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GLUCOSE CATABOLISM AND ANTHOCYANIN PRODUCTION IN APPLE FRUIT

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Key Word Index—*Malus domestica*; Rosaceae; anthocyanin synthesis; pentose phosphate pathway.

Abstract—The catabolism of glucose via the pentose phosphate pathway (PPP) has been associated with anthocyanin production in apple fruit. Chemical diversion of carbon flow into this pathway was attempted and the effect on anthocyanin synthesis assessed, using isolated discs of epidermal fruit tissue. Malonate increased PPP activity, but did not increase anthocyanin production. Alar neither increased PPP activity nor anthocyanin production. Inhibiting Krebs cycle activity does not necessarily enhance anthocyanin production *in vitro*. Sodium chloride, however, stimulated PPP activity and increased anthocyanin content by 53%. A possible mechanism for the salt effect is discussed.

INTRODUCTION

Anthocyanin production in the epidermis of apple fruit may be affected by a variety of chemical and environmental factors. The manner in which these factors influence anthocyanin synthesis is not well understood, however, perhaps because anthocyanins are synthesized from several interacting metabolic pathways. While the regulation of the activity of the enzyme phenylalanine ammonia-lyase [1], and of a sequence of enzymes specifically involved in flavone glycoside biosynthesis [2] have been shown to be central to the control of anthocyanin production, there may exist other regulatory steps prior to the formation of *trans*-cinnamic acid. Faust [3], for example, demonstrated that the natural development of red colour in apple is associated with an increasing role of the pentose phosphate pathway (PPP) in the catabolism of glucose.

Malonate, which blocks succinate dehydrogenase activity, has been found to increase red colour development of fruit [3], and more recently Alar (butanedioic acid mono 2,2-dimethylhydrazide), a plant growth regulator used commercially to stimulate fruit colour development, has also been shown to inhibit succinate dehydrogenase *in vitro* [4]. Perhaps by inhibiting Krebs cycle activity, carbon flow is shifted into the PPP, the products of which form essential precursors for the synthesis of anthocyanins. The purpose of this investigation was to determine the potential for chemical manipulation of carbon flow into the PPP, and to assess the effect of such treatments on anthocyanin synthesis using isolated discs of epidermal fruit tissue. Specific yields of CO_2, after incubation of fruit discs in radiolabelled glucose, were used to obtain C-6/C-1 ratios in order to estimate the extent to which the PPP is involved in the catabolism of glucose. Increased C-1 decarboxylation relative to C-6 decarboxylation (i.e. a lower C-6/C-1 ratio) indicates increased PPP activity relative to carbon flow through the Embden–Meyerhof–Parnas pathway [5].

RESULTS AND DISCUSSION

Glucose metabolism

With no other additions to the reaction medium, glucose uptake into epidermal apple fruit discs was linear for at least 2 hours (data not shown). Also, both the percentage of carbon taken up by the discs that was released as CO_2, and the C-6/C-1 ratio, became constant after 90 min (data not shown). Any changes in reported C-6/C-1 ratios were therefore due to changes in glucose catabolism induced by chemical treatments. Glucose catabolism studies were conducted with malonate, Alar, and sodium chloride. The latter compound was used because in peas increasing salinity altered carbohydrate metabolism by decreasing the C-6/C-1 ratio [6].

In the presence of 5 mM malonate, the C-6/C-1 ratio decreased from 0.53 to 0.20 (Fig. 1). Glucose uptake was inhibited by 27% (data not shown), but the specific yield of CO_2 from C-1 labelled glucose was greatly stimulated by this treatment (Fig. 1). By contrast, metabolism of C-6 labelled glucose was relatively unaffected (Fig. 1).

The C-6/C-1 ratio was reduced from 0.50 to 0.36 in the presence of 50 mM sodium chloride (Fig. 2). Glucose uptake was inhibited by 13%, and the shift in the C-6/C-1 ratio was due, as with malonate, to a stimulation in C-1 decarboxylation. In contrast to malonate and sodium chloride, 25 mM Alar increased the C-6/C-1 ratio to 0.63 by decreasing the specific yield of CO_2 from C-1 labelled glucose by 15%.

Anthocyanin synthesis

Dark pre-treatment of discs reduced the level of anthocyanin production during a subsequent 4 day light treatment (Table 1). Sucrose stimulated anthocyanin production over the level found in discs incubated in buffer alone. This stimulation was more pronounced in dark pretreated discs (229%) than with discs incubated in the light

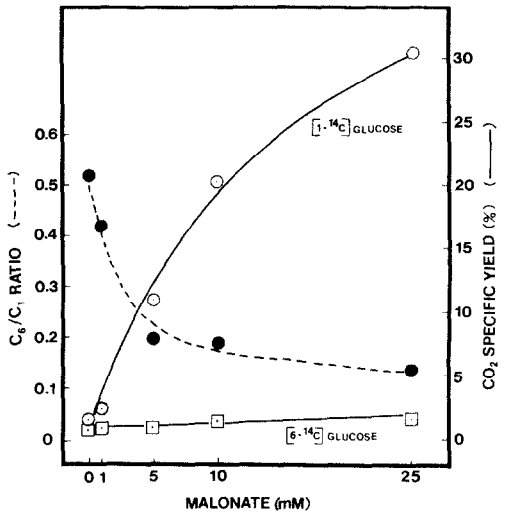

Fig. 1. The effect of malonate on glucose catabolism in isolated discs of epidermal fruit tissue. Each point represents the average of four measurements.

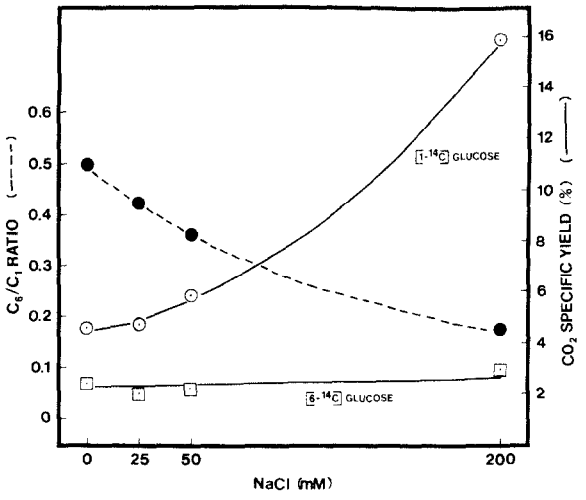

Fig. 2. The effect of NaCl on glucose catabolism in isolated discs of epidermal fruit tissue. Each point represents the average of four measurements.

without pre-treatment (53%) (Table 1). These data indicate that anthocyanin production by apple skin discs incubated in the light was limited by carbon availability, and that sucrose stimulated anthocyanin production by providing increased substrate for the requisite synthetic pathways.

In dark pre-treated discs, salt stimulated anthocyanin production by 36%. In discs transferred directly to light, in which carbohydrate reserves were not reduced, anthocyanin synthesis was 53% greater in the presence of salt than with discs in buffer alone (Table 1). In addition, salt further enhanced sucrose-stimulated anthocyanin synthesis. Taken together with the effect of salt on glucose metabolism (Fig. 2), these data suggest that salt stimulates

Table 1. Effect of chemical treatments on anthocyanin production in isolated epidermal fruit discs

Treatment	Anthocyanin (nmol idaein/cm^2)	
	Dark pre-treatment*	No pre-treatment
Buffer	7 ± 0.6†	15 ± 1.0
NaCl (25 mM)	11 ± 1.3	23 ± 0.9
Sucrose (300 mM)	23 ± 1.7	23 ± 2.3
NaCl + sucrose	37 ± 3.3	34 ± 7.0
Malonate (12.5 mM)	5 ± 0.2	6 ± 0.5
Alar (25 mM)	1 ± 0.3	10 ± 0.7
Malonate + sucrose	8 ± 1.6	—
Alar + sucrose	9 ± 0.1	—

*Discs were incubated for 2 days in buffer at room temperature in the dark.

†Mean ± s.e. (n = 3) after 4 days light.

anthocyanin synthesis by inducing greater carbon flow through the PPP, and that the magnitude of this stimulation is dependent upon the availability of carbohydrate for catabolism.

Neither malonate nor Alar stimulated anthocyanin synthesis; in fact the amount of anthocyanin was less than that found in buffer alone (Table 1). Malonate and Alar also prevented the sucrose stimulation of anthocyanin synthesis in dark pre-treated discs (Table 1). Perhaps malonate and Alar fail to increase anthocyanin synthesis in epidermal fruit discs because they alter cell metabolism in such a way that the capacity for anthocyanin synthesis becomes limited by factors other than carbon flow through the PPP.

Both sodium chloride and malonate stimulated C-1 decarboxylation of glucose. This reaction is catalysed by the first two enzymes of the PPP, glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase. Both enzymes require NADP, and Butt and Beevers [7] have suggested that the NADP/NADPH ratio regulates carbon flow through the pathway. Malonate and sodium chloride may shift glucose catabolism into the PPP by increasing the availability of NADP. In corn roots [8], malonate causes the utilization in the Krebs cycle of malate synthesized in the cytoplasm. Succinate accumulates and is radiolabelled when malonate-treated tissue is incubated in ^{14}C-bicarbonate. The accumulation of succinate may drive malate synthesis in the cytoplasm, which would lead to the regeneration of NADP. Increased malate synthesis in the cytoplasm may also explain the salt effect. If cation uptake is coupled to proton extrusion, malate synthesis may be stimulated to maintain cytoplasmic pH [9]. Again NADP will be generated, driving glucose catabolism via the PPP.

Only in the case of salt was anthocyanin synthesis promoted by a compound which stimulated glucose C-1 decarboxylation using the *in vitro* system. Malonate reduced the C-6/C-1 ratio, but inhibited anthocyanin synthesis. Reduced levels of ATP may result from malonate treatment and this may decrease the capacity of the tissue to synthesize anthocyanin.

Alar did not increase C-1 decarboxylation as expected, and in fact decreased the specific yield of CO_2 from C-1 labelled glucose. Alar is known to inhibit respiration in

apple fruit [10]. Perhaps in fruit epidermal discs, the decrease in ATP resulting from reduced respiratory activity prevents the potential promotive effect of Alar.

Despite the reported role of Alar as an inhibitor of succinate dehydrogenase activity [4], the fruit epidermal tissue clearly responds to malonate and Alar in a different manner. Alar must therefore have additional effects which prevent the stimulation of C-1 glucose decarboxylation, or Alar does not act at the succinate dehydrogenase step when fed to intact tissue.

In conclusion, stimulation of PPP activity by sodium chloride led to increased anthocyanin production. However, diversion of carbon flow into the PPP in the presence of Krebs cycle inhibitors does not necessarily result in increased anthocyanin production in epidermal fruit discs. In addition, the effect of Alar on colour development in apple cannot be explained by this mechanism based upon the data obtained with the *in vitro* system.

EXPERIMENTAL

C-6/C-1 studies. Discs of epidermal tissue (8 cm diam., 5/treatment replicate) were cut from the green area of rinsed fruit [*Malus domestica* (Borkh) 'MacIntosh'] and the cortex removed with a razor blade. Discs were rinsed with H_2O, surface blotted and floated (cuticle side up) on 10 ml O_2-satd reaction medium containing 20 mM MES–NaOH, pH 5.5, and either [1-^{14}C]- or [6-^{14}C]-glucose (1.64 μM, 61.1 μCi/μmol). Discs were incubated in a 50 ml Erlenmeyer flask at 25° for 120 min on a shaking H_2O bath with a wick (0.9 × 2.4 cm Whatman 1 filter paper satd with 50 μl 2 N NaOH) suspended on a paper clip from the flask stopper. The radioactivity taken up by both the discs (rinsed under a stream of H_2O, surface blotted and placed in 0.5 ml EtOH), and the wick were determined by liquid scintillation spectrometry.

Anthocyanin synthesis. Discs (5/treatment) were placed on filter paper in a petri dish containing 8 ml 20 mM MES–NaOH, pH 5.5, and incubated under a bank of fluorescent lights (110–120 $\mu E \cdot sec^{-1} \cdot m^{-2}$) at 18°. Discs were transferred to new petri dishes after 2 days, and the anthocyanin determined after 4 days by extracting overnight at 20° in 5 ml 1% HCl in MeOH. The extraction soln was decanted and centrifuged at 2000 *g*. Anthocyanin was determined spectrophotometrically at 535 nm using the ε (3.4×10^4) for idaein [11].

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