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The Role of Calcium in Fruit Ripening and in Alleviating Post-Harvest Physiological Disorders

R. Ben-Arie, M. Faust

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The role of calcium in fruit ripening and in alleviating post-harvest
physiological disorders

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TABLE OF CONTENTS

	<u>Page</u>
1. ABSTRACT.	1
2. INTRODUCTION.	2
3. EXPERIMENTAL.	3
3.1. Calcium effects on the physiology of whole fruits.	3
3.1.1. Ethylene evolution.	3
3.1.2. Importance of calcium in alleviating physiological disorder in stored apples	.12
3.2. Calcium effects on physiology of fruit discs	.28
3.2.1. Cyanide insensitive respiration in apple fruits	.28
3.2.2. Deferral of senescence in calcium treated apple fruit discs	.37
3.2.3..Temperature dependent inhibitory effects of calcium on ethylene biosynthesis.	.46
3.3. Calcium effects on biochemical and physical properties of cellular membranes.	.47
3.3.1. Membrane changes during ripening.	.47
3.3.2. Membrane changes during storage	.57
3.3.3. Characterization of a plasmalemma ATPase from apple fruits.	.67
3.3.4. Membrane permeability and microviscosity.	.68
4. EVALUATION OF THE RESEARCH ACHIEVEMENT.	.79
5. DESCRIPTION OF COOPERATION.	.80
6. EXAMINATION OF A HYPOTHESIS	.81
7. PUBLISHED REPRINTS AND LIST OF SUBMITTED MSS.	.83

1. ABSTRACT

The model used for this study was the 'Calville de San Sauveur' apple cultivar, widely grown in Israel, which shows different susceptibilities to the development of physiological disorders during storage when grown under different climatic and edaphic conditions. The results have indicated that these differences in susceptibility might be attributable to the differences in membrane composition and functioning, and that one of the sites of calcium involvement in fruit quality is the plasma-membrane. The parameters of membrane functioning most affected by calcium were found to be membrane permeability and microviscosity. However, the effects of calcium in the cell are not limited entirely to cellular membranes. The well-known effect of calcium on deceleration of the softening rate of fruits is a result of its binding to the pectic fractions of the cell wall and causing their insolubilization. Moreover, the activities of both soluble and membrane-bound enzymes, such as polyphenol oxidase and ATPase, have been found to be inhibited by calcium. It is therefore questionable whether there is any single site of calcium binding in the cell or any fundamental function of this ion that can be attributed with the role of controlling fruit ripening and deterioration.

From an applied point of view, the most significant result of this study is the recommendation to orchardists in the Golan Heights to apply post-harvest calcium dip treatments to their fruits, instead of the presently used practice of spraying their orchards 5-6 times prior to harvest. It was found that post-harvest dip treatments with an effective surfactant can increase the calcium level in the fruit by 150%. However, raising the calcium content of the fruit was not always beneficial and the effects on ripening were rarely noticeable. A hypothesis was therefore proposed that the role of calcium is to protect the fruit tissues under stress conditions, and that in their absence, the advantage of calcium treatment is not apparent. Stress conditions might be extreme temperatures, mineral imbalances, restricted respiration, etc. The results of preliminary trials with some stress conditions on fruit discs lend support to this hypothesis.

2. INTRODUCTION

The beneficial effect of calcium to the postharvest life of the apple fruit is now a well-known fact, even though the underlying reasons for this effect still remain somewhat ambiguous. One of the objectives of this proposal was to understand the role of calcium in fruit metabolism by studying its physiological effects at three different levels of organization; i.e. intact fruits, tissue slices (or discs) and sub-cellular particles, chiefly membranes. Most of the work done in Israel was with the 'Callville de San Sauveur' cultivar, which was chosen due to its contradictory behaviour in two different growing seasons. The fruit of orchards grown in the hot, humid, low-lying Huleh valley is of excellent keeping quality in storage, whereas fruit from orchards grown on the volcanic soils in the relatively cool, dry climate of the Golan Heights, is not only morphologically different, but also behaves differently in storage in that it is highly susceptible to disorders such as bitter pit, internal breakdown and core-flush. As at least two of these disorders can be alleviated by calcium treatment, the cultivar from the two regions was deemed to be a good model for studying the role of calcium in the postharvest metabolism of the apple.

The second objective of the proposal was to improve the keeping quality of this apple cultivar by developing methods to increase the uptake of calcium by the fruit. Though this objective has been achieved, in that the calcium content of the fruit can be noticeably increased according to the method of treatment employed, we have also found that there is a limit to the beneficial effects of calcium infiltration that are obtainable. A hypothesis to explain this finding will be presented at the end of this report and may serve as the basis for continued research on this subject.

3. EXPERIMENTAL

3.1. Calcium Effects on the Physiology of Whole Fruits

The response of intact fruit to added calcium was studied at the level of changes incurred in ripening processes, such as respiration, ethylene evolution and fruit softening, and at the level of the keeping quality of the fruit in storage, with regard to the development of physiological disorders. The methods employed to raise the calcium content of the fruit varied, ranging from pre-harvest orchard sprays to post-harvest pressure infiltration. As none of the methods employed appeared to produce physiologically different or commercially superior results to those obtained with the standard post-harvest dip commonly employed, emphasis will not be placed on the methodology, but rather on the effects achieved.

3.1.1. Ethylene evolution

The effects of calcium on ethylene evolution in higher plants are varied. Inhibition of ethylene evolution by calcium has been reported by Poovaiah and Leopold (1) in connection with inhibition of leaf abscission, by Wills and Tirmazi (2) after calcium infiltration under pressure into tomato fruits, at all stages of ripening, and by Tingwa and Young (3) in avocado fruits infiltrated under pressure. In the latter two instances, fruit respiration along with other parameters of ripening were also retarded, as has also been shown with apple fruits (4,5). However, stimulation of ethylene biosynthesis has also been reported in certain tissues, such as mungbean hypocotyls (6), subhook epicotyl segments, apple tissue slices and shake cultures of Penicillium digitatum (7). With the mungbean (6) stimulation was slight unless in the presence of kinetin and synergism between the two was described. With the other three tissues (7) stimulation by calcium was explained as a result of

removal of phosphate, which was shown to inhibit ethylene biosynthesis. In other studies with apple fruit slices, calcium was shown initially to inhibit ethylene production for up to 6 h, but its more lasting effect was a stabilization of ethylene production, which declined rapidly in the untreated tissue (8,9,10). Here we present data describing the inhibitory effect of calcium on the evolution of ethylene by whole fruits, without any apparent effect on the total respiration processes.

Materials and Methods

Warm Golden Delicious apples were immersed either fully or partially (one cheek of the fruit) in cold 4% CaCl_2 solution, containing the surfactant X-77 (0.1%) for 1 h. The temperature differential was 20-22°C. Another method employed to infiltrate calcium into whole fruits was by activating 10 p.s.i. of pressure for 2 minutes on fruits submerged in 4% CaCl_2 or in water (control).

Following treatment ethylene and carbon dioxide evolution by the fruit was monitored by enclosing one or two fruits in hermetically sealed jars for 1 h. Head space samples of 10 ml were analysed by gas chromatography.

Ethylene evolution by tissue plugs, weighing 2 g, excised from the opposite halves of the partially dipped fruits was measured 30 minutes after excision. Preliminary trials showed that both ethylene and CO_2 evolution had more or less reached a steady state at this time (Fig. 1). Individual plugs were enclosed in a 25 ml gas-tight syringe for 20 min, after which a 8 ml sample was withdrawn for gas analysis.

The calcium content of the fruit was determined by atomic absorption after freeze-drying a sample of peel and pulp tissue and ashing the dried

Fig. 1. Evolution of CO_2 and C_2H_4 by tissue plugs excised from apple fruit pulp.

powder.

Results and Discussion

Calcium infiltration of fruits always resulted in reduced ethylene evolution by whole fruits irrespective of the method used (Fig. 2). The extent of inhibition was, however, affected by the mode of application. Thus, after pressure infiltration, inhibition was greater than that caused by immersion. This might be due to infiltration of greater quantities of calcium by the former method.

In spite of the inhibition in ethylene evolution, Ca^{+2} treatment had no effect on the respiration rate or the timing of the climacteric peak (Fig. 3). These results appear to contradict in part those of Faust and Shear (4) and Bramlage et al. (3), who reported reduced respiration rates with increasing calcium content, though no effect was observed on maturity or timing of the climacteric peak. Their data, however, were collected from randomly chosen fruits harvested from trees with low or high leaf calcium contents, and the results could be an indirect effect of varying nutritional status of the trees. This seems likely as there were also significant differences in other parameters, such as fruit size and colour. In the data presented here, only the calcium contents of uniformly chosen fruits was raised and as a result, the respiration rate of the fruit remained unchanged, indicating the uniformity of the population of fruits used in the experiments.

A partial fruit dip, which raised the level of calcium only in the submerged portion of the fruit (Table 1), had the same effect on the rate of ethylene evolution by the whole fruit as did the immersion of the entire fruit (Fig. 4). These data indicate no correlation between the extent of inhibition and the amount of additional calcium taken up by the fruit.

Figure 2. The effect of CaCl_2 infiltration into apple fruits by different methods on their ethylene evolution

Fig. 3. The effect of post harvest immersion of warm apple fruits (cv. Golden Delicious) in cold 4% calcium chloride on CO_2 and C_2H_4 evolution at 20°C .

Figure 4. Decrease in ethylene production by total or partial immersion of apples in CaCl_2 solution.

Table 1. The increase in calcium content of warm Golden Delicious apples submerged or partially immersed in cold calcium chloride

Treatment	Tissue sampled	Calcium content (ppm)		% Increase
		Water	CaCl ₂	
Submergence	Whole fruit	437 ± 39	898 ± 44	105.5
Partial immersion	Exposed half	368 ± 19	429 ± 43	16.6
	Immersed half	371 ± 19	570 ± 44	53.7
% Increase		-	32.9	

Table 2. Ethylene evolution by tissue plugs excised from Golden Delicious apples following partial or total immersion in a cold CaCl₂ solution

Treatment	C ₂ H ₄ evolution (ml/g/h)	
	Water	4% CaCl ₂
Submergence	23.0 ± 3.2	14.7 ± 0.47
Partial immersion		
Exposed half	-	14.3 ± 0.25
Immersed half	-	13.8 ± 0.25

Assay of ethylene evolution by plugs excised from the opposite sides of the partially immersed fruits, indicated no differences between the two halves, but the similar values of both were significantly lower than ethylene evolution from plugs excised from the non-treated fruits (Table 2).

It appears therefore that the immobile calcium could be setting off a series of reactions which have a far reaching effect in the fruit, and eventually cause an inhibition of ethylene evolution.

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3.1.2. The importance of calcium in alleviating physiological disorders in stored apples

A low concentration of calcium in apple fruit (Malus sylvestris) has been implicated in many storage disorders and, conversely, raising the fruit calcium content has been credited with alleviating these disorders (22,24). Bitter pit (BP) of apples has been reduced or controlled in numerous cultivars with preharvest and postharvest application of calcium nitrate or chloride (3,14,17,20,21,22). CaCl_2 is also used commercially as a dip or drench treatment to reduce storage breakdown of 'Jonathan', 'Spartan' and 'Cox's Orange Pippin' apples (1,9,13,19). The incidence of internal breakdown (11) has been related to the final Ca concentration of the fruit (3,16). Uptake of the Ca solution by fruit was primarily through lenticels and increased with higher concentration of solution: absorbed Ca moved inward through tissues but remained highest near the surface (2,7,12,13,15).

The addition of various surfactants, thickeners such as Keltrol, and lecithin to postharvest applications of CaCl_2 has often increased the effectiveness of the treatment, delayed softening (3,7,11,14,15,17,21), or reduced Ca injury (7,11). For some cultivars both vacuum and pressure infiltration of CaCl_2 solution may improve the control of breakdown and bitter pit over that achieved by a simple dip (5,20).

The apple cultivar 'Calville de San Sauveur' is a soft, warm-climate cultivar, generally rather susceptible to bitter pit. The commercial practice of applying five or six calcium nitrate sprays is generally quite effective. Cultivation of this cultivar in the cooler climate and different soils of the Golan Heights has increased the susceptibility of the fruit to bitter pit (BP) as well as to internal breakdown (IB) and core-flush (CF), which were virtually unknown beforehand.

The objective of this study was to evaluate the keeping quality and Ca content of 'Calville de San Sauveur' apples cultivated on the Golan Heights. Attempts were made to improve the calcium content with sprays during the growing season, and with postharvest dips (Ca alone or in combination with various substances) to enhance Ca uptake.

Materials and Methods

Trials were conducted during 3 years to improve the calcium uptake by 'Calville de San Sauveur' apples grown in an orchard on volcanic soil poor in calcium.

a) Orchard trials. The standard commercial treatment of five or six sprays of 1% $\text{Ca}(\text{NO}_3)_2$ applied every 10-15 days until harvest was compared with 9-12 weekly sprays and with unsprayed controls. (In 1981, the orchard was inadvertently sprayed twice before the start of the experiment.) Other approaches for increasing calcium uptake were (a) changing the pH of the spray solution by adding $\text{Ca}(\text{OH})_2$, and (b) reducing the surface tension by adding the surfactant L-77. The effect of calcium phosphate sprays was examined each year with a different compound: calcium monophosphate, tri-calcium phosphate and Seniphos (Hamerhill International Ltd, U.K.). The surfactant Triton X-100 (0.025%) was added to all sprays, which were applied to runoff with a knapsack motor-sprayer.

Each spray trial was conducted in five random blocks consisting of one tree per treatment, with intervening non-treated trees. At harvest 70 fruits from each tree, representative of the dominant size, were sampled from all sides of the tree. Ten fruits were used for maturity studies (firmness, total soluble solids, acidity, and iodine test), ten for mineral

analysis, and 50 for storage studies (25 being examined upon removal from storage, and 25 after shelf-life).

b) Postharvest trials. Fruit harvested from the trees of the standard spray treatment was dipped on the day after harvest in 2000 ppm diphenylamine (DPA) and different variations of calcium chloride solutions. In the first season the effect of adding 0.3% Keltrol to 2% or 4% CaCl_2 was examined. In the second and third seasons, room temperature (25°C) 2% CaCl_2 solutions were compared with cold (0°C) solutions, with and without the addition of the surfactant L-77. The control dip (DPA only) and warm dips lasted 20 seconds, and the cold dips 15 min. Each treatment consisted of three or four replicates of approximately 50 fruits for each storage period. Half of the fruits were examined upon removal from storage, and the other half after shelf-life.

Examination of the fruit consisted of sorting for physiological disorders, measurement of firmness, T.S.S. and acidity, and assay of mineral content. Fruit with three or fewer small BP spots is not included in the % BP presented in the tables. The CF index is calculated as the percentage of fruit with slight CF, plus the percentage of fruit with severe CF x 2. The maximum value is therefore 200. The severity of IB was not evaluated as light or severe, since even when slight this disorder disqualifies the fruit from use.

c) Storage conditions. All fruit was stored in a controlled atmosphere of 1.5% CO_2 and 2% O_2 , at a temperature of 0° or -1°C ; for some treatments both temperatures were tested. The relative humidity was 90-95%. The storage period was 6-7 months and was followed by shelf life at 20°C for 5 or 6 days.

d) Mineral assays. Sampling for mineral assays was done in the first year

by peeling the fruit and removing four 13-mm plugs with a corkborer around the equator of the fruit. Each plug was divided into three samples, approximately 8 mm in length; beneath the peel (2-10 mm), the central flesh (12-20 mm), and the core section (22-30 mm). In the following two seasons, longitudinal sections were removed from the two opposite sides of each fruit (approximately one-eighth of the fruit), peeled, cored, and cut into small pieces. The samples were dried either at 85°C or freeze-dried and powdered. 500 mg of dried powder was digested in 4% H_2SO_4 , catalysed with H_2O_2 . The digest was brought to volume with double-distilled water and analysed for N and P with an auto-analyser, for K with a flame photometer, and for Mg and Ca by atomic absorption. Ca was determined in the presence of 1% La_2O_3 .

Results

a) Orchard sprays. Increasing the number of calcium nitrate sprays above the standard five or six commercial applications did not increase the calcium content of the fruit during two consecutive seasons (Table I, treatments 2 and 3). Likewise, neither fruit firmness at harvest nor the physiological disorders which developed during storage were affected by increasing the number of sprays. The standard commercial application of calcium nitrate significantly increased the calcium content of the fruit in comparison with non-treated fruit (in 1980) and also significantly affected fruit firmness and IB incidence after 7 months of storage. However, when compared with two sprays only (in 1981), the standard treatment did not have a significant effect on any of these parameters. Even when the calcium content of the fruit was significantly increased by additives to the standard calcium nitrate treatment (calcium hydroxide or L-77), no additional effect on fruit firmness

Table 1. The effect of variations in the calcium nitrate spray regime on the keeping quality of 'Calville de San Sauveur' apples in controlled atmosphere storage

Treatment	Flesh Ca (ppm)	Firmness (kg)		Titrat. acidity %, at harvest	BP %	IB %	CF index*
		At harvest	After storage				
1979, 6 mos at 0°C							
2. 1% Ca(NO ₃) ₂ x5 (Standard)	377	7.1	5.2	0.82	27	17a	3
3. 1% Ca(NO ₃) ₂ x9	295	7.2	5.3	0.80	20	14a	4
4. 1% CaHPO ₄ x5	338	7.0	4.9	0.85	31	43b	5
	N.S.	N.S.	N.S.	N.S.	N.S.		N.S.
1980, 7 mos at -1°C							
1. No spray (Control)	333a	6.7	4.4a	0.81ab	2	60a	40
2. 1% Ca(NO ₃) ₂ x6 (Standard)	357b	6.6	4.8b	0.82b	2	16b	15
3. 1% Ca(NO ₃) ₂ x12	324a	6.5	4.9b	0.82b	2	28b	16
4. 1% Ca ₅ (PO ₄) ₃ OH x6	331a	6.6	4.9b	0.79a	1	22b	45
5. 1% Ca(NO ₃) ₂ + 1% Ca(OH) ₂ x6	385c	6.6	4.8b	0.87c	2	15b	48
		N.S.			N.S.		N.S.
1981, 7 mos at -1°C							
1. 1% Ca(NO ₃) ₂ x2	382ab	7.0	6.0	0.91	7	40	15
2. 1% Ca(NO ₃) ₂ x6 (Standard)	348a	7.4	5.9	1.01	2	27	10
4. 1% Ca(NO ₃) ₂ + Seniphos x4	425b	7.2	5.8	1.01	4	36	8
5. 1% Ca(NO ₃) ₂ + 1% Ca(OH) ₂ x4	417b	7.1	6.0	1.00	10	31	17
6. 1% Ca(NO ₃) ₂ + 0.1% L-77 x4	476c	6.9	5.9	1.02	1	30	17
		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

* CF index = % fruit with slight CF + 2 (% fruit with severe CF).

a,b,c Mean separation in columns for each experiment by Duncan's multiple Range Test, 5% level.

or development of physiological disorders in storage was observed (1980,1981). BP was insignificant in both these seasons, but IB and CF were not reduced further by higher calcium content. The various calcium phosphate sprays applied (treatment 4) did not have a beneficial effect on the keeping quality of the stored fruit, and 1% CaHPO_4 even significantly enhanced IB (in 1979).

b) Post harvest dip treatments. Since spraying the trees during the growing season with calcium did not eliminate the physiological disorders, various methods of dipping the apples after harvest were tested. The first year two concentrations of CaCl_2 , with or without the thickener Keltrol, were examined. The distribution of calcium through the apple is shown in Fig. 1. Dips in 2% or 4% CaCl_2 raised the calcium content in all parts of the apple. The increment in calcium content of all fruit layers was more or less proportionate to its concentration in the dipping solution. Keltrol improved the uptake of the 2% CaCl_2 solution and made it equivalent to that of the 4% solution. However, the uptake from the 4% solution was not improved by Keltrol. Both Keltrol treatments caused some lenticel injury, but especially so with 4% CaCl_2 .

In the second season two approaches were tried: applying the calcium solution in the presence of different surfactants, and having a temperature differential between the fruit and the dipping solution. The weight increase of apples dipped from 5 to 15 min in a calcium solution containing various surfactants or detergents is shown in Fig. 2. The most effective materials were L-77 and Amway. Since Amway caused damage to the dipped fruit, only L-77 was investigated further. The effect of increasing concentrations of L-77 and increasing the length of time of immersion is shown in Fig. 3. Apples increased in weight as the concentration was raised and as the length

Fig. 1. Distribution of Ca^{2+} in apple flesh cross-section in response to different post-harvest CaCl_2 dips.

a,b,c - Mean separation in columns for each region by Duncan's Multiple

Range Test, 5% level.

Fig. 2. The effect of surfactants in the dipping solution on the amount absorbed during 15 min at 25°C. All solutions have 4% CaCl_2 .

Fig. 3. The effect of different concentrations of the surfactant L-77 with a 4% CaCl_2 solution, on the uptake by apple fruits immersed for different time lengths.

Fig. 4. The effect of temperature differential between fruit and dipping solution (2% CaCl_2 plus 0.2% L-77) on the uptake by apple fruits. Initial temperature differentials given.

of immersion time was increased. At a concentration higher than 0.1%, L-77 caused internal browning in the fruit.

The second approach was that of immersing apples held at ambient temperature in calcium solutions held at 0°C. As the apples cool, the air in the free space contracts and liquid is pulled into the fruit. The effect of various temperature differentials on increase in fruit weight is shown in Fig. 4. A temperature differential of 25° or 30°C gave very similar results while 20° was less effective.

The effect of these attempts to infiltrate calcium on the physiological disorders and condition of fruit during storage, is shown in Table 2. Comparing Tables 1 and 2 for the same season, it is seen that the dips were considerably more effective than the sprays in raising the fruit calcium level. In the 1979 season the most effective spray gave 377 ppm Ca, while the 4% CaCl_2 dip gave 474 ppm Ca. Likewise, in 1980, the sprays increased the calcium level from 333 ppm in the control to 385 ppm in the best case, while a dip of 2% CaCl_2 plus 0.1% L-77 gave 587 ppm Ca. This higher calcium concentration evidenced itself in firmer fruit. During shelf-life the fruit did not soften, whereas in the control fruit there was considerable softening.

The dips also generally reduced the incidence of the physiological disorders BP and IB when compared with the control, without affecting CF. What is interesting, however, is that the amount of reduction achieved by the dips shows no correlation to the increase effected in the fruit's calcium content. Moreover, cold dips significantly increased the incidence of rots caused by Penicillium expansum, even with the addition of a fungicide, and 0.1% L-77 caused a certain amount of lenticel injury, due to increased calcium uptake.

Table 2. The effect of postharvest calcium chloride dips on the keeping quality of 'Calville de San Sauveur' apples in controlled atmosphere storage

Treatments	Flesh Ca ppm	Firmness (kg)		BP %	IB %	CF index
		At removal from storage	After 6 days shelf-life			
1979, 6 mos at 0°C						
1. Control (DPA)	304a	5.2a	4.0a	27b	17b	77
2. CaCl ₂ 2%	344ab	5.8b	5.2ab	17a	9a	6
3. CaCl ₂ 2% + 0.3% Keltrol	376bc	5.6ab	5.5c	16a	2a	12
4. CaCl ₂ 4%	474d	5.4ba	5.4bc	13a	0a	4
5. CaCl ₂ 4% + 0.3% Keltrol	427cd	5.5ba	5.4bc	13a	0a	8
						N.S.
1980, 7 mos at -1°C						
1. Control (DPA)	329a	3.9a	2.6c	8	91b	44
2. CaCl ₂ 2%, 25°C	406b	4.6b	4.6b	4	3a	63
6. CaCl ₂ 2%, 0°C	502c	4.2b	4.3b	7	15a	57
7. CaCl ₂ 2%, 0°C + 0.01% L-77	523c	4.6b	4.7b	11	10a	67
8. CaCl ₂ 2%, 0°C + 0.1% L-77	587d	4.5b	4.7b	5	12a	60
				N.S.		N.S.
1981, 7 mo s at -1°C						
1. Control (DPA)	344a	4.7	4.6	24a	47b	16
2. CaCl ₂ 2%, 25°C	350a	4.7	4.5	14b	22a	25
6. CaCl ₂ 2%, 0°C	392b	5.0	4.6	16ab	25a	17
8. CaCl ₂ 2%, 0°C + 0.1% L-77	392b	4.8	4.7	20a	30a	10
		N.S.	N.S.			N.S.

a,b,c - Mean separation in columns for each experiment by Duncan's Multiple Range Test, 5% level.

c) Storage conditions. In order to ascertain whether the internal breakdown occurring in the 'Calville de San Sauveur' cultivar is induced by senescence or chilling, and if the latter, then amenable to treatment by calcium, fruit was stored in a controlled atmosphere at 0°C and at -1°C. BP development was unaffected by storage temperature, and the effect on IB and CF was inconclusive (Table 3). In 1979, CF tended to be somewhat higher at -1°C and with calcium treatment, but in 1981, the lower temperature significantly reduced the incidence of CF and calcium had no effect. On the other hand, IB was induced by low temperature storage in 1981 and its extent was reduced by calcium treatments. Fruit firmness was greater at 0°C than at -1°C, due to the reduced IB. Although the disorder IB occurred more or less equally at both storage temperatures in 1979, it was still significantly reduced by calcium treatments.

Discussion

A number of workers have found a correlation between the Ca level in apples and physiological disorders such as BP (10,25,26). Martin et al. (10) and Turner et al. (25) found that in 'Cleopatra' apples statistics indicated that Ca played the predominant role in inhibiting BP and that it was also the only mineral of importance as an indicator of other disorders. Van Lune and van Goor (26) found a correlation between K/Ca level, (K & Mg)/Ca level, as well as Ca level alone and BP. Lewis et al. (6) found in mineral uptake that Ca and K showed an inverse relationship. If fruit increased their Ca uptake, the K content decreased and the incidence of BP decreased as well. Apparently, there are exchange-binding sites in the cell for these two elements. Our research does not support the findings that Ca content can

Table 3. The effect of storage temperatures on physiological disorders in cv. 'Calville de San Sauveur' apples, and their response to calcium treatments

Postharvest treatment	Firmness at removal		IB		CF	
	from storage		(%)		index	
	0°C	-1°C	0°C	-1°C	0°C	-1°C
<u>1979 season</u>						
Control (DPA)	5.6	5.6	32b	17b	0	15
4% CaCl ₂ + 0.3% Keltrol	5.8	5.9	0a	0a	16	25
	N.S.				N.S.	
<u>1981 season</u>						
Control (DPA)	5.1ab	4.7a	2a	47c	58c	20ab
2% CaCl ₂ , 25°C	5.2b	4.7a	0a	22b	33bc	25ab
2% CaCl ₂ , 0°C	5.4b	5.0ab	0a	25b	53c	0a

a,b,c - Mean separation in columns for each experiment by Duncan's Multiple Range Test, 5% level.

be a reliable indicator for susceptibility to physiological disorders for the 'Calville de San Sauveur' cultivar. The normal Ca levels of the fruit are 300 ppm or higher, and they are quite susceptible to storage disorders, whereas the researchers mentioned here suggest that raising Ca level above 200 ppm will successfully control these disorders.

We also did not find that fruit quality was increasingly improved by successively increasing the Ca level. This is in agreement with Scott and Wills (19) who dipped 'Jonathan' and 'Cox's Orange Pippin' apples in Ca and achieved good control of BP. However, a longer dip raised the fruit Ca level more, without a further reduction in BP.

In cv. 'Calville de San Sauveur', sprays during the growing season were not particularly effective in controlling storage disorders, although they did sometimes increase fruit firmness and acidity. Doubling the number of $\text{Ca}(\text{NO}_3)_2$ sprays increased fruit N without increasing Ca. Lewis et al. (6) found that increasing fruit N increased BP. Sprays containing P were in some cases detrimental to fruit quality in this study. There are conflicting reports on the benefit of calcium phosphate sprays. Lewis et al. (6) found BP not affected by doubling P in 'Merton Worcester' apples. In 'Cox's Orange Pippin', increasing the P content reduced IB and BP (4). However, Turner et al. (25) found that a spray containing both Ca and P increased BP more than did a spray containing Ca alone, although this was still less than the amount of BP in unsprayed fruit. This is what was found here in the cv. 'Calville de San Sauveur'.

The different methods of postharvest dipping show more promise in affecting storage quality. Thickeners have been used successfully by a number of researchers (2,3,7,11,14,15,27). All except Betts and Bramlage (2) found

That adding Keltrol to a 4% CaCl_2 dip solution increased Ca uptake, fruit firmness, and in some cases, storage quality. In our experiments, although increasing the calcium concentrations and adding Keltrol improved uptake, no difference was found in the keeping quality of the fruit between dips of 2 or 4% CaCl_2 , with or without Keltrol. An improvement in Ca uptake was obtained also when surfactants were used, whereas previous researchers had found that Ca uptake decreases in the presence of surfactants (7,15). Here again, there was no concomitant benefit to fruit quality.

Utilizing a cold dip solution and uncooled fruit to create a local internal vacuum and facilitate Ca penetration has been tried on various cultivars (5,7,19): 'Jonathan', 'Cox's Orange Pippin', 'Spartan', 'McIntosh', and 'Golden Delicious'. Lee and Dewey (5) found a linear relationship between the increase in weight of the fruits submerged in Ca solutions and measured increase in the Ca content of the flesh. A linear relationship was also found in the temperature differential between the fruit and the solution on the one hand, and the amount of solution taken up by the fruit on the other hand, using fruit held at 20°C or below. Our experiments were successful in raising the Ca level and increasing fruit firmness by this method, although less successful in improving storage quality.

In summary, we can say that although it appears that calcium plays a part in alleviating BP and IB of the 'Calville de San Sauveur' cultivar, it is not the sole factor responsible for the development of these disorders. Above a certain level of calcium within the fruit, other, as yet unknown, factors involved in disease development become predominant. Our problem is therefore no longer to increase the uptake of calcium by fruits (the methods we have at present are quite effective), but to discover the other relevant factors involved.

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3.2. Calcium effects on physiology of fruit discs

In a study of the fundamental cellular processes affected by calcium, it is necessary to be certain that the element has been uniformly distributed throughout the tissue. Application to the intact fruit is unlikely to achieve this condition, even when pressure or vacuum infiltration are employed. Tissue discs or plugs immersed in calcium salt solution, with or without vacuum infiltration, are more likely to ensure that calcium can reach all cells, and this method was therefore employed for the study of calcium effects on respiration and ethylene evolution. In some instances calcium was applied, for comparison with intact fruits, by pressure or vacuum infiltration, and measurements were made on discs or plugs excised from the treated fruits.

3.2.1. Cyanide insensitive respiration in apple fruits

Apple fruits can be kept in storage for many months at temperatures between -1°C and 3°C , depending on the cultivar. During this time respiration, along with other metabolic functions, is greatly depressed and the ATP requirement is small. However, even at these low temperatures, reducing compounds are needed in order to prevent oxidative activity which leads to ageing. The regeneration of NADPH and NADH through malic enzyme or the pentose pathway requires respiration. It has been shown in pear buds that during the period of winter dormancy the alternate pathway of respiration is more active than the cytochrome pathway, and is less affected by low temperatures. As the buds prepare to break dormancy, respiration switches to the cytochrome pathway (2). Apples in storage may also utilize this alternate pathway to maintain cellular functions.

Calcium has been found to improve apple storage through a mechanism which as yet is poorly understood. The site of its involvement in preventing

internal breakdown would appear to be the cellular membrane. Marinos (3) concluded from his ultrastructural study of calcium-deficient plants that calcium is essential for the maintenance of cell membranes, a role that has also been used to explain its effect on respiration in fruits (1). In the present study we have investigated the possibility that calcium, in attaching to the mitochondrial membranes, alters the balance between the functioning of the normal cytochrome pathway and the alternate pathway of respiration, and in this way affects apple storage. We have also sought to determine the extent of involvement of the alternate pathway during different stages of apple ripening.

Materials and Methods

Apples, Malus sylvestris cv. Gala and cv. Calville de San Sauveur, were used, the former in the ripening experiments and the latter in the experiments and various inhibitors. Gala apples were placed individually in one-liter containers, and their respiration and ethylene production monitored automatically using a computerized system developed by Watada and Massie (9). Preclimacteric apples were defined as those whose ethylene production was around 1 $\mu\text{l/kg}$ fresh weight/h and did not show a daily increase. Apples were regarded as midclimacteric if their ethylene levels were increasing daily within a range of 7 to 11 $\mu\text{l/kg}$ fresh weight/h, and as climacteric if their ethylene levels were high - 18 to 22 $\mu\text{l/kg}$ fresh weight/h - and beginning to decline.

The apples chosen for the experiment were removed from the automatic monitor, cut in half lengthwise, and sealed on the cut surface with paraffin. One half was put into a modified pressure cooker with the skin side down in

4% CaCl_2 and subjected to a pressure of 6 p.s.i. for 10 min to allow the infiltration of calcium. The other half was not subjected to calcium treatment. Cylindrical apple portions were cut out using a cork borer, and then sliced into discs such that 6 discs weighed 1 g. The discs were placed in Gilson respiration flasks containing appropriate solutions as detailed below. The flasks were placed in a 25°C water bath in a Gilson Differential Respirometer. The flasks were shaken for 30 min to achieve temperature equilibration, and oxygen readings were taken at half-hour intervals for two hours.

Each flask contained 3 ml of phosphate buffer, 0.05 M pH 6.5. The center well contained 0.2 ml of 10% KOH to absorb CO_2 . KCN, when used, was also added to the center well (4). SHAM (an inhibitor of the alternate pathway) and the uncoupler CCCP were dissolved in ethanol and added to the phosphate buffer; and where used, the control flasks received an equivalent amount of ethanol only. Antimycin A and propyl gallate, inhibitors of the cytochrome pathway and the alternate pathway, respectively, were dissolved in the surfactant DMSO, while the control flasks received DMSO only. Concentrations used are mentioned in the text.

The extent of operation of the alternate pathway (the factor ρ) was determined using the method developed by Theologis and Laties (6). Respiration was first measured in the presence of increasing concentration of SHAM. Following the addition of a very low concentration of KCN (5 μM) to block the utilization of electrons by the cytochrome pathway, respiration was again measured in the presence of increasing concentrations of SHAM. Respiration was then monitored in the presence of high concentrations of SHAM and KCN (1.7 μM and 2 mM, respectively) to determine the amount of residual respiration,

and this was subtracted from each of the first two respiration curves. The first curve, V'_T , represents the contribution of the cytochrome pathway to total respiration plus the contribution of the alternate pathway at various concentrations of SHAM. The second curve (SHAM plus KCN) is $g(i)$, which represents the contribution of the alternate pathway as it underwent inhibition by increasing concentrations of SHAM. A plot of V'_T against $g(i)$ should give a straight line having a slope of ρ and the intercept V_{cyt} at $g(i) = 0$. The equation expressing this is:

$$V_T = \rho \cdot g(i) + V_{cyt} + V_{res}$$

where: V_T is total respiration, $g(i)$ is the contribution of the alternate pathway, ρ represents the fraction of the alternate pathway which is functioning, V_{cyt} is the cytochrome pathway and V_{res} is residual respiration after both cytochrome and alternate pathways have been fully inhibited. Subtracting V_{res} from both sides gives:

$$V'_T = \rho \cdot g(i) + V_{cyt}$$

Results and Discussion

Calville de San Sauveur apple discs, coupled and uncoupled, were titrated against SHAM with or without KCN. The results are presented in Figure 1. It can be seen that even in the absence of cyanide SHAM inhibited respiration, while in the presence of a low cyanide concentration the effect of SHAM was even more pronounced. In discs without the uncoupler CCCP, residual respiration measured in the presence of 1.7 mM SHAM and 2 mM KCN was 2 μ l/g fresh weight/h; in the presence of CCCP it was 8 μ l/g fresh weight/h. Theologis and Laties found that residual respiration was absent in mitochondrial

Figure 1. The effect of respiratory inhibitors KCN and SHAM and the uncoupler CCCP on apple fruit disc respiration. A, the effect of increasing concentrations of SHAM in the presence of 0, 0.005, and 2 mM KCN; B, the effect of 0.01 mM CCCP on the experiment in A; C, calculation of V'_T and $g(i)$ from the data presented in A; D, calculation of V'_T and $g(i)$ from the data presented in B.

preparations, and considered it to be extra-mitochondrial in origin (6). We found that in the presence of CCCP respiration was enhanced by 67% over the control. When the data in figure 1A and B were replotted as V'_T against $g(i)$ (as explained in Materials and Methods), the results shown in Figure 1C and D were obtained. In figure 1C the straight line has a slope, ρ , of 0.55, indicating that the alternate pathway was operating at 55% of its capacity. In uncoupled respiration the contribution of the alternate pathway increased to a value of 0.9, indicating almost maximal engagement of the alternate pathway.

Similar experiments were performed on Gala apples, some of which were subjected to calcium infiltration at different stages of ripening. Table I summarizes the respiratory fluxes of these apples and shows that total respiration (V_T) increased as the apple matured. Although the contribution of the alternate pathway became more pronounced in the post-climacteric fruit, the climacteric rise in respiration cannot be attributed to an increased involvement of this pathway in apples, as has also been shown for bananas and avocados (7). The maximum possible rate of respiration through the alternate pathway was fairly similar to its maximum rate through the cytochrome pathway at all stages of ripening (comparing V_{alt} and V_{cyt}).

Calcium infiltration slightly inhibited respiration, a finding which has been reported previously (1), but it also altered the relative contributions of the cytochrome and the alternate pathways to the total respiration, V_T . The effect of calcium infiltration on ρ was greatest in preclimacteric apples, where it resulted in 25% of the alternate pathway being utilized as compared with 5% in untreated apples. In apples measured just after the climacteric peak (postclimacteric) the ρ factor was the same (0.1) in both treated and

TABLE I. Effect of calcium on apples at different stages of ripeness on cytochrome pathway and alternate pathway respiration

	V_T	V_{alt}	ρ	$\rho \times V_{alt}$	V_{cyt}	V_{res}
	$\mu\text{l O}_2/\text{g fr wt/h}$				$\mu\text{l O}_2/\text{g fr wt/h}$	
Preclimacteric						
-Ca	27.4	24.2	.05	1.2	20.4	5.8
+Ca	24.5	24.0	.25	6.9	12.7	5.8
Midclimacteric						
-Ca	45.2	35.5	.04	1.4	38.1	5.8
+Ca	36.4	33.0	.1	3.3	31.5	1.6
Postclimacteric						
-Ca	43.3	44.0	.15	4.4	35.5	3.4
+Ca	38.4	43.0	.15	4.3	32.0	2.1

untreated sections, representing a rise in use of the alternate pathway in the untreated apples and a fall in the treated ones.

The effects on the apple discs of various respiratory inhibitors were tested (Table II). The inhibitors of the cytochrome pathway, KCN and antimycin A, slightly enhanced respiration at the highest concentrations used. This suggests that when the alternate pathway is inhibited in apple mitochondria the cytochrome pathway cannot entirely compensate for it, whereas when the cytochrome pathway is inhibited the alternate pathway is able to compensate.

TABLE II. Effect of various respiratory inhibitors
on apple tissue respiration

Experiment	$\mu\text{l O}_2/\text{g fr wt/h}$
A. Control	43.9 ± 0.42
KCN, 2 mM	49.0 ± 3.69
B. Control	37.4 ± 1.92
Antimycin A, 2 μM	47.3 ± 1.69
C. Control	46.2 ± 0.99
SHAM, 1.7 mM	41.0 ± 2.25
D. Control	40.8 ± 0.59
Propyl gallate, 1 mM	36.6 ± 2.85

These data show that calcium, besides decreasing respiration in apple fruit, also allows more intensive functioning of the alternate pathway. The

3.2.2. Deferral of senescence in calcium treated apple fruit discs

Having shown in the previous section that the inhibition of ethylene evolution resulting from infiltration of calcium into whole apple fruits (see section 3.1), is possibly enacted via a direct effect of calcium on membrane fluidity, two questions were asked: 1) Can calcium delay senescence of the fruit tissue by inhibiting ethylene evolution? 2) Is the effect specific to calcium? Previous observations had shown that calcium treated apple discs retained their firmness and their colour during 24 hours incubation, whereas non-treated discs turned brown and lost their coherence, becoming soft and mushy. The processes underlying these observations were therefore studied.

Materials and Methods

Apple fruit discs were excised and incubated for 24 hours with or without calcium or magnesium as described in the previous section. Ethylene evolution was also monitored in the same manner at 2, 4, 6 and 24 hours after excision. Browning potential of the tissue was monitored after 24 hours by assaying the amount of phenols accumulated and the activity of the enzymes polyphenoloxidase (PPO) and peroxidase (PO). The softening process was monitored by assaying the changes in the pectic fractions and the activity of the enzyme polygalacturonase (PG).

Results and Discussion

1) Ethylene production

In contrast to the increased rate of ethylene production, which is a sign of maturation and senescence in intact fruits, aged apple fruit discs show a

decline in ethylene evolution. This decline is considered to be an indication of the loss of membrane integrity, which is necessary for the activity of the ethylene synthesising system. Figure 1 shows that though Ca^{2+} initially inhibited ethylene production by excised discs, the decline in its production which accompanied ageing of the discs was decelerated in the presence of Ca^{2+} so that, after 24 hours, treated discs were producing 4 times as much ethylene as the control discs. Though a similar effect could also be obtained with Mg^{2+} , it was of much lower intensity (Table 1). Generally, post-climacteric fruit responded to both Ca^{2+} and Mg^{2+} to a greater degree than pre- or mid-climacteric fruit, though with regard to Mg^{2+} this was not obvious from the data presented in table 1.

Table 1. Magnesium and calcium effect on ethylene production from apple discs after 24 h incubation at 25°C

Ion	Conc. (mM)	Ethylene production (% of control)	
		Mid-Clim.	Post-Clim.
Ca	1	100	232
	10	136	566
	100	1200	3133
Mg	1	96	93
	10	115	120
	100	240	184

2) Tissue browning

The inhibition of apple slice browning in the presence of CaCl_2 solutions

Fig. 1. The effect of calcium in the incubation medium on ethylene^{production} by apple discs from pre-climacteric and post-climacteric fruits.

might be a result of the inhibition of polyphenol oxidase at concentrations of 1 mM or more (Fig. 2). Mg^{2+} had no such effect on either browning or PPO activity. Treatment of tissue slices incubated in 10 mM $CaCl_2$ solution with 10 ppm ethylene, did not affect the rate of tissue browning, indicating that the inhibition of ethylene production by $CaCl_2$ was not responsible for the inhibition of tissue browning.

Apart from the direct effect of calcium on PPO activity, inhibited browning of tissues might also be a result of the reduced permeability of the membranes. The 'tightening' of the membranes might inhibit the increased contact between the enzyme and its substrate, which naturally occurs upon excision of the tissue slices.

3) Tissue softening

The inhibited softening of apple tissue slices incubated in a calcium salt solution was due to an increase in the EDTA soluble pectin fraction (VSP), which is predominantly calcium pectate (Table 2). It appears that the calcium insolubilizes the water-soluble pectin (WSP). With the Calville de San Sauveur cv. this was more apparent than with the Anna cv. In the latter case, there was a higher content of total pectic substances (TPS) due to the rise in the VSP fraction, as the amounts of WSP and insoluble pectin (ISP) remained unaltered. A possible explanation for this is that the natural depolymerization of WSP to alcohol soluble galacturonic acid by exo-polygalacturonase during the incubation period was inhibited by calcium and the WSP, which would have accumulated, was instead precipitated as calcium pectate. A tendency, not statistically significant, of increasing amounts of WSP was indeed observed in preclimacteric fruit with increasing calcium concentrations.

Depletion of calcium within Golden Delicious tissue slices, achieved by incubation with 0.1 M EDTA, caused a softening and disintegration of the tissue.

Fig. 2. The effect of calcium chloride on activity of polyphenoloxidase from apple fruit.

Table 2. The effect of CaCl_2 incubation media on the pectin fractions extracted from apple pulp discs

Cultivar	CaCl_2 mM	(Pectic fraction as percent of fresh weight)							
		W.S.P.		V.S.P.		I.S.P.		T.S.P.	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Anna	0	0.239 ^{ab}	0.296 ^y	0.016 ^a	0.044 ^y	0.370 ^a	0.221 ^z	0.625 ^a	0.561 ^z
	1	0.214 ^{ab}	0.261 ^y	0.027 ^b	0.059 ^y	0.355 ^a	0.211 ^z	0.596 ^a	0.531 ^z
	5	0.204 ^a	0.268 ^y	0.027 ^b	0.086 ^z	0.373 ^a	0.234 ^z	0.604 ^a	0.571 ^z
	10	0.273 ^a	0.282 ^y	0.036 ^b	0.082 ^z	0.424 ^a	0.205 ^z	0.733 ^b	0.568 ^z
Calville de San Sauveur	0	0.038 ^b	0.031 ^a	0.260 ^b	0.329 ^b	0.244 ^a	0.256 ^a	0.305 ^{ab}	
	1	0.012 ^a	0.029 ^{ab}	0.203 ^a					
	5	0.010 ^a	0.041 ^{ab}	0.205 ^a					
	10	0.009 ^a	0.045 ^b	0.251 ^{ab}					

WSP - water soluble pectin; VSP - EDTA (versene)soluble pectin; ISP - insoluble pectin;

TPS - Total pectic substances; Pre - pre-climacteric fruit; Post - post-climacteric fruit.

a,b,c or x,y,z - Figures in each column are significantly different at $p = 0.05$ according to Duncan's multiple range test.

Fractionation of the pectic substances (Table 3) showed an increase in the WSP at the expense of the VSP, as was to be expected when calcium was removed from the tissue and vice-versa, when calcium was added to the tissue. The ISP and TPS were not affected by these treatments.

Table 3. The effect of calcium on the fractionation of pectic substances in apple tissue discs incubated in CaCl_2 or EDTA. (Pectic fraction as percent of fresh weight.)

Treatment	(Pectic fraction as percent of fresh weight)							
	WSP		VSP		ISP		TSP	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Control (0.7 M mannitol)	0.042 ^b	0.032 ^y	0.034 ^b	0.025 ^y	0.183 ^{ab}	0.154 ^a	0.259 ^a	0.212 ^a
10 mM CaCl_2	0.011 ^a	0.009 ^x	0.051 ^c	0.051 ^z	0.239 ^b	0.173 ^a	0.301 ^b	0.233 ^a
10 mM EDTA	0.067 ^c	0.061 ^z	0.010 ^a	0.011 ^x	0.207 ^a	0.157 ^a	0.284 ^{ab}	0.230 ^a

Calcium content of the pectic fractions (ppm)

Control (0.7 M mannitol)	1.04 ^a	0.99 ^y	9.87 ^b	7.22 ^y	9.78 ^a	7.34 ^y
10 mM CaCl_2	0.86 ^a	0.81 ^y	15.33 ^c	15.41 ^z	9.37 ^a	7.68 ^{yz}
10 mM EDTA	1.33 ^b	1.62 ^z	6.52 ^a	6.94 ^y	9.64 ^a	8.93 ^z

WSP - water soluble pectin; VSP - EDTA (versene) soluble pectin ISP - insoluble pectin:
 TPS - total pectic substances; Pre - pre-climacteric fruit; Post - post-climacteric fruit.
 a,b,c or x,y,z - Figures in each column significantly different at $p = 0.05$ according to Duncan's multiple range test.

Assay of exo- and endo-polygalacturonase from these tissue discs showed that the softening of the incubated tissue during 24 hours incubation was not a result of inhibited or enhanced enzyme activity (Table 4), as no significant difference in activity was discernable on a fresh weight basis. Both CaCl_2 and EDTA significantly reduced the amount of protein in the enzyme extracts, so that calculation of the specific activity of both enzymes showed significant increases in both treatments compared to the control. As tissue discs for the same fruits were used for all treatments, the enzymal activity calculated on a fresh weight basis appears to be a more legitimate basis for comparison in this case.

Table 4. Polygalacturonase activity in Golden Delicious tissue discs incubated for 24 hours in CaCl_2 or EDTA

Treatment	Endo-Pg (units)		Exo-PG (units)	
	<u>Pre</u>	<u>Post</u>	<u>Pre</u>	<u>Post</u>
Control (0.7 M mannitol)	4.7	3.3	103.3	61.3
10 mM CaCl_2	10.7	2.9	109.1	61.1
10 mM EDTA	12.3	4.2	114.1	58.6

Endo-PG unit = 1% reduction in viscosity of 1% sodium polypectate in 1 h by extract from 1 g tissue.

Exo-PG unit = $1\mu\text{M}$ galacturonic acid released by extract from 1 g fresh tissue within 1 hour.

The conclusion to be drawn from these experiments is that calcium

maintains tissue firmness and integrity by insolubilising the soluble pectic substances in the cell wall of the fruit.

The delayed senescence of the apple fruit, which has been found by many workers, could therefore be the result of calcium effects on a number of parameters, which include 1) inhibition of ethylene production, 2) insolubilization of water soluble pectin in the cell wall and 3) inhibition of certain enzyme activities, such as polyphenol-oxidase and ATPase (see below).

3.2.3. Temperature-dependent inhibitory effects of calcium on ethylene biosynthesis in apple discs

In order to elucidate whether the effect of calcium and magnesium ions on the membrane bound biosynthetic pathway of ethylene evolution is connected with their binding capacity as divalent cations, a comparison was drawn between the effects of the polyvalent cation spermine and of calcium. The results have already been published and therefore a copy of the paper is herewith presented.

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239

TEMPERATURE-DEPENDENT INHIBITORY EFFECTS OF CALCIUM AND SPERMINE ON ETHYLENE BIOSYNTHESIS IN APPLE DISCS CORRELATE WITH CHANGES IN MICROSOMAL MEMBRANE MICROVISCOSITY*

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SUMMARY

Inhibition of ethylene biosynthesis in apple fruit discs by calcium and spermine differed in character in at least three respects: (i) as the fruit ripened inhibition due to spermine decreased whereas that by calcium increased; (ii) inhibition by calcium was transitory, whereas that by spermine was persistent; (iii) at temperatures below 12°C calcium inhibited more than spermine whereas above 12°C the reverse was true.

Temperature-dependence of the spermine and calcium effect on ethylene biosynthesis was correlated with specific changes induced by them in the microviscosity of microsomal membranes of apple tissue. Pretreatment with calcium resulted in lower microviscosity of the membranes compared to those of buffer-treated discs and in both cases microviscosity increased with increase in temperature of incubating solution containing fruit discs. Spermine stabilized membrane fluidity in apple discs incubated at temperatures above 4°C, thereby preventing the natural increase in microviscosity which occurred with increasing incubation temperature. These effects of calcium and spermine on membrane microviscosity required preincubation of fruit discs with the test compound since addition of spermine or calcium directly to microsomal membranes immediately before assay had no effect on their microviscosity.

3.3. Calcium effects on biochemical and physical properties of cellular membranes.

An indication that the inhibition by calcium of ethylene biosynthesis is effected via its action on membrane micro-viscosity was presented in the previous chapter. The effect of calcium on membrane properties was further studied with regard to physical properties such as membrane permeability and microviscosity and chemical properties such as enzyme activity and lipid composition. In order to study the effects of calcium on these parameters it was necessary first of all to define the changes which naturally occur in the properties of cellular membranes from apple fruits during ripening and storage.

3.3.1. Membranal changes during the ripening of apple fruit.

During fruit ripening, similarly to senescence of other plant organs, progressive loss of membrane function occurs. It has been suggested that the changes in membrane structures at the onset of ripening lead to increased permeability of membranes resulting in a decompartmentalisation of cellular components and increased catabolic processes causing eventual senescence (17). In climacteric fruits this becomes pronounced in the climacteric and post-climacteric stages.

The passive permeability characteristics of lipoprotein membranes are governed by the lipid component (7) and a change in permeability should be reflected by a change in either the composition or arrangement of membrane lipids. This in turn should be seen as a change in the membrane fluidity. There are several reports indicating a correspondence between changes in membrane function and changes in membrane fluidity (8,9,19). There is also evidence of changes in membrane viscosity - the converse of fluidity - as plant tissues senesce (13,2), but no work has been done on changes in

ripening fruit.

This paper describes the lipid composition of the apple fruit as it ripens, and compares it with changes in the membrane viscosity and permeability. Because of practical difficulties associated with isolation of adequate amounts of pure plasmalemma membrane, we have worked with a microsomal fraction which was termed "plasmalemma enriched" fraction (11). Other researchers have estimated that microsomal membranes consist of around 70% plasmalemma (11).

Materials and Methods

Apples, Malus sylvestris, cv. Calville de San Sauveur, were used for the experiments. Individual apples were placed in one-liter containers at 20°C with air flow of 60 ml/min and their respiration and ethylene were measured on a F10 gas chromatograph (Packard) with an alumina column. At the appropriate stage of ripeness, as shown by the fruit's ethylene production pattern, apples were removed for analysis.

The apples were peeled, the core removed, and 50 flesh ground in a blender for 1 min in 100 ml of 250 mM sucrose, 25 mM tris, 3 mM EDTA and 1mM dithiothreitol. The slurry was filtered through 6 layers of cheesecloth, brought to pH 7.2 with NaOH and centrifuged for 15 min at 13000 x g. The supernatant was centrifuged for 30 min at 80000 x g and the pellet resuspended in 1 ml of 10 mM Tris-HCl (pH 7.2), 1mM dithiothreitol. Protein was determined by the method of Bradford (5).

Microviscosity was measured in an Elcint polarized fluorometer using the fluorescent membrane probe 1,6 diphenyl-1,3,5-hexatriene (DPH) after the method of Shinitzky and Barenholz (18). A sample containing 10-20 µg protein/ml was incubated with 1 µM DPH in 0.1 M phosphate buffer pH 7.2 for

20 min in a shaker at room temperature. The polarized fluorescence was then read at 25°C. The formula for calculating the apparent microviscosity ($\bar{\eta}$) measured by the fluorescent probe DPH is

$$\bar{\eta} = \frac{2\rho}{0.46-\rho} = \frac{\ell_{||}/\ell_{\perp}-1}{0.73-0.27\cdot\ell_{||}/\ell_{\perp}}$$

where ρ is the fluorescence polarization and $\ell_{||}/\ell_{\perp}$ is the ratio of the light emission which is oriented parallel or perpendicular to the direction of polarization of the excitation light. A full derivation of this formula can be found in Shintzky and Barenholtz (18).

After measuring membrane microviscosity the microsomal fraction was extracted for lipids using a chloroform:methanol (8:5 v/v) mixture according to the method of Renkonen et al. (16). The total membrane lipid fraction obtained was dried in a rotarivaporator, redissolved in 1 ml hexane, and stored under N_2 at -20°C.

Aliquots were taken for total phospholipid and sterol content. The mole content of phospholipids was determined by phosphorus analysis after conversion to phosphoric acid with 70% $HClO_4$ at 190°C for 60 min by the method of Bottcher et al. (4). Sterol in the lipid extracts was determined colorimetrically by the method of Chiamori and Henri (6) using cholesterol as a standard.

A final aliquot of the total lipid fraction was used for fatty acid analysis. It was run on a precoated silica gel G TLC plate (Merck) 250 μm thickness. The plates were run in hexane:ether:acetic acid (80:20;1) for 2-4 hours and then air dried. The origin containing the phospholipids was removed, washed with 5 ml chloroform:methanol,(1:1), and then dried under N_2 . The phospholipid fraction was prepared for fatty acid analysis by methylation in 4 ml 5% H_2SO_4 in methanol (v/v) held at 80°C for 1 h. The

fatty acid methyl esters were partitioned into hexane and analyzed using a Packard FID gas chromatograph equipped with temperature programming. Separations were done on a 6 ft 2 mm ID glass column (Supelco= packed with GP 10% SP-2330 on 100/120 Chromosorb W. The heating program was 160°-100°C, 5°/min. Reference methylated fatty acids were run for identification purposes.

Leakage of potassium was measured by slicing apples at appropriate stages of ripeness and with a cork borer extracting 8 discs weighing about 2 g. These were incubated in 25 ml of 0.6 M mannitol for 2 h and the leakage into the medium measured with a potassium ion electrode (Orion). The discs were frozen, thawed and boiled for 20 min to extract total potassium and this was measured in the same manner.

Results

The course of apple fruit ripening was followed by monitoring the ethylene production of the fruit. The pattern was that of a typical climacteric fruit - ethylene increased to a peak production and then declined (Table I). At the different stages of this ripening process the viscosity and semipermeable properties of microsomal membranes were measured (Table I). It is seen that there are no viscosity changes in the membranes until the fruit reaches its peak climacteric stage. Then there is a sudden increase in membrane viscosity and this increases further as the fruit enters the post climacteric phase. In parallel the membranes become more permeable to potassium ions as the climacteric is reached.

The changes in lipid components of the membranes show a similar picture. The ratio of sterol to phospholipid (Table II) does not alter in the preclimacteric

Table I. Ethylene production of apples at different stages of ripening and the microviscosity of the microsomal membranes of these apples

Ripening stage	Expt.	C ₂ H ₄ μl/kg apple/h	Microviscosity η-poise	% K ⁺ leakage in 2 h
Early climacteric	A	54	3.17	20.5
	B	51	3.75	20.6
Midclimacteric	A	61	3.11	-
	B	67	3.67	-
Peak climacteric	A	100	4.05	23.2
	B	102	4.30	24.2
Post climacteric	A	55	4.45	23.9
	B	27	4.67	25.7

Table II. Effect of stage of ripeness of apple on the sterol and phospholipid content of microsomal membranes

Stage of ripeness	Sterol μmoles/50 g fresh weight	Phospholipid g fresh weight	St/PL ratio
Early climacteric	1.08	6.77	0.16
Midclimacteric	1.39	8.91	0.16
Peak climacteric	1.48	6.43	0.23
Post climacteric	2.87	8.75	0.33

and midclimacteric stages, but increases in the climacteric and continues to rise in the postclimacteric. This change in ratio is caused by an increase in the sterol content of the membranes rather than a decrease in the phospholipid fraction.

There are also changes occurring in the fatty acids of the membrane phospholipids as seen in Table III. The two major changes are in palmytic (16:0) and linoleic (18:2), the former increasing as the fruit ripens and the latter decreasing. In experiment A the major change in these two fatty acids is between the climacteric and post climacteric stages. In experiment B the change begins before the climacteric peak and continues into the post climacteric.

By expressing the fatty acid data as various ratios found in the literature (Table III), a clearer pattern emerges. The ratio of linoleinic (18:3) to linoleic (18:2) acid does not change in a clear cut manner as the fruit ripens. The ratio of saturated to unsaturated fatty acids remains relatively constant until the post-climacteric stage when there is a large increase. This is a reflection not just of increase in palmytic acid as a percent of the total, but decreases in all three of the unsaturated 18 carbon chain fatty acids. However, perhaps the best reflection of the changes in the components of the phospholipids fatty acids is the double bond index. This shows a steady decrease from stage to stage of fruit ripeness which means a steady decrease in degree of unsaturation of the fatty acids.

Discussion

In apple fruits there are changes in the lipid components of the microsomal membranes as the fruit ripens. The most dramatic change takes place between the midclimacteric and climacteric peaks. This is when there is a sharp increase in membrane viscosity, and the membrane sterol to phospholipid

ratio mirrors this change. The fact that there are no large changes at earlier stages of ripening suggests that these membrane changes are a consequence and not a cause of the ripening process as had been suggested previously (17).

Between the climacteric peak and post climacteric stage the membrane viscosity continues to increase and the sterol content of the membranes to rise. This transition is also accompanied by a large change in the ratio of saturated to unsaturated fatty acids in the membrane phospholipids.

Ripening of fruits has many processes in common with senescence of other plant organs. From the literature it appears that there are similarities as well as differences in the behavior of the membrane lipids. In work done on senescing flower tissue it was found that phospholipid content began to decline even before signs of senescence were evident (1,3). In flower petals this decrease in phospholipid content was accompanied by an increase in membrane permeability (20). Borochoy et al. (2) found that as flowers aged the membrane viscosity increased and this was correlated with an increase in the free sterol:phospholipid ratio. This was due to a decrease in the phospholipid content, while the sterol fraction remained essentially constant. It is of interest to note that their ratios are higher than those found in apple fruit, varying from .28 in young petals to .44 in aged petals. In seeds it has been well documented that as they age there is an increase in solute leakage and a decrease in phospholipid content (10,14,15).

Thompson's group, using x-ray diffraction, demonstrated in senescing bean cotyledons, as well as algae, that senescence of membranes involves a phase change whereby the proportion of gel to liquid-gel lipid progressively increases (13,21). This changed physical state of lipid represents a substantial decrease in membrane fluidity and contributes to loss of membrane function in senescing

tissue. In algae cultures the unsaturated to saturated fatty acid ratio of the membrane lipid, while fluctuating with age, did not show any consistent trend that could be related to the change in lipid membrane state. On the other hand, in bean cotyledons the neutral lipids, including sterol esters, were felt to be the cause of the membrane phase changes.

The general conclusion appears to be that the major changes in the lipid components of the membranes during senescence are in the sterol:phospholipid ratio. This may be due to loss of phospholipids, as in flowers and seeds, or as we found in ripening apple fruit, due to increase in sterol content. There have been thus far only negative reports on the involvement of the phospholipid fatty acids in the senescence-related changes in membrane function or structure. We have found, however, that in the transition from climacteric to post climacteric the increased saturation of fatty acids also contributes to increased membrane viscosity. In terms of the double bond index there are changes throughout the ripening process and in terms of saturated-unsaturated ratio there is a large increase in ratio at later stages of ripening. Therefore, we conclude that both the sterols and the phospholipids play an important role in the membrane changes that accompany ripening.

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3.3.2. Membranal changes during apple storage.

Cellular membranes are thought to be the site of primary lesions in chilling injury (11,24). Interruption of membrane-associated metabolic activity accompanying lipid phase transitions is a principal effect of chilling damage at temperatures above freezing (11). Fukushima et al. (10) found that chilling injury of cucumber stored at 5°C was similar to that produced by hydrating the fruits, whereas chilling injury at 0°C was similar to stresses induced by dehydration.

Changes in both membrane lipid composition and viscosity have been observed in some plants during acclimation to variations in growth temperatures (14,18). These changes are thought to be a response of the cells to maintain optimal conditions for membrane-associated enzyme function (19). Typical mechanisms of viscosity regulation involve changes in fatty acid unsaturation and sterol composition of the cellular membranes. Homeoviscous regulation of membrane fluidity is well documented and is considered to be an important aspect of temperature acclimation in many animal and bacterial systems(13,22,26). The situation in plants is not so clear, and in fruit and vegetables stored at low temperatures it has not been investigated, to the best of our knowledge.

Apple is a fruit that is normally stored at low temperatures for a period of months after harvest. In controlled atmosphere storage can be up to 10 months. The difference between growth temperature of 25-35° and storage temperatures of -1°C undoubtedly requires adaptation on the part of the tissue. It was of interest to investigate this and to compare it to what is already known of whole plant acclimation to low temperature.

Materials and Methods

Apples, Malus sylvestris cv. Calville de San Sauveur, were used for the

experiments. The fruits were harvested during August 1981, and placed in both air and controlled atmosphere (CA) (2% O₂ and 1.5% CO₂) cold storage at -1°C for 4 and 6 months, respectively. Samples consisting of 5 apples were taken at harvest and after 7, 12, 16 and 24 weeks of storage. The microsomal membrane fraction was prepared from 50-100 g of apple flesh and the microviscosity of the membranes was measured. Then the lipids were extracted from the membranes and total sterols, total phospholipids, and fatty acid composition of the phospholipids were determined. At harvest and after 6 months of storage, membrane permeability was measured on apple discs by leakage of electrolytes into an iso-osmotic medium.

Preparation of microsomal membrane fraction: Apples were peeled, the core removed, and 50 or 100 g of fruit flesh was ground in a blender for 1 min in 100 or 200 ml of 250 mM sucrose, 25 mM Tris, 3 mM EDTA and 1 mM dithiothreitol. The slurry was filtered through eight layers of cheesecloth, brought to pH 7.2 with NaOH, and centrifuged for 15 min at 13,000 x g. The supernatant was centrifuged for 30 min at 80,000 x g and the pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.2), 1 mM dithiothreitol. Protein was determined by the method of Bradford (4).

Microviscosity measurement: Microviscosity was measured in an Elcint polarized fluorometer using the fluorescent membrane probe 1,6 diphenyl 1,3,5-hexatriene (DPH) after the method of Shinitzky and Barenholz (21), as reported by us previously (2).

Arrhenius plots of the microviscosity were performed by cooling the sample to +1°C, heating at the rate of 2°/min, and taking readings every 0.5°C. The temperature of the sample was measured by a thermocouple immersed in the sample. Each experiment was repeated twice.

Lipid extraction: Lipid extraction was performed in a chloroform:methanol (8:5, v/v) mixture according to Renkonen et al. (20). The membrane total lipid fraction obtained was dried in vacuo, redissolved in 1 ml hexane, and stored under N₂ at -20°C.

Sterol and phospholipid measurement; Aliquots from the total lipid fraction were taken for sterol and phospholipid content. The mole content of phospholipids was determined by phosphorus analysis after conversion to phosphoric acid with 70% HClO₄ at 190°C for 60 min by the method of Bottcher et al. (3). A molecular weight of 750 for phospholipid was used for calculation purposes. Sterol in the lipid extracts was determined colorimetrically by the method of Chiamori and Henri (5), using cholesterol as a standard.

Fatty acid determination: A further aliquot of the total lipid fraction was used for fatty acid analysis. It was run on a precoated silica gel G TLC plate (Merck), 250 µm in thickness. The plates were run in hexane:ether:acetic acid (80:20:1) for 2-4 hours and then air-dried. The origin containing the phospholipids was removed, washed with 5 ml chloroform:methanol (1:1, v/v), and then dried under N₂. The phospholipid fraction was then prepared for fatty acid analysis by methylation in 5 ml of 5% H₂SO₄ in methanol (v/v) and incubated at 80°C for 1 h. The fatty acid methyl esters were partitioned into hexane and analyzed using a Packard FID gas chromatograph equipped for temperature programming. Separations were done on a 6-ft long, 2-mm ID glass column (Supelco) packed with GP 10% SP-2330 on 100/120 Chromosorb W. The heating program was 160-200°C, 5°/min. Reference fatty acid methyl esters were run for identification purposes.

Membrane permeability: Electrolytic leakage from apple discs was measured by monitoring the conductivity of the medium after various times of incubation

using an El-Hama Instruments conductivity bridge. Eight 1-cm discs of apple fruit weighing together about 2.0 g were washed in 0.6 M mannitol and placed in 25 ml of 0.6 M mannitol at 25°C. The conductivity of the incubation medium was measured after 1, 3 and 5 h of incubation at 25°C and compared with total electrolytes obtained by freezing and then boiling the discs. Results are expressed as percent of total electrolytes.

Results

Examination of the microsomal microviscosity and solute leakage from discs of apples used for the membrane preparation, shows that during storage the membrane viscosity decreased progressively, as did its permeability to electrolytes (Table 1). At harvest, membrane viscosity was high and the greatest decrease occurred between harvest and the first measurement at 7 weeks. Consequently, there was a slight further decrease as storage continued. The electrolyte leakage from apple discs also decreased from 21.5% at harvest, to 18.5% after 6 months storage in controlled atmosphere. This decrease in membrane viscosity could reflect adaptation of the fruit to storage conditions and, if so, should be reflected by changes in the membrane composition which might account for the reduced permeability.

Table II shows the phospholipid and sterol contents of the microsomal membrane fraction. There appears to be a small increase in sterols as storage progresses. The total phospholipid content, while fluctuating experimentally, shows essentially no change during the 6 months' storage period. The ratio of sterol to phospholipid shows an irregular but progressive increase, reflecting the change in sterol levels.

The fatty acid content of the membrane phospholipids is shown in

Table I. Membrane viscosity and leakage of electrolytes of apple fruit discs at harvest and during storage at -1°C. Data is presented with standard deviation

	Storage conditions	Microviscosity	% Leakage (5 h)
Harvest	-	4.38 ± .14	21.5 ± .57
7 weeks	air	3.85 ± .14	-
12 weeks	air	3.72 ± .19	-
16 weeks	air	3.65 ± .21	-
24 weeks	Controlled atmosphere	3.53 ± .21	18.8 ± .41

Table II. Changes in sterol and phospholipid contents of microsomal membrane fraction from apple fruit during storage. Data is presented with standard deviation.

	Storage conditions	Sterol μ moles	Phospholipid μ moles	Sterol/phospholipid ratio
Harvest	-	.736 ± .26	6.68 ± 2.78	0.116 ± .03
7 weeks	air	.433 ± .27	4.84 ± 1.55	0.104 ± .03
11 weeks	air	1.039 ± .45	6.37 ± 1.99	0.160 ± .02
16 weeks	air	1.062 ± .13	8.21 ± 1.16	0.132 ± .02
24 weeks	controlled atmosphere	1.166 ± .21	6.70 ± 1.50	0.171 ± .03

Table III. Samples were taken from two different orchards in Israel. The

Table III. Fatty acid content of phospholipids from the microsomal fraction of apple fruits at harvest and during storage

Duration of storage (weeks)	Storage conditions	Fatty acid (% of total)					$\frac{18:3}{18:2}$	$\frac{\text{Unsat.}}{\text{sat.}}$	Double bond value
		16:0	18:0	18:1	18:2	18:3	Ratio		
A. Orchard 1									
0	--	28	4	-	47	16	.340	1.972	1.32
12	air	20	7	8	43	14	.325	1.912	1.36
16	air	17	2	2	41	25	.609	3.095	1.59
24	controlled atmosphere	17	7	3	41	24	.585	2.832	1.57
B. Orchard 2									
0	--	26	19	-	42	10	.238	1.107	1.14
12	air	7	14	7	42	14	.333	1.808	1.33
16	air	13	2	1	43	24	.358	3.246	1.59
24	controlled atmosphere	20	3	3	42	23	.547	2.724	1.56

main changes which appeared between harvest and the first observation during storage were a drop in the proportion of palmitic acid (16:0) and the appearance of oleic acid (18:1). During the further course of storage there was a steady increase in the proportion of linolenic acid (18:3), while there was no change in linoleic acid (18:2). The general result was an increase in unsaturation of the phospholipid fatty acids during storage time and this was reflected in the ratios and double bond index commonly utilized to express changes in fatty acid composition (Table III). The 18:3/18:2 ratio

increased during the fourth month of regular cold storage and was of the same value after 6 months in controlled atmosphere storage. The ratio of unsaturated to saturated fatty acids also increased up to 4 months of storage and here again the values differed only slightly when apples were stored for 6 months in CA. The double bond index increased during storage as well.

Membrane viscosity was measured as a function of temperature at harvest and after 6 months of storage to see if changes in the physical-chemical characteristics of the lipid bilayer could be found which might correlate with membrane adaptation during storage. The data are shown in Figure 1. At harvest three breaks in the temperature curve were found, at 9°, 15° and 25°. After 6 months' storage the lowest break had disappeared and the other two breaks were at 12° and 25°. This may be due to the changes in lipid composition and arrangement which had taken place during the time of low temperature storage.

Discussion

It appears that the largest physical change in microsomal membrane properties of apple fruit occurs during the first few weeks of storage. The greatest change in microviscosity is during this time. These changes may involve a structural rearrangement of the membrane components, a change in the components or a combination of the two. In regard to this, much work has been done on the development of cold tolerance in grain seedlings.

When wheat plants are exposed to cold temperature, level of linolenic acid increases and the level of linoleic acid decreases (1,7,9). This was found in rye roots (6), but in addition their adaptation to lower temperature involved also a large increase in phospholipids. A similar increase

Figure 1. Arrhenius plots of the microviscosity of microsomal fractions from 'Calville de San Sauveur' apples extracted at harvest and after storage.

in phospholipids was found in overwintering strawberry leaves (15) which had adapted to low temperatures, but it was not accompanied by any major changes in lipid or fatty acid composition. In apples there is no corresponding increase in phospholipids during low temperature storage, but we find an increased degree of unsaturation of the fatty acids, due mostly to a higher proportion of linolenic acid. It is possible that harvested fruit lacks the full synthesizing ability present in whole plants and is more limited in its responses to environmental changes.

These changes in membrane composition are consistent with an alternation of the physical properties of membranes; they do not necessarily correlate with the development of low temperature adaptation. In comparing soybean - a chill-sensitive plant, and broccoli - a chill-resistant plant, Markart et al. (12) found that soybean roots contained more unsaturated fatty acids than broccoli roots. However, when placed at a low temperature, the broccoli roots showed a larger increase in unsaturation than did the soybean roots. the authors concluded that changes in fatty acid unsaturation are an important aspect of plant acclimation and that the degree of unsaturation is not a good indicator when comparing different species.

The Arrhenius plot data (Fig. 1) show an interesting correlation with data obtained by Dickens and Thompson (8) on algae cultures. When algae were grown at 39°C, the microviscosity temperature dependence curves of microsomal membranes (using the polarized fluorescence technique utilized here) showed breaks at 28° and 15°. Algae cultured at 15°C showed breaks at 8° and 20°C. Lower temperatures were not examined. They postulated that the shape of these curves was due to interactions between two coexisting lipid phases and that the break points did not necessarily represent the onset or a completion of a simple phase separation. In correlating these temperature breaks with the membrane composition they found that the main change

in membrane lipid was in the fatty acid fraction, where the double bond index changed from 1.26 in 39°C-cultured cells to 1.62 in 15°C-cultured cells. This is very similar to the changes obtained here for apples at harvest or after low temperature storage, although the distribution of the fatty acids is very different between algae and apple fruit.

Studies of break points in a temperature curve of membranes may, however, yield contradictory data. Pike and Berry (16), using the polarized fluorescence technique, compared winter and summer species from the Mojave Desert, and found that winter-active species showed a break at a lower temperature than did the summer active species. Also, a recent study of several wheat and rye cultivars shows that the viscosity of chloroplast membrane lipids, measured by spin labelling, decreased during hardening, and the magnitude of the change correlated with the freezing tolerance of the cultivars (25). On the other hand, this was not confirmed by Pomeroy and Raison (17), who found no alteration in the break points of a temperature curve of wheat membrane lipids. Furthermore, calorimetric studies of membrane lipids from winter rye seedlings indicated that the phase transition temperature was not altered significantly during hardening, though an increase in membrane lipid unsaturation occurred (23). We also found little change in the temperature of the break points except for the disappearance of the lowest one.

In summary, it appears dangerous to try to extend the findings for one plant or tissue to another without thorough investigation. The membrane lipids do play a role in cold hardening or prevention of chilling injury, but the role of the various classes of lipids may differ from one system to another.

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3.3.3. The effect of calcium on enzyme activity of cellular membrane from apple fruits

ATPase appeared to be the membrane protein candidate most likely to be affected by calcium, as it is an enzyme well known to be regulated by monovalent and divalent ions. Though the effects of calcium on the activity of this membrane-bound enzyme extracted from apple fruits were unexpected, they could not be correlated with the beneficial effects of the ion on the keeping quality of the fruit. The results of this investigation have been published and are presented herewith.

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CHARACTERIZATION OF PLASMALEMMA ATPase FROM APPLE FRUIT

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Key Word Index *Malus sylvestris*; Rosaceae; apple fruit; plasmalemma ATPase; cation effects.

Abstract The ATPase found in the 80000g pellet of apple fruit showed a pH optimum of 6, was inhibited by divalent cations and stimulated by monovalent cations. The enzyme was specific for ATP and inhibited by diethylstilbestrol and dicyclohexylcarbodiimide, while unaffected by oligomycin and the uncoupler SF 6874. The K_m for ATP was 0.48 mM, V_{max} 1.2 μ mol Pi/mg protein/min. Mg^{2+} was a competitive inhibitor to ATP; K_i 0.7 mM. As the apple ripened from preclimacteric to postclimacteric, the ATPase activity increased more than two-fold.

3.3.4. The effects of calcium on membrane permeability and microviscosity.

The previous three sections showed that the chemical and physical changes which occur in the cellular membranes of apple fruits as they ripen after harvest are not of necessity of the same nature as those that occur during storage. In sections 3.1 and 3.2 it was shown that calcium affects both ripening and physiological processes during storage, and the connection between the two effects is not readily manifest. Short-term and long-term effects of calcium on apple fruit tissues were therefore studied by examining the changes incurred in membrane permeability and microviscosity following infiltration of whole fruits with calcium chloride prior to storage, in comparison with those caused by direct treatment of excised fruit discs. Experiments were conducted with Calville de San Sauveur, Jonathan, Golden Delicious and Anna apples by the methods described earlier for determination of membrane permeability (K^+ leakage) and microviscosity.

Results and Discussion

Infiltration of calcium into whole fruits

Electrolyte leakage from apple tissue discs excised from fruits that had been infiltrated with 2% CaCl_2 prior to storage, was significantly reduced by the increased level of endogenous calcium (Fig. 1). When different levels of calcium were obtained in the fruit by various post-harvest dipping treatments, a highly negative correlation was found between the level of endogenous calcium and the extent of electrolyte leakage (Fig. 2). The stage of ripening at which the discs were excised from the stored fruit had little to no effect on the permeability of the membranes in comparison with the marked effect of the endogenous calcium level. However, the microviscosity of the cellular

Fig. 1. Potassium leakage from apple fruit discs excised from fruits infiltrated with 2% CaCl_2 or water prior to storage (cv. Jonathan).

Fig. 2 Effect of endogenous calcium level on electrolyte leakage from apple discs and on membrane microviscosity in preparation from the same fruits.

membranes apparently bore no relation to their permeability, and was differently affected by calcium levels at different stages of maturity. In preclimacteric fruit added calcium tended to maintain the membranes in a more fluid state (Fig. 2A), but this effect was obliterated by the increase in microviscosity which occurred with ripening (Fig. 2B). Cellular membranes prepared from stored Jonathan apples that had been infiltrated with CaCl_2 prior to storage, showed significantly lower values of microviscosity with the DPH probe (2.958 ± 0.14 poise) than membranes from water infiltrated fruit (3.162 ± 0.15 poise) upon removal from cold storage at a pre-climacteric stage.

Infiltration of calcium into fruit discs

Calcium reduced the leakage of electrolytes generally, or potassium specifically, at a concentration of 1 mM (Fig. 3). During the first 4 hours of rapid leakage (Stage I), the effect of calcium was negligible, irrespective of the concentration; but thereafter (Stage II) leakage was virtually stopped in the presence of calcium, declining from 25% of the control after 8 h to 15% after 24 h. This was found even where the fruit discs were transferred to fresh medium every 4 hours. The increase in K^+ leakage during Stage II, which occurred as the fruit ripened at room temperature after storage, was completely prevented in the presence of calcium, and remained at the low level of between 1 and 2% throughout the climacteric rise (Fig. 4).

Magnesium also inhibited K^+ leakage from Jonathan fruit discs at all stages of ripening but to a lesser degree than calcium (Fig. 5). However, with a different cultivar, Calville de San Sauveur, Mg^{2+} had little effect on K^+ leakage during 12 h incubation and thereafter even accelerated the leakage rate (Fig. 6). The effect of Ca^{2+} on leakage from both cvs. was identical.

In contrast to the effect of Ca^{2+} in maintaining the fluidity of cellular

Figure 3. The effect of CaCl_2 at different concentrations on the rate of K^+ leakage from apple pulp discs (cv. Anna).

Fig. 4. The effect of 2% calcium chloride in the incubation medium on potassium leakage from apple discs at different stages of ripening (cv. Calville de San Sauveur).

Fig. 5. The effects of CaCl_2 and MgCl_2 in the incubation media on the leakage of potassium from pulp tissue discs excised from apples at different stages of the climacteric.

Figure 6. The effects of CaCl_2 and MgCl_2 on potassium leakage from apple fruit discs excised at different stages of the climacteric (cv. Calville de San Sauveur).

membranes, when applied to intact fruits, Ca^{2+} treatment of fruit slices resulted in an increased microviscosity of the isolated membranes (Fig. 7). The maximum effect was obtained with 40 mM CaCl_2 in both pre-climacteric and climacteric tissue slices. The employ of two different fluorescent probes showed that the increase in viscosity caused by Ca^{2+} was not only on the surface of the membrane (DNSP) but also within the membrane (DPH) (Fig. 8). Fig. 8 also shows that similar, but less marked effects on membrane fluidity were obtained following treatment with Mg^{2+} .

From this set of data, it would appear that the inhibition of electrolyte leakage caused by Ca^{2+} and Mg^{2+} might result from a 'tightening' of membrane structure and reduced fluidity, due possibly to the binding nature of these ions. However, the data obtained with intact fruits, in which calcium also inhibited K^+ leakage but maintained the membrane in a fluid state, suggests that a different explanation for reduced electrolyte leakage is required.

Fig. 7. The effect of CaCl_2 on the microviscosity of membrane fractions isolated from apple fruit discs after 4 or 24 hours incubation. (A - Peak climacteric fruit, B - preclimacteric fruit)

Figure 8. The effects of CaCl_2 and MgCl_2 in incubation medium on the surface (A) and internal (B) microviscosity of membranes isolated after 24 h from apple fruit discs at different stages of ripening.

4. Evaluation of the Research Achievement

The objective of the research, as stated in the proposal was "to understand the role of calcium in fruit metabolism, by studying its physiological and chemical effects on cellular membranes, on tissue slices and on whole fruits, and to discover possible ways the same high fruit quality can be achieved with lower levels of calcium."

Today, though we do not yet fully understand the role of calcium in fruit metabolism, we have more knowledge of its involvement and relative importance in the behaviour of the fruit. The original concept, that calcium should have a very basic role with a specific site of binding and action on the membranes, has not found much support in this research, though it cannot be entirely refuted. However, the added knowledge acquired seems to make it more difficult to conceive of a basic mechanism which could control all the widely different effects that have been observed on different compartments of the cell. Though microviscosity of cellular membranes has been found to be affected by calcium, and thus was considered to be a good candidate for a fundamental function of calcium, we have not been able to demonstrate that this effect is in any way connected to the functioning or malfunctioning of the cell. A difficulty which arose here, and which still has to be understood and overcome, is that the viscosity of cellular membranes has responded in opposite directions to added calcium, when applied to whole fruit in comparison to fruit slices.

From a practical point of view, the results of this research indicate that Israeli orchardists in the Golan Heights can replace orchard sprays with more economical post-harvest dip applications of calcium chloride with a suitable surfactant.

5. Description of Cooperation

The following list of contributions to this research will bear witness to the extent of cooperation.

Section 3.1.1. was executed in BARC by Giora Zauberman, Carl Sams and Miklos Faust.

Section 3.1.2. was executed in BARC by Susan Lurie and Miklos Faust.

Sections 3.1.3., 3.2.1., 3.3.1., 3.3.2. and 3.3.3. trials were conducted by Ruth Ben Arie and Susan Lurie in Israel.

Section 3.2.2. was performed in Israel by Ruth Ben-Arie and Lillian Sonogo.

Section 3.3.4. is part of a M.Sc. thesis being done in Ruth Ben-Arie's laboratory by Talia Mur.

Dr. Zauberman and Dr. Lurie were guest scientists in Dr. Faust's laboratory where they conducted their research. The principal investigator twice visited Dr. Faust, during the second and the third Years of the project's term, to plan and later to summarize the results of their cooperation.

6. Examination of a Hypothesis

The inconsistent responses obtained with calcium treatment of whole fruits and the occasionally apparently contradictory data resulting from physiological and biochemical assays of calcium treated tissues, have led to the following proposal: calcium is effective in maintaining proper cell functioning under stress conditions, due to its binding capacity to cellular membranes and its resultant effect on their physical properties. Tissues not

under stress conditions do not necessarily respond to added calcium, even if they contain small amounts of the cation, and conversely, tissues with high calcium contents might still respond favorably to treatment, if they are subjected to unusual stresses.

The following experiments were aimed at substantiating this hypothesis. The stresses employed were: 1) Ageing - apple pulp discs were held partially immersed in an iso-osmoticin for 24 hours. 2) Temperature - apple discs were maintained under similar conditions at temperatures ranging from 0° to 35°C. 3) Osmotic stress - the discs were held in sorbitol solutions ranging from 0 to 0.9 M.

The parameters of membrane integrity which were measured were:

1) permeability - by monitoring the leakage of K^+ ions from the tissue discs into the incubation medium; 2) ethylene evolution - ethylene was allowed to accumulate in flasks sealed for 1 h at various times during the incubation period; 3) microviscosity - membranes were isolated after 24 h incubation period and microviscosity was measured by the method described in previous sections.

The results obtained to date, support this hypothesis. During apple disc ageing calcium prevented the increased leakage of solutes through cellular membranes, and the loss of the ethylene producing capacity, which occurred in non-treated tissue discs. The microviscosity of the membranes was increased by calcium treatment and might be responsible for the reduced leakage.

Stress injury incurred in tissue slices by high temperatures began to be apparent at 25°C and was also expressed as increased solute leakage and inhibited ethylene production. Under these conditions calcium again inhibited leakage and maintained the ability to produce ethylene. The fluidity of the

membrane, which increased drastically with the rise in temperature, was maintained at a constant level due to calcium treatment. At low temperatures, around 0°C, no injurious effects were apparent, and calcium treatment hardly had any effect on the parameters measured.

When apple discs were placed under osmotic stress, solute leakage was considerably increased by hypotonic incubation media. Calcium reduced solute leakage to a constant value for both hyptonic and hypertonic solutions. The percentage of reduced leakage was thus highest for the greatest stress effect. The endogenous calcium of non-treated apple fruits was also found to be inversely correlated with the extent of solute leakage induced by hypotonic and hypertonic stress conditions.