Carboxylation of β-methylcrotonyl coenzyme A by a purified enzyme from chicken liver

In an earlier study[1,2] of leucine metabolism in crude heart extracts, data were obtained indicating that β-methylcrotonyl CoA is acted upon by enoyl hydrase (crotonase) and that the resulting β-hydroxyisovaleryl CoA is carboxylated enzymically in the presence of ATP to yield β-hydroxy-β-methylglutaryl CoA. This product is known[3] to be attacked by a specific cleavage enzyme to furnish equimolar amounts of acetoacetate and acetyl CoA. LYNEN AND KNAPPE[4-6] have recently made the interesting discovery, however, that β-methylcrotonyl CoA is carboxylated directly in crotonase-free enzyme preparations from *Mycobacterium* spp. β-Methylglutaconyl CoA, the product of this carboxylation reaction, is hydrated to yield β-hydroxy-β-methylglutaryl CoA by the action of methylglutaconase, a specific hydrase present in microorganisms and in animal tissues[7].

During the past two years we have been engaged in the purification of the relatively unstable carboxylase from chicken liver in the hope that interfering enzymes could be removed and the nature of this reaction in animal tissues could be established with finality. With a 100-fold purified preparation of the carboxylase, a requirement for methylglutaconase in the overall conversion of β-methylcrotonyl CoA to acetoacetate has been established (Table I). Furthermore, by employing carboxylase and cleavage-enzyme preparations preincubated with *p*-chloromercuribenzoate to inhibit the residual crotonase, β-methylcrotonyl CoA, rather than β-hydroxyisovaleryl CoA, has been identified as the true substrate for the chicken-liver carboxylase (Table II). The further unexpected finding has been made that β-methylvinylacetyl CoA also gives rise to acetoacetate in this carboxylase system in the absence of added crotonase. The absorption maximum characteristic of α,β-unsaturated thiol esters (λ = 267 mμ for β-methylcrotonyl CoA) does not appear when

TABLE I

REQUIREMENT FOR METHYLGLUTACONASE IN CONVERSION OF β-METHYLCROTONYL CoA TO ACETOACETATE

Expt.	*System*	*Enzyme absent from reaction mixture*	*Acetoacetate formed (μmole)*
1	Complete	None	0.14
2	No pig-heart fraction	None	0.14
3	No chicken-liver fraction	Carboxylase	0.00
4	No ox-liver fraction	Methylglutaconase	0.02 §

The complete reaction mixture contained 200 μmoles $KHCO_3$, 5 μmoles glutathione, 2.5 μmoles ethylenediaminetetraacetic acid, 25 μmoles $MgCl_2$, 2.5 μmoles ATP, 1.3 μmoles β-methylcrotonyl CoA, 100-fold purified CO_2-activating enzyme[8] (0.2 mg protein), 100-fold purified carboxylase from chicken liver (1.2 mg protein), a pig-heart fraction (shown to be free of methylglutaconase) containing 0.2 units of β-hydroxy-β-methylglutaryl CoA cleavage enzyme, and an ox-liver fraction containing methylglutaconase and 0.2 unit of β-hydroxy-β-methylglutaryl cleavage enzyme, in a final vol. of 2.2 ml. After incubation for 1 h at 38° under N_2 the reaction mixtures were deproteinized with trichloroacetic acid and aliquots were taken for the estimation of acetoacetate[9].

§ The quantity of cleavage enzyme in Expt. 4 was adequate for full activity in the assay system, as indicated in Expt. 2.

Abbreviations: CoA, coenzyme A; ATP, adenosine triphosphate.

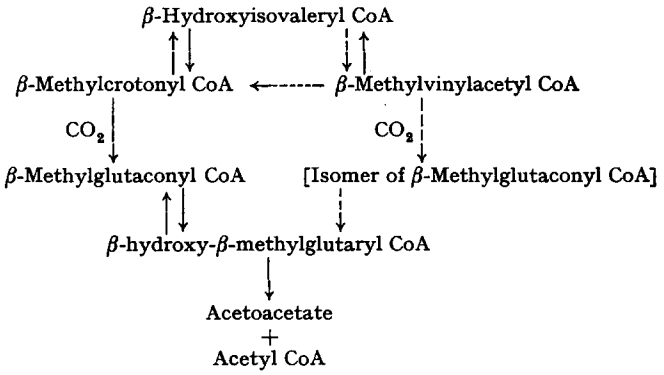
TABLE II

SUBSTRATES FOR THE CARBOXYLATION REACTION

Substrate	Acetoacetate formed (μmole)	
	Crotonase added	Crotonase absent
β-hydroxyisovaleryl CoA	0.13	0.01
β-methylcrotonyl CoA	0.07	0.11
β-methylvinylacetyl CoA	0.06	0.09

The complete reaction mixture contained $KHCO_3$, glutathione, ethylenediaminetetraacetic acid, $MgCl_2$ and ATP as shown in Table I, 1.3 μmoles of the thiol ester indicated, 80-fold purified CO_2-activating enzyme (0.1 mg protein), β-hydroxy-β-methylglutaryl CoA cleavage enzyme partially purified from ox liver (0.3 mg protein), 100-fold purified carboxylase from chicken liver (0.4 mg protein), and crystalline crotonase (0.04 mg protein) where indicated, in a final vol. of 1.5 ml. The carboxylase and cleavage-enzyme preparations were preincubated with *p*-chloromercuribenzoate (10^{-3} *M*) for 15 min at 0° to inactivate the crotonase present. Incubation. 1 h at 38°.

β-methylvinylacetyl CoA is incubated with the carboxylase alone. These observations suggest that, in the absence of crotonase, β-methylvinylacetyl CoA is either carboxylated directly or is transformed into β-methylcrotonyl CoA by the action of an isomerase[10] in the reaction mixture, as indicated in the accompanying scheme. The conversion of β-methylvinylacetyl CoA to β-hydroxyisovaleryl CoA by the action of crotonase has been established previously[11].



In view of the finding that β-methylcrotonyl CoA is carboxylated in the absence of crotonase in liver preparations, as in preparations of *Mycobacterium* spp.[4–6], attempts are in progress to determine whether methylglutaconase plays any role in the carboxylase system from pig heart.

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Soluble pyridine nucleotide nucleosidase from seminal vesicles

Pyridine nucleotide nucleosidases present in animal tissues are generally regarded as being intimately bound to the cell structure. In order to extract these enzymes it was necessary in the past to apply procedures involving the use of organic solvents[1,2]. In this communication we wish to report on the properties of a pyridine nucleotide nucleosidase which occurs in the seminal vesicles and vesicular secretion of the bull. In the course of purification it was found that the enzyme occurred in a soluble form and that the preparation possessed marked TPNase activity which equalled or even exceeded DPNase activity.

The secretion from the seminal vesicles of young mature bulls (one and a half years old) was expressed manually. Both the residual vesicular tissue and secretion, if not immediately utilised, were stored at —20°. In order to extract the enzyme from the seminal-vesicle tissue, one part of mince, prepared by passing the seminal-vesicle tissue twice through a meat mincer, was mixed with 3 vol. water and centrifuged at low speed. The aqueous extract was used for further purification and the precipitate was discarded. Both the aqueous extract from the seminal vesicles and the vesicular secretion showed DPNase as well as TPNase activity. DPNase values, in units/ml, ranged from 37 to 48 (equivalent to 148–192 in the mince) for the extract from vesicles, and from 121 to 263 for the secretion. TPNase activity in the aqueous extracts of vesicles was approximately 35 units (equivalent to 140 in the mince), and in the secretion it ranged from 75 to 335. Nearly all of the enzymic activity was present in the soluble form. This could be shown by the fact that when the aqueous extract and the secretion, respectively, were centrifuged in a Spinco Model L at 100,000 $\times$ g for 30 min, the resulting clear supernatant from the tissue extract retained 76 % of the DPNase activity present before centrifugation, and in the centrifuged secretion all of the activity remained in the supernatant.

It is obvious that from the bull seminal-vesicle tissue and secretion it is practicable to purify pyridine nucleotide nucleosidase without tedious solubilising procedures which are known to affect the enzyme yield adversely. The main steps in the purification, which was carried out in the cold room, are shown in Table I. The values given in that table were obtained with the use of 299 ml of vesicular secretion as starting material. The secretion was first brought with 0.1 M acetic acid to pH about 4.5. After centrifuging in a refrigerated centrifuge at 5500 rev./min for 10 min, 290 ml of clear, deep-red fluid were obtained. After neutralisation with 1 M NH_4OH,