ENZYMATIC HYDRATION OF CITRACONATE TO (-)CITRAMALATE*

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The existence of a cis-trans(aconitate) isomerase was reported from this Laboratory (Rao & Altekar 1961): other cis-trans isomerases are known to exist in nature (Edwards & Knox 1956; Sugiyama et al 1958; Otsuka 1961). In an attempt to study other possible cis-trans transformations, a search was made for a possible biological conversion of citraconate (methylmaleate) into mesaconate (methylfumarate). However, instead of this, an enzyme converting citraconate into (-)citramalate (α-methylmalate) was found in a microorganism capable of growth on citraconate as the sole carbon and energy source. This preliminary communication describes the characterization of the product of enzymatic hydration and of citraconase. (-)Citramalic acid is known to occur in apple peels (Hulme 1954).

A fluorescent pseudomonad was isolated from soil by the routine enrichment procedure. Growth of cells on a large scale and preparation of cell-free enzyme extracts were essentially according to the procedure described

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previously (Rao & Altekar 1962). The resulting enzyme solution was processed at 0 to 3°C. It was centrifuged at 12,000 x g and used as such or after dialysis against 50 volumes of water or ammonium sulfate fractionation. The acids were prepared by the standard procedures; citraconic and mesaconic (Shriner et al 1947) and citramalic (Barker & Blaire 1962). The acids were detected by paper chromatography (Butanol:formic:water = 4:1.5:1, Ethyl acetate:formic acid:water = 10:1:3; and, ethanol:ammonia:water = 8:1:1). Mesaconic and citraconic acids were determined quantitatively according to Wachsman (1956). Citramalate content of deproteinized samples was assayed according to Barker and Blaire (1962).

In one typical experiment, 5 μmoles citraconate or mesaconate (pH 7), 100 μmoles cysteine, 10 μmoles ferrous sulfate and 25 mg protein (of undialyzed cell-free extract) in 10 ml total volume were incubated at 28°C for 12-24 hrs with toluene and chloroform as preservatives. The reaction mixture was then treated according to Barker's procedure (1962); the continuous ether extracts contained only two non-volatile acids, viz., citramalate and smaller amounts of citraconate/mesaconate. The solvent was evaporated and the residual syrup made upto 10 ml and 9 ml were used for isolation of the pure acids by Dowex-1 chromatography (Busch et al 1952). The acid eluting first was made upto 10 ml and had the same R_f as citramalic acid. 1 ml was used for polarimetry in presence of ammonium molybdate (final vol. 11 ml; 2 dm tube). The acids isolated from reaction mixtures

containing citraconate and mesaconate had rotations -6.7° and +5.7° respectively. Phenacyl esters had m.p.104-106° and authentic DL-citramalate phenacyl esters m.p.114°. The infrared and the n.m.r. spectra of the methyl esters of authentic DL-citramalic acid and of the isolated acids were identical. The spectral and other properties of these acids and the methods of their preparation will be described in detail elsewhere. In smaller-scale routine experiments, 25 μmoles of substrate, 1 to 5 mg enzyme protein, 10 μmoles cysteine, 1 μmole Fe^{++} were incubated for 1 to 3 hrs at 30°C; volume, 1 ml.

The undialyzed enzyme solutions catalyze the hydration of both citraconate and mesaconate into (-) and (+) citramalate respectively in the absence of any added co-factors. Citraconase activity is stable to dialysis. Mesaconase activity is almost completely lost on dialysis; addition of boiled extract and/or ferrous ions and cysteine does not restore the activity. In our experiments with cell-free extracts of Clostridium tetanomorphum, mesaconase seems to be stable to dialysis and cell-free extract does not seem to contain either citraconase or malease activity. On fractionation with ammonium sulfate, citraconase activity of cell-free extracts of our isolate was found exclusively in the 40 to 60 fraction. Both SH-compounds and ferrous ions are essential for citraconase activity of dialyzed solutions or ammonium sulfate fractions. It is thus clear that the two activities, citraconase and mesaconase

are distinct and that mesaconate is not an intermediate in the hydration of citraconate to (-)citramalate. For, (i) citraconate yields citramalate under conditions in which mesaconate does not, and besides, (ii) the two compounds yield two optical antipodes. The undialyzed cell-free extracts contain feeble malease activity which disappears during dialysis and further processing. The purification of citraconase and its properties will be described elsewhere.

It is not clear from the experiments of Wang et al (1961) and of Cooper & Kornberg (1962) on the metabolism of itaconate by liver mitochondria and by a pseudomonad respectively whether (-) or (+) citramalyl CoA is involved in the reactions. The pathway of (+) citramalate dissimilation has been elucidated by Barker (1956). Whether (-) citramalate is metabolized directly or via its CoA derivative is not clear: its dissimilation is being studied further, particularly in relation to the metabolism of other C_5-dicarboxylic acids (Wang et al 1961; Cooper & Kornberg 1962; Brightman & Martin 1961; Losade et al 1960).

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