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Sugars and Organic Acids of Vitis Vinifera[1]

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Summary. Glucose, fructose, galactose, sucrose, maltose, melibiose, raffinose, and stachyose were identified in the leaves, bark, roots, and berries of *Vitis vinifera* L. var. Thompson Seedless. In addition to these sugars, verbascose and manninotriose were found in the leaves and bark.

Malic, tartaric, citric, isocitric, ascorbic, *cis*-aconitic, oxalic, glycolic, glyoxylic, succinic, lactic, glutaric, fumaric, pyrrolidone carboxylic, α-ketoglutaric, pyruvic, oxaloacetic, galacturonic, glucuronic, shikimic, quinic, chlorogenic, and caffeic acids were identified in the leaves, bark, roots, and berries.

Glucose, fructose, sucrose, malate, tartrate, and citrate were determined quantitatively in the leaf, petiole, xylem, bark, tendril, bud, puduncle pedicel, berry, lateral roots, and main roots at 4 separate physiological stages of growth. In addition, changes in the concentrations of fructose, glucose, malate, and tartrate in leaves were measured during a 36-day period starting from budburst.

Previous reports (13, 14, 15, 16) have dealt with seasonal changes in the concentration of glucose, fructose, sucrose, raffinose, malate, and tartrate in leaves, flowers, and berries of several varieties of *Vitis vinifera* grapevines. Quantitative seasonal changes in the organic acids and sugars in other parts of the grapevine have not been adequately investigated. Neither has there been a detailed investigation to identify the entire range of sugars and organic acids in the various parts of the grapevine. Earlier work by Kliewer and Nassar (17) showed that 12 to 27 % of the total acidity and 0.5 to 12 % of the reducing sugars in grape leaves is not accounted for by malic and tartaric acids and glucose and fructose, respectively. The introduction of chromatography has greatly facilitated the study of sugars and organic acids by offering a relatively simple method of separating and identifying individual components of rather complex mixtures of these substances. For a review of the literature pertaining to organic acids and sugars in grapevines, see Amerine (1, 2), Amerine and Winkler (3), and Kliewer (13,14, 15).

In the present study, 11 different parts of the grapevine were investigated for their free organic acid and sugar components. Various tests were conducted to identify individual organic acids and sugars. Glucose, fructose, sucrose, malate, tartrate, and citrate were determined quantitatively in the various parts of the plant at several different stages of development.

Materials and Methods

The source of plant material was 9-year-old cane-pruned vines of *Vitis vinifera* L., var. Thompson Seedless (syn. Sultanina), growing in an irrigated vineyard at Davis. Samples were taken from 20 vines at 4 different dates and divided into the following parts: mature leaf blades, petioles, shoot bark, shoot wood, buds, tendrils, peduncles, pedicels, berries, lateral roots, and main roots. In addition, immature leaves were sampled 4, 8, 15, 22, 29, and 36 days after budburst, and leaf areas were determined with a planimeter. The sampling dates and corresponding physiological stage of fruit growth were: June 30 (green berry), August 18 (ripening), September 28 (ripe), and November 1 (overripe). The mature leaves were healthy and green at each sampling dates except the last, by which time the leaves had become senescent. Dry weight determinations were made for all samples.

Immediately after sampling, the various parts of the grapevines were cut into small pieces and mixed, and 20 to 50 g (fr wt) were extracted with ethanol by a procedure described previously (13). A sample of the ethanol extract, equivalent to 10 g fresh weight, was passed through cation- and anion-exchange columns by methods previously described, thus separating the amino acids, organic acids, and sugars. The organic acids and sugar fractions were concentrated to 10 ml. Aliquots of the organic acid and sugar fractions were concentrated another 10 to 50-fold for

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identification of trace amounts of sugars and organic acids by paper chromatography.

The sugars listed in table I were separated by descending 1-dimensional chromatography on Whatman No. 1 paper. The identity of the sugars was checked with 3 solvent systems: *n*-propyl alcohol, benzyl alcohol, 85 % formic acid and water (50:72:20:20 v/v) (9); 1-butanol, ethanol, and water (40:11:19, v/v) (11); and ethyl acetate, acetic acid, formic acid, and water (18:3:1:4, v/v) (11). Each of these irrigants was run for 48 to 80 hours at room temperature to obtain the desired separation. The sugars were detected by spraying the air-dried chromatograms with a modified p-anisidine solution (21) and partially identified by comparison with ^{R}glucose (^{R}gl) values of known sugars in each of the 3 solvent systems. The identity of the sugars was further confirmed by elution from the chromatograms and conducting the test indicated in table I.

The organic acids listed in table I were separated by both 1- and 2-dimensional chromatography. The solvent systems employed included 1-butanol, formic acid, H_2O (10:1:10, v/v); and pentanol, 5 M formic acid (1:1, v/v). For 2-dimensional chromatography, each of these solvents systems was used in combination with 80 % redistilled phenol and ethanol, ammonia, water (85:5:15, v/v). The organic acids were detected by spraying with bromcresol green adjusted to pH 4.5. Methods used for identifying the organic acids are given in table I. For cochromatography and chemical test, spots corresponding to individual organic acids were cut out, the acid was eluted from the paper with water, and the solution concentrated at room temperature in a rotary evaporator mixer under reduced pressure.

The keto acids were obtained by extracting and preparing 2,4-dinitrophenyl-hydrazone derivatives according to the method of Isherwood et al. (12). The keto acid hydrazone derivatives were separated by paper chromatography methods of Meister and Abendschein (20). The tests used for identifying the organic acids are given in table I.

Radioactive sugars and organic acids were obtained from Thompson Seedless leaves and fruit clusters treated with $^{14}CO_2$ by methods described previously (17). The labeled sugars and organic acids were separated by using the solvent systems described above, and radio-autographs were prepared of the developed chromatograms.

The methods employed for quantitative determination of glucose, fructose, sucrose, malate, and tartrate have been described (14, 15). Citrate was determined by the colorimetric procedures of Natelson et al. (22).

In the present report, the stages of berry growth are indicated as green, ripening, ripe, and overripe, terms defined by Winkler (28). The sampling dates and corresponding stage of berry development were as follows: June 30 (green berry), August 18 (ripening), September 28 (ripe), and November 1, (overripe).

Results

Ten sugars and 23 organic acids were separated by paper chromatography and identified in grape leaves (table I). All the sugars and organic acids, except verbascose and manninotriose, were eluted from chromatograms and cochromatographed with known standards. Invertase treatment of eluted sugars, corresponding to verbascose, stachyose, raffinose, and sucrose, yielded D-fructose in each case, along with the complementary stachyose, manninotriose, melibiose, and D-glucose, respectively. Other tests employed for identifying specific sugars and organic acids are given in table I.

Most of the sugars and organic acids identified in the leaves were also found in the bark, wood, berries, and roots (table I). Verbascose and manninotriose, however, were not found in the wood, berries, or roots. All of the organic acids found in the leaves were also detected in the bark, berries, and roots. Isocitric, *cis*-aconitic, glycolic, glyoxylic, glyceric, glutaric, fumaric, lactic, pyruvic, and oxalacetic were not found in the woody tissue.

A radioautogram of the organic acid fraction extracted from grape leaves are shown in figure 1. Twenty-seven organic acids contained sufficient ^{14}C to be detectable on the radioautograms. The intensity of the spots indicate the relative amount of activity present in the various organic acids. At least 6 organic acids, visible on the radioautogram, were not identified. Radioautograms of sugar fractions from $^{14}CO_2$ treated leaves, extracted 8 hours after applying label, indicated that 9 to 10 sugars were generally decernable. All the sugars in table I, except maltose, were tentatively identified on the radioautograms. Glucose and fructose contained about 91 % of the total activity in the sugar fraction, sucrose and raffinose about 7 % and the 5 remaining sugars accounting for approximately 2 %.

The amounts of glucose, fructose, and sucrose in various parts of the grapevine at 4 different stages of growth are given in figures 2 to 5. Except during the green berry stage, glucose and fructose were present in about equal amounts in the various parts of the vine, except the bark and tendrils, where glucose concentration was 2 to 3 times that of fructose.

Several trends may be noted in the sucrose, glucose, and fructose contents of the various parts of the grapevine with increasing maturity. Sucrose usually increased during the season in the leaf blade, petiole, wood, bark, bud, peduncle, and berry, and remained about the same in the tendrils, lateral roots, and main roots. Likewise, glucose and fructose increased in the pedicel, lateral roots, and main roots, but decreased in the bark and buds. Glucose also decreased in the petiole, xylem, and peduncle during maturation, while fructose changed relatively little in the petiole, xylem, tendril, and peduncle.

Figures 6 to 9 show the concentrations of tartrate, malate, and citrate in the various parts of the grapevine at 4 different stages of development. Tartrate

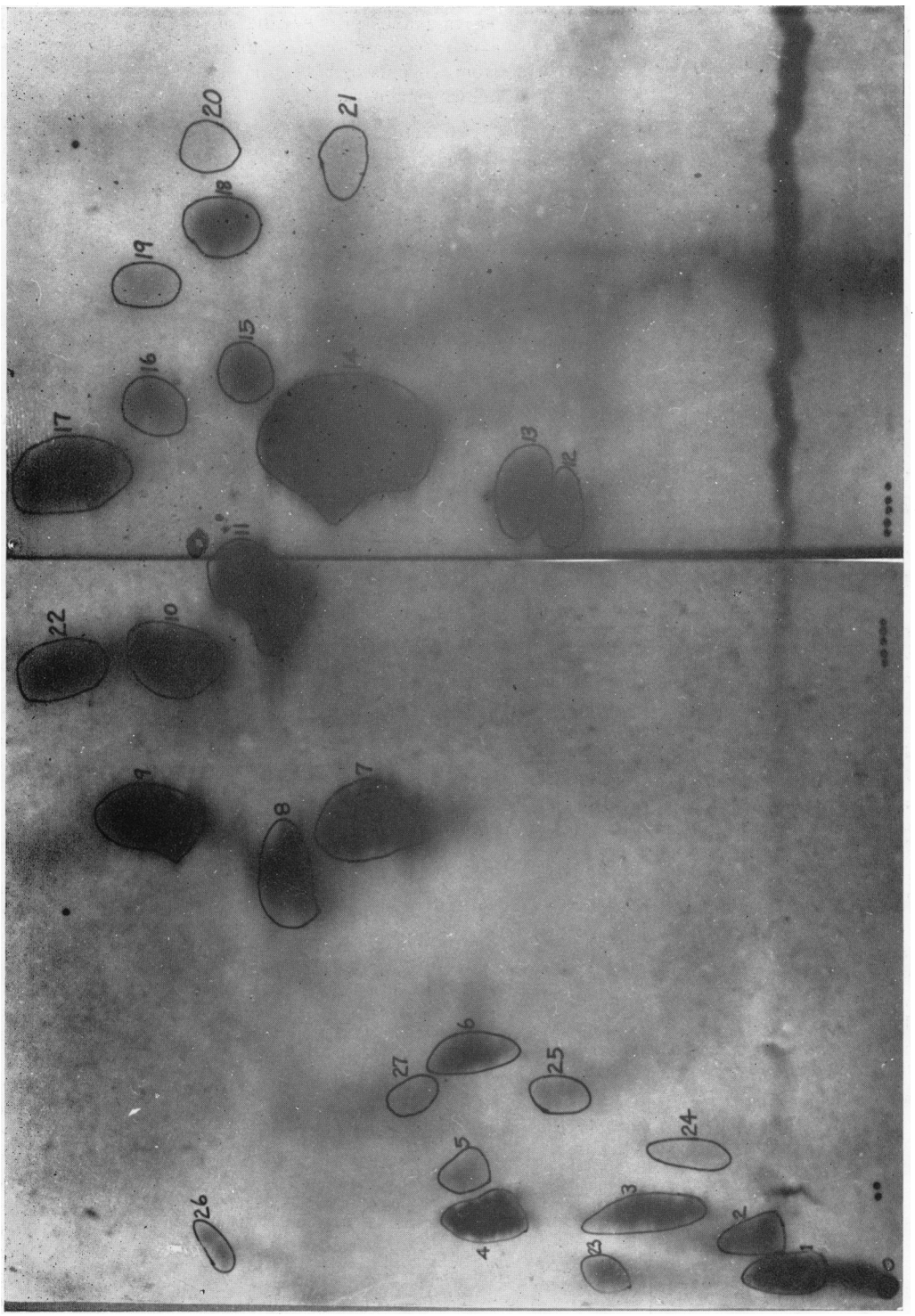

FIG. 1. Autoradiograph of a paper chromatogram showing the various organic acids present in leaves of Thompson Seedless 8 hours after treatment with $^{14}CO_2$. The organic acids corresponding to the numbers are 0 (origin), 1 (galacturonic), 2 (glucuronic), 3 (aspartic), 4 (glutamic), 5 (chlorogenic), 6 (oxalic), 7 (tartaric), 8 (ascorbic), 9 (quinic), 10 (shikimic), 11 (glyceric), 12 (iso-citric), 13 (citric), 14 (malic), 15 (α-ketoglutaric), 16 (glycolic), 17 (pyrrolidone carboxylic), 18 (succinic), 18 (glyoxylic), 20 (glutaric), 21 (fumaric) and 22–27 (unidentified). The solvent systems employed were ethanol, ammonia, water (85:5:15, v/v) for the vertical direction and N-butanol, formic acid, water (10:1:10, v/v) in the horizontal direction.

Table I. *Identifying Test Applied to the Sugars and Organic Acids in Leaves of Thompson Seedless Grapevines and Distribution of These Compounds in the Plant*

All sugars and organic acids, except pyruvic and oxalacetic acids, were separated by descending paper chromatography with 3 different solvent systems. Their R_F values were compared with known standards and used for partial identification. In addition, all compounds, except verbascose and manninotriose, were cochromatographed with authentic standards. See Materials and Methods for further details.

Compounds	Parts of vine in which compound was detected*	Enzyme and chemical tests of identity
Sugar		
Verbascose	L, B	Invertase treatment and cochromatography of products
Stachyose	L, B, W, F, R	Invertase treatment
Manninotriose	L, B, F	Melibiase treatment and cochromatography of products
Raffinose	L, B, W, F, R	Invertase treatment, negative Benedict's test and positive resorcinol and mucic acid test
Melibiose	L, B, W, F, R	Positive Benedict's and mucic acid test
Maltose	L, B, W, F, R	Positive Benedict's test and negative mucic acid test
Sucrose	L, B, W, F, R	Invertase treatment; negative Benedict's and mucic acid test; positive resorcinol and Barfoed's tests
Galactose	L, B, W, F, R	Positive galactose oxidase reaction
Glucose	L, B, W, F, R	Positive glucose oxidase reaction
Fructose	L, B, W, F, R	Positive resorcinol reaction of Roe (4)
Organic acid		
Malic	L, B, W, F, R	Positive malic dehydrogenase reaction (23); yellow color with 2-7-dihydroxynaphthalene-H_2SO_4 (7)
Tartaric	L, B, W, F, R	Red color with metavanadate (19)
Citric	L, B, W, F, R	Pentabromoacetone derivative (22)
Ascorbic	L, B, W, F, R	Positive 2,6-dichlorophenol-indophenol reaction (6)
Oxalic	L, B, W, F, R	Conversion to glycolic acid (7)
Isocitric	L, B, F, R	Positive isocitric dehydrogenase reaction (23)
cis-Acontic	L, B, F, R	
Glycolic	L, B, F, R	Brown color with naphthoresorcinol-H_2SO_4 (7); red color with 2,7-dihydroxynaphthalene-H_2SO_4 (7)
Pyrrolidone carboxylic	L, B, W, F, R	
Succinic	L, B, W, F, R	
Lactic	L, B, F, R	Positive lactic dehydrogenase reaction (10)
Glyceric	L, B, F, R	Blue color with naphthoresorcinol-H_2SO_4 (7)
Fumaric	L, B, F, R	Visible on chromatograms under ultraviolet light
Glyoxylic	L, B, F, R	Cochromatography of 2,4-dinitrophenyl-hydrazone derivative (20); positive glyoxylic reductase reaction (18)
α-Ketoglutaric	L, B, W, F, R	Cochromatography of 2,4-dinitrophenyl-hydrazone derivative (20)
Pyruvic	L, B, F, F	Cochromatography of 2,4-dinitrophenyl-hydrazone derivative (20)
Oxalacetic	L, B, F, R	Cochromatography of 2,4-dinitrophenyl-hydrazone derivative (20)
Galacturonic	L, B, W, F, R	Positive reaction with cysteine (4) and carbazole (4)
Glucuronic	L, B, W, F, R	Positive reaction with thio-glycolic acid (4)
Shikimic	L, B, W, F, R	Red color with aniline (29)
Quinic	L, B, W, F, R	
Chlorogenic	L, B, W, F, R	
Caffeic	L, B, W, F, R	

* The following symbols were used to describe parts of the vine: L (leaf), B (bark), W (wood), F (fruit), and R (roots).

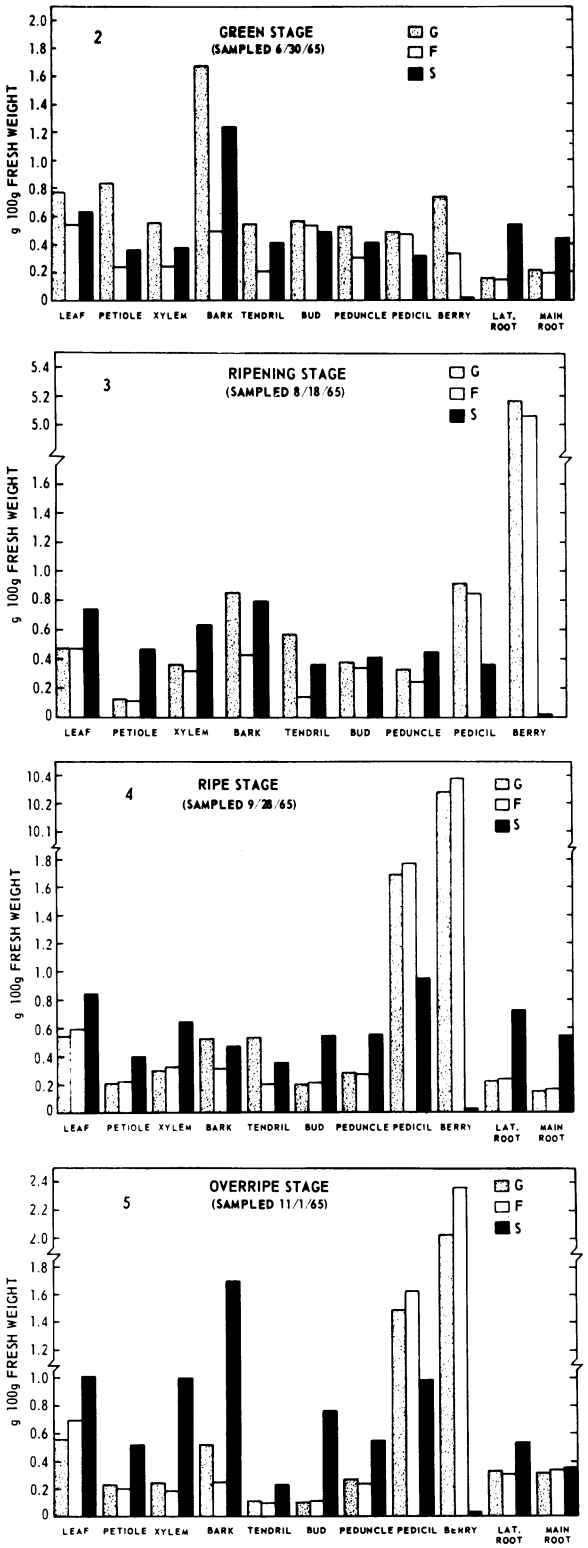

FIG. 2–5. The concentration of glucose (G) fructose (F), and sucrose (S) in the various parts of Thompson Seedless grapevines at 4 different stages of growth. The parts of the vine designated as xylem also included the pith region.

was the main organic acid in all parts of the vine at each stage of growth except the overripe period, when malates exceeded tartrates in the petiole and xylem. Citrate was present in very small amounts in all parts of the vine except the lateral and main roots, which contained nearly as much of this acid as tartrate.

The amounts of the different organic acids in the various parts of the grapevines changed considerably during maturation (fig 6–9). The xylem and peduncle generally showed increases in tartrate (on a percentage basis) during the season, while the petiole, tendril, bud, pedicel, and berry decreased in content of this acid. The amount of tartrate in the

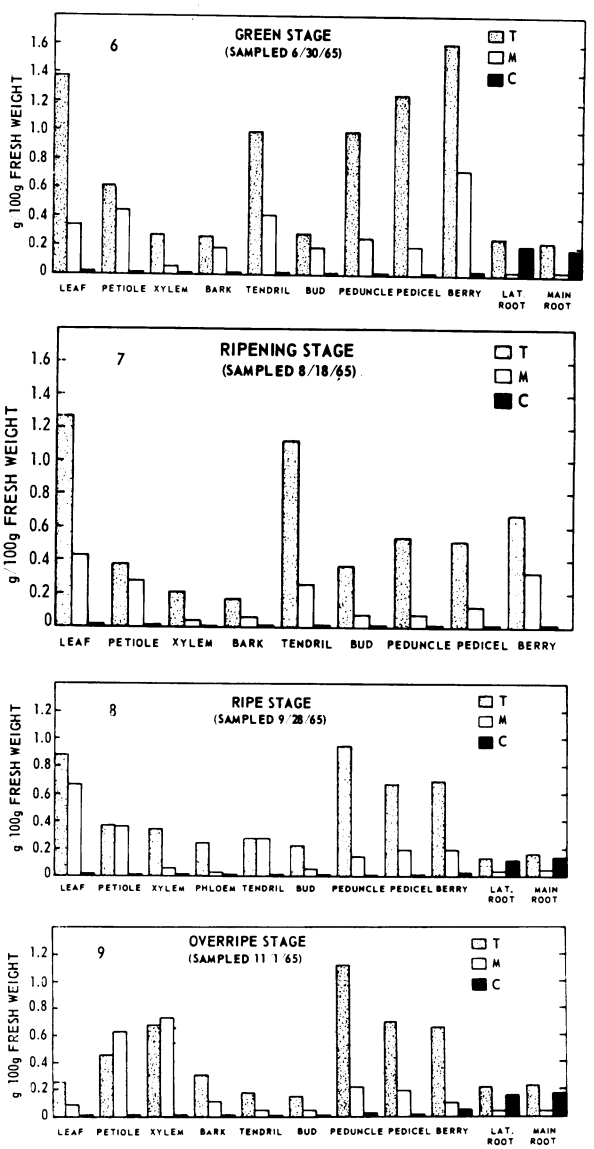

FIG. 6–9. The concentration of tartrate (T), malate (M), and citrate (C) in the various parts of Thompson Seedless grapevines at 4 different stages of growth. The part of the vine designated as xylem also included the pith region.

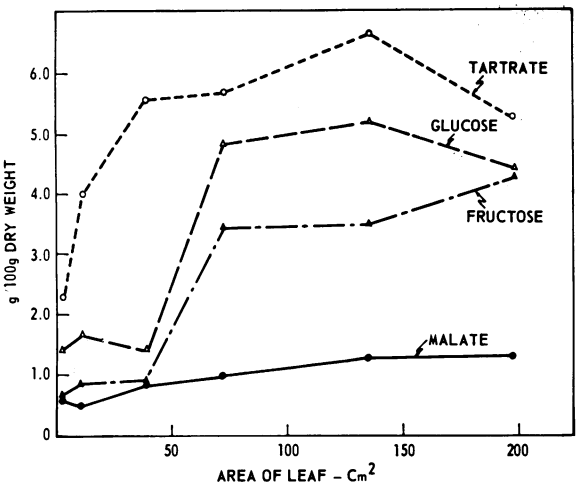

FIG. 10. Changes in the concentration of tartrate, malate, glucose, and fructose in Thompson Seedless leaves during the period of development.

bark and lateral and main roots changed very little during the period from June to November. The tartrate content of the leaves also remained relatively constant during the growing season until the end of September, when the leaves became senescent. Both tartrate and malate, however, declined rapidly in the leaves from September 28 to November 1. Malate increased with maturity in the petiole, xylem, and leaf, and decreased in the bark, bud, tendril, and berry. The amount of malate in the peduncle, pedicel, and roots changed relatively little during the season. The citrate content of the berries increased with maturity. The amount of this acid in other parts of the vine did not fluctuate much during the season.

Figure 10 shows changes in the concentration of tartrate, malate, glucose, and fructose during leaf development. The amount of malate and tartrate in immature leaves increased 2- to 3-fold from the fourth to the thirty-sixth day after budburst. During this period of rapid growth the concentration of tartrate was 5 to 7 times that of malate. During this period the average area of the leaves increased from 3 to nearly 200 cm^2. Glucose and fructose were present in relatively small amounts during the first 15 days after budburst (leaf area about one-fifth the maximum size). Enlargement of leaf area from 40 to 70 cm^2 (15–22 days after budburst) was accompanied by a 3- to 5-fold increase in glucose and fructose. During the period of rapid leaf growth the concentration of glucose exceeded fructose 1.3 to 2 times, and tartrates exceeded malates 5 to 7 times.

Discussion

All of the sugars in table I except verbascose and manninotriose were shown in a previous report to be present in the berries of several varieties of *V. vinifera*. The present study besides confirming the earlier work, shows that the sugars are widely distributed in the grapevine. The earlier work failed to show free galactose in the leaves and bark of grapevines. However, running the irrigant solvents for periods up to 80 hours brought about a clear separation of glucose and galactose, resulting in detection of the latter sugar in all parts of the vine. The 7 oligosaccharides identified in table I consisted of various combinations of 3 hexoses, glucoses, fructose, and galactose. The raffinose family of sugars, which include verbascose, stachyose, and raffinose, has been found in many woody plants (8, 26, 30) and has recently been shown to take an active part in carbohydrate translocation (26, 30). Melibiose and manninotriose can be formed by invertase hydrolysis of the glucose-fructose bond of raffinose and stachyose, respectively. Stoev et al. (25) recently found maltose in all parts of the grapevine in fairly large quantities during the dormant period. The metabolic origin of maltose in the grapevine is not known. Notably absent in detectable amounts in grapevines were free pentoses. Several investigators have reported free pentoses in wine (2). This may indicate that yeast, bacteria, or both degrade hexoses or oligosaccharides containing hexoses, into pentoses. Pentoses may also be formed by hydrolysis of pectins or other polysaccharides that contain 5-carbon sugars.

Paper chromatographic studies indicated that there are at least 27 organic acids in grapevines, with concentrations of 0.0001 g/100 g fresh weight, or greater. Twenty-three of the organic acids were identified in the leaves, bark, berries, and roots, and 14 in the woody tissue (table I). As far as the author is aware, isocitric, *cis*-aconitic, glutaric, fumaric and pyrrolidone carboxylic acids have never been identified in the grapevine, and this is the first instance in which the entire series of organic acids has been reported to be present in any single organ of this plant. Each of the organic acids listed in table II has been found in at least one other higher plant (5). This and other investigations (14, 17) have shown that malic and tartaric acids and their salts make up 68 to 92 % of the total acid in the berries and leaves of grapevines.

Malic and tartaric acids accounted for the bulk of the total acidity in all parts of the grapevine except the roots. Citric acid composed 30 to 40 % of the total acidity in the roots, and less than 2 % of the acidity in other parts of the grapevine. Oxalic, succinic, glyceric, glutaric, α-ketoglutaric, glycolic, pyrrolidone carboxylic, quinic, shikimic, galacturonic, and glucuronic acids comprise the second most abundant group of acids in grapevines. These compounds could usually be detected on paper chromatograms without concentrating extracts prior to application on paper. Isocitric, ascorbic, *cis*-aconitic, glyoxylic, lactic, fumaric, pyruvic and oxaloacetic acids were present in extremely small amounts requiring concentration of 10 to 50 times before they could be detected.

The large number of organic acids in grapevines

suggests that several metabolic cycles are present. All the intermediates of Krebs and glyoxylic acid cycles were found, indicating the probable operation of these cycles in grapevines. The presence of pyruvic, glyceric, and lactic acids is suggestive of the glycolytic pathway, and shikimic and quinic acids of the shikimic pathway. The biochemical mechanisms for the formation of tartaric, oxalic, chlorogenic, and caffeic acids in plants are unknown.

Glucose was the major sugar during the green stage in all parts of the grapevine except the roots. As the different parts of the vine matured, sucrose became the predominant sugar, except in the pedicels and berries, which accumulated large amounts of glucose and fructose. Free glucose and fructose in grapevines are believed to be formed by hydrolysis of sucrose. If that is the case then the glucose-fructose ratio should be 1. Figures 2 to 5 indicate that the glucose-fructose ratio is about 1 for all stages of growth except the green berry stage, and for all parts of the vine except bark and tendrils. Previous investigations (15, 17) have also shown that immature berries and leaves contain more glucose than fructose. The reason is not known for the greater amounts of glucose than fructose in the bark and tendrils and in other parts of the vine during the green berry stage. Hydrolysis of starch reserves into glucose, conversion of fructose into glucose, or preferential metabolism of fructose could account for the differences; which if any of these hypotheses is correct, however, remains to be shown experimentally.

The sugar content of the bark, wood, pedicel, and berry changed greatly during the season (fig 2–5). During the green berry stage the bark contained large amounts of sugar which were partly depleted during the ripening and ripe stages of fruit development. As the vines approached dormancy (November 1) there was a large increase of sucrose in both the bark and wood.

The biochemical formation of tartrate and malate in grapevines appear to be unrelated. Figure 10 shows that tartaric acid is formed very rapidly during the initial period of leaf growth. The concentration of tartrate in leaves did not change much after the leaves reached about half their maximum size. The malate concentration of leaves, on the other hand, increased for over 4 months after budburst. Unpublished data by Nassar and Kliewer showed that when $^{14}CO_2$ was presented to immature leaves, over half of the label in the organic acid fraction was found in tartaric acid. A similar experiment performed on fully mature leaves showed only 2 to 12 % label in tartaric acid and 70 to 90 % of the total activity in the organic acid fraction in malic acid. These 2 organic acids also differ greatly in their ability to be broken down by the plant. More than 50 % of radioactive malic acid administered to grape leaves was converted to other substances in 24 hours, whereas less than 2 % of labeled tartaric acid was metabolized during the same period (unpublished data). Vickery and Palmer (27) also found that tartaric acid is used sluggishly or not at all in tobacco plants. The data in figures 6 to 9 also indicate that the malate content of berries decreased considerably more than tartrate during the growing season. Several other investigators have reported similar findings with other varieties of grapes (3, 24).

Between September 28 and November 1 there were approximately 3- and 7-fold decreases in tartrate and malate, respectively, in the leaves. During this same period the tartrate content of the wood doubled and the malate concentration increased nearly 10-fold (fig 8, 9). There were also smaller increases of tartrate and malate in the petiole. These data suggest that tartrate and malate were translocated to the woody tissue when the leaves became senescent.

The roots were the only part of the grapevine containing more citrate than malate. The relatively large accumulation of citrate and the small amount of malate in the roots indicates that this part of the vine contains enzyme systems considerably different from the rest of the plant.

Acknowledgment

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