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CONTROL OF GLYCOLYSIS IN RIPENING BERRIES OF *VITIS VINIFERA*

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Abstract—The concentrations of glycolytic intermediates, acid components and adenosine nucleotides were determined at half-weekly intervals during development and ripening of grape berries. Based on distinctive non-equilibrium conditions and enzymic activities which are not controlled by substrate availability at the levels of phosphoenolpyruvate/pyruvate and fructose-6-phosphate/fructose diphosphate it is concluded that these two sites represent the major control points in the reaction sequences between sugar and acid pools in this fruit.

INTRODUCTION

The accumulation of malic and tartaric acids, which characterizes the immediate post-anthesis phase of grape berry development is followed by a quantitatively more significant rise in hexose concentration as the berries ripen. Ripening is also accompanied by a marked decrease in malic acid [1].

The results of recent work on enzymes of malate metabolism in grapes [2] suggest that the rate as well as the direction of carbon flow in glycolysis before and after the onset of ripening might play an important role in mediating the acid-sugar interrelationship. Whilst green berries show no transformation of ^{14}C-malate to sugar [3], suggesting a glycolytic flux towards pyruvate, ripening berries show appreciable gluconeogenic activity [4]. However, neither the nature of the compounds which bring about these changes in the developing berries, nor the essential regulatory mechanisms are known.

In this study the concentrations of glycolytic intermediates and of some acids, which are of interest with respect to malate metabolism, were determined during the period of grape berry development in which changes in the direction of carbon flow were expected to occur. This included periods before and after the stage of rapid sugar accumulation. Control points in the glycolytic sequence of grape berries were sought by evaluating the mass-action ratios of the relevant reactions [5, 6].

RESULTS

The changes in the contents of malic acid, citric acid and oxalacetic acid (OAA) during growth and development of Sultana grape berries, determined at intervals ranging from 3–7 days, are shown in Fig. 1a. In agreement with previous results [7] malic acid accumulated during the first 6 weeks of berry development with a subsequent large decrease in concentration after the onset of ripening. Citric acid also reached the highest level 6 weeks after anthesis but diminished later only to *ca* half its maximal value.

Calculated on a per berry basis, in order to take into account the dilution effect due to the rapid increase in berry volume at the beginning of ripening, malate content reached its peak at the time of berry softening and highest net disappearance rates were observed between week 7 and 8 after anthesis (Fig. 1b). The OAA level in these grapes followed a similar pattern, the fall preceding that of malate by about a week. The decrease in citric acid concentration (Fig. 1a) on the other hand, can be almost entirely attributed to the simultaneous increase in berry volume, resulting in a biphasic curve if expressed on a per berry basis. The plateau in the citrate content/berry (Fig. 1b) extended over the interesting phase of the start of ripening which occurred between weeks 6 and 8.

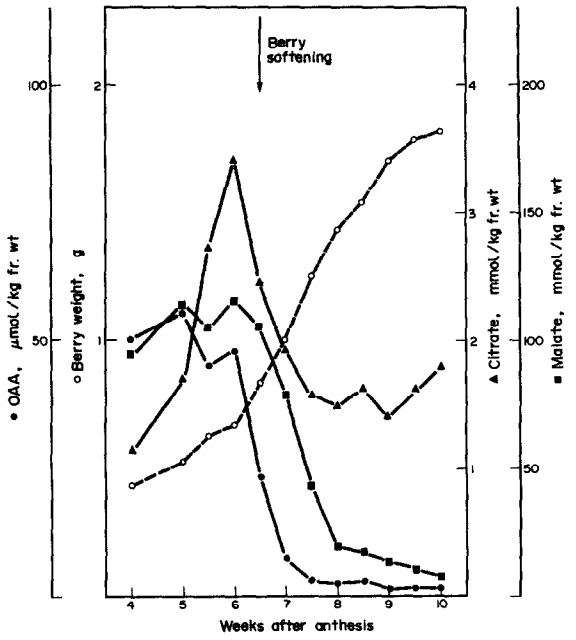

Fig. 1a. Developmental changes in the contents of malic (■), citric (▲) and oxalacetic (●) acids (note the different scales) and weight (○) of Sultana berries.

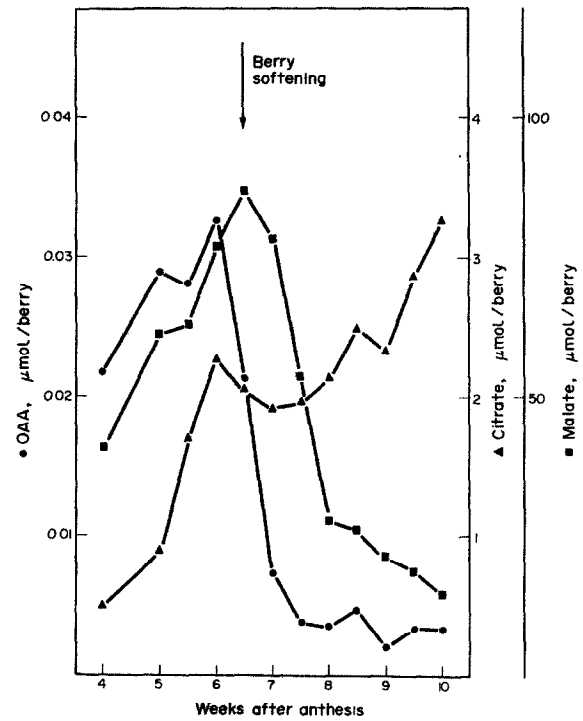

Fig. 1b. Contents per berry of malic (■), citric (▲) and oxalacetic (●) acids (different scales) throughout development of Sultana berries.

Fig. 2 shows the content of glucose-6-phosphate (G6P; note the larger scale), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (FDP) and triose phosphate (TP) at the same stages of fruit development. While no continuous trend in F6P concentrations could be found, G6P contents increased steadily, the G6P/F6P ratio going from less than 3 to *ca* 5 with progressing maturity. The TP concentrations decreased from week 4 to week 6 but then began to accumulate at the same time as the level of soluble solids (mainly sugar) in the berries started to increase and reached a maximum at the time of rapid net malate disappearance. A similar pattern was observed in the case of FDP.

The situation with respect to the products of glycolysis, phosphoenolpyruvate (PEP) and pyruvate (PYR) is represented in Fig. 3. Highest PYR to PEP ratios were observed during the initial green stage of berry growth, but later PEP tended to accumulate while the PYR concentration decreased, the difference being maximal between week 7 and 8 when PEP concentration was found to be nearly double that of PYR. At the end of the phase of rapid malate breakdown, a marked drop in PEP concentration occurred.

If we accept the definition of a regulatory enzyme as "an enzyme, which catalyses a non-equilibrium reaction and whose activity is controlled by factors other than the substrate concentration" [5, 6], we first have to identify the non-equilibrium reactions in grape glycolysis by comparing their mass-action ratios (Γ) with the equilibrium constant of the reaction. The relevant values for phosphoglucoisomerase, phosphofructokinase, fructosediphosphatase, aldolase and pyruvate kinase are given in Table 1. It is evident that the isomerase reaction is close to equilibrium, whereas phosphofructokinase and pyruvate kinase catalyse reactions which are far off equilibrium. The status of the other two reactions is difficult to assess since they fall within a range which cannot be unequivocally termed 'equilibrium' or 'non-equilibrium' [5]. The second part of the above definition implies that the flux, through the pathway, and

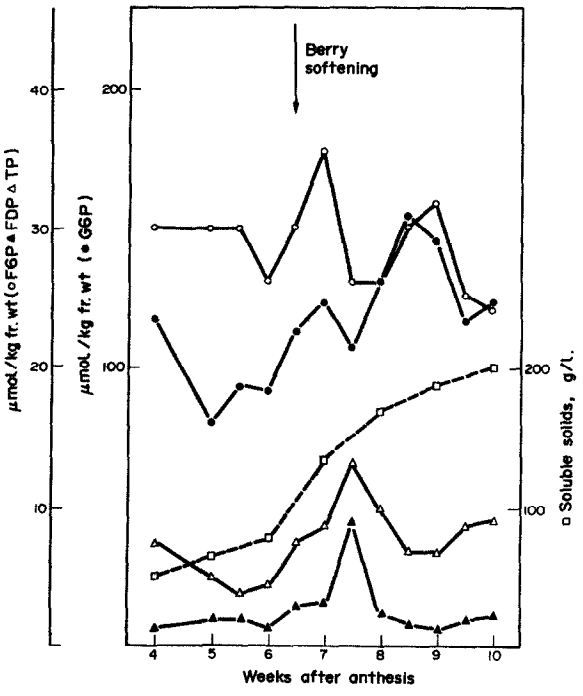

Fig. 2. Contents of fructose-6-phosphate (○), fructosediphosphate (▲), triose phosphates (△), glucose-6-phosphate (●) (larger scale) and soluble solids (□) of developing Sultana berries.

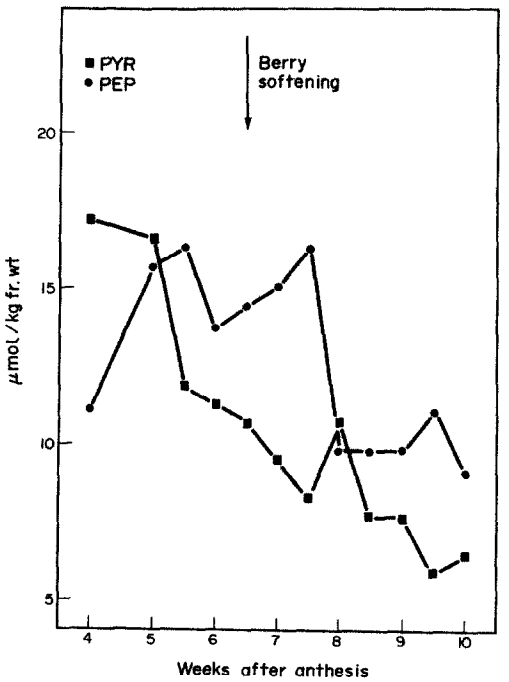

Fig. 3. Pyruvate and phosphoenolpyruvate contents of Sultana berries.

Table 1. Comparison of apparent equilibrium constants with mass-action ratios for some glycolytic reactions in grapes during growth and ripening

	K'* approx.	Weeks after anthesis 4		5		6		7		8		9	
$\frac{(F6P)}{(G6P)}$	0.4	0.24	0.37	0.32	0.29	0.27	0.29	0.27	0.20	0.19	0.22	0.22	0.20
$\frac{(FDP)\cdot(ADP)}{(F6P)\cdot(ATP)}$	1000	0.01	0.027	0.036	0.022	0.042	0.026	0.153	0.027	0.015	0.009	0.015	0.029
$\frac{(F6P)\cdot(ATP)}{(FDP)\cdot(ADP)}$	500	101.0	37.1	27.8	45.5	23.8	38.5	06.5	36.0	66.7	111.0	66.7	34.5
$\frac{(TP)^2\cdot 22^*}{(FDP)\cdot 529}$	100	2.03	0.6	0.34	0.69	0.9	1.1	0.89	2.06	1.36	1.7	1.84	1.82
$\frac{(PYR)\cdot(ATP)}{(PEP)\cdot(ADP)}$	10000	6.43	2.42	1.24	1.72	1.62	2.12	1.14	3.22	2.7	3.46	2.53	2.2

* according to [6].

substrate levels have to change in an opposite way in order for an enzyme to have regulatory characteristics. Based on the accumulation pattern of OAA, citrate and malate we assume that glycolysis in green grape berries operates steadily up to week 6 after anthesis (Fig. 1). Then the rate seems to decrease considerably. Simultaneous with this change in flux, the concentrations of F6P and PEP start to increase (Figs. 2, 3) which indicates that phosphofructokinase and pyruvate kinase are not substrate-controlled enzymes.

In addition to substrates, activities of two enzymes, phosphofructokinase (EC 2.7.1.11) and fructose diphosphatase (EC 3.1.3.11) were also determined at weekly intervals from week 4 to week 9 after anthesis. As previously reported [2] phosphofructokinase activity did not change markedly, fluctuating from 0.6 to 0.95 μmol/g fr. wt/hr (0.55–0.80 nkat/mg protein) in developing Sultana berries. Also fructose diphosphatase activity varied only little with values between 0.27 and 0.43 μmol/g fr. wt/hr (0.20–0.38 nkat/mg protein). Although rather low, the activity of the dephosphorylating enzyme was sufficient to account for the maximal malate disappearance of *ca* 0.4–0.5 μmol/g fr. wt/hr, particularly since two C_4-molecules are needed for a gluconeogenic transformation of malate to FDP.

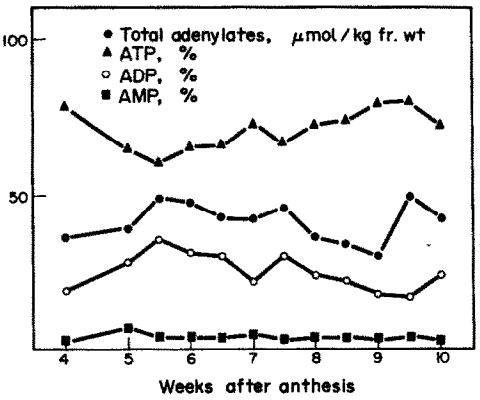

Fig. 4. Content of total adenylates and AMP, ADP and ATP as percentages of the total adenylates in developing Sultana berries.

Fig. 4 shows the concentration of the adenyl nucleotides as determined along with the other compounds. The AMP levels fluctuated between 1.23 and 2.30 μmol/kg fr. wt or 3% to 7% of total adenylate compounds, while ADP and ATP represented 17–36% and 60–78% respectively. There was no clear cut correlation between the ADP/ATP ratio and the interesting stages of berry development. However, the resulting energy charge values [8] (0.87; 0.79; 0.78; 0.78; 0.81; 0.84; 0.82; 0.84; 0.85; 0.89; 0.88; 0.85) compiled from the adenyl nucleotide data obtained at the sampling times indicated in Fig. 4 showed a sharp fall after the first stage of growth and then tended to increase within the physiological limits of 0.8–0.9 with progressing maturity.

DISCUSSION

The practically irreversible reactions catalysed by phosphofructokinase and pyruvate kinase are generally believed to be the regulatory sites in glycolysis [9].

Studies on ripening tomatoes [10] and germinating castor beans [11] not only confirmed this point of view, but also led to an inclusion of gluconeogenic regulation into the general scheme. Ap Rees [12] in a recent review pointed out the technical pitfalls and the difficulties in the interpretation of data based on the determination of unstable metabolic compounds in uncertain cellular compartments. However, at the same time he stressed the need for additional information. Our recovery experiments show that we have largely overcome the technical problems by using a quick-freezing, acid extraction technique and analysis of unfractioned extracts. The glycolytic intermediates we use for determining mass-action ratios are believed to be located in the cytoplasm, and we found no evidence for glyoxysomal enzyme activity in grape extracts. We thus consider that the compartmentalization with respect to the relevant compounds within the berries was minimal. These facts, coupled with the magnitude of the changes in concentrations of intermediates at the beginning of ripening, allow localization of the enzymic regulation of glycolysis and gluconeogenesis in this fruit.

Fig. 1 shows that OAA, citric and malic acid formation came to a standstill after about 6 to 7 weeks after anthesis, which, in combination with decreasing respira-

tion rates [1], indicates a decline in glycolysis. A determination of the mass-action ratios for some glycolytic reactions throughout grape development (Table 1) indicates that phosphofructokinase and pyruvate kinase reactions are far removed from equilibrium and thus constitute possible control points in grape glycolysis. Since the decrease in glycolytic flux coincides with an increase in substrate concentrations for these two reactions, the activity of the enzymes must be regulated by factors other than substrate availability. Thus the enzymes are regulatory [5, 6].

As mentioned previously, a change from glycolysis to gluconeogenesis takes place in grapes during transition from the green to the ripening stage [3, 4, 13]. This change in direction of carbon flow between hexoses and acids occurs at about the time of the beginning of sugar accumulation (Fig. 2).

With the onset of rapid net malate (*ca* half a week after berry softening: Fig. 1b) TP and FDP concentrations started to increase (Fig. 2). Since the activity of fructose diphosphatase was found to be low throughout grape development, this blocking of the gluconeogenic flux indicates that the enzyme is possibly rate limiting at this stage [6]. Evidence against the possibility of differential loss of enzyme activity in grape extracts from developing material has been presented in a previous study [14]. In the following ripening period of less intensive malate breakdown with concomitant reduction of gluconeogenic activity [4], fructose diphosphatase seemed to be able to metabolize its substrate as supplied. No evidence for a change back to glycolysis was found.

Since citric acid is known to be a very effective regulator of plant pyruvate kinase [9], a potent inhibitor of grape phosphofructokinase [2] and in addition has been found to modulate the latter enzyme in other organisms [8], it is thought that citric acid concentrations have an influence on *in vivo* rates of grape glycolysis, possibly in combination with the energy status of the cell [15]. It was found in other plants [9] that inhibition of pyruvate kinase and phosphofructokinase is a prerequisite to exclude futile cycling of compounds during gluconeogenesis at the respective sites. The factors which actually induce this 'reversal of glycolysis' in grapes—apart from an increase in PEP-carboxykinase activity—are not known, but the increasing availability of chemical energy in the berries as indicated by higher energy charge values with progressing fruit development, would undoubtedly favour an energy utilizing process such as gluconeogenesis.

The extent to which gluconeogenesis contributes to sugar increase during ripening is almost impossible to determine because of the continuous influx of assimilatory sugars from the leaves while the fruits are on the vine and the failure of berries to ripen after premature harvest. In our experiments, the most rapid decrease in malate resulted in the loss of 50 μmol malic acid/berry over a period of 10 days compared with a hexose accumulation rate of *ca* 500 μmol/berry/10 days. The contribution of gluconeogenesis to this massive sugar storage could thus at best amount to 5%, assuming that no fresh malate is formed during this period. Since malate was further found to be metabolized via respiration [1, 13], gluconeogenesis must be considered a mechanism which mediates the flow of carbon between the two groups of compounds, rather than a quantitatively important pathway in respect to hexose accumulation.

EXPERIMENTAL

Berries of *Vitis vinifera*, cv. Sultana syn. Thompson Seedless (Clone H_4) growing in the field were used for expts, since seeds were found to complicate the subsequent quick freezing method.

Extraction procedure. Parts of grape bunches were collected at random from 6 vines, berries cut from the pedicels with a razor blade and 10 g of this material compressed and frozen immediately between two Al blocks, precooled in liquid N_2 [16]. The frozen pieces were dropped into a mortar with liquid N_2 and ground. The visually homogeneous powder was transferred into a beaker containing 10 ml 0.6 M $HClO_4$ and 1 g Polyclar AT and allowed to thaw with continuous mixing. The slurry was filtered through a sintered glass funnel and the filtrate brought to pH 6.5–7 with solid K_2CO_3 added slowly with vigorous stirring. After adding 1% Carbowax 4000 the $KClO_4$ was precipitated at 27000 *g* for 10 min and the clear supernatant was immediately used for substrate determinations. Throughout the procedure the extract was kept below 2°. This method yielded results for the glycolytic intermediates comparable to the charcoal treatment used for similar investigations of ripening tomatoes [10] but the present procedure allows nucleotides to be assayed in the same extracts without further treatment. Known amounts (0.5 μmol) of G6P, F6P, FDP, PEP, PYR, ADP, ATP and OAA, added to 10 g frozen material before grinding, gave yields of 94, 95, 95, 102, 97, 106, 84 and 63% respectively. All intermediates were analysed at 25° by standard enzymatic methods [16] adapted for grape extracts and the changes in *A* at 340 nm were followed in a double beam spectrophotometer with digital display. The determinations were carried out in triethanolamine buffer (TRA, pH 7.6, total vol. 3 ml) containing (concn mM in brackets) for the determination of: *PYR*: TRA (85); NADH (0.1); sample 1.5 ml and 5 μg lactate dehydrogenase. *PEP*: Same plus: $MgSO_4$ (2); KCl (25); ADP (0.3) and 10 μg pyruvate kinase. *OAA*: TRA (90); NADH (0.1); sample 1.5 ml and 10 μg malate dehydrogenase. *TP*: Same plus: 10 μg glycerol-3-phosphate dehydrogenase/triosephosphate isomerase. *FDP*: Same plus: 20 μg aldolase. *G6P*: TRA (90); $MgSO_4$ (5); NADP (0.1); sample 1.0 ml and 10 μg glucose-6-phosphate dehydrogenase. *F6P*: Same plus: 7.5 μg phosphohexose isomerase. *ATP*: TRA (85); $MgCl_2$ (5); glucose (5); NADP (0.1); sample 1.0 ml; 10 μg glucose-6-phosphate dehydrogenase and 20 μg hexokinase. *ADP*: TRA (65); $MgSO_4$ (2); KCl (50); PEP (1) NADH (0·1); 10 μg lactate dehydrogenase and 4 μg pyruvate kinase. *AMP*: Same plus: 4 μg myokinase. *Citrate*: TRA (90); $ZnSO_4$ (0.2); NADH (0.1); sample 0.1 ml; 10 μg lactate dehydrogenase; 10 μg malate dehydrogenase and 50 μg citrate lyase. *Malate*: Hydrazine/glycine buffer, pH 9.6 (350); NAD (2.75); sample (diluted 1:100) 0.2 ml and 50 μg malate dehydrogenase. Variability of assays between comparable samples of grape berries was less than 10%. The concns of the intermediates were calculated [16] from an average moisture content of the berries of 90% [7].

Enzyme extractions and estimations of acid concentrations, soluble solid content and size of the berries were performed as described previously [2]. Fructose diphosphatase activity was assayed in a mixture containing HEPES buffer, pH 7.2 (50); $MgCl_2$ (8); FDP (2); NADP (0.1); 3 μg phosphohexose isomerase; 2 μg glucose-6-phosphate dehydrogenase and the reaction started (after allowing for the oxidation of hexose monophosphate impurities in the system) by adding 0.1 ml grape extract.

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