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Effect of Citric Acid-Cycle Intermediates on Oxaloacetate Utilization and Succinate Oxidation

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Evidence that oxaloacetate plays an important rate-regulating role in metabolism through the citric acid cycle is growing, particularly through studies on both animal and plant tissues, demonstrating that the rate of succinate oxidation under different conditions is inversely related to the oxaloacetate concentration and thus influenced by factors affecting the production and removal of the keto acid (Pardee & Potter, 1948; Brummond & Burris, 1953; Tyler, 1955*a*, *b*; Wolff & Ball, 1957; Avron & Biale, 1957; Corwin & Schwarz, 1959). This paper reports the results of examination of some other factors affecting the metabolism of oxaloacetate and its capacity to inhibit succinic dehydrogenase. It will be shown that oxaloacetate utilization is inhibited by the presence of some citric acid-cycle intermediates and this inhibition is reflected in a potentiation of the inhibition of succinate oxidation.

EXPERIMENTAL

Kidney, brain, liver and heart removed from decapitated rats were chilled, rapidly weighed and homogenized (Tyler, 1955*a*) at 0° in 9 parts of 0·9% NaCl containing mM-ethylenediaminetetra-acetic acid (EDTA; disodium salt three times recrystallized). Incubations, in a Dubnoff metabolic shaker, were at 28° (a few were at 38°). The reaction mixture contained 0·5 ml. of the homogenate, 0·2 ml. of 0·2M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), pH 7·4, 0·1 ml. of 0·25% cytochrome *c*, water to make a final volume of 2 ml. and the substrates and cofactors in the concentrations indicated in the tables. The cofactors were used in excess of the concentrations that gave maximum rates under these conditions, so that they were not rate-limiting. Reaction was stopped at 15 min. by the addition of 2 ml. of ice-cold 10% trichloroacetic acid. 'Total keto acid' was determined colorimetrically (Friedemann & Haugen, 1943), pyruvic acid being used as standard. Utilization rates were calculated from differences in concentrations at zero time and after incubation for 15 min. When α-oxoglutaric acid and oxaloacetate were added together in a reaction mixture the concentrations of each at the end of incubation for 15 min. were determined by a slight modification of the Friedemann & Haugen (1943) procedure, taking advantage of the fact that the 2:4-dinitrophenylhydrazones of oxaloacetate are readily decarboxylated by heating over boiling water to the 2:4-dinitrophenylhydrazones of pyruvate whereas those of α-oxoglutarate are relatively stable to such treatment. After heating, the 'total keto acid' is determined and a portion is extracted with xylol, a solvent which preferentially extracts the 2:4-dinitrophenylhydrazones of pyruvate in a mixture with those of α-oxoglutarate.

Succinoxidase activity was determined manometrically (Tyler, 1955*a*). Cytochrome *c*, adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN) were obtained from Sigma Chemical Co. and methods of preparation of material are described elsewhere (Tyler, 1955*a*).

RESULTS

Effect of succinate on oxaloacetate utilization. The addition of succinate (final concentrations 0·01–0·05M) to a reaction mixture containing 3 mM-oxaloacetate caused a significant decrease in the utilization of the keto acid in all tissues studied, the inhibition increasing with increasing concentrations of succinate (Table 1). This inhibition is a true one, not an apparent one due to the formation of new oxaloacetate during succinate oxidation. Under the conditions of these experiments (but with oxaloacetate omitted), 25 mg. of kidney formed 8, 11 and 12 μg. of keto acid from 0·01, 0·02 and 0·05M-succinate respectively. To correct for this new formation, these values were added to the zero-time values. Such corrections are probably an unnecessary precaution; in the presence of added oxaloacetate, succinate oxidation is markedly decreased and so there should be little, if any, new formation of the keto acid. However, the correction is a small one and has little effect on the results.

To examine the nature of this inhibition further a few experiments were carried out with varying concentrations of oxaloacetate, with and without added 0·02M-succinate. Treatment of these results, according to the method of Wilson (1949), by using the expression

$$\frac{V}{V_1} = 1 + \frac{K_s}{K_i}\left[\frac{(I)}{K_s + (S)}\right]$$

gave a plot with a common intercept and slopes suggestive of a competitive type of inhibition (Fig. 1) (K_i 4·7 mM).

Table 1. *Effect of succinate on oxaloacetate utilization*

Beakers contained the following: EDTA (0·25 mM), tris, pH 7·4 (0·02 M), inorganic orthophosphate (1·5 mM), $MgSO_4$ (1 mM), adenosine triphosphate (1 mM), cytochrome *c* (0·05 mM), oxaloacetate (3 mM). The reaction was started by the addition of 0·5 ml. of a 5% homogenate containing 0·4–0·9 mg. of nitrogen. Oxaloacetate utilization is given as μmoles of the keto acid disappearing in 15 min. at 28° in the presence of 25 mg. (wet wt.) of tissue. Figures in parentheses are percentage changes from control utilization values, without added succinate.

	Concn. of succinate (M)			
Tissue	0	0·01	0·02	0·05
Brain	1·70	1·30 (−24)	0·95 (−44)*	0·55 (−68)*
Liver	2·75	1·20 (−56)*	0·95 (−66)*	0·70 (−73)*
Kidney	3·65	2·40 (−34)*	1·95 (−47)*	1·80 (−51)*
Heart	4·15	2·20 (−47)*	1·90 (−54)*	1·50 (−64)*

* $P < 0·01$.

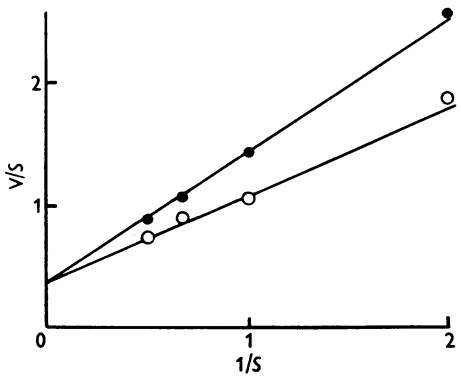

Fig. 1. Inhibition of oxaloacetate utilization by succinate. Kidney homogenate; conditions were as described in Table 1. *S*, Substrate concn. (mM); *V*, rate of disappearance of oxaloacetate (μmoles/15 min.); K_i, 4·7 mM; K_m, 1·86 mM. ○, System without succinate; ●, system with 0·02 M-succinate.

Effect of L-*malate and fumarate on oxaloacetate utilization.* L-Malate and fumarate inhibit oxaloacetate utilization, the former being somewhat more effective than the latter. In both cases the inhibition is greater at 38° than at 28°. Results with L-malate are summarized in Table 2 (the effect of fumarate is similar and is not shown). Corrections were made for possible new formation of oxaloacetate from these substances, as was done for succinate. Increasing the concentration of L-malate increases inhibition but, in contrast with the action of succinate, inhibition is independent of oxaloacetate concentration.

Effect of α-oxoglutarate on oxaloacetate utilization. When these two substances are added together in a reaction mixture the total utilization of keto acid is less than the sum of the individual utilization rates (Table 3). This holds for concentrations of the separate keto acids from 0·5 to 4·0 mM, and the decrease varies from 19 to 37%. Concentrations of ATP and magnesium are not rate-limiting as the decrease occurs not only in the absence of these cofactors but also when they are present in considerable excess (Table 4). The decrease is largest during the first 5 min. of the reaction and then rapidly falls off (Table 5). The concentration of each keto acid at 15 min. (not shown), found by a modification of the Friedemann & Haugen (1943) method, suggests that an approximately equal percentage decrease in the rate of utilization of both occurs. Also, this inter-relationship between two keto acids is not seen if pyruvate is used in a mixture with oxaloacetate or α-oxoglutarate. In such experiments the resulting utilization of 'total keto acid' is equal to the sum of the individual rates or, under certain conditions, is actually greater.

Effect of citric acid-cycle intermediates on succinate oxidation. Of all the cycle intermediates only oxaloacetate has a striking inhibitory action on succinate oxidation (Table 6). Three intermediates: fumarate, L-malate and α-oxoglutarate, which inhibit the rate of oxaloacetate disappearance, also potentiate the capacity of oxaloacetate to inhibit succinate oxidation (Table 6, lines 7–9).

DISCUSSION

Two points of particular interest are the rate-limiting effect of the presence of other citric acid-cycle intermediates on oxaloacetate utilization and the reflexion of such inhibition on the level of succinate oxidation. Differences in oxaloacetate disappearances in homogenates may be due to enzymes other than the condensing enzyme and all of them may be rate-limiting. It also is possible that a single enzyme may be involved in some of the conditions used here. However, in this report we are chiefly concerned with the effect of various additions on the rate of disappearance of oxaloacetate from the reaction mixture, not with the specific reactions or enzyme system(s) that may be involved. The decrease in utilization occurring in a

Table 2. *Effect of* L-*malate on oxaloacetate utilization*

Beakers contained the reaction mixture as given in Table 1. Oxaloacetate and L-malate concentrations were as indicated. Oxaloacetate utilization is expressed as μmoles of the keto acid disappearing in 15 min. at 38° in the presence of 25 mg. (wet wt.) of kidney. Figures in parentheses are the percentage changes from control utilization without added L-malate.

Concn. of L-malate (mM)	Concn. of oxaloacetate (mM)		
	1	5	10
0	1·45	3·45	5·25
1	1·45	3·30 (−4)	4·40 (−15)
5	1·15 (−23)	2·90 (−17)	3·80 (−28)*
10	0·85 (−41)*	1·95 (−40)*	3·00 (−43)*

* $P < 0·01$.

Table 3. *Oxaloacetate and α-oxoglutarate utilization when single and mixed*

Beakers contained the reaction mixture as given in Table 1. Oxaloacetate and α-oxoglutarate concentrations are indicated below. Utilization is given as μg. of keto acid disappearing in 15 min. at 28° in the presence of 25 mg. (wet wt.) of kidney. Line 3 is a theoretical utilization rate obtained by adding the utilization rates in lines 1 and 2.

	Concn. of keto acid (mM)			
	0·5	1·0	2·0	4·0
1. Utilization of oxaloacetate (when present as single substrate)	110	180	290	510
2. Utilization of α-oxoglutarate (when present as single substrate)	70	105	120	125
3. Theoretical utilization (when mixed)	180	295	410	635
4. Actual utilization found when oxaloacetate and α-oxoglutarate are mixed	145	205	280	495
5. $\frac{\text{Line 3} - \text{line 4}}{\text{Line 3}} \times 100$	−19%*	−31%*	−32%*	−22%*

* $P < 0·01$.

Table 4. *Effect of adenosine triphosphate and magnesium on the utilization of oxaloacetate and α-oxoglutarate*

Keto acid concentration in the beaker was 2 mM. Other conditions are as given in Table 3. Utilization is given in μg. of keto acid disappearing in 15 min. in the presence of 25 mg. (wet wt.) of kidney.

	Without additional ATP or Mg^{2+} ions	With 5 mM-ATP and 5 mM-Mg^{2+} ions
1. Utilization of oxaloacetate when present as single substrate	225	295
2. Utilization of α-oxoglutarate when present as single substrate	35	95
3. Theoretical utilization (when mixed)	260	390
4. Utilization found when both keto acids are mixed	170	245
5. Percentage change	−35*	−37*

* $P < 0·01$.

mixture of α-oxoglutarate and oxaloacetate may be due to a limited availability of one or more co-factors common for the utilization of both keto acids. However, a combination of 0·5 mM-oxaloacetate and 0·5 mM-α-oxoglutarate shows a total utilization rate less than that found for oxaloacetate at double the concentration (1 mM), despite a large excess of all known cofactors. Similar effects are found with other concentrations (Table 3). Thus it would appear that the common rate-limiting factor(s) that may exist for these two keto acids is not one of the cofactors added in this experiment.

Large amounts of succinate can inhibit oxaloacetate utilization. This effect also could be mediated through a variety of secondary reactions, all of which may be rate-limiting. The results (Fig. 1) also suggest the possibility that the two substrates may have an affinity for each other's enzymes or for a common enzyme. However, if such a mutual affinity exists oxaloacetate is a much more powerful inhibitor of succinate oxidation (K_i 1·5 μM; Pardee & Potter, 1948) than succinate is of oxaloacetate (K_i 4·7 mM; Fig. 1).

This study is part of an investigation of the possibility that oxaloacetate is an important rate-regulator of oxidative metabolism through the citric acid cycle. Therefore some relation of these results to this hypothesis is indicated. The idea of a regulatory mechanism at the enzyme–substrate level has been considered by others (Rosenthal, 1937; Pardee & Potter, 1948; Davies & Kun, 1957). Although many reactions may be rate-limiting on oxidative metabolism it has been shown that oxaloacetate has a number of characteristics that allow it to play a major role (Tyler, 1957). The inverse relationship between the level of succinate oxidation and the concentration of oxaloacetate is generally represented as a negative 'feed-back'. Fumarate and L-malate check succinic-dehydrogenase activity not only as precursors of oxaloacetate but also by retarding utilization of the keto acid. In view of the well-known negative 'feed-back' between succinic-dehydrogenase activity and oxaloacetate concentration it is somewhat paradoxical to find that succinate, in turn, inhibits the disappearance of oxaloacetate, thus tending to cancel out the negative 'feed-back'. Nevertheless, these seemingly paradoxical effects are better understood if it is borne in mind that a striking characteristic of the enzyme system *in vitro* which oxidizes succinate is its tendency to operate unrestrained by mechanisms which control the use of succinate in the cell (Tyler, 1954). This results in very high activity in homogenates but is apparently restrained in the intact cell by an efficient braking system. Oxaloacetate supplies this restraint very effectively, and any condition which interferes with its production or utilization affects the level of succinoxidase activity accordingly. Thus the results of these and other studies (Tyler, 1955*a*; Wolff & Ball, 1957; Corwin & Schwarz, 1959) indicate that there are finely adjusted mechanisms in the cell geared to the level of oxaloacetate which, in turn, controls succinate oxidation under a variety of circumstances. They also suggest that most substances affecting succinate oxidation (except for frank poisons) probably do so indirectly by acting on oxaloacetate metabolism.

Table 5. *Utilization of oxaloacetate and α-oxoglutarate at 5 min. intervals*

Conditions were as given in Table 3; concentration of keto acids was 1 mM; utilization is given in μg. of keto acid disappearing in the presence of 25 mg. (wet wt.) of kidney.

Time (min.)	Oxalo-acetate	α-Oxo-glutarate	Both keto acids	Change (%)
5	80	30	45	−59
10	50	30	70	−13
15	45	35	60	−25

SUMMARY

1. The rate of oxaloacetate utilization by tissue homogenates is inhibited, under certain conditions, by the presence of succinate, L-malate, fumarate and α-oxoglutarate.

Table 6. *Effect of citric acid-cycle intermediates on succinate oxidation*

Warburg flasks contained: 0·5 ml. of a 5% kidney homogenate in 0·9% NaCl, phosphate (0·01 M), pH 7·4, cytochrome *c* (0·05 mM), succinate (0·02 M). Other substrates were added as indicated. The final reaction mixture volume was 2 ml.; temperature, 38°; gas phase, air. After a preliminary equilibration period of 10 min. oxygen consumption was measured for 15 min. For the controls (line 1) the oxygen consumption (μl./mg. of N) during the first 15 min. was: liver, 60; kidney, 75; heart, 105. These data are expressed as 100 (line 1) and the results of all experimental conditions are calculated relative to these values.

Additions	Relative consumption of O_2		
	Kidney	Liver	Heart
1. None	100	100	100
2. Oxaloacetate (0·02 mM)	55	50	40
3. α-Oxoglutarate (0·5 mM)	90	85	90
4. Fumarate (0·5 mM)	100	100	100
5. Malate (0·5 mM)	115	90	100
6. Citrate (0·5 mM)	100	105	105
7. Oxaloacetate (0·02 mM) + α-oxoglutarate (0·5 mM)	25*	15*	—
8. Oxaloacetate (0·02 mM) + malate (0·5 mM)	30*	20*	—
9. Oxaloacetate (0·02 mM) + fumarate (0·5 mM)	30*	30*	—

* $P < 0·01$ (difference between line 2 and lines 7–9 respectively).

2. The inhibition by the last-named three substances potentiates the inhibition by oxaloacetate of succinate oxidation.

3. The role of oxaloacetate as a rate-regulator of oxidative metabolism through the citric acid cycle is discussed.

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Stimulation of Fatty Acid Synthesis by Oestradiol *in vitro*

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Previous experiments in this Laboratory demonstrated the presence in human placenta of an enzyme system which responds to oestradiol (Villee & Hagerman, 1953). This system, originally thought to be an *iso*citric dehydrogenase which required diphosphopyridine nucleotide as hydrogen acceptor (Villee, 1955), was subsequently found to require minute amounts of triphosphopyridine nucleotide and was shown to be a transhydrogenase (Talalay & Williams-Ashman, 1958; Villee & Hagerman, 1958). The transhydrogenation reaction, TPNH + DPN → DPNH + TPN, can be coupled to glucose 6-phosphate dehydrogenase as well as to *iso*citric dehydrogenase, and presumably can be linked to any dehydrogenase requiring triphosphopyridine nucleotide (Villee & Hagerman, 1958).

Talalay & Williams-Ashman (1958) suggested that this system is mediated by a 17β-oestradiol dehydrogenase with dual pyridine nucleotide specificity which alternately oxidizes and reduces oestradiol:

$$\text{oestradiol} + \text{DPN} \rightleftharpoons \text{oestrone} + \text{DPNH},$$
$$\text{oestrone} + \text{TPNH} \rightleftharpoons \text{oestradiol} + \text{TPN}.$$
$$\text{Sum:} \quad \text{DPN} + \text{TPNH} \rightleftharpoons \text{DPNH} + \text{TPN}.$$

Recent experiments in this Laboratory (Hagerman & Villee, 1959) have shown that the transhydrogenase stimulated by oestrogen and the oestradiol dehydrogenases are separate and distinct enzymes. They differ markedly in their response to thyroxine, to adenosine 2′-monophosphate and to sulphydryl inhibitors; they have different rates of thermal inactivation; and they may be separated physically by electrophoresis on a starch block or on a filter-paper curtain or by adsorption on to and elution from diethylaminoethylcellulose columns. Talalay & Williams-Ashman (1958) had postulated that all steroid hormones produce their effects by way of hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity that rapidly and reversibly oxidize and reduce the steroid. Our recent evidence renders it unlikely that this is the mechanism of action of oestrogens. Other evidence against this theory has been presented by Villee & Spencer (1959) with respect to the mode of action of testosterone and by Bush & Mahesh (1959) with respect to the mode of action of 11β-hydroxysteroids.

The reaction mediated by the transhydrogenase stimulated by oestrogen should be reversible. In experiments reported earlier (Villee & Hagerman, 1958) we were unable to couple the transhydrogenation reaction with a system in which a diphosphopyridine nucleotide-linked dehydrogenase was presented with substrate amounts of triphosphopyridine nucleotide and catalytic amounts of diphosphopyridine nucleotide. However, the trans-

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