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PYRUVATE METABOLISM DURING MATURATION OF HAMLIN ORANGES

BONGWOO ROE, PAUL L. DAVIS* and JOSEPH H. BRUEMMER

U.S. Citrus and Subtropical Products Laboratory†, P.O. Box 1909, Winter Haven, Florida 33883, U.S.A.; *U.S. Horticultural Research Station†, 2120 Camden Road, Orlando, Florida 32803, U.S.A.

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Abstract—The roles of the pyruvate decarboxylation pathway and TCA metabolic cycle in activation of anaerobic metabolism in ripening Hamlin oranges were investigated. Oranges were harvested weekly from October to February during the 1980–81 and 1981–82 growing season. Juice vesicles from each weekly sample were assayed for pyruvate decarboxylase, alcohol dehydrogenase, malic enzyme, phosphoenolpyruvate carboxylase, malate dehydrogenase, citrate synthase, isocitrate dehydrogenase and cytochrome oxidase. Also, juice was assayed for ethanol, acetaldehyde, pyruvate, oxalacetate, malate and citrate. In December when ethanol accumulated rapidly in the fruit, pyruvate decarboxylase and alcohol dehydrogenase increased markedly. During the same month, the pyruvate level declined, suggesting that the increases in enzyme levels activated the conversion of pyruvate to ethanol.

INTRODUCTION

Davis [1] observed that ethanol slowly accumulated in the juice of citrus fruit during the growing season but increased rapidly as the fruit became ripe. Earlier, Bain [2] noted an increase in respiration of Valencia orange as the fruit grew to maturity. However, after reaching the ripe stage, respiration per fruit declined. Respiration in Satsuma mandarin reached a maximum when acidity was highest in mature fruit and declined as acidity decreased [3].

Ethanol is a product of anaerobic metabolism so its accumulation indicates that some substrates of energy metabolism are passing through the anaerobic pathway. A decline in respiration at the stage when ethanol accumulates suggests that ripening is characterized by a change from aerobic to anaerobic metabolism.

In citrus fruit, pyruvate is the substrate for aerobic metabolism. It is oxidized by the pyruvate dehydrogenase complex to acetyl CoA [4], which enters the TCA cycle by condensation with oxalacetate to form citrate. Pyruvate is also a substrate for anaerobic metabolism through pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) [5]. Thus, dehydrogenase and decarboxylase are competing for pyruvate at a metabolic branch point. The affinities of the enzymes for pyruvate and the concentration of coenzymes and other reaction effectors determine the reaction rates of the two pathways [6]. The affinity of each enzyme for pyruvate is regulated by the concentration of the reaction products, acetaldehyde and acetyl CoA, and the affinity for each at the regulating site on the enzymes. Because the concentrations of acetaldehyde and acetyl CoA also depend on enzymes and products of the alcohol dehydrogenase and citrate synthase reactions, ultimate control of the dehydrogenase and decarboxylase reactions is determined by substrates, products and enzymes of both aerobic and anaerobic pathways.

We have examined substrate levels and activities of a number of enzymes in the decarboxylase and TCA pathways in Hamlin fruit during maturation. The increased activity of the decarboxylase enzyme and change in levels of pyruvate and oxalacetate suggest that mature fruit divert available pyruvate to ethanol through the decarboxylase pathway. This paper discusses results of that study.

RESULTS AND DISCUSSION

Accumulation of ethanol in juice of Hamlin oranges during the 1980–81 season followed the pattern described by Davis [1] for the 1969–70 season, i.e. ethanol increased sharply in December (Table 1). The difference between the November and December values are statistically significant ($P < 0.01$). The acetaldehyde level also increased during the growing season. The equilibrium between substrate and product for the alcohol dehydrogenase reaction was constant from December to February as indicated by the acetaldehyde–ethanol ratios. December marked a change in equilibrium; before the sharp increase in ethanol the ratio was higher and variable. The fruit reached an acceptable flavor ratio of solids to acid [7] in December and this ratio increased slightly until the January 1981 freeze. The freeze accelerated an increase in both the solids–acid ratio and the ethanol and acetaldehyde levels.

Ethanol values for Hamlin orange juice during the 1981–82 season were similar to the earlier season values; the greatest increase occurred during December (Table 2). The difference between November and December values

†Southern Region, Agricultural Research Service, U.S. Department of Agriculture.

Table 1. Accumulation of ethanol and acetaldehyde in Hamlin orange during maturation 1980–81

	Ethanol (mM)	Acetaldehyde (mM)	Acetaldehyde–ethanol ratio ($\times 10^{-3}$)	Acid (%)	°B/A
Sept	0.24±0.05	0.008±0.001	33	1.32±0.03	7.3±0.5
Oct	0.88 0.19	0.020 0.002	23	1.06 0.05	10.3 0.9
Nov	0.92 0.11	0.028 0.006	30	0.87 0.02	12.8 0.3
Dec	3.66 0.53	0.043 0.005	12	0.84 0.01	14.2 0.1
Jan	5.34 0.21	0.068 0.001	13	0.81 0.02	15.4 0.2
			1981 Freeze		
Jan	12.83 0.15	0.119 0.004	10	0.72 0.12	18.3 1.9
Feb	12.75 0.04	0.134 0.008	10	0.57 0.05	19.7 0.3

Each value is the mean of four analyses ± s.e. of the mean.

Table 2. Accumulation of ethanol, acetaldehyde and substrate levels in Hamlin orange during maturation 1981–82

	Ethanol (mM)	Acetaldehyde (mM)	Acid (%)	°B/A	Pyruvate (μM)	Oxalacetate (μM)	Malate (mM)	Citrate (mM)
Oct	1.2±0.5	0.027±0.007	1.22±0.02	9.2±0.1	68.3±2.6	35.8±2.1	4.5±0.2	39.9±0.7
Nov	1.8 0.7	0.033 0.004	1.07 0.03	10.7 0.2	63.5 3.4	25.2 2.1	5.4 0.2	38.6 0.6
Dec	4.7 0.7	0.047 0.006	0.95 0.05	12.4 0.4	46.8 1.4	24.5 1.7	6.8 0.3	38.3 0.7
Jan	6.3 0.4	0.058 0.004	0.95 0.03	12.8 0.6	47.7 1.3	19.5 2.9	6.7 0.3	38.2 0.4
Feb	10.3 1.3	0.071 0.002	0.84 0.05	15.7 1.2	48.7 1.1	17.9 2.4	7.0 0.1	36.3 0.7
Mar	7.8 0.8	0.081 0.009	0.79 0.07	16.8 1.3	ND	ND	ND	ND

Each value is the mean of four analyses ± s.e. of mean. ND, Not determined.

are statistically significant ($P < 0.01$). The ratio of acetaldehyde and ethanol in the alcohol dehydrogenase reaction changed between November and December. The acetaldehyde–ethanol ratios ($\times 10^3$) for October through March were as follows: 22, 18, 10, 9, 7 and 10. The freeze of January 1982 had no apparent effect on fruits in the location from which these were harvested.

Acetaldehyde is in equilibrium with pyruvate in the cytoplasm through the pyruvate decarboxylase reaction (Fig. 1). Pyruvate is also in equilibrium with acetyl CoA in the mitochondria through the dehydrogenase reaction. Although pyruvate values in Table 2 include cytoplasmic and mitochondrial sources, the drop in pyruvate level from November to December represents a decline in substrate available to pyruvate decarboxylase because pyruvate is formed in the cytoplasm from glycolysis and must be transported into the mitochondria for the dehydrogenase reaction. Oxalacetate level in the juice declined steadily over the growing season. Malate and citrate are present in vacuoles of the juice vesicles and so these values do not represent substrate levels for the enzymes. The malate level increased until December while the citrate level was steady throughout the season.

Pyruvate decarboxylase activity during the 1980–81 (Table 3) and 1981–82 (Table 4) seasons was markedly higher in December compared to November. The difference between the values are statistically significant ($P < 0.05$). The higher pyruvate decarboxylase activity would increase competition with the pyruvate dehydrogenase complex for pyruvate and thereby increase decarboxylation to acetaldehyde. The higher pyruvate decarboxylase activity could thus explain the decline in pyruvate and increase in acetaldehyde and ethanol in December. The 30% higher activity for alcohol dehydrogenase in December compared to November (Tables 3 and 4) would increase the competition with pyruvate decarboxylase for acetaldehyde and its availability for reduction to ethanol. Thus, the higher alcohol dehydrogenase level in December could explain the lower concentration ratio of acetaldehyde to ethanol that occurred that month. Malic enzyme (EC 1.1.1.40) increased steadily during the 1981–82 season which probably influenced the equilibrium between malate and pyruvate in the cytoplasm; malate increased and pyruvate decreased over the season (Table 2). Phosphoenolpyruvate carboxylase (EC 4.1.1.32) increased markedly during the early part of the 1980–81 and 1981–82 seasons (Tables 3 and 4). However, because malate dehydrogenase (EC 1.1.1.37) was much more active than phosphoenolpyruvate carboxylase (> 10-fold), the increase probably had little effect on the equilibrium concentration of oxalacetate which declined during the season (Table 2). The freeze in January 1981 markedly lowered enzyme activities (Table 3).

In mitochondria, oxalacetate is in equilibrium with malate through malate dehydrogenase and with citrate through citrate synthase (EC 4.1.3.7) (Fig. 1). Competition for oxalacetate by the enzymes could affect its concentration. From October to November in both seasons citrate synthase increased markedly and malate dehydrogenase was relatively constant (Tables 5 and 6). The increase in synthase activity would increase the demand for oxalacetate and could account for the decline in

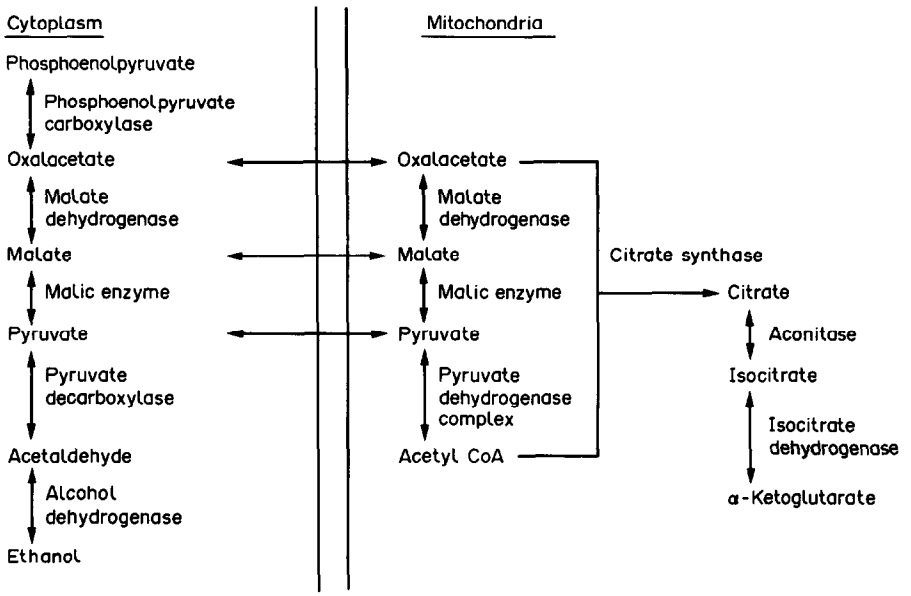

Fig. 1. Pyruvate metabolism in citrus fruit.

Table 3. Enzymes of malate and pyruvate metabolism in Hamlin orange during maturation 1980–81; U/mg protein

	Pyruvate decarboxylase	Alcohol dehydrogenase	Malic enzyme	Phosphoenol-pyruvate carboxylase	Malate dehydrogenase
Sept	15±1	79±1	47±1.3	11.2±1.2	1949±51
Oct	26 1	122 7	80 1.2	15.5 0.5	2218 67
Nov	27 1	133 2	80 2.1	20.3 1.1	2536 36
Dec	48 2	172 5	79 1.5	33.5 1.5	2811 16
Jan	53 1	126 4	59 4.3	28.8 0.5	3548 107
			1981 Freeze		
Jan	15 3	20 2	18 0.5	5.2 2.1	1760 45
Feb	6 2	15 1	8 0.2	2.3 0.2	57 7

U is defined as n mol of substrate consumed in the reaction per min at 25°. Each value is the mean of four analyses ± s.e. of the mean.

Table 4. Enzymes of malate and pyruvate metabolism in Hamlin orange during maturation 1981–82; U/mg protein

	Pyruvate decarboxylase	Alcohol dehydrogenase	Malic enzyme	Phosphoenol-pyruvate carboxylase	Malate dehydrogenase
Oct	10.7±0.4	77.2±1.2	22.5±2.5	8.9±0.7	1785± 35
Nov	14.8 1.3	88.5 3.5	34.3 6.2	15.6 1.3	2135 135
Dec	32.3 2.1	112.5 2.5	48.7 1.2	30.2 3.2	2261 18
Jan	46.5 1.5	143.3 4.1	62.5 2.5	31.5 0.5	2475 26
Feb	47.5 2.5	127.5 2.5	46.2 4.1	29.5 0.5	2481 81

U is defined as n mol of substrate consumed in the reaction per min at 25°. Each value is the mean of four analyses ± s.e. of the mean.

Table 5. Enzymes of TCA cycle in Hamlin orange during maturation 1980–81; U/mg protein

	Malate dehydrogenase	Citrate synthase	Isocitrate dehydrogenase
Sept	272 ± 29	3250 ± 50	7.4 ± 0.7
Oct	728 21	9000 20	7.8 0.1
Nov	900 4	13000 500	7.5 0.3
Dec	898 2	15600 1000	9.9 0.1
Jan	935 26	15550 250	12.5 0.2
		1981 Freeze	
Jan	400 88	3850 350	1.12 0.1
Feb	97 1	1150 50	0.17 0.04

U is defined as nmol of substrate consumed in the reaction per min at 25°c. Each value is the mean of four analyses ± s.e. of the mean.

oxalacetate level during that period. Isocitrate dehydrogenase (EC 1.1.1.42) activity was relatively constant over the season (Tables 5 and 6). Cytochrome oxidase (EC 1.9.3.1) was as active in December as in November. Thus, the level of the terminal oxidase was not a factor in the abrupt increase in anaerobic metabolism.

The general pattern of change in phosphoenolpyruvate carboxylase, malate dehydrogenase, isocitrate dehydrogenase and malic enzyme that we observed is similar to changes in these enzymes during maturation of the Satsuma mandarin described by Hirai and Ueno [3]. However, citrate synthase activity in the Hamlin reached a plateau in the middle of the season, whereas activity in the Satsuma continued to rise after respiration declined.

Stimulation of the pyruvate decarboxylase reaction as a result of an increase in the enzyme level could result in a decrease in pyruvate and increase in ethanol in ripe Hamlin fruit. The increase in alcohol dehydrogenase level could also increase conversion of acetaldehyde to ethanol. However, pyruvate decarboxylase in the orange is present as the inactive complex [8] and its activation depends on pyruvate concentration. The plant enzyme also responds to transient pH changes; it is activated by lower pH [9]. The pyruvate dehydrogenase complex, the competing enzyme for pyruvate, is also present in plant tissue in the inactive form [10] and the reaction is controlled by ratios of NADH/NAD, ATP/ADP and the product, acetyl CoA. Thus, the cofactors for these reactions regulate the rate and competition for the substrate, pyruvate, and may be more important in control of metabolism of pyruvate than the level of the enzymes.

EXPERIMENTAL

Hamlin oranges were obtained from commercial groves near the Horticultural Field Station, USDA, Orlando, Florida. All enzymes, coenzymes and substrates were obtained from Sigma and all other chemicals were obtained from Fisher. Twelve oranges were selected randomly from weekly harvests of 24 fruits. They were peeled and frozen in liquid N_2. The frozen juice vesicles were separated from seeds and coarse section membranes, mixed thoroughly to form a composite sample and stored in two 1 l. jars at −100°. Samples were removed from storage after 60–90 days for enzyme and substrate assays.

Cytoplasmic enzyme assays. A 50 g portion of frozen juice vesicles from each sample was pulverized in a micro mill at −196° (liquid N_2) with a measured amount of 1 M Tris calculated to raise the pH of the thawed extract to 7. The neutralized powder was thawed, strained through two layers of cheesecloth and centrifuged at 10000 *g* for 15 min. The supernatant was saturated with solid $(NH_4)_2SO_4$, equilibrated for 2 hr at 4° and centrifuged at 15000 *g* for 15 min. The pellet was dialysed against H_2O at 4° overnight. The resulting suspension was centrifuged at 15000 *g* for 15 min. The clean supernatant was assayed for phosphoenolpyruvate carboxylase [3], malate dehydrogenase [11], malic enzyme [12], pyruvate decarboxylase [13] and alcohol dehydrogenase [14]. Protein was estimated by the Potty modification of the Lowry method [15].

Mitochondrial enzyme assays. A mitochondrial fraction was prepared by the method of ref. [16]. The washed pellet was suspended in 10 vols of 0.1 M KPi buffer (pH 7) containing 0.1% Triton X-100 and 5 mM glutathione. After addition of glycerol to a final concn of 50%, the suspension was centrifuged again at 10000 *g* for 15 min [3]. The supernatant was assayed for citrate synthase [17], isocitrate dehydrogenase [18], malate dehydrogenase [11] and cytochrome oxidase [19].

Substrate assays. A 50 g portion of frozen juice vesicles from each sample was pulverized with 3.4 ml of 40% $HClO_4$ at −196° and 2 g of Polyclar AT was added to the frozen powder. The mixture was then thawed, strained through two layers of cheesecloth, and centrifuged at 15000 *g* for 20 min. The supernatant was raised to pH 6.5–7.0 by adding solid K_2CO_3. After addition of 1% Carbowax-4000 the $KClO_4$ was pelleted by centrifugation at 25000 *g* for 15 min [20] and the clear supernatant was assayed for citrate [21], malate [22], pyruvate [23] and oxalacetate [24]. Juice was hand reamed from 10 fruit samples for analysis of EtOH and CH_3CHO by GC analysis of headspace [1, 25]. Soluble solids values, °Brix (°B), were calculated from refractive indices of the fresh filtered juice. Titratable

Table 6. Enzymes of TCA cycle in Hamlin orange during maturation 1981–82; U/mg protein.

	Malate dehydrogenase	Citrate synthase	Isocitrate dehydrogenase	Cytochrome oxidase
Oct	469 ± 31	5033 ± 633	2.7 ± 0.1	23.2 ± 4.2
Nov	462 8	12950 550	5.9 0.3	44.2 0.9
Dec	497 3	13500 100	7.3 0.2	44.6 0.9
Jan	702 54	12600 800	9.2 0.2	52.3 0.8
Feb	469 107	8500 700	6.0 2.2	31.5 0.5

U is defined as n mol of substrate consumed in the reaction per min at 25°. Each value is the mean of four analyses ± s.e. of the mean.

acidity was calculated from the titrated vol. of standard NaOH required to adjust the juice to pH 8.2 and is expressed as % anhydrous citric acid. Differences between means for November and December were analysed statistically by Student's *t*-test.

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