

# METABOLIC PROCESSES IN CYTOPLASMIC PARTICLES OF THE AVOCADO FRUIT

## V. EFFECT OF OXALACETATE ON THE OXIDATION OF PYRUVATE AND SUCCINATE

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Studies on the oxidation of Krebs' cycle acids have been complicated, in some cases, by the inhibitory effects of one acid on the oxidation of another. The best known inhibition of this type is that of oxalacetate on the oxidation of succinate, which was first reported by Das (1) who used the Thunberg technique. Swingle *et al.* (2) confirmed the observation of Das with manometric methods. A thorough study of this inhibition was reported by Pardee and Potter (3) who observed it in rat liver homogenates and also to some extent in other animal tissues. They noted that the inhibition of succinate oxidation by oxalacetate was slowly relieved with time. Since their preparations were devoid of oxalacetate oxidase activity and since the concentration of total keto acids did not change throughout the process, they attributed the decrease in inhibition to the decarboxylation of oxalacetate to pyruvate, which was ineffective in causing inhibition. They also reported that adenosine triphosphate (ATP) reversed the inhibition while calcium ions enhanced it. Tyler (4) determined the effect of calcium, magnesium, and ATP on the succinoxidase system inhibited by oxalacetate. He found that the reversal of the inhibition was due mainly to magnesium and that ATP had an enhancing effect. In the presence of magnesium and ATP, the inhibition could be restored by the addition of calcium ions.

This paper deals with some observations on the effects of oxalacetate on the oxidation of pyruvate and succinate by cytoplasmic particles (mitochondria) of the avocado fruit.

### EXPERIMENTAL

Mitochondrial preparations from fruits of the avocado, *Persea americana*, were made in a manner similar to that described by Biale *et al.* (5).

Paper chromatograms were run by the procedure of Lugg and Overell (6) or by a modification (7). The solvents employed were, accordingly, butanol-water (1:1)-formic acid (4 M), or pentanol-water (1:1)-formic acid (5 M). The reaction mixtures were treated with 1 drop of 0.5 N HCl and

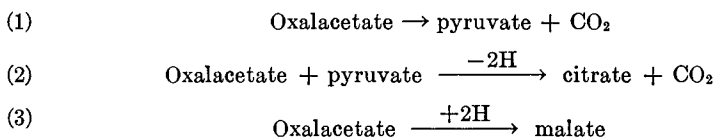
placed in a boiling water bath for 1 to 2 minutes. The denatured proteins were then centrifuged, and the supernatant fluid was applied directly to the paper. Under these conditions, neither pyruvate nor oxalacetate shows up on the paper unless present in high concentrations.

Water redistilled from glass was used throughout. Sodium pyruvate was prepared from commercial pyruvic acid and recrystallized. Oxalacetate was bought from the California Foundation for Biochemical Research. When tested chromatographically, no traces of the other acids of the cycle were found as impurities in the preparations used.

### Results

#### *Effect of Oxalacetate on Pyruvate Oxidation*

We reported earlier (8) that avocado particles were capable of oxidizing most acids of the tricarboxylic acid cycle at relatively high rates. It was observed, however, that, when oxalacetate was used as the sole substrate, no oxygen uptake was obtained manometrically; but, when the reaction mixture was chromatographed, after incubation for 120 minutes, citrate and malate clearly appeared as products (Fig. 1). The reaction sequence must accordingly be one which could account for the production of citrate and malate from oxalacetate without requiring any net uptake of oxygen. One such reaction sequence is the following.



If this were the actual mechanism by which the above results were obtained, one would predict that these consequences should follow: (a) Since the first reaction serves only to produce pyruvate to be used in the second, it would be expected that addition of increasing quantities of oxalacetate to a reaction mixture containing pyruvate should cause an increasing lag in the oxygen uptake. Fig. 2 shows that this was the case. (b) Arsenite has been shown to abolish the oxidation of pyruvate by avocado particles (8). It would be expected, then, that, by blocking Reaction 2, arsenite should completely inhibit the production of citrate and malate from oxalacetate. As shown in Fig. 1, no products could be observed chromatographically when arsenite was present in the reaction mixture. (c) Although the above formulation does not require any net oxygen uptake, CO<sub>2</sub> evolution from both Reactions 1 and 2 should be apparent from the start. In addition, arsenite should abolish the CO<sub>2</sub> evolution arising from Reaction 2 but not from Reaction 1, and thus a decreasing rate of CO<sub>2</sub> evolution would

be expected in the presence of arsenite. Fig. 3 illustrates both of these effects in agreement with the proposed mechanism. (d) If malate were produced, as postulated, by the reduction of oxalacetate and not via the usual route in the tricarboxylic acid cycle, one would expect no inhibition of malate production upon the addition of malonate. Chromatographically, no difference in the amount of malate produced could be detected in

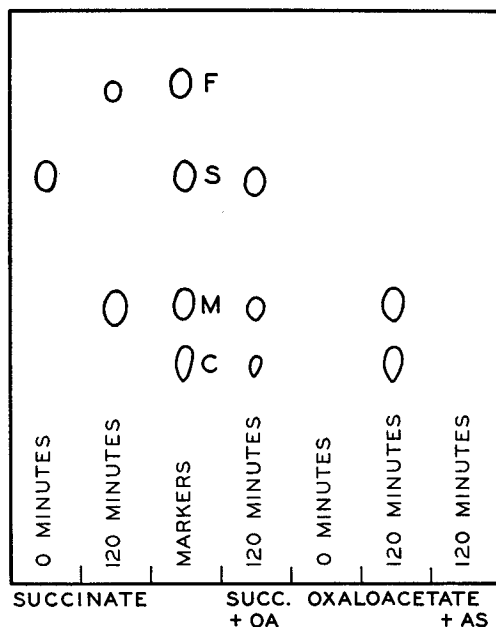
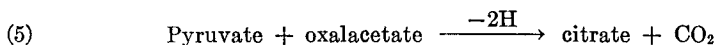
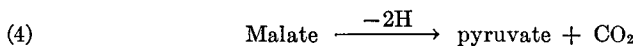


Fig. 1. Chromatographic separation of products in reaction mixtures of avocado particles and the following: succinate, succinate + oxalacetate, oxalacetate, and oxalacetate + arsenite. *F* = fumarate, *S* = succinate, *M* = malate, *C* = citrate, *As* = arsenite. The reaction mixture for succinate and succinate + oxalacetate was the same as that in Fig. 5 and for oxalacetate and oxalacetate + arsenite was the same as that in Fig. 3.

the presence or absence of malonate. All of these results are in complete agreement with the proposed mechanism.

Another sequence of reactions, in which the malic enzyme serves as the decarboxylating agent, is also in agreement with the data presented thus far. This sequence could be formulated as follows:



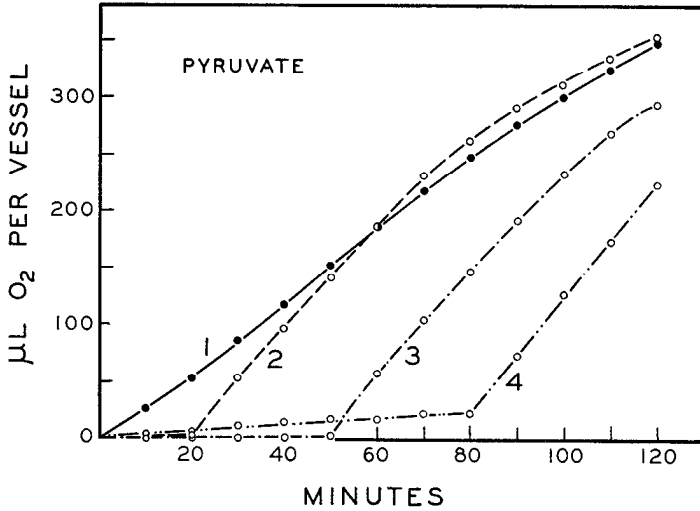


FIG. 2. Oxidation of pyruvate by avocado particles in relation to oxalacetate concentration. Curve 1, pyruvate + 0.001 M oxalacetate; Curve 2, pyruvate + 0.005 M oxalacetate; Curve 3, pyruvate + 0.010 M oxalacetate; Curve 4, pyruvate + 0.018 M oxalacetate. The reaction mixture contained, in micromoles, pyruvate 60, phosphate 25, adenosine diphosphate (ADP) 3, magnesium 3, glucose 60, coenzyme A 0.07; 0.5 ml. of particles; total volume 3.0 ml. of 0.25 M sucrose; pH 7.0; temperature 20°; gas phase air.

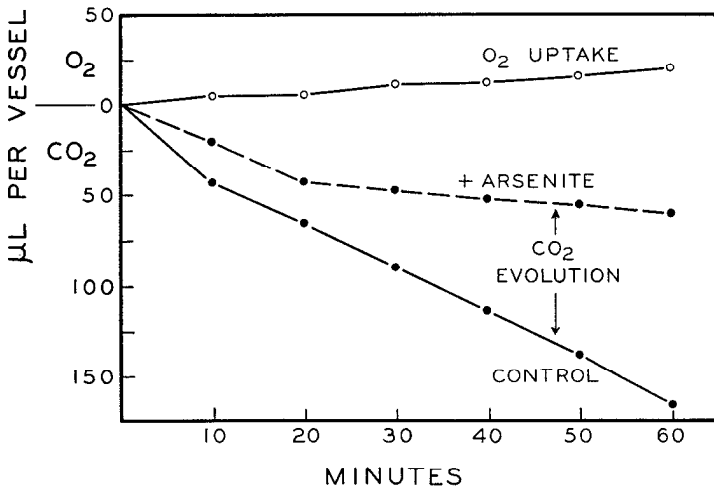


FIG. 3. Oxidation and decarboxylation of oxalacetate by avocado particles. The reaction mixture contained, in micromoles, oxalacetate 30, phosphate 25, ADP 3, magnesium 3, glucose 60; 0.5 ml. of particles; total volume 3.0 ml. of 0.25 M sucrose; arsenite  $3 \times 10^{-3}$  M; pH 7.0; temperature 20°; gas phase air.

According to this hypothesis, but not the previous one, addition of increasing quantities of oxalacetate to a reaction mixture which is oxidizing malate should cause an increasing lag in the oxygen uptake. Thus, curves similar to the ones presented in Fig. 2 are to be expected if this sequence were the one operating. Fig. 4 shows that this was not the case, since addition of increasing quantities of oxalacetate did not seem to affect the oxidation of malate. Thus, it can be concluded that the first sequence of reactions best represents the mechanism by which the observed results were obtained.

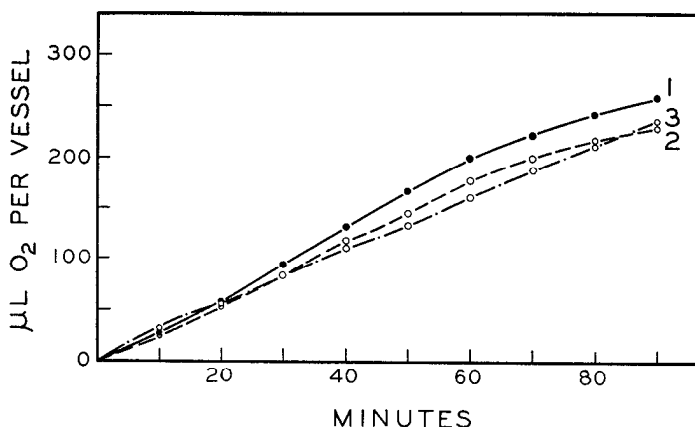


FIG. 4. Effect of oxalacetate on the oxidation of malate by avocado particles. Curve 1, malate; Curve 2, malate + 0.002 M oxalacetate; Curve 3, malate + 0.008 M oxalacetate. The reaction mixture contained 48  $\mu$ moles of malate in place of pyruvate; otherwise it was the same as that in Fig. 2.

#### *Effect of Oxalacetate on Succinate Oxidation*

When oxalacetate was added to a reaction mixture containing succinate, a lag period was evident (Fig. 5). The duration of the lag was a function of the concentration of oxalacetate, higher concentrations causing longer lag periods. The manometric picture, then, seemed rather similar to the one observed upon addition of oxalacetate to pyruvate.

Upon chromatographic separation of the reaction products from a vessel containing 0.08 M oxalacetate (Fig. 1), it was clearly seen that, although succinate has completely disappeared from the control, during the incubation period, the reaction mixture containing oxalacetate still showed a large succinate spot. The malate and citrate spots shown are probably the result of the breakdown of the added oxalacetate via the reaction sequence discussed earlier.

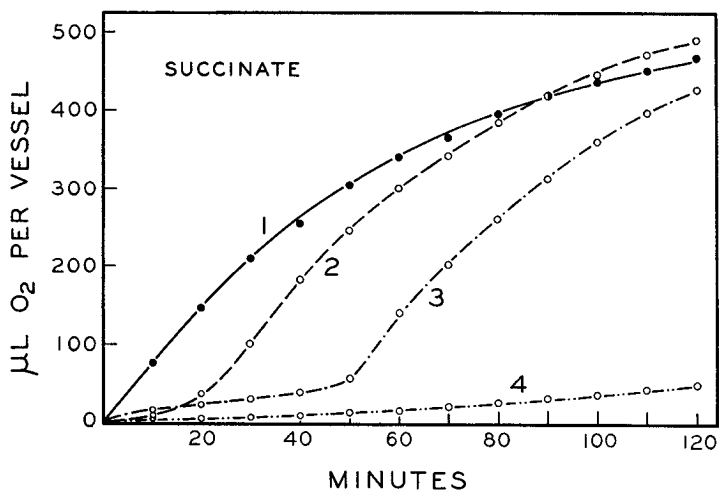


FIG. 5. Oxidation of succinate by avocado particles in relation to oxalacetate concentration. Curve 1, succinate; Curve 2, succinate + 0.002 M oxalacetate; Curve 3, succinate + 0.004 M oxalacetate; Curve 4, succinate + 0.008 M oxalacetate. The reaction mixture contained, in micromoles, succinate 60, phosphate 25, ADP 3, glucose 60; 0.5 ml. of particles; total volume 3.0 ml. of 0.25 M sucrose; pH 7.0; temperature 20°; gas phase air.

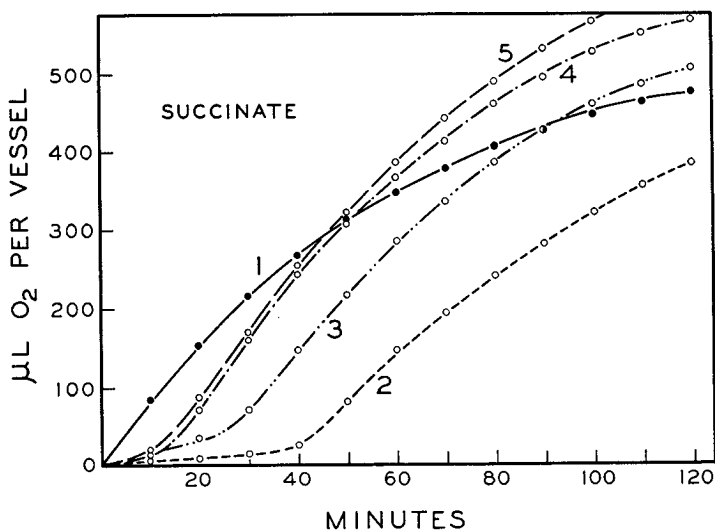


FIG. 6. Effect of magnesium and ATP on the oxalacetate-inhibited succinoxidase system. Curve 1, succinate; Curve 2, succinate + 0.004 M oxalacetate; Curve 3, succinate + 0.004 M oxalacetate + 0.001 M ATP; Curve 4, succinate + 0.004 M oxalacetate + 0.001 M magnesium; Curve 5, succinate + 0.004 M oxalacetate + 0.001 M ATP + 0.001 M magnesium. The reaction mixture of the control was the same as that in Fig. 5.

It thus seems that in this case the inhibition is real and is not due to oxidation and reduction reactions which proceed simultaneously and result in no net oxygen uptake. The reversal of the inhibition with time (Fig. 5) can be attributed to the breakdown of oxalacetate as the reaction proceeds, which thus lowers the effective concentration of the inhibitor. As the concentration of oxalacetate dropped below that critical for the inhibition of succinate oxidation, oxygen uptake was observed manometrically.

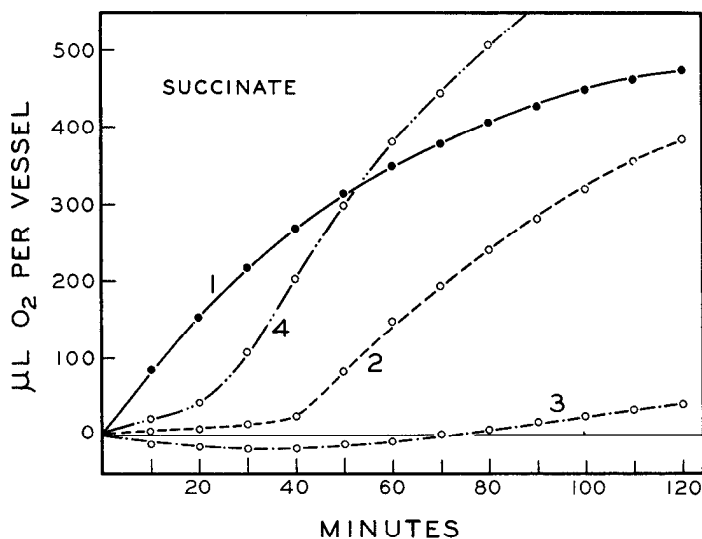


Fig. 7. Effect of calcium on the oxalacetate-inhibited succinoxidase system. The reaction mixture of the control was the same as that in Fig. 5. Curve 1, succinate; Curve 2, succinate + 0.004 M oxalacetate; Curve 3, succinate + 0.004 M oxalacetate + 0.001 M calcium; Curve 4, succinate + 0.004 M oxalacetate + 0.001 M calcium + 0.001 M magnesium + 0.001 M ATP.

The effects of magnesium ions and ATP on this inhibition are shown in Fig. 6. It is clear that magnesium at 0.001 M reversed the inhibition almost completely. ATP, at the same concentration, caused a partial reversal of the oxalacetate effect, but was not as effective as magnesium ions. The addition of ATP to magnesium did not significantly increase the reversal of the inhibition.

As seen in Fig. 7, the addition of calcium ions at 0.001 M to a succinoxidase system inhibited by oxalacetate markedly accentuated the inhibition. It is of interest, though, that even this accentuated inhibition could be reversed upon the addition of magnesium ions and ATP. It should be noted that neither calcium ions nor ATP had any effect on succinate oxidation in the absence of oxalacetate. Magnesium ions did have a small enhancing effect upon the oxidation rate.

## DISCUSSION

The effect of oxalacetate on pyruvate oxidation is of special interest, since it represents a situation where oxalacetate is able to compete with the electron transport system so as to abolish oxygen uptake almost completely. Thus, the electrons released by the pyruvate oxidase system are being utilized for the reduction of oxalacetate to malate, rather than being transported via the usual route to oxygen.

The occurrence of the coupled reactions proposed above has been described for animal tissues (9) under anaerobic conditions. They do not, presumably, occur under aerobic conditions. Moreover, in animal tissue, oxalacetate is readily oxidized with a high rate of oxygen uptake (9, 10).

Several indirect observations have been reported on the presence in plant material of the postulated coupled oxidation-reduction reaction. Davies (11), in a study of the tricarboxylic acid cycle in pea seedlings, presented a chromatogram obtained from the products of a reaction mixture containing pyruvate and oxalacetate. In addition to citrate, a spot for malate is clearly visible. According to our hypothesis, this malate was produced by the reduction of the oxalacetate present in the reaction mixture via the coupled reactions proposed. Brummond and Burris (12), in their study on the operation of the tricarboxylic acid cycle in white lupine mitochondria, observed a very low rate of oxygen uptake when oxalacetate was used as the substrate. In addition, they tried to use oxalacetate as an initiator of pyruvate oxidation and stated that "as oxaloacetate inhibited oxygen uptake for the first 30 minutes, L-malate was subsequently used as the sparking acid." This, again, agrees with our observations and fits the proposed mechanism.

Recently, Walker and Beevers (13) reported a thorough study of this phenomenon. In studies of a particulate fraction from the endosperm of germinating castor beans, they observed typical lag periods like those reported here upon the addition of oxalacetate to a reaction mixture containing pyruvate. Omission of cofactors from the reaction mixture always lengthened the lag period observed. The lag could be shortened by restoring the required cofactors. Similar observations were made on a particulate preparation from potatoes. In discussing the results, the authors mention Dr. Price as suggesting the reaction sequence proposed here as a possible explanation of their results.

It would thus seem that the occurrence of these coupled oxidation-reduction reactions is rather widespread among higher plants and is not at all limited to particles obtained from the avocado fruit.

The inhibition of succinate oxidation by oxalacetate does not seem to involve pathways similar to the ones postulated in its effect on pyruvate oxidation. It could be due to the conversion of the oxalacetate to malo-



nic acid as reported by Vennesland *et al.* for animal (14, 15) and possibly also plant tissues (16). This possibility, however, seems rather unlikely in view of the absence of malonate after an incubation of the particles with oxalacetate (see Fig. 1). With the chromatographic technique used, malonate would appear as a definite spot with an  $R_F$  intermediate between those of malate and succinate (8). In addition, when malonate is added to a reaction mixture which is oxidizing succinate, the rate of oxidation is diminished (0.01 M malonate used for a substantial inhibition), but no lag is observed, nor is the inhibition reversed with time. It is our opinion that a more likely explanation would involve the direct inhibition of the succinoxidase system by oxalacetate. The striking effects of magnesium, calcium, and ATP on the reaction could most simply be ascribed to one of two general mechanisms: (a) accelerating or decelerating the breakdown of oxalacetate or (b) formation of a complex which would be more or less effective as the inhibitor. The data available do not permit a decision as to which of the two mechanisms is operating.

#### SUMMARY

The effects of oxalacetate on pyruvate and succinate oxidations were investigated. In both cases, oxalacetate caused a lag in the oxygen uptake which was relieved with time. In the case of pyruvate, however, the lag was ascribed to an oxidation-reduction system in which pyruvate was oxidized to citrate concomitantly with reduction of oxalacetate to malate. In the case of succinate, an actual inhibition is thought to prevail as long as oxalacetate is present in the reaction mixture in inhibitory concentrations. Magnesium ions or adenosine triphosphate was found to reverse the inhibition of succinate oxidation by oxalacetate while calcium ions enhanced it.

The general occurrence of these phenomena in plant and animal tissues is discussed.

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