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Metabolic Processes in Cytoplasmic Particles of the Avocado Fruit. IX. The Oxidation of Pyruvate and Malate during the Climacteric Cycle[1, 2]

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Summary. Mitochondria isolated from preclimacteric avocado fruit oxidize pyruvate at a much lower rate than those separated from climacteric fruit. The external addition of thiamine pyrophosphate (TPP) increased the rate of pyruvate oxidation in both cases.

The study of the influence of TPP on the rate of oxidation of malate by mitochondria obtained from both preclimacteric and climacteric fruit indicated that the effect of this cofactor could be understood by assuming that malate was converted to pyruvate. TPP stimulation of malate oxidation was prevented by arsenite, an inhibitor of keto acid oxidation. The addition of glutamate increased the rate of malate oxidation through the transamination of oxaloacetate. This suggests that the rate of oxidation of malate is highly dependent upon mechanisms which remove oxaloacetate efficiently.

Incubation of mitochondria from preclimacteric fruit with malate-U-^{14}C resulted in the labeling of oxaloacetate and the accumulation of labeled pyruvate. Addition of TPP to this system induced the rapid formation of citrate. This conversion was completely inhibited by arsenite.

The results indicate that the ability to carry out the oxidative decarboxylation of α-ketoacids improves as the ripening process progresses. The idea was advanced that TPP available to the mitochondria plays an important controlling role.

Recent improvements in the techniques of isolation and assay of plant mitochondria have made possible the preparation of particles in a state where they retain a satisfactory degree of biochemical integrity (8, 23). Mitochondria which show respiratory control by ADP when oxidizing succinate, malate and α-ketoglutarate have thus been prepared from avocado fruit at different stages of ripeness, from the early preclimacteric conditions to the postclimacteric stage (11, 12, 15, 24). The opportunity opened up of following the variations in the oxidative abilities of the mitochondria during the overall ripening process. In the course of this study it was found that the rate of oxidation of malate was highly dependent on the stage of the climacteric of the fruit from which the mitochondria had been extracted. Moreover, the addition of thiamine pyrophosphate (TPP) to preclimacteric fruit particles in the presence of malate resulted in a considerable enhancement of the rate of oxidation of this substrate. At this stage of maturation the metabolism of α-ketoglutarate was also highly sensitive to the external addition of TPP (15), a situation which was not observed when the mitochondria were prepared from fruits reaching the climacteric condition.

[1] Preliminary reports of this work have appeared (10, 16).

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These observations prompted further research on the mechanism of action of TPP in increasing the rate of malate oxidation. This paper will also discuss the possible role of TPP as a controlling factor in the onset of the climacteric rise in respiratory activity of the avocado fruit during the ripening process.

Materials and Methods

Preparation of Mitochondria. Mitochondria were extracted from avocado fruit (*Persea gratissima*, Gaertn.), var. Fuerte, grown in southern California, according to a modification of a method of Wiskich et al. (24) which included the addition of a fatty acid poor bovine serum albumin at all stages of preparation and assays. This method has been published (11, 15) and was used without modification. In this study mitochondria from the yellow portion of the fruit were used exclusively because it was found (12) that this tissue yielded particles with better respiratory control than the green part of the fruit.

The fruit were kept in a dark room at 8° and used as early preclimacteric during 8 to 10 days after picking (11). For the climacteric stage they were transferred into respirometer jars at 20° and their O_2 uptake was followed continuously by means of a paramagnetic O_2 analyzer (25). Fruit were used when the respiratory rate reached the maximum intensity on the climacteric curve (4, 5).

Mitochondrial Assays. The oxygen uptake by mitochondria oxidizing malate or pyruvate was measured at room temperature using a Clark oxygen electrode as described previously (11, 15). The reaction medium in the cell was that of Wiskich et al. (24) to which a standard amount (0.75 mg/ml) of bovine serum albumin has been added.

The mitochondrial nitrogen was determined by the method of Thompson and Morrison (20) as adapted by Biale et al. (6). Due allowance was made for the presence of tris buffer and albumin in the medium in which the mitochondria were suspended.

Organic Acid Analysis. L-Malate-U-^{14}C was added to the reaction mixture and samples withdrawn after definite periods of oxidation in the presence of various compounds affecting malate oxidation. Organic acids derived from the oxidation of malate were determined by withdrawing 0.3 ml of the assay medium and adding it to 0.5 ml of methanol made 2 M in formic acid. After removal of the protein precipitate by centrifugation, 100 μl aliquot of the supernatant fluid was spotted on Whatman No. 1 filter paper and chromatographed using the upper phase of a solvent mixture consisting of *n*-butanol, formic acid and water, 1/1/1 (v/v). Radiochromatograms were made, the radioactive spots located on the paper, cut out and the radioactivity measured using a liquid scintillation counter. Pyruvate and oxaloacetate, which are not recovered quantitatively in this procedure, were separated as the phenylhydrazones of the α-ketoacids (7, 13). A sample of 0.3 ml of the reaction mixture was inactivated by addition to 0.5 ml of 47 % ethanol which was also 0.1 N with respect to HCl and contained 5 mM 2,4-dinitrophenylhydrazine. The formation of the hydrazones was allowed to proceed for half an hour after which the resulting mixture was extracted 3 times with 1 ml of chloroform-ethanol, 85/15 (v/v). The combined volumes were then extracted 3 times with a 10 % sodium bicarbonate solution. The resulting volume was acidified to pH 3 and extracted again 3 times with a mixture of chloroform-ethyl ether, 85/15 (v/v). The resulting extract was then evaporated to dryness and redissolved in 0.1 ml of propyl acetate. The phenylhydrazones of oxaloacetate and pyruvate were separated on Whatman 3 MM filter paper buffered at pH 8.0 with 0.05 M phosphate buffer, using the upper phase of the solvent mixture *tert*-amyl alcohol/ethanol/water, 50/10/40 (v/v). After separation of the hydrazones, radiochromatograms were produced and the radioactivity of pyruvate and oxaloacetate measured as previously described.

All organic acids were obtained from California Corporation for Biochemical Research. Uniformly labeled ^{14}C L-malic acid (15 mc/mmole) was obtained from Nuclear Chicago Corporation.

Results

Oxidation of Pyruvate. It is well known that the oxidation of pyruvate can proceed only if a catalytic amount of sparker acid is present in the reaction medium (1, 3, 17). TPP is an absolute requirement for the oxidative decarboxylation of pyruvate to acetyl CoA.

Figure 1 shows the influence of malate and of TPP on the oxidation of pyruvate by mitochondria extracted from climacteric fruit. These particles are normally able to oxidize malate and α-ketoglutarate at a high rate (11, 15). Curve A shows the oxidation of malate itself as a function of the concentration of this substrate. If pyruvate is also present in the medium (curve B) the rate of oxidation is only slightly higher than with malate alone. When TPP (curve C) is added to the reaction mixture the rate of oxidation of malate is markedly enhanced. Finally in the complete system (curve D) very small concentrations of malate (about 1 mM) were able to induce a high rate of oxidation of pyruvate, though malate itself at these concentrations was oxidized only very slowly. However, it should be noted that when substrate level concentrations of malate (about 10 mM) were present together with TPP (curve C), the rate of oxidation is very close to that of the complete system. If we assume that by some reac-

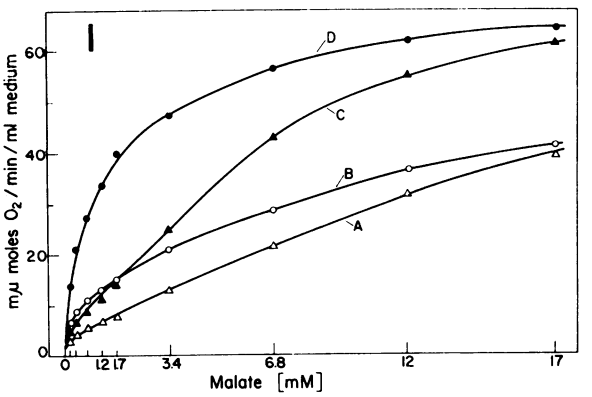

FIG. 1. Effectiveness of malate in promoting the oxidation of pyruvate by climacteric avocado mitochondria in the presence or absence of TPP. O_2 uptake measured polarographically in a final volume of 3 ml medium containing 0.25 M sucrose, 10 mM potassium phosphate buffer, 10 mM tris-HCl buffer, 5 mM magnesium chloride, 0.5 mM EDTA, 0.75 mg/ml bovine serum albumin and a sufficient amount of ADP to maintain a state 3 rate during the experiment. pH Adjusted at 7.20. Malate was added to the mitochondria in successive additions and the rate of oxidation recorded during a 2 minute period. The lowest concentrations of malate used are 0.17, 0.34, and 0.68 mM. Mitochondrial nitrogen: 75 μg/ml medium. A) oxidation of malate, B) medium containing malate and 17 mM pyruvate, C) medium containing malate and 133 μM TPP, D) containing malate, 17 mM pyruvate and 133 μM TPP.

tion pyruvate can be produced from malate, at high concentrations of malate the conditions in curves C and D are then much alike and similar results are to be expected. This hypothesis is also supported by the fact that at substrate level concentrations of malate (curve A) the rate of O_2 uptake is notably increased by TPP (curve C) and only slightly by pyruvate (curve B). These results suggest that TPP is the limiting factor for the oxidation of both malate and pyruvate.

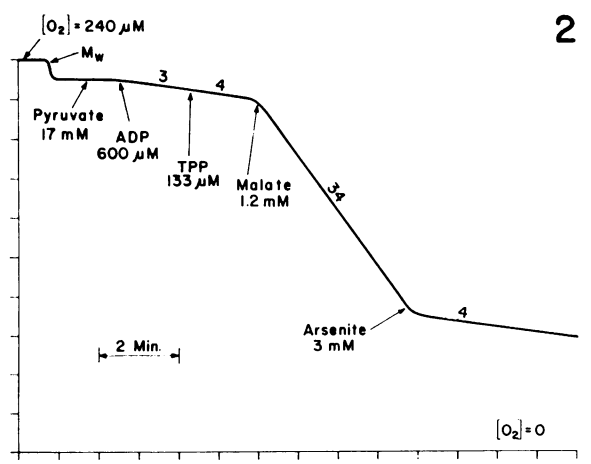

FIG. 2. Oxygen electrode trace representing the oxidation of pyruvate by preclimacteric avocado mitochondria. Same conditions as in figure 1. Additions are shown as final concentrations. The numbers on the trace represent rates of O_2 uptake expressed in mμmoles O_2 min^{-1} ml^{-1} medium. Mitochondrial nitrogen: 150 μg/ml medium.

In contrast to the activities of mitochondria obtained from climacteric peak fruit, particles from early preclimacteric avocados oxidized pyruvate only very slowly (fig 2). It is only when both TPP and sparker concentration of malate were present that pyruvate oxidation took place. A similar result was obtained when malate was added before TPP. Moreover, this figure shows that pyruvate oxidation was completely inhibited by arsenite which interferes at the lipoic acid level with the pyruvate oxidase complex (18). Other experiments have shown that the amount of TPP required in the medium to secure a high rate of pyruvate oxidation can be very small. As little as 2 to 5 μM TPP was sufficient, but in that case the response to the addition of TPP was not immediate.

It has also been found that the effect of TPP on the oxidation of malate and α-ketoglutarate by avocado mitochondria was highly dependent on the stage of maturation of the fruit used to prepare the mitochondria (11, 15). The ability of particles extracted from preclimacteric or climacteric fruit to oxidize pyruvate in the presence or absence of malate or TPP has thus been investigated and the results of this experiment are reported in table I. The 2 types of particles have very different abilities to oxidize malate, the climacteric particles showing about 3 times more activity than the preclimacteric ones. The most striking difference at substrate concentration of malate was produced by the addition of TPP which increased the rate of malate oxidation by more than 200 % for the preclimacteric but by less than 50 % for the climacteric particles. Pyruvate stimulated markedly oxygen uptake at the sparker concentration of malate but very little at the substrate level. When pyruvate and TPP were added together the presence of malate at sparker concentrations promoted greatly

Table I. *Oxidation of Pyruvate by Preclimacteric and Climacteric Avocado Mitochondria*

Same experimental conditions as in figure 1. Results are the averages of 4 different experiments in both cases. No significant oxygen uptake in the absence of malate was observed. Results are given for a sparker (1.2 mM) and for a substrate (17 mM) concentration of malate.

		μl O_2 hr^{-1} mg^{-1} N			
		Preclimacteric		Climacteric	
Addition	Malate (mM)	1.2	17	1.2	17
None		27	185	162	745
Pyruvate (17 mM)		68	190	264	740
TPP (133 μM)		95	500	262	1105
Pyruvate (17 mM) + TPP (133 μM)		222	720	620	1140

the oxidation of pyruvate by both types of particles. It thus appears that the mitochondria extracted from preclimacteric fruit have a restricted ability to oxidize malate and pyruvate and are more dependent on the addition of TPP than those isolated from climacteric fruit. The oxidation of α-ketoglutarate by preclimacteric mitochondria indicated a similar dependence on TPP (15).

Oxidation of Malate. In order to establish whether malate is converted to pyruvate, the antagonistic effect of TPP and arsenite has been assayed on mitochondria oxidizing malate. It is known (15) that when climacteric or even post-climacteric fruit particles oxidize malate, they show a very strong increase in the rate of oxidation in response to ADP with concomitant respiratory control. However, the reaction has particular features: A) the rate of oxidation decreases progressively as the reaction proceeds, and B) the state 4 rate is much lower than the initial rate of oxidation (before the addition of ADP), but after a short delay a normal state 4 rate is attained (fig 3A). This suggests that when the oxidation of malate proceeds at a high rate, an intermediate product is formed faster than it is removed and causes inhibition of the overall reaction. This assumption accounts for both the decrease in state 3 rate and the inhibited state 4. Oxaloacetate, the immediate product of malate oxidation, could be involved in the inhibitory effect observed (15, 23, 24).

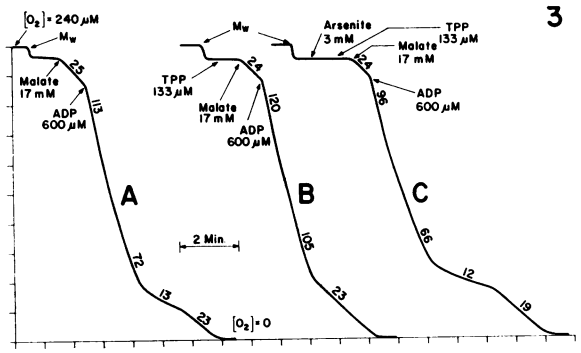

FIG. 3. Antagonistic effect of TPP and arsenite on the oxidation of malate by climacteric avocado mitochondria. Same conditions as in figures 1 and 2. Mitochondrial nitrogen: 105 µg/ml medium.

When TPP was present in the medium, the state 3 rate was more linear and the transient inhibited state 4 was suppressed (fig 3B). However, when arsenite was included at the same time (fig 3C), the effect of TPP was entirely abolished and the features of the reaction were very similar to the original one (fig 3A). Thus TPP was an effective agent in the removal of the inhibitory product arising from the oxidation of malate which could be caused to accumulate again by the addition of

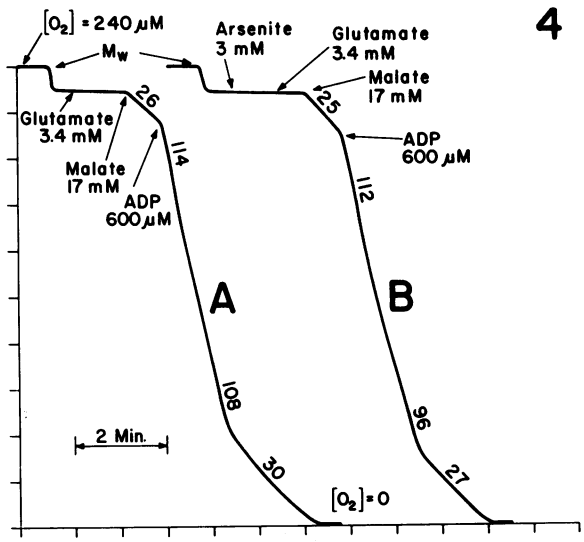

FIG. 4. Effect of glutamate on the oxidation of malate by climacteric avocado mitochondria. Same conditions as in figures 1 and 2. Same preparation as in figure 3. Mitochondrial nitrogen: 105 µg/ml medium. Trace A of figure 3 is the control trace for this figure.

arsenite. This experiment does not indicate whether the action of TPP is on a reaction involving oxaloacetate itself or on the reaction of a product of oxaloacetate.

The assumption that oxaloacetate is probably involved in slowing down the rate of malate oxidation is supported by the effect of glutamate on this system. Normally glutamate is oxidized only very slowly by avocado mitochondria (24). Using the same control trace as before (fig 3A), figure 4A shows that when a small concentration of glutamate was added to the medium the course of the reaction was very similar to that of figure 3B. It is assumed that under these circumstances, a transamination reaction between glutamate and oxaloacetate gave rise to aspartate and α-ketoglutarate and removed the inhibitory effect of oxaloacetate. As expected, arsenite had no effect in this case (fig 4B).

The study of the effect of TPP and arsenite on the oxidation of malate by preclimacteric fruit mitochondria which normally oxidize malate very slowly (table I) gives further support to the hypothesis of the formation of pyruvate during the reaction (fig 5). These particles do not respond to the addition of ADP (11, 15), but when TPP is added the rate of oxidation increases several-fold. The addition of arsenite brought the rate of oxidation down to the original value, whereas even in this complex system glutamate was still able to increase the rate of the reaction. Clearly, the rate limitation on the oxidation of malate may be removed by TPP or glutamate, which may serve to accelerate the depletion of accumulated oxaloacetate.

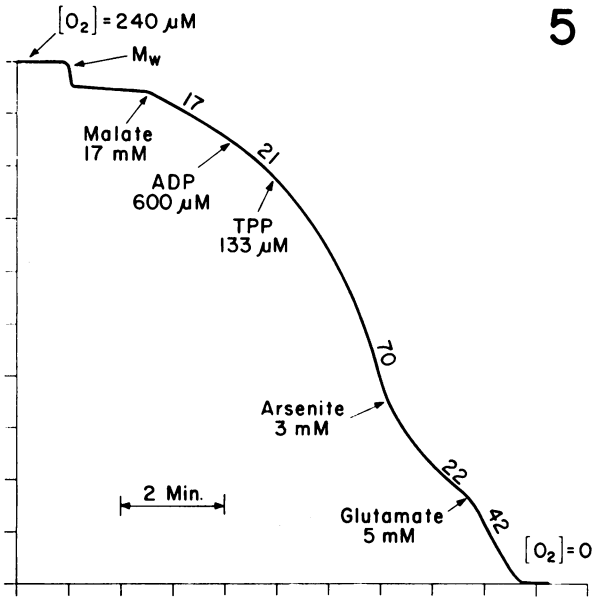

FIG. 5. Effect of TPP, arsenite and glutamate on the oxidation of malate by preclimacteric avocado mitochondria. Same conditions as in figures 1 and 2. Mitochondrial nitrogen: 150 μg/ml medium.

Oxidation of ^{14}C-Labeled Malate. The direct way to test the pathway of oxidation of malate was to perform experiments with labeled malate and identify the products of the reaction. Table II gives the results of such an experiment with preclimacteric avocado mitochondria conducted in a way similar to the experiment described in figure 5, except that it was performed in an open vigorously aerated vessel. Samples were withdrawn just after the addition of the mitochondria and after successive periods of oxidation in the presence of ADP, TPP, arsenite and glutamate.

The results show that label accumulated in both oxaloacetate and pyruvate. Calculated on the basis of specific activity in malate, the concentration of oxaloacetate was only 15 to 20 μM and was independent of the pyruvate concentration. Table II also shows that in the presence of ADP, when the reaction was proceeding at a rather slow rate (fig 5), much activity accumulated in pyruvate (A). When TPP was added to the medium pyruvate did not accumulate but was converted into citrate, in which the activity increased markedly (B). The addition of arsenite brought about a new increase in the pyruvate label whereas the production of citrate was stopped (C). When glutamate was added labeled aspartate was recovered, while the activity in pyruvate and citrate remained at a steady level (D). Finally the appearance on the radiochromatograms of a highly radioactive spot indicated that another acid was formed during the reaction. This was identified as fumarate resulting from the action of fumarase. The amounts of fumarate increased steadily during the experiment.

Table III reports the results of an experiment designed to follow the variations in oxaloacetate and pyruvate concentrations during the rapid oxidation of malate by climacteric fruit mitochondria. During this experiment, which was similar to that depicted in figure 3A, samples were withdrawn at

Table III. *Formation of Oxaloacetate and Pyruvate during the Oxidation of ^{14}C-malate by Climacteric Avocado Mitochondria*

Experiment conducted in a way similar to that depicted in figure 3A. Mitochondrial nitrogen: 475 μg/ml medium. Samples taken, A) after a 5 minute oxidation period in the absence of ADP, B) at the point where respiratory control occurs following the addition of 1 mM ADP, C) at the point where the rate of oxidation increases after the transitory inhibited state 4. Specific activity of malate: 480 dpm/mμmole.

	Distribution of radioactivity (dpm/ml medium)		
	A	B	C
Oxaloacetate	6000	18,000	10,000
Pyruvate	66,000	234,000	252,000

Table II. *Distribution of Radioactivity during the Oxidation of Uniformly Labeled ^{14}C Malate by Preclimacteric Avocado Mitochondria*

Experiment conducted in a way similar to that depicted in figure 5. Mitochondrial nitrogen: 245 μg/ml medium. Samples taken successively after 5 minute oxidation periods in the presence of A) 1 mM ADP, B) 133 μM TPP, C) 3 mM arsenite and D) 5 mM glutamate. Specific activity of malate: 410 dpm/mμmole. Initial concentration of malate: 8.5 mM (3,485,000 dpm/ml medium).

	Distribution of radioactivity (dpm/ml medium)			
	A	B	C	D
	ADP	TPP	Arsenite	Glutamate
Oxaloacetate	6600	7000	8400	6900
Pyruvate	129,000	133,000	220,000	200,000
Malate	3 360,000	3,100,000	2,740,000	2,710,000
Citrate	18,300	264,000	216,000	200,000
Aspartate	...	...	...	47,000
Fumarate	93 500	161 000	218,000	282,000

3 steps of the reaction: after a 5 minute reaction in the absence of ADP (A), at the point where respiratory control occurs after a fast but continuously decreasing rate of oxidation following the addition of ADP (B), and at the point where the inhibited state 4 ends (C). Table III shows that the activity in oxaloacetate builds up during the state 3 to reach a maximum when the respiratory control takes place. When the normal state 4 rate resumes about half of the oxaloacetate has been removed. Pyruvate increased greatly during the fast state 3 rate. It should be noted that as in the case of preclimacteric particles the absolute concentrations of oxaloacetate in the medium remained very low. Computed from the specific activity of malate, this concentration was about 40 μM when maximum inhibition was observed. Concentrations of pyruvate were much higher and the experiment showed that the labeling found in pyruvate was almost accounted for by the disappearance of labeled malate.

Discussion

It is clear from the results of this study that the oxidation of malate by avocado mitochondria isolated from preclimacteric or climacteric fruits proceeds in such a way that the formation of large amounts of pyruvate occurs. The reactions involved under all the experimental conditions have been summarized in figure 6. In this scheme is shown that malate oxidation by avocado mitochondria yields pyruvate via oxaloacetate. The evidence for this is based on the formation of aspartate when glutamate is transaminated. Direct analysis indicates that small amounts of labeled oxaloacetate are present in the reaction mixture when oxidation of labeled malate is carried out.

The mechanism of the conversion of oxaloacetate to pyruvate has not been studied, but it is assumed that either an oxaloacetate decarboxylase is present in the mitochondria or that the acid is decarboxylated in a nonenzymic way in the presence

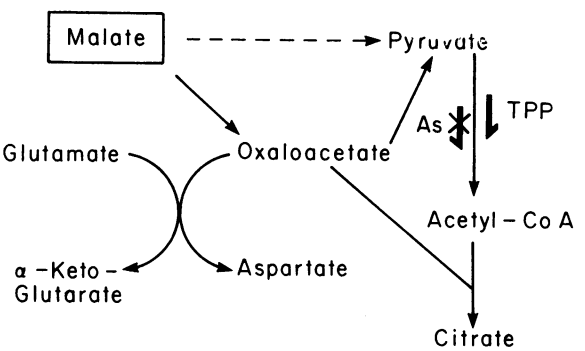

FIG. 6. Pathways of malate oxidation in avocado mitochondria in the presence of TPP, arsenite, and glutamate.

of divalent ions such as Mg^{++} (19, 21). Whatever the actual mechanism is, it is known that either of these reactions is possible in plant mitochondria. When oxaloacetate is added to mitochondria, there is generally a very long lag phase before the oxygen uptake starts, but during this time the production of carbon dioxide occurs (2, 22). In fact, with avocado mitochondria Avron and Biale (2) demonstrated that oxaloacetate was converted to malate and citrate by a dismutation reaction without consumption of oxygen.

The molecular mechanism of the malate dehydrogenase reaction is highly specific (9, 14). The enzyme shows substrate activation and product inhibition. It is thought that when oxaloacetate is in excess it binds at another site on the enzyme, probably at the NAD site, and in that way inhibits the reaction. The very low concentrations of oxaloacetate, found to range from 20 to 40 μM on the basis of isotopic estimation, could very well represent the amounts of the acid which are bound to the enzyme itself, and the depression observed during the inhibited state 4 could be explained in this way. It also appears from the results of these experiments that oxaloacetate does not accumulate in the mitochondria. It seems that the molecule of oxaloacetate leaves the catalytic site of the enzyme to undergo immediately a new reaction: decarboxylation to pyruvate, condensation with acetyl-CoA or transamination with glutamate. Consequently the rate of malate oxidation is greatly dependent on the rate at which oxaloacetate is removed.

Though the proposed scheme implies oxaloacetate as an intermediary step in the formation of pyruvate from malate, it is possible that some other pathways might give the same overall result (14). While malic enzyme can bring about the conversion of malate to pyruvate directly, this mechanism is believed not to be involved because the enzyme is not normally found in the particulate fraction. Moreover, the malic enzyme requires NADP and Mn^{++} which had no effect on avocado mitochondria. There was also no evidence for phosphoenolpyruvate as an intermediate. The pathway which requires 2 enzymes, phosphoenolpyruvate kinase and phosphoenolpyruvate carboxylase, can also lead to pyruvate from oxaloacetate. But generally this pathway is working irreversibly in the direction of oxaloacetate production (21). The fact that labeled oxaloacetate is recovered favors the scheme proposed in figure 6.

The site of action of TPP in increasing the rate of oxidation of malate is the pyruvate oxidase complex. The possibility of a direct action of TPP on oxaloacetate itself was dismissed because malonate was not found in the experiments with labeled malate.

The scheme proposed in figure 6 also provides an explanation for the differences observed in the oxidation of malate between mitochondria isolated

from preclimacteric or climacteric fruit. In the former case, the rate limiting step in the oxidation of malate is the production of acetyl-CoA from pyruvate. Only when TPP is added, the oxidative decarboxylation of pyruvate takes place at a sufficient rate. Of all the co-factors which have been tried to improve the rate of this reaction (CoA, NAD), only TPP proved to be effective, because presumably it is the limiting factor of the overall process. Mitochondria extracted from climacteric fruits are able to oxidize malate at a high rate. It is only during the state 3 rate of oxidation that TPP helps in removing oxaloacetate. It should be pointed out that in this case the rates of production of oxaloacetate and of pyruvate as well are very high. Under these circumstances the pyruvate oxidase complex is presumably working at full capacity and is still limited by TPP. All these observations on the oxidation of malate show that the climacteric fruit mitochondria have a lesser requirement for exogenous TPP than the preclimacteric ones. It is supposed that the amounts of TPP present in the mitochondria limit the rate of the reaction.

These observations have to be related to the rise in respiratory activity which takes place during the climacteric phase of ripening. Whether this rise is related to the capacity to carry out the oxidative decarboxylation of α-ketoacids and whether it is controlled by the amounts of TPP present inside the mitochondria is an open question. But the results reported here at least suggest that all these aspects are very closely interrelated. It is also conceivable that the control by TPP is exerted at the level of α-ketoglutarate. There is in fact a difference of reactivity between the 2 systems in response to TPP. The requirement for TPP of the α-ketoglutarate decarboxylase disappears earlier during the maturation than that of the pyruvate oxidase complex, as can be seen by comparing α-ketoglutarate and malate oxidation during the preclimacteric phase of maturation. This has been the basis to divide the preclimacteric stage into 2 subdivisions: early and late preclimacteric (11). While differences in levels of TPP are suggested as a possible reason for some of the observed responses of mitochondria from the 2 stages, one must allow for other factors and conditions in explaining the striking differences in the oxidative activities of the particles with the progress of ripening. Changes in compartmentation, enzymic capacity to utilize substrates and the access of substrate to the sites of action might also play an important role.

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