146. THE FATE OF OXALOACETATE IN ANIMAL TISSUES

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ACCORDING to the theory of the citric acid cycle the following anaerobic reactions are expected to take place when oxaloacetate is added to pigeon breast muscle [see Krebs, 1940].

- (1) Oxaloacetate + "triose" $\rightarrow$ pyruvate + malate.
- (2) 2 Oxaloacetate + pyruvate $\rightarrow$ citrate + CO_2 + malate.
- (3) Oxaloacetate + citrate $\rightarrow$ α -ketoglutarate + CO_2 + malate.

The net effect of these reactions is:

- (4) 4 Oxaloacetate + triose $\rightarrow$ 3 malate + α -ketoglutarate + 2 CO_2 .

In addition to 1, 2 and 3, occurs the non-enzymic decomposition of oxaloacetate:

- (5) Oxaloacetate $\rightarrow$ pyruvate + CO_2 ,

and this reaction can provide the pyruvate required in reaction 2. If 1 is completely replaced by 5, the net effect of 5, 2 and 3 is

- (6) 4 Oxaloacetate $\rightarrow$ 2 malate + α -ketoglutarate + 3 CO_2 .

In these reactions, malate stands for the system fumarate $\rightleftharpoons l(-)$ malate, "citrate" for the system citrate $\rightleftharpoons$ *cis*-aconitate $\rightleftharpoons$ isocitrate, and " CO_2 " for the system $\text{CO}_2 \rightleftharpoons \text{HCO}_3'$.

The experimental results obtained with pigeon breast muscle, pigeon liver, sheep's heart and guinea-pig kidney, agree well with scheme 6 and this is further evidence in favour of the occurrence of the citric acid cycle in these tissues.

In brain and testis (tissues in which the occurrence of the citric acid cycle has been uncertain) very similar results were obtained. Oxaloacetate forms in these tissues, as in muscle, α -ketoglutarate, as predicted by schemes 4 and 6. The obvious assumption is that the mechanism of the formation of ketoglutarate from oxaloacetate is the same as in muscle, i.e. that reactions 2 and 3 take place in these tissues; and it would then follow that the citric acid cycle also occurs in brain and testis. At the same time it is certain that other reactions take place in these tissues in addition to those formulated in the citric acid cycle and, as long as the nature and the extent of the other reactions are unknown, it seems premature to discuss the importance of the citric acid cycle in brain and in testis.

Methods

The methods used in this work were those described in previous publications [Krebs & Eggleston, 1940; Krebs, 1940; Krebs *et al.* 1940]. The tissue was suspended in phosphate saline and shaken anaerobically at 40° in the presence of oxaloacetate. After varying periods of incubation the amounts of succinate [Krebs, 1937], α -ketoglutarate [Krebs, 1938; 1940], fumarate and *l*(-)-malate [Krebs *et al.* 1940], pyruvate [Westerkamp, 1933], citrate [Pucher *et al.* 1936] and CO_2 [Warburg & Yabusoe, 1924] were measured. Oxaloacetate was also

determined [Ostern, 1933; Edson, 1935] but no oxaloacetate was found in the tissue suspensions after incubation.

Anaerobic conditions were ensured by placing yellow phosphorus in the centre well of the cups. The oxaloacetate was measured into a side arm and added to the tissue suspension after a 10 min. period of shaking at 40°. During this period O₂ was completely removed from the system by the phosphorus.

A complete experiment required at least 10 conical manometer vessels provided with one or two side arms and a centre well; they were filled as follows:

Manometer no. ...	1, 2, 3	4, 5, 6	7, 8	9	10
Side arm 1	0.4 ml. 0.2 <i>M</i> oxaloacetate	—	—	0.2 ml. 0.2 <i>M</i> oxaloacetate	0.2 ml. 0.2 <i>M</i> oxaloacetate
Side arm 2	—	—	0.3 ml. 2 <i>N</i> HCl	0.3 ml. 2 <i>N</i> HCl	—
Centre well	Phosphorus	Phosphorus	Phosphorus	Phosphorus	—
Main compartment	4 ml. tissue suspension	4 ml. tissue suspension	4 ml. tissue suspension	2 ml. tissue suspension	4 ml. 0.5 <i>N</i> HCl
Gas	N ₂	N ₂	N ₂	N ₂	Air

The manometers were rapidly shaken at 40°. After 10 min., during which thermal equilibrium was established, the oxaloacetate was mixed with the contents of the main compartment in cups 1, 2, 3, 9 and 10, and the acid was added from the side arm in 7, but not in 8 and 9. No readings were taken in 1, 2, 3, 4, 5 and 6. These manometers were shaken for 1 hr., after which they were removed from the bath. The contents of 1 were rapidly poured into a 25 ml. measuring cylinder containing 2 ml. 10 % Na tungstate. The cup was rinsed with water (10–15 ml.) and the washings were added to the suspension. Enough H₂SO₄ was added to precipitate the protein (pH 5). The volume was made up to 25 ml. and filtered into another 25 ml. measuring cylinder. Succinic acid was determined in a measured volume of the filtrate. The contents of cup 2 were treated in the same way, but the filtrate was treated in the extractor with KMnO₄, in the presence of MnSO₄ and H₂SO₄, before succinic acid was extracted [see Krebs, 1940]. The result of the succinic determination in this cup represented the sum of the succinate and α -ketoglutarate present. Cup 3, in which fumarate and malate were to be determined, was emptied into a measuring cylinder kept at 40° and the water used for washing was also warmed to 40° in order to avoid a change in fumarate-malate equilibrium. To the combined suspension and washings 5 ml. 5 % metaphosphoric acid were added. Fumarate was then determined [Krebs *et al.* 1940]. Cups 4, 5 and 6 were controls to which no oxaloacetate was added; they were treated in the same way as 1, 2 and 3 respectively.

Readings were taken in 7, 8, 9 and 10, the first being made after 10 min. of thermal equilibration, before the addition of acid (in 7) or of the oxaloacetate (in 9, 10). A large rise in pressure occurred in 9, and it was therefore necessary to set the level of the meniscus in the left manometer limb at a very low level. Half-quantities of suspension and substrate were used in this cup, in order to keep the volume of gas produced within limits which could be measured.

Readings were continued at 10 min. intervals in 7, 8, 9 and 10. 5 min. before the end of the incubation period, the meniscus in 9 was reset at low level, to allow for the determination of the bound CO₂. The very small evolution of CO₂ during these 5 min. was calculated by extrapolation. At the end of the incubation period readings were taken again and the acid was added to the contents of 8 and 9. Readings in these two manometers were continued till the pressure

due to the liberation of the bound CO_2 became constant (10–15 min.). The contents of the cup were then made up to 25 ml. and used for the determination of pyruvate and citrate. 4 ml. of the 25 ml. were used for the determination of pyruvate by the carboxylase method. To adjust the reaction to pH 5.0, 0.095 ml. 3M Na acetate (3 times equivalent to the HCl present) and 0.2 ml. 3M acetate buffer pH 5.0 were added. The remaining solution was deproteinized with trichloroacetic acid and used for the determination of citric acid according to Pucher *et al.* [1936].

The CO_2 liberated in 7 was the bound CO_2 present in the suspension at the beginning of the experimental period. In 8 and 9 the production of free CO_2 during the experimental period, and the bound CO_2 present at the end were measured. In 10 the bound CO_2 present in the oxaloacetate solution was determined. In this cup a very rapid evolution of CO_2 due to bicarbonate was followed by slow rise in pressure due to the decarboxylation of oxaloacetic acid. The amount of bicarbonate was calculated by extrapolation. The calculated quantity and the bicarbonate found in 7 provided the data required for the calculation of the initial bound CO_2 in 9.

When further products were to be determined, e.g. lactate or oxaloacetate, more manometers, filled as 1 and 4, were used. The oxaloacetate solution was prepared immediately before use. The acid was neutralized with the calculated amount of M NaHCO_3 and freed from CO_2 in an evacuated Thunberg tube.

It is essential in these experiments that the metabolic reactions should be stopped rapidly at the end of the experimental period, and especially that the suspension should not come into contact with O_2 when the cups are detached from the manometer. To facilitate rapid handling, manometers 1–6 were placed in the bath at 2 min. intervals. This made it possible to deal with each manometer at once at the end of the incubation period.

EXPERIMENTS ON PIGEON BREAST MUSCLE

Formation of pyruvate from oxaloacetate. The formation of pyruvate from oxaloacetate, being a non-enzymic reaction, is independent of the metabolic activity of the tissue. If the tissue is "dead", owing to heating to 100° or to poisoning, pyruvate is formed quantitatively [see also Breusch, 1939]. If the tissue is metabolically active varying amounts of pyruvate appear depending on the type of tissue, on the proportion of tissue to oxaloacetate and on other experimental circumstances. The quantities of pyruvate formed are generally small, and sometimes even negligible when small "physiological" amounts of oxaloacetate are added. In our experiments it was necessary, however, to add comparatively large amounts in order to obtain effects which could be accurately measured. Therefore it was not possible to avoid completely the undesirable side reaction 5. Data showing the magnitude of the reaction are contained in Tables 2 and 4.

Since this paper is concerned with the *metabolic* reactions of oxaloacetate, a distinction is made between "oxaloacetate decomposed" according to 5 and "oxaloacetate metabolized". The amount of the latter is the difference between oxaloacetate added and pyruvate found. The yields of the metabolic products are calculated as percentage of the "oxaloacetate metabolized".

Formation of carbon dioxide. Large quantities of CO_2 are rapidly formed when oxaloacetate is added to muscle suspensions. In the example shown in Table 1, Q_{CO_2} , calculated for the first 20 min., equals 51. The bulk of the CO_2 cannot be formed by reaction 5 since no equivalent amounts of pyruvate appear. Of the total CO_2 of 1317 $\mu\text{l.}$ only 436 $\mu\text{l.}$ are accounted for by reaction 5 and

Table 1. *CO₂ formation from oxaloacetate in pigeon breast muscle*

300 mg. wet pigeon breast muscle in 4.0 ml. phosphate saline; oxaloacetate 0.02 *M*; "CO₂" includes bicarbonate and free CO₂. Method of Warburg & Yabusoe [1924].

Time after the addition of oxaloacetate min.	Total CO ₂ formed in the presence of oxaloacetate (1792 μ l.) μ l.	Blank CO ₂ μ l.	Pyruvate formed from oxaloacetate μ l.
5	320	6	—
10	588	8	—
20	1013	12	490
30	1224	13	—
40	1299	14	—
50	1321	14	—
60	1337	14	436

14 μ l. by the blank. The amount of CO₂ due to metabolic reactions of oxaloacetate is thus 867 μ l. or 65% of the oxaloacetate metabolized. Further data are given in Table 2.

Table 2. *Fate of oxaloacetate in pigeon breast muscle under anaerobic conditions*

The data refer to 4 ml. muscle suspension. Period of incubation 1 hr. The concentration of the suspension was in Exp. 1: 1 part muscle, 14 parts medium; in Exp. 2: 1 part muscle, 10 parts medium; in Exp. 3: 1 part muscle, 9 parts medium. Quantities in μ l.

	Exp. 1		Exp. 2		Exp. 3	
	Control	Exp.	Control	Exp.	Control	Exp.
Observed:						
Oxaloacetate added	0	1792	0	1792	0	1344
Pyruvate found	0	436	0	260	0	582
CO ₂	58	1327	129	1629	57	978
Succinate	46	56	0	220	52	109
α -Ketoglutarate	8	344	44	90	147	299
Fumarate + malate	37	1032	0	780	0	408
Citrate	6	22	0	32	—	—
Calculated:						
Oxaloacetate metabolized	1356		1532		762	
Yield of succinate	0.8%		14.3%		7.5%	
Yield of α -ketoglutarate	25%		5.9%		20.0%	
Yield of fumarate + malate	73%		51%		53.6%	
Yield of citrate	1.2%		2.1%		—	
Yield of CO ₂	61.5%		81%		45.5%	
Recovery:						
Oxaloacetate metabolized, in μ l. C	5440		6128		3048	
Sum of the products re- covered, in μ l. C	6449		6390		2948	
% recovery	120		104		97	

Balance sheet of oxaloacetate metabolism. Typical experiments in which the various metabolic products of oxaloacetate were determined, as described in the section "methods", are recorded in Table 2. The figure for CO₂ includes the free CO₂ formed during the actual experiment and the CO₂ liberated from bicarbonate after acidification, but the bicarbonate present in the oxaloacetate solution has been deducted. The figure for citrate includes isocitrate and cis-aconitate [see Krebs, 1940]. The lower part of the table contains the yields of the

metabolites, as percentage of the oxaloacetate metabolized. The figures were calculated as follows:

$$\text{yield} = \frac{\text{metabolite found with oxaloacetate} - \text{metabolite found in control}}{\text{oxaloacetate metabolized}} \times 100.$$

A special correction was necessary in the case of CO_2 to allow for the CO_2 formation by the non-metabolic reaction 5. The CO_2 formed in this way was taken to be equivalent to the pyruvate formed and the metabolic CO_2 was therefore calculated by deducting the pyruvate found from the total CO_2 found.

To calculate the total recovery of the oxaloacetate metabolized the quantities of all the substances were expressed in terms of " $\mu\text{l. carbon}$ ". The figures were obtained by multiplying " $\mu\text{l. substance}$ " with the number of C-atoms in the molecule (Table 2).

The table shows the variations which occur in the type of experiment. The yield of α -ketoglutarate was usually near 25%. When a low yield was obtained, as in Exp. 2, a higher proportion of succinate and CO_2 was found. This suggests that reaction 6 was followed in this experiment by the conversion of α -ketoglutarate into succinate and CO_2 (see below). The yield of "malate" was usually near 50%, as expected according to 6; it was higher, up to 75%, in other experiments (e.g. Exp. 1). Such a yield is in accordance with 4. The yield of citrate is low (1–2%) because citrate formed reacts under the experimental conditions to form α -ketoglutarate.

The total recovery of the oxaloacetate metabolized was usually near 100%, within the limits of error. In Exp. 1 the recovery was 120% and this is to be expected if reaction 1 takes place and if scheme 4 holds. In this case 1 triose equivalent enters the reaction in addition to the 4 mol. of oxaloacetate and the total yield of carbon expressed in terms of oxaloacetate metabolized should be 119%.

The data thus agree, on the whole, with the schemes 4 and 6. It is probable that 4 holds when the tissue contains much of the reactive form of "triose", and 6 when "triose" is absent. Considerable variations in the concentrations of carbohydrates and their split products are very likely to be encountered in muscle suspensions.

Previous investigators [Breusch, 1939; Thomas, 1939] found roughly the same quantities of "malate", citrate and pyruvate. Their balance sheets were however incomplete since the formation of α -ketoglutarate, succinate and CO_2 was not measured.

Origin of succinate. In order to examine the origin of the succinate in the above experiment α -ketoglutarate and fumarate were added to muscle suspensions under the same conditions and succinate was determined after anaerobic incubation. Both substances form succinate [see also Weil-Malherbe, 1937] and these results do not therefore establish the source of succinate.

Table 3. *Formation of succinate from α -ketoglutarate and fumarate in pigeon breast muscle*

0.3 g. muscle, 2.7 ml. phosphate saline, pH 7.4; 40°; 1 hr.

Substrate added (final conc.)	$\mu\text{l. succinate found}$
—	12.6
0.01 M α -ketoglutarate	34.2
0.02 M α -ketoglutarate	67.8
0.01 M fumarate	40.8
0.02 M fumarate	115

EXPERIMENTS ON HEART, KIDNEY, TESTIS AND BRAIN

In tissues in which the citric acid cycle occurs, citrate or α -ketoglutarate should appear among the end-products when oxaloacetate is anaerobically metabolized. The examination of the products of the anaerobic metabolism of oxaloacetate therefore offers an approach to the problem as to whether or not the citric acid cycle may occur in any given tissue. With this in view we have examined the anaerobic fate of oxaloacetate in various organs. Typical experiments are recorded in Table 4. The main results are very similar to those

Table 4. *Fate of oxaloacetate in various tissues under anaerobic conditions*

Minced tissue suspended in phosphate saline. Period of incubation 60 min. Quantities in μ l.

Tissue	...	Sheep testis	Guinea-pig kidney	Sheep heart	Sheep brain
Conc. of suspension		1 part tissue + 2 parts medium	1 part tissue + 9 parts medium	1 part tissue + 9 parts medium	1 part tissue + 6.5 parts medium
Quantity of suspension		5 ml.	4 ml.	4 ml.	4 ml.
		Con- trol	Oxalo- acetate	Con- trol	Oxalo- acetate
Observed:					
Oxaloacetate added		0 2240	0 1792	0 1792	0 1792
Pyruvate found		0 1308	0 400	0 290	0 1120*
CO ₂		—	—	160 1478	—
Succinate		15 18	54 12	16 42	0
α -Ketoglutarate		206 384	72 284	94 308	44.5 148.5
Fumarate + malate		122 455	110 1210	96 1048	0 213
Citrate		22 134	10 64	0 58	0 19.5
Lactate		257 349	—	—	112 190
Calculated:					
Oxaloacetate metabolized		932	1392	1502	672
Yield of succinate		0.3%	3.0%	1.7%	0
Yield of α -ketoglutarate		19.1%	15.2%	14.2%	15.5%
Yield of fumarate + malate		35.7%	79.0%	63.4%	31.6%
Yield of citrate		12.0%	3.9%	5.8%	2.9%
Yield of lactate		9.9%	—	—	11.6%
Yield of CO ₂		—	—	68.4%	—

obtained with pigeon breast muscle. All the tissues examined form α -ketoglutarate in amounts of the expected order of magnitude. In brain and testis the decomposition of oxaloacetate into pyruvate is greater than in the other tissues. This may be due to the fact that the rates of metabolism are lower in these tissues.

The formation of α -ketoglutarate was to be expected in heart and kidney, since it was already known that the citric acid cycle occurs in these tissues [Breusch, 1939; Smyth, 1940]. For brain and testis the result is somewhat unexpected since several observations seemed to suggest that here the citric acid cycle does not occur [see Banga *et al.* 1939]. Although the formation of α -ketoglutarate from oxaloacetate cannot be regarded as final proof of the occurrence of the citric acid cycle (there might be an alternative pathway from oxaloacetate to α -ketoglutarate) it becomes highly probable that the citric acid cycle, or at least some of its component reactions, plays a part in the metabolism of brain and testis.

The data published by Evans [1940] show that oxaloacetate reacts in pigeon liver in the same way as in pigeon breast muscle.

SUMMARY

In pigeon breast muscle, sheep heart and guinea-pig kidney, added oxaloacetate yields CO_2 , fumarate, malate, α -ketoglutarate, citrate, succinate and pyruvate (when the conditions are anaerobic). These substances account for all the added oxaloacetate. The quantities formed are in agreement with the citric acid cycle theory.

Sheep testis and sheep brain also form citrate and α -ketoglutarate from oxaloacetate and this in our opinion suggests that the citric acid cycle, or at least some of its component reactions, can occur in these tissues. It is pointed out, however, that it is not yet possible to assess the quantitative significance of these reactions in the normal metabolism of brain and testis.

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