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Comparison of Mitochondria from Tomato Fruits at Various Stages of Ripeness[1]

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The properties of mitochondria from ripening fruits have been studied by several workers in an effort to account for the climacteric rise in respiration (8, 15, 20). The climacteric rise in apple and avocado fruits does not seem to be accompanied by uncoupling of oxidative phosphorylation or the loss of respiratory control (8, 20). Mitochondria from climacteric avocados oxidize α-ketoglutarate, pyruvate, and malate less rapidly than mitochondria from preclimacteric fruits, but the rate of succinate oxidation remains about the same (15). A soluble supernatant factor restored the oxidation of α-keto acids to preclimacteric rates.

Only limited information is available where tomato fruits are concerned. Lyons, et al. (12) recently reported that mitochondria from immature tomatoes demonstrated greater swelling than mitochondria from dark pink fruits. Oxidative activity was low, and phosphorylation was not measured. In postclimacteric tomato fruits the rate of phosphate esterification declines rapidly and reaches zero on the fourth day past the climacteric peak (13). However, high-energy phosphates, predominantly ATP, are present at all stages of ripeness from mature green to soft ripe (16). It has not been reported whether isolated mitochondria from postclimacteric tomatoes retain the ability to phosphorylate.

In connection with studies of enzymatic processes associated with ripening, we felt the need for more detailed knowledge of respiration and phosphorylation by tomato mitochondria. In this paper we report isolation of actively phosphorylating tomato mitochondria and a comparison of mitochondria from fruits at various stages of ripeness.

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Materials and Methods

Tomato fruits (*Lycopersicon esculentum* Mill., var. Kc109) were picked at the mature green stage or at the turning stage (pink at stylar end) and held at 20°. Mature green fruits were used within 1 day, and turning fruits were held until the desired degree of ripeness was attained. Mature green fruits at 20° required about 5 days to reach the turning stage and another 3 days to reach the climacteric maximum (A. Abdul-baki, unpublished data). The fruits were red ripe and soft by 14 days after turning. Fruits were harvested on a schedule providing several stages of ripeness for each experiment.

The fruits were sliced and the highly acid locular contents (14) were discarded. All subsequent steps were done at 0 to 4°. The tissue was thoroughly homogenized in a mortar with a grinding medium (1 ml grinding medium/g tissue) containing 0.5 M sucrose, 0.01 M EDTA, 0.5 M KH_2PO_4 and 0.5 M Tris. The pH of the grinding medium was adjusted with KOH or H_3PO_4 to give the desired pH in the homogenate (pH 7.5 except for experiments in table III). The high buffer concentration in the grinding medium was needed to neutralize the fruit acids. The homogenate was strained through cheesecloth and cleared of cell debris by centrifugation at $1,000 \times g$ for 5 minutes. The mitochondria were sedimented at $20,000 \times g$ for 15 minutes, washed once in 0.5 M sucrose, and suspended in 0.5 M sucrose. Nitrogen content of the suspension (18) provided an estimate of the amount of mitochondria. Protein content of the mitochondrial and soluble fractions was determined by the method of Lowry, et al. (11).

Heavy and light mitochondrial fractions were obtained by first centrifuging the strained homogenates at $4,600 \times g$ for 5 minutes. The resulting supernatant fluid was centrifuged at $20,000 \times g$ for 15 minutes to sediment the light fraction. The heavy fraction was obtained from the $4,600 \times g$ pellet by suspending the pellet in 0.5 M sucrose, centrifuging at $500 \times g$ for 5 minutes to remove debris, and then sedimenting the heavy mitochondria with $20,000 \times g$ for 15 minutes.

Oxygen consumption was measured manometrically (18) at 30° with air as the gas phase, and phosphorylation was determined from the disappearance of P_i (5). Duplicate Warburg flasks were used for each treatment. Each flask contained 300 μmoles sucrose, 100 μmoles glucose, 2.5 μmoles $MgSO_4$, 50 μmoles KH_2PO_4, 20 μmoles potassium pyruvate, 40 μmoles l-malic acid, 0.2 mg thiamine pyrophosphate, 0.4 mg NAD, 1.5 mg ATP, 0.1 mg coenzyme A, 20 K.M. units of hexokinase (all biochemicals from Sigma Chemical Company), and 0.5 ml mitochondrial suspension. The total volume was 2.5 ml and pH was 7.5. These flask contents were used in all experiments unless otherwise specified. The malate-pyruvate substrate and cofactors were included in the reaction mixture to obtain maximum mitochondrial activity and to provide substrates similar to those available in vivo. Tomato fruits contain much malate (2), and pyruvate would be produced by glycolysis. Preliminary experiments showed malate oxidation was greatly enhanced by the presence of pyruvate.

Malic dehydrogenase activity was measured spectrophotometrically (7). The reaction mixture contained 0.15 μmole NADH, 0.76 μmole oxalacetic acid, 69 μmoles Na_2HPO_4, 1.5 mmoles sucrose, and 0.010 ml mitochondria in a final volume of 3 ml, pH 7.5. The reaction took place at 25° and was initiated by adding oxalacetate. One unit of enzyme caused a decrease in OD of 0.01 per minute at 340 mμ. Enzyme activity was calculated from the change in OD between 30 and 60 seconds after starting the reaction.

In the digitonin treatments, a weighed quantity of digitonin (Nutritional Biochemicals) was dissolved in 0.5 ml (0.2–0.3 mg N) of mitochondria using a chilled teflon homogenizer. Other chemicals were polyvinyl pyrrolidone (PVP) from Calbiochem, and bovine albumin, fraction V powder from Nutritional Biochemicals.

Results

After methods for isolating active tomato mitochondria were developed, mitochondria were isolated from fruits at 4 stages of ripeness. As shown in table I ripening and senescence were accompanied by a progressive decline in capacity to oxidize malate and pyruvate. Uncoupling of oxidative phosphorylation did not occur however. The P/O ratio remained unchanged except for a small decrease at 14 days

Table I. *Changes in Mitochondrial Activity and Protein Content During Ripening*

The values are means of 4 experiments.

Stage of ripeness	$Q_{O_2}(N)$	P/O	Protein content		Mitoch./Sol
			Mitoch.	Soluble*	
			(mg/100 g fr wt of tissue)		
Mature green	1740	2.31	17.9	29.5	0.61
3 days past turning	1590	2.43	8.3	23.5	0.35
7 days past turning	1180	2.28	7.0	21.9	0.32
14 days past turning	916	2.00	6.0	19.2	0.31

* Protein content in $20,000 \times g$ supernatant fraction.

Table II. *Distribution of Mitochondria in Mature Green and Ripe (7 Days Past Turning Stage) Fruits*
These values are means from 3 experiments.

	Green		Ripe	
	Heavy fraction	Light fraction	Heavy fraction	Light fraction
$Q_{O_2}(N)$	1271	2854	495	2282
P/N*	253	607	82	430
P/O	2.25	2.38	1.91	2.11

* μmoles P_i esterified/hr mg mitochondrial N.

after turning. During ripening the yield of mitochondrial protein declined more rapidly than did the yield of soluble protein. This result indicates degradation of the particulate fraction of the cells during ripening.

Similar experiments were conducted using succinate (40 μmoles/flask) as substrate instead of malate. The Q_{O_2} (N) values for mature green and 7-day preparations were 1180 and 790 respectively, with P/O ratios of 1.7 and 1.4. Thus, tomato mitochondria differ from avocado mitochondria which do not show a decline in rate of succinate oxidation (15).

The activities of heavy and light mitochondrial fractions are given in table II. The higher Q_{O_2} (N) shown by light mitochondria was probably due to decreased contamination by plastids. However, both fractions from ripe fruits showed less oxidative activity than the corresponding fractions from mature green fruits.

A decline in membrane permeability or dehydrogenase activity during ripening could account for the lower oxidative activity of ripe preparations. Accordingly, malic dehydrogenase activity was measured in mitochondrial preparations from mature green and 14-day fruits. There was little difference between green and ripe preparations (83 and 81 units/mg protein, respectively), and no differences appeared on release of enzyme by rupturing with digitonin. A low concentration of digitonin (10 mg/ml) resulted in specific activities of 400 units per mg protein in the green and 410 units per mg protein in the ripe. A higher concentration of digitonin (40 mg/ml) gave 500 units per mg protein in the green and 530 units per mg protein in the ripe. Hence, there was no detectable loss of enzyme or decrease in membrane permeability during ripening.

The presence of inhibitors in homogenates from ripe fruits was investigated by mixing equal volumes of homogenates from mature green and 7-day fruits. Mitochondria were isolated from green and ripe homogenates and from the mixture. The $Q_{O_2}(N)$ of the mixture (2470) was approximately that expected (2360) based on the activity of the green (2740) and ripe (1650) preparations and their mitochondrial content. The P/O ratios were not affected.

The isolation medium was supplemented with several substances reported to increase mitochondrial activity. The addition of 5% polyvinyl pyrrolidone (9), 5% bovine serum albumin (17), or 0.5% cysteine (3) did not increase mitochondrial activity, and the differences between green and ripe were not affected.

The effect of isolation pH on mitochondrial activity was investigated because low pH during isolation has an adverse effect on mitochondria from 5-day corn scutella (6). The effect on corn mitochondria is due to binding of inhibitory proteins. The effects of isolation pH on mitochondrial preparations from fruits which were mature green and 14-day are given in table III. The pH had little effect on green preparations, but ripe preparations were extremely sensitive to pH 6.5. The effect was not due to increased extraction of nitrogen at the low pH which might be expected on the basis of results from isolation of apple mitochondria (10). Isolation of ripe mitochondria at pH 8.4 did not give significantly greater oxidative activity than isolation at pH 7.5, and phosphorylation was adversely affected by the higher pH.

Table III. *The Relation Between pH of the Isolation Medium and Mitochondrial Activity*
These values are means of 3 experiments.

	$Q_{O_2}(N)$	P/O	Yield of mitochondrial N (μg/ml clarified homogenate)
Mature green			
pH 6.5	2270	2.36	19.6
pH 7.5	2500	2.44	20.9
pH 8.4	2350	2.46	20.5
Ripe			
pH 6.5	240	1.25	5.5
pH 7.5	644	2.40	5.5
pH 8.4	773	2.04	4.3

When ripe mitochondria isolated at pH 6.5 were washed in 0.5 M sucrose at pH 8.5 there was a slight increase in activity, but the sucrose wash was not inhibitory to green fruit mitochondria.

DNP (2,4-dinitrophenol) at concentrations ranging from 10^{-6} to 10^{-3} M was applied to mitochondria from green and ripe fruits to determine if respiration could be increased by uncoupling. DNP concentrations of 10^{-4} M or higher were required to uncouple phosphorylation. DNP concentrations which uncoupled phosphorylation did not stimulate respiration; only inhibition was observed.

An external source of phosphate acceptor was required to obtain maximum rates of oxidation by green and ripe preparations. Omitting hexokinase and ATP from the standard flask contents reduced the $Q_{O_2}(N)$ of a mature green preparation 65 % (from 2300–810), and $Q_{O_2}(N)$ of a ripe preparation (7 days after turning) was reduced 51 % (from 1310–650). P/O ratios in the green and ripe were reduced to zero from 2.4 and 1.7, respectively.

Polarographic (4) measurements of respiration showed that the method of preparation described resulted in mitochondria lacking respiratory control. Rates of malate and succinate oxidation were stimulated by addition of ADP, but state 4 rates were not subsequently obtained. Mitochondria isolated from mature green fruits by methods similar to those of Wiskich and Bonner (19) exhibited respiratory control ratios as high as 2.7, but the quantity of mitochondria was so small that the method is not yet satisfactory.

Discussion

During ripening and senescence there was a pronounced decline in the capacity of tomato mitochondria to oxidize substrates, but coupling of electron transport to phosphorylation was relatively unaffected. These results support the observation of Rowan, et al. (16) that high-energy phosphates were present throughout ripening. Therefore the events associated with ripening do not result from a lack of metabolic energy. The isolated mitochondria were extremely active, in some instances giving P/N values exceeding 600 and P/O ratios as high as 2.7. The post-climacteric decline in whole fruit respiration parallels the declining oxidative activity of isolated mitochondria (1). However, no parallel to the climacteric rise can be found with isolated mitochondria.

The cause of the observed decrease in oxidative activity has not been determined. It is not due to a lower concentration of malic dehydrogenase. The specific activity of malic dehydrogenase was the same in intact mitochondria from green and ripe, indicating that permeability changes are not involved. The lowered $Q_{O_2}(N)$ is not due to an increased proportion of nonmitochondrial particulates. The decline in yield of mitochondrial protein indicates that degradation and not synthesis of particulates occurs during ripening; the malic dehydrogenase data show that there is no dilution of the mitochondria by nonmitochondrial particles.

Since DNP did not accelerate respiration, the passage of electrons through coupling sites does not seem to be differentially inhibited. The mitochondria show a level of dependence on phosphate acceptor comparable to most reported values, with the exception of some values for tightly coupled mitochondria (19). The lack of respiratory control in the preparations reported here may be due to the high buffer concentration used during isolation.

The finding that ripe preparations were strongly inhibited by isolation at pH 6.5 suggests that during ripening an inhibitor appears which binds to the mitochondria at low pH and is partially removed by isolation at high pH. However, we have not yet conclusively demonstrated the presence of an inhibitor. Fatty acids, polyphenols, or sulfhydryl groups are probably not involved since isolation with PVP, bovine serum albumin, and cysteine did not increase respiration rates of ripe preparations.

Summary

Mitochondria isolated from mature green tomato fruits are extremely active in oxidation and phosphorylation with malate and pyruvate as substrates. Oxidation rates decline during ripening, but P/O ratios are maintained. There is also a decrease in mitochondrial protein as a percentage of cytoplasmic protein, the greatest decrease occurring between the mature green and climacteric stages of ripeness. The decrease in $Q_{O_2}(N)$ was not due to declining malic dehydrogenase or the presence of inert nitrogenous compounds. Respiration rates were not increased by 2,4-dinitrophenol. Isolation with bovine serum albumin, polyvinyl pyrrolidone, and cysteine did not enhance activity. During ripening mitochondria became sensitive to isolation at pH 6.5, suggesting the presence of an inhibitor which binds or penetrates at low pH.

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Physiological Effects of Gibberellic Acid. VIII. Growth Retardants on Barley Endosperm [1, 2]

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Introduction

Several types of growth-retarding chemicals have been described in recent years. The first was 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride (Amo-1618) and some related compounds (7, 21). It was followed by (2-chloroethyl) trimethylammonium chloride (CCC or Cycocel) (19), tributyl-2, 4-dichlorobenzylphosphonium chloride (Phosfon D) (13) and by N-dimethylamino maleamic acid (C-011) and N-dimethylamino succinamic acid (B-995) (14).

These compounds differ somewhat in their effectiveness and the range of species which they affect. When they are active, however, they usually produce dwarfed plants with shortened, in extreme cases almost rosette-like stems, and dark-green, thickened leaves. These plants are essentially normal in other respects. The substances have therefore been called dwarfing agents or growth retardants. Their overall effect is the opposite of the growth effects produced by the gibberellins, and when a retardant and a gibberellin are applied together, the retardant effects may be overcome, resulting, at least in some cases, in normal growth (6, 16, 20). Some authors have therefore called the retardants antigibberellins, although

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