FORMATION AND ACCUMULATION OF CITRIC ACID IN *ASPERGILLUS NIGER*

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STUDIES on the mechanism of citric acid formation in *A. niger* started as early as 1913 when it was detected in *A. niger* by Zahorski.[1]

Various hypotheses have been formulated for the conversion of the straight chain form of glucose into six carbon acid possessing a branched chain. But the evidence available is not conclusive enough to permit a decision in favour of any one of them. Of all the theories put forward, that of Raistrick[2] deserves discussion, as evidence has been accumulating in recent years to establish the essential principle of his theory. In 1919, Raistrick and Clark[2] suggested that the molecule of glucose, suffering a loss of the element of two molecules of water, together with oxidation at terminal carbon atoms, may give a reactive α, γ diketoadipic acid which, on hydrolysis, should yield acetic acid and oxalacetate, the aldol condensation of the two giving citric acid. Even though sufficient proof has not been obtained for the formation and detection of α, γ diketoadipic acid, recent findings indicate that C_2-C_4 condensation takes place in the formation of citric acid.

Several suggestions have been put forward regarding the C_2 and C_4 acids which take part in citric acid formation. But the recent findings of the author[12–14] suggest that active acetate and oxalacetate are the C_2 and C_4 acids which condense together to form citrate. The pathway of the formation of C_2 acid from carbohydrate is not yet well established. Several workers have felt that the Embden-Meyerhof scheme may be operating, as alcohol and C_2 acid are always found to be present together in the sugar medium in which *A. niger* is grown. But Johnson, Knight and Walker[3] showed that in the presence of 0·002 M iodoacetate, which inhibits the formation of alcohol, citric acid can be produced Hence they felt that a pathway different from the Embden-Meyerhof scheme may occur. Recently it has been suggested that the Embden-Meyerhof scheme may continue up to the stage of pyruvate which when formed is oxidatively decarboxylated to form acetate.[4] This view has been further strengthened by the recent findings of Jagannathan and Kartar Singh[5] and Shu, Funk and Neish.[6] But Cleland and Johnson,[7] by studying the fermentation of glucose 3, 4 C^{14} by *A. niger*, have suggested a different pathway leading to the splitting of hexose into two C_3 fragments and decarboxylation of one of these C_3 fragments giving rise to C_2 acid. Even though there is sufficient evidence to prove that pyruvate is formed from hexose and that oxidative decarboxylation of pyruvate gives active acetate, the pathway of the formation of pyruvate from hexose has not yet been clearly understood.

As regards the C_4 acid, there are many pathways by which it can be formed. Recently Halliwell[8] found that by passing air with 2% CO_2, 180% citrate is formed. Further it was observed that whenever excess citric acid is formed, the ratio of CO_2 evolved to oxygen consumed is always the smallest. This suggests that Wood-Werkman reaction may be operating in *A. niger* by which CO_2 can be fixed in pyruvate to form oxalacetate. This observation is strengthened by the findings of Foster *et al.*[9] and Martin and Wilson[10] who found that *A. niger* placed on a sugar solution in presence of CO_2 labelled with radioactive carbon produces citric acid with labelled C in carboxyl groups But the enzyme responsible for Wood-Werkman reaction has not yet been detected in *A. niger* Similar suggestions have also been made as regards the operation of Thunberg reaction in *A. niger* in which two acetates may condense together to form succinate. But this type of reaction has not yet been established in *A. niger*, and acceptance of this theory has to wait until the appropriate enzyme is isolated and the formation of labelled succinate is confirmed using that enzyme and labelled acetate. There is a possibility of one more pathway for the formation of C_4 acid. While investigating the enzyme systems in *A. niger* during citric acid accumulation, the author observed that the concentrations of α-keto glutaric oxidase and fumarase of the mold increased several folds, and this suggests the possibility of formation of C_4 acid from glutamate.[11] Thus it seems there are many pathways for the formation of C_4 acid.

Several people suggested the occurrence of Krebs' citric acid cycle as the reason for the formation of citric acid in *A. niger*, since the intermediates of Krebs' cycle have been detected at different times. But none of them studied

the stepwise reactions of the cycle. Recently Ramakrishnan and Martin[12] studied the enzymes involved in the formation of citric acid in *A. niger* with a view to find out how far the cycle operates during formation and accumulation of citric acid in *A. niger*. The citric acid producing strain of *A. niger*, N.R.C. 233 has been grown in non-citrate accumulating medium containing malt extract, yeast extract and glucose in shake flasks for 18 hours and the mat obtained. The different enzymes have been extracted from the mat. Recently, the condensing enzyme has been isolated with a high degree of purity and it has been successfully established that the enzyme can affect the synthesis of citrate from acetyl phosphate, coenzyme A and oxalacetate according to the reaction acetyl-phosphate + coenzyme A → acetyl-coenzyme A + P; acetyl-coenzyme A + oxalacetate → citrate + coenzyme A. The condensing enzyme in *A. niger* appears to be different from the one isolated from animal tissues in that it is inhibited by Mg^{++} whereas it has been found essential for the latter.[13] It has been possible to detect the presence of all the enzymes of Krebs' tricarboxylic acid cycle in the cell free extracts of *A. niger*.[14] Thus, evidence for the operation of Krebs' cycle in *A. niger* when grown in a non-citrate accumulating medium has been obtained

Since the medium used in these investigations is a non-citrate accumulating medium which is different from the one used in industry for large-scale production of citric acid, mold pellets from the actively fermenting molasses medium (in which citric acid is formed and accumulated) were taken out at different periods of citric acid production, the cell free extracts prepared and tested for the presence of the enzymes of Krebs' citric acid cycle. It was found that in the initial stages when no citric acid accumulated, all the enzymes of Krebs' cycle were present and as citric acid started accumulating, aconitase and isocitric dehydrogenase activities became zero. It seems Krebs' cycle enzymes are present in *A. niger* during non-accumulation of citrate and the cycle gets broken down at aconitate and isocitrate levels when citrate starts accumulating in the medium. Even though the reason for the inhibition of aconitase has not been worked out, it is found that addition of excess citrate inhibits isocitric dehydrogenase.[11] From the above discussions, it would seem logical to assume that the formation and accumulation of citric acid in *A. niger* is the net outcome of several reactions like stoppage of operation of Krebs' cycle at a definite stage. Since most of the enzymes required for all these reactions have been detected and many of them purified, it will be possible to study the reactions stepwise, using isotopic and chemical methods, and come to a definite conclusion as regards the mechanism of formation and accumulation of citric acid in *A. niger*.

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'ATOMS FOR PEACE' EXHIBITION

DR. K. S. KRISHNAN, Director, National Phyical Laboratory, declared open the "Atoms for Peace" exhibition in New Delhi on March 10, 1955. The exhibition was organised by the U.S. Information Service with the help of the U.S. Atomic Energy Commission, to illustrate and explain the peaceful uses of atomic energy, depict the various phases of atomic energy development through pictorial panels and charts, miniature working models of Geiger counters, atomic reactors and atomic power plants, and practical uses of various forms of atomic energy. The exhibition will be shown in 50 towns and Universities in India during the next two years.