

The role of citric acid in intermediate metabolism in animal tissues

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During the last decade much progress has been made in the analysis of the anaerobic fermentation of carbohydrate, but very little is so far known about the intermediate stages of the oxidative breakdown of carbohydrate. A number of reactions are known in which derivatives of carbohydrate take part and which are probably steps in the breakdown of carbohydrate; we know furthermore, from the work of SZENT-GYÖRGYI²⁰⁾ that succinic acid, fumaric acid and oxaloacetic acid play some role in the oxidation of carbohydrate, but the details of this role are still obscure.

In the present paper experiments are reported which throw new light on the problem of the intermediate stages of oxidation of carbohydrate; in conjunction with the work of SZENT-GYÖRGYI²⁰⁾, STARE and BAUMANN¹⁹⁾ and MARTIUS and KNOOP^{13, 14)} the new experiments allow us to outline the principal steps of the oxidation of sugar in animal tissues.

I. Methods.

1. Tissue. Pigeon breast muscle was used for the majority of the experiments described in this paper. The tissue was minced in a LATAPIE mincer immediately after killing and usually suspended in 3—7 volumes of 0.1 M sodium phosphate buffer (ph = 7.4). On further dilution of the suspension the rate of metabolism decreased.

2. Quantitative determination of citric acid. The method of PUCHER, SHERMAN and VICKERY¹⁷⁾ was used; citric acid is oxidised to pentabromoacetone which is subsequently converted by means of sodium sulphide to a coloured material suitable for colorimetric determination. As pointed out by PUCHER c.s. the method is suitable for the determination of quantities between 0.1 and 1 mg. It is fairly specific; there are only a few other substances which yield also a yellow coloured material, and the only substance which interfered in our experiments was oxaloacetic acid. In pure solution 0.5 millimol oxaloacetate yielded $1.98 \cdot 10^{-3}$ millimol „citric acid”. The yield of „citric acid” is increased by 50 % if pyruvic acid is present at the same time and this suggests that bromine, like hydrogen peroxide, (MARTIUS and KNOOP¹³⁾) brings about a synthesis of citric acid from oxaloacetic and pyruvic acid. This interfering reaction can be removed if oxaloacetic acid is decomposed by heating the solution for one hour in neutral or weakly acid medium after deproteinisation. Although oxaloacetate is not completely decomposed if heated in pure solution the destruction is practically complete in the deproteinised tissue extract. This effect may be explained by the observations of POLLAK¹⁶⁾ and LJUNGGREN¹⁰⁾ which demonstrate a catalytic influence of amino compounds on the decompositions of β -ketonic acids.

3. Quantitative determination of succinic acid. The method used was a modification of SZENT-GYÖRGYI'S²⁰⁾ manometric method. The details will be described in full elsewhere.

4. Quantitative determination of α -ketoglutaric acid. The α -ketoglutaric acid was quantitatively determined by estimation of the amount of succinic acid formed on oxidation. In an aliquot sample the preformed succinic acid is determined and the figure obtained is subtracted from the sample treated with the oxidising agent. Suitable oxidising agents are cold permanganate or ceric sulphate in acid medium. Under our conditions both reagents gave identical results. The ceric sulphate method is better if significant amounts of α -hydroxyglutaric acid or glutamic acid are present, since these two substances react more readily with permanganate than with ceric sulphate to yield succinic acid. Citric acid, iso-citric acid and cis-aconitic acid do not yield succinic acid if treated with permanganate or ceric sulphate.

5. Quantitative determination of succinic acid in the presence of malonic and α -ketoglutaric acid. Malonic acid interferes with the manometric determination of succinic acid and has therefore to be removed first. The removal is brought about by a principle suggested by

WEIL-MALHERBE 22). To the neutral solution containing the three substances are added 1 ccm 2 M sodium sulphite and 2 ccm 3 M tartaric acid and the solution is then extracted continuously with ether in a KUTSCHER-STEUEDEL 9) extractor. Under our conditions extraction of succinic acid was complete in 30 minutes, whilst α -ketoglutaric acid remains quantitatively in the aqueous phase. The ethereal extract which also contains malonic acid is freed from ether, dissolved in water and treated with permanganate in acid solution in order to destroy the malonic acid and is then extracted with ether again. This second ethereal extract contains the succinic acid ready for the manometric determination.

6. Metabolic Quotients. According to the usual convention the quantities of metabolites are expressed in μ l even if they are not gases; 1 millimol citric acid, for instance, is considered equivalent to 22400 μ l. The rate of metabolic reaction is expressed by the quotient $\frac{\text{substrate metabolised}}{\text{mg dry tissue} \times \text{hour}}$. In the case of muscle the dry weight was considered to be 20 % of the wet weight.

II. Catalytic effect of citrate on respiration.

If muscle tissue is minced and suspended in 6 volumes of phosphate buffer a high rate of respiration is observed initially, but after 20–40 minutes the rate begins to fall off. If citrate is added the rate of respiration is often increased and the falling off of respiration is always much retarded. This effect is brought about by small quantities of citrate, and comparing the extra respiration with the citrate added we find that the extra oxygen uptake is by far greater than can be accounted for by the complete oxidation of citrate. An example is the following experiment:

TABLE I.
Effect of citrate on respiration of minced pigeon breast muscle.
(Manometric experiment)

Time (min.)	μ l O ₂ absorbed by 460 mg muscle (wet weight) suspended in 3 ccm phosphate saline	
	No substrate added	0,15 ccm 0,02 M sodium citrate added
30	645	682
60	1055	1520
90	1132	1938
150	1187	2080

In this experiment the citrate caused an increased respiration of 898 μ l, whilst 302 μ l O₂ are calculated for the complete oxidation of the citrate added.

The magnitude of the effect of citrate shows considerable variations from experiment to experiment; the effect appears to be dependent on the amounts of citrate and other substrates preformed in the tissue. The effect is more pronounced if glycogen, or hexosediphosphate, or α -glycerophosphate are added to the muscle, and we presume therefore that the substrate the oxidation of which is catalysed by citrate, is a carbohydrate or a related substance.

An example in which citrate promotes catalytically the oxidation of α -glycerophosphate is given in Table II. There is only a small effect of citrate in this experiment if added alone to the suspension, but a very considerable effect is observed if α -glycerophosphate is present.

SZENT-GYÖRGYI 20) and STARE and BAUMANN 19) have shown that fumarate, oxaloacetate, and succinate have a similar catalytic effect under the same experimental conditions, a fact of great importance to which we shall refer later.

The problem of the mechanism of this citrate catalysis can be approached in various ways.

TABLE II.

Effect of citrate on the respiration of pigeon breast muscle in the presence of α -glycerophosphate.

(40°; 140 min.; 460 mg muscle (wet weight) in 3 ccm phosphate saline per flask; manometric experiment)

Substrates added	$\mu\text{l O}_2$ absorbed
—	342
0,15 ccm 0,02 M citrate	431
0,3 ccm 0,2 M glycerophosphate	757
0,3 ccm 0,2 M glycerophosphate + 0,15 ccm 0,02 M citrate	1385

We have chosen the investigation of the intermediate stages of the breakdown of citrate in the tissues. If all the stages are known the mechanism of the catalytic effect will be clear.

III. Rate of disappearance of citric acid in muscle.

Since citric acid reacts catalytically in the tissue it is probable that it is removed by a primary reaction but regenerated by a subsequent reaction. In the balance sheet no citrate disappears and no intermediate products accumulate. The first object of the study of intermediates is therefore to find conditions under which citrate disappears in the balance sheet. We find that some poisons bring about this effect, for instance arsenite (Table III) or malonate. If one of these two substances is present, very large amounts of citric acid disappear provided that oxygen is available. Obviously the poisons leave the breakdown of citric acid unaffected whilst they check the synthesis of citric acid.

TABLE III.

Disappearance of citric acid in pigeon breast muscle in the presence of arsenite ($3 \cdot 10^{-3}$ mol.).

(3 ccm muscle suspension containing 750 mg wet muscle were shaken for 40 min. at 40°)

μl citrate added	μl citrate found after 40 min.	μl citrate used	Q_{citrate}
1120	30	1090	—10,9
2240	972	1268	—12,7
4480	2790	1690	—16,9

IV. Conversion of citric acid into α -ketoglutaric acid.

The oxidation of citric acid in the presence of arsenite or malonate is not complete. Only one or two molecules of oxygen are absorbed for each molecule of citric acid removed and the solution must therefore contain intermediate products of oxidation of citric acid.

Although it has long been known that citric acid is readily metabolised (see ÖSTBERG 15), (SHERMAN c.s. 18)), the pathway of the breakdown remained obscure until early 1937, when

MARTIUS and KNOOP^{13, 14}) working with citric dehydrogenase from liver discovered that the oxidation of citric acid by methylene blue yields α -ketoglutaric acid. We are able to confirm MARTIUS and KNOOP's results with other tissues and with molecular oxygen as the oxidising agent.

In previous work it had been shown that arsenite is a specific inhibitor for the oxidation of α -ketonic acid in animal tissues. It had been possible, for example, to stop the oxidation of glutamic acid⁶) and of proline²³) at the stage of α -ketoglutaric acid. We find now that large quantities of α -ketoglutaric acid are present in those suspensions in which citric acid was oxidised in the presence of arsenite.

For example: 46 grammes (wet weight) of minced muscle was suspended in 145 ccm phosphate buffer and shaken in an atmosphere of oxygen for one hour in three large flasks of the shape described previously⁽⁵⁾ with 11,5 ccm 1 M citrate and 6 ccm 0,1 M arsenite. After one hour 40 ccm of trichloroacetic acid (30 %) was added and 190 ccm filtrate was treated with 1 gramme 2,4 dinitrophenylhydrazine dissolved in 100 ccm 2 N HCl. A precipitate was formed immediately. It was collected after two hours on a sintered glass filter, and thoroughly washed with 0,1 N hydrochloric acid and water. The precipitate weighed 1,199 grammes and proved to be practically pure 2,4-dinitrophenylhydrazone of α -ketoglutaric acid (M.P. 217° C). On recrystallisation from aqueous alcohol a substance was obtained which gave the correct melting and mixed melting points (222° C). The calculated total yield of dinitrophenylhydrazone is $1,199 \frac{249}{190} = 1,57$ grammes, or 4,82 millimol.

The quantitative analysis of the deproteinised filtrate with the manometric method gave no succinic acid, but 0,0612 millimol α -ketoglutaric acid per 3 ccm filtrate or 5,07 millimol in the total volume. Both methods, isolation and manometric method, thus agree very well, the former as expected giving a slightly lower figure.

The determination of citric acid in another aliquot of the filtrate showed that 4,64 mg of citric acid were left per ccm liquid, or 6,02 millimol in the total volume. The amount of citric acid added was 11,5 millimol so that the amount metabolised was 5,48 millimol. The yield of α -ketoglutaric acid as will be seen from Table 4 is thus as complete as could be expected in the circumstances.

TABLE IV.

Citric acid metabolised	5,48 millimol	
α -Ketoglutaric acid formed (manometrically)	5,07	„ (yield 93 %)
α -Ketoglutaric acid formed (as hydrazone) .	4,82	„ (yield 88 %)

V. Conversion of citric acid into succinic acid.

In the presence of malonate the oxidation of citrate is checked at the stage of succinic acid as shown by the following experiment: 7,5 grammes (wet weight) minced pigeon muscle were suspended in 22,5 ccm phosphate buffer (0,1 M; pH = 7,4) and 3 ccm 0,2 M sodium citrate and 1 ccm 1 M malonate were added. The suspension was shaken for 40 min. in an atmosphere of oxygen, and then deproteinised by adding 34 ccm water, 2 ccm 50 % sulphuric acid and 2 ccm 15 % sodium tungstate. In the filtrate succinic and α -ketoglutaric acids were determined manometrically. 3 ccm contained 472 μ l succinic acid and 80 μ l α -ketoglutaric acid; α -ketoglutaric acid was also identified by the isolation of the 2,4-dinitrophenylhydrazone.

VI. Synthesis of citric acid in the presence of oxaloacetic acid.

The new results of the citric acid breakdown, in conjunction with previous work on the oxidation of succinic acid in tissues may be summarised by the following series: citric acid $\rightarrow$ α -ketoglutaric acid $\rightarrow$ succinic acid $\rightarrow$ fumaric acid $\rightarrow$ *l*-malic acid $\rightarrow$ oxaloacetic acid $\rightarrow$ pyruvic acid.

If it is true that the oxidation of citric acid is a stage in the catalytic action of citric acid then it follows that citric acid must be regenerated eventually from one of the products of oxidation. We are thus led to examine whether citric acid can be resynthesised from any of the intermediates of the citric acid breakdown.

Systematic experiments show that indeed large quantities of citric acid are formed if oxaloacetic acid is added to muscle anaerobically, whilst all the other intermediates, including pyruvic acid yield no citric acid under the same conditions. It is because the synthesis of citric acid from oxaloacetic acid does not require molecular oxygen and because citric acid is stable in the tissue anaerobically that it is possible to demonstrate the synthesis of citric acid in a simple experiment.

Minced pigeon breast muscle was suspended as usual in 8 volumes phosphate buffer and 3 ccm suspension were measured into a conical manometric flask the sidearm of which contained 0.3 ccm 1 M oxaloacetate. In the centre chamber a stick of yellow phosphorus was placed and the gas space was filled with nitrogen. After the removal of oxygen the oxaloacetate was added to the tissue and the flask was shaken in the water bath for 20 mins. During this period about $1000 \mu\text{l CO}_2$ were evolved. After the incubation, the suspension was quantitatively transferred into 25 ccm 6 % trichloroacetic acid and the volume was made up to 50 ccm. Citric acid was determined in the filtrate and 0.0131 millimol ($293 \mu\text{l}$) citric acid were found. Qcitrate is thus $\frac{293 \times 3}{150} = 5.86$. No citrate was present in the controls.

This experiment shows that muscle is capable of forming large quantities of citric acid if oxaloacetic acid is present and the question arises from which substance the two additional carbon atoms of the citric acid molecule are derived. Addition of various possible precursors such as acetate, or pyruvate, or of α -glycerophosphate had no effect on the rate of citric acid synthesis, but this negative result is no proof against the participation in the synthesis of one of these substances. Pyruvic acid and acetic acid arise rapidly from oxaloacetic acid and it may be that the tissue is already saturated with these substances if oxaloacetic acid alone has been added.

The fact that the catalytic effect of citrate is more pronounced if glycogen, or hexose-monophosphate, or α -glycerophosphate are present suggests that the substance condensing with oxaloacetate is derived from carbohydrate. We may term it provisionally as „triose“, leaving it open whether triose reacts as such or as a derivative for example as a phosphate ester, or pyruvic acid or acetic acid.

A synthesis of citric acid from a C_4 -dicarboxylic acid and a second substance has often been discussed, especially with reference to the citric acid fermentation of moulds (see (1, 24)), though it has not been shown before to occur in animal tissues.

MARTIUS and KNOOP¹²⁾ showed recently that citric acid is formed in vitro if oxaloacetate and pyruvate are treated with hydrogen peroxide in alkaline medium. This model reaction is an interesting analogy and it suggests that the synthesis of citric acid may be a comparatively simple reaction *).

VII. Role of citric acid in the intermediate metabolism.

1. **Citric acid cycle.** The relevant facts concerning the intermediate metabolism of citric acid may now be summarised as follows:

1. Citrate promotes catalytically the oxidations in muscle tissue, especially if carbohydrates have been added to the tissue.

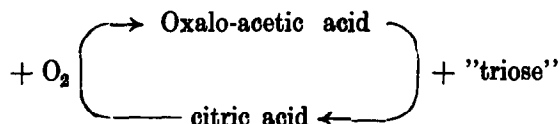
2. Similar catalytic effects are shown by succinate, fumarate, malate, oxaloacetate (SZENT-GYÖRGYI²⁰⁾, STARE and BAUMANN¹⁹⁾).

3. The oxidation of citrate in muscle passes through the following stages: citric acid $\rightarrow$ α -ketoglutaric acid $\rightarrow$ succinic acid $\rightarrow$ fumaric acid $\rightarrow$ *l*-malic acid $\rightarrow$ oxaloacetic acid.

4. Oxaloacetic acid reacts with an unknown substance to form citric acid.

*) See also the work of CLAISEN and HORI³⁾.

These facts suggest that citric acid acts as a catalyst in the oxidation of carbohydrate in the following manner:



According to this scheme oxaloacetic acid condenses with „triose” to form citric acid, and by oxidation of citric acid oxaloacetic acid is regenerated. The net effect of the „citric acid cycle” is the complete oxidation of „triose”.

The synthesis of citric acid from oxaloacetic acid as well as the oxidation of citric acid to oxaloacetic has been experimentally verified. The only hypothetical point in the scheme is the term „triose”, though we may consider it as certain that the substance condensing with oxaloacetic acid is related to carbohydrate.

The proposed scheme outlines a pathway for the oxidation of carbohydrate. Many details must necessarily be left open at the present time, but a few points will be discussed in the following sections.

2. Origin of the C₄-dicarboxylic acid. According to the scheme succinic acid or a related compound is necessary as „carrier” for the oxidation of carbohydrate and the question of the origin of succinic acid arises. We have shown previously (8) that succinic acid can be synthesised by animal tissues in small amounts if pyruvic acid is available. The physiological significance of the synthesis is now clear: it provides the carrier required for the oxidation of carbohydrate.

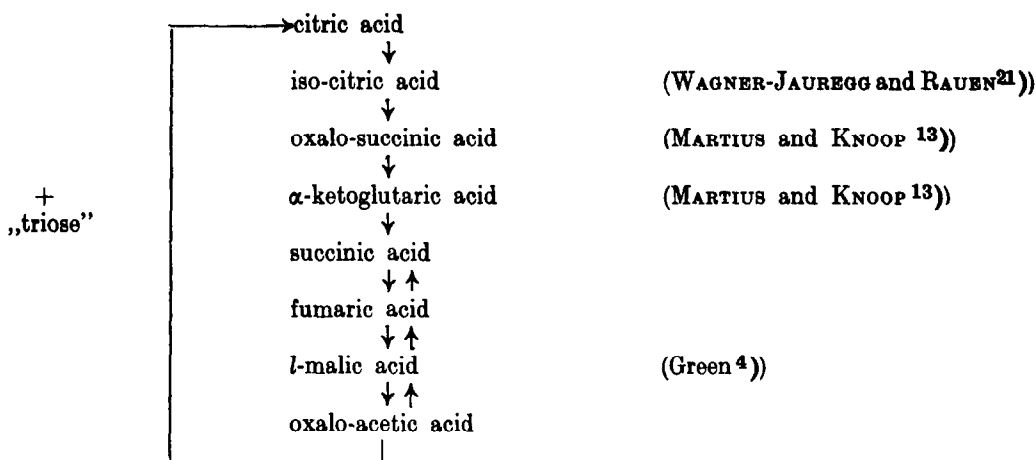
3. Further intermediate stages. (a) iso-Citric acid. WAGNER-JAUREGG and RAUEN (22) and MARTIUS and KNOOP (13, 14) have suggested that iso-citric acid is an intermediate in the oxidation of citric acid. We find that iso-citric acid is indeed readily oxidised in muscle, the rates of oxidation of citric acid and iso-citric acids being about the same.

(b) cis-Aconitic acid. cis-aconitic acid, discovered by MALACHOWSKI and MASLOWSKI (11), was first discussed as an intermediate by MARTIUS and KNOOP (13) and MARTIUS (14) showed that it yields readily citric acid with liver. We have examined the behaviour of cis-aconitic acid in muscle and other tissues and find that it is oxidised as readily as citric acid. The conversion of cis-aconitic acid into citric acid is also brought about by tissue extracts. One milligramme muscle tissue (dry weight) converts up to 0,1 mg cis-aconitic acid into citric acid per hour (40°; pH = 7,4).

MARTIUS and KNOOP (13, 14) assume that the reaction cis-aconitic $\rightleftharpoons$ citric acid is reversible and believe that it plays a role in the breakdown of citric acid. It cannot yet be said, however, whether the reaction is an intermediate step in the breakdown or in the synthesis of citric acid.

(c) Oxalo-succinic acid. The oxidation of iso-citric acid would be expected to yield in the first stage oxalo-succinic acid (MARTIUS and KNOOP). This β -ketonic acid is only known in the form of its esters, since the free acid is unstable in a pure state. In acid solution it is readily decarboxylated and yields α -ketoglutaric acid (BLAISE and GAULT (2)).

(d) Detailed citric acid cycle. The information available at present about the intermediate steps of the cycle may be summarised thus:



4. **Reversible steps.** Succinic acid arises according to our scheme by oxidative reactions from oxaloacetic acid, via citric and α -ketoglutaric acids. Anaerobic experiments, however, show succinic acid can also be formed by reduction from oxaloacetic acid (see also SZENT-GRÖRGYI). The reactions succinic acid $\rightarrow$ fumaric acid $\rightarrow$ l-malic acid $\rightarrow$ oxaloacetic acid are thus reversible under suitable conditions.

The outstanding problem in this connection is the question of the oxidative equivalent of the reduction. At least a partial answer may be given. The synthesis of citric acid as shown in section VI takes place anaerobically, although it is an oxidative process. A reductive process equivalent to the oxidation must therefore occur at the same time. The reduction of oxaloacetic acid to succinic acid is the only reduction of sufficient magnitude (see the next section) known so far to occur simultaneously with the citric acid synthesis and we assume therefore it is the equivalent for the synthesis of citric acid.

5. Effect of malonate. It follows from the preceding paragraph that succinic acid can arise from oxaloacetic acid in two different ways (a) oxidatively via citric and α -ketoglutaric acids (b) reductively via *l*-malic and fumaric acids. That two different ways and therefore two different enzymic systems bring about the conversion of oxaloacetic into succinic acid can be demonstrated with the aid of malonate. Malonate inhibits specifically the reaction succinic acid $\rightleftharpoons$ fumaric acid. Aerobically it will therefore increase the yield of succinic acid from oxaloacetic acid since it prevents its secondary breakdown. Anaerobically, on the other hand, it will inhibit the formation of succinic acid, since in this case the succinic dehydrogenase is concerned with the formation of the succinic acid. The following experiment shows that the results are as expected.

TABLE V.

Effect of malonate on the aerobic and anaerobic conversion of oxaloacetic into succinic acid.

(0,75 grammes wet muscle in 3 ccm phosphate buffer; 40° C; ph = 7,4)

Experimental conditions (final concentration of the substrates)	μl succinic acid formed in 40 min.
1. O_2 ; 0,1 M oxaloacetate;	1086
2. O_2 ; 0,1 M oxaloacetate; 0,06 M malonate	1410
3. N_2 ; 0,1 M oxaloacetate;	1270
4. N_2 ; 0,1 M oxaloacetate; 0,06 M malonate	834

6. Citric acid cycle in other tissues. We have tested the principal points of the citric acid cycle in various other animal tissues and find that brain, testis, liver and kidney of the rat are capable of oxidising citric acid as well as synthesising it from oxaloacetic acid. Of these four tissues testis shows the highest rate of synthesis and this is of interest in view of the work of THUNBERG's school on the occurrence of citric acid in spermatid fluid. 1 mg (dry weight) rat testis forms anaerobically up to 0.02 mg citric acid per hour if oxaloacetic acid is present.

Whilst the citric acid cycle thus seems to occur generally in animal tissues, it does not exist in yeast or in *B. coli*, for yeast and *B. coli* do not oxidise citric acid at an appreciable rate.

7. Quantitative significance of the citric acid cycle. Though the citric acid cycle may not be the only pathway through which carbohydrate is oxidised in animal tissues the quantitative data of the oxidation and resynthesis of citric acid indicate that it is the preferential pathway. The quantitative significance of the cycle depends on the rate of the slowest partial step, that is for our experimental conditions the synthesis of citric acid from oxaloacetic acid. According to the scheme one molecule of citric acid is synthesised in the course of the oxidation of one molecule of „triose”, and since the oxidation of triose requires 3 molecules O_2 , the rate of citric acid synthesis should be one third of the rate of O_2 consumption if carbohydrate is oxidised through the citric acid cycle. We find for our conditions:

Rate of respiration (Q_{O_2}) = -20

Rate of citric acid synthesis (Q_{citrate}) = + 5.8

The observed rate of the citric acid synthesis is thus a little under the expected figure (-6.6), but it is very probable that the conditions suitable for the demonstration of the synthesis (absence of oxygen) are not the optimal conditions for the intermediate formation of citric acid, and that the rate of citric acid synthesis is higher under more physiological conditions. This is suggested by the experiments on the aerobic formation of succinic acid from oxaloacetic acid (Table IV). $Q_{\text{succinate}}$, in the presence of malonate and oxaloacetate is +14.1, and if citrate is an intermediate stage the rate of citrate formation must be at least the same. But even the observed minimum figures of the rate of the synthesis justify the assumption that the citric acid cycle is the chief pathway of the oxidation of carbohydrate in pigeon muscle.

8. The work of SZENT-GYÖRGYI. SZENT-GYÖRGYI²⁰) who first pointed out the importance of the C_4 -dicarboxylic acids in cellular respiration, came to the conclusion that respiration, in muscle, is oxidation of triose by oxaloacetic acid. In the light of our new experiments it becomes clear that SZENT-GYÖRGYI's view contained a correct conception, though the manner in which oxaloacetic acid reacts is somewhat different from what SZENT-GYÖRGYI visualised. The experimental results of SZENT-GYÖRGYI can be well explained by the citric acid cycle; we do not intend, however, to discuss this in full in this paper.

Summary.

1. Citric acid catalytically promotes oxidations in muscle, especially in the presence of carbohydrate.

2. The rate of the oxidative removal of citric acid from muscle was measured. The maximum figure for Q_{citrate} observed was - 16.9.

3. α -Ketoglutaric acid and succinic acid were found as products of the oxidation of citric acid. These experiments confirm MARTIUS and KNOOP's results obtained with liver citric dehydrogenase.

4. Oxaloacetic acid, if added to muscle, condenses with an unknown substance to form citric acid. The unknown substance is in all probability a derivative of carbohydrate.

5. The catalytic effect of citrate as well as the similar effects of succinate, fumarate, malate and oxaloacetate described by SZENT-GYÖRGYI and by STARE and BAUMANN are explained by the series of reactions summarized in section VII 3 d.

6. The quantitative data suggest that the „citric acid cycle” is the preferential pathway through which carbohydrate is oxidised in animal tissues.

We wish to thank the Medical Research Council and the ROCKEFELLER Foundation for grants and Professor E. J. WAYNE for his help and advice.

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