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Short Communications

Stereochemistry of the Methylcitric Acids Formed in the Citrate Synthase Reaction with Propionyl-CoA

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Examination of the methylcitric acid present in the urine from patients with propionic acidaemia shows two chromatographic peaks of approximately equal intensity.[1] By correlation with hydroxycitric acids of known stereochemistry, the absolute configurations of the acids corresponding to these peaks have been shown to be 2*S*, 3*S*† (*1*) and 2*R*, 3*S* (*2*).[2]

Radioactive propionate was found to be incorporated *in vivo* into *1* and *2*, suggesting that their biosyntheses involve condensation of propionyl-CoA with oxaloacetate,[1] a reaction similar to that occurring in the biosynthesis of citric acid. Stern had previously noted that oxaloacetate was consumed when mixed with propionyl-CoA and citrate synthase, and this lends support to the hypothesis that citrate synthase is the enzyme responsible for the biosynthesis of methylcitrate. The V_{max} for the reaction involving propionyl-CoA was, however, found to be only 10^{-4} times that for acetyl-CoA.[3] Later, methylcitric acid of unknown stereochemistry was detected in a reaction catalysed by pig heart citrate synthase.[4]

We have now found that *1* and *2*, in a total yield of 10 – 15 % calculated from propionyl-CoA, are the products from the pig heart citrate synthase mediated reaction between propionyl-CoA and oxaloacetate. It is therefore likely that the acids *1* and *2* produced by propionic acidaemia patients are biosynthesised in this side reaction of citrate synthase. The time course of the reaction is shown in Fig. 2, from which it can be seen that the rate of reaction is, under the experimental conditions, about one order of magnitude lower than reported.[3] The optical purities of *1* and *2* obtained in the *in vitro* experiments are high but, due to the small amounts investigated, no accurate figures can be given. The ratio of *1* to *2* was found to vary between approximately 0.3 and 3.

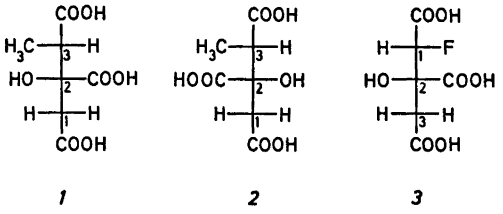

Fig. 1. Stereostructures of the methylcitric acids (*1*, *2*) formed in the citrate synthase reaction with propionyl-CoA. Proposed [15] stereostructure of the fluorocitric acid (*3*) formed in the citrate synthase reaction with fluoroacetyl-CoA.

† The numbering is that of 2-hydroxy-1,2,3-alkanetricarboxylic acids.

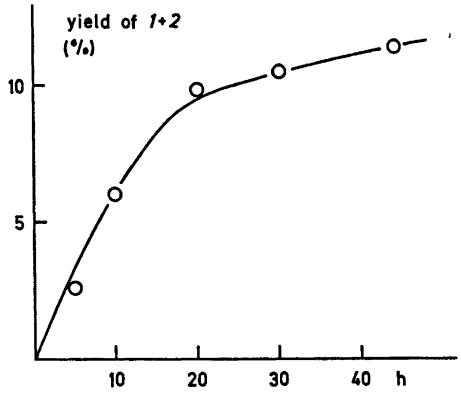

Fig. 2. Time course of the formation of methylcitric acids in the citrate synthase mediated reaction of propionyl-CoA with oxaloacetate. The ratio of acids *1*/*2* was approximately 3 at all determined points. The yield is calculated from propionyl-CoA.

The stereostructures of the methylcitric acids formed show that in this case citrate synthase is apparently unspecific with respect to *re* or *si* attack on oxaloacetate, but stereospecific with respect to reaction with the propionyl-CoA moiety. A specific methylcitrate synthase which only synthesises *1* occurs in a yeast.[2,5] The biosynthesis of *1*, but not that of *2*, is stereochemically analogous to the biosynthesis of citric acid, known to proceed in a *si* fashion [6] except in the case of a few anaerobic bacteria.[7,8] One explanation of the lack of stereospecificity in the present case is that the steric effects of the extra methylene group in propionyl-CoA inhibit the proper binding of oxaloacetate to the enzyme which occurs in the synthesis of citric acid from acetyl-CoA; thus the reaction involving propionyl-CoA leads to both *re* and *si* attack on the keto carbonyl group of oxaloacetate.

Methylcitrate formation could arise either from a minor contaminant in the enzyme preparation or by involvement of citrate synthase itself at a site different from, or identical with, the normal active site. Evidence in favour of the latter interpretation comes from the observation that propionyl-CoA has a high affinity for citrate synthase and competes with acetyl-CoA for its binding site.[4,9] A site different from the normal active site seems less likely as binding studies indicate that only one site per subunit is available for the substrates.[10–13] After treatment with butanedione,[14] which modifies arginine, the modified enzyme shows a significant difference ($p < 0.001$) in susceptibility to the two reactants (Fig. 3). This means at least that the reaction mechanisms are different, as is also obvious from the stereostructures of the products. The methylcitrate formation is less inhibited by butanedione modification than is citrate formation and this can be explained if it is assumed that arginine is necessary for the close binding of oxaloacetate to the enzyme which occurs only during citrate formation.

Since only one orientation of the methyl group in the propionyl moiety seems to be possible during the condensation to methylcitric acid, one would expect the fluorine atom in fluoroacetyl-CoA to be similarly oriented during the condensation leading to fluorocitric acid, but this is possibly not the case. Only one stereoisomer of the latter acid has been detected in the reaction mixture and the 1*R*,2*R* configuration has been ascribed to this isomer (*3*).[15] As seen in Fig. 1, the structure of *3* does not correspond to the structures of either *1* nor *2*. The absolute configuration of *3* has not been rigorously proved, however. In the choice between two enantiomers, the one demanding a *si* attack on oxaloacetate was regarded as the most probable.

Experimental. Pig heart citrate synthase (Boehringer) was used either as received or after removal of salts by gel filtration. Acetyl-CoA and propionyl-CoA were prepared from the corresponding acid anhydrides and CoA (Sigma).[16] Oxaloacetic acid (Sigma) was dissolved in 1 % $NaHCO_3$, stored at 0 °C and used within 1 day. The incubation mixtures consisted of 10 μN citrate synthase (1 mg), 8 mM propionyl-CoA and 15 mM oxaloacetate in 2 ml of 30 mM potassium phosphate buffer and had final pH values 7.3 – 7.7. Reaction mixtures were left at 22 °C for 20 h and lyophilised either directly or after gel filtration. A control experiment was performed with heat denatured enzyme. The methylcitric acids formed were isolated and characterized as in previous work.[2] For the quantitative determinations a simplified GLC internal standard procedure was used; after the methylcitric acids had been converted to trimethyl esters by reaction with diazomethane in ether, a sufficient amount of a standard solution (containing trimethyl citrate) was added so that the ratio sample/standard was close to unity. The mixture was then analysed by GLC.

Kinetic investigation. Using parallel mixtures, reactions were interrupted at various times by addition of 0.5 M NaOH and the samples were lyophilised and methylated before determination of the extent of reaction by GLC as described above.

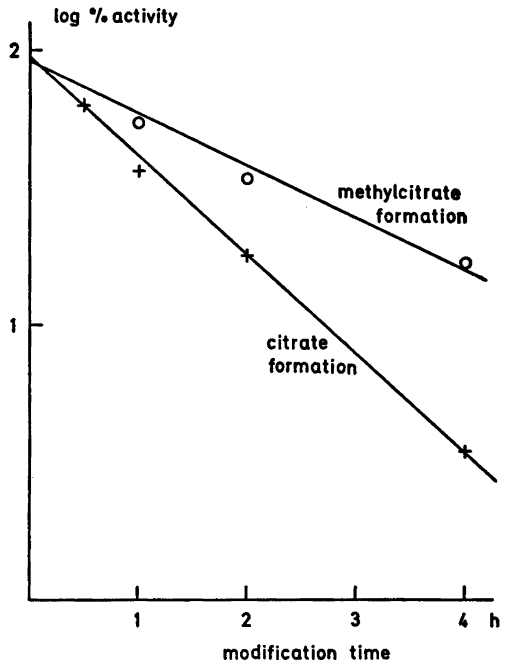

Fig. 3. Effect of butanedione modification on the activity of citrate synthase.

Arginine modification. Citrate synthase at 20 μN was reacted with 60 mM butanedione in 1 ml 0.1 M borate buffer pH 7.5.[14] The modification was stopped by gel filtration of the mixture on a column of Sephadex G-15 (Pharmacia), using 0.125 M borate pH 7.5 as eluent. An aliquot was treated similarly but without butanedione. The enzyme was assayed for residual citrate synthase activity [17] and methylcitrate synthase activity by incubation for 20 h as above. To prevent restoration of essential arginine groups, the latter reaction was performed in 0.1 M borate pH 7.5 instead of phosphate buffer.[18]

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Reduction of 3,3,5,5-Tetramethylcyclopentane-1,2-dione and its Reaction with Methylmagnesium Bromide

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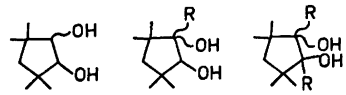
In connection with our interest in reactions [1] of 3,3,5,5-tetramethylcyclopentane-1,2-dione [2] and especially the equilibria [3] in alkaline solutions we found it desirable to synthesize a series of cyclopentane-1,2-diols (Scheme 1).

We report here the preparations of *cis*- and *trans*-3,3,5,5-tetramethylcyclopentane-1,2-diols by reduction of 3,3,5,5-tetramethylcyclopentane-1,2-dione and the spectrometric [4–11] determination of configurations of these new diols. We also describe the reaction of the title compound with methylmagnesium bromide.

Preparation of 3,3,5,5-tetramethylcyclopentane-1,2-diols. When 3,3,5,5-tetramethylcyclopentane-1,2-dione was reduced with sodium borohydride in methanol at room temperature, the only isolated product was *trans*-3,3,5,5-tetramethylcyclopentane-1,2-diol (*trans-1*). The crystallized product showed only one peak analyzed by glass capillary gas chromatography.

The diketone was hydrogenated at about 100 atm. and 60 – 70 °C to give exclusively the diol *cis-1*. Hydrogenation at lower temperatures was incomplete.

Determination of configurations. In some cases it is possible to assign the configurations by comparing the IR spectra of the isomers in the OH stretching region.[5,6,12,13] In the case of cyclopentane-1,2-diols only the *cis*-isomer is capable of forming an intramolecular hydrogen bond thus showing both the absorptions of free and bonded OH groups in dilute (< 0.005 M) solutions.



1 *2* *3*

The IR spectra of *cis-1* and *trans-1* were measured by using 0.005 and 0.0025 M CCl_4 solutions. The *cis*-isomer shows two OH bands at 3643 (free) and 3576 cm^{-1} (bonded) the intensity ratios being equal in both concentrations.

The spectrum of *trans-1* shows only one OH band (3630 cm^{-1}). When the concentration is 0.005 M, the *trans*-isomer also shows a band at *ca.* 3400 cm^{-1} due to an intermolecular hydrogen bond, which does not exist at higher dilution (0.0025 M).