Nonvolatile Organic Acids and Core Breakdown of Bartlett Pears

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The nonvolatile organic acids in Bartlett pears stored at 31° F. for several months in five levels of CO_2 were studied both qualitatively and quantitatively. A quantitative method for use in comparative analyses of the major acids in plant tissues is outlined. The results of the investigations show that the flesh tissue of Bartlett pears stored in high levels of CO_2 accumulate succinic and citric acids. Succinic acid increases significantly in the core tissue of CO_2-stored pears. At the same time, however, malic acid decreases. Inhibition of a succinic acid oxidase system is indicated. The increase in succinic acid is correlated with CO_2-induced core breakdown in the fruit. Pears from the 1960 harvest accumulated more succinic acid than those from the 1959 harvest, and considerably more CO_2 injury was found in the 1960 fruit.

O F THE PAPERS that have appeared on the effect of CO_2 on the organic acids in fruits, none has been concerned with CO_2-induced core breakdown of pears. Allentoft *et al.* (*1*) analyzed McIntosh apples stored in $C^{14}O_2$ and reported the C^{14} was incorporated into malic acid. Young and Biale (*21*) reported that lemons stored in 10% labeled CO_2 had 10 times more radioactivity in malic than in citric acid and that succinic acid was not radioactive. In studying the beneficial effect of CO_2 in storage on Bartlett pears, Ulrich and Landry (*23*), using chromatographic techniques, showed qualitatively that fruit stored in 10% CO_2 progressively accumulated malic and succinic acids, but no mention was made of the presence of CO_2 injury in these fruits. The results of analyses made by Hulme (*10*) showed that CO_2 injury to apples stored at low temperature was accompanied by an increase in succinic acid in the tissue.

In view of the above information, it seemed desirable to study changes in the acid content of Bartlett pears in connection with CO_2-induced core breakdown. Since pears stored in 5 to 10% or higher CO_2 for a few months often show CO_2 injury (*9*), an attempt was made to correlate changes in malic, citric, and succinic acids with core breakdown in fruit that was stored in air, 2.5, 5, 10, 15, and 20% CO_2.

A distinction is made between CO_2-induced core breakdown and senescent core breakdown. The former "CO_2

injury" or "brown core" occurs in preclimacteric fruit stored in CO_2 atmospheres sufficiently high to cause injury. This injury is not always confined to the core but frequently occurs in the flesh tissue. Senescent core breakdown is not related to a particular CO_2 storage atmosphere but occurs in postclimacteric fruit.

Methods and Materials

The method used for the organic acid analyses was a combination and modification of those employed by Lugg and Overell (*11*), Palmer (*17*), Hulme and Wooltorton (*11*), and Lamb (*13*). The Bartlett pears (*Pyrus communis* Linn.) used in this research during the 1959 and 1960 pear seasons were harvested at 17 to 18 pounds pressure test measured with a Magness-Taylor pressure tester equipped with a $\frac{5}{16}$-inch plunger. They were obtained from mature trees located on the Tree Fruit Experiment Station plots at Wenatchee, Wash. Pears were matched for size and color, and only the $2\frac{1}{2}$- to $2\frac{5}{8}$-inch diameter fruit free of blemishes and of fairly uniform green color were used in the experiments. The fruit were transported to Pullman, Wash., by truck and placed in storage at 31° ± 1° F. The 1959 fruit were harvested on August 24, and controlled air flows over the fruit were begun on September 10. The 1960 fruit were harvested on August 18, and controlled air flows were started on August 24. The pears were stored in 16-gallon containers each holding a few more than 200 fruit. Ten containers were used, and duplicates of five continuous flow controlled atmospheres

were employed. The storage air mixtures for the 1959 experiments consisted of duplicate atmospheres of air, 2.5, 5, 10, and 15% CO_2. In the 1960 experiments, two 20% CO_2 atmospheres were substituted for the two 2.5% CO_2 atmospheres. The amount of CO_2 in the containers was checked at intervals throughout the storage period to ascertain that the desired gas levels were being maintained. The rate of air flow over the fruit in each container was regulated by manometers at 13.0 to 13.7 liters per hour. The manometers were similar to those described by Claypool and Allen (*5*).

Five color-matched fruits from each of the controlled atmosphere chambers were removed from storage each month. The fruits were halved, cores removed with a hand pear-coring knife, and composited samples totaling 25 grams of core and 25 grams of flesh tissue from the five fruit were extracted in 200 ml. of 85% ethanol. Seeds were removed from the cores prior to extraction. The pear halves were peeled, and tissue surrounding the core area was used for the flesh tissue extractions. Only healthy-appearing tissue was taken for all analyses. The same sampling procedures were used with the ripening fruit, except that after removal from storage these fruit were held in a constant temperature ripening room (20° C.) until the desired stage of ripeness for sampling was reached. The samples were extracted for 5 minutes in a Waring Blendor with 200 ml. of 85% ethanol. The slurry was filtered through a Whatman No. 2 filter paper in a Buchner funnel and brought to a final volume of 250 ml. with distilled water. The extracts were

Table I. Percentage Recovery of Organic Acids from a Known Mixture

Sample No.	Eluted from Dowex[a] 1 × 4			Streaked Directly on Filter Paper		
	Succinic	Malic	Citric	Succinic	Malic	Citric
1	101	91	93	102	99	98
2	102	94	94	100	103	96
3	100	95	93	93	100	96
4	95	95	90	97	98	97
5	103	92	93	103	97	99
6	96	95	97	103	96	93
7	93	93	94	94	98	94
8	103	94	92	98	101	97
9	98	94	95	92	97	97
10	105	96	96	93	99	95
Mean	99.6	93.9	93.7	97.5	98.8	96.2
Range	93–105	91–96	90–97	92–103	96–103	93–99

[a] Obtained from Calif. Corp. for Biochem. Res., Los Angeles 63, Calif.

Figure 1. Changes in succinic acid content of Bartlett pear core tissue during storage at 31° F. in various levels of CO_2

(Fruit were harvested on August 24, 1959)

————— 0.03% CO_2 — · · · — 10.0% CO_2
— — — — 2.5% CO_2 — — — — — 15.0% CO_2
— · — · — 5.0% CO_2

stored at 31° F. until analyzed. A 100-ml. aliquot representing 10 grams of pear tissue containing approximately 1.0 meq. of total titratable acid calculated as malic was placed into a 125-ml. separatory funnel attached to an ion exchange column (7 mm. × 15 cm.) containing 2.5 ml. of wet Dowex 1 × 4 resin, 200 to 400 mesh, in the formate form. Enough resin for the entire experiment was previously converted to the formate form by passing $1M$ sodium formate through it until the eluate was free of chloride as outlined by Palmer (17). The alcohol extract was passed through the column at a rate of 1 to 2 ml. per minute. The columns were thoroughly washed with 50 ml. of double distilled water, and the acids were eluted from the resin with 20 ml. of 6N formic acid or until phosphate ion appeared in the eluate. The eluate was concentrated using the method of Hulme and Wooltorton (11) to a 2-ml. volume, and aliquots of from 0.1 to 0.3 ml., depending on the amount of acid present, were streaked on double acid-washed Whatman No. 42 filter paper and developed with the organic phase of an n-butanol-formic acid-water (10:3:10 v./v.) solvent system. The papers were steamed for 15 to 20 minutes to remove the formic acid. The guide spots corresponding to the location of the acid bands were sprayed with bromphenol blue acid-base indicator. The bands were located and cut from the paper, eluted in distilled water, and titrated to pH 8.1 with a Fischer automatic titrimeter. Since free acids and their salts often appear as double spots on the paper, and the cations lower the titration values of the acids, cation-free reagents and acid-washed filter paper were used. Blanks and standards were run with all analyses and duplicate chromatograms were made on all samples.

The precision of the method was checked by analyzing a known acid mixture. Ten aliquots, each made up to a 100-ml. volume, of a mixture of malic, citric, succinic, and phosphoric acids were passed through separate columns as outlined above. The acids were eluted from the columns until phosphate appeared in the eluate. The eluates were concentrated and streaked in duplicate on sheets of chromatography paper. The papers were developed and the individual acid bands titrated. At the same time, 10 aliquots of the known acid mixture which had not been previously passed through anion columns were streaked in duplicate on filter paper, developed, eluted, and titrated in the usual manner.

Experimental Results

Recovery of Standards. The results of the recovery trials on the known acid mixture are given in Table I. The mean percentage recovery of the acids indicates that when the samples were passed through the anion exchange columns, part of the malic and citric acids were lost. By passing an additional 5 ml. of 6N formic acid through the columns after the phosphate first appeared, small amounts of malic and citric acids were eluted. Even though the recovery was not complete by the time phosphate appeared in the eluate, the agreement between samples appeared good enough for the method to be used for comparative studies on the plant material.

Qualitative Identification of Acids. Acids which appeared on paper chromatograms from pear extracts were identified by cochromatography with known acids and by specific spot tests. Acids found in a concentrated extract from 10 grams of pear tissue were: galacturonic, quinic, shikimic, citric, malic, chlorogenic, and succinic. The R_f values of these acids are given in Table II.
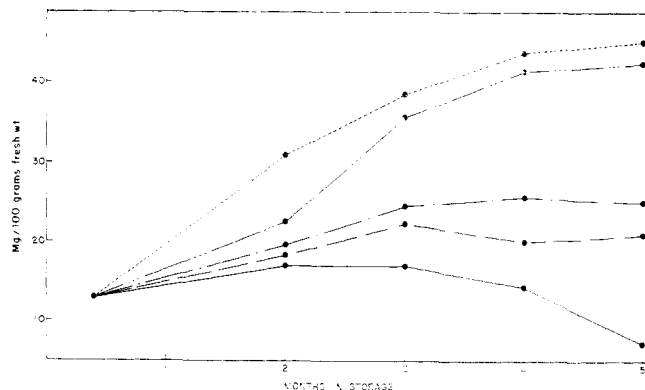
Table II. R_f Values of Pear Acids and Standards on Chromatograms Developed in n-Butanol–Formic Acid–Water (10:3:10 v./v.)

Acid	Pear Acid R_f	Standard R_f
Galacturonic	8	8
Quinic	23	23
Phosphoric	29	28
Shikimic	31	32
Citric	44	45
Malic	52	52
Chlorogenic	57	57
Lactic	..	67
Succinic	75	75

Quinic and shikimic acids were identified using the specific test of Cartwright and Roberts (3). Citric acid gave positive results with the method of Saffran and Denstedt (20). Chlorogenic acid was shown to be present by its fluorescence in ultraviolet light and by the fact that it gave a positive test with the method of Hunter et al. (12). Galacturonic acid was identified by the lead acetate method of Gee and McCready (8). Succinic and lactic acids often have similar R_f values in the formic acid solvent. Therefore, the lactic acid test using p-hydroxydiphenyl, as outlined by Feigl (7), was made on the spots identified by cochromatography as succinic acid. The test was negative for lactic acid.

To have some idea of acid changes in fruit during storage and ripening prior to making quantitative analyses, duplicates of more than 200 extracts from the 1959 harvest were spotted with a micropipet on Whatman No. 3 MM chromatography paper and developed in the n-butanol-formic acid-water solvent. The results of this survey indicated that succinic, quinic, and citric
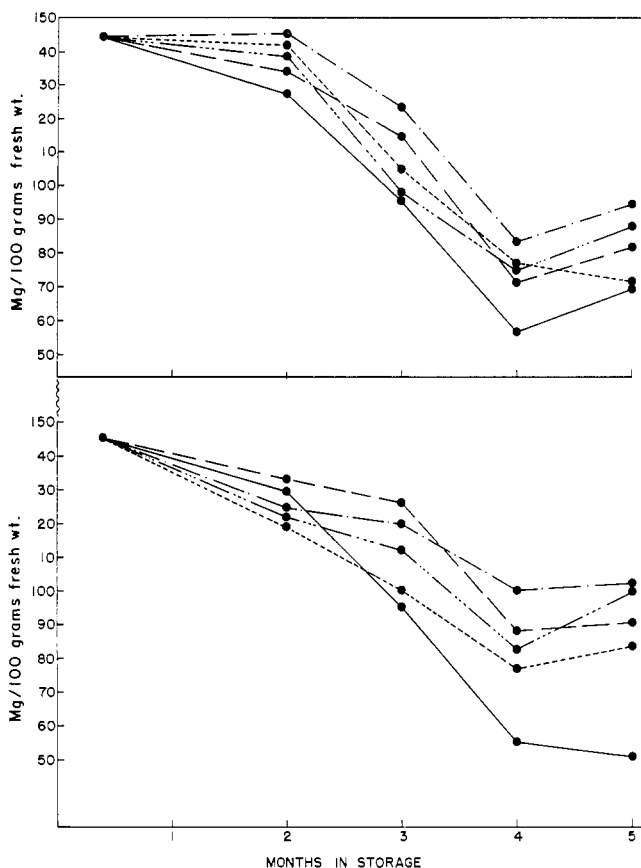
Figure 2. Changes in malic acid content of Bartlett pear flesh (a) and core (b) tissues during storage at 31° F. in various levels of CO₂

(Fruit were harvested on August 24, 1959)

——————— 0.03% CO₂   — · · · — 10.0% CO₂
— — — — 2.5% CO₂   - - - - - 15.0% CO₂
— · — · — 5.0% CO₂

Table III. CO₂ Injury in Bartlett Pears as Related to Storage Atmospheres and Succinic Acid

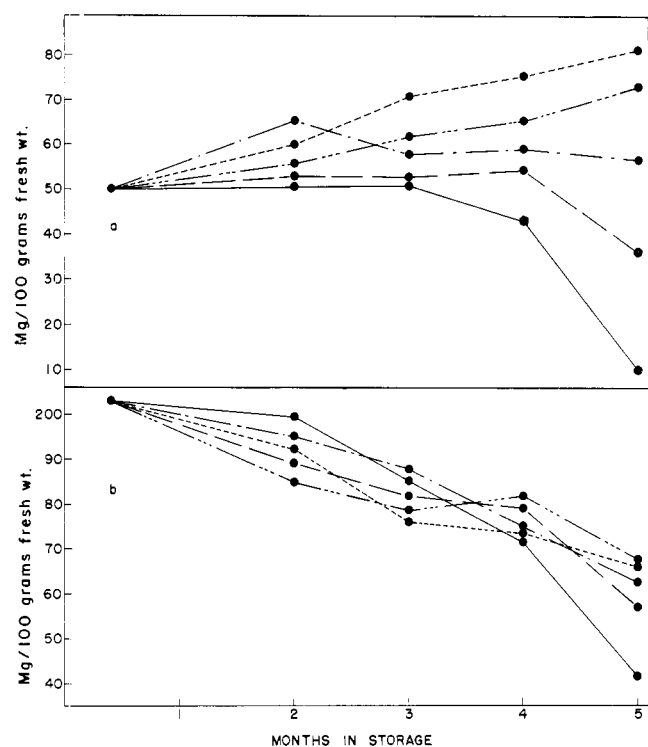|	1959 Fruit			1960 Fruit		
% CO₂ in Storage Atmosphere	% CO₂[a] injury	Mg. Succinic Acid per 100 Grams Fresh Wt. Core	Flesh	% CO₂ injury	Mg. Succinic Acid per 100 Grams Fresh Wt. Core	Flesh
0.03	0	14.56	18.18	0	10.92	9.75
2.5	0	19.08	21.10	..	...	...
5.0	3	25.78	23.64	16	28.13	22.48
10.0	12	41.39	25.70	19	56.91	35.31
15.0	15	43.18	31.78	35	77.02	36.91
20.0	..	...	...	55	103.71	49.73

[a] Based on a 40-fruit sample.

Figure 3. Changes in citric acid content of Bartlett pear flesh (a) and core (b) tissues during storage at 31° F. in various levels of CO₂

(Fruit were harvested on August 24, 1959)

——————— 0.03% CO₂   — · · · — 10.0% CO₂
— — — — 2.5% CO₂   - - - - - 15.0% CO₂
— · — · — 5.0% CO₂

acids increased in fruit stored in the high CO₂ atmospheres but appeared to remain relatively constant or to decrease in fruit stored in air. Core tissue always contained more quinic and less citric acid than flesh tissue. During storage, malic acid appeared to decrease in the core and flesh tissues of the fruit held in air and in all CO₂ atmospheres. Citric, malic, and succinic acids increased slightly in the fruit during the first part of the ripening period. When the fruit had softened to below 5 pounds of pressure, these acids decreased. The decrease in citric and malic acids was greater than in succinic. The amount of galacturonic acid increased slightly throughout ripening.

Quantitative Changes in Succinic Acid. The higher the percentage of CO₂ in the storage atmosphere, the higher the concentration of succinic acid in the fruit. The amounts of succinic acid found in the core tissue of the fruit from the 1959 harvest are presented in Figure 1. The succinic acid level in the flesh tissue followed the same trend as found in core tissue but was usually slightly lower in amount. Values for succinic acid in pears stored for 4

months in various atmospheres for the 1959 and 1960 harvest are shown in Table III. There was more CO₂ injury in the fruit from the 1960 harvest than from the 1959 harvest (Table III). Succinic acid values were in general higher for the 1960 than for the 1959 fruit stored in CO₂. Succinic acid disappeared rapidly from the CO₂-stored fruit when ripened at 69° F. in air.

Quantitative Changes in Malic Acid. Malic acid gradually disappeared from both the flesh (Figure 2a) and core (Figure 2b) tissues during the first part of the storage period. After the fourth month, however, there was a leveling off and even a slight increase in this acid in both the core and flesh tissues. The amount of malic acid in the core tissue was nearly the same as that found

in the flesh tissue. The CO₂ in the storage atmosphere appeared to retard the loss of malic acid in the fruit. The core tissue of the CO₂-stored fruit appeared to contain slightly more malic acid than did the air-stored fruit. There was only about one half as much malic as citric acid present in the Bartlett pears under study.

Quantitative Changes in Citric Acid. Citric acid was found to be the major acid present in Bartlett pears. Data for citric acid are presented in Figures 3a and 3b. There was considerably less citric acid in the core than in the flesh tissues. The amount of CO₂ in the storage atmosphere seemed to increase the citric acid in the flesh of pears stored in the 5, 10, and 15% CO₂ atmospheres. However, there was a decrease in this

acid in the core of pears during storage in all atmospheres. The greatest effect of the CO_2 seemed to be in preventing the disappearance of the acid from the fruit during storage. By the end of the fifth month, the flesh tissue of the pears stored in CO_2 was much higher in citric acid compared to that of the fruit stored in air.

Discussion

The acids shown to be present in Bartlett pears in this research have also been reported in pears by other workers. As early as 1908, Chaubin et al. (4) found that pears contained citric acid. Nelson (16) reported that in Bartlett pears two parts of citric acid were present to one part malic acid. Tavernier and Jacquin (21) compared the amounts of citric and malic acids in various pear musts. They reported considerable differences in acid ratios among varieties. Dame et al. (6) found citric acid to be the maior acid present in Bartlett pears. Ulrich and Landry (23), using paper chromatograms developed in a n-butanol–formic acid–water solvent system to study the effect of CO_2 storage on the acids of Bartlett pear, did not mention the presence of citric acid in the fruit. The authors believe, because of the results in the present investigation and the reports of others concerning citric acid, that Ulrich and Landry (23) may have incorrectly identified the citric acid spot as malic acid. The present work shows that Bartlett pear flesh tissues increase in succinic and citric acids when stored in CO_2. Core tissues increase in succinic, but decrease in citric acid during storage in CO_2. In both core and flesh tissue of the CO_2-stored fruit, the amount of malic acid decreased during storage.

The mechanism for succinic and citric acid accumulation in pear fruit can be best explained on the assumption of a citric acid (Krebs) cycle in the tissue. The presence of a Krebs cycle in pear has not been proved, but may be postulated on the basis of acids present in the fruit. The accumulation of succinic acid and the disappearance of malic acid in pears stored in CO_2 appears to be intimately associated with the inhibition by CO_2 of succinic acid oxidation. Ranson (18) stored carrot, oat, and Kalanchoe tissues in different CO_2 levels and found that the one result common to all was an accumulation of succinic and a de-

crease in malic acids in the tissues. Later, Ranson et al. (19), using mitochondrial preparations, concluded that the succinic oxidase system is sensitive to CO_2 and is rapidly inhibited in 10 to 20% CO_2. They suggested that this inhibition may account for succinic acid accumulation in tissues held in high CO_2. Work by Bendall et al. (2) with Ricinus mitochondria showed that 10% CO_2 in the Warburg reaction vessel strongly inhibited succinic acid dehydrogenase. In addition to the succinic dehydrogenase inhibition, the diphosphopyridine nucleotide, cytochrome C reductase, and cytochrome oxidase systems were inhibited, especially in CO_2 concentrations above 40%. They state that all of these inhibitions are reversible in air. The observations of these investigators seem to correlate with the changes observed in Bartlett pears stored in high CO_2 in the present research. This is especially true since the highest accumulation of succinic acid occurred in storages at the 20% CO_2 level. The disappearance of succinic acid from the fruit during ripening at 68° F. in air indicates that, here too, the inhibition is reversible. The absence of labeled carbon in succinic acid in the experiments of Allentoff et al. (1) and of Young and Biale (24) suggests that succinic acid does not arise from malic acid in the lemon and apple fruits when stored in CO_2. Such evidence supports the theory that succinic acid increases by some mechanism other than by a reversal of the Krebs cycle.

The data presented in Table III show that the buildup of succinic acid in Bartlett pears is correlated with CO_2-induced core breakdown. Whether the acid itself or some other metabolic change associated with succinic acid is responsible for the disorder remains to be determined. The work of Turner and Hanley (22), of Hulme (10), and of Neal and Hulme (15) on the effects of succinic acid on plant respiration leads one to believe that the acid buildup could upset the normal metabolism in pear cells and result in their death.

Acknowledgment

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