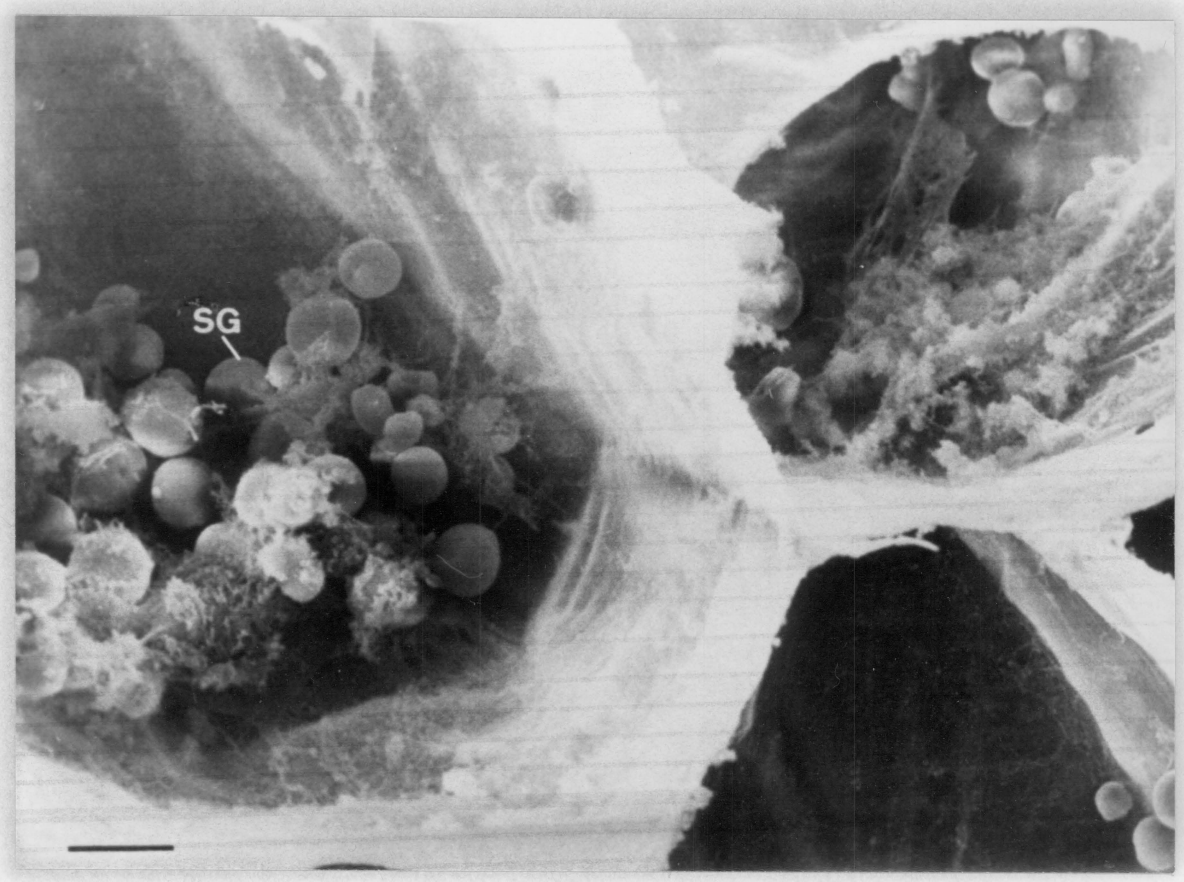

Fig. 5. Cells on the edge of bitter pit tissue in 'Delicious' apple cells. Cell walls still have a regular structure but have residual starch grains (SG) attached to the inner surface; bar = 10 μm.

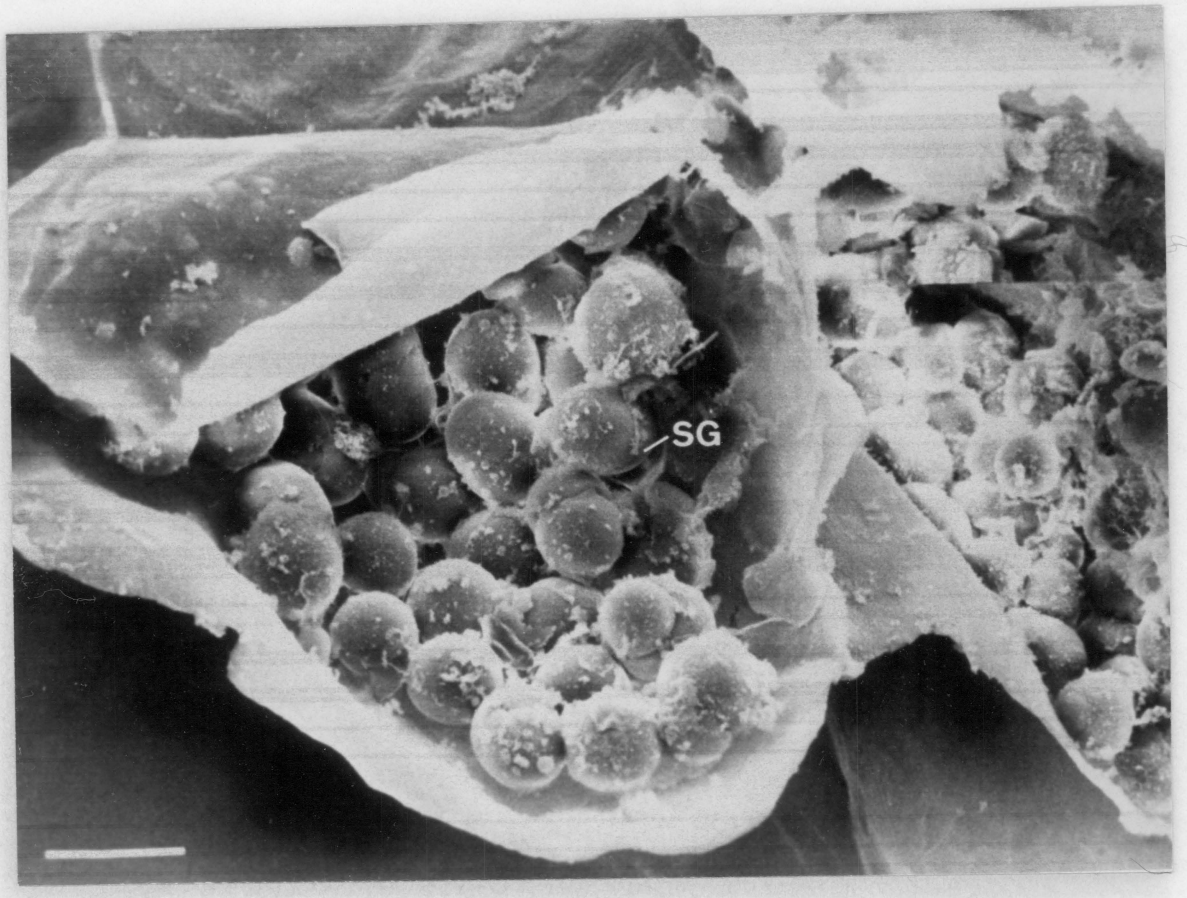

Fig. 6. Pit cells in 'Golden Delicious' showing collapsed cell walls and many spherical starch grains (SG); bar = 10 μm.

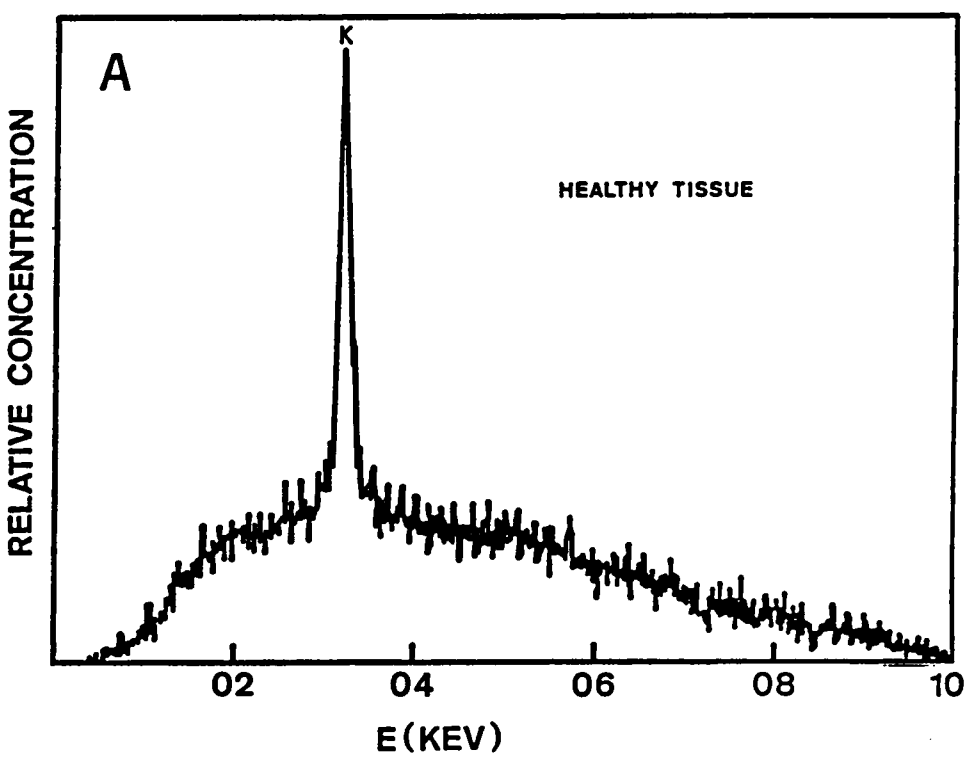

Fig. 7. X-ray micro-analysis of cation emissions by healthy cells close to a pit in 'Golden Delicious' using the EDAX 9100 system. Potassium (K) was detected while calcium and magnesium were not.

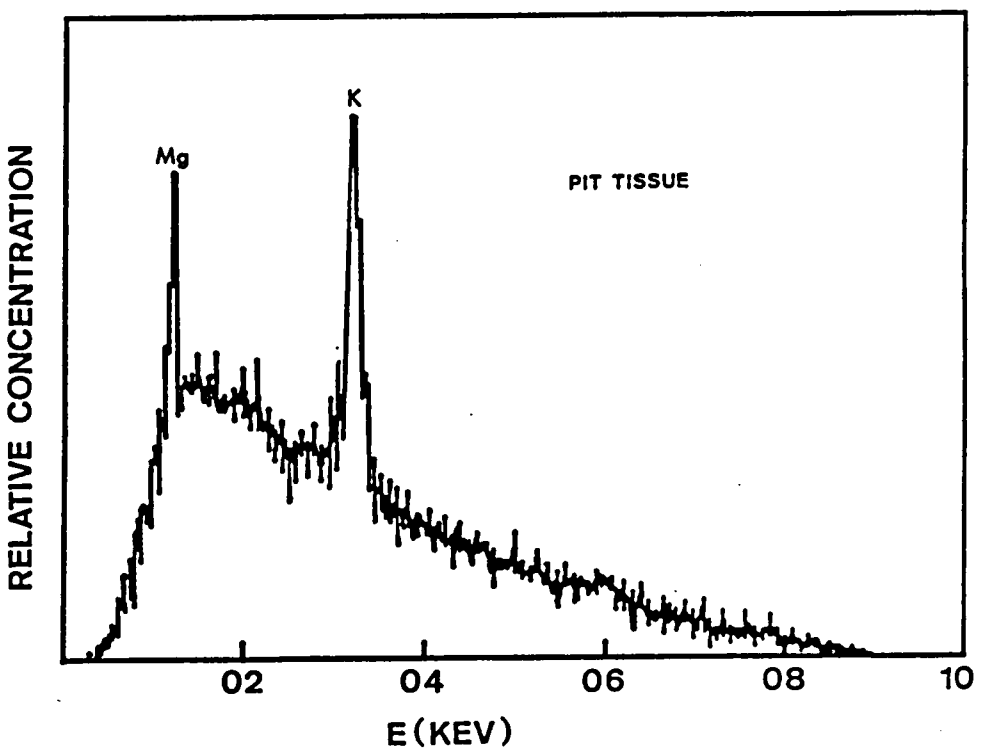

Fig. 8. X-ray micro-analysis of cation emissions by the cells of a single pit in 'Golden Delicious'. Potassium (K) was detected along with magnesium (Mg).

Discussion

The biosynthesis and physiological role of tannins in apples remains unclear, even though they are present in high concentrations (Harborne, 1980; Van Buren et al., 1966; Walker, 1962; Williams, 1960; Zocca and Ryugo, 1975). In the past, authors have circumvented the presence of tannins in plant cells by dismissing them as toxic end products of normal metabolic processes, or deterrents to foraging organisms (Harborne, 1980). However, there seems to be no ecological reason for mature apples to put maximum exposure into a foraging deterrent when the species puts so much energy into the fruit as an attractant for seed dispersal. Oxidation of these compounds is known to give fruit tissues their characteristic brown color (Kahn, 1977; Steenkamp et al., 1983; Walker, 1962). One of the most striking differences evident in healthy versus pitted tissues was the difference in tannin localization and structure. It is likely that the development of brown color in pitted tissues is the result of the exposure of tannins in these cells to polyphenol oxidase sometime during pit development (Kahn, 1977; Walker, 1962).

The TEM micrographs showed that in healthy apple tissues the tannins are localized in the cell vacuoles and have a highly organized structure. In contrast, tannins in pitted cells are not localized in the vacuole and have a globular structure. In addition, the cytoplasm in the pitted cells was missing and only residual

tannins remained, which may have given pitted tissues their characteristic dark brown color.

The authors acknowledge that the tissues studied were at an advanced stage of bitter pit, and that subcellular symptoms observed may be concomitant with the disorder rather than causal. Theories proposed for progressive bitter pit development were presented with this acknowledgement in mind. The micrographs (Fig. 1, 2, 3) suggest a physical hypothesis for the development of bitter pit symptoms in apple fruit tissues based on their high tannin content and on osmolarity considerations. In healthy tissues the geometric arrangement of tannins against the tonoplast gives them maximum surface area (Fig. 1, 2) for direct contact with any incoming or outgoing substances moving across the tonoplast. It is unlikely that this organized vacuolation and orientation of tannins in healthy tissue is simply a partitioning process to protect normal cell function. In apple cells this arrangement appears to provide all of the requirements of a charge buffering system of large capacity. Tannins are present in very high concentrations relative to other cellular constituents (Fig. 1), they are ideally localized to provide maximum absorption of substances entering or exiting the vacuole (Fig. 1), and they are known to have a high affinity for charged species (Zocca and Ryugo, 1975).

Apple cells are high in osmotically active compounds particularly sugars and ions (Quinlan, 1969; Steenkamp et al., 1983). Thus a large amount of energy may be required to maintain vacuole size, turgor pressure, and physical integrity of the

cells. Theoretically, tannin could minimize the large energy inputs required to avoid the development of radical osmotic potentials. If, however, localized regions of healthy apple tissue received an abnormal influx of charged ions the charge buffering capacity of the tannin molecules at the tonoplast could be saturated. Increased cell vacuolation would result as water entered the cell in response to a low water potential. Eventual loss of control of osmoregulation would result in extreme vacuolation, with the tonoplast and associated tannins closely oppressed to the cytoplasm and cell wall (Fig. 2). It is speculated that the change from normal tannin orientation and cell wall structure in these cells may be the result of charge saturation and physical pressure exerted within the cells. Eventual lysis and death of the cells would lead to cell collapse as the direction of wall pressure reversed. Only the remains of the stable tannin would be evident adhering to the cell walls.

Some authors have noted an accumulation of starch in pit cells (Hopfinger and Poovaiah, 1979; MacArthur, 1940; Simons, 1962, 1968; Smock, 1934; Smock and Van Doren, 1937), as was observed in SEM micrographs of pit cells (Fig. 6). This means that starch hydrolysis did not occur as it normally would during fruit maturation, and cell death preceded normal maturation processes. It was postulated very early (Carne et al., 1929) that pit cells lag behind healthy cells during maturation processes and as a result, a sharp differential in water potential is formed between pit cells, which are high in starch, and healthy cells, which are high in sugar. This would cause rapid dehydration of pit cells as water moved to healthy cells surrounding the pit, eventually resulting in the death of the de-

hydrated cells and the formation of a 'pit'. This sequence of events may account for the unusual vacuolation observed in cell surrounding pits (Fig. 2). Note that this sequence of events implies the opposite direction of water flow suggested earlier, namely dehydration of pit cells leading to death rather than excessive hydration from a loss of osmotic control leading to lysis. However, it has been reported that bitter pit symptoms may be present before starch hydrolysis occurs in healthy cells (MacArthur, 1940; Smock, 1934), and that starch persistence is a result of bitter pit rather than causal.

It is generally accepted that low Ca levels are associated with pitted fruit (Wilkinson and Fidler, 1973) and many authors have suggested that the maintenance of a cation balance is important in preventing the disorder (Ferguson et al., 1979; Perring, 1986; Wilkinson and Fidler, 1973). The balance between Ca and Mg is proposed to have a major influence on bitter pit (Hopfinger and Poovaiah, 1979). If these concepts are accepted, then the high Mg levels measured (Fig. 8) may have upset the cation balance in the pit areas and negated any preventative effect of Ca on pit development. This may have been aggravated by the already very low level of Ca encountered in the fruit (Fig. 7, 8). A more complex role of cation ratios may be in the activation or inhibition of enzymes which play a role in the development of pitting in fruit. Pyruvate kinase activity has been proposed as a marker for the potential of fruits to develop some Ca related disorders (Lavon et al., 1988). It has been established that Mg and K are required for pyruvate kinase activation, while Ca counteracts this activation (Duggleby and Dennis, 1973; Miller and Evans, 1957). It is therefore possible

that the high levels of Mg and K measured in the pit cells, together with low Ca concentrations, resulted in abnormally high activity of the enzyme. Subsequently, localized tissue decline may have resulted from the depletion of stored sugars by respiration. In healthy tissues the low level of Mg may have limited pyruvate kinase activity and therefore the tissue remained intact.

While the basic causes of bitter pit are unknown, it is evident that there are some dramatic differences between healthy and pitted apple tissues. The possible subcellular involvement of enzymes, cations, starch and tannins warrants further research.

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CHAPTER 5

CORRELATION OF PYRUVATE KINASE ACTIVITY WITH DEVELOPMENT OF BITTER PIT IN APPLE

Additional index words. ***Malus domestica***, postharvest, disorders, enzyme activity, cations

Abstract. **The relationship between pyruvate kinase activity, fruit cation concentration (Ca, Mg and K) and bitter pit in apple (*Malus domestica* Borkh.) was investigated. Beginning 5 days after full bloom, fruit were sprayed with 1% (w/v) $CaCl_2$ or 1% (w/v) $MgCl_2$ (every 10 days) and covered with paper bags in a factorial experiment designed to induce a range of poststorage bitter pit symptoms. Pyruvate kinase activity during the early stages of fruit growth was higher in fruit from treatments which developed 100% bitter pit after storage, than in fruit from treatments that developed 3% bitter pit. Fruit with a high bitter pit incidence had a lower Ca: Mg + K ratio than fruit with a low level of the disorder. There was a strong positive correlation between enzyme activity early in the season and bitter pit incidence after storage. Correlation coefficients (r) were highest in June ($r = 0.96$), declined steadily until harvest ($r = 0.67$), and were lowest after storage ($r = 0.12$).**

An assay for pyruvate kinase may be valuable for early prediction of postharvest bitter pit development.

The exact cause of bitter pit, a physiological disorder in apple, has not been fully established, but it is likely the result of a change in normal metabolic processes (Faust and Shear, 1968; Perring, 1986). Many workers have indicated that low levels of calcium, and high levels of potassium and magnesium in the fruit are important factors in bitter pit development (Perring, 1968, 1986). The same cations are also important in regulating pyruvate kinase (pyruvate-ATP phosphotransferase, EC 2.7.1.40), a key enzyme in glycolysis (Tomlinson and Turner, 1973).

Pyruvate kinase has been characterized in a number of plant tissues (Miller and Evans, 1957; Duggleby and Dennis, 1973a, 1973b; Tomlinson and Turner, 1973). Miller and Evans (1957) established that pyruvate kinase from higher plants requires K^+ or NH_4^+ for activity. Duggelby and Dennis (1973a, 1973b) and Tomlinson and Turner (1973) showed that in pea and carrot tissue, the enzyme had a requirement for K^+ and Mg^{2+} for activation, while Ca^{2+} strongly inhibited enzyme activity. Meli and Bygrave (1972) showed that rat liver mitochondria can determine the rate of pyruvate kinase by altering the ratio of Mg to Ca *in vivo*.

There is growing interest in an assay for pyruvate kinase as an indicator of physiological disorders in fruit trees due to imbalances of K, Ca and Mg. Bar-Akiva et al. (1976) showed that Mg^{2+} and K^+ deficiencies decreased pyruvate kinase activity in lemon leaves, whereas Ca^{2+} deficiency caused an increase in enzyme activity. Lavon et al. (1988a, 1988b) conducted a detailed study of the effect of K^+, Mg^{2+} , and Ca^{2+} on pyruvate kinase activity in citrus. Their results

clearly demonstrated the inhibitory effect of Ca^{2+} on pyruvate kinase activity and confirmed Bar-Akiva's suggestion that the enzyme possessed promise as an indicator of Ca deficiency.

Preliminary data indicated that amending cation levels in developing fruit produced a wide range of bitter pit symptoms. Pyruvate kinase activity early in the season was closely correlated with bitter pit after storage. It is proposed that during the early stages of fruit development, an assay for pyruvate kinase, which is sensitive to tissue cation levels, can be used to predict bitter pit in storage.

Materials and Methods

Plant Material

Apple fruit from 15-year-old 'Smoothie Golden Delicious' (***Malus domestica*** Borkh.) trees grown at the Horticulture Farm, Virginia Polytechnic Institute and State University, Blacksburg, were used in this study.

Field Treatments

In 1987, the field experiment was a 2 X 2 X 2 factorial with 16 replications of 5 fruiting spurs each in a randomized complete blocks design. Treatments were 1% (w/v) $CaCl_2$ spray, no $CaCl_2$ spray; 1% (w/v) $MgCl_2$ spray, no $MgCl_2$ spray; and bags, or no bags. Sprays were applied every 10 days with a hand held sprayer beginning 5 days after full bloom. Bags were Kraft 3 lb brown paper and were carefully stapled over spurs without damage to vegetative or floral structures. Bags were removed and replaced at each spray date, and removed completely 4 weeks before harvest. Fruit were harvested 148 days after full bloom and placed in storage (-1°C) for 6 weeks. Following storage, an assesment of bitter pit was made and results expressed as percent pitted fruit. For cation analysis, fresh tissues (a composite sample of the distal 1 cm of 3 fruit per replicate with skin) were freeze dried and ground. Each tissue sample (3g) was combusted at 490° C for 6 hr. The ash was disolved in 10 ml of 6 N HCl, and diluted

to 50 ml with distilled water. Cations (Ca, Mg, K) were then determined by atomic asorption spectrophotometry.

Enzyme Assay

Fruit were sampled every 2 weeks starting 5 days after full bloom. Fruit were washed with distilled water, and 5g of fresh fruit tissue (the outermost 2mm of equatorial peel) were fractionated in 5 ml extraction buffer, 0.5 g insoluble polyvinylpolypyrrolidone (PVPP), and 1.5 g white quartz sand, in a chilled mortar and pestle. The extraction buffer contained 50 mM imidazole-HCl (pH 7.0), 1 mM EDTA, 3 mM dithiothreitol, 5 mM ascorbic acid, 3 mM $Na_2S_2O_5$, 14 mM 2-mercaptoethanol, and 20% (v/v) glycerol (modified from Mendez et al. (1986)). The macerated tissue was filtered through cheese cloth and centrifuged at 27,000 x g for 20 min and the supernatant was used as the crude extract for the enzyme assay.

The enzyme assay was based on the procedure described by Lavon et al. (1988b) for citrus tissue. A 0.1 ml fraction of the crude extract was placed in a test tube containing 20 mM imidazole buffer (pH 7.0) with 0.25 mM Na_2MoO_4 (to inhibit phosphatase activity), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate (PEP), and 2 mM ADP in a total volume of 1.0 ml. The mixture was incubated for 15 min at 37°C in a water bath, then stopped with 1.0 ml 0.0125% (v/v) 2,4 dinitrophenylhydrazine in 2N HCl, and further incubated for 15 min at 37°C. After adding 2.0 ml 2 N NaOH, the mixture was centrifuged for 5 min at 12,500 x g and the absorbance measured at 510 nm using a

Shimadzu spectrophotometer. Blanks were run without ADP or without PEP to correct for phosphatase activity and background activity, respectively. The activity of pyruvate kinase was expressed as nmol pyruvate formed per mg dry wt per min.

Results

Pyruvate kinase activity was generally higher during the early stages of fruit growth, declined as the fruit increased in size, and then increased slightly during storage (Fig. 1). Bagged fruits had significantly higher pyruvate kinase activity throughout the sampling season (Fig. 2). Ca spray generally decreased pyruvate kinase activity, while Mg spray increased enzyme activity.

A combination of both Ca and Mg sprays resulted in slightly higher enzyme activity than in unsprayed fruit (Table 1). Overall the highest activity for pyruvate kinase over the season was obtained from fruit treated with Mg and covered with paper bags. The lowest overall seasonal enzyme activity was found in fruit sprayed with Ca alone.

At six weeks after full bloom the main effect of bags on fruit was increased pyruvate kinase activity, decreased Ca: Mg + K, and increased percent bitter pit (Table 1). Calcium sprays decreased enzyme activity, increased Ca: Mg + K, and decreased percent bitter pit. The main effect of magnesium spray was to increase pyruvate kinase activity, decrease Ca: Mg + K and increase percent bitter pit. The most significant treatment interactions were bags and Mg spray treatments which resulted in an enhancement of pyruvate kinase activity, decreased Ca: Mg + K and increased bitter pit percentage compared to either treatment alone. Significant interactions were also found between bags and Ca

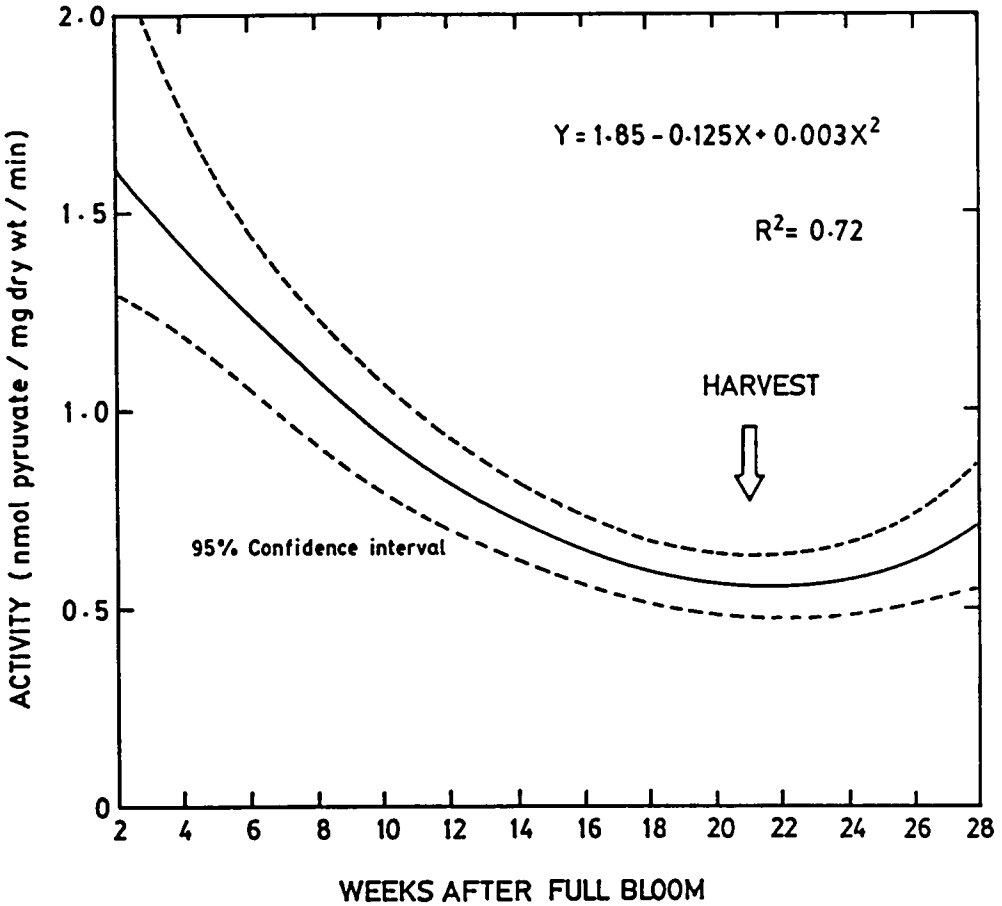

Fig. 1. Pyruvate kinase activity of 'Golden Delicious' fruit during 1988. Regression analysis of all samples.

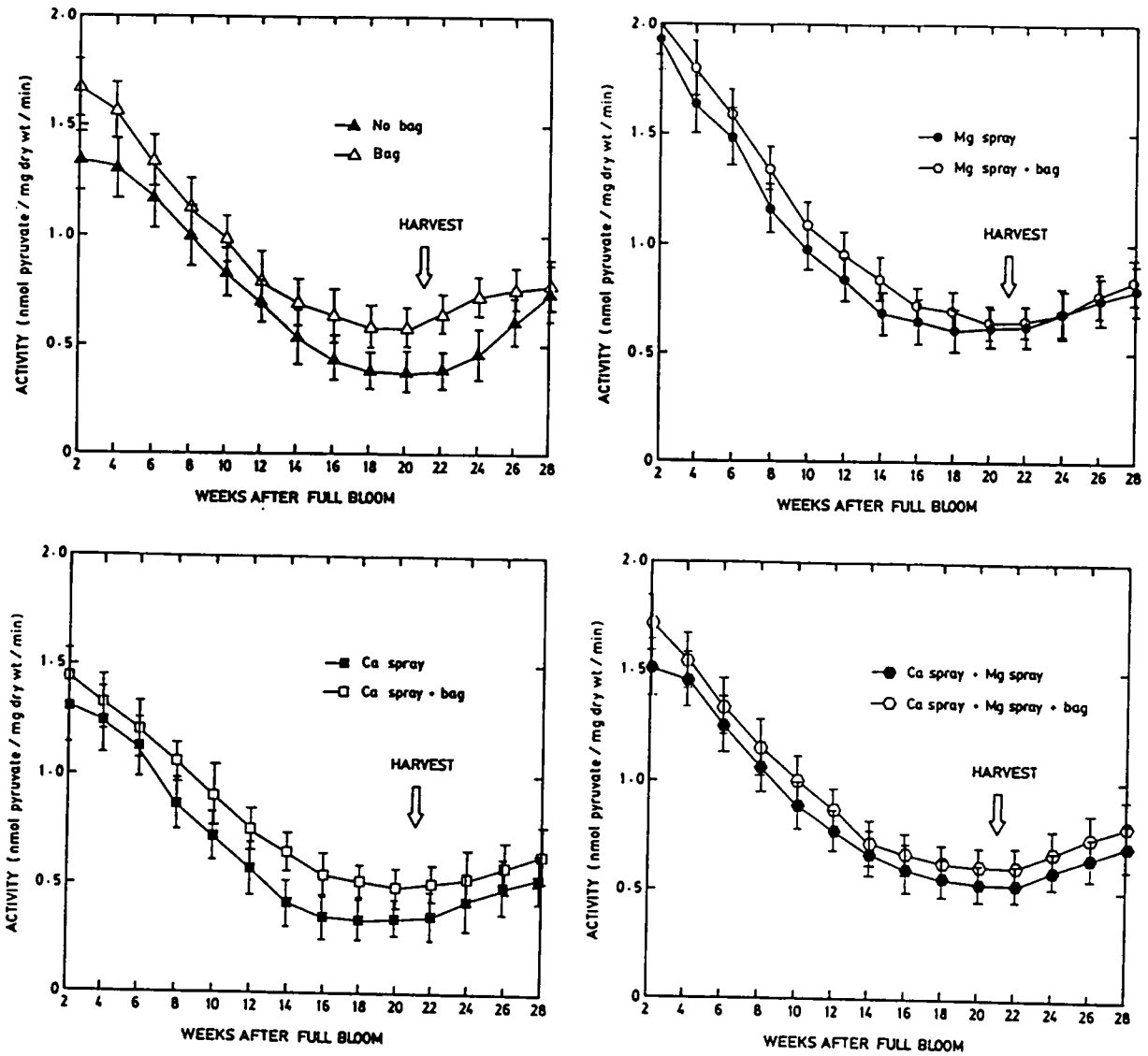

Fig. 2. Changes in pyruvate kinase activity in apple fruit during development and storage. Vertical bars = ± SE.

Table 1. Effects of $CaCl_2$, $MgCl_2$ sprays and paper bags on fruit pyruvate kinase activity and cation ratios (six weeks after full bloom) and bitter pit incidence (after storage).

	Pyruvate kinase activity (nmol/mg dry wt/min)	Ca: Mg + K ratio	Bitter pit (%)
Field treatment:			
No spray, no bag	1.19	0.056	5
Bag	1.36	0.037	42
Ca	1.13	0.068	3
Bag + Ca	1.22	0.040	13
Mg	1.49	0.035	36
Bag + Mg	1.61	0.026	100
Ca + Mg	1.25	0.051	12
Bag + Ca + Mg	1.36	0.038	38
Main effects and interactions:			
Bag	**	*	**
Ca	*	*	*
Mg	*	*	**
Bag × Ca	NS	*	*
Bag × Mg	*	*	**
Ca × Mg	NS	*	NS
Bag × Ca × Mg	NS	NS	*

NS, *, ** Nonsignificant or significant at the 5% or 1% levels respectively.

spray on cation ratio and bitter pit percentage, Ca spray and Mg spray on cation ratio, and all three factors on bitter pit percentage (Table 1). Correlation coefficients were r = 0.67 for pyruvate kinase activity and Ca: Mg + K ratio, r = 0.76 for pyruvate kinase activity and percent bitter pit, and r = 0.69 for Ca: Mg + K ratio and percent bitter pit.

Over the entire season the correlation between pyruvate kinase activity and percent bitter pit after storage exhibited a gradual decline (Fig. 3). The correlation between seasonal enzyme activity and bitter pit was highly positive in May, June and July, gradually declining to low positive correlation coefficients in September and October.

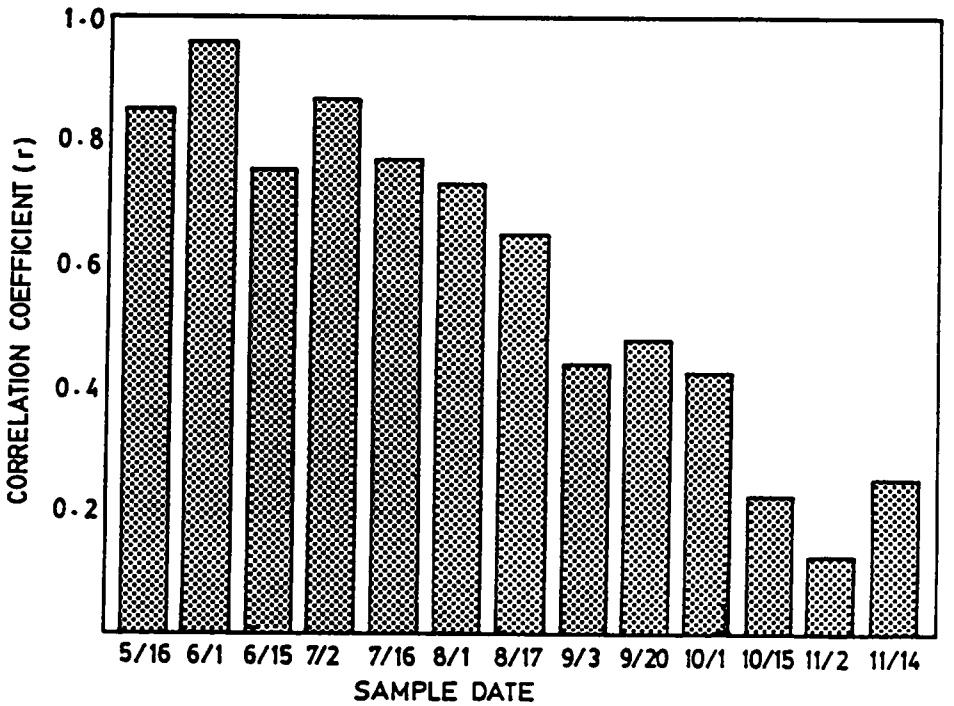

Fig. 3. Seasonal correlation coefficients of pyruvate kinase activity and percentage bitter pit after storage.

Discussion

The ability to predict bitter pit development in an apple crop early in the season has been an important objective of research workers in this field (Autio et al., 1986; Bramlage et al., 1985; Ferguson et al., 1979; Garman and Mathis, 1956; Holland, 1980; Perring, 1968, 1986; van der Boon, 1980a, 1980b). The calcium content of the developing fruit has been one of the most commonly used parameters in bitter pit prediction (Perring, 1986), along with the relative proportions of magnesium and potassium. However, there are many problems associated with predictions based on the cationic concentration of the fruit. Often there is only a loose relationship between cation level and bitter pit incidence (Perring, 1986) and predictions based on calcium content of fruit alone may sometimes be inaccurate.

Bitter pit has long been recognized as being physiological in nature (MacArthur, 1940), and closely related to metabolic processes. One enzyme intricately involved in metabolism and exerting considerable control on respiration is pyruvate kinase (Turner and Turner, 1980). The control of this enzyme is largely a function of the levels of Ca^{2+}, Mg^{2+}, and K^{+} in its environment (Meli and Bygrave, 1972), the same cations implicated in the development of bitter pit (Shear, 1975). Whether activation of pyruvate kinase is directly involved in bitter pit development is unknown, and there may be no relationship between the two, other than their coincidental mutual association with the same cations. It is dif-

ficult to envisage a system of bitter pit development involving excessive pyruvate kinase activity. One of the most obvious histological and biochemical differences between normal and pitted apple tissue is a residual accumulation of starch in pit cells (MacArthur, 1940; Witney et al., 1989). This implies cell death before starch hydrolysis occurs in pit cells, or a breakdown in starch hydrolysis leading to cell death. If there is a break in starch hydrolysis and pyruvate kinase has high activity, then stored sugars could be rapidly depleted by glycolysis leading to cell death. This is a highly speculative scenario and would be difficult to demonstrate, because sites of potential pit development are almost impossible to identify before manifestation of the disorder.

Results of this field trial and laboratory analysis clearly show a close relationship between fruit cations, pyruvate kinase activity and bitter pit incidence. Of particular importance was the high correlation early in the season between pyruvate kinase activity in the outer layer of fruit tissue and the appearance of bitter pit symptoms in storage. However, this does not implicate pyruvate kinase activity with bitter pit development. The fruit had altered cation levels very early in the season, which may have simultaneously adjusted pyruvate kinase activity and bitter pit development without involving any relation between the enzyme and the disorder.

Pyruvate kinase is activated by relative concentrations of mono- and divalent cations, which give the assay an advantage over traditional prediction techniques because it inherently accounts for cation ratios which may otherwise

require mathematical manipulation, or may escape analysis. This experiment involved altering fruit cation levels to produce a wide range of bitter pit intensities and enzyme activities. With this in mind, some caution is necessary in recommending an assay for pyruvate kinase for bitter pit prediction under normal field conditions. The results, however, show that pyruvate kinase activity may be a promising diagnostic tool for early bitter pit prediction.

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CHAPTER 6

QUALITATIVE EVALUATION OF PROTEINS DURING DEVELOPMENT OF BITTER PIT IN APPLE

Additional index words. ***Malus domestica***, electrophoresis, silver stain, phenolics

Abstract. **In an attempt to identify differences in protein populations of apple (*Malus domestica* Borkh.) fruit which developed bitter pit, the electrophoretic protein profiles from fruit were examined over the season. During fruit growth, multiple spray treatments of $CaCl_2$ and $MgCl_2$, combined with paper bag fruit covers, were applied to developing 'Golden Delicious' fruit. Bags and $MgCl_2$ spray treatments increased bitter pit incidence after storage, while $CaCl_2$ sprays suppressed the disorder. The qualitative electrophoretic pattern of soluble proteins was nearly identical from all treatments over the whole season, even once bitter pit symptoms were manifest.**

A basic explanation of the cause of bitter pit in apple has not been established (Bunemann et al., 1979; Perring, 1986). While an imbalance of fruit cations, especially Ca, Mg and K, has been implicated in the development of the disorder (Ferguson et al. 1979; Holland, 1980), the action of these cations is not well understood. Current research in bitter pit development should be aimed at an understanding of biochemical events leading to the development of the disorder (Perring, 1986; Perring and Pearson, 1987). Proteins, as the first products coded by genetic material, are ideal markers of biochemical changes associated with a morphological or physiological change (Stahmann, 1963).

The extraction of plant proteins is complicated by other plant products released during cell disruption, such as phenolics, acids, and tannins (Loomis, 1974). This is particularly pertinent in apple protein extraction, as tissues are high in phenolic compounds which bind to proteins, and denature enzymes, leading to poor electrophoretic separation (Menendez et al., 1982, 1986; Schaefer, 1987). The addition of reducing agents, polyphenol-oxidase inhibitors and synthetic polymers may overcome these problems, but their effectiveness varies with the type of tissue being studied (Kuhns and Fretz, 1978; Menendez et al., 1986; Wolfe, 1976; Schaefer, 1987). Electrophoretic conditions and staining procedures need to be optimal to achieve good separation and resolution of apple fruit proteins.

This work describes a rapid method of extracting fruit proteins and their separation by electrophoresis. A detailed examination of seasonal protein pat-

terns from apple fruit revealed no apparent differences associated with bitter pit development.

Materials and Methods

Plant material

Apple fruit from 15-year-old 'Smoothie Golden Delicious' (***Malus domestica*** Borkh.) trees grown at the Horticulture Farm, Virginia Polytechnic Institute and State University, Blacksburg, were used in the study.

Field treatments.

The field experiment was a 2 X 2 X 2 factorial with 16 replications of 5 fruiting spurs each in a randomized complete block design. Treatments were 1% (w/v) $CaCl_2$ spray, no $CaCl_2$ spray; 1% (w/v) $MgCl_2$ spray, no $MgCl_2$ spray; and bags, or no bags. Sprays were applied every 10 days with a hand held sprayer beginning 5 days after full bloom. Bags were Kraft 3 lb brown paper and were carefully stapled over spurs without damage to vegetative or floral structures. Bags were removed and replaced at each spray date, and removed completely 4 weeks before harvest. Fruit were harvested 148 days after full bloom and placed in storage (-1°C) for 6 weeks. Following storage, an assessment of bitter pit was made and results expressed as percent pitted fruit.

Protein extraction

The extraction buffer used was modified from Wendel and Parks (1982) and Menendez et al. (1986), with the following final composition: 50 mM Tris-HCl,

2 mM EDTA Na_2 , 2 mM dithioerythritol, 5 mM ascorbic acid, 3 mM $Na_2S_2O_5$, 6 mM diethylthiocarbamate, 5 mM PMSF, 14 mM mercaptoethanol, and 0.1% (v/v) Triton X-100, (pH 8.3). Fruit were sampled every 3 weeks after full bloom. Using a composite sample of 5 fruit, 5 g of fresh fruit tissue (the outermost 2 mm of equatorial peel) were frozen with excess liquid N_2, fractionated with an electric blender, and then immediately ground with a Polytron homogenizer (1 X 10 s) in the presence of hydrated PVPP (3 g/g tissue) and extraction buffer (2 ml/g tissue). The homogenate was centrifuged at 20,000 x g for 20 min, and the supernatant immediately used for electrophoresis.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was conducted in a dual vertical slab unit (140 X 140 X 1mm gels). Separating gels were 12% polyacrylamide (30% T, 2.67% C), 0.375 M Tris-HCl, (pH 8.8). Stacking gels were 4% polyacrylamide (30% T, 2.67% C), 0.125 M Tris-HCl, (pH 6.8). Gel polymerization was initiated with ammonium persulfate and Temed. Gels were run at 4°C and a constant voltage of 120V for 5 h with a 0.025 M Tris-HCl electrode buffer, (pH 8.3).

Staining

Gels were silver stained using a modification of the method described by Heukeshoven and Dernick (1985). Immediately following electrophoresis, gels were fixed in 30% ethanol and 10% acetic acid overnight, and stained using the following sequence: gels were washed in 10% ethanol (2 X 10 min) and then wa-

ter (3 X 15 min); impregnated with 0.1% $AgNO_3$ (15 min); rinsed with water (30 s); developed in 3% Na_2CO_3 and 0.02% formaldehyde (5 X 1 min); developing was stopped with 1% acetic acid (5 min); gels were washed in water (3 X 5 min); background staining was cleared with 0.5% Kodak Farmer's Reducer (30 s); the gel was washed with water (3 X 10 min); and the cycle repeated starting at $AgNO_3$ impregnation (10 min).

Results

Field treatments gave a wide range of bitter pit symptoms in fruit after storage, from 3% in $CaCl_2$ sprayed fruit, to 100% in $MgCl_2$ sprayed fruit with bag covers (Table 1). The qualitative electrophoretic pattern of soluble proteins was nearly identical for each treatment at each sample date (Figs. 1-3), and there was little change in the protein pattern from fruit over the growing season. Some change in the resolution of proteins was evident after prolonged storage (Fig. 4). However, there were no visible differences in the protein bands associated with fruit predisposed to pitting by field treatment (Fig. 2), or fruit with severe bitter pit symptoms (Fig. 5). The silver staining technique described gave excellent resolution of protein bands.

Table 1. Field treatments and bitter pit after storage.

Treatment	% Bitter pit
No Ca, no bag	5
Bag	42
Ca	3
Mg	36
Bag + Ca	13
Bag + Mg	100
Ca + Mg	12
Bag + Ca + Mg	38

All main effects and interactions significant at the 5% level except Ca x Mg.

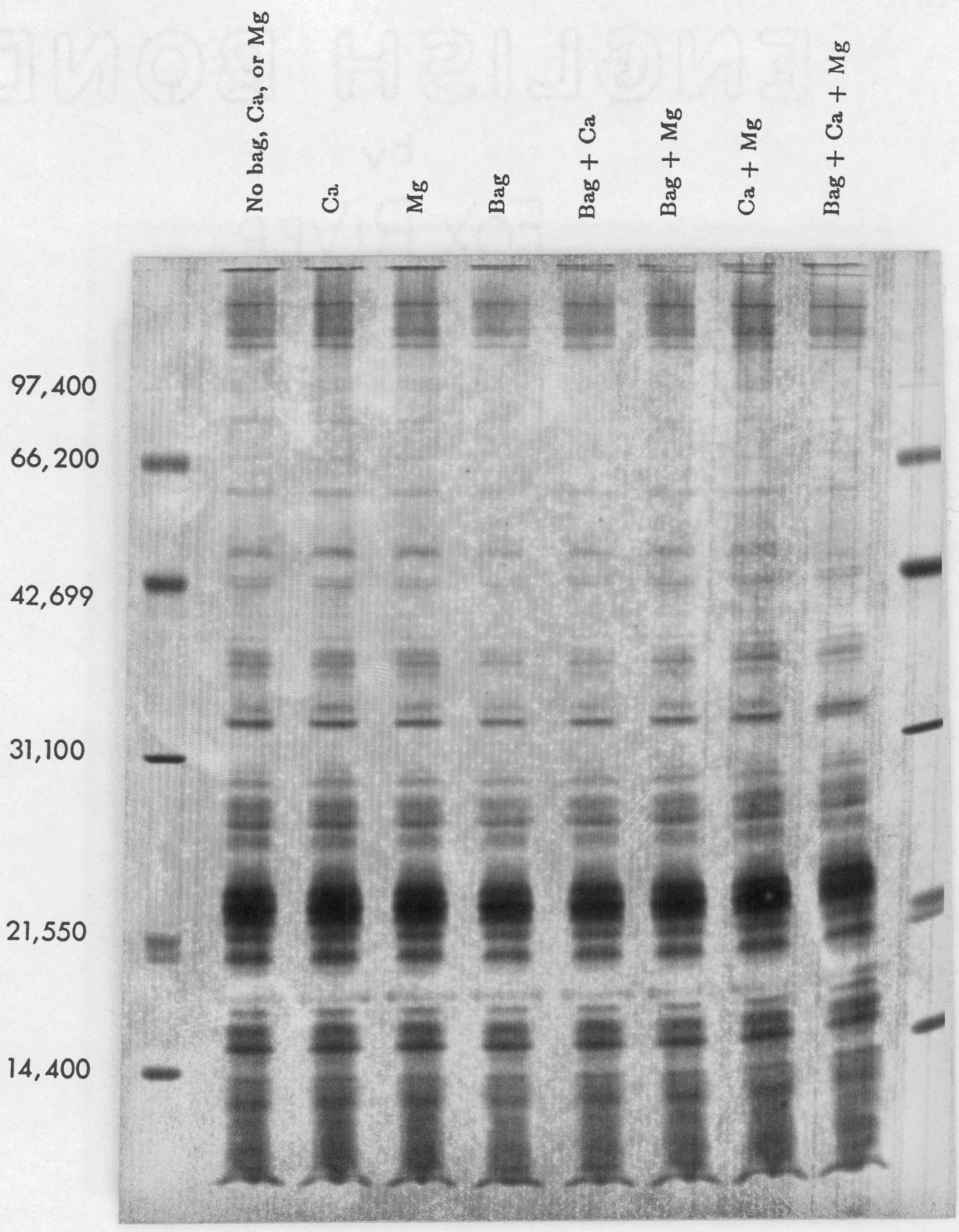

Fig 1. Electrophoretic protein profiles from apple fruit 6 weeks after full bloom.

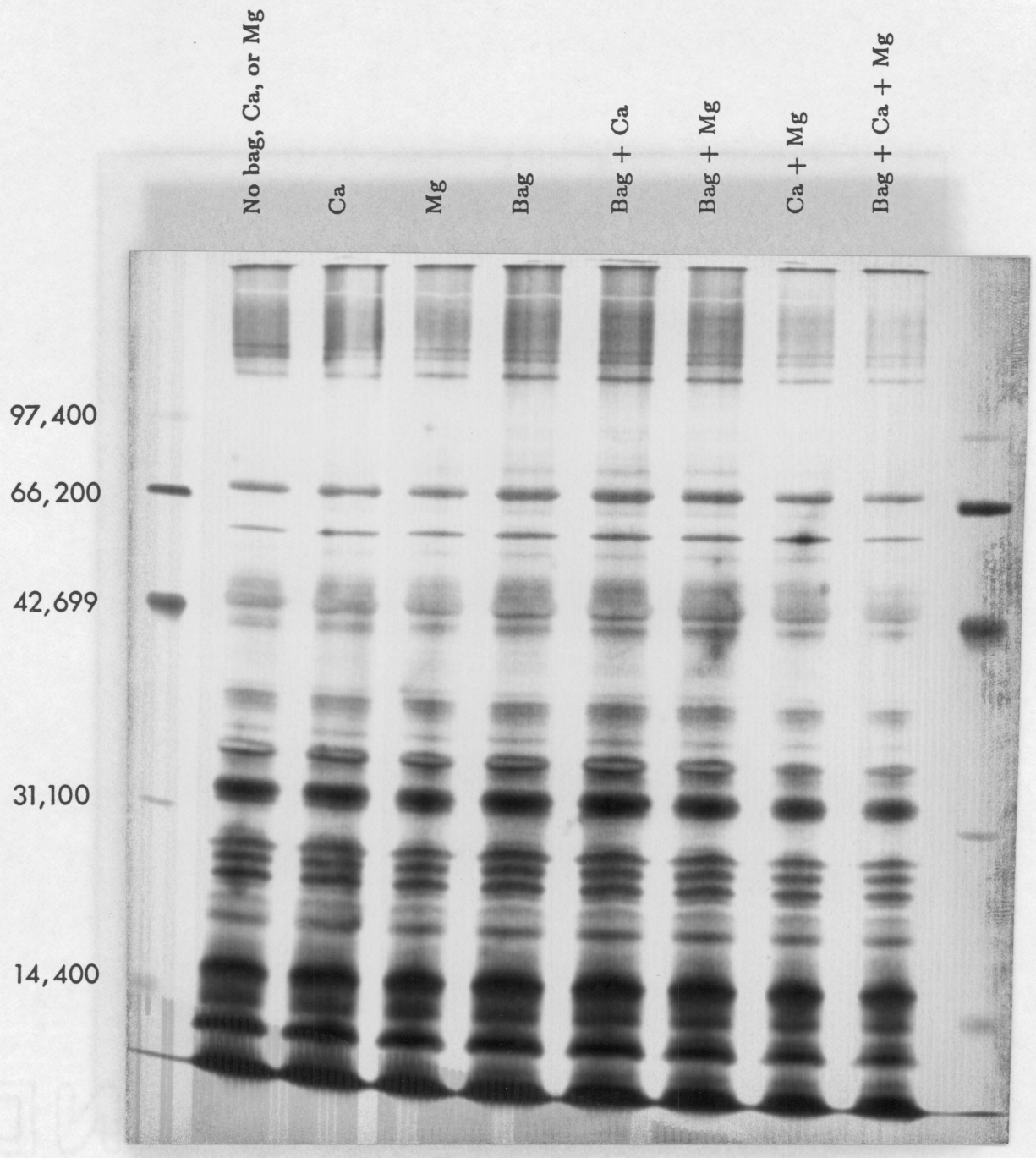

Fig 2. Electrophoretic protein profiles from apple fruit 12 weeks after full bloom.

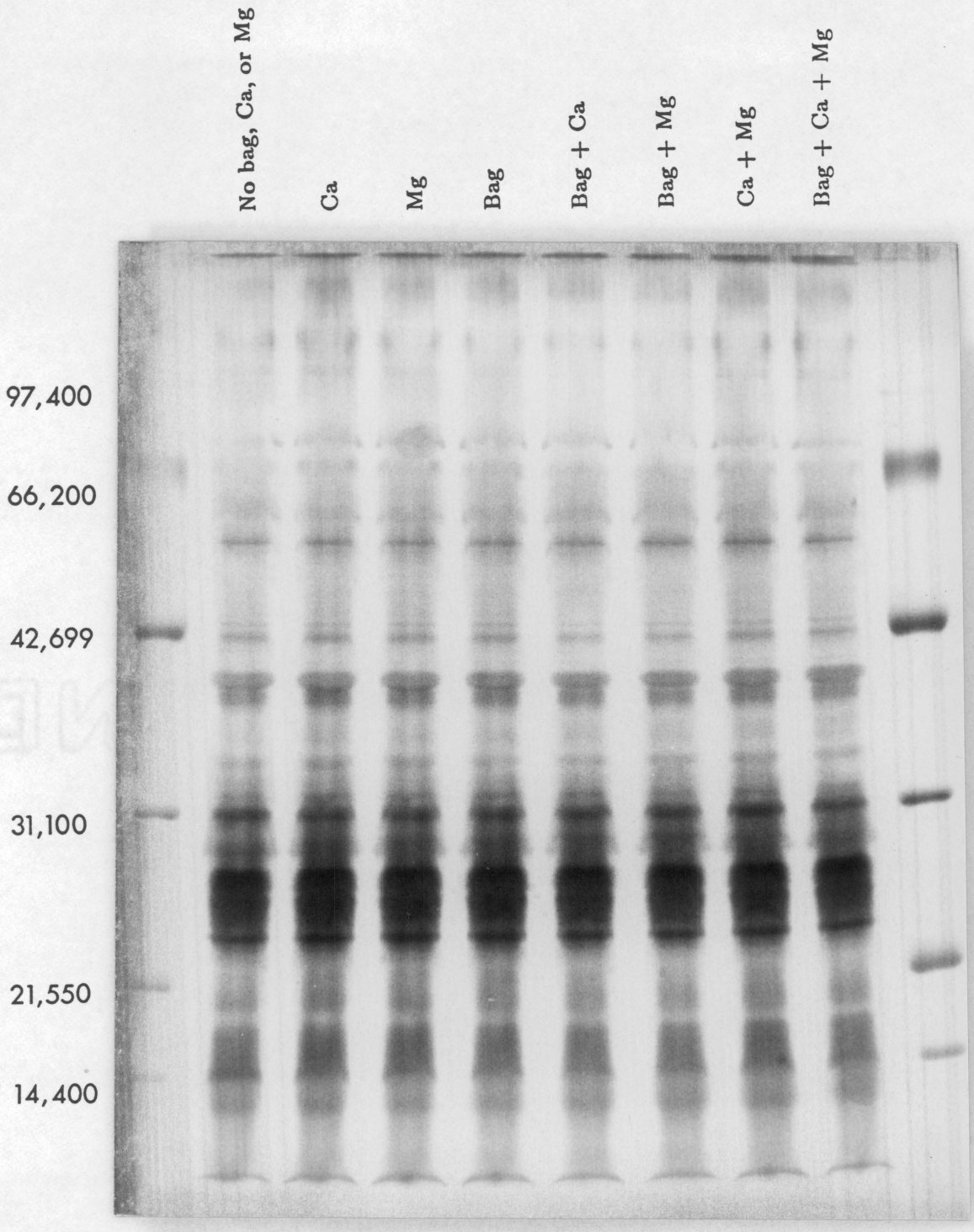

Fig 3. Electrophoretic protein profiles from apple fruit at harvest.

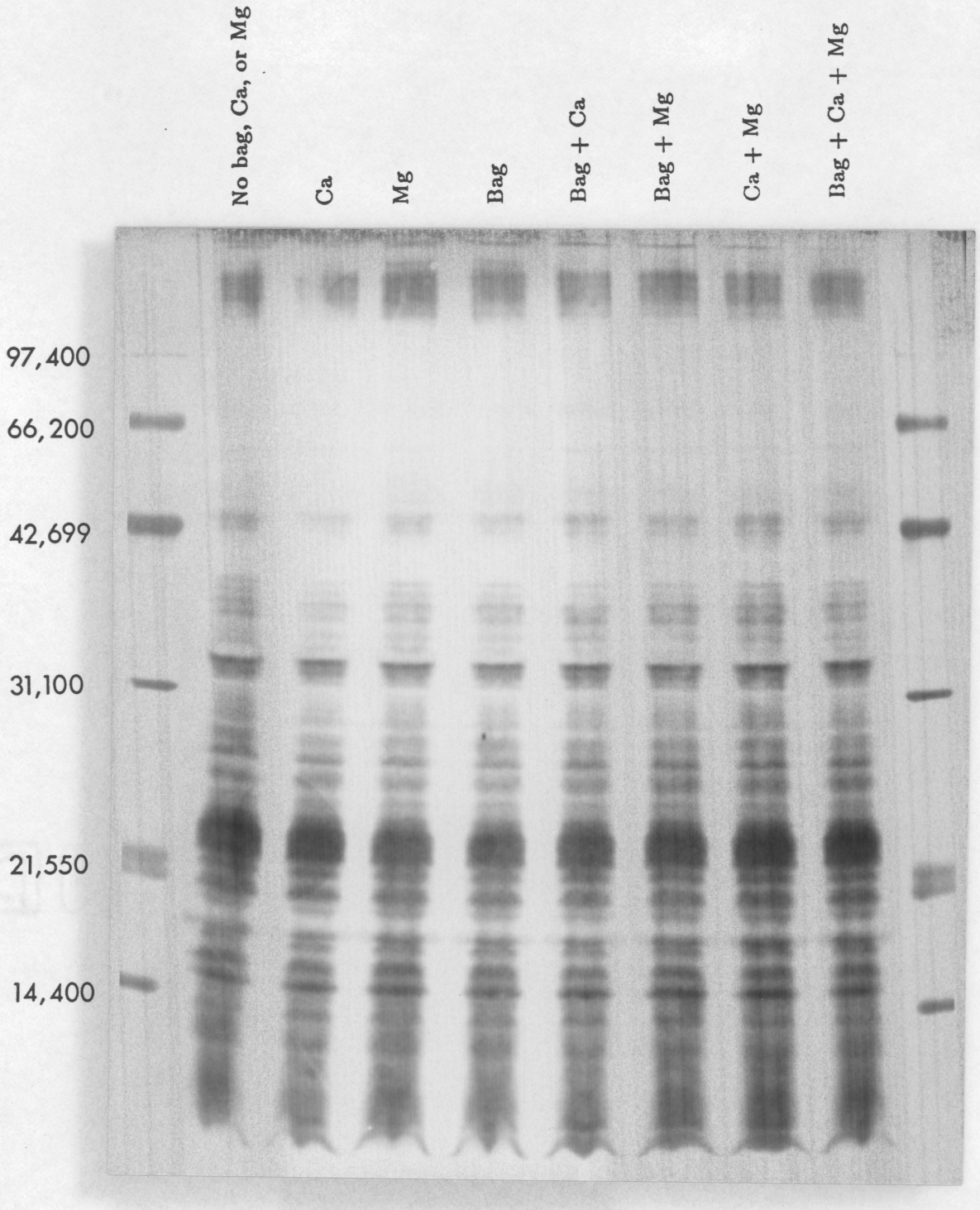

Fig 4. Electrophoretic protein profiles from apple fruit after 9 weeks storage.

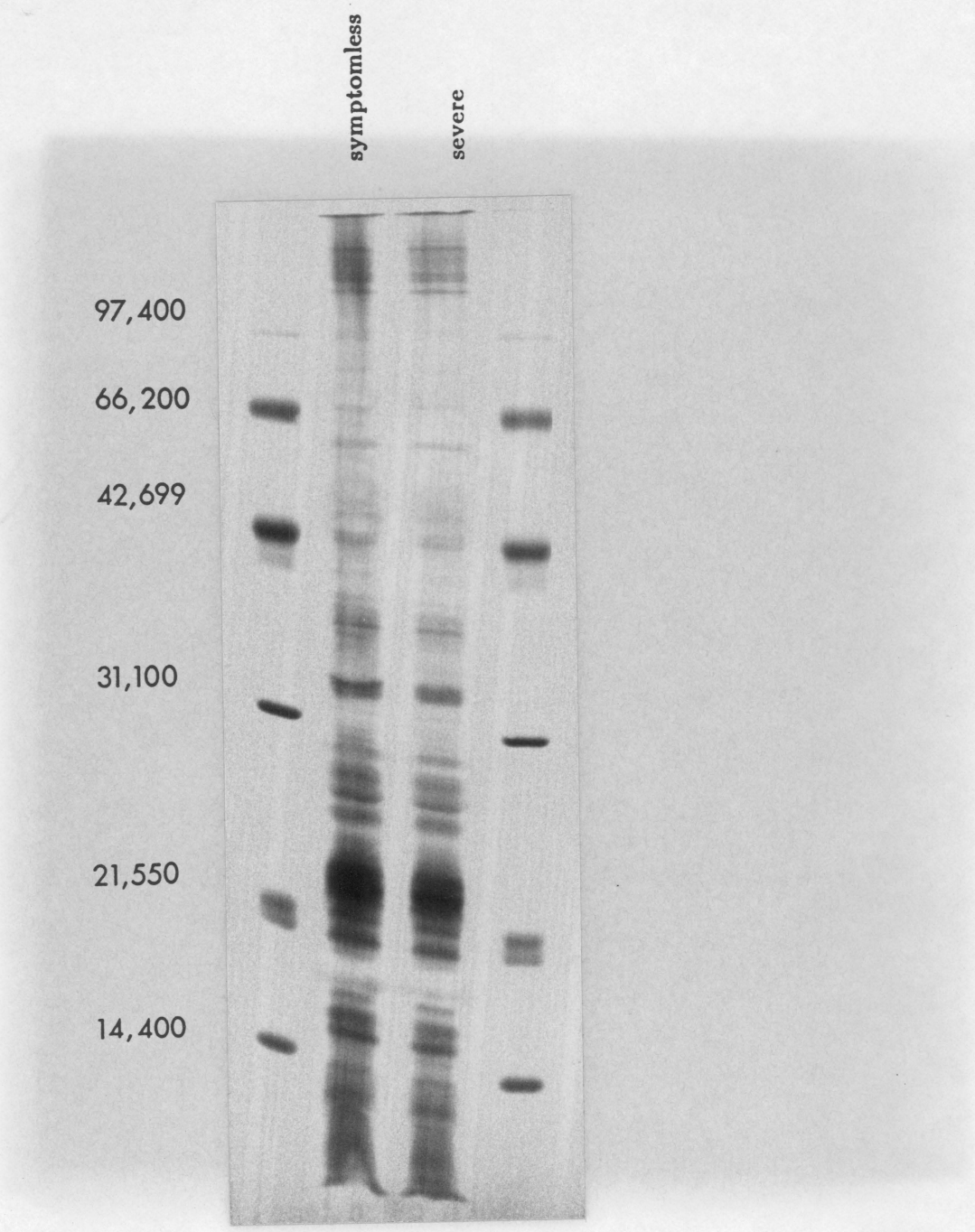

Fig 5. Electrophoretic protein profiles from apple fruit with severe bitter pit, and symptomless fruit.

Discussion

In order to study the developmental differences in protein populations in apple fruit associated with bitter pit development, it was essential to devise a system to effectively initiate bitter pit in the field. Reducing fruit transpiration with paper covers, and increasing Mg levels in fruit with $MgCl_2$ sprays, proved very effective. If these treatments are begun shortly after full bloom, they provide an ideal system for the subsequent study of bitter pit physiology.

Various methods were employed to both extract, and concentrate, fruit proteins (methods not described). Of the methods tried, the one modified from Menendez et al. (1986) as described, gave excellent results as long as samples were utilized for electrophoresis immediately after extraction. The high phenolic content of apple fruit was effectively countered by the addition of a large amount of PVPP during extraction. Concentration of proteins by ammonium sulphate precipitation did not improve resolution, proteins tended to band together in less discrete bands following this treatment. The delay in loading proteins onto gels, necessitated by concentration techniques, probably allowed residual phenolics to act on proteins in the extract.

Preliminary studies used Coomassie blue staining of gels as described Menendez et al. (1986) and Schaefer (1987), but this gave very poor resolution of bands, with only major bands evident. Silver staining modified from

Heukeshoven and Dernick (1985) allowed for visible identification of many more bands than was possible with Coomassie blue. Studies aimed at identifying small differences in plant protein populations would find the method described useful, particularly as it is less time consuming than most other staining procedures, and provides considerably improved images for photographic reproduction compared to Coomassie stains.

Results indicate that bitter pit initiation and development do not appear to be associated with a change in protein population in apple fruit. However, there were no treatments absolutely free of pitting, and the above statement is made with this limitation in mind. Being a physiological disorder, bitter pit is inextricably linked to fruit tissue metabolism. Thus, any change in enzyme (protein) action resulting in bitter pit manifestation, could have been the result of a change in the activity of certain enzymes, rather than *de novo* synthesis of new enzymes.

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