Banana Ripening: Implications of Changes in Internal Ethylene and CO₂ Concentrations, Pulp Fructose 2,6-Bisphosphate Concentration, and Activity of Some Glycolytic Enzymes

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RANDOLPH M. BEAUDRY, NACHMAN PAZ, CLANTON C. BLACK, AND STANLEY J. KAYS*
Departments of Horticulture (R.M.B., S.J.K.) and Biochemistry (N.P., C.C.B.), University of Georgia, Athens, Georgia 30602

ABSTRACT

In ripening banana (Musa acuminata [AAA group, Cavendish subgroup] cv. Valery) fruit, the steady state concentration of the glycolytic regulator fructose 2,6-bisphosphate (Fru 2,6-P₂) underwent a transient increase 2 to 3 hours before the respiratory rise, but coincident with the increase in ethylene synthesis. Fru 2,6-P₂ concentration subsequently decreased, but increased again approximately one day after initiation of the respiratory climacteric. This second rise in Fru 2,6-P₂ continued as ripening proceeded, reaching approximately five times preclimacteric concentration. Pyrophosphate-dependent phosphofructokinase glycolytic activity exhibited a transitory rise during the early stages of the respiratory climacteric, then declined slightly with further ripening. Cytosolic fructose 1,6-bisphosphatase activity did not change appreciably during ripening. The activity of ATP-dependent phosphofructokinase increased approximately 1.6-fold concurrent with the respiratory rise. A balance in the simultaneous glycolytic and gluconeogenic carbon flow in ripening banana fruit appears to be maintained through changes in substrate levels, relative activities of glycolytic enzymes and steady state levels of Fru 2,6-P₂.

As banana fruit begin to ripen, there is a rapid increase in ethylene synthesis, followed by a 4- to 5-fold increase in the rate of respiration as indicated by an increase in CO₂ production (2, 20). It is thought that this increase in CO₂ production during the respiratory climacteric is a result of an increased flux of carbon through the glycolytic pathway to the mitochondria (9, 28). An important rate-controlling step in glycolysis has been identified as the reversible phosphofrorylation of Fru 6-P² to Fru 1,6-P₂ (9, 18, 27). Three enzymes can participate in this interconversion: FBPase, ATP-PFK, and PPI-PFK (6, 8, 23). FBPase, which functions gluconeogenically, and PPI-PFK, which can catalyze the interconversion in either the gluconeogenic or the glycolytic direction, both are regulated by Fru 2,6-P₂. FBPase is inhibited by Fru 2,6-P₂ (24) while PPI-PFK is activated; the latter being activated at 10 to 20-fold lower concentrations of Fru 2,6-P₂ (6). The presence of little or no Fru 2,6-P₂ promotes a small molecular form of PPI-PFK which favors gluconeogenesis. However, as Fru 2,6-P₂ content increases, the more glycolytically active large molecular form predominates (27). PPI or urea at mM concentrations also favor maintenance of the small form of PPI-PFK (27).

Shortly after initiation of the respiratory rise, the strategy for carbon allocation in banana fruit changes as starch is converted to sugar (5, 28). Significant starch degradation begins when the respiratory rise reaches about ¾ of its maximum (i.e. approximately when ethylene synthesis peaks) (28). Starch is stored in amyloplasts and comprises 20 to 25% of the fresh weight, but sugars comprise less than 1% prior to ripening (5, 20). Starch degradation proceeds with ripening until fruits contain less than 1% starch and 20% sugar (primarily sucrose, glucose, and fructose) by weight and the fruits have respired 2 to 5% of their weight as CO₂ (5, 20). Portions of the enzymatic pathways utilized for starch production and respiration are located in the cytoplasm, thereby resulting in an increased flux of carbon in the glycolytic and gluconeogenic directions simultaneously within the same cellular compartment. It is not known how this bidirectional carbon flow is regulated, although Fru 2,6-P₂ and the aforementioned enzymes may be involved. In avocado fruit mesocarp tissue, for instance, there is approximately a 90% increase in Fru 2,6-P₂ that appears to coincide with the onset of the respiratory climacteric (3, 4).

Therefore, to investigate the relationship between ripening and the glycolytic/gluconeogenic regulator Fru 2,6-P₂, in banana fruit, a temporal study of changes in internal ethylene and CO₂ concentrations, activities of FBPase, ATP-PFK, and PPI-PFK, and levels of Fru 2,6-P₂ spanning the entire ripening period was undertaken. Changes in Fru 2,6-P₂ concentration in response to wounding and the inherent variability of Fru 2,6-P₂ in the banana fruit also were examined.

MATERIALS AND METHODS

Chemicals. All auxiliary enzymes for enzyme assays and biochemicals including Fru 2,6-P₂ were purchased from Sigma Chemical Company.

Plant Materials. Mature, green, preclimacteric banana fruit were purchased from a commercial warehouse before they had been stored and gassed with ethylene. Data reported are from two individual lots of bananas and are representative of the information gathered from a total of eight lots of fruit studied over an extended period. Fruit were incubated at 21°C and assayed at a series of preclimacteric and climacteric time intervals to determine: internal concentrations of CO₂ and ethylene; pulp...
Fru 2,6-P$_2$ concentration; pulp FBPase, ATP-PFK, and PPI-PFK activities; and peel color. Internal ethylene and CO$_2$ concentrations were monitored throughout the ripening process. Fruit were individually assayed upon reaching varying stages during the ripening process, based upon their internal ethylene concentration, e.g. 30 to 100, 300 to 1000, 2000 to 4000 and >5500 nL/L. The latter sampling range is near the peak in ethylene synthesis and was taken as d 0. Prior and subsequent sampling times were as described in the figure captions in relation to d 0. Fruits were considered to be 'climacteric' (i.e. undergoing rapid physiological changes associated with ripening) when the internal ethylene concentration rose above 30 nL/L. Data points represent the average of 4 to 5 fruit from replicates composed of 7 to 12 uniformly sized fruit randomly selected from two banana 'clusters.' Enzyme activities were determined from single tissue samples taken from a position near the fruit middle. Fru 2,6-P$_2$ concentration values were the averages of 4 or 5 fruit from which three determinations per fruit were made at the middle and approximately 4 cm from the fruit ends. Tissue samples for Fru 2,6-P$_2$ and all enzyme assays were obtained from the center of the pulp and included some degenerated ovules. Samples were excised using a stainless steel blade, weighed and immediately immersed and held in liquid N$_2$ until assayed.

**Determination of internal CO$_2$ and Ethylene Concentrations.** Glass tubes (1.7 ml volume) fitted with rubber stopper seams were attached to the surface of mature, green banana fruit using Dow-Corning 3140 RTV noncorrosive silicone rubber. This allowed the fruit's internal concentration of gases to equilibrate with the atmosphere within the tube (1). Gas samples (0.3 ml) were obtained using a gas-tight syringe after injecting 0.3 ml laboratory air into the sealed tubes and pumping the syringe 10 times. Gas samples were assayed for ethylene using a gas chromatograph (Varian 1400) with a 1.5 m × 3.2 mm column of activated alumina, 70–100 mesh, 100°C column temperature, with air, N$_2$ and H$_2$ at flow rates of 300, 50 and 50 ml min$^{-1}$ respectively) fitted with a flame ionization detector. A second gas sample was immediately withdrawn from the sealed tubes in a similar manner and assayed for CO$_2$ using a Fisher-Hamilton gas partitioner with a thermal conductivity detector. Dilution factors to ascertain the concentration of the two gases prior to sampling were determined using a standard curve for the dilution effect of repeated sampling from the glass tubes.

**Fru 2,6-P$_2$ Determination.** The technique utilized for extraction of Fru 2,6-P$_2$ were similar to those of Huber and Bickert (12). Frozen tissue was ground with washed sand and 3 ml of a buffered grinding medium (pH 7.7) with a mortar and pestle. The grinding medium contained 50 mM Hepes-NaOH, 10 mM KF, 2.0 mM Na$_2$EDTA, 2.5 mM DTT and 1% w/v insoluble PVP. The homogenate was placed in a boiling water bath for 2 min then centrifuged at 20,000g for 10 min. The supernatant was collected and quick-frozen in liquid N$_2$ then stored at −20°C. Fru 2,6-P$_2$ concentration was measured using the method of Van Schaftingen et al. (26) which is based on the ability of the extract to enhance potato tuber PPI-PFK activity. The assay mixture contained 0.1 mM Hepes (pH 8.0), 2.5 mM MgSO$_4$, 0.32 mM NADH, 1 mM Fru 6-P, 0.5 unit aldolase, 5.8 unit triose-P isomerase, 0.5 unit α-glycerol-P dehydrogenase, approximately 20 milliunits PPI-PFK, up to 50 µl of plant extract, and 1 mM Na$_2$PPI to initiate the reaction. The rate of oxidation of NADH was monitored by following the decrease in absorbance at 340 nm for 3 min after initiation using a Beckman DU-7 spectrophotometer. Fru 2,6-P$_2$ concentration was determined with respect to a standard curve.

**ATP-PFK, PPI-PFK, and FBPre Assays.** Frozen samples were ground (0.5 g tissue/4 ml medium) with washed sand in a mortar and pestle which was chilled with liquid N$_2$. The extraction medium (pH 8.0) contained 100 mM Bicine, 2 mM EDTA, 1 mM MgCl$_2$, 2% w/v PVP, 5% v/v glycerol, 10 mM DTT and 1 mM phenylmethylsulfonyl fluoride. The extract was centrifuged for 10 min at 30,000g and the supernatant was withdrawn and frozen in liquid N$_2$ until assayed as a crude extract (usually within 24 h).

The assays for FBPase, ATP-PFK, and PPI-PFK activities were conducted at 25°C in a 0.5 ml volume as described by Smyth et al. (21). The reaction medium for the ATP-PFK and glycolytic PPI-PFK assays contained 0.1 mM Hepes-NaOH (pH 8.0), 2.5 mM MgSO$_4$, 10 mM Fru 6-P, 0.16 mM NADH, 0.6 unit aldolase, 9.9 units α-glycerol-P-dehydrogenase, 5 units triose-P-isomerase, and up to 40 µl of plant extract. The rate of oxidation of NADH was monitored until it became constant (i.e. 4–8 min) then 1 mM Na$_2$PPI or 1 mM Na-ATP was added to initiate the reaction. The absorbance readings were divided by 2 to correct for the 2x amplification of the coupling system. The reaction medium for the gluconogenic PPI-PFK assay contained 0.1 mM Hepes-NaOH (pH 8.0), 2.5 mM MgSO$_4$, 0.1 mM Fru 1,6-P$_2$, 0.5 mM NADP, 9 units phosphoglucose isomerase, 2 units glucose 6-P dehydrogenase, 1 mM Pi, and up to 40 µl of plant extract. Parallel samples were assayed simultaneously in the PPI-PFK assay which included 1 µM Fru 2,6-P$_2$ in the assay medium. The assay mixture for cytoplasmic FBPase activity was similar to that for gluconogenic PPI-PFK, but used 0.1 mM Hepes-NaOH (pH 7.5), 10 mM Fru 1,6-P$_2$ and up to 80 µl of plant extract. In the gluconogenic assays, the change in absorbance due to NADP reduction was used to measure reaction rate. After the change in absorbance became steady (i.e. 4–8 min), Fru 1,6-P$_2$ was added to initiate the reaction. Parallel samples were assayed simultaneously which included 1 µM and 1 mM Fru 2,6-P$_2$ in the reaction medium for PPI-PFK and FBPase, respectively. Plant extracts routinely were boiled for 5 min to verify that enzymes were responsible for the activity recorded. Protein was determined with the Coomassie blue staining method of Bradford (7) with BSA as the standard.

**RESULTS**

Changes in Internal Ethylene, CO$_2$ and Pulp Fru 2,6-P$_2$. The internal concentrations of ethylene and CO$_2$ increased slightly over a period of 8 to 12 d prior to the onset of the logarithmic respiratory increase (Fig. 1). Two to 3 h before the rapid climacteric rise in internal CO$_2$ concentration occurred, the ethylene concentration of the fruit tissue increased dramatically (e.g. up
to 100×). The internal ethylene concentration increased for 12 to 14 h to a maximum of 10 to 12 μL/L and then declined rapidly. Trailing the rise in ethylene, the internal CO₂ concentration increased more slowly over the next 18 to 24 h to a maximum (i.e. approximately five times that of the preclimacteric respiratory minimum on d −12) and slowly decreased thereafter.

Pulp Fru 2,6-P₂ concentration exhibited a slight, transient increase concomitant with the increase in internal ethylene (i.e. 2–3 h prior to the respiratory rise) reaching approximately 1.2 times the preclimacteric level (Fig. 1, see also Fig. 3, A and B). The Fru 2,6-P₂ concentration declined after the transient rise, but increased again 24 h past the ethylene peak (d +1) and continued to increase, but at a decreasing rate until the termination of the experiment on d +7.

**Enzyme Activities.** ATP-PFK activity was essentially constant until it rose by about 60% concurrent with the respiratory rise in fruit from Lot 2 (Fig. 2A). In Lot 1 fruit, ATP-PFK activity rose at the onset of the rapid increase in the internal ethylene concentration, thus preceding the climacteric rise in respiration by 2 to 3 h (data not shown). The elevated level of ATP-PFK activity remained unchanged after ripening was initiated and was low in relation to PPi-PFK activity. ATP-PFK activity was unaffected by added Fru 2,6-P₂ (data not shown).

Maximal glycolytic and gluconeogenic PPi-PFK activity (PPi-PPK + 1 μM Fru 2,6-P₂) generally declined during ripening (Fig. 2, A and B) except for a transient increase in activity occurring concurrent with the increase in respiration. Maximal glycolytic PPi-PFK activity ranged from 2 to 3 times that of glycolytic PPi-PFK activity without added Fru 2,6-P₂ in a time-dependent manner (Fig. 3, A and B). Glycolytic PPi-PFK activity without added Fru 2,6-P₂ decreased at (Lot 1—data not shown) and just after (Lot 2) the peak in ethylene synthesis, but prior to the respiratory peak and later increased to approximately preclimacteric levels. Maximal gluconeogenic PPi-PFK activity was consistently about 1.5 times higher than gluconeogenic PPi-PFK activity without added Fru 2,6-P₂ (Fig. 3B). Gluconeogenic PPi-PFK activities (i.e. with and without added Fru 2,6-P₂) underwent similar time-dependent changes.

The activity of FBPase with or without added Fru 2,6-P₂ changed little throughout the ripening process except for an apparent transitory decrease early in the climacteric (Fig. 2B). Addition of Fru 2,6-P₂ (1 mm) consistently decreased FBPase activity to 80 to 85% that of the endogenous level prior to the climacteric (Fig. 3A). After the onset of the climacteric, added Fru 2,6-P₂ decreased activity to about 72% of the endogenous level, but the relative amount of inhibition diminished thereafter as indicated by an increase in the ratio of Fru 2,6-P₂ inhibited to Fru 2,6-P₂ uninhibited FBPase activity to about 0.9.

**DISCUSSION**

Initiation of the Respiratory Climacteric. Ethylene is known to elicit a diverse range of physiological responses including the induction of the respiratory climacteric in mature climacteric fruits (5, 20). The existence of a causal relationship between ethylene and Fru 2,6-P₂ has been examined, but evidence for a direct involvement is lacking. Very high levels of ethylene (i.e. 50 μL/L) have been shown to increase the concentration of Fru 2,6-P₂ after 24 h in some nonclimacteric storage tissues (e.g. carrot root and potato tuber [22]). An increase in Fru 2,6-P₂ has also been associated with ripening in avocado (3, 4). In pea tissue, however, increases in Fru 2,6-P₂ precede a wound-induced rise in ethylene synthesis (17). In our results, the lag between the rapid rise in ethylene synthesis at the onset of ripening in the banana and the final rise in Fru 2,6-P₂ indicates the effect of ethylene on Fru 2,6-P₂ is probably indirect through its effect on ripening per se.

The temporal relationship between the respiratory climacteric and the rise in Fru 2,6-P₂ in avocado (3, 4) has led to the suggestion that activation of PPi-PFK may be involved in the respiratory enhancement during ripening. Our data do not support this contention for banana fruit. The increase in CO₂ synthesis after the transitory rise, but prior to the final rise in Fru 2,6-P₂ does not causally implicate Fru 2,6-P₂ in the initiation of the respiratory climacteric through its effects on PPi-PFK and FBPase. Furthermore, it is doubtful that the short-lived increase in maximal PPi-PFK glycolytic activity contributes significantly to the early stages of the respiratory rise, since the increase in activity is only about 10%. The 60% increase in ATP-PFK activity at the time of the respiratory rise implies that this enzyme is more likely involved in directing carbon glycolytically for CO₂ production.

An increase in ATP-PFK activity has been reported previously
for ripening banana fruit (28), but the temporal relationship to the rise in CO₂ production was not clearly defined. The tight synchrony of changes in ATP-PFK activity and the rise in respiration in the present study argues favorably for an involvement of ATP-PFK in the initial respiratory rise. However, the respiratory increase is about 5-fold, making the increase in ATP-PFK activity too small for it to be solely responsible for the increase in CO₂ production without additional changes in substrate availability and/or regulation. In this regard, it has been proposed that anions (which have the ability to reduce the strong inhibition of ATP-PFK activity by P-enolpyruvate) may be diffusing from the vacuole in response to a loss in tonoplast integrity as ripening proceeds (9). Pi, which is abundant in the vacuole, is also effective in relieving the inhibition by P-enolpyruvate (27) and has been proposed as a probable activator of phosphofructokinase in ripening fruit (9). However, recent data indicates that cytosolic Pi levels decrease during ripening of avocado fruit although no estimates of the relative degree of change was given (3).

The synchrony of increases in PPI-PFK and ATP-PFK activity (Fig. 2) raises the possibility that both enzyme systems are responding to the same stimulus. At the time each enzyme increases in activity, the fruit is on the verge of undergoing major storage carbon interconversion (i.e., starch to sucrose, glucose and fructose) that accompanies the ripening process. The observed changes in enzyme activity may be involved in fulfilling the energy requirements (e.g., ATP and PPI) needed for the initiation of the overall ripening process via enzyme synthesis and activation, ethylene synthesis, etc.

Regulation During Sugar Production—Metabolite Conversion Model. The asynchrony of the final increase in pulp Fru 2,6-P₂ with that of CO₂ indicates that the induction of net carbon movement in the glycolytic direction is probably not the primary function of Fru 2,6-P₂ in the ripening banana. The increase in the Fru 2,6-P₂ concentration after d +0.5 does, however, indicate Fru 2,6-P₂ is probably involved in the regulation of the glycolytic/glucogenic status of the ripening tissues. Well-documented changes in the concentration of metabolites in ripening banana fruit were incorporated in the following model developed to clarify the role of Fru 2,6-P₂ in this regard.

Starch degradation is thought to take place in the amyloplasts which have the necessary enzyme compliment for conversion of (1-4) glucans to triose phosphates (14). Phosphorylated triose sugars readily pass through the amyloplast envelope (14, 15) and are, therefore, able to enter the cytosol. The involvement of phosphorylated triose sugars from starch degradation in glycolysis would therefore be similar to that envisioned for chloroplasts (23) (Fig. 4). Phosphorylated trioses would be the major carbon source for sugar production by entering a glucogenic pathway involving either FBPase or PPI-PFK while simultaneously providing a carbon source for CO₂ production glycolytically. Influx of trioses into the cytosol would be expected to result in a rise in the concentration of glucogenic intermediates. Such increases have been reported for ripening banana, including an up to 20-fold rise in Fru 1,6-P₂ (2). Given the low kₚ for PPI-PFK in the glycolytic direction (i.e., 2 to 4) (23), such an increase in Fru 1,6-P₂ could permit glucogenic carbon flow. The increase in Fru 2,6-P₂ concentration after d +0.5 would be expected to selectively enhance glycolysis and thereby oppose the effect of the increasing Fru 1,6-P₂ concentration on PPI-PFK. Thus, the reported levels of Fru 2,6-P₂ may, in fact, be an indication of the concentrations needed to maintain a balance between glycolytic and glucogenic carbon flux during the process of rapid metabolite conversion and CO₂ generation in ripening banana.

An alternative to the proposed model would be the circumvention of a direct role for FBPase or PPI-PFK via the availability of hexoses from the amyloplasts. A hexose carrier has been identified in chloroplasts of spinach (19) and, if present in the amyloplast, could permit the movement of hexoses from the amyloplasts to the cytoplasm as triose-P either for the glucogenic CO₂ production or for glucogenic sugar production in the mitochondria. The scheme for starch breakdown in the amyloplast shows Carbon flow upon work detailing starch synthesis in amyloplasts from exogenously supplied trioses (14, 15). Critical enzymes for the control of carbon flux by Fru 2,6-P₂ at the point of interconversion of Fru 6-P and Fru 1,6-P₂ are numbered: 1, FBPase; 2, ATP-PFK; 3, PPI-PFK.
amylloplasts of banana fruit, might provide a way to bypass the enzymatic step in question for hexose sugar formation. Releasing hexose-P from the amylloplast could allow for a more complete control of CO₂ production by ATP-PFK, FBPase and PPI-PFK, however, there are no data yet to support this possibility. In addition, it seems unlikely that hexoses from starch hydrolysis would be made directly available to the cytoplasm via a loss in plastid membrane integrity. Studies on other plant materials indicate starch grains in plastids are degraded in the early stages of tissue senescence (10, 11), while plastid envelopes are intact until a relatively late stage of senescence (10).

The proposed model dictates that prior to the influx of phosphorylated trioses into the cytosol, glycolytic carbon flow (i.e. that responsible for the initial respiratory increase) utilizes the enzymes ATP-PFK and/or PPI-PFK. However, as starch breakdown begins and sugars accumulate, the model necessitates an involvement of FBPase and/or PPI-PFK in the gluconicenic flow of carbon for sugar production.

The changes in gluconegenic PPI-PFK activities (approximately a 50% decrease) after d +0.5 do not suggest an obvious involvement of PPI-PFK in sugar production although relative changes in PPI-PFK activities (discussed below) indicate otherwise. The lack of significant changes in, and relatively low levels of, FBPase activity may indicate a rather limited role for FBPase in the massive accumulation of sugars as products of starch degradation. However, in the case of both PPI-PFK and FBPase, it is possible that the reaction in vivo is pushed toward sugar production without requiring a significant change in activity due to the aforementioned increase in the concentration of the substrate, Fru 1,6-P₂.

Ratios of enzyme activities (i.e. with added Fru 2,6-P₂ versus without added Fru 2,6-P₂, and glycolytic versus gluconegenic) may indicate regulatory changes not obvious when comparing activities separately. Activity ratios of PPI-PFK and FBPase are used to infer the strategy utilized by ripening banana fruit to control carbon flow as follows. If no inhibitory factors are present and the effect enzymatic regulators such as Fru 2,6-P₂ remain constant, then saturating the system with added Fru 2,6-P₂ should result in a consistent increase in PPI-PFK activity or decrease in FBPase activity relative to the enzyme activity without added Fru 2,6-P₂ regardless of the amount of enzyme present. Changes in this activity ratio indicate changes in the apparent sensitivity of the enzyme to Fru 2,6-P₂, perhaps through changes in the levels of inhibitors or activators other than Fru 2,6-P₂. Similar logic dictates that alterations in the glycolytic to gluconegenic PPI-PFK activity ratio would result from changes in regulation. An increase in the ratio would indicate a relative enhancement of glycolysis whereas a decrease would indicate gluconegenic enhancement.

In ripening banana fruit, a saturating level of added 1 μM Fru 2,6-P₂ did not result in a consistent change in glycolytic PPI-PFK activity as ripening proceeded (Fig. 3, A and B). The apparent change of PPI-PFK activity by added Fru 2,6-P₂ was approximately 2-fold until d 0 for Lot 1 (Fig. 3A) and d +0.5 for Lot 2 (Fig. 3B), whereupon it rose about 2.5- and 8.0-fold, respectively. This can be seen in Figure 2A as the more rapid drop in unenhanced PPI-PFK activity on d +0.5 relative to that with added Fru 2,6-P₂. The increase in the enhanced to unenhanced ratio of PPI-PFK activity for Lot 1 fruit (d 0, +1) occurs without a significant change in Fru 2,6-P₂ concentration from preclimacteric levels. This suggests the loss of an activator or the production of an inhibitor of PPI-PFK glycolytic activity whose effect can be overcome by addition of 1 μM Fru 2,6-P₂. Regulation of PPI-PFK glycolytic activity may be achieved via rendering the enzyme less sensitive to endogenous levels of Fru 2,6-P₂ by favoring the small molecular form of the enzyme in a manner similar to mm concentrations urea or PPI, the effects of which can also be largely overcome by additional Fru 2,6-P₂ (27). For Lot 2 fruit, the initial increase in the enhanced to unenhanced ratio of glycolytic PPI-PFK activity coincided with a decrease in endogenous Fru 2,6-P₂ levels. However, by d +1, Fru 2,6-P₂ levels returned to preclimacteric levels yet the enhanced to unenhanced ratio remained high, again implying the presence of a regulatory factor other than Fru 2,6-P₂. After d +2, the stimulatory effect of added Fru 2,6-P₂ on PPI-PFK activity began to decrease daily until it was only about 1.4 times the unenhanced level (d +7). This decreasing ratio probably reflects, in part, an enhancement of endogenous PPI-PFK glycolytic activity by the approximately five-fold increase in the Fru 2,6-P₂ concentration of ripened fruit.

The lack of significant changes in the enhanced to unenhanced gluconegenic PPI-PFK activity were probably due, in part, to the reduced sensitivity of PPI-PFK to Fru 2,6-P₂ in the gluconegenic direction compared to the glycolytic direction (note Fru 2,6-P₂ increased activity 200 to 300% glycolytically, but only 50% gluconegonically). Otherwise, the slight variation of the enhanced to unenhanced ratio of gluconegenic PPI-PFK activity indicates that the unknown factor(s) affecting glycolytic PPI-PFK activity have little or no effect on activity in the gluconegenic direction. Comparing both curves, the inhibition of glycolytic activity and the lack of a similar inhibition in gluconegenic activity suggest a net enhancement of gluconegenic activity occurs at a time which coincides with the initiation of sugar accumulation (29). The implication is that PPI-PFK is responsible for the gluconegenic flow of carbon even though CO₂ production as a result of glycolysis is relatively high.

Some animal tissues exhibit similar high gluconegenic activity when conditions suggest glycolysis should be enhanced (i.e. Fru 2,6-P₂ levels are relatively high [13]). Two possible explanations for this apparent paradox are presently being considered (13). One involves the action of an as of yet unidentified agent which reduces the inhibitory effect of Fru 2,6-P₂ on FBPase. This seems unlikely in the banana in that FBPase inhibition by Fru 2,6-P₂ during sugar production (e.g. d +1, +2) was similar to preclimacteric levels in Lot 1 of the present study (Fig. 3A). The second explanation proposes that two populations of cells exist; some glycolytically active with a high level of Fru 2,6-P₂, while others are gluconegonically active and have a low Fru 2,6-P₂ level. Theoretically, the latter means of fine control may also have a temporal component consisting of time-dependent fluctuations in Fru 2,6-P₂ concentration for the two cell populations in a manner similar to the temporal oscillations in concentration described for pea (16). The possibility of spatially separated Fru 2,6-P₂ oscillations seems plausible in view of our findings of two to 3-fold differences in Fru 2,6-P₂ concentration between adjacent 1.2 cm thick slices in banana fruit (data not shown) and the short time (approximately 30 min) in which doubling of the Fru 2,6-P₂ concentration occurs following excision (data not shown). This ability to rapidly respond to a given stimulus by adjusting Fru 2,6-P₂ levels and the observed spatial variation suggest that banana fruit have the potential to utilize Fru 2,6-P₂ as a means of rapidly adjusting carbon flow for fine control of the glycolytic pathway. It is conceivable that such fluctuations in concentration are present in ripening banana fruit as they are in pea, however, the time scale involved in the present study and averaging of data would mask their appearance. In this respect, the Fru 2,6-P₂ concentrations reported here are probably not an indication of fine control of the enzyme systems in glycolysis, but rather reflect steady state levels about which momentary, spatially distinct fluctuations may occur.

The drop in the ratio of the activity of FBPase with added Fru 2,6-P₂ to FBPase without added Fru 2,6-P₂ (i.e. maximal FBPase activity) suggests an increased sensitivity to Fru 2,6-P₂ early in the climacteric stage. Thus, a potential for relative enhancement
of glycolysis occurs at a time which coincides with the peak in ethylene synthesis, the initiation of the respiratory climacteric, and with the increase in ATP-PFK and PPI-PFK activity; again implying a common stimulus. The increase in the inhibited ratio after d +2 indicates a loss in the effectiveness of added Fru 2,6-P$_2$. As with PPI-PFK, the loss in effectiveness may be due to the effect of the increasing endogenous Fru 2,6-P$_2$ concentration upon activity.

Changes in the ratio of PPI-PFK glycolytic versus gluconeogenic activity (Fig. 5) further indicate regulation of PPI-PFK activity may have a role in predisposing ripening banana fruit toward glycolysis or gluconeogenesis. Failure of saturating levels of Fru 2,6-P$_2$ to yield a constant ratio implies PPI-PFK activity is affected by regulatory factors other than Fru 2,6-P$_2$. The drop in the ratio around d +0.5 appears to indicate a relative enhancement in gluconeogenesis at a time which coincides with the onset of sugar production. The rise in the activity ratios after d +1.0 point toward a facilitated glycolytic carbon flow through PPI-PFK; however, respiration decreased (see Fig. 1) and active production of sugar continued. In this regard, increasing Fru 2,6-P$_2$ concentrations and enzyme activity ratios for PPI-PFK might reflect an adaptation to permit glycolytic carbon flow under conditions (i.e. accumulation of substrate) which otherwise would increasingly favor gluconeogenesis.

The similarity of the shape of the endogenous Fru 2,6-P$_2$, concentration curve and enzyme activity ratio curves suggests that Fru 2,6-P$_2$ is involved in regulating the PPI-PFK glycolytic to gluconeogenic activity ratio. Fru 2,6-P$_2$ apparently acts in a manner which parallels the relative enhancements by the factors postulated above thereby assisting in regulating the direction of carbon flow. The synchrony of changes in the enzyme activity ratios and the endogenous concentration of Fru 2,6-P$_2$ may also be indicative of responses to a common effector.

Collectively, the data suggest carbon flux may be regulated by the concerted action of several interrelated factors, the net influence of which changes over time as the carbon allocation strategy of the fruit changes. During the initiation of the climacteric, some of the controlling factors appear to be increases in glycolytic enzyme activity (ATP-PFK and PPI-PFK) and an increase in the apparent sensitivity to Fru 2,6-P$_2$ (FBPase). Later in the climacteric rise, as sugar production begins, an increase in substrate levels (Fru 1,6-P$_2$), reduction in the apparent sensitivity to Fru 2,6-P$_2$ (glycolytic PPI-PFK) and relative enhancement of gluconeogenesis (PPI-PFK) appear to be factors regulating carbon flux. After the climacteric peak, the effect of still rising substrate levels (Fru 1,6-P$_2$) appears to be balanced by increasing Fru 2,6-P$_2$ levels and an increasing relative enhancement of glycolysis (PPI-PFK). Fru 2,6-P$_2$ levels reported probably reflect steady state levels needed to achieve coarse control of carbon movement in response to the changing status of the ripening fruit. However, further investigation into the role of the enzymes affected by Fru 2,6-P$_2$ is needed to clarify the means of fine control of carbon flux in a tissue which is highly active in glycolytic and gluconeogenic directions simultaneously.

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