The Climacteric in Ripening Tomato Fruit

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ABSTRACT

Phosphofructokinase is identified as the regulator reaction activated at the onset of the climacteric rise in respiration of the ripening tomato fruit (Lycopersicon esculentum Mill). The concentration of ATP in the fruit increases to a maximum value after the climacteric peak of respiration is past. Orthophosphate is proposed as the most probable activator of phosphofructokinase in the ripening fruit.

Fifteen hours after infiltrating tomato fruit with orthophosphate, the rate of respiration increased and remained high until the end of the experiment, 45 hours after infiltration. In experiments where tomato plants were grown at various nutrient levels of P, the rate of respiration when fruit harvested at the mature-green stage reached the respiratory climacteric was correlated with the concentration of orthophosphate in the fruit at the end of the experiment. These results are consistent with the hypothesis that stimulation of phosphofructokinase through increasing concentration of orthophosphate in the cytoplasm of the fruit contributes to the climacteric rise in respiration.

Hess (18) has implicated the irreversible reactions of the EMP pathway as sites of regulation of carbon flux in living cells, and many other workers have reported evidence for regulation by one or more of these reactions in a wide variety of tissues. In higher plants, Givan (16) has identified PFK as regulator in cultured cells of Acer pseudoplatanus subjected to anoxia, and Adams and Rowan (3) have reported sequential regulation of induced respiration in sliced carrot tissue of PK and PFK. During the climacteric rise in respiration of ripening fruit, G6P decreases in avocado (35), and FDP increases in banana (7). Both observations are consistent with the stimulation of PFK. The crossover theorem of Chance (14, 15) is used herein to identify PFK as the site of regulation during the climacteric rise in respiration of tomato fruits.

Stimulation of PFK in a ripening tomato fruit could be due to a decrease in the concentration of a negative effector, or the increase in concentration of a positive effector, an antagonist of a negative effector, or of either substrate. In a study of this enzyme in pea seed, Kelly and Turner (20, 21) have identified 10 effectors. In the first part of the work reported here no change in concentration of any metabolite appeared to fall in any of the above categories, and, since several workers have proposed that changes in permeability accompany the climacteric (6, 29, 30, 33), we postulate that, at the climacteric, the diffusive resistance of the tonoplast decreases, allowing Pi, a known positive effector of PFK, to diffuse down a concentration gradient from vacuole to cytoplasm, thus stimulating PFK. Phosphatase activity, which increases during the climacteric in ripening apples (25), could be responsible for stimulation of PFK by increasing the level of Pi in the cytoplasm, but our unpublished work has failed to demonstrate a positive association between phosphatase activity and the climacteric in tomato fruit.

Orthophosphate accounts for 70% of total P in the potato tuber (11, 22) and Spirodela oligorrhiza (9) of which approximately 90% was in a nonmetabolic pool not freely available to the cytoplasm. In Spirodela, this pool is depleted by imposing P deficiency, presumably by diffusion (10) from vacuole to cytoplasm. If diffusion of Pi, from a similar non-metabolic pool in tomato fruit cells is limited by impermeable membranes, any process increasing the permeability of the membranes could increase the concentration of Pi in the cytoplasm, thus inducing the climacteric rise in respiration. Although in some experiments with banana slices the respiratory increase preceded the increase in efflux (33), the changes in permeability of tonoplast and plasmalemma need not coincide, and the concentration of cytoplasmic Pi could rise before the increased efflux from the slices could be detected. Alternately, failure of a metabolic "pump" maintaining the concentration gradient, rather than an increase in permeability, would have the same effect. Invoking permeability changes of this type is restating the concept of decreasing "organization resistance" originally proposed by Blackman and Parija (12) as an explanation of the climacteric in ripening apples.

In Spirodela (10), P deficiency greatly reduced the level of total P, and the proportion of Pi in the nonmetabolic pool without greatly affecting the level of organic phosphates. Therefore, tomatoes were grown under varying phosphate regimes to modify the amount of P without the nonmetabolic pool (vacuole) potentially available to stimulate respiration. According to the hypothesis to be tested, the maximum respiration rate at the peak of the climacteric should be a function of the total concentration of P, in the fruit, providing the P concentration does not rise above the optimum for activation of PFK. This is quite distinct from correlating the increasing respiration rate before the climacteric maximum with other biochemical parameters, the approach used in previous studies by other workers.
MATERIALS AND METHODS

Plant Material. Fruits for extraction and assay of intermediates (Lycopersicon esculentum Mill) were grown at the Scoresby Horticultural Research Station, Victoria (cu. “V10”) or under glass at the Botany School, Melbourne University (cu. “Grosse Lisse”). Harvested fruits were graded into ripeness classes essentially by the method described by Rowan et al. (28). Since in experiment 1 grading into only five classes (MG, B, HR, TR, and CR) was inadequate, in experiment 2, an IG class, three grades of PB (PB1, PB2, and PB3), and two grades of HR (HR1 and HR2) were selected in addition to MG and TR (CR was not selected in this experiment). Fruits containing varying concentrations of Pi (cu. “Grosse Lisse”) were obtained by growing in sand culture using the following solutions (19): solution A: KNO3 (0.404 g per liter), Ca(NO3)2 (0.636 g per liter), MgSO4 (0.368 g per liter); solution B: NaH2PO4 (0.208 g per liter). Eight plants were used; five received 0.5 liter per day of both solutions A and B for 6 weeks, and then 1 liter per day until the initial fruit set. The remaining three plants received 0.5 liter per day of both solutions A and B for 6 weeks and thereafter 1 liter of solution A and 0.5, 0.25, and 0.125 liter respectively of solution B daily. After the initial fruit set, three of the five plants receiving full nutrients were reduced to the lower levels of solution B. When necessary, supplementary water was applied, and at weekly intervals nutrients were leached from the sand culture by exhaustive watering. Fruits were harvested at the mature green stage. Phosphofructokinase was extracted from immature fruit (1–10 g; cultivar “Grosse Lisse”) harvested from plants grown under glass at the Botany School.

Respiration Measurements. The rate of respiration was calculated by passing CO2-free air at a monitored rate through darkened respiration chambers and measuring the concentration of CO2 in the effluent airstream by a colorimetric method (13) or by using an IRGA (Grubb Parsons model SB2 or 3074). Appropriate calibration curves were constructed by preparing standard gas mixtures with a gas-mixing pump (H. Wosthoff, type 27-3). The respiration rate of single fruit was followed except in experiment 1 (Fig. 2) where samples containing eight fruits were used. The respiration rates of fruits from which glycolytic intermediates and ATP were extracted (Figs. 1 and 2) was followed continuously for 48 h before extraction. In contrast, fruits used in studying the effect of endogenous Pi, level on the climacteric maximum were harvested at the mature green stage, and the respiration rate was followed continuously while ripening in the respirometer.

Infiltration Experiments. Fruits used were grown at Geraldton, West Australia (cu. “Geraldton Smooth Skin”) and bought from local wholesalers. Experimental solutions (0.3 ml) were infiltrated into mature green fruits (100 g) by the method of Spencer (31).

Extraction and Assay of Intermediates. Acid-soluble extracts were prepared by blending 100 g of tissue in 80 or 100 ml HC104 (10%, v/v) for 2 min, filtering the blend through a pad of dry “Hyflo Super cel” over Whatman No. 541 (41H) paper and adjusting the pH to 8 with 8N KOH; the precipitate of KC104, was removed by sedimentation overnight (26). All operations were performed in a cold room at 1°C. Extracts used for determination of glycolytic intermediates were decolorized by shaking with activated charcoal for 1 min before placing in a freezing cabinet at −15°C. The water content was determined in replicate samples of tissue. Glycolytic intermediates were determined enzymatically (8). ATP was estimated using luciferin-luciferase (32) as described by Bergmeyer (8), measuring the light emitted at 562 nm with an Amino-Bowman spectrophotofluorometer coupled with an Hitachi model QPD 73 potentiometric recorder. Orthophosphate was measured by the colorimetric method of Allen (4).

Extraction and Assay of PFK Activity at Various P, Concentrations. One hundred grams of immature tomatoes were blended with a Braun juice extractor from which the tissue homogenerate flowed rapidly into a beaker containing 50 ml of extracting medium. The extracting medium (20 mm tris buffer, pH 7.5) contained sodium metabisulfite (10 mm) to prevent the formation of tannins (5) and EDTA (2 mm). The extract was filtered through “Hyflo Supercel” on Whatman No. 541 (41H) filter paper. The fraction precipitating between 33 and 45% (w/v) saturated (NH4)2SO4 was collected by centrifugation at 10,000g for 30 min. The precipitate was redissolved in a small volume of 20 mm tris buffer, pH 7.5 and was used in the PFK assay.

The reaction was carried out at 25°C in the presence of aldolase, triose phosphate isomerase, and glycerol-1-phosphate dehydrogenase in 50 mm tris, pH 7.5. Fructose-6-P was generated from G6P by phosphoglucone isomerase or F6P free of FDP was used. ATP and magnesium (final concentration 5 mm) were also added to the reaction cuvet. The concentration of NADH, between 2.5 and 5 μM in the reaction cuvet, was standardized spectrophotometrically. The rate of NADH oxidation was measured with an Amino-Bowman spectrophotofluorometer coupled to an Hitachi (series QPD 73) recorder. The excitation wavelength was 340 nm and emission was measured at 460 nm. Fluorescence was proportional to the concentration of NADH for the concentration range used. The effect of phosphate concentration on a crude preparation of PFK was investigated at two F6P levels. Phosphate (NaHPO4) was added to the assay mixture in the cuvet before starting the reaction with PFK extract.

RESULTS

Changes in EMP Intermediates and ATP. Figure 1 shows the changes in concentration of EMP intermediates in fruits from IG to TR stages and shows that the respiratory climacteric begins at stage PB, and continues to the “breaker” stage (B−Bt). The concentrations of F6P and G6P decrease between stages MG to PBt, while FDP increases sharply; all other intermediates measured increase during this interval. The concentration of pyruvate increases. Both sets of changes occur during the climacteric increase in rate of respiration. Orthophosphate increases from the start of the experiment until the PB stage. Similar changes occurred in experiment 1 but the stage at which they occurred could not be localized so accurately.

Figure 2 shows the concentration of ATP in fruits from MG to CR in experiment 1 using combined samples; the increase in ATP concentration occurs one stage later than the onset of the climacteric rise, and the climacteric peak at the HR stage also precedes the maximum concentration of ATP. ATP concentration and rate of respiration both increase by approximately 30%.

The Effect of P, Concentration on the Activity of PFK. The optimal P, concentration lies between 1 and 5 mm at both 0.8 and 3 mm F6P, but P, above 30 mm inhibits with 3 mm F6P. Near the optimal P, concentration, the effect of F6P concentration is greatly reduced (Fig. 3).

The Effect of Infiltration of P, on the Rate of Respiration of Preclimacteric Tomato Fruits. Respiration decreased rapidly after infiltration of water, followed by a small rise which slowly returned to the initial rate after about 40 hr (Fig. 4a). Infiltration with 1 mm DNP caused a similar decrease and return to the initial rate as with water, but the rate of respiration...
then increased further to a maximum after about 20 hr, followed by a decline for the remainder of the experiment (Fig. 4b). Return to the initial respiration rate was less rapid (4 hr) after infiltration with 0.7 m KCl, but the subsequent doubling of the respiration rate was extremely rapid, being complete within a further 3-hr period (Fig. 4c). Immediately after the peak was reached, the respiration rate declined to the initial rate after approximately 40 hr. After infiltration of 0.7 m phosphate buffer, pH 7.5, the rate of respiration remained below the initial level for 8 hr (Fig. 4d), then increased to approximately 50% above the initial rate. After reaching a peak 15 hr after infiltration, the rate declined but leveled off 20% higher than the initial rate for the remainder of the experiment. A similar result was obtained when this experiment was repeated.

The Relation between P, Concentration and the Respiration Rate at the Climacteric Maximum. The P, concentration and the respiratory rate at the climacteric were highly correlated (F = 19.34, p = 0.1%). Regression line A (Fig. 5) is calculated from the data obtained directly from the experiment. Regression line B includes additional data using similar plants grown in soil with optimum nutrients.

**DISCUSSION**

Figure 1 shows that the climacteric begins between the MG and PB stages. The commencement of the respiratory increase is accompanied by a crossover at PFK (Fig. 6). Between the

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**Fig. 1.** Respiratory rate at 20 C and concentration of intermediates of the EMP pathway in tomato fruits harvested at eleven stages of maturity (experiment 2).

**Fig. 2.** Respiratory rate at 20 C and concentration of ATP in tomato fruits harvested at five stages of maturity (experiment 1).

**Fig. 3.** The effect of P, concentration on PFK activity at 0.8 mM F6P (●) and 3 mM F6P (■).
stages of PB3 and B3, a crossover occurs as P3, while the rate of respiration is still increasing. Thus the increase in respiration is due in part to the sequential activation of glycolysis, initiated by the activation of PFK, and the reasons for involving P1 in this stimulation have already been discussed. The increase in ATP (Fig. 2) appears to be late to stimulate PFK. A similar increase in ATP occurs after the onset of the climacteric in fruits of other species (27, 28, 34, 35). Pyruvate kinase extracted from plants is activated by monovalent cations and Mg2+ (23) but does not readily respond to effectors active on the animal enzyme (FDP, ATP, and P1) (2). Again, increasing concentration of K+ or Mg2+ in the cytoplasm through increasing permeability of the tonoplast could activate this reaction, but would lead to simultaneous rather than sequential activation of PFK and PK. A more likely explanation is that PK is stimulated by ADP produced in the PFK reaction.

The climacteric does not have the properties of a finely regulated respiratory flux because the changes in levels of metabolites are from one steady state to another rather than continuous fluctuation (Fig. 1), and therefore it is reasonable to propose that it is the effect of release of a large pool of a single effector such as P1. Results of the infiltration experiments, while not conclusive, support this contention. The initial decrease in respiration after infiltration, also observed by Abdul-Baki et al. (1) and Spencer (31), is probably due to evacuation of CO2 from the vascular tissue and intercellular spaces of the fruit. Recovery from this decrease in respiration varies with the treatment, being rapid with water and DNP (3 hr), slower with KCl (4 hr), and requiring 12 hr after P1 treatment. The treatment with 1 mM DNP is similar to that used by Spencer (31) and Abdul-Baki et al. (1), and produced a similar response in respiration in this experiment. The lag following infiltration of phosphate buffer is probably due to P1, rather than cations, since recovery after KCl infiltration is more rapid. Salt at 0.2 M inhibits respiration (24) and, since 0.7 M KCl was infiltrated, we would expect some inhibition of respiration until the KCl solution was diluted by diffusion within the fruit. The cation concentration in the phosphate buffer was somewhat higher, but, in addition, P1 higher than 30 mM inhibits PFK (Fig. 3) and thus the infiltrated P1 would take longer than KCl to be diluted below inhibitory concentration. The increase in respiration following KCl infiltration could be "salt" respiration or stimulation of pyruvate kinase by K+.

In these infiltration experiments, the most important evidence that P1 may be limiting respiration in preclimacteric fruit comes not from the rise in respiration following the initial inhibition, but rather from the observation that the respiration rate of the P1-treated fruit does not return or begin to return to the basal rate after the rise, while it does so in all other treatments. If it is assumed that the mature green fruit

Fig. 4. The effect of treatment by vacuum infiltration on the rate of respiration (26°C) of mature-green tomato fruits. Treatments (all 0.3 ml per fruit): a: Water; b: 1 mM DNP; c: 0.7 M KCl; d: 0.7 M KNaHPO4 pH 7.5.

Fig. 5. The correlation between rate of respiration (20°C) of tomato fruits at the climacteric maximum with the concentration of total P1 in the fruits at the end of the experiment. ♦: Fruits from plants grown for the experiment at various levels of P1. Regression line A is calculated from these observations only. (F = 19.34, p = 0.1%). ■: Fruits from plants grown in later experiments at an optimum level of P1. Regression line B is calculated from all observations.
is potentially climacteric, lacking perhaps only one factor, one would expect, if the treatment was to simulate the climacteric, a relatively permanent increase in the respiration rate. Of the treatments investigated only $P_1$ had this characteristic, the other treatments producing only transitory rises. Abdul-Baki et al. (1) using similar methods, concluded that $P_1$ was not limiting the preclimacteric respiration rate of tomatoes; however, according to our data, their experiments lasted only during the period of PFK inhibition (10 hr), not long enough to observe stimulation of respiration.

The stimulation of respiration following infiltration of $P_1$ suggests that the respiration rate of preclimacteric fruit is limited by $P_1$. Furthermore, the changes in respiration rate following infiltration indicate that the concentration of cytoplasmic $P_1$ is higher at 25 hr than after 30 hr. This is consistent with the model proposed for distribution of $P_1$, in that one might expect a higher cytoplasmic concentration to precede, briefly, the final cytoplasmic concentration when equilibrium between cytoplasm and vacuole was established. If $P_1$ infiltrated into the fruits does stimulate respiration by activating PFK, this is consistent with a substantial nonmetabolic pool of $P_1$ in the cell, since the endogenous concentration of $P_1$ in the fruit is 2.5 mM, almost optimal for stimulation of PFK (Fig. 3).

In fruits grown at different levels of P and harvested at the MG stage, the respiration rate at the climacteric maximum is highly correlated with P, (Fig. 5). We must stress that this correlation is not similar to that between P and respiration in fruits harvested at different stages of maturity (Fig. 1), since the increase in P between stages GI and PB is due to import from the vine. Since fruit harvested at the MG stage ripen normally, this uptake cannot be proposed as the major source of increased cytoplasmic P, giving rise to the climacteric. However, the total amount of P, imported could determine the extent of the climacteric rise when diffuse resistance falls. In apples, the extent of the rise is a function of the time the fruit is left on the tree after the onset of the climacteric (25).

On the other hand, the data presented in Figure 5 were obtained from fruits harvested at the MG stage, which would preclude accumulation from the vine. Further, P, was extracted from these fruits after the respiratory peak had passed (CR stage) and therefore all were equivalent with respect to any changes that may have occurred after harvest. It is this $P_1$ concentration that is plotted against the climacteric maximum respiration rate, and since the variation in $P_1$ concentration was the result of preharvest treatments, respiration is more likely to be a function of $P_1$ concentration than the reverse.

The following model for the control of respiration preceding and during the climacteric in ripening tomatoes is proposed. At vacuolation, $P_1$ is accumulated in the vacuole, thus depleting cytoplasmic $P_1$, and hence causing the decline in respiration observed by Gustafson (17) at this time. The pool of nonmetabolic $P_1$, so formed would then remain inactive while the membrane system of the fruit cell maintains its integrity, and the low concentration of cytoplasmic $P_1$ would continue to control the respiration rate until the onset of ripening. The permeability changes that accompany ripening (6, 29, 30) release the accumulated $P_1$, in the vacuole to the cytoplasm, whereupon at least one (PFK) and possibly more enzymes of respiration are activated. Vacular accumulation of $P_1$ could be an ubiquitous mechanism of respiratory control in storage tissues, exerting control at a cellular level, overriding more delicate control in the cytoplasm.

LITERATURE CITED