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Development of citrus fruits: Fruit development and enzymatic changes in juice vesicle tissue [1]

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Seasonal changes in enzyme activities and some components of Satsuma mandarin and sweet lime were studied.

Although the main acid in mature Satsuma mandarin fruit is citrate, malate was predominantly accumulated in the very early stage of fruit development. In sweet lime, malate was chiefly accumulated throughout fruit development.

Juice vesicle tissue in Satsuma mandarin fruit developed in four distinctive stages. In the first stage, enzyme activities and the contents of protein and nucleic acid increased. The activity of phosphoenolpyruvate carboxylase increased most rapidly. Cell division was observed in the first half of this stage. In the second stage, acids accumulated remarkably but enzyme activities and RNA content did not change. In the third, maturation stage, the content of RNA increased again. In the fourth stage, the contents of citrate and RNA decreased, whereas the activity of NAD-dependent isocitrate dehydrogenase increased.

Compared with climacteric fruit, no remarkable increase in the activity of NADP-dependent malic enzyme was observed in citrus fruit during maturation, while activities of citrate synthetase and malate dehydrogenase increased fourfold. Respiratory activity did not rise as prominently during that time.

Acid metabolism in fruit tissue is interesting from the viewpoints of its physiology as well as commercial availability. Carboxylic acids were considered to be synthesized via CO_2 fixation catalyzed by phosphoenolpyruvate carboxylase (EC 4.1.1.31) (*2*, *17*). Ripening of fruits has been thought to occur by different acid metabolisms for climacteric and non-climacteric fruits. During ripening of apple fruit (climacteric fruit), a steep rise in respiratory activity was accompanied by an increase in the activity of NADP-dependent malic enzyme (EC 1.1.1.40), which is considered to play an important role in decreasing acid content (*10*). Similar change in malic enzyme activity was also observed in pear fruit (*7*). In contrast, little data are available to correlate acid metabolism with enzyme activity during ripening of non-climacteric fruits.

In the present paper, we describe changes in some components and enzyme activities in juice vesicle tissue during the development of citrus fruits (non-climacteric fruits) and also discuss the acid metabolism in relation to fruit development.

Abbreviations: MDH, malate dehydrogenase; NAD-IDH, NAD-dependent isocitrate dehydrogenase; GSH, glutathione; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

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Materials and methods

Plant materials

Early maturing Satsuma mandarin (*Citrus unshiu* Marcov.) Miyagawa wase was used. In 1976, fruit on leaf shoots was harvested from mature trees. For the fruit used, anthesis was from May 15 to 17 and the commercial ripening time was the end of October. Sweet lime (acid-less citrus species) was used as a reference fruit for comparison of acid accumulation.

Extraction and determination of nucleic acid and protein

Nucleic acid was extracted by the method of Ogur and Rosen (*16*) with slight modifications. RNA and DNA extractions were repeated three times. After extraction, the residue was dissolved with 1 N NaOH, then used for protein determination. RNA was assayed by measuring OD 260 nm, and DNA was determined by the method of Giles and Myers (*8*). Protein was assayed by Nessler's method (*11*).

Enzyme extraction and assay

Juice vesicle tissue was ground with mortar and pestle using sea sand and two to four times the tissue weight of the grinding medium described below. The pH was adjusted by adjusting this medium. In the very early stage of fruit development (10/VI–12/VI for Satsuma mandarin and 5/VII–6/VII for sweet lime), a glass homogenizer was used for extraction instead of a mortar and pestle. Adjustment of pH was not necessary in this case. All procedures were carried out at 4°C.

NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41): The grinding medium was composed of 0.05 M phosphate buffer (pH 7.0), 0.5 M sucrose and 10 mM isoascorbate. One molar KOH solution containing 0.5 M sucrose was used as an adjusting medium. The homogenate was centrifuged at $500 \times g$ for 10 min. The resulting pellet was washed with a medium composed of 0.05 M phosphate buffer (pH 7.0) and 0.5 M sucrose. The washed pellet was suspended in a bursting medium composed of 0.05 M phosphate buffer (pH 7.0), 0.1% Triton X-100 and 4 mM GSH. After addition of glycerol at a final concentration of 50%, the suspension was centrifuged at $15{,}000 \times g$ for 15 min. The pellet was re-extracted twice and the combined supernatant was used as the enzyme. The assay mixture for IDH contained 40 mM Hepes buffer (pH 8.2), 2 mM sodium isocitrate, 800 μM NAD, 200 μM $MnSO_4$ and the enzyme in a final volume of 0.5 ml. The increase in OD 340 nm was recorded.

Other enzymes: The grinding medium was composed of 0.2 M Tris-HCl buffer (pH 8.2), 10 mM isoascorbate and 0.1% Triton X-100. For adjusting medium, 1 M Tris solution was used. The homogenate was centrifuged at $500 \times g$ for 10 min. The pellet was washed twice with a washing medium containing 0.2 M Tris-HCl buffer (pH 8.2) and 0.1% Triton X-100. The supernatant was combined and passed through a Sephadex G-25 column. The resulting eluate was used as enzyme. Since citrate inhibits citrate synthetase and PEPC, the gel filtration could not be omitted. To assay aconitase (EC 4.2.1.3) the enzyme was preliminarily incubated with 1 mM GSH for 1 hr. The reaction mixture contained 40 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, 200 μM *cis*-aconitate and enzyme in a final

volume of 0.5 ml. Citrate synthetase (EC 4.1.3.7) was assayed according to Srere (*19*), with slight modifications. The reaction mixture contained 40 mM Tris-HCl buffer (pH 9.0), 40 μM 5,5′-dithiobis-(2-nitrobenzoic acid), 80 μM acetyl CoA and enzyme in a final volume of 0.5 ml. PEPC was assayed by the method of Lane et al. (*14*) with slight modifications. The reaction mixture contained 40 mM Tris-HCl buffer (pH 8.5), 10 mM $KHCO_3$, 2 mM $MgCl_2$, 2 mM PEP, 500 μM GSH, 150 μM NADH and enzyme in a final volume of 0.5 ml. Since the extract of citrus fruit had high MDH activity, exogenous enzyme was not necessary. MDH (EC 1.1.1.37) was assayed as described by Asahi and Nishimura (*1*). Malic enzyme was assayed by measuring OD 340 nm. The reaction mixture contained 80 mM Tris-HCl buffer (pH 7.4), 2 mM malate, 170 μM NADP, 200 μM $MnSO_4$ and enzyme in a final volume of 0.5 ml.

Determination of carboxylic acid content

To juice vesicle tissue, ethanol was added at a final concentration of 70% and the tissue was homogenized for nucleic acid extraction. The resulting supernatant of the homogenate after centrifugation was used for carboxylic acid determination. After removal of ethanol by evaporation in vacuo, the aqueous solution was washed with ether to remove lipids. A portion of the water layer was measured with a carboxylic acid analyzer (Seishin Seiyaku Co., Ltd.).

Morphological observation

To examine cell division in juice vesicles, fruit was harvested on June 4, 9, 12, 17, 21, and 26. After being fixed in Farmer's fluid (ethanol-acetate), the samples were preserved in 70% ethanol. They were embeded in paraffin then cut 15 μm thick. Delafield's hematoxylin-eosin was used for staining.

Results

Fruit development and acid accumulation

In Satsuma mandarin fruit DNA content per fruit reached maximum on June

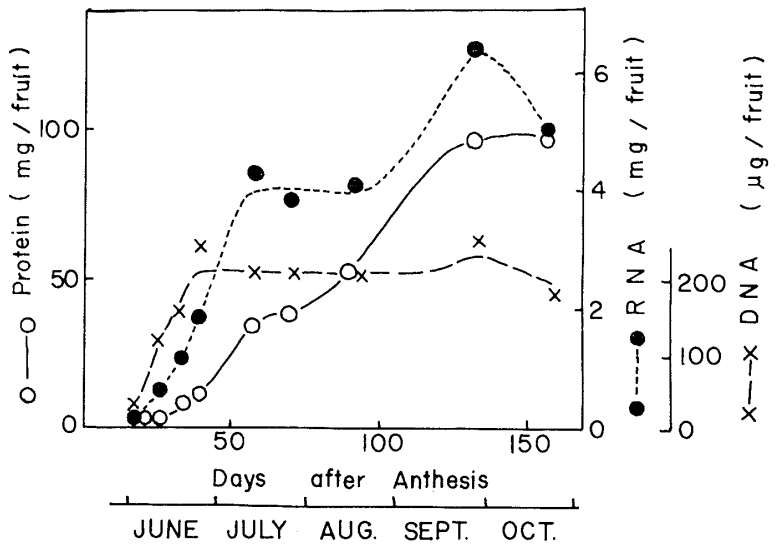
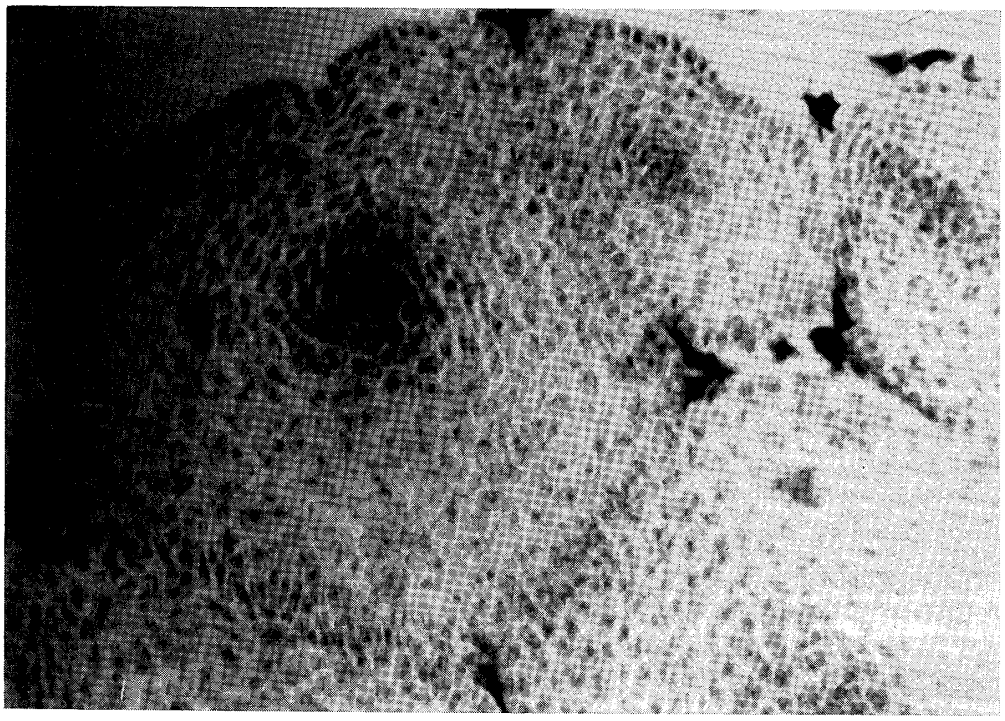
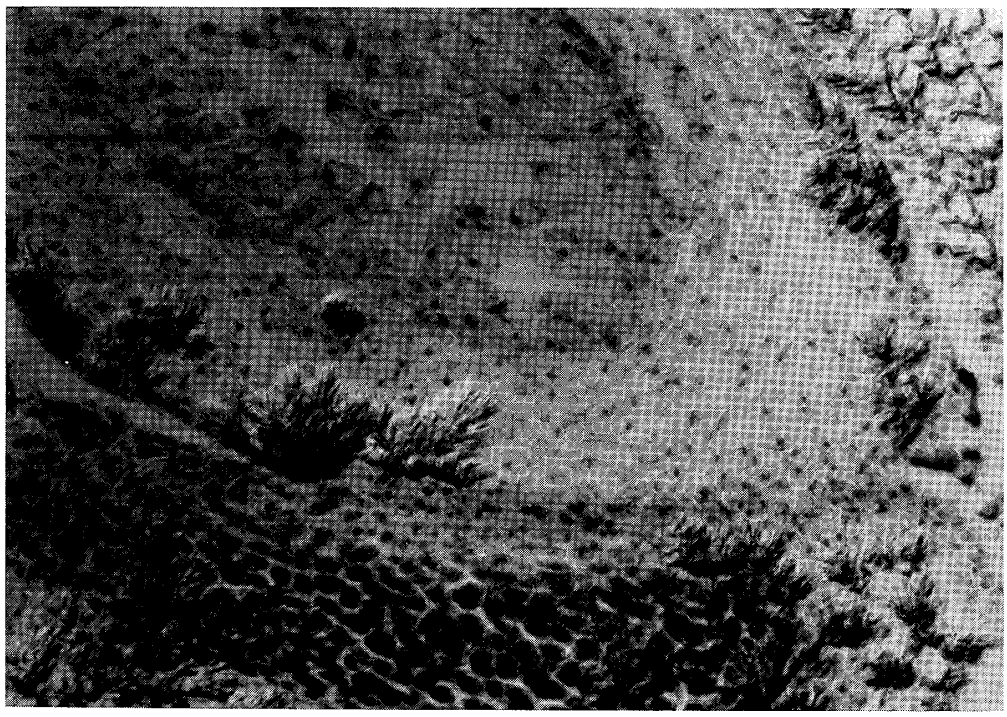


Fig. 1. *Changes in contents of nucleic acid and protein in juice vesicle tissue during development of Satsuma mandarin fruit.*

26 and thereafter scarcely changed (Fig. 1), suggesting that cell division had ceased by this time. Cell division was observed in the center of almost all juice vesicles on June 12 (Fig. 2a). From June 17 to 21 the percentage of actively mitotic juice



(a)



(b)

(c)

Fig. 2. *Sections of juice vesicles from Satsuma mandarin.* a) Juice vesicles on June 12 (×150). b) Juice vesicles on June 21. Lower vesicle shows cell division and the upper shows no division (×150). c) Juice vesicles on June 26 (×150).

vesicle decreased (Fig. 2b) and no cell division was observed on June 26 (Fig. 2c). The first rapid increase in both contents of RNA and protein was observed until mid-July and the second from mid-August to September. RNA content decreased in October, whereas protein content remained unchanged during that time (Fig. 1).

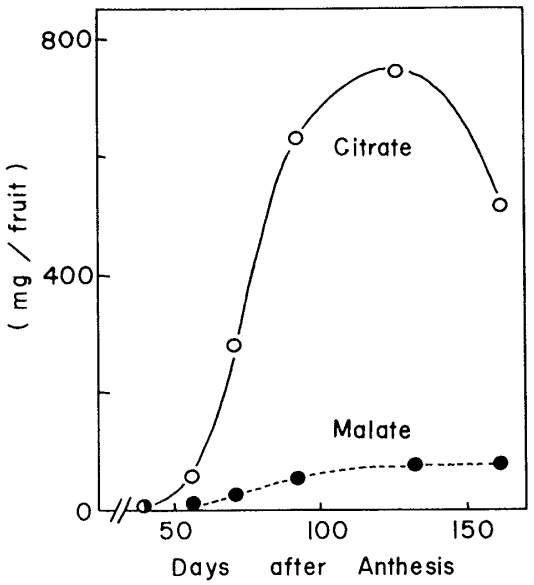


Fig. 3. *Acid accumulation in tissue of juice vesicle during development of Satsuma mandarin fruit.*

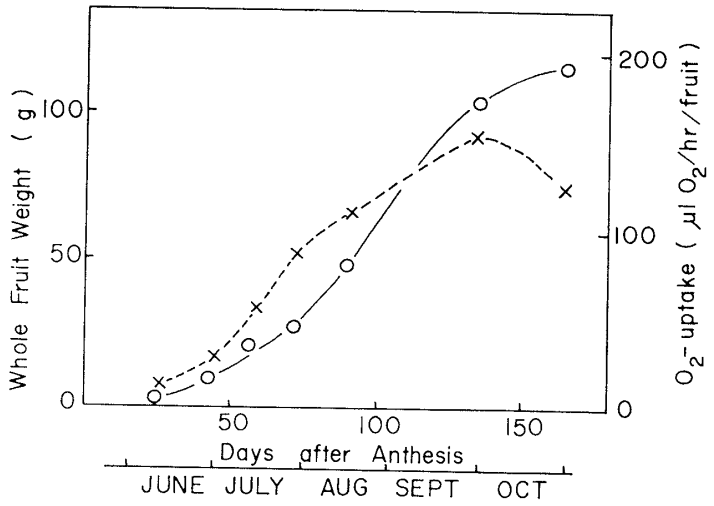


Fig. 4. *Changes in respiratory activity of juice vesicle and fresh weight of whole Satsuma mandarin fruit.* --×--, respiratory activity; -○-, whole fruit weight. Respiratory activity was measured with a Warburg manometer. Each vessel contained 1 ml of 0.1 M phosphate buffer (pH 7.0) and 1 or 2 g of juice vesicle.

Although the major acid component in mature citrus fruit is known to be citrate, in the very early stage of Satsuma mandarin development, malate was a dominant acid component (0.047 mg malate/fruit and less than 0.005 mg citrate/fruit on June 13). Citrate content surpassed malate content on June 26 (1.53 mg malate/fruit and 4.83 mg citrate/fruit). Citrate content per fruit reached its maximum in September and then decreased (Fig. 3). In sweet lime fruit malate was a major acid component throughout (0.39 mg malate/fruit and 0.11 mg citrate/fruit on July 8; 11.6 mg malate/fruit and 1.1 mg citrate/fruit on October 22).

Respiratory activity per fruit reached maximum in late September then decreased slightly. No climacteric rise was observed in juice vesicle tissue (Fig. 4).

Changes in enzyme activities

From the pattern of DNA content, the cell number of the juice vesicle appeared to be unchanged after June 26. Enzyme level was expressed as activity per fruit. It may reflect activity per cell.

Table 1 *Changes in enzyme activities in juice vesicle tissue during development of Satsuma mandarin fruit*

Date	Days after anthesis	Enzyme activities (μmoles/min/fruit)					
		PEPC	MDH	Citrate synthetase	Aconitase	NAD-IDH	Malic enzyme
10/VI–12/VI	23–25	0.119	9.27	0.180	0.456	0.090	—
29/VI–1/VII	41–44	1.61	38.7	0.768	2.38	0.255	—
13/VII–14/VII	56–57	3.11	108	2.35	1.80	0.461	9.19
27/VII–28/VII	71–72	5.64	107	2.80	3.78	1.12	18.2
16/VIII–17/VIII	90–91	6.90	60.4	2.40	4.37	0.785	21.8
30/IX–1/X	135–136	6.35	473	11.5	6.77	1.96	27.0
29/X–30/X	164–165	5.07	557	14.6	8.12	4.22	15.7

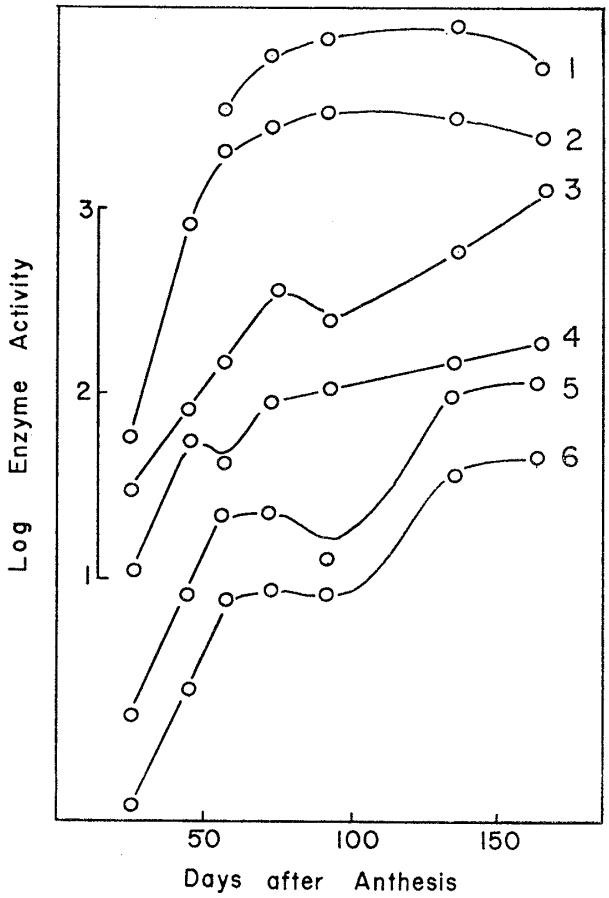


Fig. 5. *Changes in enzyme activities in juice vesicle tissue during development of Satsuma mandarin fruit.* 1: malic enzyme, 2: PEPC, 3: NAD-IDH, 4: aconitase, 5: MDH, 6: citrate synthetase. The ordinate indicates only the rate of the change in each enzyme activity and not the relation among them.

In Satsuma mandarin fruit, activities of PEPC and malic enzyme reached their maxima around July 28 and thereafter remained almost constant, while activities of citrate synthetase and MDH increased biphasically; the first phase ceased on July 15 and the second began between August 17 and September 30. The other enzymes, aconitase and NAD-IDH, did not show such trends (Table 1). These trends can be easily seen when the activities are plotted on a logarithmic scale (Fig. 5). In sweet lime fruit, the seasonal changes of PEPC and citrate synthetase were similar to those of Satsuma mandarin. On the other hand, aconitase and NAD-IDH

Table 2 *Changes in enzyme activities in juice vesicle tissue during fruit development of sweet lime fruit*

Date	Days after anthesis	Enzyme activities (μmoles/min/fruit)					
		PEPC	MDH	Citrate synthetase	Aconitase	NAD-IDH	Malic enzyme
5/VI–6/VII	42–43	0.23	12.8	0.135	0.083	0.0547	—
22/VII–23/VII	58–59	2.51	52.4	0.950	3.73	0.947	5.98
24/VIII–25/VIII	91–92	10.4	330	3.33	4.36	1.59	29.9
19/X–20/X	147–148	10.4	550	14.9	3.39	5.62	36.8

increased monophasically and biphasically, respectively. It is not clear whether MDH increases monophasically or biphasically (Table 2).

Discussion

Fruit development has been divided into several stages distinctive in metabolism and growth rate in some fruits (*15*, *18*). But no data for analysis of the development of citrus fruits has been published. During development of Satsuma mandarin fruit, four distinctive stages were observed in the changes in enzyme activities and some components. The first stage lasted until mid-July, characterized by rapid increases in enzyme activities and contents of RNA and protein. Acid accumulation was not marked. Cell division occurred in the first half of this stage. The end of cell division was roughly comparable with the data reported by Kuraoka and Kikuchi (*13*). In the second stage (from mid-July to mid-August), enzyme activities and RNA content per fruit remained almost unchanged. But the fresh weight of the fruit increased about twofold and acid accumulation was most prominent. The third stage (from mid-August to the end of September) is considered to be a maturation stage. The activities of MDH and citrate synthetase increased about fourfold. The contents of RNA and protein increased rapidly again but acid accumulation during this stage was not remarkable. In the last stage, NAD-IDH activity increased, while the activities of PEPC and malic enzyme decreased slightly. The contents of RNA and citrate decreased considerably. Thus this can be considered the stage of senescence. Sugar accumulation is thought to occur during the third and fourth stages (*12*). Similar changes in enzyme activities were observed in sweet lime fruit and may be common in the development of citrus fruits.

In the present study, the time of acid accumulation (second stage) did not coincide with that of the increase in activities of PEPC and citrate synthetase (first stage). This suggests that acid accumulation depends not only upon these enzyme levels, but also other factors. Two possibilities are related to regulation of acid accumulation. First, in Satsuma mandarin, the ratio of the enzyme activities of PEPC/NAD-IDH was high in the acid-accumulating stage. Judging from the cofactor required, NAD-IDH measured in this experiment is mitochondrial (*5*). This could suggest that NAD-IDH plays an additional regulatory role in acid accumulation of citrus fruits. Second, Wallace and his coworkers suggested inhibition of aconitase by citramalate and H_2O_2 (*2*, *4*), while Bruemmer and Roe proposed regulation of MDH activity by the coenzyme level (*3*). In addition to enzyme levels, these factors are noteworthy to be regulatory.

The increase in respiratory activity in citrus fruits was not as prominent as that of climacteric fruits in the maturation stage. In spite of this, activities of MDH and citrate synthetase in the former increased remarkably. In addition to this discrepancy, the increase in activity of NADP-dependent malic enzyme during ripening was not as remarkable as that observed in apple, pear and cherry fruits (*7*, *9*, *10*). Some differences in metabolic properties between these fruits and citrus fruits may exist.

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References

(*1*) Asahi, T. and M. Nishimura: Regulatory function of malate dehydrogenase isoenzymes in the cotyledons of mung bean. *J. Biochem.* 73: 217–225 (1973).

(*2*) Bogin, E. and A. Wallace: Organic acid synthesis and accumulation in sweet and sour lemon fruits. *Proc. Amer. Soc. Hort. Sci.* 89: 182–194 (1966).

(*3*) Bruemmer, J. H. and B. Roe: Regulation of NAD and NADP oxidoreductases in oranges. *Phytochem.* 10: 255–259 (1971).

(*4*) Clark, R. B. and A. Wallace: Dark CO_2 fixation in organic acid synthesis and accumulation in citrus juice vesicles. *Proc. Amer. Soc. Hort. Sci.* 83: 322–332 (1963).

(*5*) Cox, G. F. and D. D. Davies: Nicotinamide-adenine dinucleotide-specific isocitrate dehydrogenase from pea mitochondria. *Biochem. J.* 105: 729–734 (1967).

(*6*) Fansler, B. and J. M. Lowenstein: Aconitase from pig heart. In *Methods in Enzymology* 13. Edited by S. P. Colowick and N. O. Kaplan. p. 26–30. Academic Press Inc., New York, 1969.

(*7*) Frenkel, C., I. Klein and D. R. Dilley: Protein synthesis in relation to ripening of pome fruits. *Plant Physiol* 43: 1146–1153 (1968).

(*8*) Giles, K. W. and A. Myers: Determination of DNA by diphenylamine. *Nature* 206: 93 (1965).

(*9*) Hartmann, C. J. R.: Enzymes, maturation et comportement respiratoire d'un fruit (la cerise). In *Facteurs et Régulation de la Maturation des Fruits.* p. 235–240. Editions de C. N. R. S., Paris, 1974.

(*10*) Hulme, A. C. and M. J. C. Rhodes: Pome fruits. In *The Biochemistry of Fruits and their Products.* Edited by A. C. Hulme. p. 333–373. Academic Press Inc., New York, 1971.

(*11*) Johnson, M. J.: Isolation and properties of a pure yeast polypeptidase. *J. Biol. Chem.* 137: 575–586 (1941).

(*12*) Kubota, S., H. Fukui and S. Akao: Changes in concentrations of sugars, organic acids and amino acids in Satsuma mandarin juice during growth of fruits. *Bull. Shikoku Agric. Exp. Sta.* 24: 73–96 (1972).

(*13*) Kuraoka, T. and T. Kikuchi: Morphological studies on the development of citrus fruits I. Satsuma orange. *J. Jap. Soc. Hort. Sci.* 30: 189–196 (1961).

(*14*) Lane, D. M., H. Maruyama and R. L. Easterday: Phosphoenolpyruvate carboxylase from peanut cotyledons. In *Methods in Enzymology* 13. Edited by S. P. Colowick and N. O. Kaplan. p. 277–283. Academic Press Inc., New York, 1969.

(*15*) Nitsch, J. P., C. Pratt, C. Nitsch and N. J. Shaulis: Natural growth substances in Concord and Concord Seedless grapes in relation to berry development. *Amer. J. Bot.* 47: 566–576 (1960).

(*16*) Ogur, M. and G. Rosen: The nucleic acids of plant tissues I. The extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. *Arch. Biochem.* 25: 262–276 (1950).

(*17*) Ribéreau-Gayon, G.: Etude des mécanismes de synthèse et de transformation de l'acide malique, de l'acide tartrique et de l'acide citrique chez *Vitis vinifera* L. *Phytochem.* 7: 1471–1482 (1968).

(*18*) Romani, R. J. and W. G. Jennings: Stone fruits. In *The Biochemistry of Fruits and their Products.* Edited by A. C. Hulme. p. 411–436. Academic Press Inc., New York, 1971.

(*19*) Srere, P. A.: Citrate synthetase. In *Methods in Enzymology* 13. Edited by S. P. Colowick and N. O. Kaplan. p. 3–11. Academic Press Inc., New York, 1969.