Stereochemistry of the Reactions Catalyzed by Chicken Liver Fatty Acid Synthase†

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ABSTRACT: The stereochemistry of the four partial reactions catalyzed by chicken liver fatty acid synthase that lead to the synthesis of palmitic acid has been determined. The reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA by NADPH proceeds with the transfer of the *pro-4S* hydrogen of NADPH to form D-3-hydroxybutyryl-CoA. During the subsequent dehydration of D-3-hydroxybutyryl-CoA the *pro-2S* hydrogen and the 3-hydroxyl group are removed in a syn elimination to form crotonyl-CoA. Crotonyl-CoA is reduced to butyryl-CoA by NADPH, with the transfer of the *pro-4R* hydrogen of NADPH to the *pro-3R* position in butyryl-CoA and the transfer of a solvent hydrogen to the *pro-2S* position. The occurrence of the syn dehydration, when combined with the results of a previous study [Sedgwick, B., & Cornforth, J. W. (1977) *Eur. J. Biochem. 75*, 465–479], implies that the condensation of the enzyme-bound malonyl moiety with the enzyme-bound saturated fatty acid to form a 3-keto intermediate proceeds with inversion at C-2 of the malonyl. The stereochemistry of the hydration was derived from an analysis of the spin–spin coupling constant of 3-hydroxy[2-^{2}H]butyric acid benzylamides obtained from 3-hydroxy[2-^{2}H]butyryl-CoA synthesized by fatty acid synthase. The elucidation of the stereochemistry of the reduction of crotonyl-CoA relied on the previously established stereochemistry of pork liver acyl-CoA dehydrogenase. The source of all 28 prochiral hydrogens of the palmitic acid synthesized by chicken liver fatty acid synthase was inferred from the results of this work.

Fatty acid synthesis catalyzed by animal fatty acid synthases proceeds by condensing an enzyme-bound malonyl moiety to an enzyme-bound growing fatty acid chain to form a C-3 keto intermediate. This intermediate is reduced by NADPH to a C-3 hydroxy intermediate. The hydroxy intermediate is dehydrated to form a carbon–carbon double bond that is reduced by NADPH to give a saturated fatty acid chain that has been elongated by two carbons. This cycle is repeated 7 times to produce palmitate. Each of these four partial reactions involves bond formation at one or two (pro)chiral centers on the fatty acid. Fatty acid synthases also catalyze the reductive partial reactions and the dehydration partial reaction with the appropriate acyl-CoA[1] derivatives replacing the enzyme-bound intermediates (cf. Kumar et al., 1970).

In this study, the stereochemistry of the two reductive reactions, including the stereospecificity with respect to NADPH, and the dehydration reaction are reported for chicken liver fatty acid synthase. The results obtained can be combined with those of Sedgwick & Cornforth (1977) to deduce the stereochemistry of the condensation reaction. Determination of the stereospecificity of the four partial reactions allows the stereochemistry of palmitate synthesis to be inferred, as well as the source of all 28 prochiral hydrogens.

Materials and Methods

Enzymes. Fatty acid synthase was prepared according to Cognet & Hammes (1983) and had a specific activity of 1.5–1.6 units/mg under standard conditions (Cardon & Hammes, 1982). The glycerol and dithiothreitol contained in the storage buffer were removed by passing the enzyme through a 3-mL Sephadex G-50 centrifuge column (Penefsky, 1977), equilibrated with 0.1 M potassium phosphate, pH 7.0; for reactions performed in D_2O the column was preequilibrated with 20 mL of 0.1 M potassium phosphate, pD 7.4. [The pD is the pH, as measured with a glass electrode, plus 0.4 (Glasoe & Long, 1960).] Acyl-CoA dehydrogenase from pork liver was prepared according to Thorpe (1981), except the calcium phosphate and gel filtration columns were omitted. The enzyme had a specific activity of 1.15 units/mg in 0.05 M potassium phosphate, pH 7.6, at 25 °C with butyryl-CoA (30 μM) as the substrate, a 280:450 nm absorbance ratio of 11, and no detectable thioesterase activity when 0.1 unit was incubated with 1 mM butyryl-CoA for 24 h in the assay buffer.

Chemicals. ATP, $NADP^+$, NADPH, acetyl-CoA, malonyl-CoA, crotonyl-CoA, acetoacetyl-CoA, butyryl-CoA, *N*-acetyl-*S*-acetoacetylcysteamine, hexokinase (type V), L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxybutyrate dehydrogenase (type IV), and glucose-6-phosphate dehydrogenase from *L. mesenteroides* (type XXI) were from Sigma. D-[1-^{3}H]Glucose, 4 Ci/mmol, was from ICN. Benzylamine from Aldrich was redistilled before use. All other chemicals were high purity commercial grades, and distilled deionized water was used to prepare all solutions.

(S)-[4-^{3}H]NADPH. Five nanomoles of D-[1-^{3}H]glucose (4 Ci/mmol) was incubated with 5 units of hexokinase in 0.5 mL of 100 mM potassium phosphate, pH 8.0, containing 50 μM ATP, 2 mM $MgCl_2$, and 100 μM $NADP^+$. After 10 min 0.2 unit of glucose-6-phosphate dehydrogenase was added, and the reaction was followed to completion by measurement of the absorbance at 340 nm. The reaction was allowed to continue for at least 30 half-times since a significant primary isotope effect exists (Hermes et al., 1982). The reaction was quenched by vortexing with 2 drops of chloroform for 1 min, and the (*S*)-[4-^{3}H]NADPH was isolated by chromatograhy on DEAE-Sephacel as described below. The final product had a specific activity of 1100 cpm/pmol.

[4-^{3}H]NADP$^+$. The pooled *S*-[4-^{3}H]NADPH solutions were made 5 μM in acetyl-CoA and 10 μM in malonyl-CoA, and 1×10^{-2} unit of fatty acid synthase was added. When the NADPH fluorescence (340 nm excitation, 465 nm emission) had disappeared, the reaction mixture was titrated to pH 5.0 with acetic acid and extracted 3 times with chloroform

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[1] Abbreviations: CoA, coenzyme A; Tris, tris(hydroxymethyl)-aminomethane.

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to remove the radioactive fatty acids. The aqueous phase was titrated back to pH 7.0 with 1.0 N KOH, diluted to 20 mM potassium phosphate (ca. 130 mL total volume), and passed through a 1.5-mL DEAE-Sephacel column. Under these conditions the CoA thio esters and NADPH are retained, while the [4-^{3}H]NADP$^+$ is recovered in the eluate.

(R)-[4-^{3}H]NADPH. Glucose 6-phosphate (50 μM final concentration) and 0.2 unit of glucose-6-phosphate dehydrogenase were added to 50 mL of the eluate containing [4-^{3}H]NADP$^+$. After 30 min, the reaction was quenched by vortexing 10-mL aliquots with 0.2 mL of chloroform, and the (*R*)-[4-^{3}H]NADPH was isolated by DEAE-chromatography as described below. The (*R*)-[4-^{3}H]NADPH had a specific activity of 640 cpm/pmol.

3-Hydroxy[2,2-$^2H_2$]butyryl-CoA. Five micromoles of acetoacetyl-CoA, 200 μmol of K_2HPO_4, and 100 μmol of KH_2PO_4 were dissolved in 2 mL of D_2O (99.8%, Aldrich), lyophilized, redissolved in 3 mL of D_2O, and allowed to stand overnight. Ten micromoles of glucose 6-phosphate was dissolved in 0.5 mL of D_2O, lyophilized, and redissolved in 0.5 mL of D_2O. One unit of fatty acid synthase in D_2O, 0.2 unit of glucose-6-phosphate dehydrogenase, and 0.6 μmol of NADPH were added to the acetoacetyl-CoA solution, and five successive additions of 50 μL of the glucose 6-phosphate solution were made each time the absorbance at 340 nm decreased to 0.5. Following the last addition of glucose 6-phosphate, the absorbance had not decreased from the maximum reading (1.15) after 1 h. The reaction was quenched by vortexing with 2 drops of chloroform, and the CoA thio esters were isolated by DEAE chromatography. The pooled CoA thio ester fractions (30 mL) had a concentation of 0.1 mM as determined by absorbance measurements. The free thiol concentration was less than 5 μM based on the lack of reaction with 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M Tris, pH 8.5, at 25 °C (Ellman, 1959). After base hydrolysis (0.5 N NaOH for 30 min at 25 °C) the free thiol concentration was 110 μM, and the D-3-hydroxybutyrate concentration determined with D-3-hydroxybutyrate dehydrogenase (Delafield & Duodoroff, 1969) was 81 μM. Nuclear magnetic resonance analysis of the 3-hydroxybutyric acid benzylamide (see below) indicated better than 90% deuterium incorporation at C-2.

Separation of NADP$^+$, CoA Thio Esters, and NADPH. These products were isolated from enzymic reaction mixtures by diluting the phosphate buffer to less than 10 mM and loading the reaction mixture on a 1.5-mL DEAE-Sephacel column preequilibrated at 4 °C with 15 mM potassium phosphate, pH 7.0, in a 3-mL syringe. The column was eluted stepwise with 60 mL each 30, 55, and 130 mM potassium phosphate, pH 7.0. The flow rate was maintained at about 2 mL/min, and fractions were collected every 5 min. As shown in Figure 1, NADP$^+$ was eluted by 30 mM potassium phosphate, CoA thio esters (except malonyl-CoA) were eluted by 55 mM potassium phosphate, and NADPH was eluted by 130 mM potassium phosphate. The NADP$^+$ was identified by its reduction to NADPH in the presence of 0.1 mM glucose 6-phosphate and glucose-6-phosphate dehydrogenase in 30 mM potassium phosphate, pH 7.0, at 25 °C. The CoA esters were quantitated by titrating free thiols with 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.5 M Tris, pH 8.0, at 25 °C (Ellman, 1959) after base hydrolysis (0.5 M NaOH for 30 min). The NADPH was determined by its absorbance at 340 nm. No detectable overlap of NADP$^+$ and acetyl-CoA was observed in the eluant, but a small overlap of acetyl-CoA with NADPH occurred which did not affect any of the quantitative results since the NADPH was completely oxidized in the experiments where quantitation was imporatant.

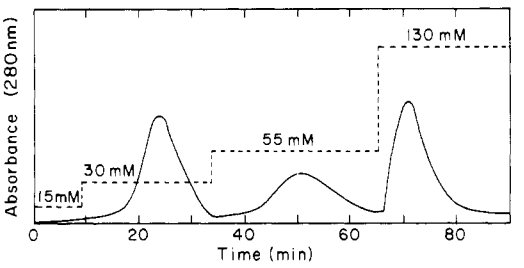

FIGURE 1: Elution pattern for the separation of 0.5 mg each of NADP$^+$ (peak 1), acetyl-CoA (peak 2), and NADPH (peak 3) on a 1.5-mL DEAE-Sephacel column at 4 °C. The absorbance at 280 nm (scale not specified) vs. the time is shown. The flow rate was ~2 mL/min, and concentrations of 15, 30, 55, and 130 mM potassium phosphate (pH 7.0) were used as the eluting buffer in step gradients as indicated by the dashed line.

Preparation and Purification of Benzylamides from CoA Thio Esters. In order to analyze the isotopic composition of the acyl portion of the CoA thio esters, they were converted to their respective benzylamides. This procedure has several advantages relative to base hydrolysis: the mild reaction conditions preclude any possible proton exchange at C-2 (Lienhard & Wang, 1968) or hydration of crotonyl-CoA to 3-hydroxybutyrate (Sedgwick & Morris, 1980); the aromatic absorption permits facile chromatographic detection and quantitation; the benzylamides may be easily separated by reverse-phase high-performance liquid chromatography; the benzyl protons provide a convenient integration standard for the nuclear magnetic resonance spectra. The CoA thio esters obtained from the DEAE-Sephacel chromatography were concentrated about 10-fold to a potassium phosphate concentration of 0.55 M, pH 7.0. The resulting solution was titrated to pH 10.3 with redistilled benzylamine. After 2.5 h at room temperature, the reaction mixture was titrated to pH 6.5 with 1 M H_3PO_4. The aqueous solution was extracted with 3 equal volumes of spectral grade chloroform. The pooled chloroform extracts were extracted once with one-fourth the volume of 0.1 M potassium phosphate, pH 7.0, and 3 times with a similar volume of water. The chloroform phase was dried over $MgSO_4$, filtered through a glass wool plug, and dried under a stream of dry nitrogen. The residue was dissolved in a minimal volume (~0.3 mL) of methanol and diluted 3-fold with water. These samples were purified by reverse-phase high-performance liquid chromatography on a 10 mm × 250 mm C_{18} column eluted with 60% methanol. Figure 2 shows the separation obtained for 3-hydroxybutyric acid benzylamide (peak 2), crotonic acid benzylamide (peak 3), and butyric acid benzylamide (peak 4). An overall yield of 85% was achieved for the conversion of butyryl-CoA to butyric acid benzylamide.

Reduction of Acetoacetyl Thio Esters. Either acetoacetyl-CoA (0.2 mM) or *N*-acetyl-*S*-acetoacetylcysteamine (1 mM) was incubated with 5 × 10^{-3} unit of fatty acid synthase and 0.5 μM stereospecifically tritiated NADPH in 0.7 mL of 0.1 M potassium phosphate, pH 7.0. The decrease in NADPH fluorescence (excitation 340 nm, emission 465 nm) was observed, and the reaction was allowed to proceed for over 30 half-times. The reactions were quenched by vortexing with 2 drops of chloroform, and the reaction products were isolated by DEAE-Sephacel chromatography as described above. The cysteamine thio esters were not retained by the column.

Hydration of Crotonyl-CoA. Varying amounts of crotonyl-CoA were incubated with 5 × 10^{-2} unit of fatty acid synthase in 1 mL of 0.1 M potassium phosphate, pH 7.0. The

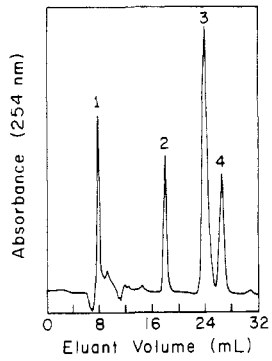

FIGURE 2: Reverse-phase high-performance liquid chromatography at room temperature on a 10 mm × 250 mm C_{18} column of a mixture of ~40 nmol of 3-hydroxybutyric acid benzylamide (peak 2), ~10 nmol of crotonic acid benzylamide (peak 3), and ~40 nmol of butyric acid benzylamide (peak 4). The absorbance at 254 nm (0.05 full scale) vs. the milliliters of eluant (60% methanol–40% water) is shown. The void volume (peak 1) is marked by the injection peak.

reaction was followed to completion by observing the absorbance at 290 nm. Crotonyl-CoA has a much larger extinction coefficient at 290 nm than hydroxybutyryl-CoA. When crotonyl-CoA was hydrated in D_2O, the buffer salts and crotonyl-CoA were first lyophilized from D_2O, and the enzyme was added as a D_2O solution. The reaction was quenched by vortexing with 2 drops of chloroform, and the protein was removed by centrifugation. The supernatant contained a mixture of 3-hydroxybutyryl-CoA and crotonyl-CoA. For derivitization by benzylamine, this mixture was made 0.55 M in potassium phosphate by addition of an equal volume of 1 M potassium phosphate, pH 7.0.

Stereochemical Analysis of 3-Hydroxybutyryl-CoA. Fifty microliters of 1.2 M Tris base and 50 μL of 50 mM NAD^+ were added to 0.4 mL of the hydrated crotonyl-CoA solution. The L-3-hydroxybutyryl-CoA was then quantitated by the addition of 5×10^{-2} unit of L-3-hydroxyacyl-CoA dehydrogenase and determination of the amount of NADH produced by measurement of the absorbance at 340 nm. A separate 0.2-mL aliquot of the hydrated crotonyl-CoA solution was hydrolyzed by adding 0.2 mL of 1 N NaOH. After 30 min, the hydrolysis mixture was neutralized with 0.4 mL of 1 M Tris-HCl and the D-3-hydroxybutyrate determined as above, except the enzyme used was D-3-hydroxybutyrate dehydrogenase. The thio ester concentration was determined by the difference in free thiol concentration (Ellman, 1959) before and after base hydrolysis.

Fatty Acid Synthase Catalyzed Exchange of a C-2 Proton of 3-Hydroxybutyryl-CoA. Twenty milliliters of 0.12 mM 3-hydroxy[2,2-$^2H_2$]butyryl-CoA in 55 mM potassium phosphate, pH 7.0, was incubated with 1.6 units of fatty acid synthase for 48 h. The reaction was quenched by vortexing 5-mL aliquots with 0.2 mL of chloroform and removing the precipitated protein by centrifugation. The 3-hydroxybutyryl moiety was isolated as the benzylamide as above.

Reduction of Crotonyl-CoA with [4-^3H]NADPH. To completely oxidize the [4-^{3}H]NADPH, 0.5 μM NADPH was incubated with 1 mM crotonyl-CoA and 0.1 unit of fatty acid synthase in 0.7 mL of 0.1 M potassium phosphate, pH 7.0. The reaction was followed by the decrease in NADPH fluorescence (340 nm excitation, 465 nm emission) and was allowed to proceed for over 20 half-lives (ca. 2 h). The reaction was quenched by vortexing with 2 drops of chloroform, and the products were analyzed by DEAE-Sephacel chromatography.

Reduction of Crotonyl-CoA in D_2O. Two milligrams of crotonyl-CoA was dissolved in 1 mL of 0.1 M potassium phosphate, pD 7.2, and lyophilized. After redissolving the crotonyl-CoA in 1.5 mL of the same buffer, 4 mg of NADPH and 1.6 units of fatty acid synthase in 0.3 mL of 0.1 M potassium phosphate, pD 7.4, were added.[2] The reaction was followed spectrophotometrically by observing the absorbance at 380 nm. After 24 h 0.77 mM NADPH had been oxidized and 0.35 mM butyryl-CoA had been formed, as determined by enzymic assay with acyl-CoA dehydrogenase (Thorpe, 1981). The reaction was quenched by vortexing with chloroform at this time since preliminary experiments indicated that extended incubations resulted in a slow decrease in the butyryl-CoA concentration. The butyryl-CoA was then isolated by DEAE-Sephacel chromatography. The butyryl moiety was isolated in a purified form as the benzylamide.

Acyl-CoA Dehydrogenase Catalyzed Exchange of the pro-2R Hydrogen of Butyryl-CoA. To 3 mL of 0.2–1 mM butyryl-CoA in 0.1 M potassium phosphate, pH (D), 7.4, was added 5×10^{-2} unit of acyl-CoA dehydrogenase. This enzyme has been shown to rapidly exchange the *pro-2R* hydrogen of butyryl-CoA with the solvent (Biellman & Hirth, 1970b). The incubations were allowed to proceed for either 1 day or 4 days after which the butyryl-CoA was reisolated by DEAE-Sephacel chromatography and the butyryl moiety was obtained as the purified benzylamide.

Oxidation of Butyryl-CoA with Acyl-CoA Dehydrogenase. Reaction mixtures contained phenazine methosulfate (1 mM), 2,6-dichlorophenolindophenol (0.03 mM), less than 0.01 mM butyryl-CoA, and 1×10^{-2} unit of acyl-CoA dehydrogenase in 0.5 mL of 0.1 M potassium phosphate, pH 7.0. The reduction of the dye was followed by observing the absorbance at 600 nM. The hydrogen atoms released to the solvent were isolated by bulb to bulb distillation.

Oxidation of [4-^3H]NADPH by Glutamate Dehydrogenase. [4-^{3}H]NADPH was incubated for 10 min with 1 mL of a solution containing 1 mM α-ketoglutarate, 10 mM NH_4Cl, 2 units of glutamate dehydrogenase, and 0.1 M potassium phosphate, pH 7.0. Control reactions with unlabeled NADPH indicated the oxidation was complete in less than 1 min. The reactions were quenched by vortexing with 2 drops of chloroform, and the products were separated by DEAE-Sephacel chromatography. Glutamate is not retained on the column in the presence of 15 mM potassium phosphate, pH 7.0.

Nuclear Magnetic Resonance. The benzylamides were prepared for nuclear magnetic resonance analysis by removing the high-performance liquid chromatography solvent by rotary evaporation. This solid was then lyophilized twice from solvents used for nuclear magnetic resonance measurements, D_2O for 3-hydroxybutyric acid benzylamide and $CDCl_3$ for butyric acid benzylamide. All spectra were taken on a Bruker WM 300. The chemical shifts are measured with respect to tetramethylsilane; however, the internal standard used was chloroform which was assumed to have a chemical shift of δ 7.26.

Scintillation Counting. Scintillation counting samples contained less than 10% water (v/v) in aqueous counting scintillant (Amersham) and were counted in a Beckman LS 250 scintillation counter.

Optical Measurements. Spectrophotometric assays were performed on a Cary 118 spectrophotometer, and fluorometric assays were performed on a Perkin-Elmer MPF-44B fluorometric spectrophotometer. The extinction coefficients used

[2] The elimination of free thiols, i.e., dithiothreitol, is essential for any reaction utilizing crotonyl thio esters as substrates since the first-order rate constant for the reaction of 2 mM dithiothreitol with crotonyl-CoA at pH 7.5 is 6×10^{-2} min^{-1}.

Table I: Stereospecificity of Reduction by NADPH

stereospecificity of [4-^{3}H]NADPH	substrates	% radioactivity eluted[a] by 15 mM	30 mM	55 mM
R	α-ketoglutarate, NH_4Cl[b]	1	96	3
S		98	2	<1
R	*N*-acetyl-*S*-acetoacetylcysteamine	6	91	3
S		97	3	<1
R	acetoacetyl-CoA	7	74	19
S		11	16	73
R	crotonyl-CoA	76	14	10
S		12	85	3
R	crotonyl-CoA (1 mM), $NADP^+$ (0.2 mM)	11	80	9

[a] Percentage of counts eluted by the concentrations of potassium phosphate indicated. $NADP^+$ was eluted with 30 mM and CoA thio esters with 55 mM potassium phosphate. About 85–95% of the radioactivity (8000–50 000 cpm) placed on the column was eluted. [b] The enzyme was glutamate dehydrogenase; for all other entries it was fatty acid synthase.

to determine concentrations were as follows: fatty acid synthase, $\epsilon_{280} = 4.82 \times 10^5\ M^{-1}\ cm^{-1}$ (Hsu & Yun, 1970); NADPH, $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$, $\epsilon_{380} = 880\ M^{-1}\ cm^{-1}$ (P-L Biochemicals, Circular OR-10); CoA thio esters (except crotonyl-CoA), $\epsilon_{260} = 15\,400\ M^{-1}\ cm^{-1}$ (P-L Biochemical Circular OR-10); crotonyl-CoA, $\epsilon_{266} = 22\,600$ (Stadtman, 1957); butyric acid benzylamides, $\epsilon_{258} = 218\ M^{-1}\ cm^{-1}$ (CRC Handbook of Chemistry and Physics); 2,6-dichlorophenolindophenol, $\epsilon_{600} = 20\,000\ M^{-1}\ cm^{-1}$ (Thorpe, 1981).

Results

Oxidation of [4-^{3}H]NADPH by Fatty Acid Synthase. The stereochemical purity of the [4-^{3}H]NADPH was checked with the well-characterized glutamate dehydrogenase reaction which transfers the *pro-4S* hydrogen from NADPH to α-ketoglutarate (Nakamoto & Vennesland, 1960). A typical experiment is summarized in Table I. However, due to decomposition stereochemical purity (>95%) could not be maintained during storage or lyophilization so the [4-^{3}H]-NADPH was used within 24 h of synthesis. The data presented in Table I indicate that the reduction of *N*-acetyl-*S*-acetoacetylcysteamine stereospecifically transfers the *pro-4S* hydrogen of NADPH: more than 90% of the radioactivity was found in $NADP^+$ when tritium was in the *pro-4R* position of NADPH, and more than 95% of the tritium appeared in the cysteamine derivative when tritium was in the *pro-4S* position. The reduction of *S*-acetoacetyl-CoA by [4-^{3}H]NADPH is not as simple. Approximately 75% of the radioactivity parallels the stereochemistry of the reduction of *N*-acetyl-*S*-acetoacetylcysteamine by [4-^{3}H]NADPH, but a significant amount of radioactivity was found in the CoA thio ester fractions when (*R*)-[4-^{3}H]NADPH was oxidized, and a comparable amount of [4-^{3}H]$NADP^+$ was isolated when (*S*)-[4-^{3}H]NADPH was oxidized. The radioactivity eluted with 30 mM potassium phosphate when both (*R*)-[4-^{3}H]NADPH and (*S*)-[4-^{3}H]-NADPH were oxidized was proven to be [4-^{3}H]$NADP^+$ by reducing the $NADP^+$ to (*S*)-[4-^{3}H]NADPH with glucose 6-phosphate and glucose-6-phosphate dehydrogenase as described in the synthesis of (*R*)-[4-^{3}H]NADPH. The radioactivity was then eluted from the DEAE-Sephacel column with 130 mM potassium phosphate, pH 7.0. These results are not inconsistent with those of Dugan et al. (1970), who reported essentially 100% stereospecificity with acetoacetyl-CoA as substrate since they determined only the radioactivity of the isolated 3-hydroxybutyrate. Apparently other radioactive thio esters are formed. A likely possibility is butyryl-CoA formed by the hydration and subsequent reduction of hydroxybutyryl-CoA. Virtually all of the hydroxybutyrate is obtained as the CoA derivative, which suggests that the steady-state reduction of acetoacetyl-CoA does not proceed through a covalent enzyme intermediate.

The results in Table I for the oxidation of (*R*)- and (*S*)-[4-^{3}H]NADPH by crotonyl CoA indicate that the *pro-4R* hydrogen is predominantly removed (>75%), but a significant amount of the *pro-4S* hydrogen appears in products other than $NADP^+$ as well. A possible explanation for the additional radioactive products is that the 3-hydroxybutyryl-CoA formed by hydration of the crotonyl-CoA [a reaction favored by an equilibrium constant of 3.5 (Stern & del Campillo, 1956)] is oxidized by the product $NADP^+$ to acetoacetyl-CoA. This acetoacetyl-CoA would then be reduced by the [4-^{3}H]NADPH with the demonstrated specificity for the *pro-4S* hydrogen. These reactions should be enhanced by including $NADP^+$ in the reaction mixture. The last entry in Table I indicates that when crotonyl-CoA is reduced with (*R*)-[4-^{3}H]NADPH in the presence of 0.2 mM $NADP^+$, [4-^{3}H]$NADP^+$ is the major product. The radioactivity eluted by 30 mM potassium phosphate was again demonstrated to be [4-^{3}H]$NADP^+$ by incubating the eluant with glucose 6-phosphate and glucose-6-phosphate dehydrogenase. The radioactive product of this reaction eluted from the DEAE-Sephacel column with 130 mM potassium phosphate. The major radioactive product formed, besides [4-^{3}H]$NADP^+$, when either (*R*)- or (*S*)-[4-^{3}H]NADPH was oxidized by crotonyl-CoA was not a CoA thio ester since it was not retained on the DEAE-Sephacel column in the presence of 15 mM potassium phosphate. The product was characterized further by high-pressure liquid chromatography (10 mM potassium phosphate, pH 2.5, 40% methanol). The largest fraction (71%) eluted with carrier butyric acid, a minor fraction (<10%) eluted at or near the void volume, and the remaining counts (22%) eluted in a well-defined peak after the methanol concentration was increased to 70%, indicating a much more hydrophobic product than butyric acid. The counts eluted from the DEAE-Sephacel column with 55 mM potassium phosphate were demonstrated to be butyryl-CoA by oxidizing the pooled fractions with acyl-CoA dehydrogenase in the presence of 10 μM carrier butyryl-CoA; 83% of the radioactivity was released to the solvent by this treatment.

Stereochemistry of Alcohol Formation. The results in Table II indicate that the alcohol formed by reduction of either

Table II: Stereochemistry of 3-Hydroxybutyryl from Fatty Acid Synthase Reactions

substrates	thio ester (μM)	L-3-hydroxybutyryl-CoA (μM)	D-3-hydroxybutyryl-CoA (μM)
NADPH, acetoacetyl-CoA	120	<1	89
NADPH, *N*-acetyl-*S*-acetoacetylcysteamine	220	<1	210
crotonyl-CoA, H_2O	118	44	49
L-3-hydroxybutyryl-CoA	21	8	10

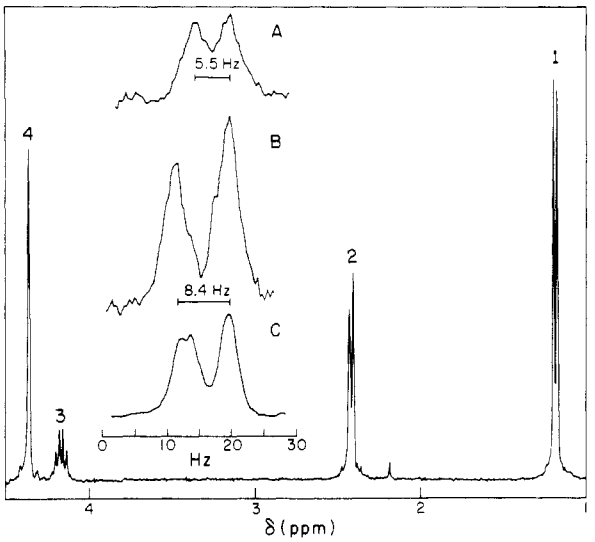

FIGURE 3: 300-MHz nuclear magnetic resonance spectrum of 3-hydroxybutyric acid benzylamide in D_2O at 25 °C. The signal intensity vs. the chemical shift with respect to tetramethylsilane is shown. The peaks labeled 1, 2, and 3 are the methyl protons, C-2 protons, and C-3 protons of hydroxybutyrate, and peak 4 is due to the benzyl methylene protons. The insets are the C-2 resonances of (*R,S*)- and (*S,R*)-3-hydroxy[2-^{2}H]butyric acid benzylamide synthesized by fatty acid synthase (A), the (*R,R*)- and (*S,S*)-3-hydroxy[2-^{2}H]butyric acid benzylamide (B), and 3-hydroxybutyric acid benzylamide (C), with the abscissa in hertz.

N-acetyl-*S*-acetoacetylcysteamine or acetoacetyl-CoA is over 99% the D (*3R*) isomer. This agrees with previous studies of fatty acid synthases (Wakil & Bressler, 1962; Lynen, 1961). The amount of total thio ester and D-alcohol agree well for the cysteamine substrate, but a significant difference exists with the CoA substrate. This is consistent with the results in Table I which suggest some butyryl-CoA may be formed when acetoacetyl-CoA is the substrate. Surprisingly, the hydration of crotonyl-CoA by fatty acid synthase produced an equimolar amount of D- and L-3-hydroxybutyryl-CoA. The formation of the L-3-hydroxybutyryl-CoA was traced to a small contaminant of crotonase in the fatty acid synthase. Because of the high turnover number of crotonase (Waterson & Hill, 1972), even a 0.01% contamination by weight would produce an equilibrium amount of L-3-hydroxybutyryl-CoA. The relative activity of the crotonase contaminant and the fatty acid synthase crotonyl-CoA hydratase activity can be determined by measuring the initial rate of production of (*S*)- and (*R*)-3-hydroxybutyryl-CoA, respectively. This is most easily approximated by quenching the reaction after 20% of the crotonyl-CoA has been hydrated and determining the relative amounts of (*S*)- and (*R*)-3-hydroxybutyryl-CoA. The ratio of (*S*)- to (*R*)-3-hydroxybutyryl-CoA varied from 7:1 to nearly 1:1 with different preparations of fatty acid synthase.

Stereochemistry of the Hydration of Crotonyl-CoA. The 300-MHz nuclear magnetic resonance spectrum in D_2O of 3-hydroxybutyric acid benzylamide is shown in Figure 3. The aromatic resonances at about δ 7.3 are not shown. The peaks labeled 1, 2, and 3 represent the methyl protons, C-2 protons, and C-3 proton of hydroxybutyrate, and the peak labeled 4 represents the benzyl methylene protons. When crotonyl-CoA is hydrated with fatty acid synthase in D_2O and the product 3-hydroxy[2-^{2}H]butyryl-CoA is derivatized with benzylamine, the C-2 peak of the 3-hydroxybutyric acid benzylamide integrates to one proton and has a spin-spin coupling constant of 5.5 Hz as shown in the expansion of the C-2 region (Figure 3, inset A). Since Mohrig et al. (1981) found that (*R,R*)- and (*S,S*)-3-hydroxy[2-^{2}H]butyrate has a corresponding spin–spin coupling constant of 9.0 Hz, the products of the hydration of crotonyl-CoA by fatty acid synthase and the contaminating crotonase are (*S,R*)- and (*R,S*)-3-hydroxy[2-^{2}H]butyryl-CoA, respectively. This indicates that the hydration involves the syn addition of the elements of H_2O across the double bond. These conclusions were confirmed by incubating D-3-hydroxy[2,2-$^2H_2$]butyryl-CoA with fatty acid synthase in H_2O for 2 days. The isolated 3-hydroxybutyric acid benzylamide had a nuclear magnetic resonance spectrum which indicated a single proton on C-2 with a spin–spin coupling constant of 8.4 Hz (inset B of Figure 3), indicating the formation of (*R,R*)- and (*S,S*)-3-hydroxy[2-^{2}H]butyryl-CoA. The nuclear magnetic resonance spectrum of the 3-hydroxybutyric acid benzylamide C-2 protons with two protons on C-2 is shown in inset C of Figure 3. As expected both spin–spin coupling constants (5.5 and 8.4 Hz) can be seen. The presence of a small crotonase contaminant does not affect these conclusions; in fact the results confirm the observation of Willadsen & Eggerer (1975) that crotonase catalyzes a syn elimination of water from 3-hydroxybutyryl-CoA. If either enzyme catalyzed an anti elimination, both spin–spin coupling constants would be seen in the nuclear magnetic resonance spectrum.

Table III: Stereochemistry of C-2 in Butyryl-CoA

substrate	acyl-CoA dehydrogenase treatment	C-2 proton intensity/ benzyl proton intensity[a]
[2-^{2}H]butyryl-CoA[b]		0.52
butyryl-CoA	1 day in D_2O	0.53
butyryl-CoA	4 days in D_2O	0.51
[2-^{2}H]butyryl-CoA[b]	1 day in H_2O	0.49
[2-^{2}H]butyryl-CoA[b]	1 day in D_2O	0.11

[a] The benzyl protons (2) of butyric acid benzylamide were used as the internal integration standard of the nuclear magnetic resonance spectra. The benzyl proton integration was from δ 4.41 to 4.50, and the C-2 proton integration was from δ 2.14 to 2.27. [b] Prepared by the fatty acid synthase catalyzed reduction of crotonyl-CoA by NADPH.

Stereochemistry of Butyryl-CoA Formation. The butyryl-CoA isolated from the reduction of crotonyl-CoA with (*R*)-[4-^{3}H]NADPH was shown to lose 83% of its radioactivity when oxidized by acyl-CoA dehydrogenase, an enzyme known to remove the *pro-2R* and *pro-3R* hydrogens from butyryl-CoA (Biellman & Hirth, 1970 a,b), indicating that the hydrogen transferred from NADPH is in the *pro-R* position. When either the acyl-CoA dehydrogenase or the electron acceptor was omitted from the reaction mixture, less than 7% of the radioactivity distilled with the water. Since the *pro-2R* proton of butyryl-CoA is exchanged with solvent in the absence of the electron acceptor (Biellman & Hirth, 1970b), this indicates that the tritium is present at the *pro-3R* position of butyryl-CoA. Integration of the nuclear magnetic resonance spectrum of the butyric acid benzylamide derived from butyryl-CoA formed by the reduction of crotonyl-CoA in D_2O (entry 1 in Table III) indicated a single proton was present on C-2. The well-defined triplet at δ 0.97 indicated the presence of two protons at C-3, confirming the conclusion that the hydrogen derived from the solvent is incorporated solely at C-2 of butyryl-CoA. The configuration at C-2 was determined by allowing acyl-CoA dehydrogenase to exchange the *pro-2R* hydrogen of butyryl-CoA in the absence of an electron acceptor. Since incubation of butyryl-CoA for either 1 or 4 days results in the exchange of only one proton from C-2 (entries 2 and 3 in Table III), the exchange with solvent is stereospecific; Biellman & Hirth (1970b) have shown the

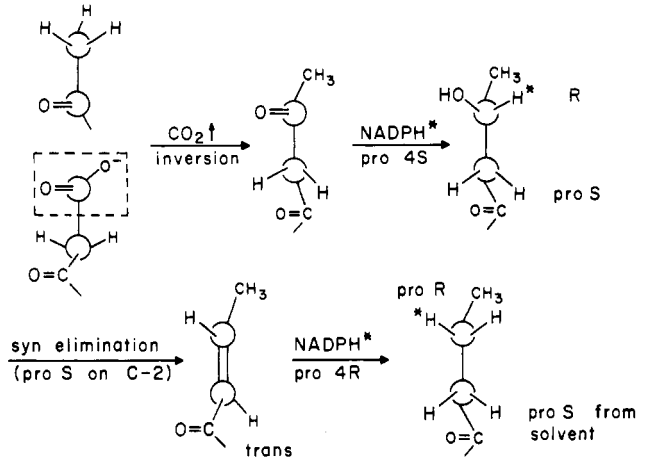

FIGURE 4: Stereochemical course of the reactions catalyzed by chicken liver fatty acid synthase derived from this work. The condensation of the malonyl and acetyl moieties to give a keto intermediate is followed by reduction of the intermediate to an alcohol with NADPH, dehydration, and a reduction of the carbon–carbon double bond by NADPH. This mechanism is discussed further in the text.

easily exchanged hydrogen is in the *pro-2R* position. The failure of acyl-CoA dehydrogenase to remove the deuterium from [2-^{2}H]butyryl-CoA formed by fatty acid synthase (entry 4 in Table III) indicates the product must be (*S*)-[2-^{2}H]-butyryl-CoA. This conclusion is confirmed by demonstrating that [2,2-$^2H_2$]butyryl-CoA is formed by allowing acyl-CoA dehydrogenase to exchange deuterium into the *pro-2R* position of the (*S*)-[2-^{2}H]butyryl-CoA (entry 5 in Table III). The small number of protons left in the [2,2-$^2H_2$]butyryl-CoA 0.22) primarily reflects the ~5% H_2O in the reaction mixture that was introduced with the acyl-CoA dehydrogenase.

Discussion

The results of the stereochemical studies are summarized by the reaction sequence in Figure 4. The first step, which is the condensation of the malonyl and acetyl moieties, proceeds with inversion at C-2. This conclusion is based on previous work that established this reaction proceeds with retention of configuration at C-2 if the subsequent dehydration proceeds by anti elimination of water or with inversion of configuration at C-2 if the dehydration proceeds by syn elimination (Sedgwick et al., 1977). Since we have shown the elimination of water is syn, the condensation must proceed with inversion at C-2. A similar conclusion was reached for yeast fatty acid synthase (Sedgwick et al., 1978).

The reduction of acetoacetyl-CoA to D-3-hydroxybutyryl-CoA (step 2) with the transfer of the *pro-4S* hydrogen of NADPH is a conserved feature of all fatty acid synthases studied thus far, namely, the enzyme from yeast (Lynen, 1961; Seyama et al., 1977b), pigeon liver (Wakil & Bressler, 1962; Dugan et al., 1970), *Brevibacterium ammoniagenes* (Seyama et al., 1977a), castor bean seeds (Saito et al., 1980), and *Chlorella vulgaris* (Saito et al., 1980).

The elimination of water in step 3 is syn, with the *pro-S* hydrogen from the C-2 being removed. The next step is the reduction of crotonyl-CoA to butyryl-CoA which proceeds by transfer of the *pro-4R* hydrogen of NADPH to the *pro-3R* position on butyryl-CoA, with the concomitant addition of a solvent proton to the *pro-2S* position of butyryl-CoA. Thus, both the hydrogens added during the reduction and the elements of water added during hydration are added to the same face of crotonyl-CoA. This stereochemistry follows the general pattern emerging for the enoyl reductase: the nucleotide specificity, either *pro-4R* or *pro-4S*, determines the stereospecificity of hydrogen incorporation at C-3, *pro-3R* or *pro-3S*,

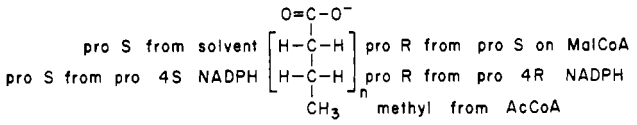

FIGURE 5: Origin of the prochiral and methyl hydrogens of fatty acids synthesized by chicken liver fatty acid synthase inferred from this work. This stereochemistry may not be strictly preserved during fatty acid synthesis as discussed in the text. (MalCoA is malonyl-CoA, and AcCoA is acetyl-CoA.)

respectively, with the proton from solvent being incorporated at C-2 in a syn addition (Saito et al., 1981). The yeast enzyme is an exception: the *pro-4S* hydrogen of NADPH is incorporated into the *pro-3S* position of the butyryl moiety, but the solvent proton is incorporated in an anti addition (Sedgwick & Morris, 1980).

The stereochemical results for the partial reactions combined with the cyclic mechanism of addition of two carbon fragments first proposed by Lynen (1961) implies that each of the four hydrogens on the even and odd carbons of the fatty acid synthesized can be traced to a unique source, with the exception of the methyl hydrogens on the terminal carbon which are derived from the primer acetyl group. As shown in Figure 5 the *pro-R* and *pro-S* hydrogen on the even carbons should be derived from the *pro-S* hydrogen of malonyl-CoA and from the solvent, respectively, while the *pro-R* and *pro-S* hydrogens on the odd carbons are derived from the *pro-4R* and *pro-4S* hydrogens of NADPH, respectively. The stereochemical purity of the even carbons of the fatty acids synthesized is limited by "post-malonate" exchange: Sedgwick & Cornforth (1977) found that less than the expected amount of label from [2-^{2}H]- or [2-^{3}H]malonyl-CoA was incorporated into fatty acids, even when the exchange of the C-2 protons of malonyl-CoA with water was accounted for. The results reported here for the addition of the solvent proton at C-2 during hydration and reduction of crotonyl-CoA show significantly greater stereospecificity than would be expected if post-malonate exchange was as extensive as observed by Sedgwick & Cornforth (1977). This suggests that the post-malonate exchange may require an intermediate with more than a four-carbon chain or may require the intermediate to be esterified to the enzyme.

The product analyses of the acetoacetyl reductase and enoyl reductase partial reactions indicate that several reaction pathways proceed at similar rates. These may include acylation of the fatty acid synthase, reduction of a covalent intermediate, and subsequent hydrolysis of the product or transesterification of free CoA, all in competition with the direct reduction of acyl-CoA's, without formation of a covalent enzyme–substrate intermediate. The enoyl reductase reaction is further complicated by competition for crotonyl-CoA with the hydration reaction which produces a substrate for the acetoacetyl reductase reaction. Some of these difficulties have been considered previously for the acetoacetyl reductase activity (Dodds et al., 1981) and for the enoyl reductase activity (Strom et al., 1979). These complexities must be taken into account when differences in the assays for the partial reactions of fatty acid synthase are interprreted.

The complete elucidation of the stereochemistry of the fatty acid synthase reactions for a single enzyme reported here provides the necessary foundation for a more detailed examination of the individual reaction intermediates.

Registry No. (*S*)-[4-^{3}H]NADPH, 20545-66-8; (*R*)-[4-^{3}H]-NADPH, 18846-69-0; [4-^{3}H]NADP$^+$, 10012-95-0; 3-hydroxy[2,2-$^2H_2$]butyryl-CoA, 89232-28-0; acetoacetyl-CoA, 1420-36-6; D-3-hydroxybutyryl-CoA, 21804-29-5; crotonyl-CoA, 992-67-6; butyryl-CoA, 2140-48-9; fatty acid synthase, 9045-77-6; 3-hydroxybutyric acid benzylamide, 89232-29-1; crotonic acid benzylamide, 89232-30-4;

butyric acid benzylamide, 10264-14-9; (*R,S*)-3-hydroxy[2-^{2}H]butyric acid benzylamide, 89232-31-5; (*R,R*)-3-hydroxy[2-^{2}H]butyric acid benzylamide, 89232-32-6.

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