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Enzymes of fatty acid metabolism: a commentary * by

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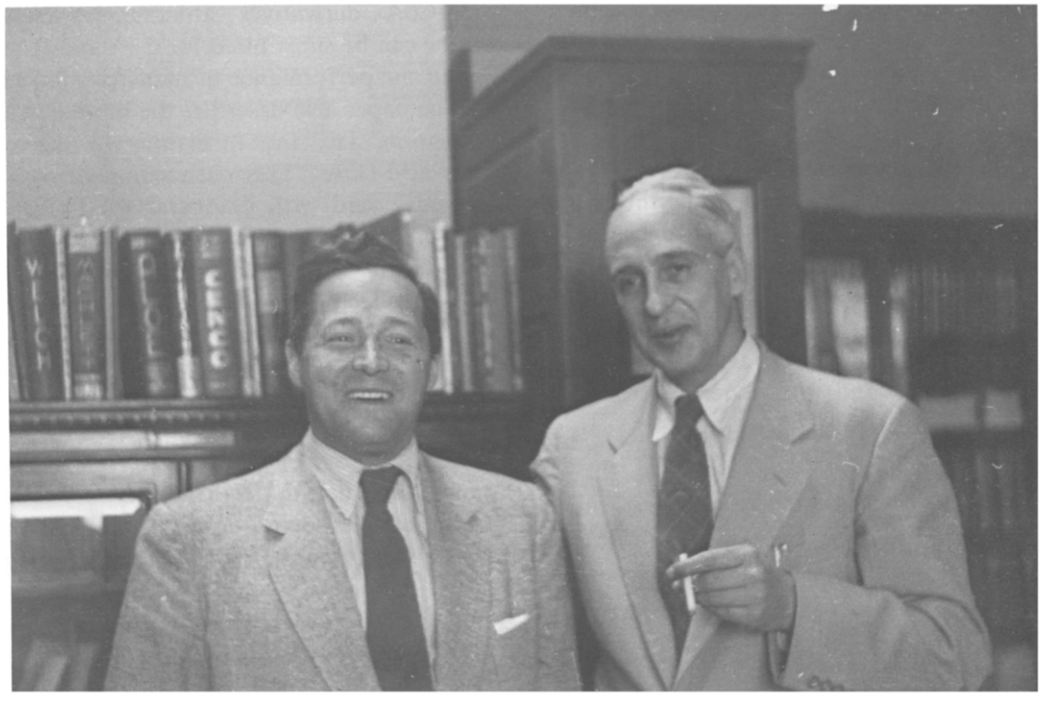
on 'Enzymes of fatty acid metabolism'
by F. Lynen and S. Ochoa
Biochim. Biophys. Acta 12 (1953) 299–314



Feodor Lynen (left) and Severo Ochoa

This is basically a review paper on the enzymes of fatty acid oxidation and synthesis, but it also contains original information on some of these enzymes through work in both the Munich and New York laboratories. A footnote acknowledgement to the Rockefeller Foundation for travelling fellowships to the authors informs the reader that their collaboration occurred not only by mail or telephone, but also by direct interaction both at Munich (Summer of 1952) and New York (Fall and Winter of the same year).

The author of this essay had long been interested in clarifying the mechanism of the so-called condensing reaction, the key reaction of the Krebs citric acid cycle. Through this reaction citrate is synthesized by interaction of oxaloacetate with an 'active' two-carbon fragment ('active' acetate). Lynen's isolation of 'active' acetate as acetyl-coenzyme A (acetyl-CoA) and the formation of this compound from acetyl phosphate by Stadtman's phosphotransacetylase (acetyl phosphate + HS-CoA ⇄ acetyl-S-CoA + orthophosphate) provided an assay for the condensing enzyme (citrate synthase) which led to its isolation from pig heart in crystalline form in our laboratory. The assay measured the rate of

* Dedicated to the memory of Fitzi Lynen, one of the most intelligent and charming men the author has known.

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citrate synthesis in the presence of enzyme, oxaloacetate and acetyl phosphate, with phosphotransacetylase and CoA in catalytic amounts. Citrate synthase, it will be remembered, catalyzes the reaction oxaloacetate + acetyl-CoA ⇄ citrate + CoA.

Fatty acids are oxidized or synthesized by a series of reversible reactions, referred to by Lynen as 'the fatty acid cycle' (Fig. 1). Synthesis starts by interaction of two molecules of acetyl-CoA to form acetoacetyl-CoA + CoA (reaction 1). Beta-keto-thiolase, the enzyme catalyzing this reaction, was so named for the reverse reaction (acetoacetyl-CoA + CoA → 2 acetyl-CoA). The synthesis of acetoacetyl-CoA is followed by its reduction to β-hydroxybutyryl-CoA. The enzyme catalyzing this reaction (a pyridine nucleotide enzyme) was named β-ketoreductase (reaction 2). Reduction is followed by dehydration to crotonyl-CoA by the enzyme crotonase (reaction 3). Finally, crotonyl-CoA is reduced to butyryl-CoA by ethylene reductase (reaction 4). Further elongation of the fatty acid chain occurs by the successive addition of single acetyl residues followed by a similar series of reactions, i.e., butyryl-CoA + acetyl-CoA ⇄ β-ketohexanoyl-CoA + CoA, and so on, mainly up to C_{16}.

The paper describes the properties of the four enzymes β-ketothiolase, β-ketoreductase, crotonase, and ethylene reductase, insofar as they were known at that time.

Two enzymes of the fatty acid cycle, namely β-ketoreductase and ethylene reductase, also react with *N*-acetylthioethanolamine, and these derivatives can conveniently be used as substrate for their assay.

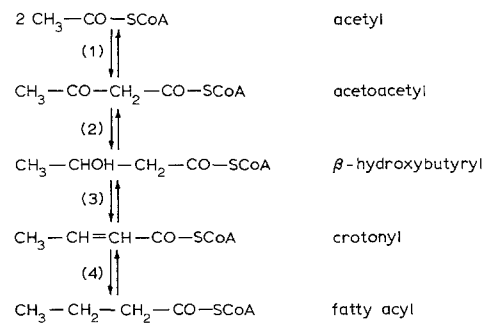

Fig. 1. Reactions of the fatty acid cycle. The above reactions are catalyzed by the following enzymes: (1) β-ketothiolase; (2) β-ketoreductase; (3) crotonase; (4) ethylene reductase.

It should be emphasized that fatty acids are metabolized only after activation to the corresponding fatty-acyl-CoA derivatives although *N*-acetylthioethanolamine can be substituted for CoA and this was convenient in the performance of many routine enzyme assays.

The paper also describes the mechanism of fatty acid activation, i.e., the formation of the corresponding fatty-acyl-CoAs. This occurs mainly by interaction of the fatty acid with CoA and ATP ($R\text{-}CH_2\text{-}COOH$ + $HSCoA$ + ATP ⇄ $R\text{-}CH_2\text{-}CO\text{-}SCoA$ + AMP + PP_i), but it can take place also by transfer of CoA from acetyl-CoA. A very active enzyme, present in heart and probably skeletal muscle, but not in liver, catalyzes the reversible transfer of CoA from succinyl-CoA to acetoacetate and is probably important in the activation of acetoacetate in peripheral tissues.

The paper by Lynen and Ochoa was one of the early papers in which the enzymes of fatty acid metabolism were studied in detail. Important studies on this subject were also carried out in D.E. Green's laboratory.

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ENZYMES OF FATTY ACID METABOLISM*

by

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Work of recent years (for reviews see [3,5,30]) has thrown much light on the mechanism of the β-oxidation of fatty acids formulated by KNOOP in 1904[32]. Through the use of isotopic tracer techniques[74,53,75] and of cell-free tissue preparations capable of oxidizing fatty acids[50,43,37-40,20,15,16] it was established that the two-carbon units removed successively from fatty acid chains during β-oxidation are identical to the two-carbon units derived from carbohydrate through the oxidative decarboxylation of pyruvic acid[39]. Further, these units can either condense with one another—or with longer fatty acid chains—to bring about fatty acid synthesis, or can undergo oxidation via the citric acid cycle[74,53,6,39,40,20]. Work with extracts of *Clostridium kluyveri* demonstrated that fatty acid synthesis occurs by a reversal of β-oxidation[61-63,28]. Evidence was also obtained that in the process of synthesis, in both bacteria[4,66] and animal tissues[1,78,52], the methyl end of "acetic acid" units is added to the carboxyl end of a fatty acid chain.

Further progress was hampered by the failure to detect intermediates during fatty acid oxidation or synthesis although from the early work of DAKIN[14] the corresponding α,β-unsaturated, β-hydroxy- and β-keto derivatives would be expected to be involved. Such a view would be in agreement with the observations that, at least in some tissues, the above compounds are oxidized at about the same rate as the corresponding fatty acids[31,20].

The finding that fatty acids are not oxidized unless they undergo a preliminary activation and the fact that this activation is dependent on the generation of energy-rich phosphate[50,43,37,38,42,20,33,61,62,63,29,15,16] suggested that the actual intermediates might not occur as the free acids[28]. The identification of the two-carbon unit as S-acetyl coenzyme A**[46,47,65,51,71,34,59,45] shed new light on the problem and strongly suggested

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** The following abbreviations are used: Coenzyme A (reduced), CoA, CoA-SH or CoA-SH; S-acyl coenzyme A derivatives, S-Acyl CoA, acyl-S-CoA, acyl-S-CoA, or simply acyl CoA; adenosine triphosphate, ATP; adenosine-5'-phosphate, AMP; pyrophosphate, PP; oxidized and reduced diphosphorpyridine nucleotide, DPN^+ and DPNH; oxidized and reduced flavin adenine dinucleotide, FAD and $FADH_2$.

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that the active intermediates might be the CoA derivatives of the fatty acids[47,3,30]. This belief was reinforced by the observation that S-acetyl CoA or the S-acyl derivatives of higher fatty acids can be generated enzymically through a reaction of the fatty acid with acyl phosphates in the presence of CoA[59,60] or with CoA and ATP[11,44,15,36,49,72,23,16,41,67]. Finally, work on the enzymic breakdown and synthesis of acetoacetate and other β-keto fatty acids[7,76,9,24,75,58,70,64,3,21,68,22] opened the way for an understanding of the mechanism whereby fatty acid chains are shortened or elongated, by the removal or addition of acetyl CoA, during fatty acid oxidation or synthesis.

By employing synthetic S-acyl analogues of the fatty acid derivatives of CoA[48,55] or the CoA fatty acid derivatives themselves[48,69,67] more recently made available by chemical or enzymatic synthesis, it has been possible to characterize and isolate from animal tissues some of the enzymes of fatty acid metabolism and obtain a clearer picture of the process as a whole. The development of rapid and sensitive optical methods of assay, whose introduction in enzymology we owe to OTTO WARBURG, has greatly facilitated the task of purifying the individual enzymes and studying their mechanism of action. The work described in this paper owes much to WARBURG's pioneering contributions which opened up new avenues of approach to dynamic biochemistry.

Fatty acid cycle

The results of the work summarized above and of the more recent work to be discussed in this paper show that fatty acid oxidation and synthesis proceed through the reactions illustrated in Fig. 1.

Fatty acid synthesis is accomplished through repetition of a cycle of four consecutive reactions: (a) condensation of two molecules of acetyl CoA to form acetoacetyl CoA and CoA–SH, (b) reduction of acetoacetyl CoA to β-hydroxybutyryl CoA, (c) dehydration of β-hydroxybutyryl CoA to crotonyl CoA, and (d) reduction of crotonyl CoA to butyryl CoA. A new cycle is started by the reaction of butyryl CoA with another molecule of acetyl CoA, to form β-ketocaproyl CoA + CoA–SH, and so forth. The cycle is repeated eight times until stearyl CoA is formed. All four reactions of the fatty acid cycle are reversible and fatty acid oxidation, once the fatty acid is activated through conversion to the corresponding acyl CoA derivative, proceeds by a reversal of the above sequence. The acetyl CoA split off at the end of each sequence is either oxidized via the citric acid cycle, by reacting with oxalacetate to form citrate and CoA–SH[51,71], or is converted to acetoacetyl CoA + CoA–SH. In liver, acetoacetyl CoA is hydrolysed by a specific deacylase with formation of CoA–SH and acetoacetate[69a,14a]. The presence of this enzyme would seem to account for the formation of free aceto-

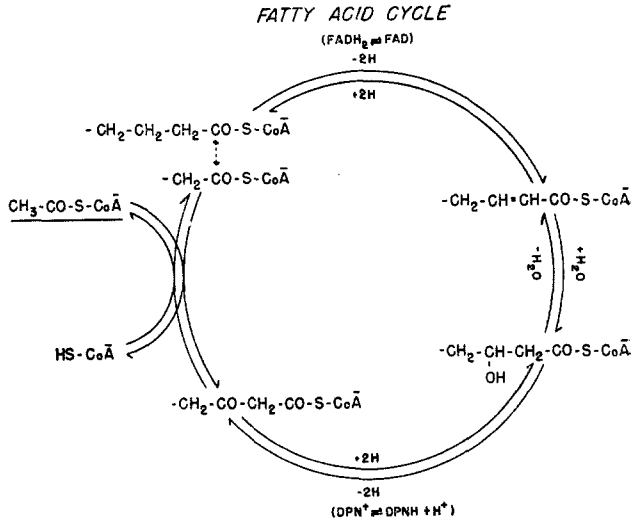

Fig. 1. Fatty acid cycle

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acetate in this organ. In either case CoA–SH is made available for activation of further fatty acid molecules.

The equilibrium of reaction (a) is predominantly in favor of the thioclastic splitting of the β-ketoacyl CoA derivatives. For this reason the name β-ketothiolase, or simply thiolase, has been proposed for this class of enzymes[48]. The equilibrium of reaction (b) favors reduction of the β-keto derivative, hence the name β-ketoreductase has been proposed for this group of enzymes[48]. The name crotonase has been suggested[67] for the enzyme or enzymes catalyzing reaction (c) and, finally, the name ethylene reductase has been used[55] to designate the enzyme or enzymes catalyzing reaction (d).

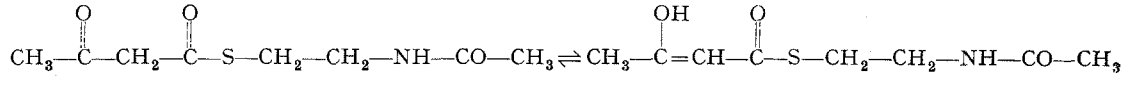
There are two main mechanisms for activation of fatty acids, *i.e.*, for the synthesis of their S-acyl CoA derivatives: (a) by a reaction with CoA–SH and ATP which, as we shall see later, results in the reversible formation of the corresponding acyl CoA, AMP and PP, and (b) by transfer of CoA from certain acyl CoA compounds such as acetyl CoA or succinyl CoA. Animal tissues, such as liver, heart, and kidney, utilize mainly the first mechanism while *C. kluyveri* utilizes the second. Extracts of this organism catalyze the reversible transfer of CoA from acetyl CoA to such fatty acids as propionate or butyrate[60]. Because of the presence of phosphotransacetylase[59], in the presence of CoA such extracts can utilize acetyl phosphate for activation.

A transferring enzyme of rather limited specificity is present in heart and probably in skeletal muscle and kidney but appears to be absent from liver. This enzyme catalyzes the reversible transfer of CoA from succinyl CoA to acetoacetate[21,68,69,22]. Through the formation of acetoacetyl CoA the enzyme activates acetoacetate, produced in the liver and carried by the blood stream to the peripheral tissues, for oxidation in these tissues via the tricarboxylic acid cycle (see reference no. 8). The latter in turn generates the necessary succinyl CoA through the oxidation of α-ketoglutarate which, as shown by recent work[26,54,27], reacts with CoA–SH and DPN^+ to form succinyl CoA, CO_2, and DPNH.

S-acyl fatty acid derivatives

Synthetic S-acetoacetyl and S-crotonyl derivatives of N-acetyl thioethanolamine[48a] have been found to act as substrates of β-ketoreductase and ethylene reductase respectively[48,55]. These structural analogues of the natural S-acyl CoA derivatives have therefore provided suitable substrates for the isolation of the two enzymes. Further, the two model compounds have characteristic absorption spectra. This made it possible not only to predict the optical properties of the corresponding natural substrates, *i.e.*, S-acetoacetyl and S-crotonyl CoA, but also to develop convenient optical methods for the assay of several enzymes. Thus, the analogues have greatly facilitated the study of individual steps of fatty acid metabolism.

S-acetoacetyl-N-acetyl thioethanolamine was obtained as a colourless crystalline compound (m.p., 60°) through reaction of N-acetyl thioethanolamine with diketene. As a solid, the compound is in the keto form but it undergoes rapid enolization in solution as can be shown with the ferric chloride reaction and by the change in its absorption spectrum. At equilibrium the percentage of enol is higher in solutions of the thioester



than in those of acetoacetic ethyl ester, a fact which was first observed by BAKER AND REID with acetoacetic thioethyl ester[2].

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The absorption spectrum of S-acetoacetyl-N-acetyl thioethanolamine is shown in Fig. 2. At pH 6.2 the compound has a band with a maximum at 233 mμ; this absorption peak is characteristic of the thioester bond[57,60]. At pH 8.0 an additional band appears with a maximum at 303 mμ. This band is to be attributed to the formation of an enolate ion and, as shown in Fig. 3, depends on the pH. The increasing absorption parallels the increasing dissociation as the pH is raised. The pK' of the compound was found to be 8.54.

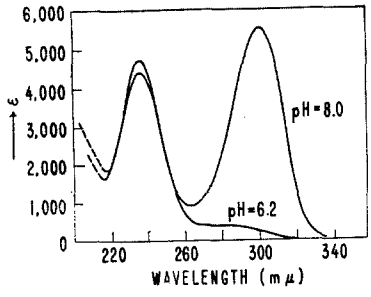
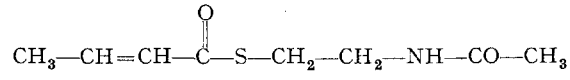


Fig. 2. Ultraviolet absorption spectrum of S-acetoacetyl-N-acetyl thioethanolamine.

S-crotonyl-N-acetyl thioethanolamine was obtained in



crystalline form (m.p., 61.5–62°) through reaction of crotonyl chloride with the lead salt of N-acetyl thioethanolamine[55]. Its absorption spectrum is shown, along with that of free crotonate, in Fig. 4. It is evident that with the binding of the unsaturated acid to sulfur there is a shift toward longer wavelengths of the absorption due to the double bond. Free crotonate has a maximum (not shown on the figure) at 204 mμ while the thioester has a maximum at 224 mμ. The thioester has an additional band at 263 mμ which is possible due to the —C(=O)—S-group. The shift of the two absorption maxima toward longer wavelengths may be a reflection of the resonance between the double bond and the thioester linkage, a fact which is of great importance for the chemical reactivity of the former.

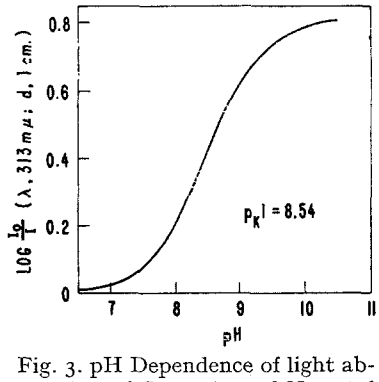


Fig. 3. pH Dependence of light absorption of S-acetoacetyl-N-acetyl thioethanolamine at 313 mμ. $c = 5 \cdot 10^{-5}\ M$; $d = 1.0$ cm.

A number of S-acyl CoA derivatives of fatty acids have now become available through chemical or enzymic synthesis. Solutions of acetoacetyl CoA can be readily prepared by the enzymic transfer of CoA from succinyl CoA to acetoacetate. This reaction has made possible the isolation of acetoacetyl CoA[69] and its routine preparation for the assay of β-ketothiolase. Succinyl CoA itself can be prepared enzymically with α-ketoglutaric dehydrogenase[54,27] or by means of the reaction between succinate, CoA, and ATP, of KAUFMAN *et al.*[26,27]. However, the compound can be obtained much more readily by the synthetic procedure of SIMON AND SHEMIN[56] with CoA–SH and succinic anhydride. The method of SIMON AND SHEMIN has further been applied to the preparation of other S-acyl CoA derivatives such as acetyl, propionyl, butyryl, and crotonyl CoA. S-acyl fatty acid derivatives have also been prepared by means of the enzymic reaction of fatty acids with CoA–SH and ATP[21a] and, in the case of the acetyl and propionyl derivatives, through the phosphotransacetylase catalyzed reaction between CoA–SH and the corresponding acyl phosphates[59]. Finally, S-acetoacetyl and S-β-hydroxybutyryl

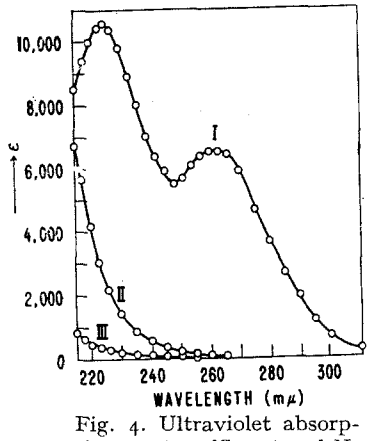


Fig. 4. Ultraviolet absorption spectra of S-crotonyl-N-acetyl thioethanolamine (I), crotonate (II), and N-acetyl thioethanolamine (III), at pH 7.0.

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CoA have recently become available through chemical synthesis. T. WIELAND[77] has succeeded in synthesizing these compounds by reaction of CoA–SH with the thioesters of acetoacetic or β-hydroxybutyric acid and thiophenol. S-acetoacetyl CoA has also been prepared through the interaction of CoA–SH with diketene[14a].

The absorption spectra of acetoacetyl CoA and crotonyl CoA are essentially the same as those of their thioethanolamine analogues. The main difference is that, in contrast to N-acetyl thioethanolamine, CoA absorbs in the 260 mμ region because of its adenine group. This does not interfere with the enol band of acetoacetyl thioesters but interferes to some extent with the 263 mμ band of the crotonyl derivatives. However, the extinction coefficient of the latter compounds at this wavelength is very high and the interference of the adenine moiety of crotonyl CoA can be eliminated by addition of adenine nucleotide, for example adenylic acid, to the blank cell.

In contrast to the optical behavior of the acetoacetyl and crotonyl CoA derivatives, β-hydroxyacyl and saturated acyl CoA compounds show only the thioester band at 233 mμ in addition to the adenine band. It is therefore possible in enzymic experiments, to follow the appearance and disappearance of β-ketoacyl or dehydroacyl CoA compounds if β-hydroxyacyl or acyl CoA compounds are involved in the reaction. At pH 8.0 the enol absorption of acetoacetyl thioesters is markedly increased by magnesium ions[67a], probably through formation of a chelate structure. This increase in absorption can conveniently be made use of to increase the sensitivity of the optical enzyme tests.

ENZYMES OF FATTY ACID METABOLISM

Activating enzymes. As already stated the main mechanism for the activation of fatty acids in animal tissues is through a reaction of the fatty acid with CoA–SH, in the presence of ATP, to yield the corresponding S-acyl CoA, AMP, and PP. This reaction requires the presence of Mg ions. The first reaction of this type to be studied in detail was the activation of acetate by an enzyme present in liver, yeast, and other tissues[11]. The mechanism of the over-all reaction was established by LIPMANN *et al.*[44] with partially purified enzyme preparations from liver and yeast (Reaction 1). Similar results were obtained by HILZ AND LYNEN (unpublished experiments) with a highly purified enzyme

$$CH_3—COOH + HS—CoA + ATP \rightleftharpoons CH_3—\overset{\overset{O}{\|}}{C}—S—CoA + AMP + PP \qquad (1)$$

from yeast and by GREEN and collaborators[21,23] with purified enzymes from heart muscle and liver. The acetate enzyme is active also with propionate.

Recent experiments[25] suggest that the over-all reaction occurs in three steps as indicated below:

(a) ATP + enzyme $\rightleftharpoons$ AMP-enzyme + PP
(b) AMP-enzyme + CoA $\rightleftharpoons$ CoA-enzyme + AMP
(c) CoA-enzyme + acetate $\rightleftharpoons$ acetyl-CoA + enzyme

The occurrence of reaction (a) is supported by the incorporation of labelled PP into ATP in the presence of a partially purified enzyme from yeast. This exchange is dependent upon the presence of Mg^{+2}. Reaction (c) is supported by the incorporation of isotopic acetate into acetyl CoA in the presence of the enzyme.

An enzyme catalyzing the activation of fatty acids from C_4 to C_{12} was isolated from ox liver in D. E. GREEN's laboratory[72]. The enzyme catalyzes Reaction 2. The same

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enzyme preparation was active on α,β-unsaturated and β-hydroxy derivatives. The formation of crotonyl- and β-hydroxybutyryl CoA by liver enzymes under similar

$$-CH_2-CH_2-COOH + HS-CoA + ATP \rightleftharpoons -CH_2-CH_2-\overset{O}{\overset{\|}{C}}-S-CoA + AMP + PP \qquad (2)$$

conditions has also been observed in other laboratories[41,67]. The activation of higher fatty acids, presumably C_{14} to $_{18}$, by ATP and HS-CoA is catalyzed by yet another enzyme discovered by KORNBERG AND PRICER[36,35] in liver. KORNBERG's enzyme catalyzes the formation of S-stearyl CoA, AMP, and PP from stearic acid, HS-CoA and ATP.

CoA transferases. In *Cl. kluyveri* extracts activation of fatty acids appears to occur predominantly by transfer of CoA from acetyl-S-CoA. The first enzyme of the CoA transferase type was discovered by STADTMAN in extracts of *Cl. kluyveri* and named CoA transphorase[60]. The enzyme catalyzes the reversible transfer of CoA from acetyl-CoA to propionate (Reaction 3).

$$CH_3-\overset{O}{\overset{\|}{C}}-S-CoA + CH_3-CH_2-COOH \rightleftharpoons CH_3-COOH + CH_3-CH_2-\overset{O}{\overset{\|}{C}}-S-CoA \qquad (3)$$

Cl. kluyveri extracts also catalyze the transfer of CoA from acetyl CoA to butyrate, vinyl acetate, and lactate[60a]. This enzyme (enzymes) is (are) similar to the succinyl-CoA-acetoacetate transferase of heart muscle but with different substrate specificity.

The reversible transfer of CoA from succinyl CoA to acetoacetate was discovered independently by GREEN and co workers[21,22] and by STERN *et al.*[68,69]. The enzyme which, as already mentioned, is present in heart and probably in skeletal muscle and kidney but not in liver, catalyzes Reaction 4. In the early stages of purification[69] the

$$HOOC-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-S-CoA + CH_3-CO-CH_2-COOH \rightleftharpoons HOOC-CH_2-CH_2-COOH + CH_3-CO-CH_2-\overset{O}{\overset{\|}{C}}-S-CoA \qquad (4)$$

enzyme assay was based on the rate of citric acid synthesis from succinyl CoA and acetoacetate in the presence of oxalacetate, an excess of thiolase, and crystalline citrate condensing enzyme, as indicated by the reactions below:

Succinyl—S—CoA + acetoacetate $\rightleftharpoons$ succinate + acetoacetyl—S—CoA (transferase)
Acetoacetyl—S—CoA + HS—CoA $\rightleftharpoons$ 2 acetyl—S—CoA (thiolase)
2 Acetyl—S—CoA + 2 oxalacetate + 2 H_2O $\rightleftharpoons$ 2 citrate + 2 HS—CoA (citrate condensing enzyme)

Sum: Succinyl—S—CoA + acetoacetate + HS—CoA + 2 oxalacetate + 2 H_2O $\rightleftharpoons$ Succinate + 2 citrate + 2 HS—CoA

A heated ammonium sulfate fraction from ox liver, free of transferase, was used as the source of thiolase. After removal of thiolase, the transferase assay was based on the increase in optical density at pH 8.1 and wavelength 305 mμ due to the formation of acetoacetyl-S-CoA. Although magnesium ions are not required for the reaction, as mentioned previously, Mg^{+2} markedly augments the light absorption and was added to the reaction mixture in order to increase the sensitivity of the assay.

The enzyme has been isolated from pig heart and purified about 700-fold over the initial phosphate extract[67a]. The purification involved ammonium sulfate and acetone fractionation, removal of inactive proteins by heat and by adsorption on Ca phosphate

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gel, fractionation with ethanol in the presence of Zn ions, and refractionation with ammonium sulfate.

Fig. 5 shows the appearance of the enol band of S-acetoacetyl thioesters when succinyl CoA is incubated with acetoacetate and transferase at pH 8.1, indicating the formation of S-acetoacetyl CoA. Within the range 290 to 330 mμ this band corresponds closely with the corresponding band of S-acetoacetyl-N-acetyl thioethanolamine. It may also be seen that most of the absorption in this region disappears after the further addition of CoA-SH and thiolase since the equilibrium position of the thiolase reaction favors the formation of acetyl CoA. The forward course and the reversal of the transferase

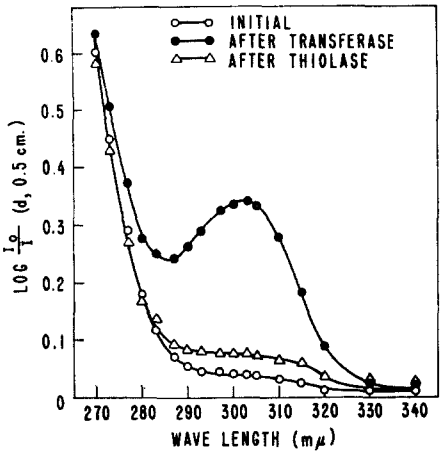

Fig. 5. Spectral changes accompanying the enzymatic synthesis and breakdown of S-acetoacetyl CoA at pH 8.1. Volume, 1.5 ml; d = 0.5 cm; temp. 25°. —○—○— S-Succinyl CoA (~ 0.1 μM). —●—●— After establishment of equilibrium on addition of acetoacetate (50 μM) and transferase (60 μg of protein). —△—△— After further addition of CoA-SH (0.15 μM) and thiolase (90 μg of protein). Acetoacetate and CoA-SH added to both blank and experimental cells. $MgCl_2$ (8.0 μM) present in reaction mixture.

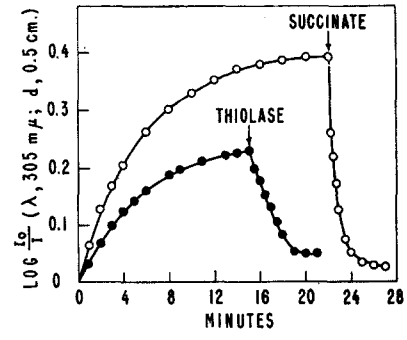

Fig. 6. Optical demonstration of transferase and thiolase activities. Volume, 1.5 ml; d = 0.5 cm; pH, 8.1; temp., 25°. Upper curve: Transferase (17 μg of protein) added at zero time to a mixture of succinyl CoA (~ 0.11 μM) and acetoacetate (50 μM); succinate (40 μM) added at the arrow. Lower curve: Transferase (17 μg of protein) added at zero time to a mixture of succinyl CoA (~ 0.06 μM) and acetoacetate (50 μM); CoA-SH (0.15 μM) and thiolase (2.7 μg of protein) added at the arrow. Other details as in Fig. 5.

reaction as followed at 305 mμ are shown in Fig. 6. The upper curve shows the increase in absorption at 305 mμ on adding transferase to a mixture of succinyl CoA and acetoacetate and the reversal of the reaction by succinate after equilibrium was established. The equilibrium constant of the transferase reaction (K_{eq} = (Succinate) (acetoacetyl-S-CoA)/Succinyl-S-CoA) (acetoacetate)) is about 10^{-2} at pH 8.1.

The acetoacetyl CoA formed by the reaction between succinyl CoA and acetoacetate, in the presence of transferase, was isolated as a crude alcohol-insoluble barium salt and further purified by paper chromatography[69]. In ethanol-acetate its R_F is 0.52 at 24°, while that of acetoacetate is 0.75. Like acetyl CoA[46,47], acetoacetyl CoA gives a positive sulfhydryl reaction with nitroprusside only after alkaline hydrolysis.

Some insight into the mechanism of action of the transferase has been gained by experiments with methylene labelled ^{14}C-succinate[19a]. When ^{14}C-succinate and succinyl-CoA are incubated with transferase a rapid exchange of free- and thioester-bound succinate occurs. This suggests the possibility that succinyl CoA, or acetoacetyl CoA, reacts with the enzyme to form a CoA-enzyme compound which retains the energy of the

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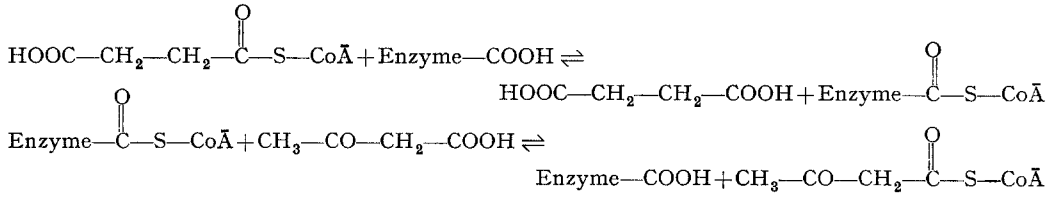
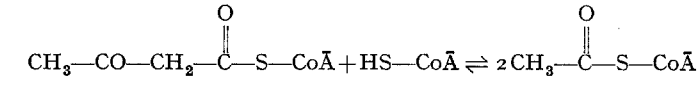
thioester bond and can transfer CoA to the free acid, acetoacetic or succinic. The reaction could be visualized as involving a carboxyl group of the enzyme as indicated below:



The best preparations of transferase so far obtained are free of thiolase and, under the conditions of the optical assay, catalyze the formation of 250 moles of acetoacetyl-CoA per minute per 100,000 g of enzyme at 25°. As assayed optically, the purified transferase catalyzes the transfer of CoA from succinyl CoA to acetoacetate, β-ketovalerate, β-keto*iso*caproate, and β-ketocaproate in order of decreasing activity. β-ketooctanoate is inactive. The enzyme catalyzes the transfer of CoA from acetoacetyl CoA to succinate but not to β-hydroxybyturate, crotonate, butyrate or octanoate.

β-keto thiolase. The enzyme catalyzing Reaction 5 has been partially purified from sheep liver[48] and more extensively from pig heart[50a]. The enzyme has also been referred to as the acetoacetate condensing enzyme[69]. In the lower curve of Fig. 6, addition of CoA-SH and thiolase to a mixture of succinyl CoA and acetoacetate previously incubated with transferase is shown to cause a decrease in optical density at 305 mμ due to cleavage of the acetoacetyl CoA formed by the CoA transfer reaction. The assay used for the purification of the pig heart enzyme was based on the decrease in optical density at



(5)

pH 8.1 and wavelength 305 mμ with acetoacetyl CoA and CoA–SH as substrates in the presence of Mg^{+2}. Solutions of acetoacetyl CoA were prepared every few days by incubating synthetic succinyl CoA and acetoacetate with purified CoA-transferase. When the reaction reached equilibrium, the pH of the mixture was brought to 5.5 with acetic acid, the solution was heated to 75° for 2 minutes to destroy the transferase, cooled, centrifuged and the supernatant adjusted to pH 8.0. Acetoacetyl CoA was stable for several days if stored at −18° when not in use.

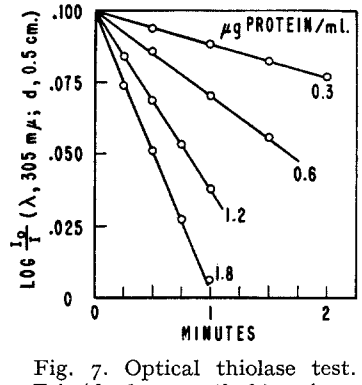


Fig. 7. Optical thiolase test. Tris (hydroxymethyl) amino - methane-HCl buffer pH 8.1, 200 μ*M*; $MgCl_2$, 8.0 μ*M*; reduced glutathione, 10.0 μ*M*; CoA-SH, 0.15 μ*M*; S-acetoacetyl CoA, ~0.03 μ*M*. Volume, 1.5 ml; *d* = 0.5 cm; temp., 25°.

The enzyme has been purified about 300 fold over the initial phosphate extract through steps involving ammonium sulfate and acetone fractionation, removal of inactive proteins at pH 5.3, refractionation with ammonium sulfate, and low temperature ethanol fractionation in the presence of Zn ions. The time course of the reaction in the optical test with varying concentrations of purified pig heart enzyme is shown in Fig. 7.

The thioclastic cleavage of acetoacetyl CoA to acetyl CoA results not only in a decrease of light absorption at 305 mμ but also in a concomitant increase in the absorption in the 240 mμ region

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due to the formation of a second thioester bond, since two molecules of acetyl CoA are formed per molecule of acetoacetyl CoA disappearing. In the presence of CoA-SH, oxalacetate, thiolase, and citrate condensing enzyme, acetoacetyl CoA yields two molecules of citrate per molecule of sulfhydryl (*i.e.*, per molecule of CoA–SH) appearing[69], according to the following reactions:

Acetoacetyl—S—CoĀ + HS—CoĀ ⇌ 2 acetyl—S—CoĀ (thiolase)
2 Acetyl—S—CoĀ + 2 oxalacetate + 2 H_2O ⇌ 2 citrate + 2 HS—CoĀ (citrate condensing enzyme)
Sum: Acetoacetyl—S—CoĀ + HS—CoĀ + 2 oxalacetate + 2 H_2O ⇌ 2 citrate + 2 HS—CoĀ

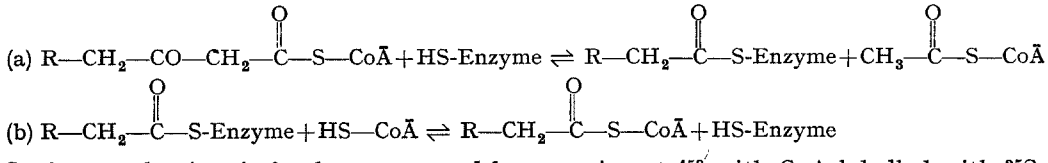
The equilibrium position of the thiolase reaction is very far toward cleavage[48,68,69]. For this reason it was not feasible to use this reaction for the isolation of acetoacetyl CoA. Determinations of the equilibrium constant (K'_{eq} = (Acetyl-S-CoA)²/(Acetoacetyl CoA)(CoA-SH)) by means of the optical method gave an approximate value of $5 \cdot 10^4$ at pH 8.1, and $1 \cdot 10^4$ at pH 9.0.

The reversibility of the reaction can be demonstrated by the optical method as previously reported[48]. The synthesis of acetoacetyl CoA from acetyl CoA can be followed directly at alkaline pH (~ 9.0) as a small increase in the optical density at 305 mμ in the presence of large amounts of acetyl CoA[48a], or indirectly through coupling with the β-keto reductase to effect the oxidation of reduced DPN[48].

The purified heart enzyme is highly specific for acetoacetyl CoA. β-Ketovaleryl CoA reacts at 20% of the rate of acetoacetyl CoA, and β-ketocaproyl- and *iso*caproyl-CoA react practically not at all. This is in contrast to the broader specificity of crude enzyme fractions[68] and indicates that there must be other thiolases, acting on S-β-ketoacyl CoA derivatives of higher chain length.

Under the conditions of the optical test, the best preparations of the heart enzyme so far obtained catalyze the cleavage of 3000 to 4000 moles of acetoacetyl CoA per minute per 100,000 g of enzyme at 25°. When coupled with β-keto reductase about 30 times more acetoacetate condensing enzyme must be used in the back reaction to reach the rates obtained in the direction of acetoacetyl CoA cleavage.

β-Ketothiolase has been found to be inhibited by sulfhydryl reagents such as iodoacetic acid or arsenoxide[48a]. This indicates that the enzyme is an "SH enzyme" and suggests the following mechanism of action:



Such a mechanism is further supported by experiments[45a] with CoA labelled with ^{35}S. On incubation of propionyl-S-CoA with ^{35}S-H-CoA, in the presence of purified heart thiolase, radioactive propionyl-S-CoA is formed indicating the occurrence of the following reaction:

Propionyl S—CoĀ + HS-enzyme ⇌ Propionyl-S-enzyme + HS—CoĀ

The above mechanism provides an explanation for the unequal isotope distribution in acetoacetate observed during oxidation of isotopic fatty acids in liver[10,13,12,19,18]. For example, octanoic acid labelled with ^{14}C in the carboxyl group can yield acetoacetate in which the ratio of the radioactivity in the carbonyl and carboxyl carbons is less than unity. Some acetoacetate labelled exclusively in the carboxyl group must arise when

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non-labelled acetoacetyl-S-CoA from the last four carbons of the fatty acid chain reacts with thiolase to give non-labelled acetyl-S-enzyme and this in turn reacts with labelled acetyl-S-CoA from the pool to yield $CH_3—CO—CH_2—C^*(=O)—S—CoA$. This is shown schematically in Fig. 8 for the case of caproic acid.

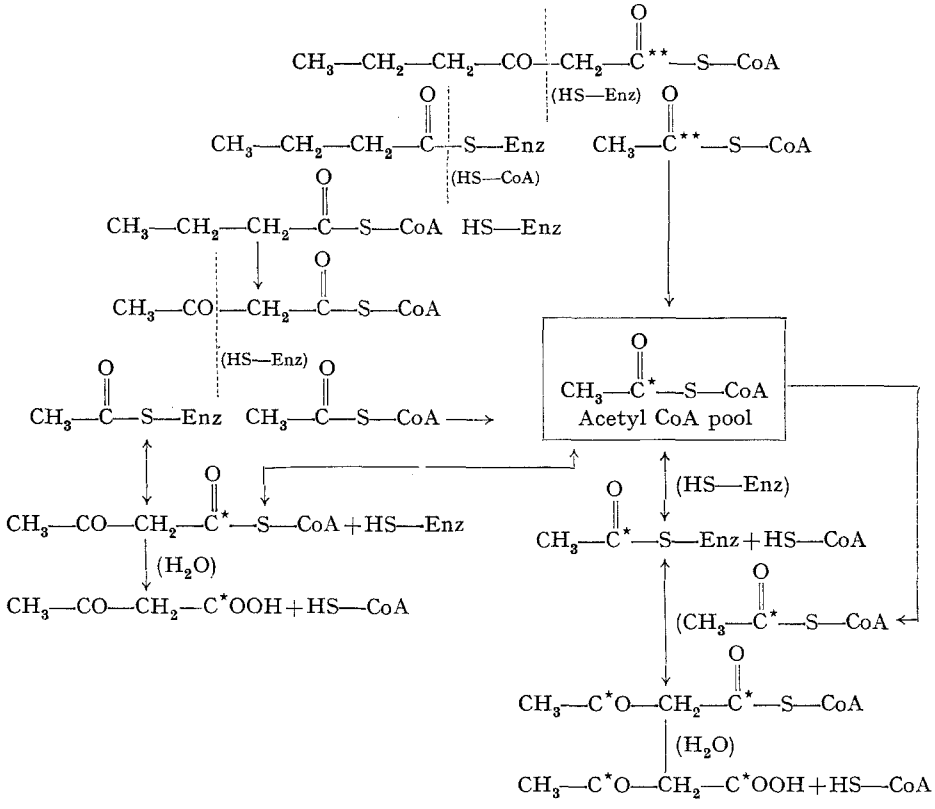

Fig. 8. Asymmetric labelling of acetoacetate from carboxyl-labelled caproic acid.

β-keto-reductase. This enzyme catalyzes Reaction 6. The finding that

$$CH_3—CO—CH_2—C(=O)—S—CoA+DPNH+H^+ \rightleftharpoons CH_3—CHOH—CH_2—C(=O)—S—CoA+DPN^+ \quad (6)$$

the acetoacetyl-S-CoA analogue, S-acetoacetyl-N-acetyl thioethanolamine, was readily reduced by DPNH in the presence of enzyme solutions from various sources afforded a convenient assay for this enzyme. Employing this assay the enzyme was purified some 300-fold from sheep liver extracts by a procedure involving three steps: precipitation with ethanol, denaturation of inactive protein at 55°, and fractionation with ammonium sulfate[48]. The time course of the reaction in the optical test with varying concentrations of the purified enzyme is shown in Fig. 9.

The reaction is readily reversible but, at pH 7.35 with equimolecular amounts of DPNH and the acetoacetyl thioethanolamine derivative, it proceeds in the direction of reduction of the latter to the extent of 95 %. The equilibrium constant of the reaction (K'_{eq} = (S-β-hydroxybutyryl compound) (DPN+)/(S-acetoacetyl compound) (DPNH))

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has been found[48a] to be $5.2 \cdot 10^2$ at pH 7.0. The enzyme does not react with free acetoacetate or with ethyl acetoacetate; it also fails to react with S-acetoacetyl glutathione. With acetoacetyl-S-CoA the reaction is much faster than with the thioethanolamine derivative. This is undoubtedly due to the much higher affinity of the enzyme for the natural compound. In fact, in kinetic studies with S-acetoacetyl-N-acetyl thioethanolamine it was not possible to reach saturation of the enzyme with the analogue[48a].

In the presence of thiolase, β-keto reductase, and DPNH, the latter is oxidized on addition of acetyl-S-CoA; DPN^+, β-hydroxybutyryl-S-CoA and HS-CoA are the reaction products[48]. This occurs according to the reactions below:

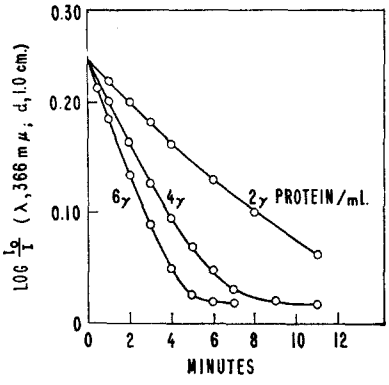

Fig. 9. Optical β-keto reductase test. Pyrophosphate buffer pH 7.4, 50 μM; DPNH, 0.13 μM; S-acetoacetyl-N-acetyl thioethanolamine, 5.0 μM. Volume, 2.0 ml; d = 1.0 cm; temp. 25°.

$$2\,CH_3{-}\overset{O}{\overset{\|}{C}}{-}S{-}CoA \rightleftharpoons HS{-}CoA + CH_3{-}CO{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}O{-}S{-}CoA \quad \text{(thiolase)}$$

$$CH_3{-}CO{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}S{-}CoA + DPNH + H^+ \rightleftharpoons CH_3{-}CHOH{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}S{-}CoA + DPN^+ \quad \text{(reductase)}$$

$$\textit{Sum:}\ 2\,CH_3{-}\overset{O}{\overset{\|}{C}}{-}S{-}CoA + DPNH + H^+ \rightleftharpoons CH_3{-}CHOH{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}S{-}CoA + HS{-}CoA + DPN^+$$

The formation of HS-CoA can be followed through the appearance of sulfhydryl groups. β-hydroxybutyryl-S-CoA was extracted from the acidified reaction mixture with *p*-cresol and converted into the corresponding hydroxamic acid by reaction with hydroxylamine. The β-hydroxybutyrohydroxamic acid was identified by paper chromatography (R_F in aqueous butanol, 0.29). On incubation of the natural β-hydroxybutyryl-S-CoA with DPN^+ and purified β-ketoreductase at pH 9.05, the reduction of DPN^+, followed at 340 mμ, is accompanied by the formation of acetoacetyl-S-CoA as shown by the increase in optical density at 303 mμ.

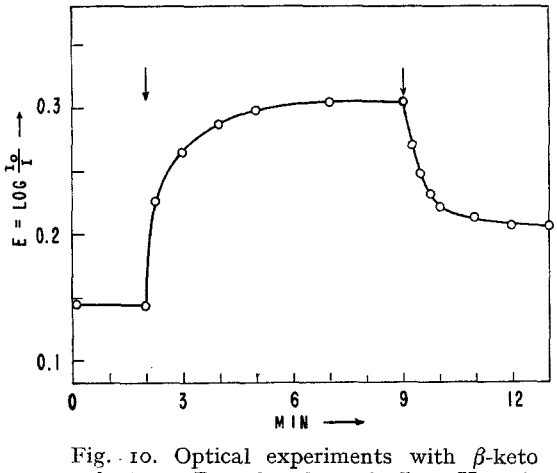

Fig. 10. Optical experiments with β-keto reductase. Pyrophosphate buffer pH 6.58, 100 μM; DPN^+, 5.0 μM; S-β-hydroxybutyryl CoA, 2.0 μM. Volume, 2.0 ml; λ, 366 mμ; d = 1.0 cm; temp. 21°. β-keto reductase (1.5 mg of protein) added at the first arrow. S-acetoacetyl CoA (0.43 μM) added at the second arrow.

As previously mentioned both β-hydroxybutyryl- and acetoacetyl-S-CoA have recently become available synthetically. The course of the β-ketoreductase reaction with these two compounds[48a] is shown in Fig. 10.

LEHNINGER AND GREVILLE[41] have recently reported the interesting observation that liver contains two different β-ketoreductases. One of them catalyzes the reversible oxidation of free *l*-hydroxybutyrate by DPN^+, the other catalyzes the reversible oxidation

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of *d*-β-hydroxybutyryl-S-CoA by DPN+. The latter reaction, which is undoubtedly catalyzed by the β-ketoreductase here described, was demonstrated by making use of the fact that liver also contains enzymes catalyzing the formation of *d*- or *l*-β-hydroxybutyryl-S-CoA in the presence of ATP, CoA-SH, and *d*- or *l*-β-hydroxybutyrate.

The chain-length specificity of the β-ketoreductase is still unknown and it is not possible to decide at this time whether more than one enzyme is concerned with the CoA derivatives of β-keto and β-hydroxy acids from C_4 to C_{18}. The purified reductase described above has been found to act rapidly on S-β-ketocaproyl-N-acetyl thioethanolamine[48a].

Crotonase. Synthetic S-crotonyl CoA is converted to S-acetoacetyl CoA, in the presence of DPN, by crude enzyme preparations from heart or liver[67]. The reaction can be followed through the appearance of the absorption band of DPNH at 340 mμ or that of acetoacetyl-S-CoA at 305 mμ. Also, on addition of HS-CoA, citrate condensing enzyme, and oxalacetate, crotonyl-S-CoA acts as an acetyl donor for citrate synthesis; the required thiolase was present in the crude enzyme preparation used. These observations, together with the fact that reduced leucosafranine is oxidized by synthetic β-hydroxybutyryl-S-CoA in the presence of partially purified preparations of ethylene reductase[55] indicate the occurrence of an enzyme catalyzing the reversible Reaction 7 below. The name crotonase has been suggested for this enzyme[67]. The enzyme has no action on free

$$CH_3—CH=CH—\overset{\overset{O}{\|}}{C}—S—CoA + H_2O \rightleftharpoons CH_3—CHOH—CH_2—\overset{\overset{O}{\|}}{C}—S—CoA \qquad (7)$$

crotonate or on the S-crotonyl derivatives of N-acetyl thioethanolamine, glutathione or thioglycolic acid.

As already mentioned the spectrum of S-crotonyl CoA is similar to that of S-crotonyl-N-acetyl thioethanolamine. This is readily apparent when the contribution of the adenine moiety of the CoA derivative is eliminated by reading S-crotonyl CoA against a solution containing an identical amount of the compound but previously subjected to alkaline hydrolysis. The difference spectrum so obtained[52a], illustrated in Fig. 11, shows absorption maxima at 224 and 263 mμ like S-crotonyl-N-acetyl thioethanolamine. The crotonyl CoA was obtained through reaction of CoA-SH with crotonic anhydride following the method of SIMON AND SHEMIN[56].

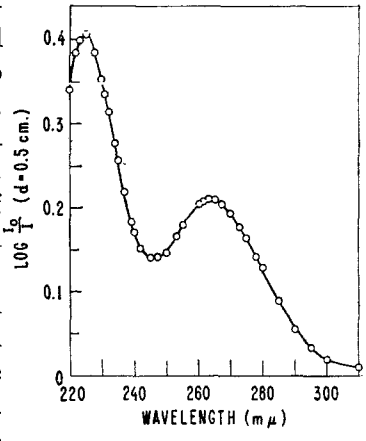

Fig. 11. Difference ultraviolet absorption spectrum of S-crotonyl CoA before and after alkaline hydrolysis of the thioester bond. $c \simeq 6 \cdot 10^{-5}$ *M* in each cell; $d = 0.5$ cm; pH, 7.5. Crotonyl CoA in blank cell previously hydrolyzed with alkali.

The decrease in light absorption at 263 mμ when crotonase acts on crotonyl CoA affords a simple method of assay for this enzyme. The purification of the enzyme from ox liver has recently been undertaken. Through steps involving denaturation of inactive proteins by acidification and heat, followed by acetone, ammonium sulfate and low temperature ethanol fractionation, preparations of the enzyme have been obtained representing about 100-fold purification over the original extract[52a]. The preparations are free of fumarase showing that fumarase and crotonase are distinct enzymes. Crotonase has a remarkably high activity as may be seen in Fig. 12. which shows the time course of the reaction in the optical test with varying amounts of the enzyme. The equilibrium

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constant of the reaction has not yet been determined but it appears to favor the S-β-hydroxyacyl derivatives. Nothing can as yet be said as to the chain-length specificity of crotonase and consequently the occurrence of several enzymes of this type is not excluded.

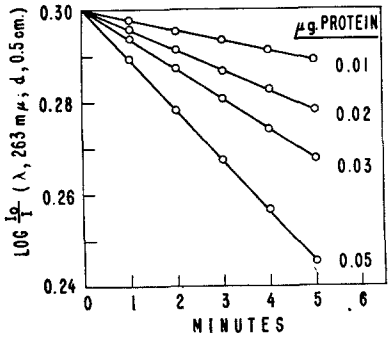

Fig. 12. Optical crotonase test. Tris (hydroxymethyl) aminomethane-HCl-buffer pH 7.5, 100 μM; egg albumin, 0.1 mg; ethylenediamine tetraacetate, 1.5 μM; S-crotonyl CoA, ~ 0.5 μM. 2.0 μM of AMP in blank cell. Volume, 1.5 ml; d = 0.5 cm; temp., 25°.

Crotonase would also appear to convert S-vinylacetyl CoA to the β-hydroxybutyryl derivative[21a]. If so, an equilibrium would be established between the S-acyl CoA derivatives of crotonic, vinylacetic, and β-hydroxybutyric acid. This might explain the observation that vinylacetate can be either oxidized or reduced by extracts of *C. kluyveri*[28,62]. The failure of crotonate to replace vinylacetate in this system[62] may have been due to failure of the bacterial extracts to activate crotonate.

Ethylene reductase. Ethylene reductase was detected in liver extracts[55] by a method similar to that employed by FISCHER AND EYSENBACH to study fumarate reductase[17]. Leucosafranine is oxidized by S-crotonyl-N-acetyl thioethanolamine, but not by free crotonate, in the presence of an enzyme from liver. The reaction is shown in Fig. 13. Here again a natural compound, in this case crotonyl-S-CoA, could be replaced by its readily synthesized thioethanolamine analogue. The

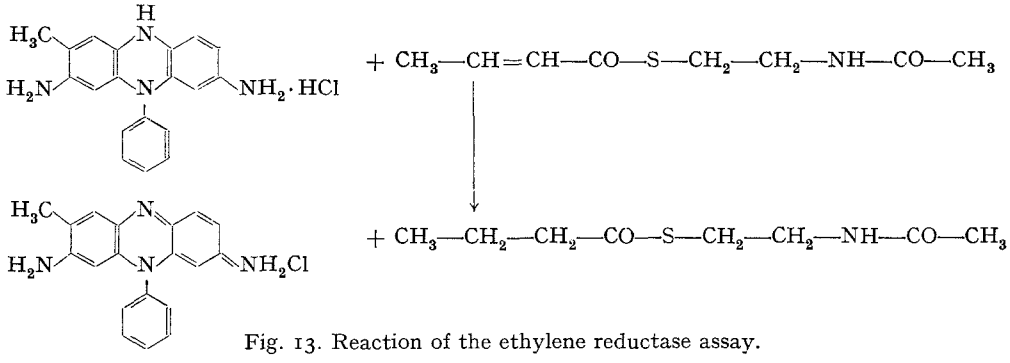

Fig. 13. Reaction of the ethylene reductase assay.

enzyme assay, in which the appearance of colour from the leucodye is followed, is illustrated in Fig. 14. By the use of this assay ethylene reductase was purified about 80-fold from sheep liver extracts through steps involving acetone fractionation, adsorption and elution from calcium phosphate gel, and ammonium sulfate fractionation. Solutions of the purified enzyme are yellow in colour. A colourless, almost inactive

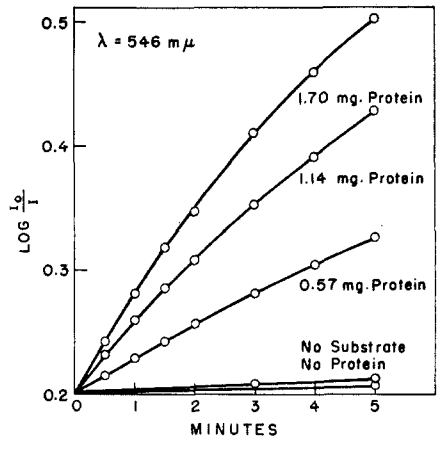

Fig. 14. Optical ethylene reductase test. Phosphate buffer pH 7.1, 140 μM; leucosafranine T, 0.5 μM; S-crotonyl-N-acetyl thioethanolamine, 2.6 μM. Volume, 2.1 ml; d = 0.5 cm; temp. 17°.

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protein can be precipitated from these solutions with ammonium sulfate at pH 3.6 as in the method of WARBURG AND CHRISTIAN[73] for the resolution of the flavoprotein D-amino acid oxidase. The activity of the protein can be restored by the addition of flavin adenine dinucleotide which has been found to be the prosthetic group of the enzyme. Thus, like fumarate reductase, ethylene reductase appears to be a flavoprotein. DPNH or TPNH cannot substitute for the leucodye.

In line with the above observations GREEN and co-workers[20a] have recently reported on the isolation of flavoproteins from ox liver catalyzing the oxidation of butyryl-S-CoA and some higher acyl-S-CoA derivatives in the presence of triphenyltetrazolium as hydrogen acceptor. The prosthetic group appears also to be FAD.

There is thus little doubt that the enzymes of the ethylene reductase class are flavoproteins. The nature of the electron transport system in the cell mediating the transfer of hydrogen from the reduced flavoprotein to molecular oxygen is still unknown. During fatty acid synthesis, hydrogens made available through oxidation of carbohydrate as reduced pyridine nucleotides must be transferred to the ethylene reductase flavoproteins to effect the reduction of the α,β-unsaturated S-acyl-CoA fatty acid derivatives. How such interaction takes place is also unknown.

SUMMARY

The intermediates in the biological breakdown and synthesis of fatty acids are S-acyl derivatives of coenzyme A.

Fatty acid synthesis is accomplished through repetition of a cycle of four consecutive reactions: a. Condensation of two molecules of acetyl CoA to form acetoacetyl CoA and coenzyme A (CoA–SH); b. reduction of acetoacetyl CoA to β-hydroxybutyryl CoA; c. dehydration of β-hydroxybutyryl CoA to crotonyl CoA, and d. reduction of crotonyl CoA to butyryl CoA. A new cycle is started by the reaction of butyryl CoA with another molecule of acetyl CoA, to form β-keto-caproyl CoA + CoA–SH, and so forth. The cycle is repeated eight times until stearyl CoA is formed.

All four reactions of the fatty acid cycle are reversible and fatty acid oxidation, once the fatty acid is activated through conversion to the corresponding S-acyl CoA derivative, proceeds by a reversal of the above sequence.

There are two main mechanisms for activation of fatty acids: (a) By a reaction with ATP and CoA to form S-acyl CoA, adenosine monophosphate and pyrophosphate, and (b) by transfer of CoA from certain acyl CoA compounds such as acetyl CoA or succinyl CoA.

The isolation and identification of some of the key enzymes of fatty acid metabolism is outlined and their mechanism of action discussed.

RÉSUMÉ

Les intermédiaires dans la dégradation et la synthèse biologique des acides gras sont des dérivés S-acylés du coenzyme A.

La synthèse des acides gras est le résultat de la répétition d'un cycle de 4 réactions consécutives: (a) condensation de deux molécules d'acétyl CoA conduisant à l'acétoacétyl CoA et au coenzyme A (CoA–SH), (b) réduction de l'acétoacétyl CoA en β-hydroxybutyryl CoA, (c) déshydratation du β-hydroxybutyryl CoA en crotonyl CoA, et (d) réduction du crotonyl CoA en butyryl CoA. Un nouveau cycle recommence par la réaction du butyryl CoA avec une autre molécule d'acétyl CoA, qui donne le β-cétocaproyl CoA + CoA–SH, et ainsi de suite. Le cycle se répète huit fois jusqu'à la formation du stéaryl CoA.

Les quatre réactions du cycle des acides gras sont réversibles et l'oxydation d'un acide gras, après son activation par transformation en dérivé S-acylé du CoA, suit le chemin inverse de la synthèse.

Il y a deux mécanismes principaux d'activation des acides gras: (a) par une réaction avec ATP et CoA qui donne du S-acyl CoA, de l'adénosine monophosphate et du pyrophosphate et (b) par transfert du CoA de certains acyl CoA, tels que l'acétyl CoA et le succinyl CoA.

L'isolement et l'identification de quelques-uns des enzymes essentiels au métabolisme des acides gras sont esquissés et leur mécanisme d'action discuté.

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ZUSAMMENFASSUNG

Die Zwischenprodukte bei dem biologischen Abbau und bei der Synthese der Fettsäuren sind S-Acylderivate des Coenzyms A.

Die Fettsäuresynthese wird erreicht durch die Wiederholung eines Kreislaufs von 4 aufeinanderfolgenden Reaktionen: a. Der Kondensation von 2 Molekülen Acetyl-CoA zu Acetoacetyl-CoA und Coenzym A (CoA–SH), b. der Reduktion des Acetoacetyl-CoA zu β-Hydroxybutyryl-CoA, c. der Dehydratisierung des β-Hydroxybutyryl-CoA zu Crotonyl-CoA und d. der Reduktion des Crotonyl-CoA zu Butyryl-CoA. Ein neuer Kreislauf wird begonnen mit der Reaktion des Butyryl-CoA mit einem anderen Molekül Acetyl-CoA unter Bildung von β-Ketocaproyl-CoA und CoA–SH, usw. Dieser Kreislauf wird 8 mal wiederholt bis Stearyl-CoA gebildet ist.

Alle 4 Reaktionen des Fettsäurekreislaufs sind reversibel und die Fettsäureoxydation verläuft über die umgekehrten Stufen der obigen Folge, wenn einmal die Fettsäure durch Überführung in das entsprechende S-Acyl-CoA aktiviert ist. Es gibt 2 Hauptmechanismen für die Aktivierung der Fettsäuren: a. Eine Reaktion mit ATP und CoA unter Bildung von S-Acyl-CoA, Adenosin monophosphat und Pyrophosphat, und b. eine Überführung des CoA von gewissen Acyl-CoA-verbindungen wie Acetyl-CoA oder Succinyl-CoA.

Die Isolierung und Identifizierung einiger Schlüsselenzyme des Fettsäurestoffwechsels wird umrissen und der Wirkungsmechanismus besprochen.

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