56. THE CITRIC ACID CYCLE

(A reply to the criticisms of F. L. BREUSCH and of J. THOMAS)

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ALTHOUGH, in the view of the author, the preceding paper [Krebs & Eggleston, 1940] removes any doubt about the occurrence and significance of the citric acid cycle in pigeon breast muscle, it was thought desirable to clarify the situation, by discussing in full some of the criticisms of the theory which Breusch [1937; 1939] and Thomas [1939] have expressed in recent papers. Both authors admit the occurrence of the citric acid cycle, but believe it to be an unimportant side reaction. Breusch argued that citrate was formed only in experiments in which the concentration of oxaloacetate was unphysiologically high and that the citrate was not formed from oxaloacetate, but from some undefined impurity. The latter argument was based on the fact that the yield of citrate in his experiments never exceeded 2% of the oxaloacetate added. Thomas [1939] observed that under certain conditions 100 mg. of added oxaloacetate yielded 16–22 mg. malate and only 1·3–1·8 mg. citrate. Breusch [1939] published at the same time somewhat similar data. Both authors concluded that the formation of citrate was of no importance in the oxidation of carbohydrate, and that the catalytic function of the C_4-dicarboxylic acids was fully explained by the theory of Szent-Györgyi. This theory, it may be recalled, states, that the catalytic functions of the C_4-dicarboxylic acids is completely described by the reaction scheme

$$\text{fumarate} \underset{+2H}{\overset{-2H}{\rightleftarrows}} \text{oxaloacetate}.$$

In contrast, the theory of the citric acid cycle states that oxaloacetate further reacts in another way as follows:

oxaloacetate + pyruvate → citrate → α-ketoglutarate → succinate → fumarate.

The following comments are made in reply to the criticisms of Breusch and of Thomas.

I. *The crucial experiment*

Both authors disregard one of the most important experimental observations in the field, viz. the oxidative formation of succinate from oxaloacetate in the presence of malonate. Since this fact was first shown [Krebs & Johnson, 1937], we have found conditions in which the aerobic formation of succinate can be demonstrated in the most striking manner. When low concentrations of oxaloacetate are used, the inhibition of the anaerobic reduction of oxaloacetate to succinate is almost complete. As shown in Table 1 only 39 μl. succinate are formed from oxaloacetate by anaerobic reduction but 6·5 times this amount (254 μl.) is formed in the presence of O_2.

Table 1. *Formation of succinate from oxaloacetate and fumarate in the presence of malonate*

Each vessel had 4 ml. muscle suspension, containing 267 mg. fresh muscle in phosphate saline; malonate added 10 min. before the addition of the other substrates; malonate concentration in all vessels 0·025 *M*; 40°; 70 min.; for further details see the preceding paper; succinate was determined as described in § XIX, 6.

Substrate added... (final conc.)	(1) 0·02 *M* pyruvate 0·005 *M* fumarate	(2) 0·02 *M* pyruvate 0·005 *M* oxaloacetate	(3) 0·02 *M* pyruvate 0·005 *M* fumarate	(4) 0·02 *M* pyruvate 0·005 *M* oxaloacetate
Gas	O_2	O_2	N_2	N_2
µl. O_2 absorbed	884	595	0	0
µl. succinate formed	*306*	*254*	*43*	*39*

Succinate thus arises in large amounts from oxaloacetate, although the direct reduction of oxaloacetate is virtually blocked. The same applies to succinate formation from fumarate (Table 1).

This is the crucial experiment because it remains unexplained by Szent-Györgyi's theory and calls unequivocally for a revision of this theory. It postulates an oxidative reaction leading from oxaloacetate to succinate.

II. *Synthesis of citrate*

The citric acid cycle (as yet the only theory which offers an explanation for the aerobic formation of succinate from fumarate and oxaloacetate) was supported by the following observations:

(1) Citrate can be formed in muscle.

(2) Citrate can be oxidized to succinate in muscle.

(3) The rates of these reactions are of the expected order of magnitude.

As was already mentioned, these results have been questioned by Breusch [1937; 1939] and by Thomas [1939] who advanced two arguments against the experiments on the synthesis of citrate. The first is that synthesis of citrate occurred only at "unphysiologically" high concentrations of oxaloacetate; the second, that the yields of citrate did not exceed 2% of the oxaloacetate added.

In reply it may be pointed out (1) that citrate is also formed when fumarate is added, i.e. when oxaloacetate gradually arises during the experiment in "physiological" concentrations, (2) that the yields of citrate under these conditions reach 15% (see the preceding paper). That the yields are not greater is due to the fact that the methods of separating the synthesis of citrate from its oxidation are still most imperfect. The rate and degree of *accumulation* of citrate are therefore only minimum values for the actual *formation* of citrate.

One of the reasons for the incomplete yields of citrate is an anaerobic reaction between oxaloacetate and citrate. Citrate disappears when oxaloacetate is added, as shown in Table 2. The conditions favouring the synthesis of citrate also accelerate its removal.

Table 2. *Anaerobic removal of citrate by oxaloacetate*

4 ml. muscle suspension containing 0·4 g. pigeon breast muscle in 4 ml. phosphate saline in each cup; N_2; 40°; procedure as in the preceding paper.

	(1)	(2)	(3)
µl. citrate added	448	448	448
µl. oxaloacetate added	448	896	—
µl. citrate recovered after 60 min. incubation	216	189	415
µl. change in citrate	*−234*	*−261*	*−33*

The reaction between oxaloacetate and citrate suggests that the Szent-Györgyi system acts as a hydrogen carrier in the oxidation of citrate (or *iso*-citrate), a point at present under investigation.

Oxidation of citrate

In his recent paper Breusch [1939, p. 1769] makes the statement that pigeon breast muscle "has only slight ability to break down citric acid" and he concludes that it cannot act as an intermediate metabolite. This statement is contradictory to the findings of numerous previous investigators [Thunberg, 1910; Batelli & Stern, 1911] including Breusch himself [1937] and Thomas [1939]. To refute Breusch's recent view, it will suffice to quote Breusch's own previous observation. He found [1937, p. 277, Table 17] that 20 ml. of muscle suspension (containing 2 g. of wet muscle) removed 20 mg. citrate in 60 min. This quantity requires 10,600 μl. O_2 for complete oxidation. Breusch found the actual uptake per hr. of 20 ml. suspension to be only about 5000 μl. O_2 (p. 270, Table 8, column 3). Thus the rate of citrate disappearance can easily account for the total respiration of the muscle.

It is obvious, because of the cyclic nature of the metabolic processes, that added citrate will not necessarily disappear from the muscle under all circumstances. In the normally respiring muscle the rates of the formation and of the oxidation of citrate, and of all the other members of the cycle, are approximately equal. Only under special conditions, which upset the physiological balance, can the ability of the tissue to oxidize citrate be demonstrated, e.g. when poisons (As_2O_3, malonate) or a large excess of citrate (0·05 M) have been added.

Miscellaneous

Several of the criticisms arise solely from a misinterpretation of the theory. Thomas as well as Breusch draw incorrect conclusions from the theory and argue against the theory when these conclusions are not confirmed by their experiments. This applies, for instance, to Thomas's statement that the formation of malate from oxaloacetate must be inhibited by malonate if the citric acid cycle is correct. In actual fact it was expressly stated that the reactions succinate $\rightleftharpoons$ fumarate $\rightleftharpoons$ malate $\rightleftharpoons$ oxaloacetate are reversible, and that oxaloacetate can be converted into the other C_4 dicarboxylic acids in two ways, either anaerobically, or aerobically, the first being unaffected by malonate. Thomas omitted the reversibility symbol ($\rightleftharpoons$) in his version of the theory (p. 232) and hence reaches incorrect conclusions. Breusch argues that fumarate must yield citrate, because fumarate yields oxaloacetate. But the theory only postulates the intermediate formation, and not the accumulation of citrate. The accumulation can only be expected under special conditions, such as those defined in the preceding paper.

Finally it may be pointed out that the O_2 uptake in many of Breusch's and of Thomas's experiments is only 20–50 % of the average figures in our experiments. This applies to the data given in Table 4 of Thomas's paper and Table 2 in Breusch's paper [1937]. The low values indicate that the experiments were carried out with tissue which had already lost the greater part of its metabolic activity; thus it is not surprising that the results were unsatisfactory. Not only did Thomas find no catalytic effect of citrate, but he was also unable to reproduce the catalytic effects of fumarate and succinate [Thomas, 1939, Table 4] demonstrated by Stare & Baumann [1936].

SUMMARY

The criticisms put forward by Breusch and by Thomas against the theory of the citric acid cycle are discussed.

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