On the enzymic mechanism of acetoacetate synthesis

It has become evident that acetoacetate synthesis can be effected *in vitro* by coupling of two enzymes known to occur in liver, the HMG–CoA condensing enzyme[1,2] (reaction (1)) and the HMG–CoA cleavage enzyme[3] (reaction (2)).

$$\text{Acetoacetyl–CoA} + \text{acetyl–CoA} + H_2O \longrightarrow \text{HMG–CoA} + \text{CoASH} \quad (1)$$

$$\text{HMG–CoA} \longrightarrow \text{Acetoacetate} + \text{Acetyl–CoA} \quad (2)$$

$$\textit{Sum}\ \text{Acetoacetyl–CoA} + H_2O \longrightarrow \text{Acetoacetate} + \text{CoASH} \quad (3)$$

Recently LYNEN *et al.*[4] have presented seemingly convincing evidence that acetoacetate synthesis in ox-liver extract does occur via this mechanism and have assigned it major physiological significance.

We wish to report that in rat-liver extracts acetoacetate synthesis proceeds unimpaired after the endogenous HMG–CoA condensing and cleavage enzymes have been completely inactivated by treatment with iodoacetamide. As shown in Table I with iodoacetamide-treated rat-liver extract, acetoacetate synthesis proceeded stoichiometrically from substrate amounts of acetoacetyl-CoA without (Expt. 1b) or with (Expt. 1c) additional iodoacetamide. The failure of acetyl-CoA to accelerate the disappearance of acetoacetyl-CoA (Expt. 1d) showed the complete inhibition by iodoacetamide[2] of endogenous HMG–CoA condensing enzyme. The presence of the latter enzyme in untreated extract was demonstrated by optical[2,4] and chemical measurements. The failure of acetyl-CoA to give rise to acetoacetate (Expt. 1e) and to be detected in Expts. 1b and 1c showed that endogenous thiolase (Expt. 2a) had been inactivated by iodoacetamide. In the untreated liver extract both acetyl-CoA and acetoacetyl-CoA formed acetoacetate. Less acetoacetate was formed from acetoacetyl-CoA by the untreated extract than by the iodoacetamide-treated extract.

TABLE I

ACETOACETATE SYNTHESIS FROM ACETOACETYL-CoA

The reaction mixture in each experiment contained 100 μmoles Tris-HCl buffer, pH 7.5, and other additions as indicated, in a final vol. of 1.0 ml. Incubation, 30 min at 38°. In Expt. 1, the enzyme was a sonic extract of washed rat-liver mitochondria[7] which had been incubated with 10^{-3} *M* iodoacetamide at 0° for 2 h before use. In Expt. 2, the enzyme was an untreated, similarly prepared extract. All values (Expt. 1b onward) have been corrected for the spontaneous AcAc–CoA deacylation (Expt. 1a). Acetoacetate was determined chemically[8] and acetoacetyl-CoA[9] and acetyl-CoA[10] by specific enzymic methods.

Expt.	*Additions*				*change (μmoles)*		
	Enzyme	*AcAcCoA*	*AcCoA*	*IAAm*	*AcAc*	*AcAcCoA*	*AcCoA*
	mg	*μmole*	*μmole*	*μmoles*			
1a		0.98		2	(+ 0.12)	(— 0.19)	
1b	2	0.98			+ 0.40	— 0.40	0
1c	2	0.98		2	+ 0.40	— 0.36	0
1d	2	0.98	0.96	2	+ 0.38	— 0.36	— 0.26
1e	2		0.96	2	+ 0.01		— 0.23
2a	2	0.98			+ 0.30	— 0.69	+ 0.51
2b	2	0.98	0.96		+ 0.27	— 0.69	+ 0.31
2c	2		0.96		+ 0.10		— 0.33

Abbreviations: CoA (or CoASH), coenzyme A; HMG, β-hydroxy-β-methylglutaric acid; IAAm, iodoacetamide; Tris, tris(hydroxymethyl)aminomethane; AcAc, acetoacetyl-; Ac, acetyl-.

This is explained by the more rapid loss of acetoacetyl-CoA by a secondary reaction, thiolysis, which can proceed when CoASH release occurs by acetoacetyl-CoA deacylation and later reaction[1]. The presence of HMG-CoA-cleavage enzyme and its complete inactivation by iodoacetamide treatment is shown by the data in Table II. Glutathione was not required for cleavage enzyme activity. It decreased acetyl-CoA accumulation because acetyl glutathione, formed by chemical transacetylation, was hydrolyzed by an endogenous thioesterase[5].

TABLE II

INHIBITION OF HMG–CoA CLEAVAGE ENZYME BY IODOACETAMIDE

The basic reaction mixture (Expt. 2) contained 100 μmoles Tris–HCl buffer, pH 7.5, 2.0 μmoles DL-HMG–CoA and enzyme as indicated. In Expt. 1, 2 μmoles iodoacetamide was also present; in Expt. 3, 13 μmoles glutathione and 20 μmoles $MgCl_2$ were added. Incubation, 30 min at 38°.

Expt.	*Enzyme*	*Acetoacetate*	*Acetyl CoA*
		μmole	*μmole*
1	Iodoacetamide-treated (2 mg)	0.004	0
2	Untreated (2 mg)	0.70	0.54
3	Untreated (2 mg)	0.57	0.16

It is noteworthy that acetoacetate synthesis from acetyl-CoA or acetoacetyl-CoA proceeded without added thiol or Mg^{++}, even though acetoacetate synthesis in this extract, when assayed in a *catalytic* system[4,6], employing acetyl phosphate, CoASH, phosphotransacetylase and thiolase to generate acetoacetyl-CoA, required a thiol and was stimulated by Mg^{++}, as originally found for ox-liver extract[4,6]. These experiments show (a) that the thiol requirement and Mg^{++} stimulation evident in the *catalytic* acetoacetate assay system do not reflect the requirements of acetoacetyl-CoA deacylation itself and (b) even in enzyme fractions possessing both HMG-CoA condensing and cleavage enzymes, acetoacetate synthesis need not proceed via HMG-CoA. They provide strong evidence that a specific acetoacetyl-CoA deacylase catalyzing reaction (3) occurs in rat-liver mitochondria, although it cannot be excluded that the acetoacetyl group is first transferred to some non-thiol contaminant of commercial CoA to form the true substrate of the hydrolytic enzyme. Identical results to those recorded here have been obtained with a partly purified ox-liver acetoacetate-synthesizing enzyme system[6] and will be reported elsewhere.

This work was supported by Grant A-739 from the U.S. Public Health Service. We thank Dr. CECIL COOPER for a supply of liver extract.

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Received July 24th, 1959