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CHANGES IN THE ACTIVITIES OF ENZYMES CONCERNED WITH SUGAR METABOLISM DURING THE DEVELOPMENT OF GRAPE BERRIES

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Abstract—Changes in activities of invertase, hexokinase, glucose 6-phosphate dehydrogenase, sucrose synthetase, sucrose phosphate synthetase and sucrose phosphatase in extracts prepared with Carbowax 4000 were studied at weekly intervals during the development of sultana grape berries. The characteristic rapid rise in the rate of sugar accumulation by the berries about halfway through development was preceded by an increase in the activity of invertase. At about the same time that the rate of sugar accumulation increased the activities of sucrose synthetase, sucrose phosphate synthetase and sucrose phosphatase increased. The activity of glucose 6-phosphate dehydrogenase decreased early in the development of the berries and remained low. The possibility that sugar accumulation in grapes occurs by a pathway involving the synthesis and hydrolysis of sucrose phosphate is discussed in terms of the changing activities of the enzymes necessary for this pathway to operate.

INTRODUCTION

RIPE grape berries contain about equal concentrations (10 g per 100 g fresh weight) of glucose and fructose while the concentration of sucrose is less than 0·1 g per 100 g fresh weight.[1] However, sucrose is the main sugar translocated in the grape-vine[2] and experiments in which whole excised berries have been fed radioactive sugars through the pedicel have shown that sucrose is both synthesized from reducing sugars and hydrolysed and resynthesized by berries.[3,4] The pathway and form in which carbohydrate is moved into the vacuoles of the sugar-storing cells of the pericarp of grape berries is not known. Some plants contain invertase which hydrolyses sucrose prior to its accumulation, e.g. sugar-cane stem tissue,[5,6] while in others it appears that sucrose is accumulated unchanged, e.g. tobacco leaf[7] and artichoke tuber.[8] Experiments similar to those carried out with other plant tissues,[5,6] which were conducted with slices of grape berries, proved unsatisfactory because, during incubation, most of the endogenous sugars and organic acids were lost from the tissue indicating either a high rate of cell rupture or cell leakiness (Hawker, unpublished results).

Arnold[9] has shown that mature grape berries contain β-D-fructofuranoside fructohydrolase (E.C. 3.2.1.26) (hereafter referred to as invertase) and the results of Hardy[3,4] indicate that at least one sucrose-synthesizing enzyme is active. Sacher[10] detected sucrose synthetase

[1] W. M. KLIEWER, *Plant Physiol.* **41**, 923 (1966).
[2] C. A. SWANSON and E. D. H. ELSHISHINY, *Plant Physiol.* **33**, 33 (1958).
[3] P. J. HARDY, *Australian J. Biol. Sci.* **20**, 465 (1967).
[4] P. J. HARDY, *Plant Physiol.* **43**, 224 (1968).
[5] J. S. HAWKER and M. D. HATCH, *Physiol. Plantarum* **18**, 444 (1965).
[6] J. A. SACHER, M. D. HATCH and K. T. GLASZIOU, *Plant Physiol.* **38**, 348 (1963).
[7] H. K. PORTER and L. H. MAY, *J. Exp. Botany* **6**, 43 (1955).
[8] J. EDELMAN and M. A. HALL, *Biochem. J.* **88**, 36P (1963).
[9] W. N. ARNOLD, *Biochim. Biophys. Acta* **110**, 134 (1965).
[10] J. A. SACHER, *Plant Physiol.* **41**, 181 (1966).

(UDP glucose:D-fructose 2-glucosyltransferase, E.C. 2.4.1.13) in bean pod tissue but did not detect sucrose phosphate synthetase (UDP glucose:D-fructose 6-phosphate 2-glucosyltransferase, E.C. 2.4.1.14). Neither of these enzymes has been extracted from fleshy fruits. Sucrose phosphatase (sucrose phosphate phosphohydrolase) has been found in leaves, stems, roots and tubers of plants[11] but has not been reported to occur in fruits. The present paper describes the changes in activities of the above enzymes during the development of sultana grape berries.

RESULTS

Effect of Carbowax on Extraction of Enzymes

The use of Carbowax 4000 in the standard homogenizing medium (see Experimental) resulted in extracts with high enzyme activity as compared to extracts prepared without Carbowax.

Enzymes Detected

Invertase, hexokinase (ATP:D-hexose 6-phosphotransferase, E.C. 2.7.1.1), glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, E.C. 1.1.1.49), glucose phosphate isomerase (D-glucose-6-phosphate ketol-isomerase, E.C. 5.3.1.9), sucrose synthetase, sucrose phosphate synthetase and sucrose phosphatase were detected in extracts of sultana berries.

The properties of invertase from sultana berries were found to be similar to those described by Arnold[9] for invertase extracted from Ohanez grape berries. The concentration of glucose necessary to saturate hexokinase was not determined and the results are therefore expressed in arbitrary units calculated from the percentage hydrolysis of 1·3 mM glucose. Under the conditions employed insignificant hydrolysis of glucose 6-phosphate occurred. The activity of glucose 6-phosphate dehydrogenase was less at pH 4·0, 5·0 and 8·5 than at pH 7·0 or 7·4. No reaction was observed in the absence of substrate or when NADP was replaced with NAD.

Sucrose and sucrose phosphate were formed when the enzyme was incubated in appropriate reaction mixtures with fructose or fructose 6-phosphate respectively. The identity of sucrose and sucrose phosphate was confirmed as described previously.[12] During the assay of sucrose phosphate synthetase more than 50 per cent of the radioactive fructose 6-phosphate supplied was converted to glucose phosphate indicating the presence of glucose phosphate isomerase.

Crude berry extracts hydrolysed sucrose phosphate. The hydrolysis at pH 6·0 and 8·3 was 60 per cent and 45 per cent respectively of that at pH 6·7 and at each pH the hydrolysis was almost completely inhibited by omitting $MgCl_2$ and adding 2·5 μmoles of EDTA to the reaction mixtures. At pH 6·7 the hydrolysis of sucrose phosphate was inhibited by 97 per cent by this treatment whereas the hydrolysis of fructose 6-phosphate was only inhibited by 40 per cent. Sucrose phosphatases from plants are inhibited by some di- and tri-saccharides and the amount of inhibition by the different sugars varies depending on the species from which the enzyme is extracted.[13] The rate of sucrose phosphate hydrolysis by grape berry extracts is inhibited most by maltose and the inhibition by sucrose and melezitose is much less while

[11] J. S. HAWKER and M. D. HATCH, *Biochem. J.* **99**, 102 (1966).
[12] J. S. HAWKER, *Biochem. J.* **105**, 943 (1967).
[13] J. S. HAWKER, *Biochem. J.* **102**, 401 (1967).

TABLE 1. EFFECT OF EDTA AND SUGARS ON THE HYDROLYSIS OF SUCROSE PHOSPHATE BY A CRUDE SULTANA GRAPE BERRY EXTRACT

Compound	Concentration (mM)	Inhibition* (%)
EDTA	30	97
Sucrose	100	22
Sucrose	250	34
Melezitose	100	22
Turanose	100	15
Maltose	100	79
Glucose	100	0
Fructose	250	0

* Values are percentage inhibitions of the hydrolysis of 71 μM (fructosyl-^{14}C) sucrose phosphate by sugars at the concentrations indicated. In one set of tubes $MgCl_2$ was replaced by EDTA.

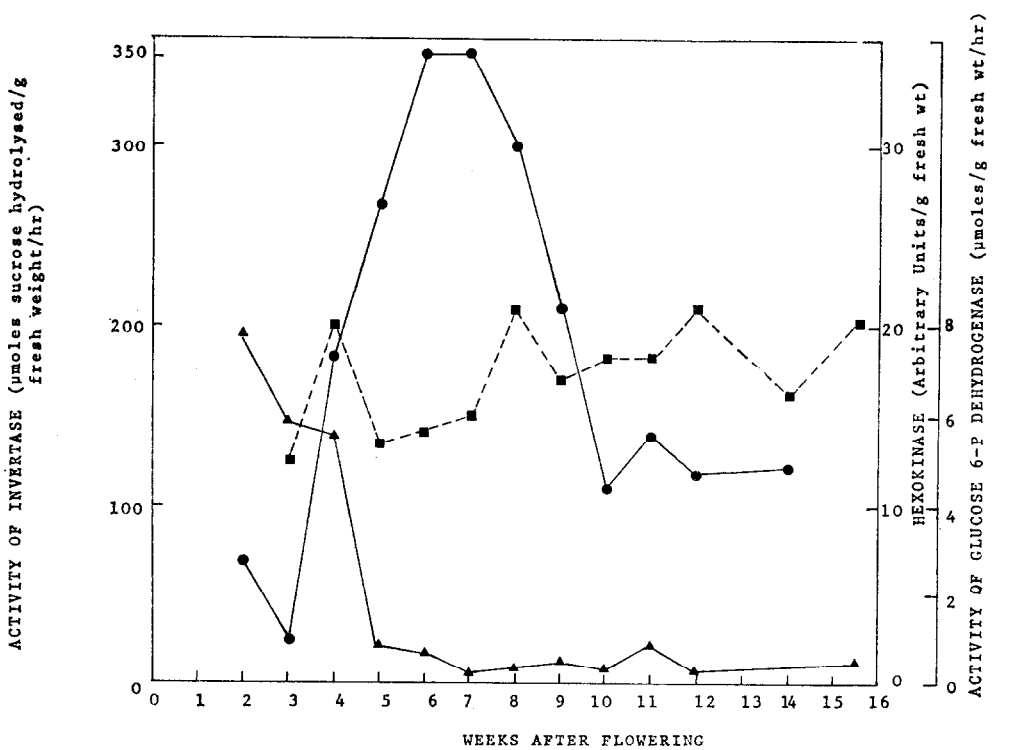

FIG. 1. ACTIVITIES PER g FRESH WEIGHT OF INVERTASE (●), HEXOKINASE (■) AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE (▲) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.

Note the different scales on the ordinate axes.

glucose and fructose have no effect (Table 1). The above results indicate the presence of a specific sucrose phosphatase in grape berries for the reasons given previously[12, 14] and allow the quantitative estimation of the activity of sucrose phosphatase in crude berry extracts.

Changes in Activities of Enzymes during the Development of Sultana Berries

Values in Figs. 1–4 are means of three determinations on three separate enzyme preparations.

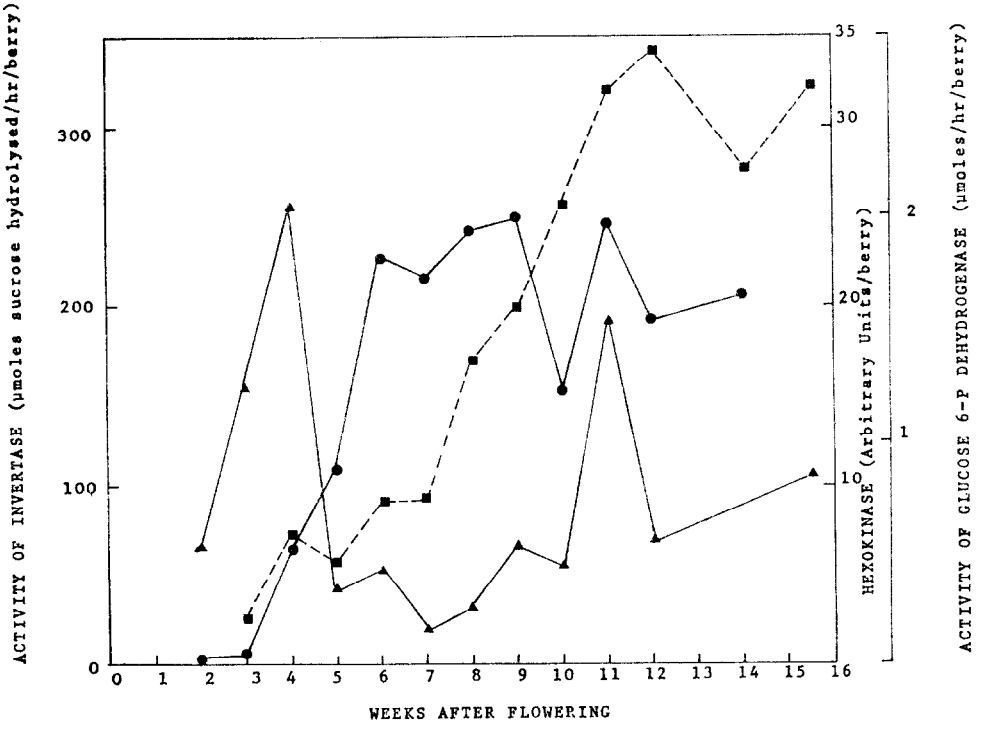

FIG. 2. ACTIVITIES PER BERRY OF INVERTASE (●), HEXOKINASE (■) AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE (▲) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.
Note the different scales on the ordinate axes.

Invertase, hexokinase and glucose 6-phosphate dehydrogenase show three patterns of activity during the development of the berry (Fig. 1). While invertase activity increases (expressed on a fresh weight basis) to a maximum and then decreases, glucose 6-phosphate dehydrogenase activity decreases early and remains low and hexokinase activity remains relatively constant. Expressed per berry the activities of invertase and hexokinase increase and remain at a maximum while glucose 6-phosphate hydrogenase activity increases early but then falls (Fig. 2).

Some weeks after the increase in invertase activity occurs, the activities of sucrose phosphate synthetase and sucrose phosphatase rise and remain at a maximum both on a concentration basis and per berry (Figs. 3 and 4). Sucrose synthetase activity, although initially

[14] J. S. HAWKER, *Phytochem.* **5**, 1191 (1966).

high, later follows the same pattern as sucrose phosphate synthetase (Figs. 3 and 4). Invertase and sucrose phosphatase activities are about 100- and 10-fold greater respectively than the activities of the other enzymes.

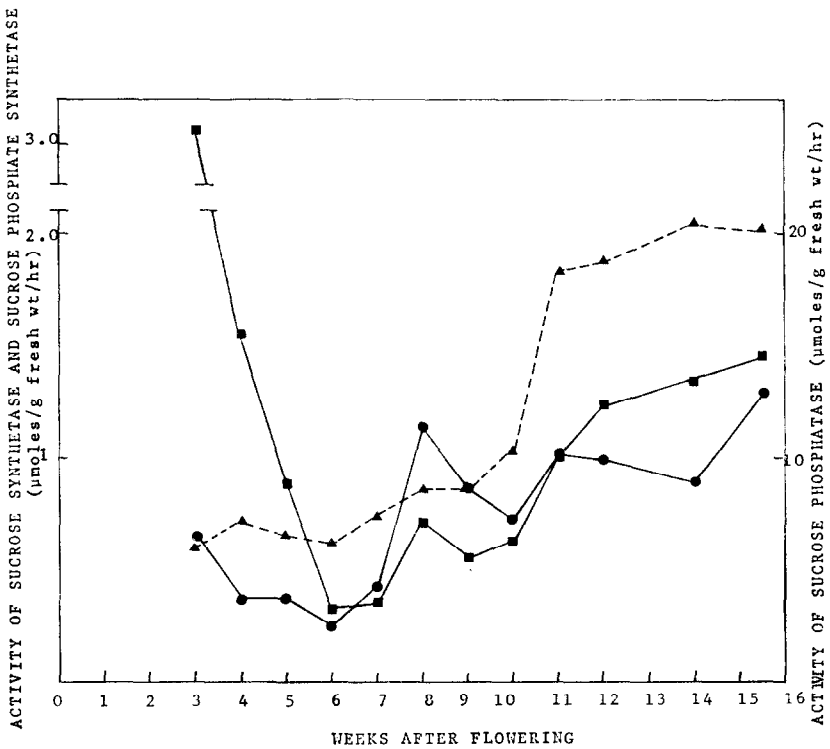

FIG. 3. ACTIVITIES PER g FRESH WEIGHT OF SUCROSE PHOSPHATASE (▲), SUCROSE SYNTHETASE (■) AND SUCROSE PHOSPHATE SYNTHETASE (●) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.
Note the different scales on the ordinate axes.

Comparison of Growth, Organic Acid Accumulation, Sugar Accumulation and Enzyme Activities during the Development of Sultana Berries

The fresh weight of the green immature berries increased steadily for 6 weeks accompanied by an increase in organic acid (Figs. 5 and 6). At the "onset of ripening" (sixth or seventh week) the berries softened, they increased in size more rapidly, organic acids began to decrease and reducing sugar began to increase, all of which are typical changes in developing grape berries.[1, 15, 16]

Examination of Figs. 1–6 shows that invertase activity reached a maximum at about the time rapid sugar accumulation began, whereas hexokinase activity generally followed the fresh weight curve and sucrose phosphate synthetase and sucrose phosphatase activities showed similar trends to reducing sugar concentration. After an initial decrease in sucrose synthetase activity which was almost the direct opposite to the increase in invertase activity, sucrose synthetase activity followed approximately the reducing sugar curve. Generally

[15] B. G. COOMBE, *Plant Physiol.* **35**, 241 (1960).
[16] J. M. HARRIS, P. E. KRIEDEMANN and J. V. POSSINGHAM, *Vitis*, in press (1968).

shade temperatures in the vine-yard were around 30° but between the harvests on the seventh and eighth weeks and between the tenth and eleventh weeks temperatures on several days were above 38°. Some of the enzymes extracted from berries on the eighth and eleventh weeks showed sudden increases in activity as compared to the previous weeks (Figs. 2 and 4).

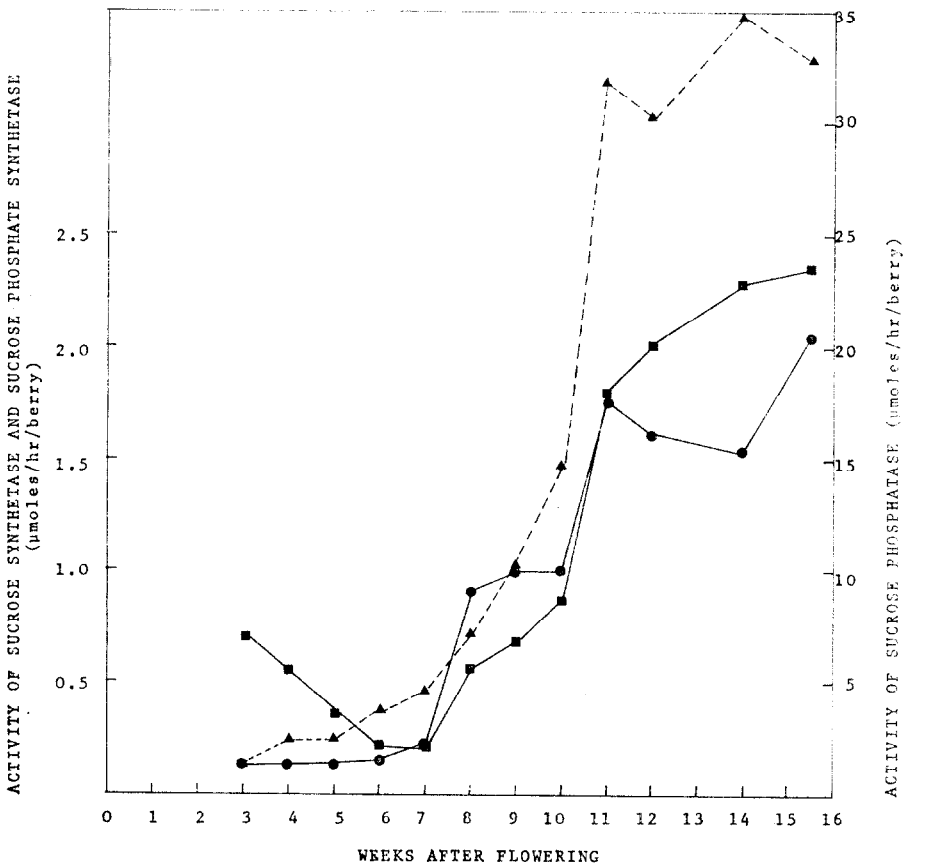

FIG. 4. ACTIVITIES PER BERRY OF SUCROSE PHOSPHATASE (▲), SUCROSE SYNTHETASE (■) AND SUCROSE PHOSPHATE SYNTHETASE (●) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.
Note the different scales on the ordinate axes.

DISCUSSION

Comparisons of activities of enzymes extracted from tissues of different chemical and physical natures, such as immature grapes and ripe grapes, are difficult.[17] The use of Carbowax, which has an affinity for tannins and which can split tannin-protein complexes, has partly overcome this difficulty.[18–20] In the present work the different patterns of activity observed for different enzymes during the development of the grape berries suggest that the

[17] M. SPENCER, *Plant Biochemistry*, p. 793, Academic Press, London and New York (1965).
[18] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. B.* **158**, 514 (1963).
[19] J. T. MEYNHARDT, *S. Afr. J. Agric. Sci.* **8**, 381 (1965).
[20] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).

activities measured may be correlated at least with amounts of enzymes present in the tissues rather than merely be a reflection of the different chemical and physical properties of the tissues. Sucrose synthetase from sugar-cane is strongly inhibited by oxidation products of phenols[21] and grapes contain phenols,[22] polyphenoloxidase[23] and tannins. The high activity of sucrose synthetase observed in immature grape extracts prepared with Carbowax 4000 suggests that the lower values observed for sucrose phosphate synthetase and sucrose phosphatase (Fig. 3) are reliable and are not due to inhibition or inactivation of these enzymes by

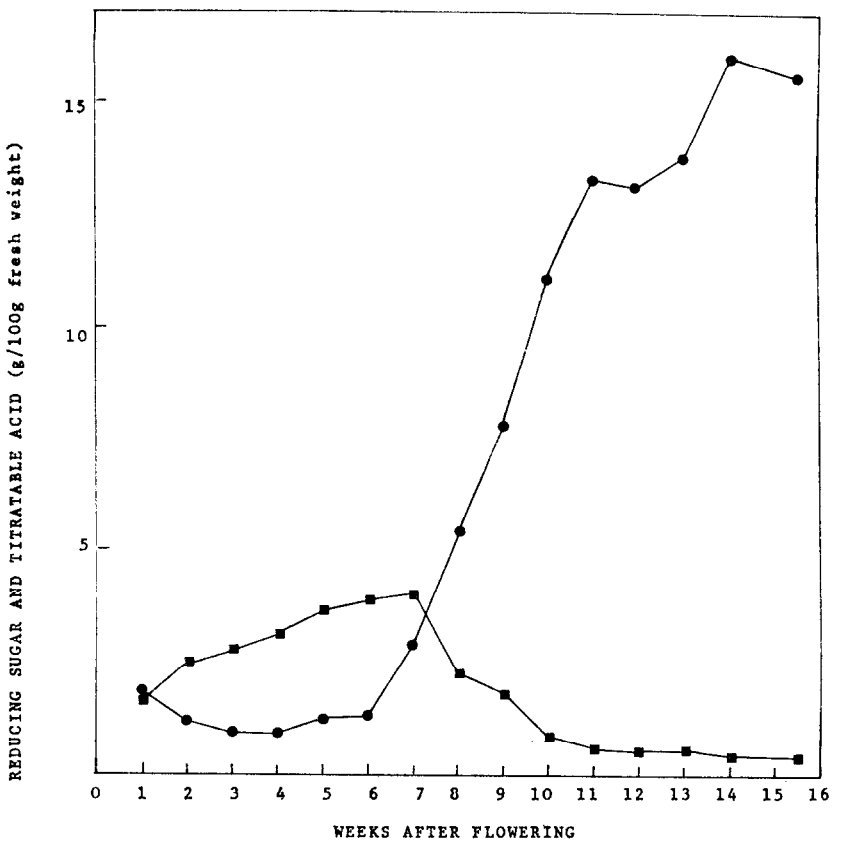

FIG. 5. CONCENTRATION OF REDUCING SUGAR (●) AND TITRATABLE ACID (■) IN DEVELOPING SULTANA GRAPE BERRIES.

phenols or tannins. Further confidence in the results is gained by comparing the pattern of glucose 6-phosphate dehydrogenase activity with sucrose phosphate synthetase and sucrose phosphatase activities (Figs. 1 and 3). Glucose 6-phosphate dehydrogenase activity was high while the activities of the other two enzymes were low and vice versa.

Although the nature and changes of carbohydrates in fleshy fruits have been studied in some detail, practically nothing is known of the enzymes or pathways involved in sugar accumulation and conversions in these fruits.[17] Experiments with slices of storage tissue suggest

[21] C. R. SLACK, *Phytochem.* **5**, 397 (1966).
[22] V. L. SINGLETON, *Am. J. Enol. Vit.* **17**, 126 (1966).
[23] F. RADLER, *J. Sci. Fd. Agric.* **15**, 864 (1964).

that sucrose can be accumulated either without prior breakdown[7,8] or after inversion by invertase.[5,6] Similar experiments with slices of grape berry were unsuccessful due to loss of solutes from the slices and to the high activity of invertase present (Hawker, unpublished results). The scheme proposed by Glasziou,[24] Sacher *et al.*,[6] Hatch[25] and Hawker and Hatch[5,11] for the accumulation of sucrose by sugarcane storage tissue involves hydrolysis of sucrose in the free space, phosphorylation of glucose and fructose, synthesis of sucrose phosphate and hydrolysis of sucrose phosphate. In immature storage tissue of sugar-cane

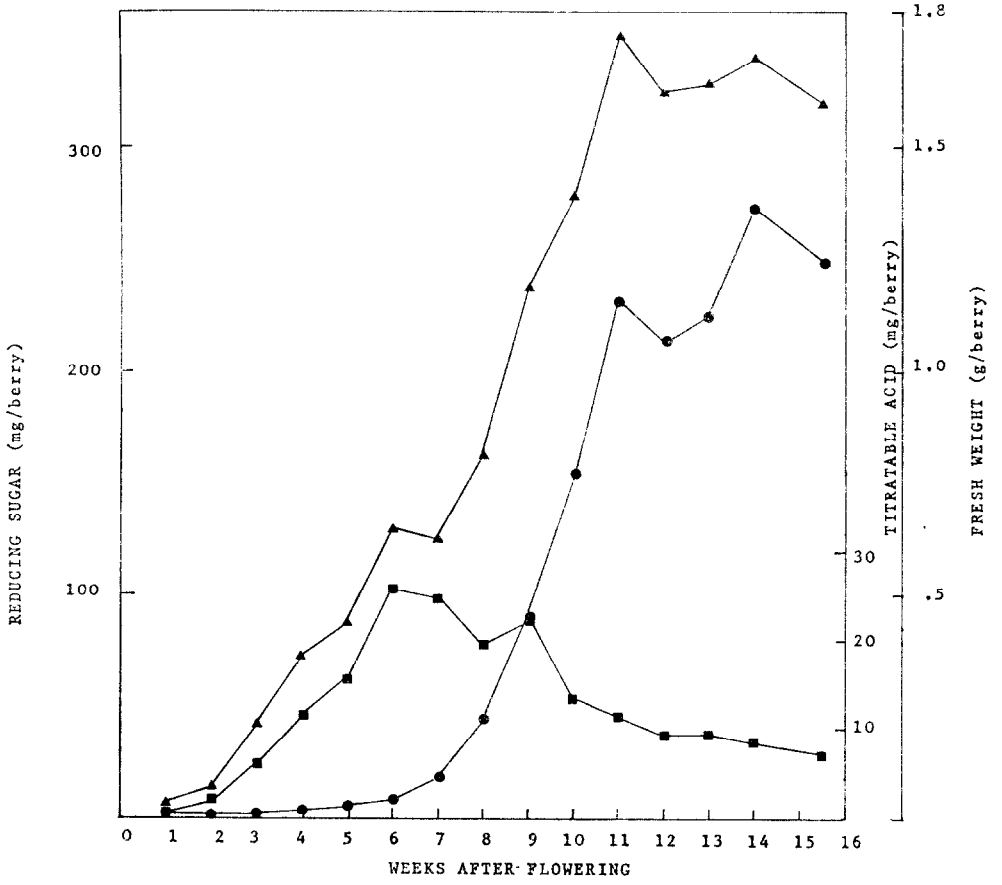

FIG. 6. FRESH WEIGHT (▲), REDUCING SUGAR (●) AND TITRATABLE ACID (■) PER BERRY IN DEVELOPING SULTANA GRAPE BERRIES.

some of the sucrose is hydrolysed in the vacuole.[6] The present work shows that many of the enzymes necessary for this pathway to operate are also present in grape berries. From Figs. 5 and 6 it can be calculated that the maximum average rate of sugar accumulation per hour is 1·1 μmoles per g fresh weight or 2·5 μmoles per berry. The activities of the enzymes assayed *in vitro* were sufficient to maintain this rate of accumulation. Although the presence of particular enzymes in a tissue does not prove that a particular pathway is operating *in*

[24] K. T. GLASZIOU, *Plant Physiol.* **36**, 175 (1961).
[25] M. D. HATCH, *Biochem. J.* **93**, 521 (1964).

vivo, the increases in enzyme activity which occurred prior to or at about the same time as rapid sugar accumulation began in grape berries and the turnover of sucrose known to occur in grape berries[3,4] suggest that reducing sugar accumulation by grapes involves more than translocation and hydrolysis of sucrose.

Unlike many fleshy fruits the grape berry will not ripen off the vine since it relies on a continuing supply of carbohydrate. Although some organic acid may be converted to sugars during ripening the bulk of the sugar accumulated must come from other parts of the plant (Fig. 6). It will be interesting to compare the changes in activities of enzymes in grape berries with possible changes which may occur in a fruit which converts starch and acid to reducing sugar during ripening after removal from the tree.

EXPERIMENTAL

Materials

Grape berries from young vines of *Vitis vinifera* L. cv. Sultanina (syn. Sultana, Thompson Seedless) growing in a vine-yard near Adelaide, South Australia, were used fresh. Zero time in the figures in the Results section was 13 November 1967.

Determination of Fresh Weight, Reducing Sugar Content and Titratable Acidity of Sultana Grape Berries

At weekly intervals a total of 200 berries from ten bunches of grapes were weighed to determine the average fresh weights. Samples of 10 g were thoroughly ground with water in a mortar and made up to 100 ml. After filtration, reducing sugar content was determined[9] and titratable acid was determined by titrating to the phenolphthalein end point with 50 mM NaOH. The results for acid are expressed as gramme of tartaric acid.

Preparation of Enzyme Extracts

Washed grapes (50 g) were homogenized at full speed for 1 min in a Serval Omnimixer containing 100 ml of 0·5 M tris-HCl buffer, pH 8·7, 150 mg of diethyldithiocarbamate, 100 mg cysteine-HCl, 0·5 ml of 0·5 M EDTA and 8 g of Carbowax 4000 (B.D.H. grade). All operations were carried out at between 0 and 4°. The homogenate was squeezed through muslin and to 60 ml of the "filtered homogenate" was added 39 g of Carbowax 4000. Following stirring for 30 min the resulting suspension was centrifuged at 30,000 *g* for 15 min. The pellet was suspended in 10 ml of 5 mM tris-HCl buffer, pH 7·0, containing 1 per cent Triton X100. After centrifugation of the suspension, 3 ml of the resulting supernatant were desalted by passage through a 15 ml column of Sephadex G-25 which had been washed with 5 mM tris-HCl buffer, pH 7·0. The desalted preparation was used for the assay of all enzymes except sucrose phosphatase.

Measurement of Enzyme Activities

Invertase. The activity of invertase at pH 4·0 and 30° was determined as described by Arnold.[9]

Sucrose synthetase. This enzyme was assayed at 30° using 30 mM fructose as described previously[12] but in the presence of 100 mM sucrose and at pH 8·5 to overcome the interference caused by invertase. The enzyme was saturated with respect to fructose at a concentration of 30 mM. Activity is expressed as μmoles of sucrose synthesized per hr.

Sucrose phosphate synthetase. Enzyme activity was determined as described previously[12] but in the presence of 15 mM fructose 6-phosphate, 30 mM EDTA and at 30°. The reaction proceeded at at least 90 per cent of its maximum velocity at 15 mM fructose 6-phosphate. Activity is expressed as μmoles of sucrose phosphate synthesized per hr.

Hexokinase. The reaction mixture used for sucrose phosphate synthetase was modified by replacing UDP-glucose, fructose 6-phosphate and EDTA with 1·3 mM (U-^{14}C) glucose, $MgCl_2$ and ATP.

Glucose 6-phosphate dehydrogenase. This enzyme was assayed at 30° and at pH 7·4.[26] Activity is expressed as μmoles NADP reduced per hr.

Sucrose phosphatase. Enzyme activity in the "filtered homogenate" was determined as described previously[11] except that the tris-maleate buffer was adjusted to pH 6·7 and the temperature was 30°. Maximum velocities were calculated as previously.[11]

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

[26] A. KORNBERG and B. L. HORECKER, *Methods in Enzymology*, Vol. 1, p. 323, Academic Press, London and New York (1955).