Respiration, Organic Acid, and Sugar Composition of Apple Fruits Collected from Apple Mosaic Virus- or Russet Ring Virus-Infected Trees

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ABSTRACT

Respiration, organic acid content, and sugar content were determined for apple fruits collected from healthy trees and from trees infected with either apple russet ring virus (RRV), which causes both foliar and fruit symptoms, or with apple mosaic virus (AMV), which induces only foliar symptoms. Fruits were collected at monthly intervals during two growing seasons from healthy and AMV- or RRV-infected McIntosh trees. No differences in respiration were detected between healthy and diseased fruits. Virus infection did not hasten or delay the onset of the respiratory climacteric in mature fruit, although there was an indication of a virus-induced increase in the height of the climacteric. Virus infection did not result in the appearance of any detectable new acids: the content of malic acid, one of the two major acids present, was slightly decreased by virus-infection; there was no apparent change in the quinic acid concentration of the infected fruits. Likewise, there was no effect of virus infection on the content of glucose, sucrose, or fructose, the three main sugars found in apple fruit.

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Possible effects of virus infection of trees on physiology of fruits may be due to direct effects of virus multiplication within the fruits, or indirect effects of virus infection on photosynthetic processes in leaf tissue. Although a few attempts have been made to determine effects of virus infection on the biochemistry of leaf tissue from apple and other fruit trees (5, 11, 12, 13, 14), no previous attempts have been made to determine the effects, if any, of virus infection on the physiology and biochemistry of fruits collected from diseased trees.

As fruits grow, the main source of nutrition is the leaf and the main translocatory substance in apples is a sugar (D-glucitol) (22, 24). The immediate precursors of organic acids are in general either sugars or other organic acids (22, 24). It seemed logical, therefore, to assume that if the photosynthetic ability of leaf tissue is impaired, as it generally is in virus infection, then this might be reflected in the organic acid and sugar composition of the fruit. Furthermore, the keeping quality of apples is influenced greatly by respiration of fruit tissues, and virus infection is known to affect respiration. Also, in many virus diseases the general pattern of metabolic changes appears to resemble an accelerated aging process. Therefore, it appeared desirable to determine whether virus infection of trees affects respiration of fruits during their development, or the onset or height of the normal respiratory climacteric; i.e., the heightened increase in respiration which occurs just prior to maturation of fruits, and which coincides with full ripeness and flavor of the fruit.

The purpose of the present study, therefore, was to determine whether or not infection of McIntosh apple trees with either apple mosaic virus (AMV), which caused foliar symptoms but no fruit symptoms (9), or with apple russet ring virus (RRV), which causes both foliar and fruit symptoms (1, 18), affects respiration of fruits during the growing season or at the respiratory climacteric. Also, analyses were made to determine whether infection by AMV or RRV alters composition of organic acids and sugars within the fruits.

MATERIALS AND METHODS.—*Source of material.*—Fruits were collected from RRV-infected and AMV-infected McIntosh apple trees, and from nearby healthy trees in the same commercial orchard. The presence of virus infections in these trees was based on symptomatology and on graft-transmissibility. Collections were made at random, although care was always taken to collect fruits from areas on the AMV-infected trees where distinct foliar symptoms were present; and in the case of the RRV-infected trees, fruits with russet rings were chosen, when possible.

Latent viruses.—To discount the possibility of latent virus infections in either the healthy or the AMV- and RRV-infected trees, mechanical inoculations were made by homogenizing 5 g of petals and/or young leaves collected from virus-diseased and healthy trees in 0.05 M phosphate buffer (pH 7.0), containing 0.01 M sodium diethyldithiocarbamate (DIECA) and 0.01 M cysteine hydrochloride. Each extract was filtered through cheesecloth and used to mechanically inoculate leaves of pre-darkened *Chenopodium quinoa* plants which had been dusted with Celite; inoculated plants were kept under observation for at least 4 wk.

Resipiration studies.—1) During the growing season: A Gilson differential respirometer was used to measure oxygen consumption and carbon dioxide evolution of tissue cylinders of apple fruits collected in June, July, August, and September, 1970, and in June, 1971, from healthy and virus-infected trees. Cylinders of pulp tissue just beneath the peel were removed using a no. 4 (9-mm diam) stainless steel cork borer. Approximately the top 1-cm of these cylinders was taken for analysis. Cylinders were taken

from or near russet rings of fruits from RRV-infected trees whenever possible. Two tissue cylinders were floated in 1.0 ml of 0.1 M citrate-phosphate buffer, pH 4.2, in which apple tissue shows a high level of respiratory activity (19). Oxygen consumption was measured by placing 0.2 ml of 20% KOH and filter paper wicks in the center well of six flasks. The center well of the other six flasks was kept empty so that CO_2 evolution minus O_2 consumption was measured. Carbon dioxide evolution was determined by adding values obtained for O_2 consumption to those obtained for $CO_2 - O_2$. The first manometer reading was recorded after a 15-30 min equilibration period, and oxygen consumption and CO_2 evolution are presented as microliters per hr per g dry wt every 15 min for 2 hr.

2) Respiratory climacteric: The respiratory climacteric of 1 kg whole mature fruit lots was determined by measuring CO_2 evolution in an Infrared Gas Analyzer (IRGA) (Mine Safety Appliances Model 200). Rate of respiration was calculated by passing CO_2-free air at a monitored rate through a respiration chamber containing fruit, and measuring concentration of CO_2 in the effluent airstream.

Fruits were collected in September 1969 and 1970, just prior to or at the time of commercial harvest. They were kept in the cold-room overnight and left for 1 day in an air-conditioned lab for temperature equilibration. Carbon dioxide measurements were begun the next day and continued each day thereafter for approximately 2 wk.

Preparation of freeze-dried extracts.—Twenty to thirty healthy or virus-infected fruits collected in June, July, August, and September of 1968 and 1970 were stored at 4 C for 1 or 2 days prior to sectioning for freeze-drying. Cylinders (minus the peel) from young fruits harvested in June and July were obtained by using a no. 12 (15-mm diam) cork borer dipped in a 1% ascorbic acid solution. One or two sections were taken from each fruit and dipped quickly in ascorbic acid solution to prevent the onset of browning. In older fruits (August and September), a commercial "apple-pear slicer" was used to cut the peeled fruits into equal sections and to remove the core. Alternate slices were cut into smaller pieces and were first quickly frozen at −46 to − 51 C (−55 to −60 F), and then freeze-dried for 2-3 days in a Thermovac Freeze-Drier. Dry wts were determined and samples were then homogenized to a fine powder and stored frozen for subsequent analyses.

Extraction procedures for organic acid and sugar analysis.—One-hundred-mg quantities of freeze-dried tissue (200-mg amounts of fruits harvested in September) were homogenized with 25 ml of boiling 80% ethanol and centrifuged at 3,000 *g* for 10 min in a refrigerated centrifuge. The supernatant was saved, and the pellet was resuspended and centrifuged twice more using 25-ml volumes of boiling ethanol. Supernatants from the three centrifugations were combined and reduced to dryness in a Buchler flash evaporator at 37 C. Dried material was redissolved in 25 ml of deionized water and passed through a 1 × 24-cm Dowex cation-exchange column (Dowex 50W x-2, 50- to 100-mesh, Baker Chemical Co. or 100-mesh, Sigma Chemical Co.), which had been washed with deionized water prior to use. The column was then rinsed twice with 25-ml volumes of deionized water, and the effluent passed through a 1.3 × 25-cm Dowex anion-exchange column in the formate form (Dowex 1 x-8, 100- to 200-mesh). The anion resin column was rinsed with 25-ml volumes of deionized water, and the effluent reduced to dryness in a flash evaporator. This dried material was redissolved in 5 ml of deionized water and frozen for subsequent analysis of sugars.

Five-ml fractions were collected from the anion resin column containing the organic acids using the formic acid gradient elution system of either Wang & Mancini (23) or Palmer (15). The fractions were placed in a 37 C water bath and reduced to dryness using a stream of air (15). One ml of deionized water and one drop of a 0.02% phenol red solution (Fisher no. SO-1-24) were added to each of the dried tubes. The contents were then titrated to the phenol red end point with 0.01 N sodium hydroxide.

Because of the nature of Palmer's system (15), the two major acids of apple fruits could be collected in 45-50 fractions, compared to 90-100 fractions with the method of Wang & Mancini (23). Therefore, Palmer's gradient elution system allowed a more rapid analysis of the organic acids, and although the system of Wang & Mancini was used to obtain most of the data from the 1968 season, the system of Palmer was employed to obtain some of the third replicates of the 1968 data and all of the 1970 data.

Gas chromatography of organic acids.—The extraction procedure described above was followed to suitably prepare the material for gas chromatography, except that 50-mg samples of the freeze-dried tissue were used. The organic acids were removed from the anion resin column by passing 100 ml of 6 N formic acid through the column. The effluent was taken to dryness in a flash evaporator and redissolved in 2 ml of methanol for transfer to a small vial. The material was then concentrated further with a stream of nitrogen gas and the acids present were esterified with boron trifluoride. The methyl esters of the acids were analyzed by gas chromatography using a 1.83 m (6-ft) coil-shaped stainless steel column [3.2-mm (1/8-inch) O.D.], packed with 6% DEGS (diethyleneglycol succinate), 3% OV 17 (silicone fluid) on 80- to 100-mesh Diatoport-S in a Hewlett Packard Research Chromatograph, Model 5750, at temperatures programmed between 79-180° C, using a flame ionization detector with a helium carrier gas flow rate of 25 ml/minute at a pressure of 3.51 kg-force/cm^2 (50 psi).

Paper chromatography and quantitative determination of sugars.—Twenty-five-μl amounts of the sugar samples were applied to the origin of Whatman no. 1 paper and the chromatograms were developed in a descending direction for 48 hr in the upper layer of a butanol-acetic acid-water mixture (4:1:5, v/v/v) (2, 20). The dried chromatograms were

sprayed with aniline hydrogen phthalate (25 mg/ml in water-saturated *n*-butanol) for spectrophotometric estimation of the concentration of individual sugars (8). The sprayed chromatograms were air-dried and heated at 115-120 C for 10 min for detection of sugars. Spots, 4 cm^2, were eluted off the paper with 5 ml of 0.7 N HCl prepared in 80% ethanol. Color differences were measured at 390 nm in a Spectronic 20. Each extract was run in triplicate.

TABLE 1. Comparison of oxygen uptake, carbon dioxide evolution, and respiratory quotients (R Q) of McIntosh apple fruits collected from apple mosaic virus (AMV)-, russet ring virus (RRV)-infected, and control trees

Sampling Date	O_2 uptake (μl/hr/gm dry wt)		CO_2 evolution (μl/hr/gm dry wt)		R Q (CO_2/O_2)	
	AMV	Control	AMV	Control	AMV	Control
6/28/70	1,073	1,180	1,280	1,440	1.19	1.22
7/28/70	413	390	674	669	1.63	1.71
8/26/70	252	243	330	319	1.31	1.31
9/26/70	199	212	312	311	1.56	1.46
	RRV	Control	RRV	Control	RRV	Control
6/28/70	920	870	1,200	1,140	1.30	1.30
7/28/70	493	506	710	670	1.44	1.32
8/26/70	251	256	361	337	1.44	1.32
9/26/70	201	206	305	323	1.53	1.56

RESULTS.—*Latent viruses.*—Observation of inoculated plants over a 4-week period revealed no viral symptoms. Chlorotic leaf spot virus (CLSV), the virus most likely to be present in a latent form within the trees, is easily transmitted mechanically from apple leaves to *Chenopodium quinoa* or *C. amaranticolor* plants (3, 7). On the other hand, only one strain of AMV has been reported to be mechanically transmitted to *Chenopodium* (25), and in our laboratory attempts to transmit it to an herbaceous host have not been successful. Moreover, RRV has been mechanically transmitted to *Chenopodium* only two or three times in several hundred attempts. Therefore, on the basis of the lack of viral symptoms, the inoculated plants, the AMV- and RRV-infected trees, and their respective controls, were judged to be free of latent virus infections.

Respiration.—1) During the growing season: Virus infection had no significant effect upon oxygen uptake or carbon dioxide evolution of either AMV or RRV-infected tissues (Table 1). Moreover, the Respiratory Quotients (CO_2/O_2) of infected and healthy fruits were also very similar (Table 1).

2) Respiratory climacteric: Measurements of carbon dioxide evolution, as determined through the use of IRGA, revealed that the climacteric rise began, in all cases, about 7 days after harvest (Fig. 1). Therefore, both AMV and RRV infections had no effect on the onset of the respiratory climacteric. On the other hand, both viruses appeared to have a considerable stimulatory effect on the height of the climacteric, with a maximum increase of 43% in the case of AMV infection, and 29% in the case of RRV infection. However, these increases were not statistically significant.

Organic acids.—Gradient elution of the anion exchange resin columns with formic acid consistently revealed the presence of only quinic and malic acids, the two major acids found in apple fruit tissue (6), regardless of whether the elution system of Wang & Mancini (23) or that of Palmer (15) was used. Citric acid, present in small amounts in apple fruit tissue (6), could not be detected with either of the gradient elution systems.

When gas chromatography was employed to determine the feasibility of its use in routine analysis of fruit acids, malic acid was routinely detected, but quinic acid was not. A small amount of citric acid was observed only by using increased attenuation (sensitivity) and by holding the program at its terminal temperature (180 C) for approximately 15-20 min. A very small amount of fumaric acid was also detected by gas chromatography. However, because of the time required to prepare the material for gas chromatography, and the failure to detect quinic acid in gas chromatograms, this method was not considered to be particularly advantageous for routine use.

Results of the gradient elution from ion-exchange columns of the organic acids in fruits collected during the 1968 and 1970 growing seasons from AMV and RRV-infected trees, revealed that the quinic acid content was unaffected by both AMV and RRV infection during the two seasons and at maturity (Table 2). The data given in Table 2 are for 1970, but

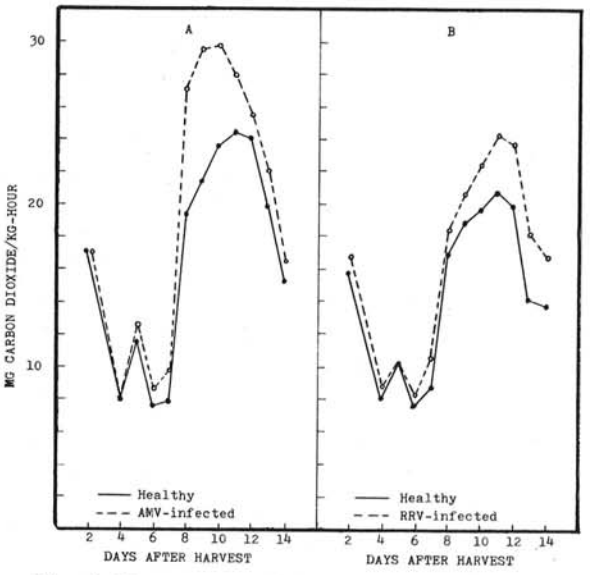

Fig. 1. Measurement of the respiratory climacteric in whole mature apple fruits collected from virus-infected (○———○) and healthy control (●———●) trees, as determined by carbon dioxide evolution detected with an Infrared Gas Analyzer. **A)** AMV-infected vs. control; **B)** RRV-infected vs. control.

the 1968 data were very similar. On the other hand, there were some small differences in the malic acid content between fruits from control and virus-infected trees, and these differences were statistically significant in some cases (Table 2). The overall trend appeared to be toward a slightly lower content of malic acid in fruits from diseased trees, and this was true whether the results were expressed on a dry- or fresh-weight basis.

TABLE 2. Content of malic and quinic acids in fruits collected during the 1970 growing season from virus-infected and healthy McIntosh trees[a]

Month	Quinic		Malic[b]	
	AMV	Healthy	AMV	Healthy
June	.026	.026	.170	.171
July	.004	.005	.107*	.127
Aug.	.001	.001	.082	.080
Sept.	.001	.001	.076	.078
Month	**Quinic**		**Malic[b]**	
	AMV	Healthy	AMV	Healthy
June	.022	.021	.149*	.165
July	.003	.004	.097**	.101
Aug.	.001	.001	.069	.076
Sept.	.001	.001	.066*	.069

[a] Each figure represents the average of three replicates and expresses the milliequivalents of acid/100 mg freeze-dried weight.

[b] * = Statistically significant at the 5.0% level. ** = Statistically significant at the 1.0% level.

In order to determine the efficiency of the resin column system employed for the detection of organic acids, the percent recovery of quinic, malic, and citric acids was measured after passage of known amounts of the acids through cation and anion exchange columns. The recovery was judged to be good, with an average of 87.6% quinic, 87.4% malic, and 82.2% citric acids recovered from the anion resin column after elution with formic acid.

In addition, for fruits harvested in 1970, the combined total of quinic and malic acids was compared to the total acidity, as determined by titration of a 1-ml aliquot of each 25-ml sample used for gradient solution, without previous passage of that aliquot through either a cation or anion exchange column (Table 3). A very close correlation was obtained for fruits collected from AMV-infected and control trees, whereas, in fruits from RRV-infected trees, the total acidity was consistently slightly greater than the combined totals of malic and quinic acids, particularly in June and July. Since this situation occurred in both diseased and healthy fruits, it was apparently not a result of virus infection; and since these differences were not observed in the case of AMV and its control, it is possible that the differences observed in the case of RRV and its control were only due to experimental error.

Sugars.—Virus infection of trees did not result in any consistent major differences in the relative concentrations of the three main sugars in fruits during either of the 2 years that determinations were made (Table 4). The data given are for 1970, but the data for 1968 were very similar. The glucose content in the September-harvested RRV-infected fruits was significantly greater than that of the control, but since this difference was not observed in the first 3 months of the growing season, nor in the 1968 data, it was not considered to be particularly meaningful.

DISCUSSION.—Information on possible biochemical changes in fruit tissues, produced for a whole growth season on trees systemically infected with a virus for many years, is desirable from a scientific and a practical point of view. However, a study aiming at obtaining such information is complicated by the lack of knowledge on the presence and multiplication of virus in the fruit and on the effects of the virus on the physiology of the leaves. A change in the latter, of course, would be expected to affect, and to be reflected in, the biochemistry of the fruit tissues.

Most virus infections increase the normal respiratory pattern of infected tissues, although the magnitude of the increase is not always the result of virus multiplication (10, 21). The present investigation revealed no significant effects of virus infection of McIntosh apple trees on the respiration of the fruits, where virus multiplication may or may not be taking place. Both AMV and RRV caused an increase in the height of the respiratory climacteric, but these increases did not prove to be statistically significant and should be considered as trends. With regard to commercial factors, the more important finding seems to be that virus infection did not result in an earlier onset of the climacteric.

TABLE 3. A comparison of the total titratable acidity with the total combined acidity of malic (M) and quinic (Q) acids in McIntosh fruits collected during the 1970 growing season from apple mosaic virus (AMV)- and russet ring virus (RRV)-infected trees, and from healthy control trees[a]

Month	AMV		Control	
	Total acidity	Acidity of M + Q	Total acidity	Acidity of M + Q
June	.188	.196	.190	.196
July	.107	.112	.119	.132
Aug.	.080	.083	.085	.081
Sept.	.082	.077	.075	.079
Month	**RRV**		**Control**	
	Total acidity[b]	Acidity of M + Q	Total acidity[b]	Acidity of M + Q
June	.193*	.171	.204	.186
July	.128*	.100	.128**	.104
Aug.	.073	.070	.081	.078
Sept.	.073**	.067	.075	.070

[a] Each figure represents the average of three replicates and expresses the milliequivalents of acid/100 mg freeze-dried weight.

[b] * = Statistically significant at the 5.0% level. ** = Statistically significant at the 1.0% level.

TABLE 4. Relative concentrations of three sugars in apple fruit collected during the 1970 growing season from apple mosaic virus (AMV)-, russet ring virus (RRV)-infected, and healthy control trees[a]

Harvest Date	AMV-infected				Control			
	S	G	F[b]	Total	S	G	F	Total
June	tr.	.15	.28	.43	tr.	.15	.29	.44
July	.03	.10	.63	.76	.03	.13	.65	.81
Aug.	.08	.16	1.02	1.26	.08	.18	.95	1.21
Sept.	.14	.17	1.04	1.35	.11	.15	1.10	1.36
Harvest Date	RRV-infected[c]				Control			
	S	G	F	Total	S	G	F	Total
June	tr.	.18	.24	.42	tr.	.16	.27	.43
July	.02	.12	.51	.65	.03	.13	.57	.73
Aug.	.08	.21	.92	1.21	.08	.18	.91	1.17
Sept.	.08	.26**	1.18	1.52	.10	.16	.86	1.12

[a] Data is expressed as relative optical density readings of 4-cm² spots eluted from the paper chromatograms with 0.7 N HCl in 80% ethanol, and read at 390 nm in a Spectronic 20 spectrophotometer.

[b] S = sucrose; G = glucose; F = fructose.

[c] ** = Significant at 1% level, as compared with control.

There is no information on the concentration of apple mosaic or russet ring viruses in fruit tissue, and in fact, only RRV exhibits fruit symptoms. It is known, of course, that in mosaic diseases virus concentration is greater, or that virus is present only, in the symptomatic areas of the leaf, while green areas have less virus or no virus at all (4). It seems likely that any direct effects of virus multiplication on the physiology of the fruits would be most likely observed in the very young fruits where the cell size is small and cell division and enlargement are rapid. After that time, if the virus is present only, or present at higher concentrations, in some (e.g., symptomatic) areas of the fruit, any effects of virus multiplication might be masked by the volume of uninfected cells. When one considers the small and often conflicting biochemical changes detected in infected leaf tissue, where there is a greater percentage of infected cells, then it is not surprising to find little or no change, with regard to organic acid and sugar composition, in the fruits where the virus concentration is unknown, and may be quite low or even absent.

On the other hand, both AMV and RRV produce foliar symptoms, and these are particularly severe in the case of AMV infection. Since the main source of nutrition for the growing fruit are the leaves, it therefore seemed likely that biochemical alterations of the leaf tissue would be reflected in the acid and sugar composition of the fruits. However, this was not borne out by the present study. Therefore, although apple mosaic virus infection, where no fruit symptoms develop, depresses yields, varying from slight reductions to as high as 55% (16, 17), the foliar manifestations of the disease on fruit quantity do not appear to be reflected in the biochemistry of the fruit.

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