METABOLIC PROCESSES IN CYTOPLASMIC PARTICLES OF THE AVOCADO FRUIT. III. THE OPERATION OF THE TRICARBOXYLIC ACID CYCLE [1]

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The evidence for the operation of the tricarboxylic acid cycle in the metabolism of plant mitochondria has been based mostly on studies of oxidative activity. The oxidation of di- and tricarboxylic acids of the cycle was demonstrated in a variety of tissues, such as etiolated seedlings of mung bean (22), pea (10, 31), Avena (32), and Black Valentine bean (4), lupin cotyledons (7), castor-bean endosperm (5, 33), pea internodes (26), cauliflower (15, 16, 17) and broccoli (18) buds, Arum spadix (13, 14), tubers of white potato (28) and of sweet potato (2, 19), avocado fruit (6, 23), spinach leaves (24) and green as well as etiolated pea seedlings and leaves (29, 30). In several of these studies the oxidation rates of acids other than citrate, α-ketoglutarate, succinate or malate were also followed (5, 10, 22, 29, 30, 32). The metabolism of pyruvate by the mediation of the cycle was the subject of investigations dealing with young and rapidly growing tissues (4, 5, 7, 10, 22, 32), and to a lesser extent with mature and stable material (2, 6, 19).

Oxidative studies alone do not furnish sufficient proof for the cycling nature and the sequence of the individual reactions. A better insight may be gained by studying the formation of reaction products along with oxidations. This was done only to a limited extent with plant materials. Davies (10) adopted this procedure for a particulate fraction from pea hypocotyl. James and Elliott (14) also cited some chromatographic evidence for mitochondria from Arum spadix.

The objective of this study was to investigate the operation of the cycle in avocado mitochondria by following rates of oxidation of acids and formation of products in the presence and absence of inhibitors.

MATERIALS AND METHODS

Avocado particles were obtained in a manner similar to that described in the first paper of this series (6). Fruits of the Fuerte variety were peeled and 150 gm of the grated tissue blended in a Waring blendor with 300 ml of a solution 0.25 M in sucrose and 0.05 M in phosphate for 1 minute at 47 volts. The brei was centrifuged at $500 \times g$ for 5 minutes to remove unbroken cells and large fragments. The resultant supernatant was centrifuged at $17{,}000 \times g$ for 15 minutes, and the final pellet suspended in 8.0 ml of the sucrose phosphate solution. This enzyme preparation contained approximately 1.1 to 1.5 mg nitrogen per 0.5 ml of suspension.

All operations preceding the measurement of activity were performed in a cold room maintained at 0 to 2° C. The manometric measurements were carried out at 20° C using conventional Warburg apparatus. The rates of oxidation are reported on an hourly basis since they were linear with time from the outset. The oxidation of fumarate formed an exception, and is treated separately below. All the results were repeated at least once, and the values given are those of a representative experiment.

Paper chromatograms were run following the procedure of Lugg and Overell (20) or its modification (8). The solvents employed were, accordingly, butanol-water (v/v)-formic acid (4 M), or pentanol-water (v/v)-formic acid (5 M). Reaction mixtures were deproteinized by adding to each one drop of 0.5 *N* HCl and placing in boiling water for 1 to 2 minutes. The denatured proteins were centrifuged off and the supernatant applied directly to the paper. With the method, as used, neither pyruvate nor oxaloacetate shows up on the paper unless present in very high concentrations.

α-Ketoglutarate was purchased from California Foundation for Biochemical Research; citrate, succinate, malate and fumarate from Eastman Company; pyruvic acid obtained from Eastman was converted into the sodium salt and recrystallized; adenylates and Co A from Pabst Laboratories; DPN and DPT from Nutritional Biochemicals.

The following abbreviations are used: adenosinemonophosphate—AMP; adenosinetriphosphate—ATP; diphosphopyridine nucleotide—DPN; diphosphothiamine—DPT; coenzyme A—Co A.

RESULTS

OXIDATION OF KREBS CYCLE ACIDS: As can be seen in table I, the avocado particulate preparation was able to oxidize, at a good rate, all the acids of the cycle which were tried. The variability in the rates between the two experiments shown is about as high as was ever observed among different particulate preparations. It should be noted, however, that a consistent pattern was obtained in every experiment, with succinate giving the highest and fumarate the lowest rates. Commercially purchased isocitric acid was also tested, but in view of the rather large amount of contaminants detected chromatographically, the values obtained are of questionable significance and were thus omitted (1). The sample of cis-aconitate used separated chromatographically into two distinct spots (fig 2). One spot agreed in its R_f value, with that of cis-aconitate, while the other was tentatively identified as trans-aconitate. Since the trans-aconitate spot did not change after an incubation with the particulate preparation, it was assumed that the mitochondria acted on cis-aconitate only.

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TABLE I

OXIDATION OF KREBS CYCLE ACIDS IN THE PRESENCE OF MALONATE AND ARSENITE

SUBSTRATE*	EXPT 1		EXPT 2	
	CONTROL	0.01 M MALONATE	CONTROL	0.001 M ARSENITE
	$\mu l\ O_2/vessel \cdot hr$			
Citrate	137	80	179	80
Cis-aconitate	160	122	224	107
α-Ketoglutarate	178	93	247	-9
Succinate	367	89	481	362
Fumarate	110	73	130	24
Malate	151	120	259	40

Complete reaction mixture of controls in micromoles: substrate - 60, phosphate - 30, AMP - 3, magnesium - 3, glucose - 60, DPN - 1.5, DPT - 0.2, Co A - 0.07, in a total of 3.0 ml of 0.25 M sucrose.

Particulate preparation contained approximately 1.0 mg nitrogen per vessel. pH 7.0; 20° C; gas phase: air.

* No oxygen uptake in the absence of substrates.

Fumarate, as contrasted with the other acids, was not oxidized at a constant rate from the outset. Its oxidation exhibited a lag period of at least 20 minutes, before significant oxygen uptake could be observed (fig 1). This lag might correspond to the time required for the formation of a sufficient concentration of malate, which is the actual oxidizable substrate. The oxidation rates of fumarate given in the literature were generally low (22, 32) with the exception of the recent work by Beevers and Walker (5) in which the addition of DPN, ATP and Co A resulted in a rate similar to that obtained with other acids.

The unusual pattern of oxaloacetate oxidation by the avocado particles has posed some new problems and is treated in a separate publication of this series (3).

COFACTOR REQUIREMENTS FOR THE OXIDATION OF KREBS CYCLE ACIDS: In order to obtain a washed preparation relatively free of adhering cofactors, the following procedure was followed. The pellet from the high speed centrifugation was resuspended with the use of a close fitting lucite homogenizer in 30 ml of the sucrose-phosphate solution. The suspension was then diluted to 180 ml with sucrose-phosphate and centrifuged again at high speed for 15 minutes. The resultant pellet was suspended in 8.0 ml of sucrose-phosphate, as in the usual preparation. The experiments reported in tables II, III, IV, and V were performed with this washed preparation. In table II single factors were omitted from the complete reaction mixture, while in table III the omission was cumulative. It is evident from both tables that magnesium was required in all cases, while the omission of AMP caused a reduction in the oxidation rates of citrate and α-ketoglutarate. In general, the requirements for the malic oxidation system were similar to those of the other acids. In no case was there a need for DPN, which presumably is particle bound.

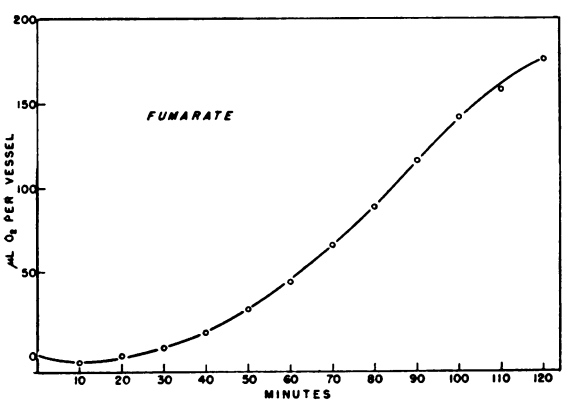

FIG. 1. Oxidation of fumarate by cytoplasmic particles of the avocado fruit. Conditions same as in table I.

TABLE II

COFACTOR REQUIREMENTS FOR THE OXIDATION OF KREBS CYCLE ACIDS (SINGLE FACTOR OMISSION)

	CITRATE	α-KETOGLUTARATE	SUCCINATE
	$\mu l\ O_2/vessel \cdot hr$		
Complete	225	248	226
Omit DPT	189	...	...
" DPN	223	201	236
" glucose	178	211	237
" magnesium	132	89	153
" AMP	152	109	215

Complete reaction mixture same as in table I.

DETECTION OF REACTION PRODUCTS: The end products of oxidation for each of the acids were determined two hours after the addition of the particulate suspension to the reaction mixture. The chromatographic patterns at the end of this period were compared with the respective patterns at 0 time, that is immediately after the addition of the enzyme suspension to the reaction mixture. A diagramatic representation of these patterns, based on the actual chromatograms, is shown in figure 2.

TABLE III

COFACTOR REQUIREMENTS FOR THE OXIDATION OF KREBS CYCLE ACIDS (CUMULATIVE OMISSION)

	CITRATE	α-KETOGLUTARATE	SUCCINATE*
	$\mu l\ O_2/vessel \cdot hr$		
Complete	265	223	253
Omit DPT	240	215	...
" DPT, DPN	234	226	210
" DPT, DPN, glucose	214	178	246
" DPT, DPN, glucose, Mg	112	60	...
" DPT, DPN, glucose, Mg, AMP	105	24	141

Complete reaction mixture same as in table I.

* For succinate oxidation only DPN, glucose, Mg, and AMP were tested.

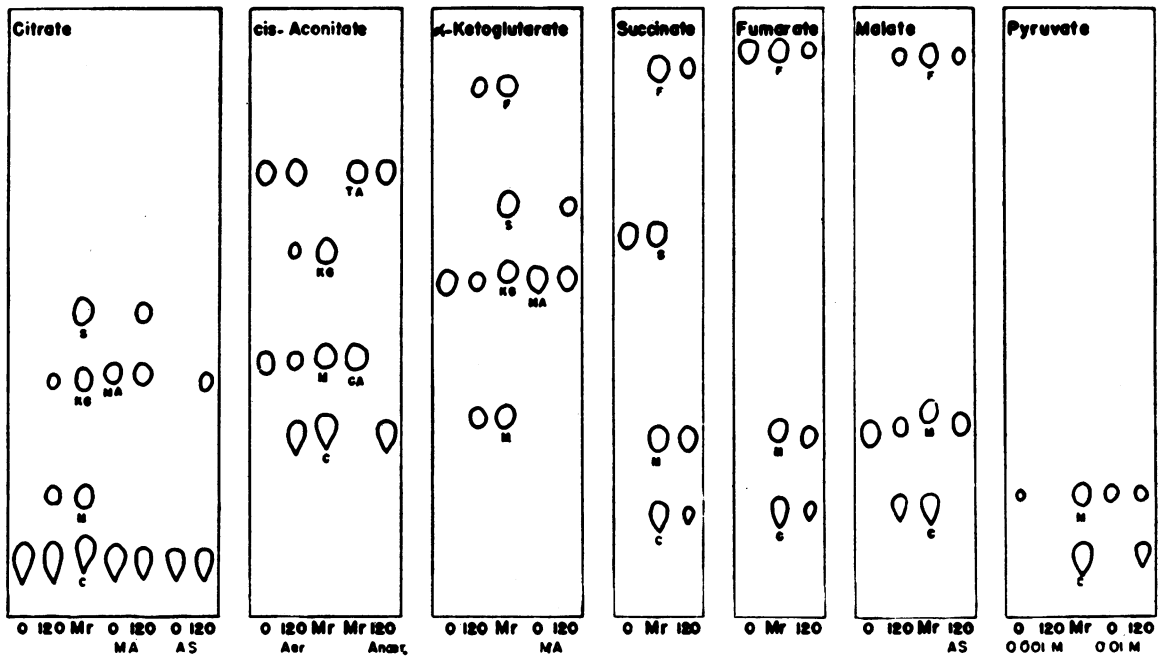

FIG. 2. Diagram of chromatographic separations of reaction products in the oxidation of Krebs cycle acids by cytoplasmic particles of the avocado fruit. Designations: Markers, Mr; citrate, C; malate, M; α-ketoglutarate, KG; succinate, S; cis-aconitate, CA; trans-aconitate, TA; fumarate, F; malonate, MA; arsenite, AS; aerobic conditions, Aer; anaerobic conditions, Anaer.

Concentrations under chromatogram for pyruvate refer to the concentration of malate used as a sparker acid.

It can be seen that after 120 minutes of incubation citrate was converted into malate and α-ketoglutarate; cis aconitate into citrate, α-ketoglutarate and possibly malate; anaerobically cis aconitate was converted to citrate only; aerobically α-ketoglutarate was converted into malate and fumarate; succinate into malate, fumarate and citrate; fumarate into malate and citrate; and malate into fumarate and citrate. All these results are in complete agreement with the operation of the tricarboxylic acid cycle.

EFFECT OF INHIBITORS: The addition of inhibitors to the reaction mixtures changed the pattern of products observed as well as the rate of oxygen uptake. The two inhibitors utilized in this study were malonate and arsenite. Malonate is thought to block chiefly the conversion of succinate to fumarate, while arsenite is considered as a potent inhibitor of pyruvate and α-ketoglutarate oxidations. Since it has been shown that dimercapto systems are much more sensitive to arsenite than single SH groupings, the effect of arsenite on the two oxidase systems has been interpreted as indicating the paritcipation of lipoic acid in one of the intermediate steps (12).

As can be seen in table I and figure 2, our results are in complete agreement with the postulated sites of action of these inhibitors. Table I illustrates that malonate affected succinate oxidation most markedly. Chromatographically (fig 2) no malate could be ob-

TABLE IV

COFACTOR REQUIREMENTS FOR PYRUVATE OXIDATION (SINGLE FACTOR OMISSION)

	μL O_2/MG N · HR
Complete	266
Omit glucose	211
" DPT	247
" DPN	193
" CoA	209
" AMP	224
" Mg	138
" Malate	18
" Pyruvate	36

Complete reaction mixture same as in table I, plus 6 micromoles of malate.

TABLE V

COFACTOR REQUIREMENTS FOR PYRUVATE OXIDATION (CUMULATIVE OMISSION)

	μL O_2/MG N · HR
Complete	334
Omit glucose	299
" glucose, DPT	244
" glucose, DPT, DPN	206
" glucose, DPT, DPN, CoA	168
" glucose, DPT, DPN, CoA, AMP	155
" glucose, DPT, DPN, CoA, AMP, Mg	116
" glucose, DPT, DPN, CoA, AMP, Mg, Malate	−19

Complete reaction mixture same as in table I, plus 6 micromoles of malate.

served as a product of citrate or α-ketoglutarate oxidations in the presence of malonate. Instead, a spot for succinate was clearly visible in both cases. In the case of arsenite, table I illustrates the marked effect of the inhibitor on α-ketoglutarate oxidation. Even at 0.0001 M α-ketoglutarate oxidation was inhibited to the extent of 75 %. When arsenite was added to a reaction mixture containing citrate, no spot corresponding to malate could be observed. The only detectable product was α-ketoglutarate. The effect of arsenite on pyruvate oxidation will be discussed in the following section.

Pyruvate Oxidation and Condensation: No evidence for the operation of the tricarboxylic acid cycle is satisfactory unless it includes proof for rapid utilization of pyruvate through a synthetic reaction involving a four carbon acid with subsequent formation of citrate. The avocado particles were able to oxidize pyruvate provided certain additions were made to the reaction mixture as shown in tables IV and V. The requirement for a sparker is absolute. Malate was used in the experiments reported here, but oxaloacetate could serve as well (3). The omission of magnesium reduced the rate markedly, while the influence of other factors could be observed only when omitted cumulatively. The combined omission of glucose, DPT, DPN, Co A and AMP reduced the rate by over 50 %, though when omitted singly they did not influence the rate greatly. The effects of the inhibitors on pyruvate oxidation are shown in table VI. Arsenite inhibited the reaction virtually completely at 0.001 M, and to the extent of 78 % at 0.0001 M. Malonate caused a 75 % inhibition at 0.01 M. The latter inhibition can be attributed to interference with the formation of the condensing partner, while arsenite presumably affects the lipoic acid step in pyruvate oxidation.

The products of oxidation of 0.02 M pyruvate sparked by 0.001 M malate could not be detected chromatographically (fig 2). Presumably, pyruvate was completely oxidized through the cycle, with none of the acids accumulating in a large enough concentration to appear on the paper. This hypothesis was strengthened by the fact that citrate could be easily detected when the sparker concentration was increased to 0.01 M (fig 2). The addition of malonate (not shown in the diagram) brought out a spot for succinate and a somewhat fainter fumarate spot. In the presence of arsenite no distinction could be made between the 0 time and the 120 minute patterns. However, when arsenite was added to a reaction mixture oxidizing malate (fig 2), no citrate could be observed as a product of the reaction. Thus, the citrate production by malate oxidation, in the absence of arsenite, proceeds most probably, through a decarboxylation to pyruvate followed by the condensation process.

Table VI

Inhibitor Effects on Pyruvate Oxidation

Inhibitor conc	Arsenite	Malonate
moles/l	*μl O_2/vessel · hr*	
0.0	223	140
1×10^{-4}	49	...
5×10^{-4}	24	...
1×10^{-3}	14	...
3×10^{-3}	− 8	...
1×10^{-2}	−14	36

Reaction mixture in control: same as in table I, plus 6 micromoles of malate.

Discussion

The avocado fruit is, to the best of our knowledge, the only ovarian tissue from which biochemically active cytoplasmic particles were obtained thus far. Attempts were made by Pearson and Robertson (25) to isolate mitochondria from the apple, but their preparations oxidized succinate at a low rate and did not respond to any of the other acids tested. On the other hand, the particles of the avocado were found to carry on both oxidation and phosphorylation (6), to be affected by the uncoupling action of dinitrophenol at certain stages of ripening (23), and to be able to reduce glutathione at the expense of the oxidation of some acids of the tricarboxylic acid cycle (34).

The mitochondrial complex of the avocado appears to be a well integrated entity which has low cofactor requirements. The negative response to exogenous cytochrome c (6) has been explained by the presence of this substance as an integral component of the particle (9). The response to adenylate and magnesium in the α-ketoglutarate oxidation and to magnesium only for the succinoxidase system was shown previously (6) and confirmed here. In this study oxidation rates were compared when one factor was omitted at a time and when the omission was cumulative. With either method the oxidation of citrate, α-ketoglutarate or succinate did not require any exogenous glucose, DPN or DPT. In general, the cofactor requirements for the avocado particles were similar to those of most plant tissues, with the exception of the castor bean endosperm (5) and the sweet potato tuber (19). The mitochondrial oxidations of the latter tissues were stimulated by DPN, DPT and Co A. It seems that neither differences in preparative procedure, nor in rates of oxidation could account for the discrepancy.

The cofactor requirements for pyruvate oxidation by particulate enzymes of the avocado were essentially the same as those for the other acids, except for the need for catalytic quantities of a sparker. An absolute requirement for a sparker acid was shown also for mungbean seedlings (22), cauliflower buds (17), lupin cotyledons (7), Arum spadix (13), oat seedlings (32), castor bean endosperm (5), Black Valentine bean (4) and sweet potato tuber (19). In several other cases malate was added to a reaction mixture containing pyruvate, but was not shown conclusively to be necessary. Malate has been used in nearly all studies, though the obvious choice for a condensing partner with pyruvate is oxaloacetate.

The difficulty experienced with oxaloacetate was probably the result of a lag in oxygen uptake observed when it was applied as a sparker. This lag was reported for lupin cotyledon (7), Avena seedlings (32), castor bean endosperm and white potato (33) and for the avocado fruit (3). In a study of this phenomenon (3) we concluded that this lag was due to the simultaneous oxidation of pyruvate and reduction of oxaloacetate, resulting in no net oxygen uptake.

The role of the sparker acid becomes evident when one follows reaction products along with oxidation rates. Davies (10) detected citrate when pyruvate and oxaloacetate were incubated with the mitochondrial preparation from pea seedlings. A similar observation was made by Lieberman and Biale (19) when pyruvate and malate were added to sweet potato particles. The chromatograms obtained from avocado mitochondrial oxidations clearly show the formation of citrate from pyruvate and malate in this study, or from pyruvate and oxaloacetate reported elsewhere (3). Citrate was also detected in this study as a product of the oxidation of malate, fumarate, or succinate.

Brummond and Burris (7) established the entry of pyruvate into the cycle by a different procedure than most workers. They incubated mitochondria from cotyledons of etiolated white lupine seedlings with pyruvate-2-C^{14} and unlabeled malate. At the end of the incubation period the molar specific activities of citrate and pyruvate were essentially the same, indicating that citrate arose from pyruvate directly. In addition, they isolated all the other acids of the cycle (using added carrier acid) and showed significant incorporation of the label in each. They concluded that the tricarboxylic acid cycle operates in white lupine mitochondria but "whether it is functional in mature tissue remains to be established." We submit that the evidence obtained since and presented here coupled with observations of others, strongly suggests the operation of the cycle in mature tissues of several species.

It is probably correct to say that not all the reactions essential for the functioning of the cycle have been sufficiently studied. This is particularly true with respect to the rather involved condensation process. Davies (11) stated that fluoroacetate failed to cause accumulation of citrate, though in non-poisoned systems citrate was observed chromatographically. Walker and Beevers (33) demonstrated the formation of acetyl phosphate from pyruvate, when trans-acetylase was added to the plant mitochondrial preparation. This strongly suggested acetyl Co A as an intermediate in pyruvate oxidation. The wide occurrence of Co A in higher plants (27) is suggestive, although by no means conclusive, of its role in pyruvate metabolism. It was also shown that solubilized preparations from a variety of plant tissues are able to form acetyl Co A from acetate, Co A and ATP (21). The participation of DPT in pyruvate oxidation was indicated by the marked increases in oxygen uptake observed upon its addition in some tissues (33, 19). The high sensitivity of the pyruvate and α-ketoglutarate oxidation systems to arsenite, demonstrated in this study, suggests that lipoic acid may also be involved, as shown previously for animal and bacterial preparations (12).

The evidence for the entry of pyruvate into the cycle has to be coupled with proof for its oxidation through the catalytic action of the acids. This can be done by the use of several techniques. One can show the ability of all the acids to serve as sparkers for pyruvate oxidation (32, 33). The technique used by Brummond and Burris (7) and discussed above provides another way. However, neither of these methods indicates the sequence in which the reactions proceed. By following the products of the reactions and the effect of inhibitors on them this additional information can be secured. In this manner, we obtained evidence regarding the acids participating in the cycle and the order in which these reactions are arranged, which is in complete agreement with the tricarboxylic acid cycle.

In conclusion, it could be said that the results of all investigations regarding the operation of the tricarboxylic acid cycle in plant tissues provide strong evidence for regarding it as a major pathway of oxidation in particles isolated from a variety of higher plant tissues.

Summary

This paper is concerned with the operation of the tricarboxylic acid cycle in particles obtained by differential centrifugation from avocado fruits. With the aid of manometric and paper chromatographic techniques the oxidation and appearance of the expected products was shown when each of the acids was incubated with the enzyme preparation.

The effect of malonate on the inhibition of oxidation and appearance of succinate as product was shown for several acids. The effect of arsenite on the oxidation of the acids and product formation was found to agree with its action on animal mitochondria-inhibiting pyruvate and α-ketoglutarate oxidation.

Cofactor requirements were studied for the oxidation of pyruvate, citrate, α-ketoglutarate, succinate and malate. Magnesium ions were required in all cases, while the requirement for the other cofactors varied with the substrate used. A sparker quantity of malate was found to be an absolute necessity before any pyruvate oxidation proceeded.

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