Phytochemistry, 1969, Vol. 8, pp. 19 to 23. Pergamon Press. Printed in England

CHANGES IN THE ACTIVITIES OF MALIC ENZYME, MALATE DEHYDROGENASE, PHOSPHOPYRUVATE CARBOXYLASE AND PYRUVATE DECARBOXYLASE DURING THE DEVELOPMENT OF A NON-CLIMACTERIC FRUIT (THE GRAPE)

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(*Received* 18 *June* 1968)

Abstract—The activities of malic enzyme, pyruvate decarboxylase, phosphopyruvate carboxylase and malate dehydrogenase were determined at weekly intervals during the development of a fruit which does not show a climacteric rise in respiration (sultana grape berries). Unlike apples, which show large increases in activities of malic enzyme and pyruvate decarboxylase, grape berries did not have large consistent increases in activities of these enzymes during the period in which malic acid concentration decreased. Phosphopyruvate carboxylase activity which was high in very young berries, decreased to a low value at about the same time that net synthesis of malic acid ceased.

INTRODUCTION

THE INCREASE in respiratory quotient (R.Q.) and increased rate of oxidation of added malate observed during the climacteric rise in respiration of apples has been partly explained by increased activities of malic enzyme and pyruvate decarboxylase at this stage of development.[1] Although grape berries do not have a climacteric rise in respiration[2–4] they do show increases in R.Q. and utilization of added malate after the stage of development called the onset of ripening.[4] At this stage the amount of organic acid begins to decrease and reducing sugar begins to accumulate rapidly.[3] Meynhardt[5] has shown that mature Barlinka grape berries contain phosphopyruvate carboxylase and malic enzyme which he suggested might be involved in the dark fixation of carbon dioxide known to occur in grape berries.[6] It is also possible that malic enzyme has another role in grape berries. The decrease in malic acid which begins at the onset of ripening[7] may be due to decarboxylation of malate by malic enzyme and subsequent decarboxylation of pyruvate as suggested for apples.[1]

Although the changes in rates of photosynthesis, dark fixation of carbon dioxide, respiration, malic acid synthesis and malic acid breakdown have been studied during the development of grape berries[3, 4, 7] nothing is known about relevant enzyme activities during these changes. The present paper describes the changes in activities of malic enzyme, phosphopyruvate carboxylase, pyruvate decarboxylase and malate dehydrogenase during the development of sultana grape berries.

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RESULTS

Enzymes Detected

Pyruvate decarboxylase (2 oxoacid carboxy-lyase, E.C. 4.1.1.1), malic enzyme (L-malate: NADP oxidoreductase (decarboxylating), E.C. 1.1.1.40), phosphopyruvate carboxylase (orthophosphate:oxaloacetate carboxylase (phosphorylating), E.C. 4.1.1.31) and malate dehydrogenase (L-malate:NAD oxidoreductase, E.C. 1.1.1.37) were detected in extracts of sultana grape berries prepared in the presence of Carbowax 4000. No reaction was observed when enzyme or substrate were omitted from the reaction mixtures for the four enzymes.

Malic enzyme was assayed by measuring the rate of reduction of NADP. That the reduction was due to malic enzyme and not to a malate dehydrogenase is shown by the following facts. The reaction was specific for NADP. The rate of reduction of NADP was greater at pH 7·4 than at pH 4·0, 5·0, 7·0, 8·4 or 9·5. Furthermore when the enzyme preparation was incubated with pyruvate, $NADPH_2$ and $NaH^{14}CO_3$, oxidation of $NADPH_2$ was observed spectrophotometrically and over 90 per cent of the ^{14}C fixed cochromatographed on paper with malic acid.

Changes in Activities of Enzymes During the Development of Sultana Berries

Values in Figs. 1, 2 and 3 are means of three determinations on three separate enzyme preparations. The concentration of titratable acid in the berries increased steadily for 6 weeks after flowering and from the seventh week onwards decreased.[8] Malic acid and tartaric acid synthesis is mainly responsible for the increase in organic acid and the breakdown of malic acid accounts for much of the decrease in titratable acid.[7] Similar results were observed for sultanas grown in Adelaide in the 1966–67 season (Hardy, unpublished results).

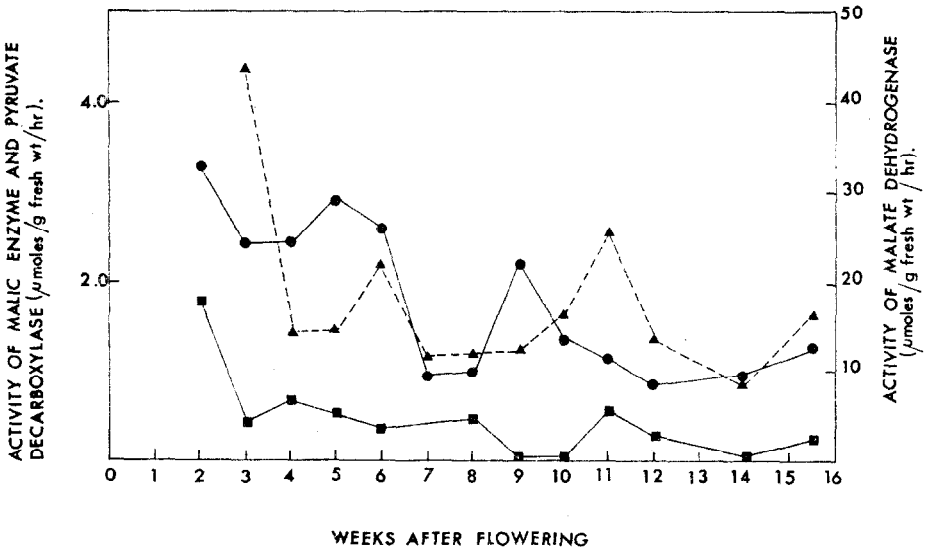

FIG. 1. ACTIVITIES PER g FRESH WEIGHT OF MALIC ENZYME (●), PYRUVATE DECARBOXYLASE (■) AND MALATE DEHYDROGENASE (▲) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.

[8] J. S. HAWKER, *Phytochem.* **8**, 9 (1968).

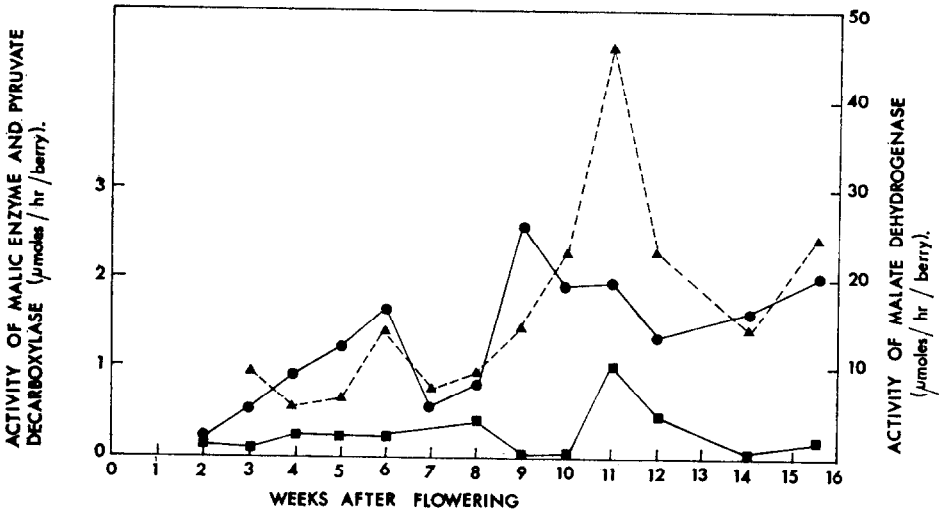

FIG. 2. ACTIVITIES PER BERRY OF MALIC ENZYME (●), PYRUVATE DECARBOXYLASE (■) AND MALATE DEHYDROGENASE (▲) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.

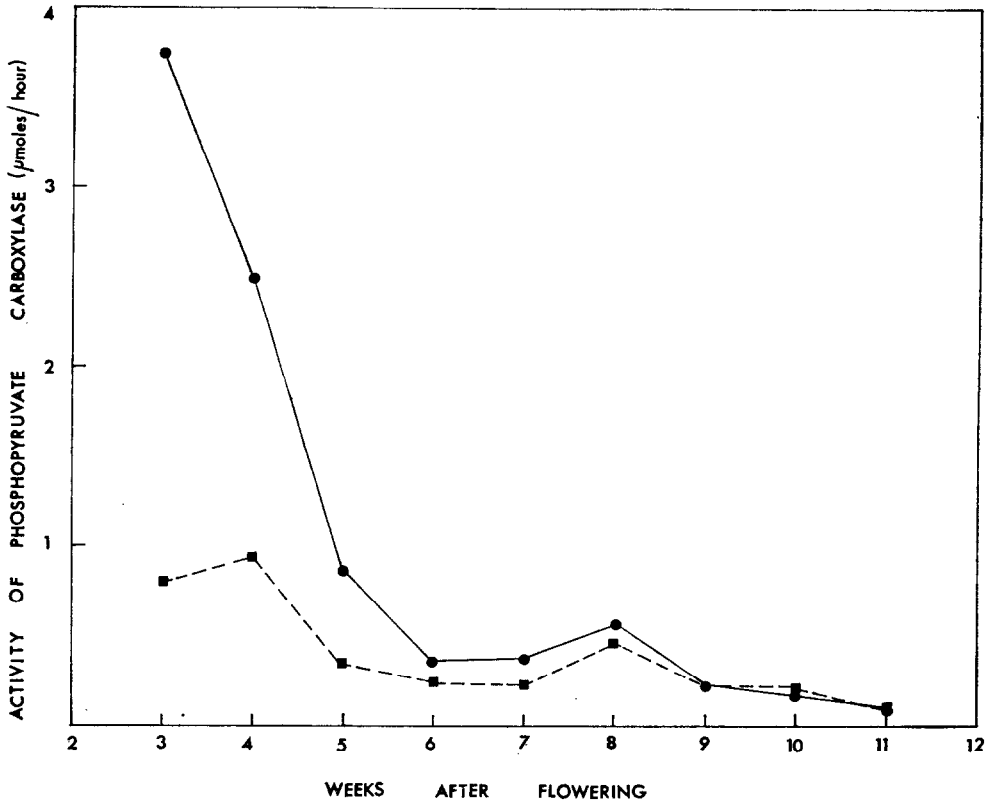

FIG. 3. ACTIVITY OF PHOSPHOPYRUVATE CARBOXYLASE PER g FRESH WEIGHT (●) AND PER BERRY (■) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.

The activities of pyruvate decarboxylase, malic enzyme and malate dehydrogenase (expressed per g fresh weight) all decreased during the development of the berries (Fig. 1). Expressed per berry the activities of malic enzyme and malate dehydrogenase showed an upward trend while pyruvate decarboxylase activity did not show any consistent changes (Fig. 2). The high activities of malate dehydrogenase and pyruvate decarboxylase observed on the eleventh week do not correspond to any known changes in metabolism of the berry. However, high activities of enzymes concerned with sugar metabolism were observed at the same time and these occurred after higher than average vineyard temperatures.

It was not possible to measure phosphopyruvate carboxylase activity in the preparations each week. Activities were determined at the end of the season in the enzyme preparations which had been stored at $-15°$. The results showed that on both a concentration basis and berry basis the activity of phosphopyruvate carboxylase decreased during the development of the berries (Fig. 3).

DISCUSSION

Evidence has been presented which suggests that the activities of the enzymes in the preparations from grapes were correlated with enzyme concentrations *in vivo* and were not due to different physical and chemical properties of the berries as they matured.[8] Grapes do not have a climacteric rise in respiration as observed in apples but, like apples, they do show increased R.Q. values and they utilize added malate after the onset of ripening.[4] (The onset of ripening occurred at about the sixth or seventh week in the sultanas used in the present work.) Also, in contrast to apples, it can be seen from the results that grapes do not show a consistent large increase in the activities of malic enzyme or pyruvate decarboxylase at or after the onset of ripening.

In the grape berry, it is possible that the changes in R.Q. without a concomitant increase in CO_2 production is due to a change in substrate for CO_2 production. Some of the malic acid in grape berries before the onset of ripening is sequestered at sites where it is unavailable for respiration.[9] At the onset of ripening, membrane permeabilities may increase allowing the stored malic acid to come into contact with malic enzyme and pyruvate decarboxylase and be metabolized. A greater permeability of membranes would also explain the increased rate of utilization of exogenously supplied malate by grape berries after the onset of ripening.[4]

Although the rate of respiration of grape berries expressed per berry decreases throughout the development of the berries[3,4] the activity per berry of at least one enzyme involved in the Krebs cycle (malate dehydrogenase) increases (Fig. 2). However, the increase may be due to synthesis of cytoplasmic malate dehydrogenase rather than mitochondrial malate dehydrogenase.

The rate of dark CO_2 fixation by sultana grape berries is high in the young berries but quickly declines and by the onset of ripening (about the sixth week after flowering) is very low.[4] The activity of phosphopyruvate carboxylase decreased in a similar manner (Fig. 3). The carboxylation catalysed by phosphopyruvate carboxylase is virtually irreversible and this reaction coupled with malate dehydrogenase provides an enzymic mechanism for the synthesis of malate.[10] It seems likely that phosphopyruvate carboxylase plays an important role in CO_2 fixation in immature grapes. Later in the development of the berries when the rate of CO_2 fixation is low and malate concentration is decreasing the activity of phospho-

[9] P. J. HARDY, *Plant Physiol.* **43**, 224 (1968).
[10] S. L. RANSON, *Plant Biochemistry*, p. 504, Academic Press, London and New York (1963).

pyruvate carboxylase is also low. The lower rates of dark fixation of CO_2 may also partly account for the higher R.Q. values observed after the onset of ripening.

Although the equilibrium in air of the reaction catalysed by malic enzyme favours decarboxylation, if malate is removed to inert pools the carboxylation reaction might proceed since the reaction is readily reversible.[10] In immature grapes, where malic acid is stored in inert pools,[9] it is possible that the reaction catalysed by malic enzyme contributes to dark CO_2 fixation. Later, when malic acid is being broken down, the enzyme may catalyse the decarboxylation of malic acid. In this connexion it is interesting to note that the activity of malic enzyme does not decrease as much as the activity of phosphopyruvate carboxylase (Figs. 1, 2 and 3).

EXPERIMENTAL

Preparation of Sultana Grape Berry Extracts

Enzyme assays were carried out using the concentrated enzyme extracts which were also used for assays of enzymes concerned with sugar metabolism.[8] These extracts were prepared from berries of *Vitis vinifera* L. cv. Sultanina (syn. Sultana, Thompson Seedless) using Carbowax 4000 to avoid interference by tannins.[8] Zero time in Figs. 1, 2 and 3 was 13 November 1967.

Measurement of Enzyme Activities

Pyruvate decarboxylase. This enzyme was assayed at 30° as described by Singer.[11] Activity is expressed as μmoles of CO_2 released per hr at pH 6·0 in the presence of 33 mM sodium pyruvate.

Malate dehydrogenase. The procedure involving the measurement of $NADH_2$ formation at pH 10 and 30° was employed.[12] Activity is expressed as μmoles of NAD reduced (which is equivalent to μmoles of malate oxidized) per hr in the presence of 13·5 mM malate and 1 mM NAD.

Phosphopyruvate carboxylase. The assay procedure of Slack and Hatch[13] at 30° was used with the addition of 2 μg pyridoxal phosphate per reaction mixture. Activity is expressed as μmoles CO_2 fixed per hr at pH 8·3 in the presence of 62 mM $NaHCO_3$.

Malic enzyme. The rate of reduction of NADP was determined spectrophotometrically at 30°. The reaction mixture contained tris-HCl buffer, pH 7·4 (250 μmoles), NADP (0·25 μmole), $MgCl_2$ (10 μmoles), malate (20 μmoles) and enzyme in a final volume of 3 ml. Activity is expressed as μmoles of NADP reduced per hr (which is equivalent to μmoles of malate decarboxylated). The reverse reaction was carried out by the method of Slack and Hatch.[13] The reaction mixture was chromatographed on Whatman No. 1 paper using the organic phase of butanol:acetic acid:water (4:1:5, by volume).

Acknowledgement—Technical assistance by Mr. B. J. Michael is gratefully acknowledged.

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[12] R. G. Wolfe and J. B. Neilands, *J. Biol. Chem.* **221**, 61 (1956).
[13] C. R. Slack and M. D. Hatch, *Biochem. J.* **103**, 660 (1967).