STUDIES ON ACETOACETATE FORMATION

by

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ABSTRACT

In recent years, two mechanisms have been proposed for the enzymatic formation of acetoacetate by liver extracts. One of these, the "HMG-CoA cycle", involves the condensation of acetyl-CoA and acetoacetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) via the action of the HMG-CoA condensing enzyme, with the release of free coenzyme A (CoASH) (reaction 1),

$$\text{Acetyl-CoA} + \text{acetoacetyl-CoA} + H_2O \rightleftharpoons \text{HMG-CoA} + \text{CoASH} \qquad (1)$$

followed by cleavage of the HMG-CoA to acetyl-CoA and free acetoacetate, via the action of the HMG-CoA cleavage enzyme (reaction 2).

$$\text{HMG-CoA} \rightleftharpoons \text{acetoacetate} + \text{acetyl-CoA} \qquad (2)$$

The second mechanism which has been proposed involves a direct deacylation of acetoacetyl CoA through the action of a specific acetoacetyl-CoA thioesterase (reaction).

$$\text{Acetoacetyl-CoA} + H_2O \longrightarrow \text{acetoacetate} + \text{CoASH} \qquad (3)$$

Evidence is presented which indicates acetoacetate formation by a soluble enzyme system from bicarbonate extracts of whole beef liver proceeds largely, if not exclusively, via HMG-CoA (reactions 1 and 2). Both the HMG-CoA condensing and cleavage enzymes have been partially purified from beef liver bicarbonate extracts, each free of contamination by the other, in good yields. The level of activity of these two enzymes is sufficiently high to account for all the acetoacetate formed by liver tissue. The possibility that the specific acetoacetyl-CoA thioesterase may play a minor

role in the enzymatic synthesis of acetoacetate is also discussed.

The intracellular and tissue localization of the enzymes of acetoacetate formation is also discussed. In liver homogenates, most, if not all, of the acetoacetate-synthesizing activity appears to be associated with the mitochondrion. Evidence is also presented that the primary reason for the inability of extrahepatic tissue preparations to catalyze the accumulation of acetoacetate may be the lack of one of the enzymes involved, i.e., the HMG-CoA condensing enzyme, and not merely further metabolic degradation of acetoacetate, as has generally been assumed.

An enzyme fraction in chicken liver extracts which inhibits the in vitro formation of acetoacetate by chicken liver homogenates has also been studied. Evidence is presented that this enzyme fraction exerts its effect through the inactivation of coenzyme A. Preliminary observations indicate that this enzyme may be a 3'-nucleotidase, removing the 3'-phosphate of coenzyme A, forming dephosphocoenzyme A.

The occurrence of a highly active β-hydroxybutyryl dehydrogenase in extracts of dry culture of C. kluyveri has been noted. This enzyme differs from the similar enzyme reported in mammalian tissues, in that it is very specific for triphosphopyridine nucleotide, and is virtually inactive with diphosphopyridine nucleotide (DPN) (reaction).

$$\text{Acetoacetyl-CoA} + \text{TPNH} + H^+ \rightleftharpoons \beta\text{-hydroxybutyryl-CoA} + \text{TPN}^+ \qquad (4)$$

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ABBREVIATIONS

The following abbreviations have been used: acetyl-P, acetyl phosphate; acyl-CoA, acyl thioester of coenzyme A; acyl-GSH, acyl thioester of glutathione; ATP, adenosine triphosphate; BAL, 2,3-dimercaptopropanol; CoASH, reduced coenzyme A; DEAE-cellulose, diethylaminoethyl cellulose; DPN and DPNH, the oxidized and reduced forms, respectively, of diphosphopyridine nucleotide; DTO, 6,8-dithioloctanoic (dihydrolipoic) acid; EDTA, ethylenediamine tetraacetic acid; GSH, reduced glutathione; HMG, β-hydroxy-β-methylglutaric acid; IAA, iodoacetamide; Pi, orthophosphate; PPi, inorganic pyrophosphate; TPN and TPNH, the oxidized and reduced forms, respectively of triphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)-aminomethane.

The physiological and pathological aspects of ketosis and ketogenesis have attracted the attention of many workers for more than a century, sparked by the accumulation of the three ketone bodies, acetone, acetoacetate and β-hydroxybutyrate, as metabolic end-products in the ketosis associated with diabetes mellitus. Over the years, this relationship between ketosis and diabetes mellitus has led to a great deal of interest in the biochemistry of ketogenesis. Although it was early recognized that fatty acids served as the dietary source of the ketone bodies, and that the chief site of ketogenesis was the liver, it is only in recent years that significant progress has been made in the clarification on the precise biochemical pathways through which their formation proceeds. Within the last decade, various enzymatic pathways for the formation of acetoacetate, the primary ketone body, have been proposed, and abundant but inconclusive evidence presented for each proposal. During the last three years, in particular, there has been considerable controversy concerning this problem. This thesis represents the results of a concerted effort to establish conclusively the ultimate pathway(s) through which acetoacetate, and thus the other ketone bodies, are formed.

HISTORICAL BACKGROUND

The story of ketosis began in 1857, when Petters (1) reported the presence of acetone in diabetic urine. Eight years later, Gerhardt (2) detected acetoacetate in diabetic urine, although he incorrectly identified the compound as ethyl acetoacetate. Tollens (3) and Diechmuller (4) later correctly identified Gerhardt's compound as the free acid. The relationship between these two ketones was pointed out by Arnold (5), who recognized that

acetoacetate was the immediate precursor of acetone.

In 1898, Geelmuyden (6) recognized that fatty acids gave rise to the ketone bodies. This led to the perfusion studies of Embden et al (7,8) with isolated dog liver. These workers found that acetoacetate was formed when livers were perfused with media containing fatty acids having an even number of carbon atoms. Perfusing the livers with fatty acids having an odd number of carbon atoms did not give rise to significant amounts of acetoacetate. These observations led to the development of the theory of β-oxidation, in which fatty acids are degraded by a stepwise removal of two-carbon units through oxidation at the β-carbon, to yield a terminal four-carbon fragment which could give rise to acetoacetate. These workers (9,10) and Friedman (11) also found that acetoacetate was formed when excised livers were perfused with acetate. This was explained as a β-oxidation - condensation process; the two-carbon units formed during β-oxidation could condense to form a four-carbon compound, which could then give rise to acetoacetate. Embden and Loeb (8) also found that acetoacetate was formed when livers were perfused with the aromatic amino acids, phenylalanine and tyrosine.

Although these early observations provided a firm foundation for further studies of ketogenesis, very little progress was made for almost 30 years. In the late 1930's, the next step in the transition from whole animal observations to the study of the individual enzymes was taken, namely the study of ketogenesis by liver slices. Jowett and Quastel (12) and Leloir and Munoz (13) showed that when liver slices were incubated with even-numbered fatty acids, acetoacetate accumulated in the medium. Various other groups found that liver slices could form acetoacetate from a wide variety of compounds, including acetate (14), crotonate (15), vinylacetate (16),

sorbate (14) and leucine (17). Quastel et al (18) showed that the oxidation of fatty acids by tissue slices prepared from spleen, testis and kidney also resulted in the accumulation of acetoacetate; however, the acetoacetate formed by these tissues amounted to only 1/40 to 1/10 that formed by an equivalent liver preparation.

The next advance, the formation of acetoacetate by cell-free preparations, was accomplished by Munoz and Leloir (19) in 1943. They found that the "easily sedimentable fraction" of liver homogenates could carry out the oxidation of fatty acids, in the presence of Mg^{++}, adenylic acid, cytochrome c and another oxidizable substrate. Ketones were the major end product. Their preparation, however, was quite labile, and would not oxidize fatty acids with chain lengths of more than six carbons. Lehninger (20) reported the preparation of a similar "easily sedimentable fraction" from rat liver which could oxidize fatty acids with chain lengths of up to 18 carbons, and which was relatively stable. In the presence of ATP and Mg^{++}, and with the addition of malonate to block terminal oxidation via the tricarboxylic acid cycle, fatty acids were quantitatively converted to acetoacetate. In addition, in the absence of a source of oxalacetate, and in the presence of malonate, pyruvate was quantitatively converted to acetoacetate. Lehninger demonstated that acetoacetate was not further metabolized in these liver preparations. Shortly after this, Kennedy and Lehninger (21,22) showed that the mitochondrion was the actual site of fatty acid oxidation and acetoacetate formation. Lehninger (23) also prepared a similar enzyme preparation from heart, which could oxidize fatty acids under the same conditions as did the liver preparation. However, there was no accumulation of acetoacetate; in its place, α-ketoglutarate and succinate accumulated, indicating that acetoacetate, if formed, was further metabolized by extrahepatic tissues. Grafflin and Green

(24), working with what they termed the "cyclophorase" system from liver and kidney, demonstrated the formation of acetoacetate from several unsaturated short-chain acids. It was later shown that the "cyclophorase" system was, to all intents and purposes, simply a mitochondrial preparation.

An important breakthrough in the study of the precise mechanism of ketogenesis occurred with the demonstration of acetoacetate formation by soluble enzymes. Soodak and Lipmann (25) reported that soluble extracts of pigeon liver acetone powders could catalyze the formation of acetoacetate from acetate, if supplemented with ATP, Mg^{++}, and coenzyme A. In 1951, Stadtman et al (26) found that pigeon liver extracts, supplemented with bacterial phosphotransacetylase and coenzyme A, could form acetoacetate from acetyl phosphate. Using this coupled enzyme system and C^{14}-labelled compounds, these workers showed that both two-carbon units condensing to form acetoacetate came from "active acetate", which Lynen (27) identified as the coenzyme A thioester of acetic acid. The reaction sequence is represented by reaction 1 (catalyzed by phosphotransacetylase) and the overall reaction 2 is catalyzed by the liver extract.

$$2 \text{ acetyl-P} + 2 \text{ CoASH} \rightleftharpoons 2 \text{ acetyl-CoA} + 2 P_i \qquad (1)$$

$$2 \text{ acetyl-CoA} \longrightarrow \text{acetoacetate} + 2 \text{ CoASH} \qquad (2)$$

$$\text{Net Reaction:} \quad 2 \text{ acetyl-P} \longrightarrow \text{acetoacetate} + 2 P_i$$

The discovery of acetoacetyl-CoA (28,29,30) and the demonstration of its formation as an intermediate in fatty acid metabolism (31,32) provided an attractive candidate for the role of the immediate precursor of enzymatically formed acetoacetate. Acetoacetyl-CoA could be formed from fatty

acids *via* β-oxidation, and also from the condensation of two molecules of acetyl-CoA *via* the action of β-ketothiolase (reaction 3).

$$2 \text{ acetyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{CoASH} \qquad (3)$$

At this same time, Stern *et al* briefly reported (29) the partial purification from beef liver extracts of an enzyme system which was capable of catalyzing the formation of acetoacetate from added acetyl-CoA, or from acetyl-CoA generated *in situ* with phosphotransacetylase, acetyl phosphate and catalytic concentrations of coenzyme A. They postulated that acetoacetyl-CoA formed by β-ketothiolase (reaction 3) was hydrolyzed by a specific deacylase to liberate free acetoacetate (reaction 4):

$$\text{Acetoacetyl-CoA} + H_2O \longrightarrow \text{acetoacetate} + \text{CoASH} \qquad (4)$$

Unfortunately, the unequivocal demonstration of the presence of this specific deacylase or thioesterase in liver extracts has proven extremely difficult.

Another mechanism for the formation of acetoacetate by tissue extracts was demonstrated by Stern *et al* (30) and also by Green *et al* (31). In the presence of succinate, various tissue extracts can catalyze the formation of acetoacetate from acetoacetyl-CoA through the action of succinyl-β-ketoacyl coenzyme thiophorase (CoA transferase, thiophorase) (reaction 5):

$$\text{Acetoacetyl-CoA} + \text{succinate} \rightleftharpoons \text{acetoacetate} + \text{succinyl-CoA} \qquad (5)$$

This enzyme is not found in mammalian liver; its physiological role appears to involve the extrahepatic activation of acetoacetate prior to its cleavage

to acetyl-CoA and its subsequent oxidation *via* the tricarboxylic acid cycle in these tissues.

A further mechanism which could be considered was reversal of the acetoacetate activation reaction (reaction 6) which had been reported in yeast (29) and pigeon liver (36).

$$\text{Acetoacetate} + \text{ATP} + \text{CoASH} \rightleftharpoons \text{acetoacetyl-CoA} + \text{AMP} + \text{PP}_i \qquad (6)$$

However, like the CoA transferase reaction, this reaction does not occur in mammalian liver, and appears to be involved only in extrahepatic activation of acetoacetate.

Based on the findings that (a) beef liver extracts hydrolyzed acetoacetyl-CoA only on the addition of glutathione, (b) acetoacetate synthesis from acetyl phosphate and coenzyme A in the coupled phosphotransacetylase-liver extract system required the addition of thiol for maximal synthesis, and (c) beef liver extracts contained a very active acetoacetyl-glutathione thioesterase, Stern and Drummond (34) postulated a mechanism for acetoacetate formation involving the transfer of the acetoacetyl moiety from coenzyme A to glutathione by a transferase (reaction 7) followed by hydrolysis of the glutathione thioester to liberate free acetoacetate (reaction 8).

$$\text{Acetoacetyl-CoA} + \text{GSH} \longrightarrow \text{acetoacetyl-GSH} + \text{CoASH} \qquad (7)$$

$$\text{Acetoacetyl-GSH} + \text{H}_2\text{O} \longrightarrow \text{acetoacetate} + \text{GSH} \qquad (8)$$

Unfortunately, although reaction 7 occurs very rapidly over a considerable range of experimental conditions, and especially at alkaline pH, the transfer

is non-enzymatic, and lacks thiol and thioester specificity. The existence of an enzyme catalyzing this reaction has never been definitely established. Although the coupling of reactions 7 and 8 constitutes a mechanism for the deacylation of acetoacetyl-CoA, there is no evidence that it has any physiological significance in acetoacetate formation. Lynen et al (35) later reported that the purification of the acetoacetate synthesizing enzyme system did not parallel the purification of the acetoacetyl-GSH thioesterase.

In 1954, Bachhawat et al (36) reported the formation of acetoacetate from HMG-CoA, a degradation product of the amino acid leucine, by the HMG-CoA cleavage enzyme, which is found in a wide variety of tissues (reaction 9).

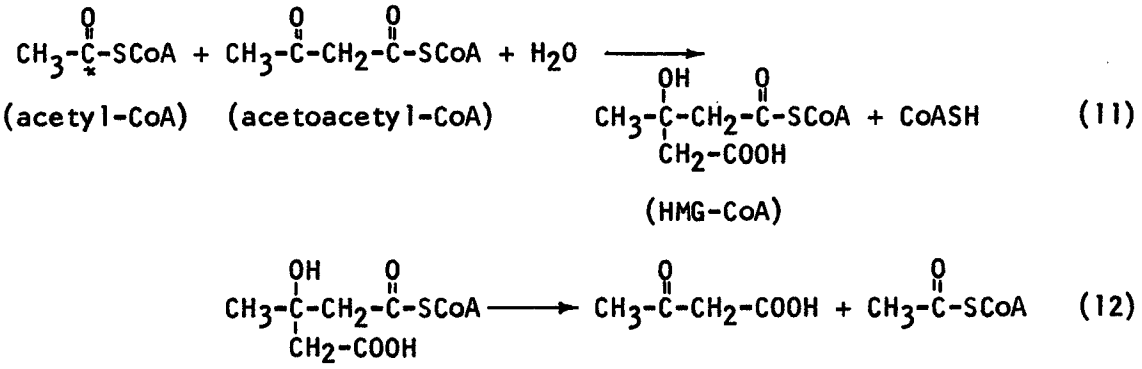
$$\text{HMG-CoA} \longrightarrow \text{acetoacetate} + \text{acetyl-CoA} \qquad (9)$$

These authors partially purified the enzyme (37), and demonstrated absolute requirements for thiol and divalent cation. The acetoacetate synthesizing system from beef liver also showed these same requirements. In 1956, Rudney (38) briefly reported the formation of radioactive HMG following the incubation of liver enzyme preparations with ATP, coenzyme A, and acetate-C^{14}. In a subsequent report (39), the same author demonstrated that the product of the reaction was an HMG-CoA thioester, and was formed by the condensation of acetoacetyl-CoA and acetyl-CoA. The enzyme was given the name "HMG-CoA condensing enzyme". Using a purified enzyme fraction from yeast, which proved to be a source of a more stable enzyme than did liver, Rudney and Ferguson (40) were able to demonstrate that the product of the condensation reaction was the HMG-CoA monothioester, and that the condensation therefore proceeded according to reaction 10. To determine which thioester bond was hydrolyzed during the condensation reaction, acetyl-1-C^{14}-CoA and unlabelled acetoacetyl-

Acetoacetyl-CoA + acetyl-CoA + H_2O

$\longrightarrow$ HMG-CoA + CoASH (10)

CoA were incubated with purified, thiolase-free HMG-CoA condensing enzyme, and the radioactive HMG-CoA isolated and incubated with the HMG-CoA cleavage enzyme of Bachhawat et al (37). The products of the cleavage reaction were isolated and analyzed for radioactivity. The C^{14} was found exclusively in the carboxyl group of the acetoacetate formed; thus, it must have been the thioester bond of acetyl-CoA which was hydrolyzed during the condensation, and the reaction must have proceeded as shown by reactions 11 and 12. A more complete report on the purification and properties of the HMG-CoA



condensing enzyme from baker's yeast has since been published (41,42).

In 1956, Lynen briefly reported (43) that Bublitz, working in the Munich laboratory, had separated an acetoacetate synthesizing enzyme system from beef liver acetone powder into two fractions, enzymes "A" and "B", both of which were required for the formation of acetoacetate from acetyl phosphate in the coupled transacetylase system described above. Later, Lynen et al (35) published a longer report on these two enzymes, identifying enzyme "B" as the HMG-CoA condensing enzyme, and enzyme "A" as the HMG-CoA cleavage enzyme. Since their enzyme "B" preparation from liver proved to be

unstable, they prepared a purified HMG-CoA condensing enzyme from baker's yeast. Using this purified yeast enzyme and a purified enzyme "A" preparation from beef liver, they carried out a series of experiments, essentially a duplication and extension of the experiment of Rudney and Ferguson as outlined above, which demonstrated that if these two purified enzymes were coupled in an appropriate assay system, acetoacetate was formed. From these experiments, they concluded that acetoacetate formation in liver extracts, with the exception of that formed from the aromatic amino acids, for which a different pathway is already known (44), proceeds exclusively <u>via</u> HMG-CoA. It should be pointed out, however, that their evidence is derived almost entirely from experiments in which <u>yeast</u> preparations were used as the source of the HMG-CoA condensing enzyme. When this fact is taken into consideration, it would appear that the statement,

> "Wir konnen daher annehmen, dass die Acetacetatbildung in der Leber, bis auf den Abbau der aromatischem Aminosauren, uber β-hydroxy-β-methylglutaryl-CoA fuhrt."[1]

is somewhat premature.

In 1960, Stern and co-workers presented evidence that acetoacetate formation by rat liver mitochondrial preparations (45) and partially purified beef liver extracts (29,46,47) from acetoacetyl-CoA need not proceed <u>via</u> HMG-CoA. It was shown that acetoacetate could be formed from substrate amounts of acetoacetyl-CoA by liver preparations in which β-ketothiolase and the HMG-CoA condensing and cleavage enzymes had been apparently totally inactivated by previous treatment with iodoacetamide. Similar findings with an untreated rat liver mitochondrial preparation have been independently

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[1] "We can therefore assume that acetoacetate formation in liver, except that formed in the degradation of the aromatic amino acids, preceeds <u>via</u> β-hydroxy-β-methylglutaryl CoA." (cf. reference 35).

reported by Segal and Menon (48); however, the validity of the latter report is very much open to question, as will be more fully discussed later.

More recently, Hird and Symons (49) have reported on the formation of acetoacetate from C^{14}-labelled substrates by sheep omasum and rumen epithelium, which Pennington (50,51,52) has shown to be fully as active as liver tissue in catalyzing the formation of acetoacetate. Their results, obtained with whole cell preparations, indicate almost beyond dispute that at least 75%, and probably more, of the acetoacetate formed in their experiments could not possibly have been formed by direct deacylation of acetoacetyl-CoA; the HMG-CoA pathway is the only known mechanism which can explain their findings. This will be discussed in more detail later.

Clearly, the precise enzymatic mechanism for the formation of acetoacetate is controversial and unsettled. This thesis represents an attempt to clarify the mechanism. Three main approaches to the problem have been employed. The first consists of attempts to purify the acetoacetate forming system in liver to a greater degree than that previously achieved. The second approach consisted of efforts to resolve the system into two or more enzymatic entities. If indeed HMG-CoA is an intermediate, it should be possible to separate the two enzymes involved, namely, the HMG-CoA condensing enzyme and the HMG-CoA cleavage enzyme. Synthesis of acetoacetate could then proceed only upon recombination of the two enzymatic entities. Finally, the third approach has been to study the mechanism by which a protein factor obtained from chicken liver inhibits acetoacetate formation in the standard assay system. The following experiments describe our results to date and strongly indicate that HMG-CoA is an intermediate. Two enzymes have been obtained, each largely free of the other, and acetoacetate synthesis proceeds only upon their recombination. A brief preliminary account of some of our

work has appeared (53). In addition, the reduction of acetoacetyl-CoA by a TPNH-specific dehydrogenase in C. kluyveri extracts is described.

MATERIALS AND METHODS

MISCELLANEOUS

ATP (dipotassium salt), coenzyme A ("75% pure"), glucose 6-phosphate (barium salt), L-cysteine HCl, glutathione (reduced), TPN, TPNH, DPN, DPNH, HMG, glucose 6-phosphate dehydrogenase and dried cultures of Clostridium kluyveri were commercial products. For some experiments, analytically pure coenzyme A was prepared from the commercial product by chromatography on DEAE cellulose (Selectacel) according to Moffatt and Khorana (54). Glucose 6-phosphate was converted to the dipotassium salt through exhaustive treatment with Amberlite IR-120, potassium form, before use. Acetyl phosphate was prepared as the dilithium salt from acetic anhydride and orthophosphate according to Avison (55) and purified (final purity: 95%) by fractional precipitation from aqueous solution with ethanol (56). Acetyl-CoA was prepared from acetic anhydride and CoASH according to Simon and Shemin (57), acetoacetyl-CoA from diketene and CoASH according to Lynen et al (35), and HMG-CoA from HMG anhydride and CoASH according to Hilz et al (58). These thioesters were used without further purification.

Coenzyme A was measured by the catalytic phosphotransacetylase assay (59). Protein was estimated by the spectrophometric method of Warburg and Christian (60) or by the modified biuret reaction of Gornall et al (61). Nucleic acid was estimated spectrophotometrically (60) or by the turbidimetric method of Jones and Lipmann (62).

Crude extracts of Clostridium kluyveri, used as a source of phosphotransacetylase and as a supplementary source of β-ketothiolase, were prepared essentially according to Stadtman and Barker (63). Vacuum-dried cells (1 g) were ground to a smooth paste with 10 ml of 0.01 M potassium phosphate buffer, pH 8.1, and incubated with frequent stirring for 4½ hours at 38°. The suspension was centrifuged for 15 minutes at 20,000 x g, 0°. The precipitate was thoroughly extracted with 5 ml of cold glass-distilled water, and the resulting suspension centrifuged as before, The two cell-free supernatant solutions were combined; 13 to 14 ml of a bright yellow extract were obtained. This extract can be shown to contain phosphotransacetylase (64) and β-ketothiolase (65) by appropriate assay procedures. An interesting side-light is that the β-ketothiolase of this bacterial extract appears to be absolutely specific for CoASH and acetoacetyl-CoA; unlike the mammalian enzyme, it cannot catalyze a thiolysis of acetoacetyl-Pn by pantetheine.

PURIFICATION OF THE ACETOACETATE SYNTHESIZING ENZYME SYSTEM

I. From Beef Liver Homogenates

The acetoacetate synthesizing enzyme system of beef liver was purified according to Stern et al (46) from bicarbonate extracts through precipitation with ammonium sulfate, adsorption of inactive protein on calcium phosphate gel, and precipitation with ethanol. Data from a typical purification procedure are presented in Table II. The purification procedure is readily reproducible, and regularly results in a five- to seven-fold purification of the system. It should be noted that if the liver used has an abnormally high lipid content, the calcium phosphate gel step may not be as successful as is reported here; in fact, it may result in a decrease, rather than increase,

in specific activity.

II. From Beef Liver Acetone Powder

Beef liver was obtained immediately after slaughter and packed in ice for transport to the laboratory. If the tissue was not to be used at once, it was frozen within two hours after slaughter. To prepare the acetone powder, partially thawed or fresh tissue was cut into small pieces and homogenized with 10 volumes of cold (-20°) acetone in a Waring Blendor operating at full velocity for 3 minutes. The suspension was then placed in a deep freeze for 15 to 20 minutes. The supernatant solution was decanted and discarded, and the residue filtered by suction on a large (25 cm) Buchner funnel, using coarse (Whatman No. 5) filter paper. The damp "cake" which was obtained was briefly homogenized in the blendor with a further 5 volumes (based on the weight of the original tissue) of cold acetone, and again filtered. The "cake" was washed on the filter with cold acetone, and dried as thoroughly as possible by suction. The damp mass was then crumbled by hand and dried *in vacuo* at 0°. The dried mass was rubbed to a fine powder and stored over anhydrous calcium sulfate ("Drierite") at -20°. Using the above procedure, 20 to 25 g of a fluffy tan-coloured powder can be obtained from 100 g of liver tissue.

Preparation of extract. A portion of the acetone powder was weighed and ground to a smooth paste in a pre-chilled mortar with 10 volumes of 0.2 M potassium bicarbonate solution, containing 0.005 M cysteine and adjusted to pH 8.2 to 8.3. This suspension was allowed to stand 30 to 45 minutes at 0°, with occasional stirring, and centrifuged for 20 minutes at 20,000 x *g*, 0°. The precipitate was then thoroughly extracted with a further 2 volumes of the same buffer, and the suspension was centrifuged. The two supernatant solutions were combined. From 10 g of acetone powder, 95 to 100 ml of

extract were obtained.

Ammonium sulfate fractionation. The bicarbonate extract was diluted with cold glass-distilled water, if necessary, to adjust the protein concentration to 30 mg per ml. Solid ammonium sulfate (21.2 g for each 100 ml of diluted extract) was added gradually over a period of 20 minutes, with mechanical stirring, to bring the solution to 30% saturation. After standing 15 minutes with continued stirring, the suspension was centrifuged for 20 minutes at 20,000 x g, 0°. The precipitate was discarded. The supernatant solution was brought to 60% saturation by addition of another 21.2 g of solid ammonium sulfate for each 100 ml of original diluted extract, in the manner indicated. The mixture was stirred and centrifuged as before, and the supernatant fluid was discarded. The precipitate was dissolved in a minimal volume of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine, and dialyzed overnight vs 3 liters of the same buffer. The results are summarized in Table III.

III. From Beef Liver Mitochondrial Acetone Powder

The large scale isolation of mitochondria from beef liver and preparation of the acetone powder were carried out according to the procedure of Mahler et al (66).

Preparation of extract. Bicarbonate extracts of the mitochondrial acetone powder were prepared by the general procedure outlined above for beef liver acetone powder.

Ammonium sulfate fractionation. The bicarbonate extract was diluted with glass-distilled water, if necessary, to adjust the protein concentration to 30 mg per ml, and stirred at 0°. Solid ammonium sulfate (24.7 g for each 100 ml of diluted extract) was added gradually over 20 minutes, to bring the solution to 35% saturation. After standing 15 minutes with stirring,

the suspension was centrifuged for 20 minutes at 20,000 x g, 0°. The precipitate was discarded. The supernatant fluid was brought to 55% saturation by the addition of another 14.1 g of solid ammonium sulfate for each 100 ml of the original diluted extract, in the manner indicated. The suspension was stirred and centrifuged as before, and the supernatant solution was discarded. The precipitate was dissolved in a minimal volume of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine, and dialyzed overnight vs 4 liters of the same buffer. The results are summarized in Table III.

IV. From Pigeon Liver Homogenates

Preparation of extract. Pigeons were stunned with a blow on the head, decapitated, and bled. The livers were rapidly removed and immediately frozen. For preparation of the crude extract, 28 g of partially thawed liver, cut into small pieces, were placed in a Waring Blendor and 140 ml of 0.2 M potassium bicarbonate, containing 0.005 M cysteine and adjusted to pH 8.2 to 8.4, were added. The suspension was homogenized for 5 minutes at full velocity, chilled in ice for 10 minutes, and homogenized 5 minutes further at full velocity. The homogenate was strained through four layers of cheesecloth to remove fat particles and centrifuged for 30 minutes at 30,000 x g, 0°. The precipitate was discarded.

Ammonium sulfate precipitation. The crude extract was diluted with an equal volume of cold glass-distilled water, and stirred in an ice bath. Solid ammonium sulfate (28.4 g for each 100 ml of diluted extract) was added slowly over a period of 20 minutes, to bring the solution to 40% saturation. After standing 15 minutes with continued stirring, the suspension was centrifuged for 20 minutes at 20,000 x g, 0°, and the supernatant fluid was discarded. The precipitate was dissolved in a minimal volume of 0.02 M

potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine, and dialyzed overnight *vs* 4 liters of the same buffer. The slightly turbid dialyzed extract was centrifuged for 30 minutes at 30,000 x *g*, 0°. The results are summarized in Table III.

V. From Pigeon Liver Acetone Powder

Pigeon liver acetone powder was prepared by the general procedure outlined above for beef liver acetone powders. Extracts were prepared as described above, using either the bicarbonate buffer already described or 0.05 M potassium phosphate buffer, pH 7.5.

Ammonium sulfate precipitation. The crude extract was diluted with cold glass-distilled water to adjust the protein concentration to 30 mg per ml, and stirred at 0°. Solid ammonium sulfate (24.7 g for each 100 ml of diluted extract) was added gradually over a period of 20 minutes, to bring the solution to 35% saturation. After standing 15 minutes with continued stirring, the suspension was centrifuged for 20 minutes at 20,000 x *g*, 0°. The supernatant solution was discarded. The precipitate was dissolved in a minimal volume of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine, and dialyzed overnight *vs* 4 liters of the same buffer. The results are summarized in Table III.

ACETOACETATE SYNTHESIZING ASSAY SYSTEM

The acetoacetate synthesizing activity of tissue extracts was measured as their ability to catalyze the formation of acetoacetate from acetyl phosphate in the presence of phosphotransacetylase and catalytic concentrations of CoASH. Two assay systems have been used in this work, both based on the method of Stern *et al* (46).

Assay system I, used in earlier experiments, consisted of Tris-HCl

buffer, pH 8.15, 200 μmoles; $MgCl_2$, 2.5 μmoles; KCl, 10 μmoles; glutathione, 20 μmoles; dilithium acetyl phosphate, 45 μmoles; coenzyme A, 0.05 μmoles; Clostridium kluyveri extract, 0.02 to 0.10 ml, depending on the phosphotransacetylase activity; enzyme to be assayed; glass-distilled water to a final volume of 2.0 ml. The glutathione was neutralized to pH 8 with KOH immediately before use. All components except the enzyme fraction to be assayed were added to tubes at 0°, and this mixture was incubated at 38° for 5 minutes. The reaction was initiated by addition of enzyme, and incubation was continued at 38°. After 60 minutes, the tubes were removed to an ice bath and 1.0 ml of 12% (w/v) trichloroacetic acid added. Denatured protein was removed by centrifugation, and suitable aliquots of the supernatant solution were taken for determination of acetoacetate by the phenylhydrazone method of Greenberg and Lester (67) as modified by Barkulis and Lehninger (68).

For Assay system II, all components of the above system were reduced to one-fourth the stated amounts, with a final volume of 0.5 ml. Acetoacetate in the deproteinized solution was measured by a modification of the method of Walker (69) similar to that reported by Kalnitsky and Tapley (70). The procedure which gave the most reproducible results, and which has been adopted for routine use, is as follows:

A suitable aliquot of the deproteinized solution was diluted to 0.05 ml with 4% (w/v) trichloroacetic acid in a glass-stoppered test tube at 0°. To this diluted aliquot were added, in rapid succession, 3.5 ml of M sodium acetate buffer, pH 5.1, and 3.0 ml of the diazotized p-nitroaniline reagent described by Walker (69). The tubes were then placed in a water bath at 38°. After 30 minutes, the tubes were placed in ice for 3 to 5 minutes. The solutions were then acidified by the addition of 6.0 ml of 5N HCl, and

the tubes were kept in ice for 8 to 10 minutes. Ethyl acetate (4.0 ml) was then added, and the formazan derivative quantitatively extracted into the organic layer by shaking the tubes by hand 35 times. The absorbance of the organic layer was then measured in a Beckman DU spectrophotometer at λ= 450 mμ. (d=0.5 cm). Under the above conditions, 0.1 μmole of acetoacetate will produce an absorbance of 0.470. Using this procedure, the colour developed was proportional to the amount of acetoacetate present over the range of 0.05 to 0.55 μmoles.

One unit of acetoacetate synthesizing activity is defined as the amount of enzyme which, under the conditions of either of the above assay systems, catalyzes the formation of 1.0 μmole of acetoacetate per hour. Specific activity is expressed as units per mg of protein.

PREPARATION AND ASSAY OF HMG-CoA CONDENSING ENZYME

The HMG-CoA condensing enzyme was purified from baker's yeast according to the procedure of Ferguson and Rudney (41). The purification was carried as far as the ammonium sulfate precipitation step following protamine treatment. The above authors found that the enzyme at this stage of the purification procedure was very unstable if stored as a solution, even when frozen. It was, however, somewhat more stable if stored as a precipitate under saturated ammonium sulfate solution. Even under these conditions, they reported that the enzyme lost 25 per cent of its activity in one week. In contrast to this, we found that if the enzyme solution was thoroughly dialyzed *vs* dilute buffer (0.01 M Tris-HCl buffer, pH 7.0) prepared from glass-distilled deionized water, it retained full activity after 5 months. In later preparations of this enzyme, the autolysis

procedure of Lynen et al (35), rather than that of Ferguson and Rudney, was used.

The HMG-CoA condensing enzyme preparations were assayed by measuring the amount of acetoacetate formed in the catalytic assay system supplemented with an excess of partially purified HMG-CoA cleavage enzyme. In the absence of HMG-CoA cleavage enzyme, the yeast enzyme was unable to catalyze the formation of acetoacetate. For our purposes, one unit of HMG-CoA condensing enzyme was defined as the amount of enzyme which, under the conditions of the assay, catalyzed the formation of 1.0 μmole of acetoacetate per hour. One of these units is approximately equivalent to one unit of enzyme "B" activity as defined by Lynen et al (35), who used a similar assay method, and is approximately equivalent to three units of HMG-CoA condensing activity as measured in the spectrophotometric assay of Ferguson and Rudney (41). Their assay is based on the disappearance of acetoacetyl-CoA enolate ion absorption at λ= 310 mμ. The spectrophotometric assay procedure is rather unreliable from a quantitative standpoint, since most of the enzyme fractions are considerably contaminated with β-ketothiolase, which will interfere with the assay.

PREPARATION AND ASSAY OF HMG-CoA CLEAVAGE ENZYME

The HMG-CoA cleavage enzyme was partially purified from beef liver acetone powder extracts by a modification of the procedure of Lynen et al (36). The procedure finally adopted for routine use is given below.

Heat treatment. A phosphate buffer extract of beef liver acetone powder, prepared by the general procedure described previously, was heated in a 55° water bath until its temperature reached 50°. It was then transferred to a 50° bath and kept there for 10 minutes. The suspension was immediately

placed in an ice bath to cool. Denatured protein was removed by centrifugation for 45 minutes at 30,000 x g, 0°.

Zinc precipitation. The heat-treated supernatant solution was diluted with 0.05 M Tris-HCl buffer, pH 7.5, to adjust the protein concentration to 15 mg per ml, and stirred at 0°. To this diluted extract, 0.25 volume of 0.1 M zinc acetate solution was added dropwise. After standing 15 minutes with continued stirring, the suspension was centrifuged for 10 minutes at 20,000 x g, 0°, and the supernatant solution discarded. The precipitate was thoroughly extracted with 0.2 M potassium phosphate buffer, pH 7.5, containing 0.003 M glutathione, with the aid of a Potter Elvjehm homogenizer. The suspension was centrifuged for 10 minutes at 20,000 x g, 0°. The residue was washed with a small volume of the same buffer and recentrifuged. The two supernatant solutions were combined.

Ammonium sulfate precipitation. The redissolved zinc precipitate was mixed with an equal volume of a neutral saturated (0°) ammonium sulfate, to produce 50% saturation. After standing 20 to 30 minutes with mechanical stirring, the suspension was centrifuged for 20 minutes at 20,000 x g, 0°. The precipitate was dissolved in a minimal volume of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.002 M cysteine, and dialyzed overnight vs 5 liters of the same buffer. The dialyzed extract was then centrifuged for 30 minutes at 30,000 x g, 0°.

The HMG-CoA cleavage enzyme preparations were assayed by measuring the amount of acetoacetate formed in the standard acetoacetate synthesis assay system supplemented with an excess of HMG-CoA condensing enzyme from yeast. In the absence of the "condensing" enzyme, the HMG-CoA cleavage enzyme fractions were unable to catalyze the formation of acetoacetate. For our purposes, one unit of HMG-CoA cleavage enzyme was defined as the

amount of enzyme which, under the conditions of the assay, catalyzed the formation of 1.0 μmole of acetoacetate per hour. One of these units is approximately equivalent to one unit of enzyme "A" activity as defined by Lynen et al (35), who used a similar assay procedure, and is approximately equivalent to 1.5 to 2.0 units of HMG-CoA cleavage enzyme activity as defined by Bachhawat et al (36), who measured the acetoacetate formed from stoichiometric concentrations of HMG-CoA.

The purification procedure outlined above is essentially that of Lynen et al (35), but with the omission of the acetone step. We found that the acetone step, while achieving a considerable purification, resulted in a considerable loss of total activity, due to "spreading" of the enzyme over a wide range of acetone concentrations and to loss of protein through denaturation. Thus, this procedure results in only a five- to seven-fold purification, compared to the 30-fold purification reported by the above authors. However, the total enzyme recovery was consistently in the region of 75%, compared to 10%. In any event, the purification achieved through this modified procedure was adequate for our purposes. A summary of a typical purification is given in Table I.

TABLE I.

Purification of HMG-CoA cleavage enzyme from beef liver acetone powder

Fraction	Protein	Specific Activity	Total Activity	Yield
	mg	units/mg	units	%
Phosphate extract	2100	0.90	1890	"100"
Heated extract	1404	1.28	1790	95
Zinc precipitate	541	2.96	1600	84
$(NH_4)_2SO_4$ precipitate	281	4.76	1340	71

PREPARATION OF RAT TISSUE HOMOGENATES

Female rats (150 g, fasted 48 hours) were stunned by a blow on the head, decapitated and bled. The heart, liver and kidney were rapidly perfused with a small volume of cold saline. The various organs were quickly excised and placed in cold aerated 0.25 M sucrose solutions. The tissues were cut into small pieces, blotted with filter paper and weighed. Homogenates (brain, 20%; all other tissues, 10%) were prepared in 0.25 M sucrose solution in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenates were stored frozen for 5 days before assay; this has been shown by Bucher et al (71) and Segal et al (72), and confirmed in this laboratory, to produce an "activation" of the acetoacetate synthesizing enzyme system, possibly by freeing the enzyme(s) from the particle-bound form.

For some experiments, mitochondria were isolated from rat liver homogenates by differential centrifugation (73). The mitochondria were also "activated" by frozen storage for 5 days.

RESOLUTION OF THE ACETOACETATE SYNTHESIZING ENZYME SYSTEM

1. Procedure of Bublitz (43)

To 110 ml of a phosphate extract of beef liver acetone powder, at 0°, 6.1 ml of N acetic acid was added, bringing the solution to pH 5.4 (glass electrode). After standing 10 minutes with gentle stirring, the suspension was centrifuged for 20 minutes at 30,000 x g, 0°. The precipitate was discarded. The supernatant solution was readjusted to pH 7.5 by the cautious addition of 1.2 ml 5N KOH.

To 120 ml of acid supernatant fraction, in a -5° bath, 30 ml of cold

(-10°) ethanol was added dropwise, with efficient stirring, to produce a final ethanol concentration of 20% by volume. After standing 10 minutes with continued stirring, the suspension was centrifuged 40 minutes at 13,000 x g, -5°. The supernatant solution (142 ml) was brought to a final ethanol concentration of 40% by volume by the dropwise addition of 47 ml of cold (-20°) ethanol, during which the temperature was gradually lowered to -15°. After stirring for an additional 10 minutes, the suspension was centrifuged 90 minutes at 13,000 x g, -15°. The supernatant solution was discarded. The two precipitates were dissolved in minimal volumes of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine, and dialyzed overnight vs 5 liters of the same buffer.

The dialyzed 0 to 20% ethanol fraction (38 ml) was brought to 32% saturation by the addition of 8.6 g of solid ammonium sulfate gradually over 20 minutes, with mechanical stirring. After standing 20 minutes with continued stirring, the suspension was centrifuged for 20 minutes at 20,000 x g, 0°. The supernatant solution was brought to 90% saturation by the addition of a further 15.6 g of solid ammonium sulfate, in the manner indicated. After standing 20 minutes, the suspension was centrifuged for 20 minutes at 20,000 x g, 0°. The supernatant solution was discarded.

The dialyzed 20 to 40% ethanol fraction (25 ml) was brought to 63% saturation by the addition of 11.1 g of solid ammonium sulfate in the manner indicated. After stirring for 20 minutes, the suspension was centrifuged as above. The supernatant solution was brought to 80% saturation by the addition of a further 3 g of solid ammonium sulfate. The suspension was stirred and centrifuged as described above. The supernatant solution was discarded.

The four ammonium sulfate precipitates were dissolved in minimal

volumes of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine, and dialyzed *vs* 3 liters of the same buffer. After 3 hours, the buffer was changed and dialysis continued for 5 hours. The dialyzed fractions were clarified by centrifugation for 30 minutes at 30,000 x *g*, 0°.

II. Ammonium Sulfate Fractionations

Ten ml of a phosphate buffer extract of beef liver acetone powder, prepared by the general procedure outlined above, was brought to 50% saturation by the dropwise addition of 10 ml of a neutral saturated (0°) solution of ammonium sulfate, with efficient stirring. After stirring for 15 minutes, the suspension was centrifuged 15 minutes at 20,000 x *g*, 0°. The supernatant solution was brought to 85% saturation by the addition of 4.9 g of solid ammonium sulfate over a period of 20 minutes, with mechanical stirring. After 15 minutes the suspension was centrifuged for 30 minutes at 20,000 x *g*, 0°. Both precipitates were dissolved in minimal volumes of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M cysteine. An aliquot of the 50 to 85% saturated fraction was immediately frozen. The balance of the 50 to 85% saturation fraction and the 0 to 50% saturation fraction were dialyzed *vs* 3 liters of the same buffer. After 2 hours, the buffer was replaced and dialysis continued for 2 hours. The buffer was again changed, and dialysis continued for a further 2 hours. The dialyzed fractions were centrifuged for 30 minutes at 30,000 x *g*, 0°.

Immediately before assay, a second set of fractions was prepared by the same procedure, but *without* dialysis. These fractions were assayed as rapidly as possible after preparation.

III. Chromatography on Calcium Phosphate Gel

Experiment 1. Calcium phosphate gel ("brushite" form, $2CaO.P_2O_5.5H_2O$) was prepared according to Tiselius (74) and thoroughly washed with 0.002 M

potassium phosphate buffer, pH 7.0. A column of the gel, 2 cm x 18 cm, was prepared in a jacketed chromatography tube. Water, chilled by passage through Tygon tubing coiled in a salt bath at -2 to -4°, was circulated through the jacket. This served to keep the effluent at 2 to 4°. The column was equilibrated for temperature in the cold room for 4 hours. Beef liver ammonium sulfate fraction (total of 325 mg protein), previously dialyzed thoroughly vs 0.002 M potassium phosphate buffer, pH 7.0, was placed on top of the column and thoroughly washed in with the same buffer. The protein was eluted with a linear gradient. The mixing flask initially contained 1 liter of 0.002 M potassium phosphate buffer, pH 7.0, and the reservoir flask 1 liter of 0.2 M potassium phosphate buffer, pH 7.0. The flow rate was maintained at 1 ml per minute by application of slight pressure to the reservoir flask from a nitrogen tank. Fractions of 5 ml were collected into tubes containing 0.05 ml of 0.1 M neutral cysteine, to give a final thiol concentration of 0.001 M. Protein concentration of the effluent was followed by light absorption at λ= 280 mμ. After 160 tubes had been collected, protein remaining on the column was eluted with 0.2 M potassium phosphate buffer, pH 7.0. (As later experiments showed, a considerable amount of protein is not eluted with 0.2 M buffer.) The eluates were pooled in lots of approximately 20 tubes (100 ml) and the protein precipitated by addition of solid ammonium sulfate (60 g for each 100 ml of solution) to produce 85% saturation. The precipitates were collected by centrifugation, dissolved in minimal volumes of 0.02 M potassium phosphate buffer, pH 7.5, and dialyzed overnight vs 6 liters of the same buffer.

Experiment 2. A column of calcium phosphate gel ("brushite") was prepared by the procedure described above, and equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. Beef liver ammonium sulfate fraction (total of

365 mg of protein) was adsorbed onto the gel as described above. Protein was removed from the gel by stepwise elution with phosphate buffer, pH 7.0, of gradually increasing ionic strength. The peaks (as determined by light absorption at λ= 280 mμ) were pooled and lyophilized. The lyophylized powders were dissolved in minimal volumes of glass-distilled water, and dialyzed vs 7 liters of 0.005 M neutral cysteine for 8 hours. The external fluid was then replaced with 7 liters of 0.005 M potassium phosphate buffer pH 7.5, containing 0.001 M cysteine, and dialysis continued for 6 hours.

IV. Zinc-ethanol Fractionation.

Ten ml of beef liver 20 to 35% ethanol fraction were diluted with 10 ml of glass-distilled water, to produce a protein concentration of 15 mg per ml. The solution was adjusted to pH 6.0 with 2 ml of potassium succinate buffer, pH 6.0. While the solution was efficiently stirred, 8 ml of 0.1 M zinc acetate solution were added dropwise. After standing for 10 minutes with continued stirring, the suspension was centrifuged for 10 minutes at 13,000 x g, 0°.

To the clear supernatant solution, 2.0 ml of cold (0°) ethanol was added, dropwise, to produce a final ethanol concentration of 6.2% by volume. The suspension was stirred for 10 minutes, and centrifuged for 15 minutes at 13,000 x g, 0°.

The resulting supernatant solution was chilled to -5° in a dry ice-ethanol bath, and 2.0 ml of cold (-5°) ethanol added dropwise, with stirring, to produce a final ethanol concentration of 11.8% by volume. After standing 10 minutes at -5° with continued stirring, the suspension was centrifuged for 15 minutes at 13,000 x g, -5°.

The supernatant solution obtained was chilled to -5°, and 2.0 ml of cold ethanol was added dropwise, with stirring, to produce a concentration

of 16.6% by volume. The suspension was stirred 15 minutes further and centrifuged for 15 minutes at 13,000 x g, -5°.

This final supernatant solution was chilled to -15°, and 15 ml of cold (-15°) ethanol was added dropwise, with stirring, to produce an ethanol concentration of 30% by volume. After standing 15 minutes at -15° with continued stirring, the suspension was centrifuged for 10 minutes at 13,000 x g, -16°.

All precipitates were immediately taken up in minimal volumes of 0.02 M Tris-HCl buffer, pH 7.5, containing 0.01 M EDTA and 0.1% glutathione, and dialyzed without centrifuging for 10 hours vs 6 liters of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA and 0.001 M cysteine. The buffer was then replaced with a similar buffer, but without EDTA, and dialysis was continued for 3 hours. The buffer was replaced by 3 liters of fresh buffer, also without EDTA, and dialysis was continued for a further 3 hours. All preparations were centrifuged to remove turbidity. (The zinc precipitate fraction contained a considerable amount of insoluble protein.)

PURIFICATION OF CHICKEN LIVER "INHIBITOR" ENZYME FRACTION

Preparation of extract. Livers of young (8 week) chickens were obtained immediately after slaughter and packed in ice. Portions of chilled liver (150 g) were placed in a Waring Blendor, and 200 ml of 0.2 M potassium bicarbonate solution, containing 0.005 M cysteine and adjusted to pH 8.2 - 8.4, were added. The suspension was homogenized at full velocity for 5 minutes, and cooled in an ice bath for 10 to 15 minutes. Another 100 ml of the same buffer was added, and homogenization continued for 5 minutes. Homogenates from several portions of liver were pooled, strained through two layers of cheesecloth, and centrifuged for 60 minutes at 13,000 x g, 0°.

First ammonium sulfate fractionation. The bicarbonate extract was diluted with 0.6 volumes of cold glass-distilled water, and stirred in an ice-bath. Solid ammonium sulfate (28.2 g for each 100 ml of diluted extract) was added gradually over 20 minutes, to bring the solution to 40% saturation. After stirring for 20 minutes, the suspension was centrifuged for 60 minutes at 13,000 x g, 0°. The precipitate was discarded. The supernatant solution was brought to 70% saturation by adding another 21.2 g of solid ammonium sulfate for each 100 ml of original diluted extract, in the manner indicated. The mixture was stirred and centrifuged, and the precipitate dissolved in a minimal volume of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.005 M cysteine. The solution was dialyzed vs 4 liters of the same buffer for 3 hours. The buffer was then replaced with 4 liters of fresh buffer, and dialysis continued for 4 hours.

Heat treatment at acid pH. The dialyzed solution was slowly warmed to room temperature, and adjusted to pH 5.5 (glass electrode) by the cautious addition of 2 N acetic acid. The preparation was placed in a bath at 55° until the temperature reached 50°, and was then transferred to a bath at 50°. After 5 minutes at this temperature the extract was chilled in an ice bath. The pH of the extract was adjusted to 7.0 - 7.5, and denatured protein was removed by centrifugation. This heat treatment serves to remove any remaining acetoacetate-synthesizing activity, and the heated fraction is considered as the starting material for purification of the "inhibitor" fraction.

Treatment with calcium phosphate gel. The heated fraction was diluted with an equal volume of 0.02 M potassium phosphate buffer, pH 7.5, and the solution was adjusted to pH 5.5 (glass electrode) by the cautious addition of 2N acetic acid. Sufficient calcium phosphate gel (dry weight, 20 mg per ml) was added in a thin stream, with efficient stirring, to provide a gel/protein

ratio of 35/100. Stirring was continued for 10 minutes, and the suspension was centrifuged at 13,000 x g, 0°, for 10 minutes. The precipitate was discarded. To the supernatant, further calcium phosphate gel (1 ml for each 100 mg of protein in the original heated supernatant) was added in the manner indicated. The suspension was stirred for 10 minutes and centrifuged. The precipitate was discarded. The supernatant solution was adjusted to pH 7.5 by the cautious addition of 5N KOH.

Second ammonium sulfate fractionation. Solid ammonium sulfate (28.2 g for each 100 ml of gel supernatant) was added gradually over 20 minutes, with mechanical stirring, to bring the solution to 40% saturation. If a significant precipitate formed, it was removed by centrifugation and discarded. The supernatant solution was brought to 70% saturation by the addition of a further 21.2 g of solid ammonium sulfate for each 100 ml of original gel supernatant, in the manner indicated. After 20 minutes, the suspension was centrifuged at 20,000 x g, 0°. The precipitate was dissolved in a minimal volume of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M L-cysteine, and dialyzed overnight vs 6 liters of the same buffer.

Zinc-ethanol fractionation. Ten ml of the dialyzed ammonium sulfate fraction was diluted with an equal volume of cold glass-distilled water, and stirred in an ice-bath. The solution was adjusted to pH 6 by addition of 1.0 ml of M potassium succinate buffer, pH 6.0, and 5.0 ml of 0.1 M zinc acetate solution added dropwise. After 5 minutes, the suspension was centrifuged at 20,000 x g, 0°. The precipitate was discarded.

The supernatant solution was placed in a bath at -3°, and sufficient cold ethanol was added dropwise, with mechanical stirring, to produce a final ethanol concentration of 18% by volume. The suspension was stirred 15 minutes at -3° and centrifuged at 20,000 x g, -3°. In a similar manner,

the resulting supernatant solution was brought to 27% ethanol by volume, while the temperature was gradually reduced to -6°. The suspension was stirred for 15 minutes at -6° and centrifuged for 15 minutes at 13,000 x g, -6°. The supernatant fluid was then brought to 37.4% ethanol by volume in a similar manner, while the temperature was gradually reduced to -15°. The suspension was centrifuged at 13,000 x g, -15°, and the supernatant solution discarded. The three ethanol precipitates were dissolved in minimal volumes of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA and 0.1% glutathione and dialyzed without centrifuging vs 2 liters of 0.02 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA and 0.005 M L-cysteine. After 2 hours, fresh buffer was added and dialysis continued for 3 hours. At this time, the external fluid was replaced by a similar buffer, but without EDTA, and dialysis was continued for a total of 5 hours vs 3 changes of 2 liters each of this buffer.

An assay system has been devised for the "inhibitor enzyme" on the basis of its interference with acetoacetate formation by beef liver enzymes. One unit of "inhibitor enzyme" has been defined as the amount of enzyme which produces a 50% decrease in the rate of acetoacetate formation by 2.0 to 2.2 units of acetoacetate-synthesizing enzyme under the standard conditions of assay system I, or 0.5 to 0.55 units under the standard conditions of assay system II. The decrease in the rate of acetoacetate formation is proportional to the amount of "inhibitor enzyme" protein added, providing that the rate is not decreased by more than 60% (cf. Figure 5).

RESULTS

PURIFICATION OF THE ACETOACETATE-SYNTHESIZING SYSTEM

Our understanding of the precise mechanism of acetoacetate synthesis has been hindered in the past due to failure of various investigators to achieve a high degree of purification of the enzyme system. Our first approach to clarifying the mechanism involved efforts to improve the degree of purity beyond that reported by Stern et al (46). These attempts using fresh homogenates of beef liver as starting material have largely met with failure. A typical purification of the acetoacetate-synthesizing system is summarized in Table II. Attempts to achieve further purification by the use of alumina C_γ gel and organic solvents (acetone, methanol, tert.-butanol, n-butanol, n-propanol) were unsuccessful. These attempts invariably resulted in fractions with less activity than the starting 20-35% ethanol fraction; the organic solvents, particularly the higher alcohols, usually produced considerable protein denaturation and very considerable loss of activity. Recombinations of various fractions obtained by the above procedures provided no evidence that more than one enzymatic entity was required for acetoacetate formation.

TABLE II.

Purification of acetoacetate-synthesizing system from beef liver extracts

Fraction	Protein	Enzyme Activity: Specific Activity	Enzyme Activity: Total Activity	Yield
	mg	units/mg	units	%
Bicarbonate extract	5780*	0.123	712	"100"**
$(NH_4)_2SO_4$, 30-60% sat.	1100	0.480	532	75
Gel supernatant	770	0.590	455	64
Ethanol, 20-35%	250	0.985	246	35

* 300 ml of diluted extract
** Arbitrarily taken as 100%

Because our attempts to improve the purification from fresh beef liver homogenates failed, we undertook purification studies using beef liver acetone powder and beef liver mitochondrial acetone powder as starting material. It might be emphasized that Lynen et al (35) used beef liver acetone powder as starting material for their studies. We have also attempted purification using fresh pigeon liver homogenates and pigeon liver acetone powder. The data on the partial purification of the system from these sources are shown in Table III. It can be seen that the crude extracts of the acetone powders of whole beef liver and of beef liver mitochondria exhibit a higher specific activity than do bicarbonate extracts of beef liver. Attempts to obtain highly active fractions from these extracts have again been unsuccessful. Pigeon liver preparations, both homogenates and acetone powder extracts, are much more active than the corresponding beef liver preparations. This appeared promising at first, but all attempts to achieve either extensive purification of the pigeon liver system or resolution of it into two or more fractions resulted in failure. All the procedures tried (use of adsorbents, salt and organic solvents) resulted only in loss of activity. Thus the attempts to obtain a highly purified acetoacetate-synthesizing system have been unsuccessful and have therefore yielded no information regarding the enzymatic mechansims.

PROPERTIES OF THE ACETOACETATE-SYNTHESIZING SYSTEM

The properties of the acetoacetate-synthesizing system have been studied by Lynen et al (35) and by Drummond and Stern (47). A reinvestigation of some of these matters is reported here. It was felt that a clear knowledge of the properties of the system could shed light on the

TABLE III.

Purification of acetoacetate-synthesizing system from other liver preparations

Source of Extract	Fraction	Protein	Specific Activity	Total Activity	Recovery
		mg	units/mg	units	%
Beef liver acetone pdr.	Bicarbonate extract	4860*	0.253	1230	"100"**
	$(NH_4)_2SO_4$ 30-60% sat.	1575	0.540	850	69
Beef liver mitochondria acetone pdr.	Bicarbonate extract	2800*	0.450	1260	"100"**
	$(NH_4)_2SO_4$ 35-55% sat.	880	0.870	766	61
Pigeon liver homogenate	Bicarbonate extract	4080***	0.430	1755	"100"**
	$(NH_4)_2SO_4$ 0-45% sat.	1185	1.340	1590	91
Pigeon liver acetone pdr.	Bicarbonate extract	2190*	0.845	1850	"100"**
	$(NH_4)_2SO_4$ 0-35% sat.	1030	1.300	1340	73
Pigeon liver acetone pdr.	Phosphate extract	2020*	2.55	5150	"100"**
	$(NH_4)_2SO_4$ 0-35% sat.	775	4.50	3490	68

* From 10 g of acetone powder.
** Arbitrarily taken as 100%.
*** From 28 g of pigeon liver.

enzymatic mechanisms involved. Of particular interest is the reported thiol and magnesium requirement (35, 47). These two factors are known to be required for HMG-CoA cleavage enzyme activity and if HMG-CoA were an intermediate in acetoacetate formation the thiol and magnesium requirement of the latter system could readily be explained.

Effect of Divalent Cations and Thiols. Enzymatic synthesis of acetoacetate by purified beef liver fractions exhibited a marked requirement for divalent cation. If divalent cation is omitted from the assay system, acetoacetate synthesis is depressed by 15 to 25% (Table IV). If divalent cation is omitted, and EDTA added to the assay system, acetoacetate synthesis is virtually completely blocked. Mn^{++} was found to be the most effective activator, with Mg^{++} only slightly less effective (Figure 1). Low concentrations of Co^{++} were found to produce a slight activation of acetoacetate

TABLE IV.

Mg^{++} stimulation and EDTA inhibition of acetoacetate synthesis

Conditions	Acetoacetate synthesized
	μmoles per hour
Complete system	0.68
No Mg^{++}	0.52
No Mg^{++}, plus 10^{-3} M EDTA	0.05

Standard assay conditions were employed, except as indicated. The 20-35% ethanol fraction of beef liver (1.1 mg protein) was used.

formation. All other divalent cations tested (Fe^{++}, Cd^{++}, Sr^{++}, Ca^{++}, Ni^{++} and Cu^{++}) were inhibitory. At high concentrations, all the divalent cations were inhibitory.

Acetoacetate synthesis by both crude and purified liver fractions was also markedly stimulated by addition of thiols. In the absence of added

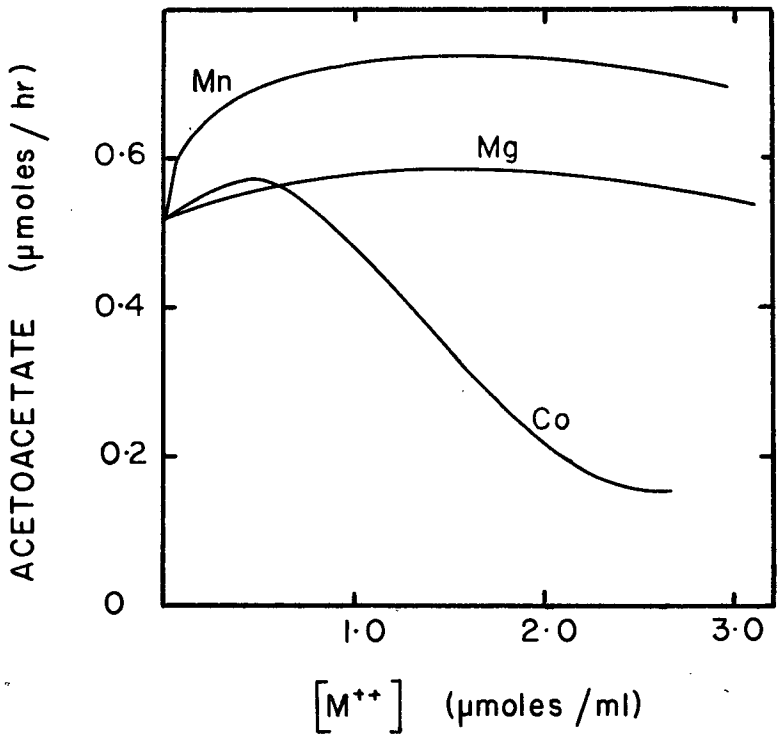

Figure 1. Effect of divalent cation concentration on the rate of acetoacetate synthesis. Standard assay conditions were employed, except as indicated. The 20-35% ethanol fraction of beef liver (1.1 mg protein) was used.

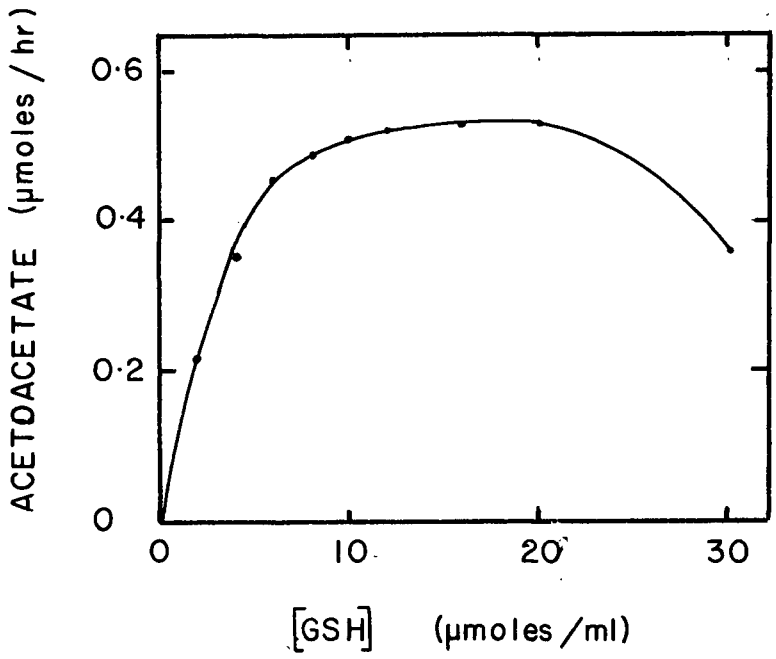

Figure 2. Effect of GSH concentration on the rate of acetoacetate synthesis. Standard assay conditions were employed, except as indicated. The 20-35% ethanol fraction of beef liver (1.1 mg protein) was used.

thiol, purified fractions were virtually inactive (Figure 2). Drummond and Stern (47) reported that several monothiols (GSH, cysteine, mercaptoethanol, mercaptoethylamine, and α-thiolglycerol) were approximately equally effective in producing maximal activation at 10^{-2} M concentration, while thiomalate and thioglycollate were partially effective, and pantetheine was inhibitory. Dithiol compounds (DTO, BAL, and reduced lipoamide) were effective at 10^{-3} M concentration. In the present study, GSH was routinely used as the thiol activator, since virtually all other thiols interfere with the estimation of acetoacetate by the method of Walker (69) by producing highly-coloured blanks. The colour produced by several thiols is given in Table V. This observation in fact raises the possibility of

TABLE V.

Thiol interference with the determination of acetoacetate by the Walker procedure

Thiol	Absorbance (λ = 450 mμ)
Glutathione	0.000
Cysteine	0.040
BAL	0.109
DTO	0.319
2-Mercaptoethanol	0.098
Thioglycollate	0.046

Ten μmoles of each thiol in 0.5 ml of 4% trichloroacetic acid was treated as described under Materials and Methods for determination of acetoacetate by the modified procedure of Walker (69).

adapting the Walker procedure for use as an assay for thiol compounds. Although the procedure is rather complex and time-consuming, it has two marked advantages over the nitroprusside method of Grunert and Phillips (75): the colour produced is quite stable, and dithiol compounds react.

This latter point may make further investigation into this procedure worthwhile, since none of the current methods available for determination of dithiol compounds is very satisfactory. Drummond and Stern (47) showed that high concentrations of dithiols depressed acetoacetate by liver enzymes. As Figure 2 shows, GSH is also inhibitory at high concentrations.

The precise mechanism by which thiols activate the enzymatic symthesis of acetoacetate is not yet definitely established. Drummond and Stern (47) demonstrated that maintenance of coenzyme A in the reduced form is not the mechanism, since reducing agents such as sodium sulfide, potassium borohydride, and sodium cyanide, which can reduce disulfides, produced only a slight activation. Although phosphotransacetylase and β-ketothiolase are activated by thiols, their activities were already excessive in the absence of added thiol. By preincubation of enzyme with thiol, followed by effective removal of the thiol by rotary dialysis or dilution and assay of the enzyme without added thiol, these workers were able to achieve a partial activation of acetoacetate synthesis, indicating the possibility that reduction of an enzyme or bound coenzyme may be involved. Considering the wide variety of thiols which can activate the system, it is unlikely that the thiol becomes bound as a coenzyme.

In a brief examination of acetoacetate synthesis by rat liver mitochondria, it was found that thiols activated the system, as with beef liver enzymes, but that addition of Mg^{++} caused a decrease, rather than an increase, in acetoacetate formation (Table VI). It was also found that 10^{-3} M EDTA, which almost totally blocked acetoacetate synthesis by beef liver enzymes, had very little effect on acetoacetate synthesis by rat liver mitochondria. Further experiments showed that higher concentrations of EDTA did produce a significant inhibition of acetoacetate formation by

TABLE VI.

Effect of Mg^{++}, GSH and EDTA on acetoacetate synthesis by rat liver mitochondria

System	Acetoacetate synthesized
	/umoles per hour
Complete	0.731
No thiol	0.296
No Mg^{++}	0.900
No Mg^{++}, no thiol	0.426
No Mg^{++}, no thiol, plus 10^{-3} M EDTA	0.352
No Mg^{++}, plus 10^{-3} M EDTA	0.845

Standard conditions of assay system II were used, except as indicated. The enzyme used was a rat liver "activated" mitochondrial preparation (1.82 mg protein).

the mitochondrial preparation (Table VII). A possible explanation of these observations could be the presence of sufficient Mg^{++} in the mitochondrial preparation to produce maximal stimulation of the enzyme system, so that

TABLE VII.

Effect of EDTA on acetoacetate synthesis by rat liver mitochondria

EDTA concentration	Acetoacetate synthesized
	/umoles per hour
1×10^{-3} M	0.449
2×10^{-3} M	0.421
1×10^{-2} M	0.323
2×10^{-2} M	0.244

Standard conditions of assay system II were used, with the omission of $MgCl_2$, and the addition of EDTA as indicated. The enzyme fraction used was a rat liver "activated" mitochondrial preparation (0.9 mg of protein).

added cation would produce an inhibitory concentration (cf. Figure 1). The fact that EDTA is inhibitory only at very high concentrations might indicate the presence of bound metal. Attempts to unequivocally demonstrate a Mg^{++} requirement with dialyzed mitochondria and with mitochondria fragmented by brief homogenization at 14,000 rpm in a Servall Omni-mixer and subsequently dialyzed were unsuccessful, as both procedures resulted in almost total loss of enzyme.

Heat Inactivation. The outstanding property of the acetoacetate-synthesizing system is its marked heat lability. The ability of liver fractions to catalyze the formation of acetoacetate is almost completely destroyed by heating the extracts for 5 minutes at 50°, at either neutral or acid pH. It has been shown by Lynen et al (35), and confirmed in this laboratory, that heated extracts can be reactivated by the addition of purified HMG-CoA condensing enzyme. This will be discussed in more detail in a later section.

Inhibitors. Acetoacetate synthesis by liver fractions is not inhibited by tetraethylpyrophosphate (5×10^{-4} M) (47), or by cyanide (10^{-2} M). Drummond and Stern (47) showed that concentrations of arsenite greater than 10^{-3} M produced significant inhibition. Pretreatment of enzyme fractions with iodoacetamide, N-ethylmaleimide or periodate produces significant inhibition of the enzyme (Table VIII, Figure 3).

TABLE VIII.

Effect of pretreatment of enzyme with inhibitors

Inhibitor	Concentration	Acetoacetate
		μmoles
None		1.380
Iodoacetamide	2.0×10^{-3} M	0.617
N-ethylmaleimide	2.0×10^{-4} M	0.795
	2.0×10^{-3} M	0.150
Potassium periodate	2.8×10^{-4} M	0.0

Beef liver $(NH_4)_2SO_4$ 30 to 60% sat., thoroughly dialyzed vs 0.02 M potassium phosphate buffer, pH 7.5, to remove thiol, was incubated at 0° with the above inhibitors at 0°. After 30 minutes, excess iodoacetamide and N-ethylmaleimide were discharged with excess cysteine, and excess periodate was discharged with excess Tris-HCl buffer, pH 7.5. Aliquots of each solution, representing 1.5 mg protein, were then taken for assay of acetoacetate synthesizing activity under standard conditions.

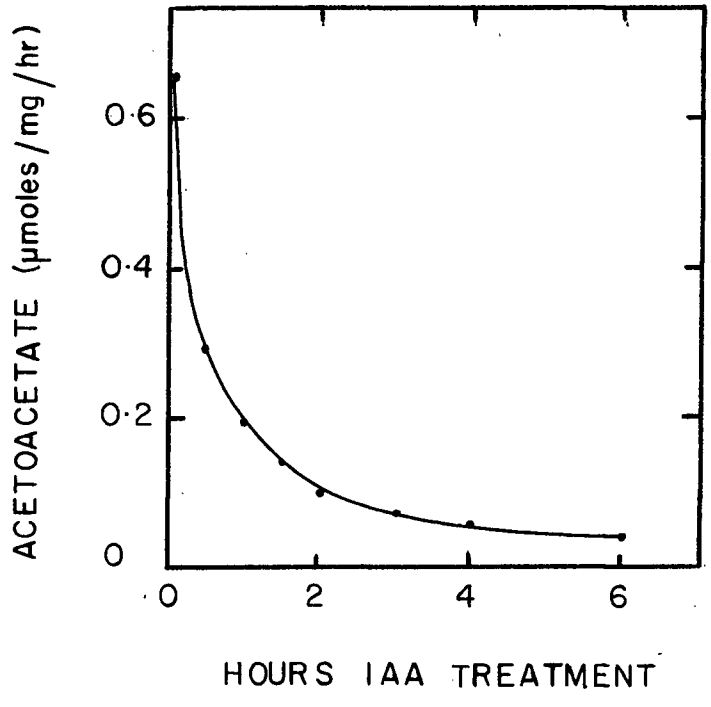

Figure 3. Inhibition of the acetoacetate-synthesizing system by treatment with iodoacetamide. Beef liver $(NH_4)_2SO_4$ 30-60% saturation fraction was diluted to a protein concentration of 10 mg per ml, and incubated at 0° in the presence of 1×10^{-3} M iodoacetamide. At intervals, as noted, aliquots were removed and remaining iodoacetamide discharged with excess cysteine. Protein was precipitated with ammonium sulfate (80% saturation), collected by centrifugation, and dissolved in 0.02 M potassium phosphate buffer, pH 7.5. The dissolved protein was then dialyzed for 2 hours vs the same buffer, containing 1×10^{-2} cysteine, and then 3 hours vs fresh buffer containing 1×10^{-3} M cysteine. The enzyme fractions were then assayed in the standard assay system.

RESOLUTION OF THE ACETOACETATE-SYNTHESIZING SYSTEM

The second major approach to the problem of clarifying the mechanism of acetoacetate formation consisted of efforts to resolve the system into two or more enzymatic entities. Complete resolution would result in loss of acetoacetate-synthesizing activity. This activity would then be restored after recombination of the required enzymatic entities. Specifically, if HMG-CoA were an intermediate in acetoacetate formation, then it should be possible to separate the two enzymes involved, i.e., the HMG-CoA condensing and cleavage enzymes. Acetoacetate formation could proceed only upon their combination. Our first experiments consisted of efforts to confirm the presence and the resolution of these two enzymes by the methods reported by the Munich group.

1. The Procedure of Bublitz

In 1956, Lynen briefly reported (43) that Bublitz, working in his (Lynen's) laboratory, had achieved the separation of the acetoacetate-synthesizing system of beef liver acetone powder extracts into two fractions, both of which were required for acetoacetate formation. No details of the exact technique used for the separation have ever been published; the only information available is a rather sketchy "flow sheet", which is reproduced in Figure 4. Using this "flow sheet" as a guide, we devised a detailed procedure in an attempt to reproduce the resolution of the system into two distinct fractions. The results are summarized in Table IX. From the data in the table, it is obvious that a complete separation had not been achieved, since 43% of the acetoacetate-synthesizing activity present in the starting material was recovered in one of the final fractions. All possible combinations of the two ethanol fractions

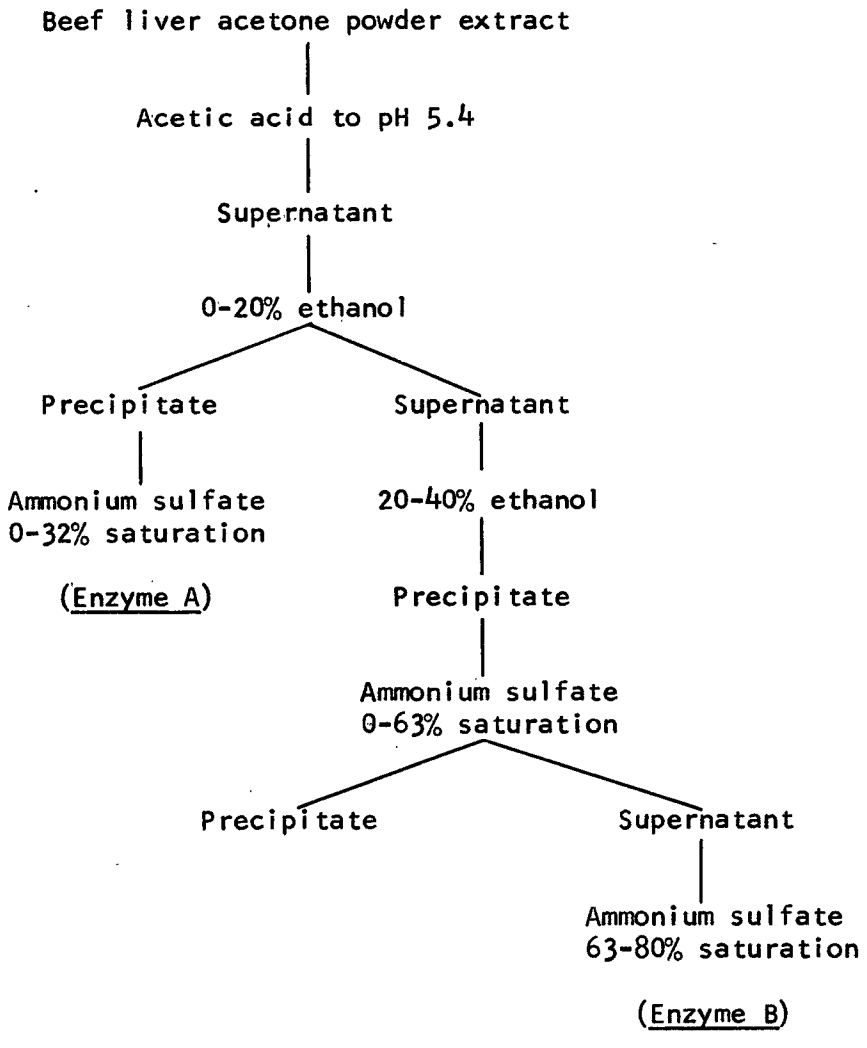

Figure 4. Outline of fractionation procedure for separation of the acetoacetate-synthesizing enzyme system. This outline is the only information available on the procedure used by Bublitz (43).

TABLE IX.

Attempt to reproduce the resolution of the acetoacetate-synthesizing system into two components by the procedure of Bublitz (43).

Fraction	Protein	Specific activity	Total activity	Yield
	mg	units/mg	units	%
1. Phosphate buffer extract	4150	0.245	1018	"100"
2. Acid supernatant	3230	0.319	1000	98
3. a. Ethanol ppt., 0-20%	494	0.110	54	5
b. Ethanol ppt., 20-40%	849	0.568	483	48
4. Low ethanol:				
a. $(NH_4)_2SO_4$, 0-32% sat.	198	0.0	0	0
b. " 32-90% sat.	216	0.185	40	4
5. High ethanol:				
a. $(NH_4)_2SO_4$, 0-63% sat.	712	0.610	435	43
b. " 63-80% sat.	26	0.0	0	0

For details of the procedure used, see under Materials and Methods.

and the four ammonium sulfate fractions were tried. In every case, there was no evidence for any degree of resolution of the system into two components; the acetoacetate formed by a combination of any two fractions was always the same as or less than the sum of the acetoacetate formed by the fractions when assayed individually. It is quite possible that our detailed procedure may differ from the exact method used by Bublitz in one or more important details; this could account for his success and our failure. In any event, we were unable to resolve the system into two components by a combination of ethanol and ammonium sulfate fractionations.

II. Ammonium Sulfate Fractionations

In 1958, Lynen et al (35) reported that a preparation of "enzyme B", which they equated with the HMG-CoA condensing enzyme, could be obtained by fractionation of a beef liver acetone powder extract with ammonium sulfate between the limits of 50 and 80% saturation. They also reported that this "enzyme B" preparation from liver extracts was very unstable.

It could not be dialyzed; it was unstable if stored at 0°, losing 50% of its activity in 8 hours and virtually all its activity in 24 hours. Although it was stable for several months at -20°, it rapidly lost activity if repeatedly frozen and thawed. A series of experiments were carried out with extracts of beef liver acetone powder fractionated at 0-50% and 50-80% saturation with ammonium sulfate, as described in Experimental Section. The results of these experiments are summarized in Table X.

From Experiment I, it can be seen that dialyzed 0-50% saturated and dialyzed 50-80% saturated fractions were approximately equally effective catalyzing the synthesis of acetoacetate. When these two fractions were assayed in combination, the amount of acetoacetate formed (2.54 μmoles) was almost exactly the sum of the acetoacetate formed by the two fractions when assayed separately (2.52 μmoles). This hardly indicates resolution of the enzyme system into two components. It was also found that freshly prepared fractions and fractions prepared the previous day and stored overnight at -18°, without dialysis, had identical activity. When the undialyzed 50-80% saturation fractions were assayed in combination with the dialyzed 0-50% saturation fraction, the acetoacetate formed was considerably less than the sum of the acetoacetate formed by the individual fractions, indicating the presence of some inhibitory factor in the undialyzed fraction.

In Experiment II, freshly prepared ammonium sulfate fractions were assayed alone, together, and with purified HMG-CoA condensing and cleavage enzymes. Again, the acetoacetate formed when the two fractions are combined was considerably less than the sum of the acetoacetate formed by the individual fractions (0.52 μmoles vs 0.84 μmoles). However, it can be seen that acetoacetate formation by the 0-50% saturation fraction was

TABLE X.

Attempted resolution of acetoacetate-synthesizing system of beef liver acetone powder extracts by salt fractionation

Expt. No.	Ammonium sulfate fractions	Acetoacetate μmoles
I	(1) 0-50% sat., dialyzed (6.1 mg protein)	1.27
	(2) 50-80% sat., dialyzed (7.8 mg protein)	1.25
	(3) 50-80% sat., undialyzed, frozen and thawed (6.8 mg protein)	0.41
	(4) 50-80% sat., undialyzed, freshly prepared (6.2 mg protein)	0.38
	(1) plus (2)	2.54
	(1) plus (3)	0.75
	(1) plus (4)	0.68
II	(1) 0-50% sat., undialyzed, freshly prepared (6.5 mg protein)	0.65
	(2) 50-80% sat., undialyzed, freshly prepared (6.1 mg protein)	0.19
	(1) plus (2)	0.52
	(1) plus HMG-CoA condensing enzyme (4 units)	2.38
	(2) plus HMG-CoA cleavage enzyme (4 units)	0.39

The ammonium sulfate fractions were prepared as described under Materials and Methods. HMG-CoA condensing and cleavage enzymes were prepared and assayed as described earlier. Standard assay conditions (assay system I) were employed.

increased almost four-fold on addition of purified HMG-CoA condensing enzyme, and that acetoacetate formation by the 50-80% saturation fraction was doubled on addition of purified HMG-CoA cleavage enzyme. This was at least a tentative indication that HMG-CoA might be an intermediate. It was also a tentative indication that perhaps the two enzymes had been partially separated. However, no firm conclusions could be drawn and, moreover, the recovery of activity was not satisfactory. We have thus

been unable to reproduce the results reported by the German workers.

The results of the above experiments indicate a possible inhibition of the acetoacetate-synthesizing system by ammonium sulfate. To settle this question, a purified beef liver enzyme and a crude extract of beef liver acetone powder were assayed in the presence and absence of ammonium sulfate (Table XI). From the results, it is obvious that the acetoacetate-synthesizing system is inhibited either by ammonium sulfate or by some impurity present in commercial ammonium sulfate. This observation points up the necessity for adequate dialysis of all enzyme fractions used in studies on acetoacetate synthesis.

TABLE XI.

Inhibition of the acetoacetate synthesizing system by ammonium sulfate

Fraction	Protein	$(NH_4)_2SO_4$ concentration	Acetoacetate
	mg	M	µmoles
Beef liver 20-35% ethanol	3.80	-	2.24
"	3.80	0.2	0.72
Beef liver acetone powder extract	6.25	-	1.17
"	6.25	0.2	0.31

Standard assay conditions (assay system I) were employed, except as indicated. The ammonium sulfate concentration refers to the final concentration in the assay system, and corresponds to 5% saturation.

A preliminary attempt has been made to determine the site of inhibition by ammonium sulfate. HMG-CoA condensing enzyme, HMG-CoA cleavage enzyme and excess C. kluyveri extract were added to purified acetoacetate-synthesizing enzyme in the presence of sufficient ammonium sulfate to depress acetoacetate formation 45% (Table XII). Acetoacetate synthesis

was restored only by the purified HMG-CoA condensing enzyme, indicating that this may be the site of inhibition. However, this is not in accord with the statement by Rudney and Ferguson (41) that the HMG-CoA condensing enzyme is not inhibited by the presence of ammonium sulfate. Lack of time has prevented further investigation of this aspect of the problem.

TABLE XII.

Site of inhibition of acetoacetate synthesis by ammonium sulfate

System	Acetoacetate μmoles
Enzyme	0.52
Enzyme + ammonium sulfate	0.29
Enzyme + ammonium sulfate + HMG-CoA condensing enzyme	0.51
Enzyme + ammonium sulfate + HMG-CoA cleavage enzyme	0.30
Enzyme + ammonium sulfate + excess C. kluyveri extract	0.31

Standard assay conditions (assay system II) were employed, except as indicated. The enzyme used was a 20-35% ethanol fraction of beef liver (1.1 mg protein). Where ammonium sulfate was added, the final concentration in the assay system was 0.05 M. Where noted, 5 units each of HMG-CoA condensing and cleavage enzymes were added. "Excess" C. kluyveri extract was produced by adding three times the normal amount of the extract.

III. Attempted Resolution by Column Chromatography

The use of column chromatography for the purification and separation of enzymes is now well established. Since previous efforts to resolve the system by classical procedures had met with little success, we explored the use of chromatography. A single attempt at chromatography on DEAE-cellulose as described by Peterson and Sober (76) was singularly unsuccessful. The only result was almost total destruction of the system. Since the standard purification procedure of the acetoacetate-synthesizing system, as outlined above, involves a calcium phosphate gel step, chromatography

of the system on calcium phosphate gel ("brushite" form), as described by Tiselius (74), was attempted. Although a more stable form of calcium phosphate gel ("hydroxylapatite") for enzyme chromatography has been described (77), the somewhat less stable "brushite" form was selected since it permits more rapid flow rates. Rapid flow rates seemed desirable, due to the reported instability of liver HMG-CoA condensing enzyme (35,39).

Experiment 1. In the first experiment, the enzyme was adsorbed on a column of "brushite" and eluted by a linear gradient of phosphate buffer, with increasing concentration as the parameter. The recovery of the acetoacetate-synthesizing system, as summarized in Table XIII, was very low (12.6% of the activity placed on the gel column was recovered). To determine whether this low recovery was due simply to destruction of

TABLE XIII.

Chromatography of the acetoacetate-synthesizing system on calcium phosphate gel ("Brushite"). Gradient elution.

Fraction	Tubes Pooled	Protein	Specific Activity	Total Activity	Yield
			units/mg	units	%
Beef liver $(NH_4)_2SO_4$ 30-60% sat.	-	325.0	0.740	240.0	
I	26-45	15.4	0	0	
II	46-65	15.8	0	0	
III	66-85	23.0	0	0	
IV	86-105	39.2	0.172	6.7	2.8
V	106-125	26.1	0.473	12.4	5.2
VI	126-144	11.5	0.397	4.6	2.0
VII	145-170	6.1	0.497	3.0	1.3
VIII	170-196	58.2	0.050	3.0	1.3
Totals		199.3		29.7	12.6

For details, see under Materials and Methods.

enzyme or to separation of the enzyme system into more than one component, various recombinations were attempted. One such recombination experiment is summarized in Table XIV. That at least a partial resolution had occurred was obvious; two fractions which when assayed individually catalyzed the formation of a total of 0.14 µmoles of acetoacetate catalyzed the formation of 0.70 µmoles of acetoacetate when combined, a five-fold increase. All the fractions eluted from the gel column were then assayed for HMG-CoA condensing and cleavage enzyme activities; the results are summarized in Table XV. The results showed that the HMG-CoA condensing enzyme was eluted from the gel at relatively low buffer concentrations, and that the bulk of the HMG-CoA cleavage enzyme was eluted at higher buffer conentrations, although some HMG-CoA cleavage enzyme activity was found in

TABLE XIV.

Partial resolution of the acetoacetate-synthesizing system by chromatography on calcium phosphate gel

Experiment	Enzyme	Acetoacetate µmoles
1	Fraction VI, 0.280 mg protein	0.10
2	Fraction VIII, 0.570 mg protein	0.04
3	Fraction VI, 0.280 mg protein plus Fraction VIII, 0.570 mg protein	0.70
4	Fraction VI, 0.280 mg protein plus Fraction VIII, 1.140 mg protein	0.78

Standard assay conditions (assay system II) were employed. For a description of the fractions, see Table XIII.

TABLE XV.

Chromatography of the acetoacetate-synthesizing system on calcium phosphate gel ("brushite"). Distribution of HMG-CoA condensing and cleavage enzymes in eluates.

Fraction	HMG-CoA condensing enzyme			HMG-CoA cleavage enzyme		
	Specific Activity	Total Activity	Yield	Specific Activity	Total Activity	Yield
	units/mg	units	%	units/mg	units	%
Beef liver $(NH_4)_2SO_4$ 30-60% sat.	0.740	240.0		1.23	400.0	
I	0	0	0	0	0	0
II	0	0	0	0	0	0
III	0	0	0	0.100	2.3	0.6
IV	0.324	12.7	5.3	0.172	6.7	1.7
V	2.780	56.3	23.4	0.473	12.4	3.1
VI	0.397	4.6	2.0	0.571	6.6	1.6
VII	0.497	3.0	1.3	1.310	8.0	2.0
VIII	0.050	3.0	1.3	1.510	82.4	20.6
Totals		79.6	33.3		118.4	29.6

For description of the fractions, see Table XIII. HMG-CoA condensing and cleavage enzymes were assayed as described under Materials and Methods. Yields are calculated on basis of % of the activity placed on the column which is recovered in the fraction.

almost all fractions. While resolution was incomplete, the recoveries were low, there was a strong indication that both the HMG-CoA condensing and cleavage enzymes were present, and that they had been partially resolved.

Experiment 2. With this encouraging indication, another chromatographic separation was attempted, using a step-wise elution technique rather than the gradient used in the first experiment. The results are summarized in Tables XVI and XVII. The recovery of acetoacetate-synthesizing activity (Table XVI) was somewhat higher than that in the first column experiment (34.3% vs 12.6%). However, as Table XVII shows, the recoveries of HMG-CoA

TABLE XVI.

Chromatography of acetoacetate-synthesizing system on calcium phosphate gel ("Brushite")

Fraction	Eluant* concentration	Protein	Specific activity	Total activity	Yield
	M	mg	units/mg	units	%
Beef liver $(NH_4)_2SO_4$, 30-60% sat.	-	334.0	0.450	150.0	"100.0"**
I	0.01	33.2	0	0	0
II	0.05	37.8	0	0	0
III	0.10	47.4	0.455	21.6	14.4
IV	0.10	35.4	0.845	29.9	19.9
V	0.30	111.4	0	0	0
VI	0.50	18.0	0	0	0
Totals		273.2		51.5	34.3

* Eluant was potassium phosphate buffer, pH 7.0. For detailed procedure, see Materials and Methods.

** Arbitrarily taken as 100%.

TABLE XVII.

Chromatography of the acetoacetate synthesizing system on calcium phosphate gel ("brushite"). Distribution of of HMG-CoA condensing and cleavage enzymes in the eluates.

Fraction	HMG-CoA condensing enzyme			HMG-CoA cleavage enzyme		
	Specific Activity	Total Activity	Yield	Specific Activity	Total Activity	Yield
	units/mg	units	%	units/mg	units	%
Beef liver $(NH_4)_2SO_4$, 30-60% sat.	0.450	150	"100.0"	0.770	257.0	"100.0"
I	0	0	0	0	0	0
II	0	0	0	0.270	10.2	4.0
III	2.010	95.4	63.6	0.455	21.6	8.4
IV	1.070	37.9	25.3	1.220	43.2	16.8
V	0	0	0	1.330	148.0	57.6
VI	0	0	0	0	0	0
Totals		133.3	88.9		223.0	86.8

For a description of the fractions, see Table XVI. HMG-CoA condensing and cleavage enzyme activities were determined as described under Materials and Methods. Yields are calculated on the basis of % of activity placed on the column which was recovered in the fraction.

condensing and cleavage enzymes were essentially quantitative, a considerable improvement over the first experiment. It can be seen from Table XVII that fraction III was essentially free of cleavage enzyme but 63.6% of the condensing enzyme activity. Fraction V was completely devoid of condensing enzyme, but contained 57.6% of the cleavage enzyme activity. Thus, a very clear resolution of the two enzymes had occurred in these two fractions with excellent recovery of the original acetoacetate-synthesizing activity. This was a very strong indication that HMG-CoA is indeed an intermediate.

All attempts to obtain a more complete resolution of the system on "brushite" columns have been unsuccessful. Tiselius has postulated that the calcium phosphate gels function as weak ion-exchangers; it would appear from our results that the specificity of the materials with respect to ion-exchange is insufficient to give a good resolution of the HMG-CoA condensing and cleavage enzymes. Another possibility is that the physical characteristics of the two enzymes are very similar; this would explain the great difficulty in separating them by conventional enzyme purification techniques.

IV. Studies on Heated Extracts

It has been mentioned previously that an outstanding feature of the acetoacetate-synthesizing system is its marked heat lability. It was reported by Lynen et al (35), and has been confirmed in this laboratory, that liver extracts lost their ability to synthesize acetoacetate in the catalytic assay system after brief heat-treatment, and that the addition of purified HMG-CoA condensing enzyme to these heated extracts restored the acetoacetate-synthesizing activity to the initial levels. The effect of heat treatment on acetoacetate synthesis by crude extracts of beef liver

and of beef and pigeon liver acetone powders, and by purified fractions from beef liver is shown in Table XVIII. As the table shows, addition of purified HMG-CoA condensing enzyme to the crude extracts (but not to the 20-35% ethanol fraction) produced a stimulation of acetoacetate-synthesizing activity. Similar observations have been reported by Wieland et al (78) and by Bucher et al (71) for rat liver extracts. Assuming that acetoacetate formation does proceed via HMG-CoA, this would indicate that that the HMG-CoA condensing enzyme is the rate-limiting factor in acetoacetate synthesis by crude extracts. Heating such extracts at neutral pH for 8 minutes destroyed completely their ability to form acetoacetate (Table XVIII). When excess purified HMG-CoA condensing enzyme from yeast was added to these heated extracts, acetoacetate-synthesizing ability was completely restored. However, addition of the yeast enzyme to the heat-treated 20-35% ethanol fraction did not restore the level of acetoacetate synthesis to that of the initial preparation. As the results of the direct HMG-CoA cleavage enzyme assays show, heating had little or no effect on this enzyme in the crude extracts, but almost completely removed it from the 20-35% ethanol fraction. The results indicate that the heat lability of the acetoacetate-synthesizing system may indeed be due to destruction of the HMG-CoA condensing enzyme, and that in the most purified acetoacetate-synthesizing fraction (20-35% ethanol fraction), the cleavage enzyme is also largely removed by heat. This is further strong evidence that acetoacetate synthesis may proceed via HMG-CoA.

V. Fractionation with Ethanol in the Presence of Zinc

A major criticism of the work of Lynen et al (35) is that most of the studies were carried out using a purified HMG-CoA condensing enzyme from yeast. Any study of the HMG-CoA pathway as the mechanism for acetoacetate

TABLE XVIII.

Effect of purified HMG-CoA condensing enzyme
on acetoacetate synthesis by heated liver fractions

Fraction	Protein	HMG-CoA cleavage enzyme	Aceto-acetate synthesis	Acetoacetate synthesis with HMG-CoA condensing enzyme	Recovery
	mg/ml	units/ml	units/ml	units/ml	%
Fresh beef liver:					
Crude extract	62.0	-	8.3	13.7	
After heat-treatment	31.6	-	0.8	14.7	107
Ethanol ppt., 20-35%	44.0	64.0	25.4	24.9	
After heat-treatment	18.2	4.3	1.4	4.2	16.6
Beef liver acetone powder:					
Crude extract	30.2	36.2	14.3	22.0	
After heat-treatment	20.2	35.4	1.2	22.0	100
Pigeon liver acetone powder					
Crude extract	31.4	-	48.7	100.0	
After heat-treatment	24.0	-	5.8	96.1	96.1

The fresh beef liver fractions were prepared as described by Stern et al (46) (cf. Table II). The acetone powder extracts were prepared as described under Materials and Methods. Heat treatment was carried out by adjusting the fractions to pH 7.5 and heating for 8 minutes at 50°, followed by cooling and centrifugation. The HMG-CoA cleavage enzyme activities were determined by the direct assay of Bachhawat et al (37), except that the Mg^{++} and thiol concentrations were reduced to produce maximal conditions, the other components were reduced by one-half, and acetoacetate was determined by a modification of the method of Walker (69), as already described. For measurement of acetoacetate-synthesizing activity, standard conditions (assay system II) were employed.

synthesis should include the isolation and identification of HMG-CoA condensing enzyme from liver extracts. Rudney (39) and Lynen et al (35) have reported that the HMG-CoA condensing enzyme of liver extracts is very unstable, and claimed that this lability precluded attempts to obtain a purified enzyme from liver extracts. However, the acetoacetate-synthesizing system of liver extracts is stable for relatively long periods. Purified acetoacetate-synthesizing extracts retain full activity for several weeks when frozen, if repeatedly thawed and refrozen, and one purified extract, frozen immediately after preparation and stored continuously at -18° without thawing, was fully active after more than one year. These two observations, the marked lability of the HMG-CoA condensing enzyme and the relative stability of the acetoacetate-synthesizing system, did not seem compatible with the concept that HMG-CoA was an obligatory intermediate in acetoacetate formation. In an attempt to shed further light on the mechanism, we have subjected the most purified acetoacetate-synthesizing preparation (20-35% ethanol fraction) to various fractionation procedures. By precipitation with zinc ion followed by increasing concentrations of ethanol, several fractions were obtained in which acetoacetate-synthesizing ability was virtually absent (Table XIX). The fractions obtained by ethanol precipitation showed no increase in activity when assayed in the presence of excess yeast condensing enzyme. These fractions, however, showed a very great increase in activity when assayed in the presence of excess HMG-CoA cleavage enzyme (Table XIX). The latter enzyme was shown by separate specific assay to be present only in very small amounts in two of these fractions. This strongly indicated that these fractions contained the HMG-CoA condensing enzyme largely free of cleavage enzyme activity. In fact, these fractions contained more than 90%

TABLE XIX.

HMG-CoA condensing enzyme from liver fractions

Fraction	Protein	Acetoacetate-synthesizing system			HMG-CoA condensing enzyme		
		Specific Activity	Total Activity	Yield	Specific Activity	Total Activity	Yield
	mg	units/mg	units	%	units/mg	units	%
Ethanol ppt., 20-35%	407.0	0.585	239.0	"100.0"	1.030	420.0	"100.0"
Zinc ppt.	53.3	0.250	13.3	5.6	0.735	39.2	9.3
Ethanol ppt., 0-6.2%	44.5	0.130	5.4	2.3	2.080	92.5	22.0
Ethanol ppt., 6.2-11.8%	60.0	0.102	6.1	2.5	2.450	147.0	35.0
Ethanol ppt., 11.8-16.6%	81.0	0.154	12.5	5.0	1.640	133.0	31.5
Ethanol ppt., 16.6-30.0%	21.5	0.081	1.8	0.7	0.500	10.8	2.6
Totals:	260.3 (64%)		39.1	16.1		422.5	100.5

For description of the fractionation procedure, see Materials and Methods. Standard assay conditions for acetoacetate-synthesizing system and HMG-CoA condensing enzyme activities were employed.

of the original HMG-CoA condensing enzyme activity of the initial 20-35% ethanol fraction, as determined by acetoacetate formation in the presence of excess HMG-CoA cleavage enzyme, and less than 10% of the initial acetoacetate-synthesizing activity. The ratio of HMG-CoA condensing enzyme activity to acetoacetate-synthesizing activity in one fraction was as high as 24. This procedure has been repeated several times, and appears to be readily reproducible. The recovery of HMG-CoA condensing enzyme in all cases has been essentially quantitative. The data are a very clear indication for the almost complete and quantitative separation of the HMG-CoA condensing enzyme from the cleavage enzyme. After several months at -18°, these "Zn^{++} -ethanol" fractions have retained their full HMG-CoA condensing enzyme activity. However, they are now totally unable to synthesize any acetoacetate in the absence of supplementary HMG-CoA cleavage enzyme. This is an indication that the small amount of cleavage activity they originally contained has deteriorated and we now have condensing enzyme preparations with absolutely no cleavage enzyme contamination.

That the "Zn^{++} -ethanol" fractions do indeed contain the HMG-CoA condensing enzyme has been further demonstrated in several ways. The first point of evidence, of course, is the assay system used: synthesis of acetoacetate in the presence of purified HMG-CoA cleavage enzyme. The fractions were also fully able to replace yeast enzyme in restoring acetoacetate synthesis in heated liver extracts (Table XX) which have been shown previously to contain the cleavage enzyme. In another experiment, HMG-CoA was accumulated by incubating a "Zn^{++} -ethanol" fraction in a modified assay system and identified as the hydroxamic acid derivative by paper chromatography (Table XXI). A procedure for the isolation and

TABLE XX.

Equivalence of HMG-CoA condensing enzyme preparations from yeast and liver

System	Acetoacetate μmoles/hour
Heat treated liver fraction	0.09
Heat-treated liver fraction plus HMG-CoA condensing enzyme (yeast)	1.00
Heat-treated liver fraction plus liver "Zn^{++} -ethanol" fraction	0.99

The heated liver fraction was an ammonium sulfate 30-60% sat. fraction of beef liver extracts, heated for 8 minutes at 50° followed by cooling and centrifugation (1.4 mg protein). Where noted, 4 units of purified yeast HMG-CoA condensing enzyme was added. The liver "Zn^{++} -ethanol" fraction was the 11.8-16.6% ethanol fraction described in Table XIX (0.75 mg protein). Standard assay conditions were employed.

TABLE XXI.

Identification of thioester formed by beef liver "Zn^{++} -ethanol" fraction

Experiment No.	System	R_f values of hydroxamic acids
1	No enzyme	0.64
2	Yeast condensing enzyme	0.65, 0.37
3	Beef liver enzyme	0.65, 0.37
	Known HMG hydroxamic acid	0.35
	Known acethydroxamic acid	0.63

All tubes contained Tris-HCl buffer, pH 8.15, 100 µmoles; KCl, 5 µmoles; $MgCl_2$, 1 µmole; glutathione, 10 µmoles; dilithium acetyl phosphate, 45 µmoles; coenzyme A, 1 µmole; C. kluyveri extract, 0.05 ml. Experiment 1 was a control, with no enzyme added. The yeast HMG-CoA condensing enzyme was 10 units, assayed as described under Materials and Methods. The beef liver enzyme was the 11.8-16.6% ethanol fraction described in Table XIX (4.9 mg protein). Final volume, 1.0 ml. The tubes were incubated for 60 minutes at 38°. The reaction was stopped by acidification to pH 4 with 2N acetic acid and heating at 100° for three minutes; this also serves to destroy residual acetyl phosphate. Thioesters present were then converted to their hydroxamic derivatives and prepared for chromatography according to Hele et al (79), and separated by ascending paper chromatography on Whatman 3MM paper according to Lynen et al (35).

identification of the HMG-CoA per se is presently being developed, but time limitations have precluded completion of the study.

The above-described "Zn^{++} -ethanol" fractionations were performed on a 20-35% ethanol fraction prepared with 0.02 M phosphate as buffer. When a similar liver preparation, with 0.02 M Tris-HCl as buffer, was

subjected to the same treatment, the fractionation was unsuccessful. This indicates the possibility that the HMG-CoA cleavage enzyme is bound to and/or destroyed by the precipitate of zinc phosphate which forms when the zinc acetate solution is added to the phosphate-buffered enzyme preparations. Although HMG-CoA cleavage enzyme can be recovered following precipitation with Zn^{++} from less purified liver fractions by extraction with high concentrations (0.2 M or greater) of phosphate, no more than 15 to 20% can be recovered when the 20-35% ethanol fractions are treated by the procedure used here. This might indicate that the HMG-CoA cleavage enzyme in this fraction is destroyed on addition of Zn^{++}. However, it is possible that the enzyme is very strongly adsorbed to the zinc phosphate precipitate, and it might still be possible to recover it by suitable treatment.

Our experiments have strongly implicated HMG-CoA as an intermediate in acetoacetate synthesis. It thus became of great interest to examine the distribution of the two enzymes concerned during the course of the usual procedure for purification of the acetoacetate-synthesizing system. Thus the various fractions obtained during theppurification procedure were assayed for their ability to synthesize acetoacetate, alone, in the presence of excess condensing enzyme, and in the presence of excess cleavage enzyme. The results are shown in Table XXII. The data clearly show that purification of the acetoacetate-synthesizing system proceeds at the expense of HMG-CoA cleavage enzyme, since in the final fraction only 10% of this enzyme remained. The procedure actually results in a purification of the condensing enzyme, the cleavage enzyme being discarded so that in the end it becomes rate-limiting. On the basis of these figures and those of Table XIX, it can be calculated that the HMG-CoA condensing enzyme of

TABLE XXII.

Fate of HMG-CoA condensing and cleavage enzymes during purification of the acetoacetate-synthesizing system

Fraction	Acetoacetate-Synthesis	HMG-CoA condensing enzyme			HMG-CoA cleavage enzyme		
	Total Activity	Specific Activity	Total Activity	Yield	Specific Activity	Total Activity	Yield
	units	units/mg	units	%	units/mg	units	%
Crude extract	712	0.123	712	"100"	0.440	2550	"100"
$(NH_4)_2SO_4$, 30-60% sat.	532	0.480	532	75	0.785	864	34
Gel supernatant	455	0.590	455	64	0.800	616	24
Ethanol, 20-35%	246	1.190	297	42	0.985	246	10

HMG-CoA condensing and cleavage enzyme activities were determined as described under Materials and Methods. Standard assay conditions (assay system II) were employed.

liver has been purified 15 to 20-fold or more, with recoveries approaching 40%.

It might be of interest to record that the data in Table XXII carry an ironical implication. In 1957, Rudney was attempting to clarify the mechanism of HMG-CoA formation from acetyl-CoA by yeast extracts and to purify the enzyme which later became known as the HMG-CoA condensing enzyme. Stern and co-workers, working directly downstairs from Rudney's laboratory, had purified the acetoacetate-synthesizing system and while doing so had, in reality, succeeded in purifying the HMG-CoA condensing enzyme from beef liver. It was not possible at that time to know that HMG-CoA might be involved in acetoacetate formation.

LOCALIZATION AND DISTRIBUTION OF THE ENZYME

A study on the intracellular localization of the acetoacetate-synthesizing system and the HMG-CoA condensing and cleavage enzymes in rat liver homogenates indicated that these enzyme activities are situated virtually exclusively in the mitochondrion. This has already been reported by Bucher et al (71). However, as these authors pointed out, HMG-CoA condensing enzyme is known to be present in microsomes, where it is thought to function in sterol synthesis. The level of this enzyme in microsomal preparations is much lower than that in mitochondrial preparations, and would not be detected by our assay method. Rudney (39) has demonstrated the presence of the HMG-CoA condensing enzyme in rat liver microsomes by the use of isotopic assay procedures, which are much more sensitive than our assay system.

The distribution of these enzyme activities in rat tissue homogenates was also studied. Acetoacetate-synthesizing activity and HMG-CoA condensing

enzyme activity were detectable only in liver homogenates; the results with kidney homogenates were somewhat equivocal, due to a high tissue blank in the absence of substrate. This is somewhat anomalous, since many workers have shown that some extrahepatic tissues can form acetoacetate (cf. Historical Background). However, as was pointed out in the previous paragraph, these enzymes could be present at levels too low to be detected in our assay system. The HMG-CoA cleavage enzyme, on the other hand, was detected in almost all tissues examined (Table XXIII). Bachhawat et al (37) also found that the HMG-CoA cleavage enzyme is found in a wide variety of tissues. Lynen et al (35) reported results similar to our findings.

TABLE XXIII.

Distribution of HMG-CoA cleavage enzyme in rat tissues

Tissue	Specific Activity units/mg protein
Liver	0.122
Heart	0.188
Kidney	0.086
Brain	0.021
Uterus	0
Ovary	0.239
Adrenal	0.224

The homogenates were prepared as described under Materials and Methods. Standard assay conditions were employed, except that 0.01 M potassium cyanide was added to the system, to block oxidative metabolism. Preliminary experiments with the purified enzyme indicated that cyanide did not interfere with the assay.

THE CHICKEN LIVER "INHIBITOR ENZYME"

Stern et al (46) reported that the rate of acetoacetate formation by crude extracts of chicken liver in the catalytic assay system was not proportional to protein concentration, and attributed this to the presence of a secondary enzyme system(s) which competed with the acetoacetate-synthesizing system for the substrate or one of the intermediates. The inhibitory factor could be largely removed from the acetoacetate-synthesizing system in this tissue by fractionation of the homogenates with ammonium sulfate. In the present investigation it was felt that an understanding of how this factor exerted its inhibitory activity might shed important light on the mechanism of acetoacetate formation. To this end, the "inhibitor enzyme" has been purified from chicken liver homogenates by a series of steps involving salt precipitation, destruction of residual acetoacetate synthesizing activity by heat treatment, adsorption of inactive protein on calcium phosphate gel, reprecipitation with salt, and precipitation with ethanol in the presence of zinc ions. The data from a typical purification are shown in Table XXIV. The procedure is readily reproducible, and a six- to ten-fold purification is regularly achieved. For convenience, this enzyme is temporarily being designated as the "inhibitor enzyme". When the precise nature of its action is definitely established, a more accurately descriptive name will be proposed.

As described under Materials and Methods, the assay system for the "inhibitor enzyme" was based on its interference with acetoacetate formation by beef liver enzymes, for the simple reason that this was the only property of the enzyme which was known at that time. As Figure 5 shows, the degree of inhibition of acetoacetate formation is directly proportional to the concentration of chicken liver protein added over a fairly wide

TABLE XXIV.

Purification of the chicken liver "inhibitor enzyme"

Fraction	Protein	Specific Activity	Total Activity	Recovery
	mg	units/mg	units	%
1. Heated supernatant	3710	0.31	1160	"100"
2. Gel supernatant	-	-	-	-
3. $(NH_4)_2SO_4$, 40-70% sat.	642	0.91	584	51
4. a. Ethanol ppt., 18-27%	102	1.05	107	9
b. Ethanol ppt., 27-37%	147	2.50	368	32
c. Ethanol ppt., 37-50%	44	1.80	80	7

For details of the purification procedure, assay method and definition of the unit, see Materials and Methods.

range, under the specified conditions.

Preliminary attempts to determine the exact means by which the "inhibitor enzyme" interferes with acetoacetate formation showed that the enzyme was not an acetyl phosphatase or an acetyl-CoA deacylase, nor was it the HMG-CoA deacylase described by Dekker et al (80). It was not a proteolytic enzyme hydrolyzing any of the enzymatic components of the catalytic assay system. In addition, the "inhibitor enzyme" did not catalyze the further metabolism of any acetoacetate formed. Two observations provided the first definite clue to its mechanism of action. The "inhibitor enzyme" was incubated with the non-enzymatic components of the assay system, in the presence and absence of the C. kluyveri extract, before addition of the acetoacetate-synthesizing enzyme (Table XXV). When no "inhibitor enzyme" was added (Expts. 1a and 2a), 2.27 µmoles of acetoacetate accumulated. When the "inhibitor fraction" was added simultaneously with the beef liver enzyme, acetoacetate synthesis was depressed approximately 45%. However, when the "inhibitor enzyme" was incubated with

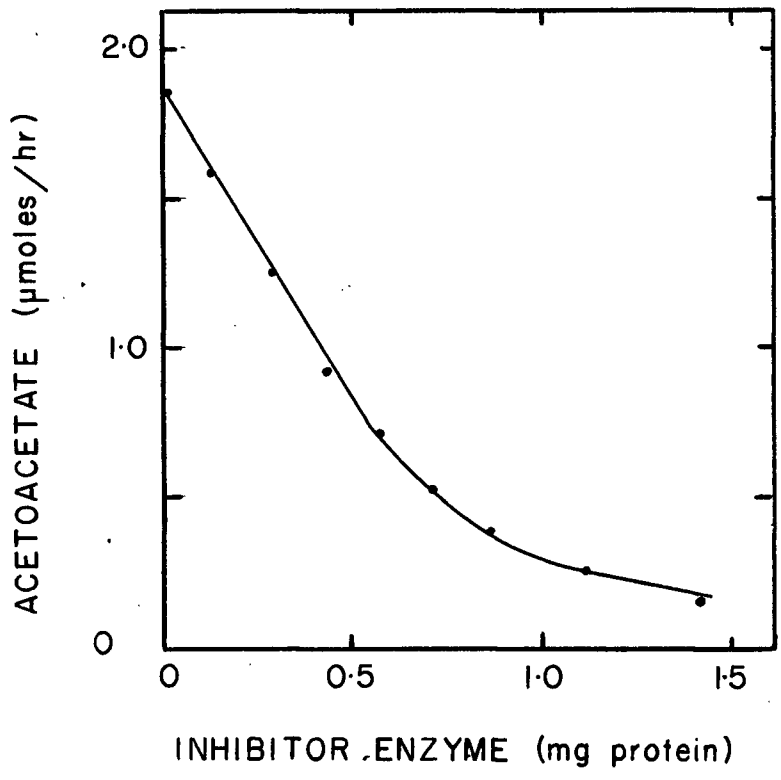

Figure 5. Interference by the chicken liver "inhibitor enzyme" with acetoacetate formation by beef liver enzymes. The beef liver enzyme was a 20-35% ethanol fraction (2.5 mg protein). The chicken liver "inhibitor enzyme" was a 27-37.4% ethanol fraction. Standard assay conditions were employed.

TABLE XXV.

Effect of pre-incubating the assay system components with the "inhibitor enzyme" before addition of the acetoacetate-synthesizing fraction

Experiment No.	Preincubation Time	Phosphotransacetylase added:	
		Start of preincubation	End of preincubation
	minutes		
1a	30	2.27	2.27
1b	30	1.27	1.28
1c	30	0.0	0.53
2a	60	2.27	2.27
2b	60	1.23	1.23
2c	60	0.0	0.12

All tubes contained all the nonenzymatic components of assay system I for acetoacetate formation. Acetoacetate-synthesizing enzyme (beef liver 20-35% ethanol fraction, 3.9 mg protein) was routinely added to all tubes at the end of the preincubation period. In experiments 1a and 2a, no "inhibitor enzyme" fraction was added. In experiments 1b and 2b, "inhibitor enzyme" (chicken liver 27-37.4% ethanol fraction, 0.49 mg protein) was added at the end of the preincubation period, simultaneously with the beef liver enzyme fraction. In experiments 1c and 2c, the same "inhibitor enzyme" fraction was added at the start of the preincubation period. C. kluyveri extract (0.05 ml, 0.8 mg protein) was added as noted. The preincubation was for 30 or 60 minutes, as noted, at 38°. Following addition of the beef liver fraction, the usual procedure was followed.

the assay components before addition of the acetoacetate-synthesizing enzyme fraction, inhibition of acetoacetate synthesis was complete (1c and 2c, first column). The significant observation in these experiments was that the extent of inhibition was decreased when the C. kluyveri extract was present during the preincubation period, indicating that the bacterial extract was "protecting" the sensitive component of the assay system in some manner.

The other important observation was that the inhibition of acetoacetate synthesis observed when the "inhibitor enzyme" was added to the assay system could be overcome by increasing the concentration of coenzyme A in the assay system. When the coenzyme A concentration was increased to six times its usual level, the effect of the "inhibitor enzyme" on acetoacetate formation by beef liver enzymes was completely blocked (Figure 6).

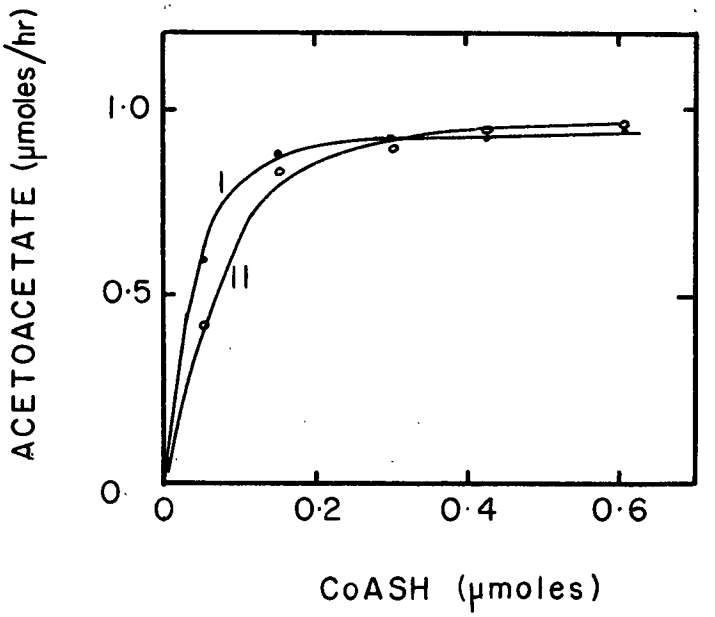

Figure 6. Effect of coenzyme A concentration on depression of acetoacetate formation by beef liver enzymes in presence of the chicken liver "inhibitor enzyme". Curve I is the rate of acetoacetate formation by beef liver enzymes (20-35% ethanol fraction, 1.1 mg protein), in the absence of "inhibitor enzyme". Curve II is the same, in the presence of "inhibitor enzyme" (chicken liver ammonium sulfate 40-70% sat. fraction, 1.4 mg protein).

These two observations indicated that the "inhibitor enzyme" might be depressing acetoacetate formation by inactivating the coenzyme A present in the assay system. To test this possibility, coenzyme A was incubated with the "inhibitor enzyme", and the deproteinized solution was assayed for coenzyme A by the phosphotransacetylase method (59) (Table XXVI). As the data in the table show, the "inhibitor enzyme" does indeed appear to be inactivating coenzyme A in some way.

TABLE XXVI.

Inactivation of coenzyme A by chicken liver "inhibitor enzyme"

Experiment No.	Enzyme	Coenzyme A added	Coenzyme A recovered
	mg	μmoles	μmoles
1a	-	0.01	0.01
1b	0.19	0.01	0
2a	-	0.02	0.02
2b	0.19	0.02	0

The reaction mixture contained Tris-HCl buffer, pH 8.15, 100 μmoles; $MgCl_2$, 1 μmole; KCl, 4 μmoles; cysteine, 4 μmoles; and coenzyme A and "inhibitor enzyme" (chicken liver, 27-37% ethanol fraction), as noted; final volume, 0.3 ml. The reaction was started by addition of enzyme, and incubation was at 38°. After 45 minutes, the reaction was stopped by acidification to pH 4 with 5N HCl and heating in a boiling water bath for 1 minute, followed by cooling. Coenzyme A in the reaction mixture was then determined by the phosphotransacetylase method (59).

A preliminary attempt has been made to determine precisely what action the "inhibitor enzyme has on coenzyme A. Coenzyme A was incubated with the enzyme, and the deproteinized supernatant solution subject to paper chromatography. The results (which will not be reported here in

detail, since they are quite inconclusive and incomplete) indicate the formation of a new compound, which exhibited ultraviolet light absorption and which reacted with the Toennies and Kolb reagent (81) for free thiol compounds. One obvious possible interpretation of this observation is that the "inhibitor enzyme" is a phosphatase or 3'-nucleotidase, removing the 3'-phosphate of coenzyme A to form dephosphocoenzyme A. The occurrence of this type of nucleotidase in plant tissue extracts has been reported (82-85). This nucleotidase has, in fact, been utilized for formation of dephosphocoenzyme A on a preparative scale (84,86). Prostatic phosphatase will also remove the 3'-phosphate of coenzyme A (84). However, our results are not yet conclusive. Time limitations have prevented a more thorough investigation of this possibility. At any rate it is quite apparent that knowledge of the mechanism of this enzyme has not assisted us in elucidating the mechanism of acetoacetate formation as was originally hoped.

β-HYDROXYBUTYRYL DEHYDROGENASE FROM C. KLUYVERI

All of our evidence on the mechanism of acetoacetate formation by liver extracts was compatible with the operation of the HMG-CoA pathway. In an attempt to obtain further conclusive evidence for the obligatory formation of HMG-CoA as an intermediate, we began to seek some means by which the HMG-CoA pathway could be <u>specifically</u> blocked. One logical approach would be to remove HMG-CoA as it formed by converting it to some inert metabolite. If acetoacetate is formed via HMG-CoA, the introduction of such a "shunt" into the assay system should prevent acetoacetate formation. In the search for a means of shunting out HMG-CoA, three enzymes were considered: β-methylglutaconase (58) which catalyzes the inter-

conversion of HMG-CoA and β-methylglutaconyl-CoA (reaction 13); the HMG-CoA deacylase of chicken liver described by Dekker et al (80) (reaction 14); and HMG-CoA reductase, described by Rudney and co-workers (87-89) and also by Knappe et al (90), which catalyzes the conversion of HMG-CoA to mevalonate in the presence of TPNH (reaction 15). The first of these,

$$\text{HMG-CoA} \rightleftharpoons \beta\text{-methylglutaconyl-CoA} + H_2O \qquad (13)$$

$$\text{HMG-CoA} + H_2O \longrightarrow \text{HMG} + \text{CoASH} \qquad (14)$$

$$\text{HMG-CoA} + 2\ \text{TPNH} \longrightarrow \text{mevalonate} + 2\ \text{TPN} + \text{CoASH} \qquad (15)$$

β-methylglutaconase, was not suitable for our purposes, since the equilibrium of the hydration-dehydration reaction lies in the direction of formation of HMG-CoA, rather than its removal. The second possibility, the HMG-CoA deacylase, was an attractive possibility, but it, too, proved to be unsuitable. The deacylase preparations described by Dekker et al (80) contain considerable HMG-CoA cleavage enzyme, and the pH of the catalytic assay system would favor the cleavage reaction rather than deacylation of HMG-CoA. In addition, the deacylase preparations would undoubtedly contain "inhibitor enzyme" activity, which would make interpretation of results virtually impossible. The third enzyme mentioned above, HMG-CoA reductase, appeared to be the answer to our problem, since it has been considerably purified from yeast (89,90) and the equilibrium of the reaction lies far in the direction of mevalonate formation.

In view of these considerations, the effect of purified yeast HMG-CoA reductase on acetoacetate formation in the catalytic assay system was determined (Table XXVII). As the data in the table show, addition of

TABLE XXVII.

Effect of TPNH and HMG-CoA reductase on acetoacetate formation by beef liver enzymes

Experiment No.	Additions	Acetoacetate
		μmoles
1	None	1.54
2	TPNH generating system plus HMG-CoA reductase	0.21
3	TPNH generating system without HMG-CoA reductase	0.12

Standard conditions of assay system I were employed, with other additions as noted. The acetoacetate-synthesizing system used was a beef liver ammonium sulfate 30-60% sat. fraction (3.0 mg protein). The TPNH generating system consisted of 1 μmole of TPN, 30 μmoles glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase (Sigma Chemical Co., Type III). HMG-CoA reductase was purified from baker's yeast as described by Knappe *et al* (90); 15 milliunits were added, where noted.

HMG-CoA reductase and a source of TPNH to the catalytic assay system produced almost complete inhibition of acetoacetate formation (Experiment 2), as had been anticipated. However, a rather surprising observation was that addition of the purified yeast enzyme appeared to be unnecessary (Experiment 3). Presumably either the liver enzyme fraction used or the *C. kluyveri* extract contained HMG-CoA reductase. To confirm that the inhibition of acetoacetate synthesis was due to reduction of HMG-CoA to mevalonate, an attempt was made to isolate the mevalonate from the reaction mixture as the hydroxamic acid derivative, as described by Lynen and Grassl (91). No mevalonate could be detected. Spectrophotometric assays of the liver and bacterial extracts for HMG-CoA reductase activity were negative. It therefore became apparent that the inhibition of acetoacetate formation was due to some reaction other than reduction of HMG-CoA

to mevalonate.

A systematic investigation into the basis of these observations led to the discovery that an extremely active acetoacetyl-CoA reductase or β-hydroxybutyryl dehydrogenase in the C. kluyveri extract was responsible for the observed inhibition of acetoacetate formation. In brief, inhibition of acetoacetate formation had occurred not because HMG-CoA was being "shunted out" to mevalonate, but because acetoacetyl-CoA was being reduced to β-hydroxybutyryl-CoA. A surprising finding was that the enzyme is virtually absolutely specific for TPNH (Figure 7). This is in contrast to the β-hydroxybutyryl dehydrogenase from liver (92), which is absolutely specific for DPNH, and the heart enzyme (93), which is only 1/10 as active with TPNH as with DPNH. Spectrophotometric examination of the product of the reaction showed only absorption due to the thioester bond and to the adenine moiety of the coenzyme A. When the deproteinized reaction mixture was subjected to paper chromatography, a new thioester spot appeared in the position reported by Stern et al (30) for β-hydroxybutyryl-CoA (Table XXVIII).

It is quite possible that this enzyme is identical with the β-hydroxypropionyl dehydrogenase described by Vagelos and Earl (94). These workers reported that C. kluyveri extracts contain an enzyme which catalyzes the reversible oxidation of β-hydroxypropionyl-CoA to malonyl semialdehyde-CoA in the presence of TPN. Further investigation of this enzyme with respect to substrate specificity might shed further light on this question. It is entirely reasonable that this enzyme may play an important role in fatty acid metabolism in this organism and this problem is certainly worth further investigation. Unfortunately, time has marched away.

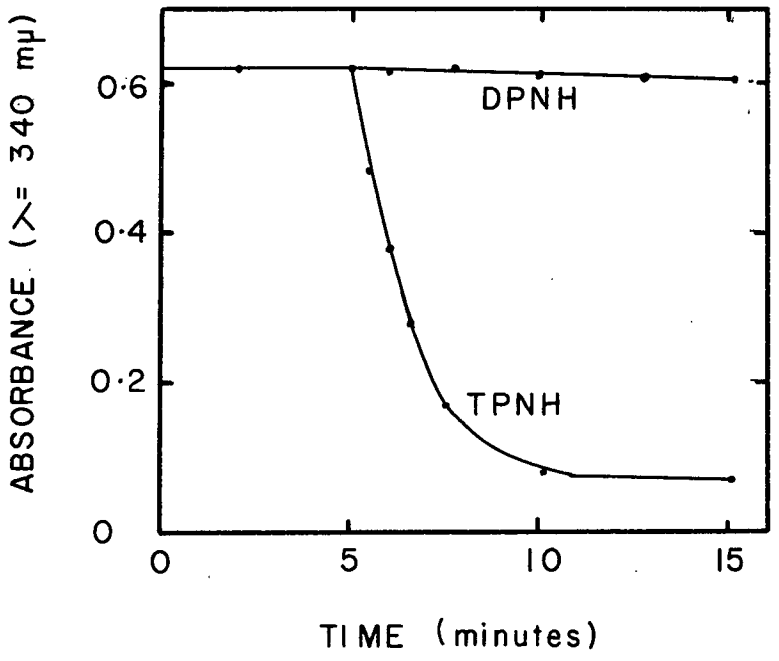

Figure 7. Pyridine nucleotide specificity of the β-hydroxybutyryl dehydrogenase of C. kluyveri. The experimental cuvette (d = 0.5 cm) contained Tris-HCl buffer, pH 7.5, 100 μmoles; and DPNH or TPNH, as noted, 0.3 μmoles. At the first arrow, 0.02 ml of C. kluyveri extract (0.35 mg protein) was added. At the second arrow, 0.5 μmoles of acetoacetyl-CoA was added to initiate the reaction. Total volume, 1.5 ml; temperature, 24°. The control cuvette contained buffer and enzyme.

TABLE XXVIII.

Chromatography of products of the ω-hydroxybutyryl dehydrogenase of *C. kluyveri*

System	R_f values of thioesters
Control	0.59
Experimental	0.45, 0.58, 0.68
Acetyl-CoA	0.47
Acetoacetyl-CoA	0.58

The reaction mixture contained Tris-HCl buffer, pH 7.5, 30 μmoles; acetoacetyl-CoA, 0.6 μmoles; TPNH, 1.4 μmoles, and *C. kluyveri* extract, 0.02 ml (0.35 mg protein); total volume 0.3 ml. The control contained all additions except enzyme. Incubation was for 20 minutes at 38°. The reaction was stopped by acidification to pH 4 and heating in a boiling water bath for 3 minutes. Denatured protein was removed by centrifugation. The entire supernatant solutions were then streaked on Whatman 3MM paper, and subjected ascending chromatography in the ethanol/acetate system (95) for 8 hours at 4°. Coenzyme A thioesters were detected by ultraviolet absorption and the delayed nitroprusside reaction (95).

DISCUSSION

The experiments reported in this thesis clearly implicate HMG-CoA as an intermediate in acetoacetate formation. By the use of techniques involving neutral heat treatment, it is possible to obtain preparations containing the HMG-CoA cleavage enzyme which have lost over 90% of their acetoacetate-synthesizing activity. This activity can be completely restored by adding back HMG-CoA condensing enzyme. The zinc-ethanol fractions clearly contain HMG-CoA condensing enzyme. These fractions are unable to synthesize acetoacetate alone and 93.2% of the original acetoacetate-synthesizing activity is restored upon adding back purified HMG-CoA cleavage enzyme or heated extracts of liver. In addition, the experiments involving calcium phosphate gel column fractionation clearly indicate the separation of the acetoacetate-synthesizing system into two fractions, each of which has little or no ability to form acetoacetate by itself. One fraction when supplemented with HMG-CoA condensing enzyme gave an almost quantitative recovery of acetoacetate-synthesizing activity. The second fraction when supplemented with HMG-CoA cleavage enzyme likewise gave an almost complete recovery of activity. Combining the two fractions also restored the ability to form acetoacetate.

The question now is whether the HMG-CoA pathway is the only mechanism functioning in the catalytic assay system. The quantitative nature of the recovery achieved on adding condensing enzyme to heated extracts, and upon adding cleavage enzyme to the zinc-ethanol fractions strongly indicates that most, if not all, of the acetoacetate formed by liver extracts in the catalytic assay system proceeds through this mechanism. This matter is discussed further below.

The HMG-CoA pathway as a mechanism for acetoacetate formation is a

very attractive one, since it provides an explanation for many of the known properties of the acetoacetate-synthesizing system. The thiol and divalent cation requirements for acetoacetate formation from acetyl phosphate and coenzyme A in the catalytic assay system (35,47) are readily explained by the requirement of the HMG-CoA cleavage enzyme for these factors (37). The relative efficacy of various thiols in activating the acetoacetate-activating system of beef liver extracts closely parallels their efficacy in activating the HMG-CoA cleavage enzyme (37). Bachhawat et al (37) found Mg^{++} to be the most effective of the divalent cations in activating the cleavage enzyme, with Mn^{++} partially effective and Co^{++} ineffective. This is in contrast with our results with the acetoacetate-synthesizing system (cf. Figure 1). However, these workers were using very high concentrations of metal ion. We have found that high concentrations of the cations are inhibitory (cf. Figure 1), and also that in order to produce maximal assay conditions the metal ion concentration in the direct HMG-CoA cleavage enzyme assay must be reduced by one-half or more from that reported by Bachhawat et al (37). This may explain the discrepancy.

The HMG-CoA pathway is also consistent with the heat lability of the acetoacetate-synthesizing system, since the HMG-CoA condensing enzyme is very sensitive to even brief heating. This has been discussed in detail earlier.

The HMG-CoA pathway also provides an explanation for the long-known "asymmetric labelling" of metabolically-formed acetoacetate. When, for example, octanoate-1-C^{14} is incubated with a liver enzyme preparation (e.g. slices, homogenates, mitochondria, etc.), the acetoacetate formed contains more isotope in the carboxyl group than in the carbonyl group. The earliest explanations of this phenomenon invoked the existence of two

different forms of "active acetate". Lynen (cf. reference (32)) and Beinert and Stansly (96) extended this theory by postulating that an S-acyl β-ketothiolase functioned as an alternate form of "active acetate". However, a simpler and more logical explanation for the asymmetric labelling is available if the HMG-CoA pathway functions as the mechanism of liver ketogenesis. When octanoate-1-C^{14} is incubated with liver preparations, one "turn" of the fatty acid β-oxidation "cycle" (or, more properly, β-oxidation "spiral") will release acetyl-1-C^{14}-CoA, thus labelling the "acetyl pool". Oxidation of the resulting hexanoate would finally yield unlabelled acetoacetyl-CoA. Condensation of this unlabelled acetoacetyl-CoA with labelled acetyl-CoA from the "acetyl pool" (reaction 11) would form HMG-5-C^{14}-CoA, which would, via the HMG-CoA cleavage enzyme reaction (reaction 12), give rise to acetoacetate labelled exclusively in the carboxyl group. However, some isotope would be incorporated into acetoacetyl-CoA through equilibration with the labelled "acetyl pool" via β-ketothiolase; the acetoacetyl-CoA thus formed would carry isotope in both the carboxyl and carbonyl carbons. Condensation of this symmetrically-labelled acetoacetyl-CoA with labelled acetyl-CoA from the "acetyl pool", followed by cleavage of the HMG-CoA thus formed, would give rise to acetoacetate labelled in both the carboxyl and carbonyl carbons. The net result would be the formation of acetoacetate labelled in both positions, but with the majority of the isotope in the carboxyl group, i.e., asymmetrically labelled. The longer the fatty acid chain being oxidized, the greater the complement of symmetrically labelled acetoacetyl-CoA would be. The end result would be a ratio of label in the carboxyl and carbonyl groups of acetoacetate approaching unity. The overall picture is summarized in Figure 8.

In addition to the above points, the HMG-CoA pathway can also be

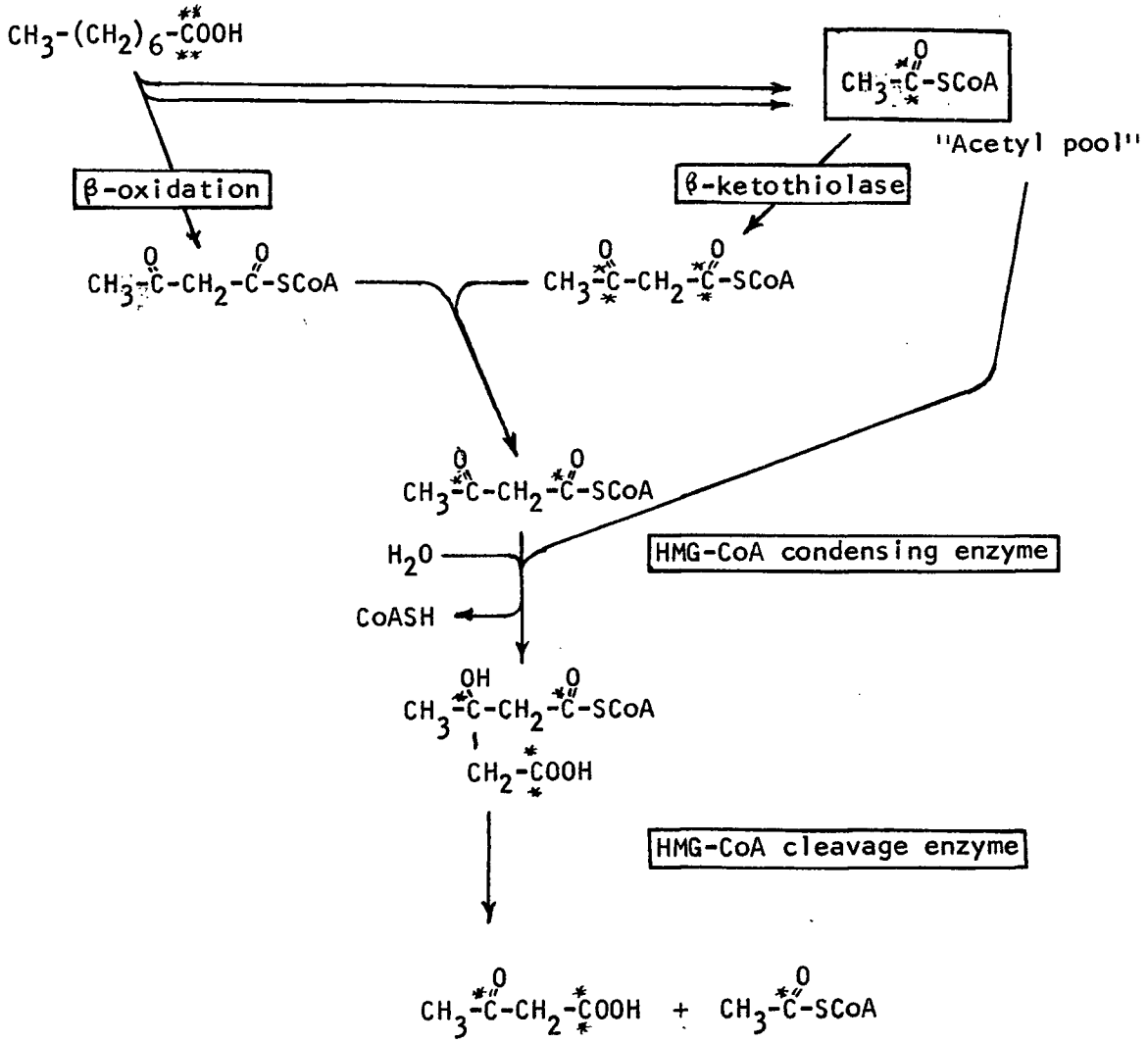

Figure 8. The HMG-CoA mechanism as an explanation for the asymmetric isotope labelling of enzymatically-formed acetoacetate. For details, see text.

used to explain the lack of accumulation of acetoacetate during oxidation of fatty acids by extrahepatic tissues. Liver, the only one of the tissues tested which forms appreciable amounts of acetoacetate, is also the only mammalian tissue which contains readily detectable levels of the HMG-CoA condensing enzyme. The fact that extrahepatic tissues lack this enzyme may well be the reason for the inability of extrahepatic tissues to cause acetoacetate accumulation, rather than the fact that these tissues can catalyze the further oxidation of acetoacetate, which has been generally considered as the reason.

The similarities between the properties of the acetoacetate-synthesizing system and those of the HMG-CoA condensing and cleavage enzymes provide a strong indication that they may be identical. The evidence presented in this thesis strongly indicates that this is so. In a recent paper, Hird and Symons (49) have presented evidence which strongly indicates that acetoacetate formation from fatty acids by intact cell preparations from sheep omasum and rumen epithelium proceeds largely, if not entirely, *via* HMG-CoA. When butyrate-1-C^{14} was incubated with their enzyme preparations, 75% of the C^{14} incorporated into the acetoacetate which accumulated in the medium was located in the carboxyl group. They also incubated their enzyme preparations with unlabelled butyrate in the presence of acetate-1-C^{14} or a source of acetate-1-C^{14} (lactate-2-C^{14}, octanoate-1-C^{14}). When the accumulated acetoacetate was analyzed for isotope distribution, between 75 and 80% of the radioactivity was found in the carboxyl group. In the first-mentioned set of experiments, utilizing butyrate-1-C^{14}, the asymmetric labelling can be explained through a partial equilibration of isotope *via* the thiolase reaction, as shown by Figure 9, without invoking the HMG-CoA pathway. Thus, the asymmetrically-labelled acetoacetate could

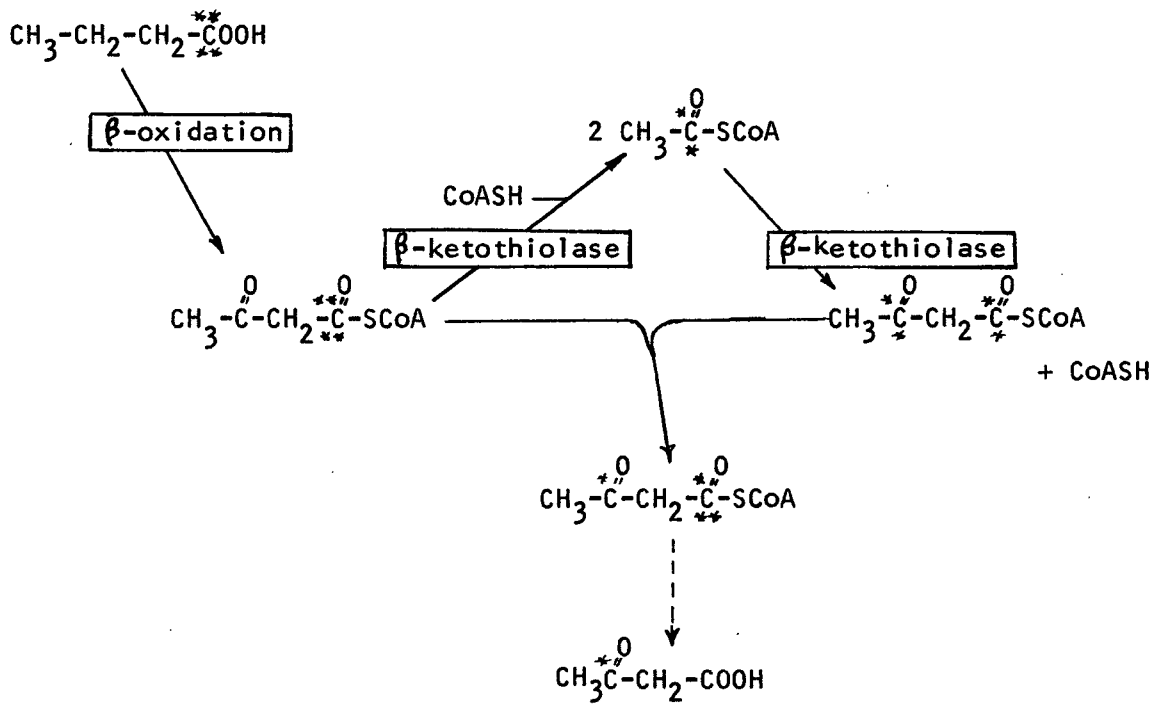

Figure 9. Asymmetric labelling of acetoacetate formed enzymatically from butyrate-1-C^{14}. For explanation, see text.

have been formed by a direct deacylation of acetoacetyl-CoA. The results of the second set of experiments, utilizing unlabelled butyrate and acetate-1-C^{14} as substrates, however, virtually exclude the direct deacylation as a major pathway for acetoacetate formation in these tissue preparations. If the acetoacetate is formed by direct deacylation of acetoacetyl-CoA, the only way in which the acetate-1-C^{14} could have been incorporated into the acetoacetate would be via the β-ketothiolase reaction, as shown in Figure 10, and this could give rise only to symmetrically-labelled acetoacetate. If, on the other hand, acetate-1-C^{14} could be incorporated both via the β-ketothiolase equilibrium and via the HMG-CoA pathway, the situation would be essentially that represented in Figure 8,

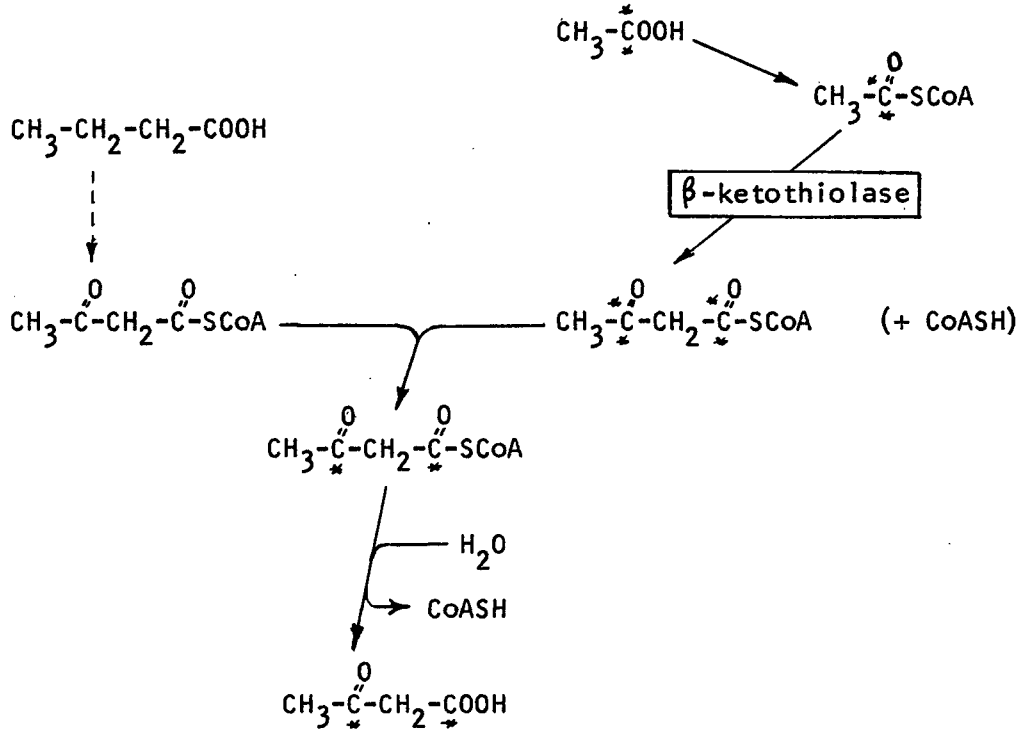

Figure 10. Incorporation of acetate-1-C^{14} into acetoacetate enzymatically formed via direct deacylation of acetoacetyl-CoA. For explanation, see text.

and the acetoacetate would be labelled predominantly in the carboxyl group. Since the acetoacetate formed in this set of experiments contained three to four times as much C^{14} in the carboxyl group as in the carbonyl group, it would appear that the acetoacetate was indeed formed via HMG-CoA. On the basis of their data, Hird and Symons were able to calculate that 75 to 80% of the acetoacetate could only have been formed via the HMG-CoA pathway. The other 20 to 25% of the acetoacetate could have been formed via a direct deacylation of acetoacetyl-CoA, or by the HMG-CoA pathway after equilibration of acetyl-1-C^{14} -CoA and acetoacetyl-CoA via the β -ketothiolase reaction, or by a combination of both.

In contrast to the evidence described above, Stern and co-workers have presented evidence for the presence of a specific acetoacetyl-CoA deacylase in liver extracts, using partially purified beef liver enzymes (47) and sonic extracts of rat liver mitochondria (45). After treatment with iodoacetamide to inactivate the HMG-CoA condensing and cleavage enzymes, these extracts were able to synthesize acetoacetate from substrate concentrations of acetoacetyl-CoA. Unlike the acetoacetate-synthesizing system of beef liver extracts described in this thesis, the deacylase system described by the Cleveland group requires neither divalent cation nor thiol. Stern apparently holds the opinion that although both the HMG-CoA condensing and cleavage enzymes are present in liver extracts, and their levels of activity are sufficiently high to account for all of the acetoacetate formed, these enzymes may not be involved in acetoacetate formation. The evidence presented in this thesis, and the evidence of other workers which has also been described here, does not definitely exclude the possibility of the existence of a specific acetoacetyl-CoA deacylase. However, there is little evidence that such a deacylase plays a major role in acetoacetate formation. It is quite possible that this deacylase does exist, and may account for a small part of the acetoacetate formed by liver extracts. For example, prolonged treatment of the acetoacetate-synthesizing system with iodoacetamide or the addition of high concentrations of EDTA to the catalytic assay system do not completely block acetoacetate formation. The existence of a specific acetoacetyl-CoA deacylase might account for the small amount of residual activity (less than 10%) under these conditions. However, the presence both of iodoacetamide and of substrate concentrations of acetoacetyl-CoA, as in the stoichiometric assay used by Stern (45,47), could hardly be considered as physiological,

and it would be rather risky to base conclusions as to the physiological mechanism of acetoacetate formation on results obtained under such conditions. In addition, the specific deacylase does not explain the very definite divalent cation and thiol requirements for acetoacetate synthesis in the catalytic assay system. Another point is that there is no adequate explanation as to why the HMG-CoA condensing and cleavage enzymes would not couple to provide a pathway for acetoacetate formation, when it has now been established that both enzymes are very definitely present.

Segal and Menon (48) have also presented evidence which they claim indicates the formation of acetoacetate via direct deacylation of acetoacetyl-CoA. They incubated rat liver mitochondrial preparations with unlabelled acetoacetyl-CoA and acetyl-2-C^{14}-CoA for short periods, and analyzed the acetoacetate which formed for isotope content; they found that very little radioactivity had been incorporated. Analysis of residual acetoacetyl-CoA showed that it, too, contained very little isotope. They also reported that their mitochondrial preparations contained virtually no β-ketothiolase activity. The validity of their results can be criticized on several points. Starting with 1 μmole of acetoacetyl-CoA, at pH 7.9, 0.41 μmole of acetoacetate formed chemically, in the absence of enzyme, and 0.52 μmoles in the presence of enzyme (100 μg. of mitochondrial protein) after ten minutes incubation. This extremely high rate of chemical deacylation does not correspond with the known stability characteristics of acetoacetyl-CoA (97). They reported that they used the Walker method (69) for determination of acetoacetate, and make no mention of any modification of the method. We have found that the original procedure, as published by Walker, is excellent when pure solutions of acetoacetate are being assayed, but is very unreliable when trichloroacetic acid supernatant

solutions from tissue preparations are being assayed. Under these conditions, a 20% variation in the assay of two identical samples was quite common. Another weak point in their evidence is that a considerable amount of the acetoacetyl-CoA (0.4 μmole) initially present is not accounted for, either *per se* or as acetoacetate. In addition, their statement that the mitochondrial preparations used in their experiments contained little or no β-ketothiolase is difficult to understand. If it had been possible to obtain an acetoacetate-synthesizing liver fraction completely or largely free of thiolase, the problem of the enzymatic mechanism of acetoacetate synthesis would undoubtedly now be only of historical interest. The main stumbling block in the study of the mechanism of ketogenesis in liver extracts has always been that in all the liver fractions obtained, both crude and purified, the disappearance of acetoacetyl-CoA *via* the β-ketothiolase reaction proceeded at a rate many times greater than the rate at which acetoacetyl-CoA was hydrolyzed to acetoacetate. From their data, it can be calculated that acetoacetate was formed "enzymatically" at a rate of 6.6 μmoles per mg protein per hour. This would make their mitochondrial preparation the most active acetoacetate-synthesizing fraction ever prepared - it would be approximately 16 times as active as an identical liver preparation reported by Stern and Miller (45). On the basis of these considerations, it would appear that a logical explanation for the lack of incorporation of isotope from acetyl-2-C^{14}-CoA into acetoacetate in their experiments would be that there was little or not acetoacetate formed enzymatically. It is the opinion of this author that the report of Segal and Menon (48) must be viewed rather skeptically, at least until further experiments are carried out under very carefully controlled conditions.

From the results reported here, this laboratory has been led to the

conclusion that certainly most, if not all, of the acetoacetate formed by liver preparations proceeds through HMG-CoA. The role of a specific acetoacetyl-CoA deacylase must be relatively minor, if it is involved at all. Its precise and quantitative role, if any, must await future studies. Our conclusions are thus very similar to those of Hird and Symons (49) who used sheep omasum and rumen epithelium for their studies. We are thus also in agreement with Lynen et al (35), even though we have been unable to reproduce their results. Finally, it can be said that the HMG-CoA pathway is particularly attractive from a physiological point of view, since it provides a biochemical explanation for the long-known physiological relationships between the metabolism of acetoacetate, cholesterol, fatty acids and branched-chain fatty acids.

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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 contain g 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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