The Conversion of Citrate into *cis*-Aconitate and *iso*Citrate in the Presence of Aconitase

By H. A. KREBS and OLGA HOLZACH
Medical Research Council, Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield, 10

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Martius & Lynen (1950) and Friedrich-Freksa & Martius (1951) have expressed doubts on the intermediary formation of *cis*-aconitate in the aconitase system because a study of the kinetics of the conversion of citrate into *iso*citrate failed to show a lag period. The authors argue that if the conversion followed the original scheme of Martius (1937)

$$\text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate}$$

the rate of formation of *iso*citrate should gradually rise until the optimal concentration range of *cis*-aconitate was built up, and they conclude from the absence of a lag period in their experiments that the interconversion of citrate and *iso*citrate does not necessitate the formation of *cis*-aconitate.

The experiments reported in this paper were designed to re-investigate the question of the existence of a lag period in the aconitate system. Dilute heart-muscle aconitase preparations were mixed with citrate, and the time course of the formation of aconitate and *iso*citrate was measured at 25°. Dilution and a relatively low temperature draws out the time curve and thus favours the detection of a lag period.

EXPERIMENTAL

Pig heart (17 g.), minced in a domestic mincer, was suspended for 5 min. in 20 vol. 0·9% NaCl. The supernatant was removed by centrifugation and the washed muscle was homogenized with 250 ml. water in a Waring Blendor. The object of washing the muscle was to remove substances, mainly phosphates, lactic acid and unsaturated compounds, which interfere with the determinations of either *cis*-aconitate or *iso*citrate. A mixture of 120 ml. heart-muscle suspension, 120 ml. M-sodium citrate, 150 ml. 0·1 M-$NaHCO_3$ and 210 ml. water was incubated at 25° with continuous mechanical stirring. A stream of 5% CO_2 in N_2 (v/v) was passed through the suspension throughout so that pH was 7·4. A control suspension contained the same reagents except citrate. At intervals 25 ml. samples were removed for analysis. The sample for the determination of aconitate was acidified with 6·5 ml. 20% (w/v) metaphosphoric acid, that for the determination of *iso*citric acid with 2·5 ml. glacial acetic acid. These quantities were sufficient for duplicate analyses.

Aconitic acid was determined manometrically according to Johnson (1939) by quantitative catalytic hydrogenation. The acid was extracted with ether from a 13 ml. sample in a Kutscher-Steudel apparatus after addition of 2 ml. 50% (w/v) H_2SO_4. The extracted material, after evaporation of the ether, was dissolved in a total volume of 2·5 ml. water. The main compartment of a conical Warburg manometer contained 25 mg. of the palladium catalyst of Köppen (1932) and 3 ml. 3% (w/v) metaphosphoric acid, the side arm 0·9 ml. of the ethereal extract and 0·1 ml. 20% (w/v) metaphosphoric acid. The gas space contained cylinder hydrogen washed with pyrogallol and acid dichromate. The bath temperature was 38°.

*iso*Citric acid was determined polarimetrically. To 27·5 ml. of the acidified solution 22·5 ml. 29% (w/v) ammonium molybdate were added. After filtration the rotation was measured at 18° in 2 dm. tubes. The concentration of *iso*citrate was calculated on the basis of the $[\alpha]_D$ values given by Eggleston & Krebs (1949).

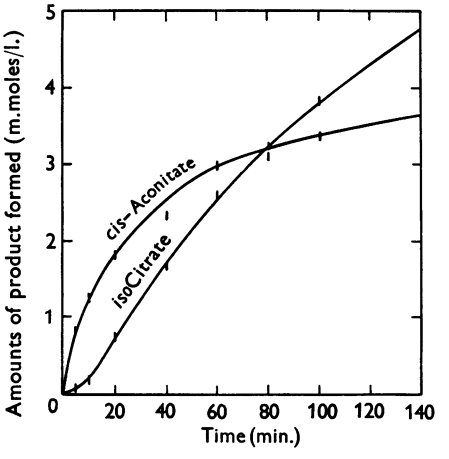

Fig. 1. Time curve of the formation of *cis*-aconitate and *iso*citrate from citrate in the presence of aconitase. For conditions see text. Additional data: amounts of *cis*-aconitate after 180 min. 4·16, after 270 min. 4·25, after 24 hr. 5·80 m-moles/l.; amounts of *iso*citrate after 180 min. 6·02, after 270 min. 8·08, after 24 hr. 12·4 m-moles/l.

The results of a representative experiment are as in Fig. 1. The curves clearly demonstrate the existence of a lag period before the formation of *iso*-citrate reaches maximum velocity. Hardly any

*iso*citrate is formed in the first 5 min. The rate rises progressively and reaches a maximum after about 40 min. In contrast, the velocity of *cis*-aconitate formation is maximal at the start and decreases gradually. Similar curves were obtained in five other experiments. They are in accordance with the assumption that *cis*-aconitate is an intermediate in the conversion of citrate into *iso*citrate and there is therefore no reason for changing the theory of aconitase action originally put forward by Martius (1937).

SUMMARY

The rate of formation of *iso*citrate and *cis*-aconitase from citrate in the presence of heart-muscle aconitase was measured. Contrary to findings by Friedrich-Freksa & Martius (1951) the rate of *iso*citrate formation was not maximal at the start, but showed a distinct lag period. The observations are in agreement with Martius's earlier assumption (1937) that *cis*-aconitate is an intermediate in the conversion of citrate into *iso*citrate.

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