


H A N S A . K R E B S

## The citric acid cycle

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In the course of the 1920's and 1930's great progress was made in the study of the intermediary reactions by which sugar is anaerobically fermented to lactic acid or to ethanol and carbon dioxide. The success was mainly due to the joint efforts of the schools of Meyerhof, Embden, Parnas, von Euler, Warburg and the Coris, who built on the pioneer work of Harden and of Neuberg. This work brought to light the main intermediary steps of anaerobic fermentations. In contrast, very little was known in the earlier 1930's about the intermediary stages through which sugar is oxidized in living cells. When, in 1930, I left the laboratory of Otto Warburg (under whose guidance I had worked since 1926 and from whom I have learnt more than from any other single teacher), I was confronted with the question of selecting a major field of study and I felt greatly attracted by the problem of the intermediary pathway of oxidations. These reactions represent the main energy source in higher organisms, and in view of the importance of energy production to living organisms (whose activities all depend on a continuous supply of energy) the problem seemed well worthwhile studying.

The application of the experimental procedures which had been successful in the study of anaerobic fermentation however proved to be impracticable. Unlike fermentations the oxidative reactions could not be obtained in cell-free extracts. Another difficulty was the rapid deterioration of the oxidative reactions which occurred when tissues were broken up by mincing or grinding, and by suspending them in aqueous solutions.

We now know the reasons for this loss of activity. The chief is the destruction of coenzymes of the nucleotide-type under the influence of hydrolysing enzymes. In the intact tissues these cofactors are apparently separated from the enzymes that can attack them but they become exposed to their action when the tissue structure is destroyed.

It is nowadays possible to take countermeasures against these interfering reactions. Rapid fractionation of the tissue by centrifugation near freezing point separates the destructive enzymes; co-factors which are also removed are known and can be replaced by addition of the pure substances. But this

information was not available to the earlier workers and their methods of approach were somewhat indirect.

### *Early work*

The first major investigation into the intermediary metabolism of oxidation was that of Thunberg<sup>1</sup>, who examined systematically the oxidizability of organic substances in isolated animal tissues. Between 1906 and 1920 he tested the oxidation of over 60 organic substances, chiefly in muscle tissue. He discovered the rapid oxidation of the salts of a number of acids, such as lactate, succinate, fumarate, malate, citrate, and glutamate. Thunberg's results were confirmed and extended by Batelli and Stern<sup>2</sup> and later investigators. Batelli and Stern appreciated already in 1910 some of the significance of these findings when they wrote, "Man kann annehmen, dass der die Oxydation dieser Sauren bewirkende Prozess mit dem der Hauptatmung der Gewebe identisch ist."

However the data of Thunberg and of Batelli and Stern remained isolated observations because they could not be linked to the chief oxidative process of muscle tissue, the oxidation of carbohydrate. Another twenty years had to elapse before they could be incorporated into a coherent account of respiration.

An important development came from the laboratory of Szent-Györgyi<sup>3</sup> of Szegedin in 1935, who discovered that pigeon breast muscle - the chief flight muscle and therefore particularly powerful - is especially suitable for the study of oxidative reactions because this tissue maintains its oxidative capacity well after disintegration in the "Latapie" mill and suspension in aqueous media. He confirmed on this material the rapid oxidation of the C<sub>4</sub>-dicarboxylic acids - succinic, fumaric, malic, and oxaloacetic acids - and arrived at the new conclusion that part of the action of these substances was of a catalytic nature. Final proof of this *catalytic* effect (as opposed to the oxidation in which the acids serve as substrate) was provided by Stare and Baumann<sup>4</sup> in December 1936. These workers showed that very small quantities of the acids suffice to effect an increase in respiration, and that the increase is a multiple of the amount of oxygen necessary for the oxidation of the added substance (Table 1). Moreover the added C<sub>4</sub>-dicarboxylic acid was not used up when it stimulated oxidations and could be subsequently detected in the medium. There thus remained no doubt that the acids can act as catalysts in

Table 1. Catalytic effect of fumarate on the respiration of minced pigeon breast muscle. (Stare and Baumann, 1936.)

<i>Concentration of added fumarate</i> ( <i>M</i> )	<i>Average increase in O<sub>2</sub> uptake observed</i> ( $\mu$ l)	<i>Amount of O<sub>2</sub> calculated for complete oxidation of added fumarate</i> ( $\mu$ l)
0.0001	151	20
0.0002	235	40

respiration, but neither Szent-Györgyi, nor Stare and Baumann could offer a satisfactory explanation of the catalytic effect. They assumed that the dicarboxylic acids served as hydrogen carriers between foodstuff and cytochrome.

### *Crucial experiments*

The next step was the discovery, made in Sheffield early in 1937, that citrate can act as a catalyst in the same way as succinate. A decisive contribution to the field was made in March 1937 by Martius and Knoop<sup>5</sup>, who elucidated the fate of citrate when undergoing oxidation in biological material. Whilst it has long been known that citrate can be oxidized in plants, animals, and micro-organisms, the intermediary steps remained obscure until Martius and Knoop discovered  $\alpha$ -ketoglutarate as a product of citrate oxidation. They showed this reaction in extracts of liver and of cucumber seeds and suggested that *cis*-aconitate and isocitrate were intermediates, as formulated in Fig. 1. This interpretation was in accordance with the earlier discovery of Wagner-Jauregg and Rauen<sup>6</sup> that isocitrate behaves in the same way as citrate in extracts of cucumber seeds.

Further relevant observations were made in the Sheffield laboratory between March and June 1937<sup>7</sup>. Firstly, the reaction which Martius and Knoop had demonstrated in liver was found to occur at a rapid rate in muscle and other tissues. The rate was found to be sufficient to justify the assumption that the reaction constitutes a component of the main respiratory process of the tissue. The formation of ketoglutarate from citrate could be demonstrated by two procedures, either by the addition of arsenite or by raising the concentration of citrate to a high level. Arsenite preferentially inhibits the oxidation of ketonic acids, presumably by reacting with the sul-

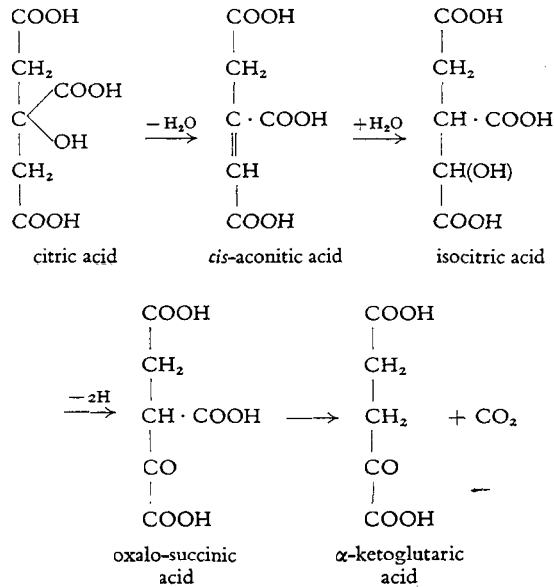


Fig. 1. Conversion of citric into  $\alpha$ -ketoglutaric acid according to Martius and Knoop (1937).

phosphoryl group of coenzyme A which is essential for the metabolism of  $\alpha$ -ketonic acids. A high substrate concentration causes a competitive inhibition of the oxidation of other substances. When malonate was added, succinate was found to be a major product of the oxidation of citrate. Of major significance was another new observation: citrate was not only broken down at a rapid rate but was also readily formed in muscle and in other tissues provided that oxaloacetate was added. This could be explained by the assumption that some oxaloacetate was broken down to pyruvate or acetate, and that the formation of citrate was the result of a combination between the remaining oxaloacetate on one hand, and pyruvate or acetate on the other. The discovery of the synthesis of citrate from oxaloacetate and a substance which could be derived from carbohydrate, like pyruvate or acetate, made it possible to formulate a complete scheme of carbohydrate oxidation<sup>7</sup>. According to this scheme (Fig. 2) pyruvate, or a derivative of pyruvate, condenses with oxaloacetate to form citrate. By a sequence of reactions in which *cis*-aconitate, isocitrate,  $\alpha$ -ketoglutaric, succinic, fumaric, malic, and oxaloacetic acid are intermediates, one acetic acid equivalent is oxidized and the oxaloacetic acid required for the condensing reaction is regenerated. The concept explained the catalytic action of the di- or tricarboxylic acids, the oxidizability of these

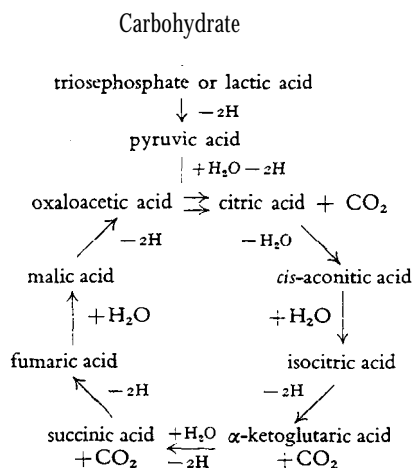


Fig. 2. The original citric acid cycle. (Krebs and Johnson, 1937; Krebs, 1943.)

acids in tissues which oxidize carbohydrates, and the similarity of the characteristics of the oxidation of these substances and of the main respirations already noted by Batelli and Stem in 1910. The scheme describes in detail the fate of the carbon atoms of the substrate and the stages where hydrogen is removed and  $\text{CO}_2$  is released.

It is convenient to use a brief term for the kind of scheme. Its essential feature is the periodic formation of a number of di- and tricarboxylic acids. As there is no term which would serve as a common denominator for *all* the various acids, it seemed reasonable to name the cycle after one, or some, of its characteristic and specific acids. It was from such considerations that the term "citric acid cycle" was proposed in 1937.

### *Additional evidence*

The evidence in support of the cycle mentioned so far comes under two main headings: firstly, all the individual stages of the cycle have been demonstrated to occur in animal tissues, and their rates are high enough to comply with the view that they are components of the main respiratory process. Secondly, di- and tricarboxylic acids have been shown under suitable conditions to stimulate oxidations catalytically.

A third set of experiments supporting the concept employs the inhibitor malonate, first recognized by Quastel<sup>8</sup> as a specific agent for succinic de-

hydrogenase. Malonate competitively prevents succinate from reacting with the enzyme, an effect due to the similarity between the structures of the two compounds. The blockage of succinic dehydrogenase in any system where succinate is an intermediate causes an accumulation of succinate. It is a special advantage of the inhibitor technique that it can be applied without serious interference with the natural conditions, e.g. without disintegrating the tissue (although in some cases permeability barriers may prevent the penetration of the inhibitor to the site of the enzymes). Malonate has been found to inhibit the respiration of all animal tissues, and the expected accu-



mulation of succinate is found even when the inhibitor is injected into the intact organism<sup>9,10</sup>. These observations may be taken as independent proof of the participation of succinic dehydrogenase in the respiration of animal tissues.

More recently Peters<sup>11</sup> has discovered another valuable specific inhibitor. Injection of fluoroacetate into the intact organism leads to an accumulation of citrate in animal tissues. The actual inhibitor is probably a fluorotricarboxylic acid which arises from fluoroacetate and oxaloacetate and appears to prevent competitively the metabolic removal of citrate. The accumulation of citrate in the poisoned organism, by analogy with the accumulation of succinate, may be taken as an indication of its normal intermediary formation.

### *Further intermediates*

Since the original formulation of the cycle in 1937, three additional intermediates have been identified. In 1948 Ochoa<sup>12</sup> and independently Lynen<sup>12</sup> finally established oxalosuccinic acid as an intermediate, as already postulated by Martius and Knoop. They demonstrated the presence of a specific decarboxylase converting oxalosuccinate to  $\alpha$ -ketoglutarate. A very major achievement was the identification of the derivative of pyruvate which con-

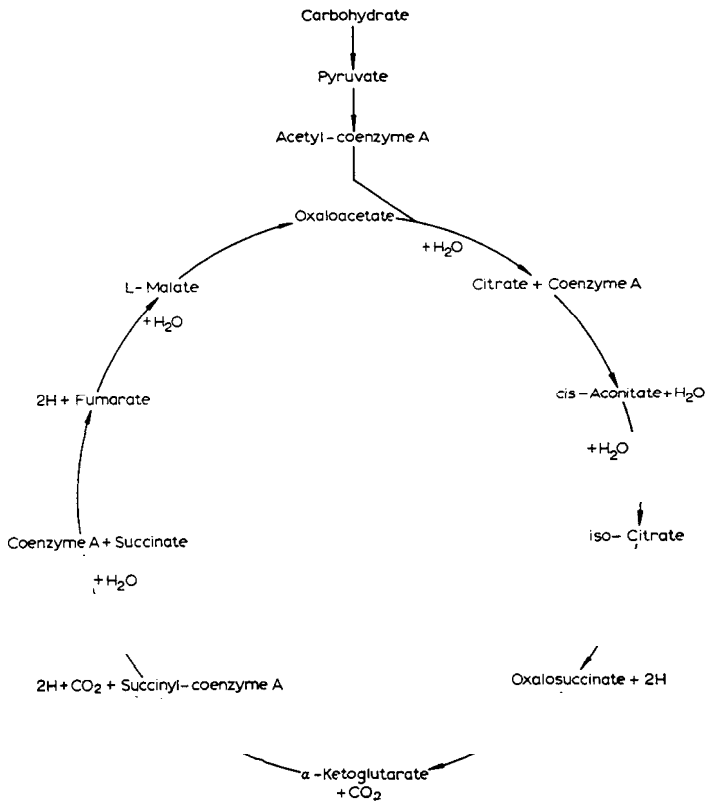


Fig. 3. Extended version of the citric acid cycle.

denses with oxaloacetate to form citrate. Thanks to the work of Lipmann's, Stem and Ochoa<sup>14</sup> and Lynen<sup>15,16</sup> this is now known to be acetyl coenzyme A. As Dr. Lipmann will himself deal with the biochemistry of coenzyme A I will not further discuss the matter. Finally, experiments of the schools of Ochoa<sup>17</sup> and Green<sup>18</sup> have shown that coenzyme A also participates in the conversion of α-ketoglutarate to succinate and that succinyl coenzyme A is an intermediary stage. The place of these three intermediates in the cycle is shown in Fig. 3.

*Common pathway of foodstuff oxidation*

The concept of the citric acid cycle was originally put forward as a scheme of the oxidation of carbohydrate. It was however clear from the beginning

that the cycle must also play a major part in the oxidation of a considerable fraction of the protein molecule. Of the 20 amino acids that are commonly found as protein constituents, three - glutamic acid, aspartic acid, and alanine - form derivatives which are intermediate in the citric acid cycle. Five other amino acids - histidine, arginine, citrulline, proline, hydroxyproline - are known to form glutamic acid in the animal body and they can therefore enter the citric acid cycle via  $\alpha$ -ketoglutaric acid. Five further amino acids - the three leucines, tyrosine, phenylalanine - yield acetyl coenzyme A and malic acid; the 13 amino acids listed constitute a large proportion of the common proteins, e.g. more than 75% in the case of casein. The metabolic fate of the carbon chain of the chief remaining amino acids is not yet fully established and it is likely that future work will reveal further connections between these amino acids and the citric acid cycle. It is thus evident that a substantial proportion of protein molecules pass through the citric acid cycle when undergoing oxidation.

Since 1943 it has become evident that the citric acid cycle also comes into play in the later stages of the oxidation of fatty acids. The classical work on the  $\beta$ -oxidation of the higher fatty acids had shown that the carbon skeleton of the fatty acids is broken down by the removal of pairs of carbon atoms and that acetoacetic and  $\beta$ -hydroxybutyric acids are products of oxidation. These "ketone bodies" were presumed to arise from the last four carbon atoms of the chain. Earlier work on isolated tissues by Quastel<sup>19</sup>, Edson<sup>20</sup>, Stadie<sup>21</sup> and others produced much confirmatory evidence in support of  $\beta$ -oxidation and added the new observation that, contrary to the older assumption, more than one molecule of ketone body could be formed from one fatty acid molecule. It is further of interest to record that inhibitor experiments with malonate by Quastel and Wheatley<sup>8</sup>, and Edson and Leloir<sup>20</sup> suggested already in 1935 that the oxidative removal of the ketone bodies was associated with the metabolism of the  $C_4$ -dicarboxylic acids. Decisive progress in this field dates from 1943 onwards. In that year Breusch<sup>22</sup> and Wieland and Rosenthal<sup>23</sup> reported independently that acetoacetate could cause a formation of citrate in animal tissues if oxaloacetate was present. The evidence presented by these workers was not conclusive because oxaloacetate alone forms much citrate and the increased yield could be explained without assuming a direct participation of acetoacetate in the formation of the tricarboxylic acids<sup>24</sup>. But isotope work from 1944 onwards by American workers has removed any doubts; they show conclusively that the carbon atoms of fatty acids, and of acetoacetate, appear in the acids of the citric acid



cycle, and that these acids are thus intermediates in the complete oxidation of fatty acids<sup>25</sup>.

This has been confirmed by the more recent investigations with enzyme preparations carried out by the school of Lynen, Lipmann, Ochoa, Stem, and Green<sup>26,30</sup> which have demonstrated the details of the pathway that leads from fatty acids to the citric acid cycle. Acetyl coenzyme A is the form in which all carbon atoms of fatty acids enter the cycle.

It is indeed remarkable that all foodstuffs are burnt through a common terminal pathway. About two-thirds of the energy derived from food in higher organisms is set free in the course of this common pathway; about one-third arises in the reactions which prepare foodstuffs for entry into the citric acid cycle. The biological significance of the common route may lie in the fact that such an arrangement represents an economy of chemical tools. An analysis of the energy giving reactions (which I cannot here pursue in detail; see Krebs<sup>31</sup>) shows that in spite of a multitude of sources of energy the number of steps where energy is utilized is astonishingly small - only seven. The common pathway of oxidation is one of the devices reducing the number of steps where special chemical tools for the transformation of energy are required.

### *The cycle in different types of cells and organisms*

The main experiments on which the concept of the citric acid cycle is based were carried out on striated muscle, chiefly of pigeon-breast muscle, and on pigeon liver. The crucial experiments have been repeated with many other animal materials and they suggest that the cycle occurs in all respiring tissues of all animals, from protozoa to the highest mammal. It is true that some tissues and material at first appeared to give negative results. These were later found to be due to special complications such as permeability barriers, or destruction of enzyme systems as a result of manipulating the tissues. These experimental difficulties have been overcome in many instances by improved methods of handling respiring material, and wherever this was possible the occurrence of the cycle has been demonstrated.

The component reactions of the citric acid cycle have also been shown to occur in many micro-organisms<sup>32</sup> and in plants. In some materials the rates of the individual steps are sufficiently rapid to justify the assumption, supported by isotope data, that the cycle represents the main terminal pathway

of oxidation. This applies to organisms of widely different types such as *Azotobacter*, *Micrococcus leisodeicticus*, and *Rhodospirillum rubrum*, and the seedlings of beans and peas<sup>33</sup>. But there are other materials where the evidence in support of the cycle falls short in respect to the quantitative aspects. In yeasts, for example, the activities of the enzymes that oxidize the malate and citrate are at most 5-25% of the expected order. Moreover when <sup>14</sup>C-labelled acetate is oxidized by baker's yeast the intracellular dicarboxylic acids remain unlabelled, an observation which argues against the participation of the dicarboxylic acids in the oxidation of acetate<sup>32</sup>. It is true that these results may not be looked upon as conclusive because permeability barriers might prevent the mixing of substances arising as intermediates with those that are present in other compartments of the cell, and at present it is best to regard the terminal pathway of oxidation in yeast, and certain other micro-organisms, e.g. *E. coli*, as an open problem, even though the reactions of the cycle occur in these materials.

#### *The role of intermediates of the cycle in synthetic processes*

If, for the sake of argument, it is assumed that in some cells the cycle is not the main mechanism by which energy is produced but if nevertheless the enzyme systems responsible for the component reactions of the cycle occur, the problem of the physiological significance of the cycle in these materials presents itself. It is relevant with regard to this problem that in addition to the energy-giving mechanisms there is another major set of chemical changes in rapidly growing organisms: the synthetic processes connected with growth. As far as the turnover of carbon is concerned both types of reactions can be of the same order of magnitude. The reactions of the cycle can supply an important intermediate for a number of syntheses, e.g.  $\alpha$ -ketoglutaric acid, which is a precursor of glutamic acid and other amino acids, as well as of the porphyrins required for the synthesis of the cytochromes and the blood pigments. Many observations<sup>32</sup>, especially from isotope experiments, support the view that in some micro-organisms the cycle primarily supplies intermediates rather than energy, whilst in the animal and most other organisms it supplies both energy and intermediates.

*Common features of different forms of life*

Before I conclude I would like to make an excursion into general biology, prompted by the remarkable fact that the reactions of the cycle have been found to occur in representatives of all forms of life, from unicellular bacteria and protozoa to the highest mammals. We have long been familiar with the fact that the basic constituents of living matter, such as the amino acids and sugars, are essentially the same in all types of life. The study of intermediary metabolism shows that the basic metabolic processes, in particular those providing energy, and those leading to the synthesis of cell constituents are also shared by all forms of life.

The existence of common features in different forms of life indicates some relationship between the different organisms, and according to the concept of evolution these relations stem from the circumstance that the higher organisms, in the course of millions of years, have gradually evolved from simpler ones. The concept of evolution postulates that living organisms have common roots, and in turn the existence of common features is powerful support for the concept of evolution. The presence of the same mechanism of energy production in all forms of life suggests two other inferences, firstly, that the mechanism of energy production has arisen very early in the evolutionary process, and secondly, that life, in its present forms, has arisen only once.

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