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ESTERIFICATION OF PHOSPHATE IN RIPENING FRUIT[1]

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The biochemical evidences of changes which mark the physiological development of fruit are associated with distinct periods in the life-history of the fruit. These periods are: 1) cell division; 2) cell enlargement; 3) maturation; 4) "autogenous climacteric"; and 5) senescence. The climacteric (4) refers to a respiratory pattern observed in many fruit following maturation, which is characterized by a sudden, sharp increase in respiratory rate to the "climacteric maximum." This maximum is reached by some kinds of fruit, while attached to the plant, or by other fruit as a post harvest phenomenon. The period of senescence, which terminates the development of all fruits, precedes final deterioration and decay of the natural product.

Robertson and Turner (7) proposed that the occurrence of limited respiration during the pre-climacteric period of fruit development may be due to the availability of phosphate acceptors. Thus, these workers implicated a controlling role for the process of oxidative phosphorylation during the later periods of fruit ripening. Pearson and Robertson (6) and Millerd, Bonner, and Biale (5) demonstrated that 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, increased respiration in pre-climacteric fruit (apple slices and avocado slices), but had no effect on fruit at climacteric and post climacteric stages. Analogous to the effect of DNP on avocado slices is the effect of naturally occurring uncouplers isolated from climacteric avocados by Millerd et al (5). These substances depressed P/O ratios of isolated mitochondria, and caused a corresponding increase in respiration. In view of these effects on respiration in both pre-climacteric fruit and isolated mitochondria, the hypothesis was proposed (5) that fruit ripening results from an uncoupling of oxidative phosphorylation.

In view of the evidence cited above, it was anticipated that a measurement of the status of phosphate esterification in intact fruit during the sequence of stages in its life-history would show whether a loss of phosphorylative capacity did, in fact, precede ripening. The ripening process is considered, in this paper, to embrace the periods of climacteric and senescence.

Measurements of the incorporation of radioactive phosphate into organic phosphate esters has shown significant esterification by tomato fruit during maturation, post climacteric, and senescent periods. It was possible to prevent phosphorylation by the uncoupling action of dinitrophenol, but complete disappearance of phosphorylative capacity did not occur naturally until the termination of senescence (apparent in tomato fruit from deep red skin coloration and the soft, structural condition of a completely ripened fruit). These findings, therefore, have established the occurrence of phosphorylation in whole fruit beyond the climacteric maximum of respiration and well into the post climacteric period of fruit ripening. Quantitative information concerning the phosphorylative capacity of tomato fruit are presented in this paper as a basis for the evaluation of the role of phosphorylation in the ripening processes of fruit.

MATERIALS AND METHODS

Tomato fruit were used for the experiments reported here. These were obtained from local gardens and from greenhouse plants (Ohio Wilt Resistant) grown under the supervision of the Ohio State University Department of Horticulture. Solutions used for the various treatments were injected into the locules of the tomatoes by means of a hypodermic syringe fitted with a two-inch needle. All incubations were at room temperature. Bruising injury was produced by rolling the fruit (under some mechanical pressure) prior to injection of labeled phosphate. Care was taken not to puncture the skin of the fruit during this treatment. After incubation, the fruit was sectioned and extracted by grinding in a mortar with sufficient trichloroacetic acid to bring the final concentration to 3 %. The clear supernatant fraction ob-

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tained by centrifugation was spotted on S and S No. 589 filter paper and the phosphorylated compounds chromatographed according to the procedure of Bandurski and Axelrod (2). Autoradiographs of these chromatograms were obtained on Eastman No-screen x-ray film. Quantitative determinations of the distribution of the labeled phosphates were made by cutting out the discrete spots indicated by the autoradiographs and assaying for radioactivity with an end window (1.4 mg/cm) Geiger-Müller tube and standard scaling circuit.

Experimental Results

Esterification Rate in Intact Fruit: Radioactive phosphate was injected into whole tomato fruit (ca 100 gm) at a rate of 0.5 ml phosphate (0.01 M) containing 100 microcuries of radioactivity. The uptake of inorganic phosphate proceeds rapidly, and reaches a steady state equilibrium in less than 24 hours (fig 1). All of the phosphate esters appear at about the same rate, and there is no observable tendency for the accumulation of large quantities of any single ester.

Phosphorylation Capacity of Intact Fruit: The capacity of tomato fruit for the incorporation of P^{32}-orthophosphate into organic esters was measured over the entire range of physiological ages. Size and skin color were a useful index of age, with climacteric respiratory behavior just preceding the onset of pink color in the fruit. Maximum esterification of P^{32}-phosphate was observed in green fruit, early in maturation, and likewise, was maximal in full-sized green, mature fruit at the "climacteric maximum" of respiration. The P^{32}-esterification curve for post climacteric fruit (fig 2) parallels the sharp decline in respiration

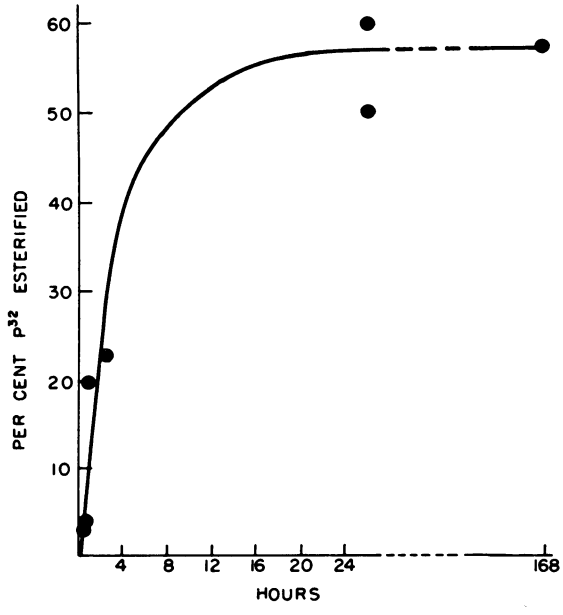

Fig. 1. Rate of phosphate esterification in whole fruit.

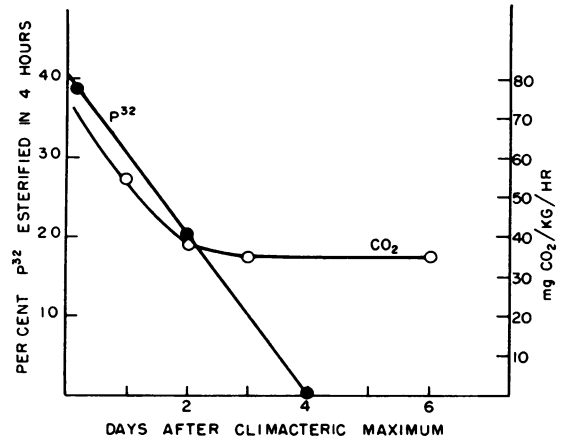

Fig. 2. P^{32} esterification and respiration (CO_2 evolution) of post climacteric tomato fruit. CO_2 data from Alban (1).

(CO_2 evolution) during the early post climacteric period. It would appear, therefore, that the early post climacteric decline in phosphorylative capacity is not the result of an uncoupling of oxidative phosphorylation. However, in late post climacteric stages, phosphorylative capacity completely disappears.

Sensitivity of Phosphorylation to DNP: A complete disappearance of phosphorylation pattern occurs when DNP, sufficient to produce a concentration of 10^{-4} M in the treated portion, is injected with P^{32}-orthophosphate into whole tomato fruit. The typical phosphorylation pattern shown in figure 3 A shows a phosphate ester component which moves at 54 % of the rate of inorganic phosphate (methanol–formic acid–water (2)). This has been tentatively identified as glucose-6-phosphate by co-chromatography with authentic glucose-6-phosphate in several solvents, but no attempt has been made to identify the other components. Figure 3 B shows the pattern obtained after DNP-treatment. No P^{32} component other than orthophosphate can be detected in the extracts of DNP-treated green or pink-green fruit, or in extracts of fully ripe fruit injected with P^{32}-orthophosphate alone (fig 3 C). The complete absence of any component in the TCA extract of DNP-treated fruit which could be attributed to substrate level phosphorylations probably indicates a rapid utilization of such esters which prevents their detection by the procedures used.

Effect of Bruising Injury on Phosphorylation Capacity of Both Pre-climacteric and Climacteric Green Tomato Fruit: A loss of phosphorylation capacity can also be induced by bruising injury (fig 4). The loss may be partial or complete, depending upon the severity of the bruise and the time elapsed after bruising. Complete loss of phosphorylative capacity occurs in a few hours, even though the bruised area is restricted to one part of the fruit.

Loss of Phosphorylative Capacity after DNP-Treatment: Inasmuch as fruit injected simultaneously with P^{32}-phosphate and DNP produced no de-

tectable organic phosphate ester, several green, mature fruit were labeled initially by injecting with P^{32}-phosphate and incubating for three days. DNP was then injected into one locule of each fruit, and the fruit extracted after 1, 4 and 24 hours to measure the rate of disappearance of P^{32} esters. Separate extracts were made for the tissue in direct contact with the DNP added and that tissue which was not in contact with DNP. The rate of disappearance of esters was identical in both extracts (fig 5). It appears, therefore, that bruising injury and DNP treatment are similar in that both cause a loss of phosphorylative capacity throughout the entire fruit even though the treatments are localized. These data indicate that both DNP and bruising bring about the release (or production and release) of a diffusible substance which results in the uncoupling of oxidative phosphorylation. These effects are summarized in figure 6.

Effect of DNP on Normal Ripening: In view of the uncoupling action of dinitrophenol on the phosphorylation of green, mature fruit, it became of interest to determine the character of ripening in a DNP-treated fruit. If the observed incorporation of P^{32}-phosphate into esters provides a source of energy required to carry out the ripening process, including degradation of chlorophyl and formation of red pigments (lycopenes), a green fruit uncoupled with DNP would be expected to remain green indefinitely. Green mature fruit were harvested, injected with DNP, and allowed to incubate for several weeks at room temperature. Control fruit ripened normally, during this period and developed the deep red color of fully ripe fruit. In contrast, DNP-treated fruit were essentially unchanged in appearance from the green, freshly harvested product. The requirement for an energy source, in the form of oxidative phosphorylation, would appear, therefore, to be a necessary factor for the successful achievement of "ripening."

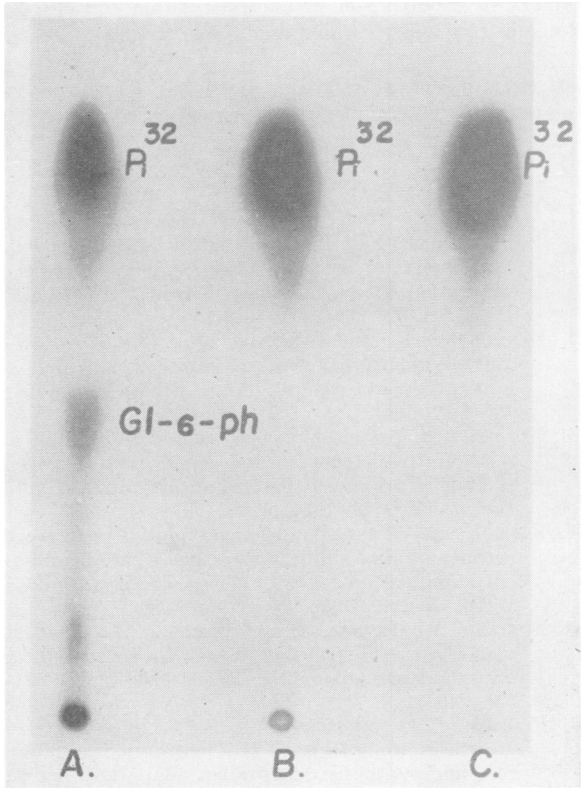

Fig. 3. Autoradiograph of a paper chromatogram of TCA extracts of tomato fruit. A. Extract of green, mature tomato fruit, injected with P^{32}-orthophosphate. B. Extract of green, mature tomato fruit, injected with P^{32}-orthophosphate plus DNP. C. Extract of fully ripe, tomato fruit, injected with P^{32}-orthophosphate.

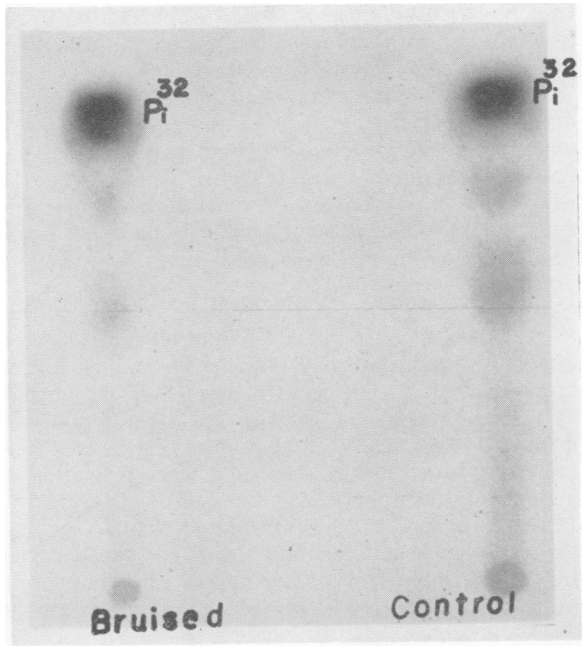

Fig. 4. Autoradiograph of chromatogram of TCA extracts of green mature tomato fruit. *Left.* Extract of bruised fruit, injected with P^{32}-orthophosphate. *Right.* Extract of normal fruit, injected with P^{32}-orthophosphate.

Discussion

The concept of the ripening process in fruit which emerges from these studies is one of dynamic processes continuing through a major part of the post climacteric decline. Thus, the senescing fruit actively produces energy (as phosphate esters) and presumably is still carrying on biochemical work and biochemical syntheses. The correlation, made by previous workers, of respiratory increases and natural uncoupling would be invalidated by the existence of dinitrophenol-sensitive phosphorylation at climacteric maximum. Rather, the suggestion advanced by Hulme (3), that climacteric may be induced by increased availability of ADP provided by systems synthesizing new protein (enzymes), would be more consistent with our observations. Further evidence of an efficient oxidative phosphorylation system in climacteric

fruit has been obtained by Biale (unpublished) in recent studies with mitochondria prepared from avocado in different physiological stages.

Inasmuch as the inhibition of phosphorylation with dinitrophenol prevents fruit at the climacteric maximum of respiration from ripening normally, the energy required for ripening probably depends upon the oxidative phosphorylation observed in post climacteric fruit. As ripening is completed, phosphorylative capacity can no longer be detected.

Summary

The occurrence of phosphate esterification has been established, by means of P^{32} tracer techniques, for intact tomato fruit during early periods in maturation (pre-climacteric), and likewise has been established during the post climacteric period of senescence. The failure of fruit to ripen normally, when formation of high-energy phosphate esters is inhibited, either by DNP-treatment or bruising injury, further sustains the apparent requirement for oxidative phosphorylation by ripening fruit. The role of phosphorylation in fruit ripening would thus seem to point toward the existence of energy requiring syntheses, which are essential to the completion of ripening by senescing fruit.

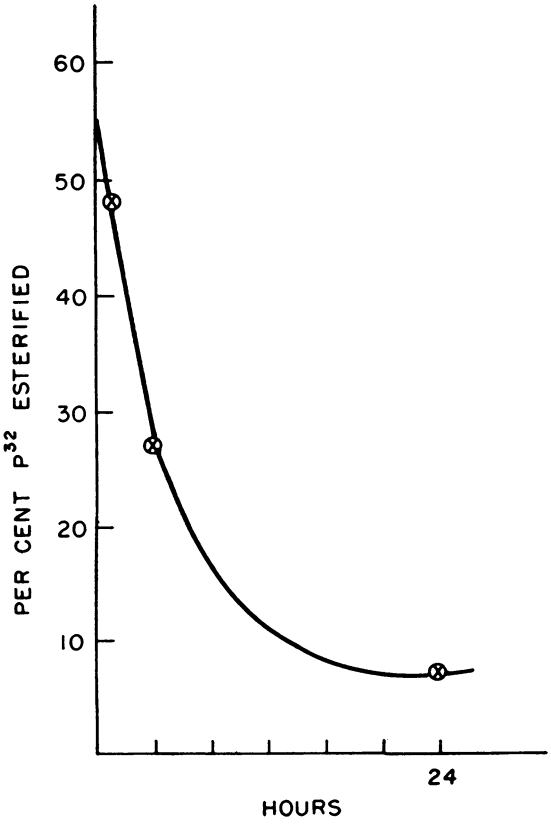

Fig. 5. Rate of disappearance of P^{32}-phosphate esters after DNP-treatment. Circles indicate rate in portions of fruit in direct contact with injected DNP. Crosses indicate rate in portions which contained no visible traces of injected DNP.

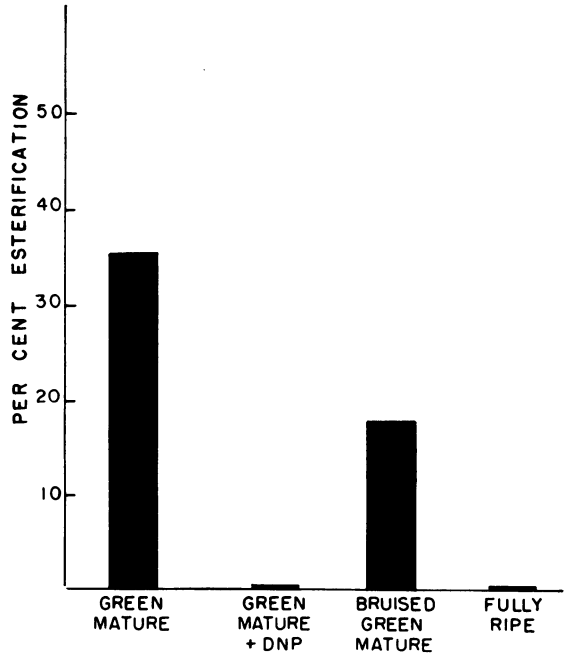

Fig. 6. Comparison of phosphorylation capacity by tomato fruit under various conditions.

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