Rat Hepatic Microsomal Acetoacetyl-CoA Reductase

A β-KETOACYL-CoA REDUCTASE DISTINCT FROM THE LONG CHAIN β-KETOACYL-CoA REDUCTASE COMPONENT OF THE MICROSMAL FATTY ACID CHAIN ELONGATION SYSTEM*

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The present study provides evidence for a new rat liver microsomal enzyme, a short chain β-ketoacyl (acetoacetyl)-CoA reductase, which is separate from the long chain β-ketoacyl-CoA reductase component of the microsomal fatty acid chain elongation system. This microsomal reductase converts acetoacetyl-CoA to β-hydroxybutyryl-CoA at a rate of 70 nmol/min/mg of protein; the enzyme has a specific requirement for NADH and appears to obtain electrons directly from the reduced pyridine nucleotide without the intervention of cytochrome b₅ and its flavoprotein reductase. The apparent Kₐ of the enzyme of the acetoacetyl-CoA was 21 μM and for the cofactor, 18 μM. The pH optimum was broad, ranging from 6.5 to 8.0. The product formed is the L-isomer of β-hydroxybutyryl-CoA. High carbohydrate fat-free diet resulted in a small but significant (35%) increase in microsomal acetoacetyl-CoA reductase activity. The cytosol also contains this enzyme activity, measuring approximately 57% of that found in the microsomes. The mitochondrial activity which is 20–25% higher than the microsomal activity appears to be due to L-β-hydroxyacyl-CoA dehydrogenase which converts acetoacetyl-CoA to L-β-hydroxybutyryl-CoA. The microsomal acetoacetyl-CoA reductase activity was extracted from the microsomal membrane by 0.4 M KCl, resulting in an 8–10-fold purification; in addition, the long chain fatty acid elongation system was unaffected by this extraction procedure. Employing β-hydroxyhexanoyl-CoA as a substrate, evidence is also provided for a separate dehydratase which acts on short chain substrates. Lastly, the liver microsomes had no detectable acetoacetyl-CoA synthetase or acetyl-CoA acetyltransferase activities. Hence, the possible involvement of the rat hepatic microsomal short chain β-ketoacyl-CoA reductase, short chain β-hydroxyacyl-CoA dehydratase, and the previously reported short chain trans-2-enoyl-CoA reductase in the hepatic utilization of acetoacetyl-CoA and in the synthesis of butyryl-CoA for hepatic lipogenesis is discussed.

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The liver is considered to be the major site of production of the ketone bodies, acetoacetate, and β-hydroxybutyrate (1), while peripheral tissues like developing brain (2–6), lactating mammary gland (7), adipose tissue (8), and developing lungs (9) have been shown to readily utilize acetoacetate for energy and lipogenesis. The utilization of acetoacetate in these tissues appears to require activation to its thiol ester, either via cytoplasmic acetoacetyl-CoA synthetase or mitochondrial 3-oxoacid-CoA transference, followed by thiolase cleavage to acetyl-CoA (3, 5–7). In 1971, Stern (10) reported the existence of an acetoacetyl-CoA synthetase in liver cytoplasm and mitochondria, and although its presence in cytoplasm was confirmed (11, 12), it was generally concluded that the enzyme activity was too low to be of physiological importance in providing acetyl-CoA for hepatic lipid synthesis. A reinvestigation (13–15) of the cytoplasmic enzyme activity under optimal conditions revealed 4–14 times higher activity than previously reported (10, 11), which would be sufficient to make a substantial contribution to lipogenesis. Furthermore, in an isolated perfused rat liver study, it was shown that ketone bodies could contribute up to 22% of carbon units incorporated into fatty acids (14).

During our studies on enzymes of rat hepatic microsomal elongation we discovered a new NADPH-specific short chain trans-2-enoyl-CoA reductase which could reduce crotonyl-CoA to butyryl-CoA and trans-2-hexenoyl-CoA to hexanoyl-CoA, respectively (16). Recent kinetic evidence indicated that this short chain reductase was different from the enoyl-CoA reductase which is involved in the hepatic microsomal fatty acid elongation of palmitoyl-CoA to stearoyl-CoA (17). In addition, we also reported the ability of hepatic microsomes to convert acetoacetyl-CoA to butyryl-CoA at a rate of 3–5 nmol/min/mg of protein (16). These results suggested the presence of a microsomal β-ketoreductase, which can reduce acetoacetyl-CoA to β-hydroxybutyryl-CoA, and a dehydratase to convert the latter compound to crotonyl-CoA in addition to the previously reported short chain trans-2-enoyl-CoA reductase.

In the present study, evidence is provided that the rat hepatic microsomal short chain β-ketoacyl-CoA reductase (acetoacetyl-CoA reductase) is distinct from the long chain β-ketoacyl-CoA reductase component of the microsomal fatty acid chain elongation system. Several biochemical properties of the acetoacetyl-CoA reductase, including cofactor specificity, product identification, apparent Vₘₐₓ and Kₐ values for acetoacetyl-CoA and NADH, protein concentration, and pH optimum are presented. In addition, the transfer of reducing equivalents from NADH to the short chain β-ketoacyl-CoA reductase does not require cytochrome b₅ in contrast to the long chain system. High carbohydrate fat-free diet marginally stimulates the short chain ketoreductase activity, while the

1 Throughout the text the name acetoacetyl-CoA reductase is identical to short chain β-ketoacyl-CoA reductase; we do not wish to imply that the reductase is specific for acetoacetyl-CoA, as at the present time we do not know whether other β-keto-CoA derivatives are substrates for the enzyme.
long chain enzyme activity is markedly enhanced. Unlike the long chain β-ketoacyl-CoA reductase, the short chain reductase is extracted from the microsomal membrane by high salt concentration, suggesting surface topography. The presence of a separate dehydratase which can act on short chain substrates is also reported. Furthermore, acetacetyl-CoA reductase activity was also present in the cytosolic and mitochondrial fractions, the latter ostensibly due to the 3-hydroxyacyl-CoA dehydrogenase of the β-oxidation system. Finally, the possible involvement of the rat hepatic microsomal short chain β-ketoacyl-CoA reductase, the short chain β-hydroxyacyl-CoA dehydratase, and the short chain trans-2-enoyl-CoA reductase in the hepatic utilization of acetacetyl-CoA and in the synthesis of butyryl-CoA for hepatic lipogenesis is discussed.

MATERIALS AND METHODS

Chemicals—The following compounds were obtained from Sigma: oxalyl chloride, methyl acetacetate, palmitic acid, anhydrodesodium sulfate, coenzyme A, palmityl-CoA, stearoyl-CoA, acetyl-CoA, malonyl-CoA, β-hydroxybutyryl-CoA, l-a-phosphatidylcholine (dilauryl), pyruvic acid, acetic acid, β-hydroxybutyric acid, crotonic acid, heptanoic acid, enzymatically reduced NADH and NADPH, NAD+, L-3-hydroxyacyl-CoA dehydrogenase, lactic acid dehydrogenase, β-hydroxybutyrate dehydrogenase, and bovine serum albumin. Sodium borohydride, hexanoic acid, α-ketoglutaric acid, and malonic acid were purchased from New England Nuclear.

Isolation of Mitochondria and Microsomes—Male Sprague-Dawley rats, 175-225 g (5-6 weeks old) were given access ad libitum to Purina Rat Chow or high carbohydrate diet ("fat-free test diet," Nutritional Biochemicals), following starvation for 24 h. Forty-eight h after the fat-free diet, the mitochondrial and microsomal fractions was carried out as described earlier (18), with one modification; following the initial 100,000 X g centrifugation the microsomal pellet was washed and resuspended in 20 mM Tris buffer. The protein of all the subcellular fractions was determined by a modification of the method of Lowry et al. (19).

Chemical Synthesis—The procedure described by Stoffel and Fras (20) was used to synthesize 3-hydroxy fatty acids. Briefly, the fatty acid was reacted with oxalyl chloride to obtain the acyl chloride, which is then reacted with sodium borohydride to form the β-hydroxy acid methyl ester. The coenzyme A derivatives were synthesized as described by Fong and Schulz (21). Methyl α-ketoglutarate was purchased from Aldrich, and [1-14C]malonyl-CoA was purchased from New England Nuclear.

Determination of the β-Ketopalmityl-CoA and β-Ketohexanoyl-CoA—The procedure described by Stoffel and Fras (20) was used to synthesize 3-hydroxy fatty acids. Briefly, the fatty acid was reacted with oxalyl chloride to obtain the acyl chloride, which is then reacted with sodium borohydride to yield the β-keto ester. The β-ketopalmityl or hexanoate ester was then reduced by sodium borohydride to form the β-hydroxy ester of the corresponding β-keto acid. The coenzyme A derivatives were synthesized as described by Fong and Schulz (21). β-Hydroxyhexanoyl-CoA was used without further purification, while β-hydroxypalmitoyl-CoA was purified as described by Al-Arif and Bleacher (22).

The procedure used to obtain β-ketopalmityl-CoA is a modification of the procedure of Stoffel et al. (20), as previously described (24). Determination of the Mg2+-Enolate Complex of β-Ketopalmityl-CoA or Acetacetyl-CoA—The assay mixture in the sample cuvette contained 100 mM Tris-HCl, pH 8.3, 100 μM of the enzymatically synthesized β-ketopalmityl-CoA (150 μM, final concentration) or acetacetyl-CoA (200 μM), and 0.2 mg/ml of NADH in a total volume of 2.5 ml; in the reference cuvette, MgCl2 was omitted. Formation of Mg2+-enolate was determined in an Amino DW-2 UV/VIS spectrophotometer in the dual wavelength mode as described earlier (24, 26).

Enzymatic Synthesis—The procedure described by Stoffel and Pruss (20) was used to synthesize β-hydroxy fatty acids. Briefly, the fatty acid was reacted with oxalyl chloride to obtain the acyl chloride, which is then reacted with sodium borohydride to form the β-hydroxy acid methyl ester. The coenzyme A derivatives were synthesized as described by Fong and Schulz (21). β-Hydroxyhexanoyl-CoA was used without further purification, while 6-hydroxypalmitoyl-CoA was purified as described by Al-Arif and Bleacher (22).

Concentration of NADH oxidized/min/mg of protein using extinction coefficient of 0.2 mM-1 cm-1

Product Identification—Verification of the conversion of acetacetyl-CoA to β-hydroxybutyryl-CoA was determined by method of Williams and Mellanby (27). Microsomes were incubated for 7 min with 500 μM acetacetyl-CoA and 500 μM NADH. Following incubation, the reaction was terminated, the contents saponified as described previously (26), and following centrifugation, varying aliquots were used for the assay (27). The enzyme employed in the procedure, β-hydroxybutyrate dehydrogenase, catalyzes only the conversion of the β-isomer of β-hydroxybutyrate to acetocetate.

When the product was tested with L-β-hydroxyacyl-CoA dehydrogenase, microsomes were preincubated as indicated above. The reaction was terminated with HClO4 (5% final concentration), centrifuged to remove the protein, and then neutralized with 1.0 M NaOH. Varying aliquots of the supernatant were added to a reaction mixture containing 5 units of the dehydrogenase and 500 μM NAD+. In some experiments, the L-β-hydroxyacetyl-CoA dehydrogenase was replaced with our purified acetacetyl-CoA reductase preparation.2 β-Hydroxyhexanoyl-CoA Dehydratase Activity (Short Chain β-Hydroxyacyl-CoA Dehydratase)—The short chain dehydratase activity was assayed by measuring the formation of hexanoyl-CoA by gas-liquid chromatography. The assay mixture (total volume of 1.0 ml) contained 100 mM Tris-HCl, pH 7.4, 150 μM β-hydroxyhexanoyl-CoA, and 200 μM NADPH, and the reaction was initiated with 1 mg of microsomal protein. After 1-3 min of incubation at 37° C, the reaction was terminated by the addition of 0.5 ml of 15% metaphionic KOH. Fifty nmoi of heptanoic acid dissolved in ethanol were added and the internal standard, 0.5 μl of 4 n HCl was added, and fatty acids were extracted into 2×2.5 ml of HPLC grade hexane. The pooled hexane phase was evaporated to approximately 25 μl by nitrogen, and a 3-μl aliquot was then analyzed by gas-liquid chromatography as described previously (16, 26).

Conversion of Acetacetyl-CoA to Crotonyl-CoA and Butyryl-CoA—The procedure employed to determine the formation of crotonyl-CoA and butyryl-CoA was previously described (18). Hepatic microsomal conversion of acetacetyl-CoA to crotonyl-CoA was observed only when NADH was used. Replacement of NADH by NADPH resulted

2 M. R. Prasad, L. Cook, R. Vieth, and D. L. Cinti, manuscript in preparation.

3 The abbreviations used are: HPLC, high performance liquid chromatography; FFD, fat-free diet; HMG-CoA, 3-hydroxy-3-methylgly- taryl coenzyme A.
in no product formation, while the presence of both NADH and NADPH led to butyryl-CoA formation.

**Hepatic Microsomal Condensation and Elongation of Palmitoyl-CoA**—The rate of hepatic microsomal condensation and elongation of palmitoyl-CoA was measured as described previously (26). To determine the effects of short chain acyl-CoA on the fatty acid elongation system, 100 μM acetoacetyl-CoA or 100 μM β-hydroxyhexanoyl-CoA was included in the reaction mixture.

**RESULTS**

**Acetocetyl-CoA Reductase Activity**—Recently, our laboratory reported the ability of rat hepatic microsomes to convert acetocetyl-CoA to butyryl-CoA (16). Since this conversion occurred only in the presence of both NADH and NADPH and the reduction of crotonyl-CoA required specifically NADPH, it became evident that the initial reduction of acetocetyl-CoA to β-hydroxybutyryl-CoA required the pyridine nucleotide, NADH. Fig. 1A shows the increased rate of NADH oxidation following the addition of 200 μM acetocetyl-CoA to a suspension of liver microsomes; this represents a rate of approximately 70 nmol of NADH oxidized/min/mg of protein. When the NADH concentration was decreased 5-fold to 10 μM (Fig. 1B), the rate of oxidation was markedly reduced. When NADH was replaced by NADPH, acetocetyl-CoA did not stimulate the endogenous NADPH oxidation rate (Fig. 1C) indicating that the rate of reduction of acetocetyl-CoA is NADH specific. The microsomal acetocetyl-CoA reductase activity could only be measured in the presence of the coenzyme A derivative; for example, free acetoacetic acid did not stimulate the microsomal oxidation of NADH. Furthermore, neither acetyl-CoA alone nor in the presence of malonyl-CoA stimulated NADH oxidation (Fig. 1D) suggesting that the microsomes do not have the enzymatic machinery to generate acetocetyl-CoA via the condensation reaction.

As seen in Fig. 2, acetocetyl-CoA reduction is linear up to almost 1 mg/ml of microsomal protein. A plot of the initial velocity of the reaction versus the substrate concentration resulted in a hyperbolic curve which gave an apparent $K_m$ of 21 μM and apparent $V_{max}$ of 74 nmol/min/mg of protein (Fig. 3, inset). The acetocetyl-CoA reductase activity appeared to reach maximum at a NADH concentration of 50 μM (Fig. 4). The apparent $K_m$ for the NADH as determined from a Lineeweaver-Burk plot was 18 μM, while the apparent $V_{max}$ was 80 nmol/min/mg. The pH optimum for the reductase activity was very broad, with little change in activity between pH 6.5 and 8.0; significant loss of activity occurred below pH 6.0 and above pH 8.5.

**Identification of Reaction Products of Microsomal Metabolism of Acetocetyl-CoA**—Scheme 1 illustrates the hepatic microsomal pathway for the conversion of acetocetyl-CoA to butyryl-CoA. To determine that the NADH oxidation represented a conversion of acetocetyl-CoA to β-hydroxybutyryl-CoA, hepatic microsomes were incubated with acetocetyl-CoA and NADH for 10 min. Following saponification to release the β-hydroxybutyric acid from the CoA, an aliquot of the supernatant was added to a mixture containing NAD+ and β-hydroxybutyrate dehydrogenase (28). In this reaction, β-hydroxybutyrate (not the CoA form) is converted by the dehydrogenase to acetocetic acid with the formation of NADH. This is precisely what we observed with our supernatant. Furthermore, increasing the amount of supernatant to the reaction mixture resulted in a proportionate increase in NADH formation. However, it should be pointed out the stoichiometric quantities of NAD+ and β-hydroxybutyrate (1:1) cannot be obtained at this time because a portion of the
generated $\beta$-hydroxybutyryl-CoA is quickly converted to crotonyl-CoA. Our laboratory is presently working on an HPLC assay which will be used to quantitate acetoacetate, $\beta$-hydroxybutyrate, and crotonate. Preliminary studies with HPLC show an absorbance peak (210 nm) with a retention time of 31.7 min, which is identical to the retention time obtained with a pure sample of crotonic acid.\(^2\) Hence, although we cannot quantitate the crotonate peak at this time because of base-line noise, the peak is not present in the zero time samples; however, the peak is observed in those preparations containing only NADH and in those samples containing NADH plus NADPH. We have already reported (16) the microsomal conversion of crotonyl-CoA to butyryl-CoA by the trans-2-enoyl-CoA reductase in the presence of NADPH.

It should be emphasized that stoichiometric measurements of the product of the first reduction reaction must await not only a sensitive assay procedure but also a specific inhibitor of the dehydratase or purification of the $\beta$-keto (acetoacetyl-CoA) reductase.

The above results suggest that the microsomes convert acetoacetyl-CoA to the $\alpha$-isomer of $\beta$-hydroxybutyryl-CoA, since $\beta$-hydroxybutyrate dehydrogenase is stereospecific for $\beta$-hydroxybutyrate. This conclusion is supported by the experiment in which L-$\beta$-hydroxyacyl-CoA dehydrogenase added to the neutralized microsomal supernatant, obtained following incubation with NADH and acetoacetyl-CoA (see "Materials and Methods"), resulted in no NADH formation. However, the addition of our partially purified reductase preparation did result in the reduction of NAD$^+$, suggesting the presence of the $\beta$-isomer in the microsomal supernatant.

Are Acetoacetyl-CoA Reduction and $\beta$-Ketopalmitoyl-CoA Reduction Catalyzed by the Same Enzyme?—The first indication that two separate $\beta$-ketoacyl-CoA reductases existed in rat hepatic microsomes was the observation that short chain $\beta$-ketoacyl-CoAs such as acetoacetyl-CoA (Fig. 1) undergo reduction only in the presence of NADH, whereas total fatty acid elongation is supported by either NADPH or NADH, with NADPH being the more effective cofactor (29). Additional evidence for two separate $\beta$-ketoacyl-CoA reductases was obtained by examining the involvement of cytochrome $b_5$ in the reduction of acetoacetyl-CoA and $\beta$-ketopalmitoyl-CoA. We have recently reported (24) that cytochrome $b_5$ participates only in the first reduction step of fatty acid chain elongation. As shown in Fig. 5, although $\beta$-ketopalmitoyl-CoA was capable of stimulating the rate of reoxidation of cytochrome $b_5$, acetoacetyl-CoA had no effect on the reoxidation rate. The stimulation of the rate of reoxidation of cytochrome $b_5$ by $\beta$-ketopalmitoyl-CoA also occurred under anaerobic conditions and in the presence of 1 mM KCN indicating that the results cannot be explained by stimulation of the desaturase pathway (24). Thus, unlike the long chain $\beta$-ketoacyl-CoA reductase, acetoacetyl-CoA reductase can accept reducing equivalents directly from NADH without the intervention of the cytochrome $b_5$ system.

To further establish two separate enzymes, we examined the effect of acetoacetyl-CoA on the NADH-dependent microsomal elongation of palmitoyl-CoA. In the presence of microsomes, palmitoyl-CoA undergoes condensation with malonyl-CoA to yield $\beta$-ketostearoyl-CoA. This intermediate is then converted to $\beta$-hydroxystearoyl-CoA by the microsomal $\beta$-ketoacyl-CoA reductase in the presence of NADH;
ultimately stearoyl-CoA would be synthesized. If the \( \beta \)-ketocoyl-CoA reductase component of the chain elongation system and the acetoacetyl-CoA reductase were identical enzymes, the addition of acetoacetyl-CoA to an assay mixture containing microsomes, palmitoyl-CoA, malonyl-CoA, and NADH should result in competitive inhibition of elongation of palmitoyl-CoA. As shown in Table I acetoacetyl-CoA, at a concentration 7-fold greater than that of palmitoyl-CoA, did not inhibit the elongation of palmitoyl-CoA (Table I). However, under these conditions, \( \beta \)-hydroxyhexanoyl-CoA did not inhibit the elongation of palmitoyl-CoA.

**TABLE I**

Effect of acetoacetyl-CoA and \( \beta \)-hydroxyhexanoyl-CoA on microsomal condensation and NADH-dependent elongation of palmitoyl-CoA.

| Addition                  | Condensation | Elongation |
|---------------------------|--------------|------------|
| None                      | 0.58         | 1.12       |
| Acetoacetyl-CoA (100 \( \mu \)M) | 0.61         | 1.02       |
| \( \beta \)-Hydroxyhexanoyl-CoA (100 \( \mu \)M) | 0.56         | 1.20       |

The assay mixture (1.0 ml total volume) contained 0.2 \( \mu \)M \[^{14}C\]palmitoyl-CoA, 15 \( \mu \)M palmitoyl-CoA, 100 \( \mu \)M Tris, pH 7.4, and when present, either 100 \( \mu \)M acetoacetyl-CoA or 30 \( \mu \)M \( \beta \)-hydroxyhexanoyl-CoA; the reaction was initiated with microsomes (1 mg/ml of microsomal protein) from rats on a fat-free diet, incubated at 37 °C for 3 and 5 min, and terminated with 0.5 ml of 15% KOH/methanol. Condensation and elongation were determined as described under *Materials and Methods*.

**TABLE II**

| Group | Acetoacetyl-CoA reductase activity | Long chain \( \beta \)-ketocoyl-CoA reductase activity |
|-------|-----------------------------------|-----------------------------------------------|
|       | nmol NADH oxidized/ min/mg protein | nmol cytochrome b intact/ min/mg protein       |
| Control | 71.3 ± 10.9*                      | 0.61 ± 0.07                                 |
| Fat-free diet | 97.0 ± 13.7* (35%) | 1.63 ± 0.30 (240%) |

* Both control and fat-free diet animals were initially starved for 24 h followed by refeding for 48 h and put to death; control rat group received Purina rat chow.

**Effect of High Carbohydrate FFD**—Rats were starved for 24 h followed by refeding for 48 h, either normal rat chow or the fat-free diet. As seen in Table I, the acetoacetyl-CoA reductase activity in liver microsomes obtained from rats on the FFD was increased from 71.3 to 97.1 nmol/min/mg of microsomal protein or a 35% stimulation, whereas the long chain \( \beta \)-ketocoyl-CoA reductase activity was markedly stimulated (240%) by the FFD, when \( \beta \)-ketocoyl-CoA was employed as a substrate. Apparently the two reductases respond differently to the high carbohydrate diet; whether longer term feeding of FFD markedly stimulates the acetoacetyl-CoA reductase is unknown at the present time.

**Acetoacetyl-CoA-stimulated NADH Oxidation in Rat Liver Cytosolic and Mitochondrial Fractions**—We have recently reported (16) that cytosol contamination of the microsomes was less than 1% based on two different cytosol marker enzymes, lactate dehydrogenase and malate dehydrogenase. Under these same isolation conditions, we compared the acetoacetyl-
CoA reduction by NADH in microsomes versus cytosol. To our surprise, the liver cytosol contained significant amounts of reductase activity, 43.3 ± 8.8 nmol of NADH oxidized per min per mg of cytosolic protein obtained from three different cytosolic fractions. Although the cytosolic activity was high, the microsomal fraction obtained from the same three animals averaged 76 nmol/min/mg of microsomal protein or 75% higher activity in the microsomal fraction than in the cytosolic fraction. Interestingly, the acetoacetyl-CoA reductase activity in the cytosol was not enhanced by the 2-day FFD. In fact, the activity was slightly decreased to 33.4 ± 4.3 nmol/min/mg of cytosolic protein, a reduction of 23%. Certainly, cytosolic contamination could not account for the acetoacetyl-CoA reductase activity observed in the microsomal fraction; rather it appears that the enzymatic activity exists in both fractions.

The mitochondrial fraction was also active in the reduction of acetoacetyl-CoA. Under the same conditions as for the microsomal fraction, the addition of 200 μM acetoacetyl-CoA to the mitochondrial fraction (from untreated rats) resulted in a NADH oxidation rate of 88 ± 11 nmol/min/mg of mitochondrial protein for two different mitochondrial preparations measured in triplicate, a rate that is 24% higher than that observed in the microsomes. Since the mitochondrial possessed slightly higher activity than the microsomes, the extent of contamination of the latter fraction by the mitochondria was determined by measuring succinate cytochrome c reductase and glutamate dehydrogenase activities (30), mitochondrial markers, in both fractions. While these activities were 295 and 116 nmol/min/mg of protein, respectively, in the mitochondria, the microsomal activities were 5.0 and 4.6 nmol/min/mg of protein, indicating a 1.7 to 4.0% contamination. Hence, a 4% contamination would contribute a total of only 3.5 nmol/min/mg of protein to the microsomal activity. The mitochondrial activity is most likely attributed to the β-oxidation enzyme, L-β-hydroxyacyl-CoA dehydrogenase, which in the presence of NADH can convert acetoacetyl-CoA to β-hydroxybutyryl-CoA. However, this enzyme generates only the β-isomer. As noted earlier, the product of the microsomal reaction is the D-isomer.

**Extraction of Acetoacetyl-CoA Reductase Activity from Rat Liver Microsomes**—In our attempt to isolate the short chain β-ketoacyl-CoA reductase from the microsomes, we observed that high concentrations of KCl were capable of stripping a significant portion of the enzyme activity from the microsomes. As shown in Table III, homogenization of microsomes in 0.4 M KCl resulted in significant removal (82 and 84%) of acetoacetyl-CoA reductase activity from liver microsomes from both untreated and FFD animals, respectively. This initial isolation step has resulted in a 8- to 10-fold purification. Interestingly, increasing the KCl concentration to 0.6 M did not remove the additional 10 to 12% residual reductase activity. However, reducing the KCl concentration below 0.4 M resulted in a proportionate decrease in reductase activity extracted from the microsomes. It was also observed that under conditions where we extracted 84% of the acetoacetyl-CoA reductase activity, there was absolutely no effect on the microsomal fatty acid chain elongation of palmitoyl-CoA (Table III). This activity was measured in the FFD animals because this diet induces chain elongation activity. These results also support the existence of at least two β-ketoacyl-CoA reductases.

**Attempts to Detect Acetoacetyl-CoA Synthetase and Acetyl-CoA Acetyltransferase (Thiolase) Activities in Hepatic Microsomes**—Cytosolic acetoacetyl-CoA synthetase is one of the key enzymes in the utilization of ketone bodies in lipogenesis (3, 5–7). Having discovered the ability of microsomes to convert acetoacetyl-CoA to butyryl-CoA, the site of acetoacetyl-CoA synthetase becomes an obvious question. Of the several pathways that can synthesize acetoacetyl-CoA (15), we examined liver microsomes for the presence of two activities, i.e. acetoacetyl-CoA synthetase and acetyl-CoA acetyltransferase (thiolase). The detection or measurement of acetoacetyl-CoA was followed by the absorption of its Mg⁺⁺-enolate complex which occurs at 303 nm. The assay mixture contained 100 μM acetoacetic acid, 100 μM CoASH, 500 μM ATP, 0.1 M Tris-HCl, pH 8.2, 50 mM KCl, 1 mg/ml of microsomal protein, and 50 mM MgCl₂. The spectrum from 250 to 350 nm was continuously recorded from 0 to 10 min. Under these conditions, there was no peak formation at 303 nm. However, when microsomes were replaced by the cytosolic fraction, a significant peak was formed at 303 nm, which decreased with the time of incubation. This disappearance of the 303-nm peak was most likely due to the thiolase or hydroxylase activity reported to be present in the cytosol (15).

Similarly, the presence of acetyl-CoA acetyltransferase activity in microsomes was examined by incubating 20 or 50 μM acetyl-CoA, 0.1 M Tris-HCl buffer, pH 8.2, 50 mM MgCl₂, and 1 mg/ml of microsomal protein in a total volume of 2.5 ml. Again, under these conditions no formation of Mg⁺⁺-enolate complex occurred. As a control, 1 mg of microsomal or cytosolic protein was added to a Mg⁺⁺-enolate complex of acetoacetyl-CoA. In 12 min, only 40% of the complex decreased in the presence of microsomal protein, whereas within 3 min the Mg⁺⁺-enolate complex disappeared in the presence of cytosolic fraction. These results indicate that the inability to observe a Mg⁺⁺-enolate complex with the microsomal fraction is not due to the presence of a very active hydroxylase or thiolase activity in microsomes, but rather due to an absence in microsomes of acetoacetyl-CoA synthetase and acetyl-CoA acetyltransferase activities. It should be noted that the method employed was capable of detecting as little as 1.0 μM acetoacetyl-CoA.

**DISCUSSION**

The present study provides evidence for the existence of a new rat hepatic microsomal enzyme which catalyzes the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA in the presence of the cofactor NADH. This enzyme has been designated short chain β-ketoacyl-CoA reductase or specifically acetoacetyl-CoA reductase. It differs from the well known long chain β-ketoacyl-CoA reductase component of the fatty acid chain elongation system in several ways: 1) the acetoacetyl-CoA reductase has a specific requirement for NADH whereas the long chain reductase utilizes either NADPH or NADH; 2) the acetoacetyl-CoA reductase obtains its electrons directly from NADH without the intervention of cytochrome b₅₆₇ and its flavoprotein reductase, while the long chain keto-reductase receives its electrons from cytochrome b₅ (24); 3) high salt (KCl) concentrations can extract the acetoacetyl-CoA reductase from the microsomal membrane whereas the long chain enzyme is unaffected by such concentrations; and 4) acetoacetyl-CoA, the substrate for the short chain enzyme, had no effect on the microsomal chain elongation of palmitoyl-CoA.

The microsomal enzymatic activity in the presence of acetoacetyl-CoA is quite high, approximately 70 nmol/min/mg of protein; the apparent Kₘ for the substrate was 21 μM, and for the cofactor, 18 μM. Product identification using β-hydroxybutyrate dehydrogenase, L-β-hydroxyacyl-CoA dehydrogenase, and partially purified reductase indicated that acetoacetyl-CoA was reduced to the β-hydroxybutyryl-CoA; indeed, the CoA derivative rather than the free
Acetoacetate, another enzyme, the thiolase, forms 2 acetyl-CoA molecules and the synthase generates HMG-CoA. A fourth enzymatic activity must now be added to this group: the NADH-specific short chain \( \beta \)-ketoacyl-CoA (acetoacetyl)CoA reductase. Three of the enzymes are found in the mitochondrial and cytosolic fractions; the thiolase and synthetase are predominant in mitochondria and the hydrolase is predominant in cytosol. The newly discovered reductase activities are present in the microsomal and cytosolic fractions.

The hepatic synthesis of acetoacetyl-CoA is most probably derived from two sources, the mitochondrial \( \beta \)-oxidation of long chain fatty acids and the cytosolic conversion of acetoacetate to acetoacetyl-CoA in the presence of ATP (10, 11, 13, 14).

The question which must be addressed and to which an answer must be sought is: what is the physiological role of the microsomal short chain \( \beta \)-ketoacyl-CoA reductase? The enzyme appears to be loosely bound to the membrane surface, based upon the ease with which the enzyme can be stripped from the microsomal membrane without the intervention of detergents. This is a reasonable location for the enzyme since the substrate is hydrophilic. Since the cytosol already contains three acetoacetyl-CoA-utilizing enzymes (hydrolase, thiolase, and HMG-CoA synthase) all of which have significant activities (0.41, 6.78, and 0.11 \( \mu \)mol/min/g of liver, wet weight, respectively) what is the need for an additional acetoacetyl-CoA-metabolizing enzyme which appears to be found in both microsomes and cytosol? Even if the rat liver cytosolic acetoacetyl-CoA synthetase (13) were to function optimally, the rate of generation of acetoacetyl-CoA would only approximate 0.3–0.5 \( \mu \)mol/min/g, wet weight. This represents just 10% of the rate of the microsomal NADH acetoacetyl-CoA reductase activity. Aragon and Lowenstein (15) have calculated that livers of fed rats contain a concentration of acetoacetyl-CoA that is in the low nanomolar range; however, Menahan et al. (33) have estimated the concentration to be 1–10 \( \mu \)M. These concentrations are well below the apparent \( K_m \) of the substrate for any of the aforementioned enzymes, for example, an apparent \( K_m \) of 33 \( \mu \)M for the thiolase (34), 25 \( \mu \)M for the hydrolase (15), and 21 \( \mu \)M for the microsomal \( \beta \)-ketoreductase. Furthermore, there appear to be at least two forms of cytosolic

### Table III

| Preparation | Acetoacetyl-CoA reductase activity* | Reductase activity extracted | Total elongation activity* |
|-------------|-----------------------------------|-----------------------------|---------------------------|
|             | nmol/min/mg protein               | %                           | nmol/min/mg protein       |
| I. Untreated rat |                                   |                             |                           |
| A. Control microsomes | 71.3 ± 10.9                     | ND                          |                           |
| B. Microsomes after extraction with 0.4 M KCl | 19.3 ± 1.49                  | 81.5                        |                           |
| C. 100,000 \( \times \) g supernatant obtained after microsomes were treated with 0.4 M KCl | 786.7 ± 74.2                 |                             |                           |
| II. FFD rat |                                   |                             |                           |
| A. Control microsomes | 97.0 ± 13.7                     | 1.359 ± 0.268              |                           |
| B. Microsomes after extraction with 0.4 M KCl | 15.4 ± 5.14                    | 84.1                        | 2.97 ± 0.157              |
| C. 100,000 \( \times \) g supernatant obtained after microsomes were treated with 0.4 M KCl | 780.4 ± 113.0                 |                             |                           |

* Assay mixture contained 0.1 M Tris, pH 7.4, 5 \( \mu \)M rotenone, 200 \( \mu \)M acetoacetyl-CoA, 50 \( \mu \)M NADH, and 1 mg/ml of microsomal protein in a total volume of 2.5 ml. The reaction was run at 36.5 °C.

Fatty acid elongation was measured using palmitoyl-CoA as substrate, as described previously (26).

Values represent means ± S.D. obtained from 3 animals per group.

ND, not determined.
HMG-CoA synthase and both forms have apparent $K_m$ values less than 3 $\mu M$ (35). With such a low $K_m$, one would expect rapid conversion of any generated acetoacetyl-CoA to HMG-CoA.

All of the values, of course, have been obtained from in vitro conditions; the in vivo situation may be quite different, however. Now regulatory factors, such as hormones, binding proteins, inhibitory products, or intermediates may markedly influence the enzyme activities. Indeed sufficient acetoacetyl-CoA must be formed since Endemann et al. (14) have shown that ketone bodies are incorporated into sterols as well as fatty acids to a significant degree.

Notwithstanding the aforementioned results, one of the functions of the microsomal $\beta$-ketoreductase in concert with the dehydratase and enoyl-CoA reductase may be to provide butyryl-CoA for lipogenesis. Lin and Kumar (36) have shown that both rat mammary gland and liver do prefer butyryl-CoA over acetyl-CoA as the primer in fatty acid synthesis. In 1969, Nandekar and Kumar (37) reported the presence in the cytosol of lactating rabbit mammary gland, a NADH-dependent $\beta$-ketoadipin CoA reductase, in addition to a cytosolic thiolase and enoyl-CoA hydratase. They found that these three enzymes plus fatty acid synthetase, which provided the acetyl-CoA reductase activity associated with it, synthesized butyryl-CoA from acetyl-CoA. Lin and Kumar (36) demonstrated that the rate of incorporation of butyryl-CoA, as primer, during fatty acid synthesis in rat liver is 1.26 nmol/min/mg of protein. We have previously (16) reported that microsomes generated 3.0 nmol of butyryl-CoA/min/mg of microsomal protein and more recently, 4–5 nmol/min/mg of protein. If liver can synthesize 8–14 $\mu$mol of palmitate/g of tissue (38), then 8–14 $\mu$mol of primer is necessary; our microsomal system can provide approximately 17 $\mu$mol of butyryl-CoA precursor/h of liver. Hence, our microsomal system may play a role in providing the liver with butyryl-CoA for lipogenesis.

At this time it is difficult to assess the link between hepatic ketone body formation, cytosolic synthesis of acetoacetyl-CoA and $\beta$-hydroxybutyryl-CoA, and microsomal reduction and dehydration of these coenzyme A forms. During abnormal intermediary metabolism as a result of a pathophysiological condition, such as diabetes, in which there can arise excess production of ketone bodies, can this newly discovered microsomal system participate in the utilization of the ketone bodies? Is this enzyme system inducible in such pathophysiological conditions as diabetes? Additional work is necessary before providing answers to these questions. If a connection exists between ketone body utilization and the microsomal reduction system, the cytosol must also be involved since it is this site where the ketone bodies are activated. We observed no acetoacetyl-CoA synthetase or acetyl-CoA acetyltransferase activities in the liver microsomal fraction.

The reason for the $\beta$-ketoaoyl-CoA reductase activity in the cytosol is unknown presently. We observed that contrary to the microsomal enzyme, the cytosolic activity was not inducible by the fat-free diet; in fact, there was a small reduction in reductase activity in the cytosol. Since the cytosol was capable of generating only 0.6 nmol of butyryl-CoA/min/mg of protein (16), a role for the enzyme in providing this primer is questionable.

Finally, our results with $\beta$-hydroxyhexanoyl-CoA, in which this substrate was dehydrated and converted to hexanoyl-CoA by the microsomes in the presence of NADPH and in which it did not inhibit chain elongation of palmitoyl-CoA strongly suggest that at least two dehydratases exist in the endoplasmic reticulum, the short chain enzyme, and the well known component of the chain elongation system.

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