ENZYMES OF ACETOACETATE FORMATION

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Two mechanisms have been proposed for the formation of acetoacetate. Using extracts prepared from acetone powders of beef liver Lynen and coworkers (1958) obtained evidence that acetoacetate formation occurred via HMG-CoA as intermediate and involved the HMG-CoA cleavage and HMG-CoA condensing enzymes as shown in reactions (1) and (2).

(1) Ac-CoA + AcAc-CoA + H_2O ⟶ HMG-CoA + CoASH

(2) HMG-CoA ⟶ Acetoacetate + Ac-CoA

In the assay system used, acetyl-CoA was generated from catalytic amounts of CoA in the presence of excess acetyl phosphate and bacterial phosphotransacetylase. Acetoacetyl-CoA is formed by thiolase present in liver fractions and in the bacterial extract. Using a similar assay system, and also substrate amounts of acetoacetyl-CoA, Drummond and Stern (1960) suggested that acetoacetate formation in extracts prepared from fresh beef liver could take place by a direct deacylation of acetoacetyl-CoA (reaction 3).

(3) AcAc-CoA + H_2O ⟶ Acetoacetate + CoASH

Very recently, Segal and Menon (1960) have reported that acetoacetate synthesis in mitochondrial rat liver preparations occurs exclusively by this latter mechanism.

We now wish to report that acetoacetate formation in extracts of fresh beef liver requires two enzymes each of which have been obtained relatively free of the other. The data strongly suggest that the HMG-CoA pathway accounts for all the acetoacetate formed in these liver extracts as measured in the catalytic assay system.

When a number of beef liver fractions were subjected to heating at 50°, aceto-

acetate synthesis was largely destroyed. Activity was completely restored by addition of purified yeast HMG-CoA condensing enzyme (Table I). Lynen and co-workers (1958) concluded from similar observations that loss in acetoacetate synthesis was due to heat lability of the condensing enzyme. These fractions all contain HMG-CoA cleavage enzyme as determined by direct assay and this enzyme is stable to heat in all fractions except the 20-35% ethanol (the most purified aceto-acetate synthesizing preparation of Stern et al (1960)). In accord with this, addition of yeast condensing enzyme gave only very small recovery of activity in this heated preparation (Table I). With the exception of the 20-35% ethanol, acetoacetate synthesis in most of the unheated fractions is increased by addition of condensing enzyme, presumably because this enzyme is limiting. Wieland and co-workers (1960) have made similar observations in crude rat liver extracts. The results indicate that loss of acetoacetate forming activity by heat may indeed be due to destruction of the condensing enzyme, and that in the 20-35% ethanol fraction the cleavage enzyme is also largely removed by heat.

In an effort to obtain further information regarding the mechanism involved, our most purified preparation (20-35% ethanol) was subjected to several fraction-ation procedures. By precipitation with zinc ion, followed by increasing concen-trations of ethanol, several fractions were obtained in which acetoacetate synthesizing activity was virtually absent (Table II). The fractions obtained by ethanol precipitation showed no increase in activity when assayed with excess yeast condensing enzyme. Three of the fractions, however, showed a very great increase in activity when assayed in the presence of excess cleavage enzyme (Table II, column 5). This strongly indicated that these fractions contained the condensing enzyme largely free of cleavage enzyme. The latter enzyme was shown by separate specific assay to be present only in very small amounts in two of the fractions (Table II, column 2). The cleavage enzyme is known to be precipitated by zinc (see footnote Table II).

Furthermore acetoacetate synthesizing activity in the zinc-ethanol fractions was now restored by adding a heat-treated preparation (Table II, last column).

Table I

Effect of yeast HMG-CoA condensing enzyme
on acetoacetate synthesis in heated beef liver fractions

(1) Fraction	(2) Protein	(3) HMG-CoA cleavage activity	(4) Aceto-acetate synthesis	(5) (4) + yeast condensing enzyme	Recovery
	mg/ml	units/ml	units/ml	units/ml	per cent
Crude $KHCO_3$ extract	62	-	8.3	13.7	
Heated $KHCO_3$ extract	31.6	-	0.76	14.7	107
30-60% $(NH_4)_2 SO_4$	74.0	61.3	43.6	-	
Heated 30-60% $(NH_4)_2 SO_4$	46.2	57.0	2.8	35.2	
20-35% ethanol	44	64.0	25.4	24.9	
Heated 20-35% ethanol	18.2	4.34	1.4	4.2	16.6
Extract acetone pwdr. beef liver	30.2	36.2	14.3	22.0	
Heated acetone pwdr. extract	20.2	35.4	1.22	22.0	100
Extract of acetone pwdr. pigeon liver	31.4	-	48.7	100.0	
Heated acetone pwdr. pigeon liver	24.0	-	5.75	96.1	96.1

The crude bicarbonate extract, the 30-60% saturated $(NH_4)_2 SO_4$, and the 20-35% ethanol fractions were prepared by the method of Stern et al (1960) and represent three of the steps in the purification of the acetoacetate synthesizing system. The acetone powder extracts were prepared by the method of Lynen and coworkers (1958). Heat treatment was carried out by adjusting the fractions (2 ml) to pH 7.5 and stirring in a bath at 50° for 8 minutes, followed by cooling and centrifugation. Protein was determined by the method of Warburg and Christian (1941). Acetoacetate synthesis was assayed by the method of Stern et al (1960) except that all components were reduced by one-fourth and acetoacetate was determined by the method of Walker (1954). Specific activity is defined as units per mg protein. Yeast HMG-CoA condensing enzyme was purified by the method of Ferguson and Rudney (1959). The ammonium sulfate precipitate obtained after protamine treatment was exhaustively dialyzed against .02 M Tris, pH 7.0. An amount was added which catalyzed the formation of at least 4 μmoles of acetoacetate in the presence of excess cleavage enzyme. HMG-CoA was prepared by the procedure of Hilz et al (1958). HMG-CoA cleavage enzyme was assayed by the system of Bachhawat et al (1955) except that all components were reduced by one-half and the Walker method used for acetoacetate determination. The per cent recovery is calculated on the basis of acetoacetate formed in the original unheated extracts in the presence of added condensing enzyme, because this enzyme is limiting.

The total recovery of units was 93.2% of the original 20-35% ethanol when these fractions were supplemented with a heat treated acetone powder extract. Clearly, recovery of the condensing enzyme was virtually quantitative Identical results

Table II

Effect of HMG-CoA condensing and cleavage enzymes on acetoacetate formation in zinc-ethanol fractions of beef liver

(1) Fraction	(2) Total protein	(3) HMG-CoA cleavage activity	(4) Acetoacetate synthesis total activity	(5) (4) + condensing enzyme	(6) (4) + cleavage enzyme	(7) (4) + heated beef acetone pwdr. extract
	mg	units	units	units	units	units
20-35% ethanol	440	640	253	249	455	455
Extract of Zn^{++} ppt.	53.3	-	13.0	12.5	-	39.0
0-6.2% EtOH	44.5	-	14.2	8.7	94.7	92.5
6.2-11.8% EtOH	60.0	18.2	8.0	10.6	147.0	147.0
11.8-16.6% EtOH	81.0	20.0	20.3	15.2	133.0	133.0
16.6-30% EtOH	21.5	-	1.7	1.2	-	10.7
Total recovery %	60.0	-	12.5	10.5	-	93.2

20-35% ethanol of beef liver fraction (44 mg/ml) (10 ml) in .02 $MKPO_4$ pH 7.5 was diluted with 10 ml water and, while stirring in ice, 2 ml M K succinate pH 6.0 was added, followed by 8 ml of 0.1 M zinc acetate. The heavy precipitate was removed by centrifugation. The supernatant was fractionated with ethanol between the limits indicated in the table. The 0-6.2% fraction was obtained at 0°, the 6.2-11.8 and the 11.8-16.6% fractions at -5° C and the remaining fraction at -15°. All precipitates were taken up in 0.02 M Tris pH 7.5 containing 0.1% glutathione. Only a small amount of the zinc precipitate dissolved. It was recentrifuged and the precipitate discarded.* All fractions were dialyzed overnight against 6 l of 0.02 M phosphate pH 7.5 containing 1 mM EDTA and 1 mM cysteine, and for a further 5 hours against 4 l of the same buffer without EDTA. Acetoacetate synthesis was measured as described in Table I. HMG-CoA cleavage enzyme was purified from liver by the method of Lynen and coworkers (1958) and sufficient enzyme was added to catalyze the formation of at least 2 µmoles of acetoacetate per hour in the presence of excess yeast condensing enzyme. The heated beef liver acetone powder extract was that shown in Table I. Recoveries are calculated on the basis of the original 20-35% ethanol assayed with added cleavage enzyme, since, in this fraction this enzyme is limiting.

* Lynen and coworkers (1958) found that the cleavage enzyme was solubilized from a zinc precipitate only by extraction with high phosphate concentration. In a more recent experiment we have confirmed that this enzyme can indeed be recovered from the zinc precipitate.

were obtained when the heated 30-60% ammonium sulfate fraction was used. The heated 20-35% ethanol preparation, however, did not restore activity obviously because the cleavage enzyme had been largely destroyed. Addition of purified cleavage enzyme to the original 20-35% ethanol increases acetoacetate synthesis, indicating that in this fraction, cleavage enzyme is limiting (see Table II).

The condensing enzyme in these fractions can also be demonstrated by the optical test system described by Ferguson and Rudney (1959). The enzyme is quite stable even after repeated freezing and thawing. The condensing enzyme from yeast, in contrast to the findings of the above authors, is also stable. This may be due to exhaustive dialysis in dilute buffer using deionized glass distilled water.

It thus seems likely that acetoacetate synthesis in these liver fractions occurs by the combined action of HMG-CoA condensing and cleavage enzymes. Each enzyme has been obtained largely free of the other. The quantitative nature of the recovery on combination of the two enzymatic entities indicates that this system accounts for most, if not all, of the acetoacetate formed in these extracts as measured in the catalytic assay system.

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